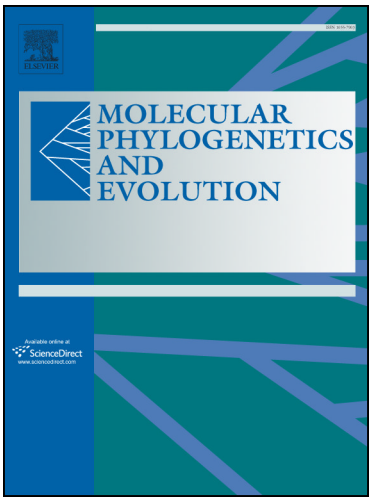

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PII: S1055-7903(18)30387-7
DOI: https://doi.org/10.1016/j.ympev.2018.10.002
Reference: YMPEV 6290

To appear in: *Molecular Phylogenetics and Evolution*

Received Date: 15 June 2018
Revised Date: 25 September 2018
Accepted Date: 1 October 2018

Please cite this article as: Galaska, M.P., Li, Y., Kocot, K.M., Mahon, A.R., Halanych, K.M., Conservation of mitochondrial genome arrangements in brittle stars (Echinodermata, Ophiuroidea), *Molecular Phylogenetics and Evolution* (2018), doi: https://doi.org/10.1016/j.ympev.2018.10.002

Conservation of mitochondrial genome arrangements in brittle stars (Echinodermata, Ophiuroidea).

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Keywords

mtDNA, Ophiuroidea, brittle star, mitochondrial genome

Abstract

Brittle stars are conspicuous members of benthic ecosystems, fill many ecological niches and are the most speciose of all classes of echinoderms. With high levels of biodiversity, elucidating the evolutionary history of this group is important. Understanding of higher-level relationships within Ophiuroidea has been aided by multilocus nuclear data and DNA barcoding. However, the degree of consistency between mitochondrial and nuclear data within ophiuroids remains unclear and deserves further assessment. In this study, 17 mitochondrial genomes spanning the taxonomic breadth of Ophiuroidea were utilized to explore evolutionary relationships through maximum likelihood analyses, Bayesian inference and comparative assessment of gene order. Our phylogenetic analyses, based on both nucleotide and amino acid residues, support recent findings based on multilocus nuclear data and morphology, in that the brittle star clades Ophintegrida and Euryophiurida were recovered as monophyletic with the latter comprising Euyalida, Ophiuridae and Ophiopyrgidae. Only three different arrangements of the 13 protein coding and 2 ribosomal RNA genes were observed. As expected, tRNA genes were more likely to have undergone rearrangement but the order of all 37 genes was found to be conserved in all sampled Euryalida and Ophiuridae. Both Euryalida and the clade comprised of Ophiuridae and Ophiopyrgidae, each had their own conserved rearrangement of protein coding genes and ribosomal genes, after divergence from their last common ancestor. Euryalida has a rearrangement of the two ribosomal RNA genes, *rrnS* and *rrnL*, in contrast to Ophiuridae and Ophiopyrgidae, which had an inversion of the genes *nad1*, *nad2*, and *cob* relative to Ophintegrida. Further, our data support the gene order found in all sampled Euryalida as the most likely ancestral order for all Ophiuroidea.

1. Introduction

Ophiuroids, or brittle stars, occur in all the world's oceans from the deep sea to intertidal zones and are more speciose than other extant lineage of echinoderms (Stöhr et al., 2012). Ophiuroids fill a wide array of ecological niches including suspension feeders (Emson et al., 1991), scavengers, and even opportunistic generalists that will consume anything from detritus to smaller individuals of their own species (Fratt and Dearborn, 1984). Additionally, ophiuroids possess multiple types of reproductive strategies (Heimeier et al., 2010a; Mladenov et al., 1983; Tominaga et al., 2004). Because of their great diversity and ecological importance, evolutionary relationships within Ophiuroidea are of great interest (O'Hara et al., 2014; Stöhr et al., 2012). Traditionally, Ophiuroidea was thought to comprise two extant lineages, Ophiurida and Euryalida (Smith et al., 1995). Fossil evidence suggests this classification may be incorrect, as Euryalida appears to have evolved more recently (Smith et al., 1995). Recent studies employing transcriptome data (O'Hara et al., 2014) and target-capture approaches (O'Hara et al., 2017), along with morphological data (O'Hara et al., 2018), have greatly improved understanding of ophiuroid evolutionary history and suggest Ophiuroidea is comprised of two clades Euryophiurida and Ophintegrida. Euryophiurida is comprised of Euryalida, and some members of the group formerly called Ophiurida, specifically Ophiuridae, Ophiopyrgidae and the *Ophiomusium* complex. Ophintegrida is comprised of all remaining families formerly assigned to Ophiurida.

Mitochondrial genomes are an excellent molecular marker for phylogenetics (Boore and Brown, 2000; Cameron, 2014; Egger et al., 2017; Li et al., 2014). Additionally mitochondrial data, specifically *cox1*, is widely utilized for barcoding of species along with use in population genetics, biogeography and phylogenetic studies (Galaska et al., 2017a, 2017b; Hajibabaei et al., 2007; Heimeier et al., 2010b). Further, rare genomic changes among mitochondrial genomes such as gene rearrangements and inversions can be studied in a comparative fashion, shedding further light on the evolutionary history of a group of organisms (Boore and Brown, 1998, 2000; Chen et al., 2018; Li et al. 2015; Zhong et al., 2008). Currently there are only seven publicly available ophiuroid mitochondrial genomes, six of which are published (Perseke et al., 2008, 2010; Scouras et al., 2004), out of the approximately 2,100 currently recognized species (Stöhr et al., 2017). All of these complete Ophiuroidea mitochondrial genomes are circular and contain all 37 genes found in the typical bilaterian mtDNA genome (Boore and Brown, 2000; Perseke et al., 2010). However, ophiuroid mitochondrial genomes have been suggested to have accelerated rates of evolution with significant rearrangements of gene order in comparison to other classes of echinoderms, specifically echinoids, asteroids, and holothuroids (Scouras et al., 2004).

Understanding the degree of congruence between nuclear (O'Hara et al., 2014, 2017) and mitochondrial (Perseke et al., 2008, 2010; Scouras et al., 2004) evolutionary histories has implications for the utility of mitochondrial genomes for animal phylogenetics (Moore, 1995; Boore and Brown, 1998). Currently there are 32 recognized families of ophiuroids (O'Hara et al. 2017), with six having publicly available mitochondrial genomes. In this study, we sequenced 10 new ophiuroid mitochondrial genomes more than doubling previously available data for brittle

stars. These new species include two previously unrepresented families (four individuals in Ophiopyrgidae and one individual in Ophiolepididae) and increased taxonomic coverage within Ophiuridae and Gorgonocephalidae. We test two hypotheses: 1) the mitochondrial rearrangements of protein-coding genes and ribosomal RNA genes will remain conserved within the major ophiuroid clades identified by O'Hara et al (2014, 2017) and, 2) the inferred phylogenetic relationships recovered from mitochondrial gene sequences will be consistent with that of the O'Hara et al. (2014, 2017) nuclear data set.

2. Methods

2.1. *Collection, genome assembly, annotation and mapping*:

Collection and locality information for the 10 ophiuroid specimens sampled are given in Table 1. Specimens were collected from the Southern Ocean using Blake trawls, preliminarily identified on the ship by Chester Sands, Matthew Galaska and Ken Halanych, and subsequently confirmed back in the laboratory with appropriate literature (e.g., McKnight, 1967; Sieg & Waegele, 1990). Samples were preserved in either >90% ethanol or frozen at -80°C. Seven additional ophiuroid mitochondrial genomes were downloaded from NCBI (Table 1) for inclusion in this study.

Genomic DNA was extracted using Qiagen's DNeasy® Blood and Tissue kit (Valencia, CA) following manufacturer's protocol. Library preparation and paired-end sequencing was performed by The Genomic Services Lab at Hudson Alpha Institute in Huntsville Alabama. Sequencing employed the Illumina HiSeq 2500 platform using 2 x 125 paired-end v4 chemistry for all specimens except *Ophionotus victoriae*, which was sequenced earlier using 2 x 100

paired-end v3 chemistry. *De novo* assemblies of paired-end reads were performed using Ray 2.2.0 (Boisvert et al., 2012) with a k-mer of 31. Mitochondrial genomes were identified using BLASTn (Altschul et al., 1990) with the mitochondrial genome of *Ophiocomina nigra* (Perseke et al., 2010) serving as the bait sequence. Contigs were initially annotated using the MITOS web server (Bernt et al., 2013) and annotations were checked manually using Artemis (Rutherford et al., 2000). Translation from nucleotides to amino acids used the echinoderm mitochondrial translation code (NCBI translation code 9). For leucine, L1 and L2 were coded by CTN and TTR, respectively and for serein S1 and S2 were coded by AGN and TCN, respectively. Comparisons of the rearrangement of coding genes were visualized using Mauve (Darling et al., 2004). Reconstruction of gene order evolution was performed using TreeREx 1.85 with default parameters (Bernt et al., 2008).

2.2. *Phylogenetic analyses*:

Phylogenetic analyses were performed on 17 ophiuroids (Table 1) along with two asteroids, *Acanthaster brevispinus* and *Acanthaster planci* (GenBank Accessions AB231476 and AB231475, respectively; Yasuda et al., 2006), which represent the sister taxon of Ophiuroidea (Cannon et al. 2014). Analyses were conducted on amino acid (AA) sequences from the 13 mitochondrial protein-coding genes (*cox1, cox2, cox3, cob, atp6, atp8, nad1, nad2, nad3, nad4, nad4l, nad5, and nad6*) and nucleotide sequences from the same 13 genes plus both ribosomal RNA genes (*rrnS* and *rrnL*). All genes were individually aligned using MAFFT (Katoh et al., 2005) under default parameters in the TranslatorX software package (Abascal et al., 2010). Resulting alignments were manually evaluated and minor corrections were made by hand. To remove any

ambiguously aligned regions, alignments were trimmed using Gblocks V. 0.91b (Talavera and Castresana, 2007) with default settings. Resulting trimmed alignments for each gene were concatenated using FASconCAT (Kück and Meusemann, 2010) for use in phylogenetic analyses. To select an appropriate partition scheme and the best-fitting substitution model for each partition, ModelFinder (Kalyaanamoorthy et al., 2017) was used. Maximum likelihood (ML) analyses in IQ-TREE v1.6.6 (Nguyen et al., 2015) were used to infer phylogenetic relationships, using 1,000 replicates of ultrafast bootstrapping (UFBoot2; Hoang et al., 2018) to evaluate nodal support. Bayesian inference was conducted using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) with each partition assigned its own best-fit nucleotide substitution model. The Markov chain Monte Carlo (MCMC) was run for 20,000,000 generations (sampling every 1,000 generations) to allow adequate time for convergence. At the end of the run, the standard deviation of split frequencies was less than 0.01. All parameters were checked with Tracer v 1.5 (Drummond and Rambaut, 2007). After omitting the first 20% “burn in” trees, the remaining sampled trees were used to estimate the 50% majority rule consensus tree and the Bayesian posterior probabilities (PP).

3. Results

3.1. *Mitochondrial genome composition*:

Complete mitochondrial genomes, which included all 13 protein-coding genes, 22 tRNA genes and 2 ribosomal RNA genes, were recovered for all 10 newly-sequenced ophiuroids. Each sampled ophiuroid was found to have genes on both strands, consistent with previously available ophiuroid mitochondrial genomes (Perseke et al., 2008, 2010; Scouras et al., 2004).

Mitochondrial genome sizes (Table 2) within the sampled Euryalida were fairly conserved with *Astrospartus mediterraneus* having the smallest genome at 16,238 bp and *Astrotoma agassizii* having the largest one at 16,524 bp. Ophintegrida had a larger range in mitochondrial genome size, from 15,845 bp in *Ophiacantha linea*, up to 17,383 bp in *Ophiocomina nigra.* The sister clades Ophiuridae and Ophiopyrgidae had highest variation in mitochondrial genome sizes, ranging from *Ophionotus victoriae* at 15,932 bp to *Ophioplinthus gelida* with 18,387 bp.

3.2. *Phylogenetic analyses*:

Our results (Figure 1) recovered a branching order consistent with that of O'Hara et al (2014; 2017). Maximum likelihood analyses run on both amino acid and nucleotide alignments returned identical branching orders with strong nodal support. Best-fit partition models can be found in Supplementary Table 1. Our analyses recovered three main clades with Euryalida sister to the clade Ophiuridae and Ophiopyrgidae within Euryophiurida, and all other families of ophiuroids comprised another clade, Ophintegrida. The two species of *Ophiura* and *Ophiosteira* were recovered as monophyletic but the two *Ophioplinthus* species were not. Our analyses further confirm recent work by O'Hara et al (2018), which characterized morphological diagnoses of higher taxon relationships within Ophiuroidea while also reaffirms that more recent relationships, such as *Ophioplinthus*, may need further evaluation. Bayesian AA analyses differed from ML with the basket stars *Astrospartus mediterraneus* and *Gorgonocephalus chilensis* not recovered as monophyletic (Supplemental Figure 1).

3.3. *Gene order conservation*:

Organization of the 13 coding genes and 2 ribosomal RNA genes was conserved within Euryalida and Ophiuridae (Figure 2). Conversely, samples from Ophintegrida exhibited one of two differing arrangements with variable placement of ribosomal genes. One arrangement was recovered in *O. aculeata*, *A. squamata*, and *O. nigra* and was unique to Ophintegrida. The mtDNA arrangement of Ophintegrida and Euryalida differs in the order of *rrnS* and *rrnL* genes. In comparison, mtDNA genomes of Ophintegrida differ from Ophiuridae and Ophiopyrgidae in the strand placement of *nad1*, *nad2*, and *cob*. Euryalida differs from Ophiuridae and Ophiopyrgidae in both the order of *rrnS*, *rrnL*, and the strand location of *nad1*, *nad2*, and *cob*. Ophiuridae and Ophiopyrgidae's unique arrangement of *nad1, nad2,* and *cob*, may be due to a block inversion of these onto the opposite strand as the transcriptional order is maintained (Figure 2). Multiple non-coding regions were located across all sequenced Ophiuroidea and are detailed within their GenBank entries (Table 1).

Across all three clades, the arrangement of the block of genes from *cox1* through *nad5* and associated tRNAs, approximately 8,000 base pairs, is conserved. Interestingly, all Euryalida sampled have the same gene order, including tRNAs. Although *Ophiacantha linea* and *Ophioceres incipiens* from Ophintegrida shared the same relative arrangement of coding and ribosomal genes as Euryalida, the arrangement of tRNA genes differed between these two species and from Euryalida species. The relative order of tRNA genes within Ophintegrida has been presented by Perseke et al. (2010). The three specimens of Ophiuridae (*Ophionotus victoriae*, *Ophiura albida*, and *Ophiura lutkeni*) all possess identical arrangement of all 37 mitochondrial genes. Within Ophiopyrgidae, there are two unique arrangements of tRNA genes. *Ophioplinthus brevirima*, *Ophiosteira antarctica*, and *Ophiosteira sp*. all possessed the

same unique mitochondrial gene arrangement, which differed from Ophiuridae only in the relative position of *trnL1*. *Ophioplinthus gelida* had the second unique arrangement within Ophiopyrgidae with the placement of the tRNA genes *trnL1*, *trnY* and *trnV*.

Inference of gene order evolution from TreeREx based on the protein-coding and ribosomal genes, recovered the order found in all sampled Euryalida as the most likely ancestral order of all Ophiuroidea (Figure 1). These results suggest that two separate transposition events of the ribosomal genes occurred within sampled Ophintegrida clades. Further, a transposition and inversion of the *nad1*, *nad2*, and *cob* genes apparently occurred in the most recent common ancestor of Ophiuridae and Ophiopyrgidae but after the most recent common ancestor of the Euryophiurida.

4. Discussion

Similar to most other ambulacrarians (i.e., echinoderms and hemichordates) all ophiuroids investigated in this study possessed a conserved gene arrangement of the block *cox1* through *nad5*, signifying that this region is under strict selection, presumably for functional reasons. Euryalida shares the same ancestral protein coding and ribosomal RNA gene order with *Ophioceres incipiens* and *Ophiacantha linea* of Ophintegrida, but the arrangement of tRNAs is more variable. The recovered phylogenetic tree from this study and O'Hara et al (2014) both suggest that *Ophioceres incipiens* and *Ophiacatha linea* are from separate clades within Ophintegrida. Thus, rearrangement of *rrnS* and *rrnL* has occurred independently at least twice to account for the mitochondrial gene order found in *Ophiopholis aculeata*, *Amphipholis*

squamata, and *Ophiocomina nigra*. With rearrangements of the ribosomal RNA genes, the transcriptional order is likely not under strong selection as long as they are transcribed together. Within Euryalida, there is no difference in arrangement of any of the 37 mitochondrial genes, suggesting that this order is either strongly conserved within this group or that sampling was not extensive enough to reveal additional variation. The arrangement within Ophiuridae was also conserved for all 37 mitochondrial genes. In Ophiopyrgidae, only tRNAs showed signs of rearrangement, including within the two species of the genus *Ophioplinthus*.

Although ophiuroid phylogenetic relationships based on mitochondrial analyses have shown inconsistencies (Littlewood et al., 1997; Scouras and Smith, 2001), our recovered relationships within Ophiuroidea are consistent with recent analyses of nuclear loci (O'Hara et al., 2014, 2017) (Figure 1). The three conserved clades provide further support for the two proposed orders within Ophiuroidea, Euryophiurida and Ophintegrida which were supported with a 100% bootstrap support, consistent with O'Hara et al 2017. This work also further supports the recent reinstatement of Ophiopyrgidae which was assigned to Ophiuridae. Previous work (Perseke et al., 2010) concluded that the ancestral gene arrangement in Ophiuroidea was the same as that of *Ophiocomina nigra*, a member of clade Ophintegrida, but additional sampling coupled with analyses in TreeREx, did not support these findings. If the gene order of *Ophiocomina nigra* were the ancestral order for Ophintegrida, the arrangement found in Euryalida can be explained by an inversion of *rrnS* and *rrnL* in the common ancestor of Euryalida. Similarly, the arrangement of Ophiuridae and Ophiopyrgidae could also be explained by the transposition of *nad1*, *nad2*, and *cob*, in their common ancestor.

Euryalida diverged from Ophiuridae and Ophiopyrgidae a minimum of 180 million years ago (Ma) (O'Hara et al., 2017), after the end-Permian mass extinction and subsequent radiation of Ophiuroidea species (Chen and McNamara, 2006). Using O'Hara's (2017) estimated divergence times for Ophiuroidea, we can estimate the relative timing of gene rearrangements. Specifically, the two independent rearrangements of the ribosomal RNA genes in the Ophintegrida occurred within the last ~175 Ma for *Ophiacantha linea* and ~205 Ma for *Ophiopholis aculeata* and *Amphipholis squamata*. Further sampling within Ophiuroidea clades could further refine these estimates. Within approximately the same time scale, ~180 Ma (Tsang et al., 2014), analyses of brachyuran crabs (Sun et al., 2005), and gastropods (Grande et al., 2008), have shown more significant mitochondrial rearrangements. The sampling presented here is from across a wide evolutionary range of Ophintegrida and thus the two main arrangements of the 13 protein coding genes and 2 ribosomal RNA genes recovered, are likely representative of patterns within group. In general, rearrangement of tRNA genes was absent within recognized families with the exception of *Ophioplinthus gelida* which surprisingly differed from that of *Ophioplinthus brevirima* (Ophiopyrgidae). Ultimately, three arrangements of the 13 protein-coding genes and 2 ribosomal RNA genes were recovered which is less conserved than other groups such as insects (Cameron, 2014), Demospongiae (Wang and Lavrov, 2007), and the cnidarian Octocorallia (Brockman and McFadden, 2012), but similar to that of annelids (Zhong et al., 2008). Within echinoderms, Echinoidea has the most conserved gene order and Ophiuroidea was found to be the most rearranged (Perseke et al., 2010). Although ophiuroid mitochondrial genomes are considered to be more extensively rearranged,

within ophiuroids, arrangements are fairly conserved and consistent with our current understanding of brittle star phylogeny and taxonomy.

Acknowledgements

Funding from the National Science Foundation (NSF ANT- 1043670 to ARM, NSF ANT- 1043745 & OPP- 0132032 to KMH) is gratefully acknowledged. This research was made possible with assistance from the Captains and crews of the *RV/IB Nathianel B. Palmer* (NBP12- 10) and ASRV *Laurence M. Gould* (LMG13- 12, LMG04- 14, and LMG06- 05). We would like to thank Chester Sands for his morphological identifications. This is Molette Biology Laboratory contribution XX and Auburn University Marine Biology Program contribution XXXX.

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Figure 1. Phylogenetic relationships recovered by Maximum Likelihood analyses utilizing both nucleotide and amino acid alignments. Support values not shown have 100% bootstrap support for both analyses. Nucleotide support values are on the left, amino acid support values are in the middle and Bayesian inference support is on the right. Colored circles show the corresponding gene arrangement of the 13 protein coding and 2 ribosomal RNA genes. Gene order of nodes was estimated using TreeREx, with "I" representing an inversion and "T" representing a transposition. Genes on different lines are encoded by different strands. The "SA" and "SO" following *Astrotoma agassizii* represents South American and Southern Ocean origination of the sample.

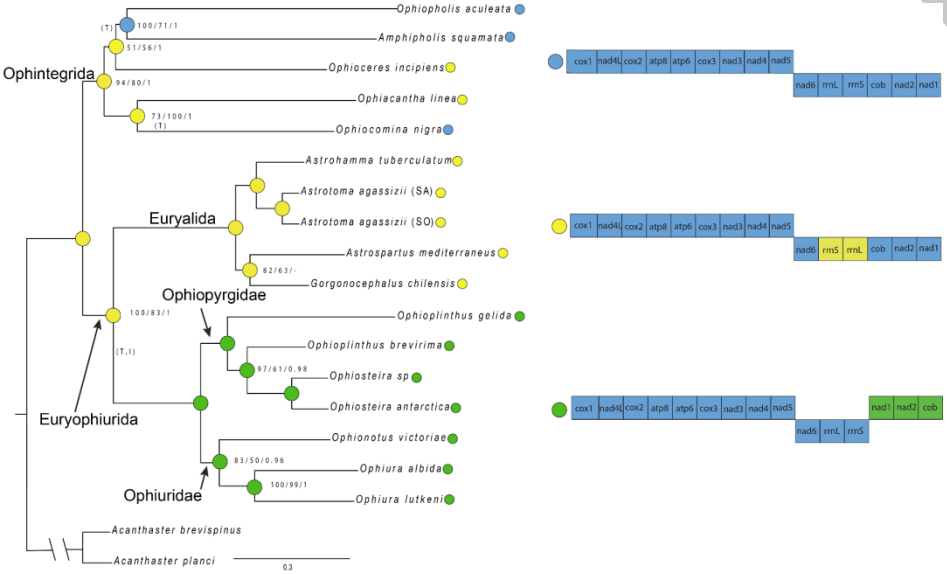

Figure 2. Gene order of all 37 mitochondrial genes for all 17 specimens. Gene orders with more than one specimen are noted above the order. Underlined genes signify their location on the minor strand.

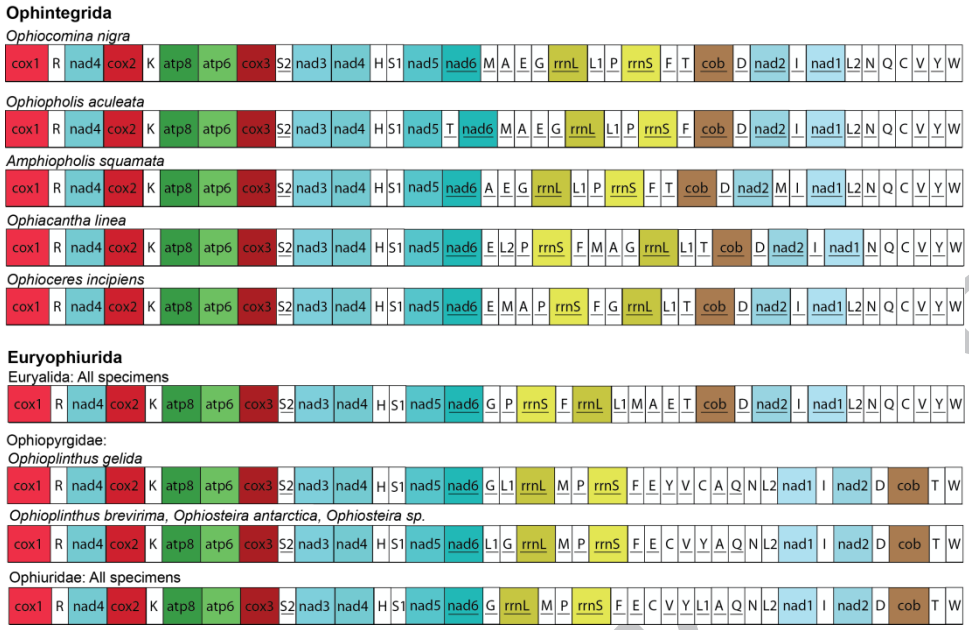

Table 1. Taxa employed, GenBank accession number, and collection information (depth, and coordinates of novel samples). The "SA" and "SO" following *Astrotoma agassizii* represents South American and Southern Ocean origination of the sample.

Taxonomy	**Family**	**Species**	**mtDNA genome**	**Depth (m)**	**Latitude**	**Longitude**
Ophiurida	Amphiuridae	*Amphipholis squamata*	NC_013876			
Euryalida	Gorgonocephalidae	*Astrohamma tuberculatum*	MH671876	612	72°12.25 S	103°35.78 W
		Astrospartus mediterraneus	NC_103878			
		Astrotoma agassizii (SA)	MH671877	854	53°47 S	49°33 W
		Astrotoma agassizii (SO)	MH671878	457	76°28.76 S	165°44.26 W
		Gorgonocephalus chilensis	MH671879	664	64°24.67 S	61°57.79 W
Ophiurida	Ophiacanthidae	*Ophiacantha linea*	NC_023254			
	Ophiolepididae	*Ophioceres incipiens*	MH671880	277	63°23.05 S	60°03.40 W
	Ophiocomidae	*Ophiocomina nigra*	NC_013874			
	Ophiactidae	*Ophiopholis aculeata*	AF314589			
	Ophiopyrgidae	*Ophioplinthus brevirima*	MH671882	228	63°23.31 S	60°07.20 W
		Ophioplinthus gelida	MH671875	228	63°23.31 S	60°07.20 W
		Ophiosteira antarctica	MH671883	570	75°19.77 S	176°59.10 W
		Ophiosteira sp	MH671884	570	75°19.77 S	176°59.10 W
	Ophiuridae	*Ophionotus victoriae*	MH671881	122	67°44.42 S	69°17.37 W
		Ophiura albida	NC_010691			
		Ophiura lutkenii	AY184223			

Table 2. Genome size and nucleotide composition of assembled Ophiuroidea mitochondrial genomes. For species included in this study, we have provided the number of sequences that passed filtering and the mean quality score for those sequences.

Species	Length	GC%	Number of Sequences*	Mean Coverage
Amphiopholis squamata	16,907	33.25		
Astrohamma tuberculatum	16,438	26.34	25,078,450	67.6
Astrospartus mediterraneus	16,238	28.76		
Astrotoma agassizii (SA)	16,464	28.10	291,477,874	2096.7
Astrotoma agassizii (SO)	16,524	29.13	392,707,946	36.4
Gorgonocephalus chilensis	16,361	27.93	37,074,420	68.4
Ophiacantha linea	15,845	31.43		
Ophioceres incipiens	18,107	39.49	22,594,598	45.9
Ophiocomina nigra	17,383	39.42		
Ophionotus victoriae	15,932	33.70	76,927,024	34.7
Ophiopholis aculeata	16,472	36.35		
Ophioplinthus brevirima	15,967	31.57	25,940,978	91.8
Ophioplinthus gelida	18,387	34.01	43,117,214	323.4
Ophiosteira antarctica	16,979	30.63	35,292,608	80.6
Ophiosteira sp	16,664	31.12	44,273,144	148.3
Ophiura albida	16,580	31.51		
Ophiura lutkenii	17,329	34.13		

* Mean quality score greater than 34 in Illumina's quality score for all runs.

- 17 mtDNA genomes spanning Ophiuroidea used to explore phylogeny and gene order evolution.
- mtDNA data support monophyletic Ophintegrida and Euryophiurida.
- Only 3 mtDNA gene order arrangements observed and all sampled Euryalida with same order.
- Euryalid gene order likely ancestral for Ophiuroidea.