The queen conch mitogenome: intra- and interspecific mitogenomic variability in Strombidae and phylogenetic considerations within the Hypsogastropoda

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*Aliger gigas* is an economically important and vulnerable marine species. We present a new mitogenome of *A. gigas* from the Mexican Caribbean and use the eight publicly available Strombidae mitogenomes to analyze intra- and interspecific variation. We present the most complete phylogenomic understanding of Hypsogastropoda to date (17 superfamilies, 39 families, 85 genera, 109 species) to revisit the phylogenetic position of the Stromboidea and evaluate divergence times throughout the phylogeny. The *A. gigas* mitogenome comprises 15,460 bp including 13 PCGs, 22 tRNAs, and two rRNAs. Nucleotide diversity suggested divergence between the Mexican and Colombian lineages of *A. gigas*. Interspecific divergence showed high differentiation among Strombidae species and demonstrated a close relationship between *A. gigas* and *Strombus pugilis*, between *Lambis lambis* and *Harpago chiragra*, and among *Tridentarius dentatus*/Laevistrombus canarium*/Ministrombus variabilis*. At the intraspecific level, the gene showing the highest differentiation is ATP8 and the lowest is NAD4L, whereas at the interspecific level the NAD genes show the highest variation and the COX genes the lowest. Phylogenomic analyses confirm that Stromboidea belongs in the non-Latrogastropoda clade and includes Xenophoridea. The phylogenomic position of other superfamilies, including those of previously uncertain affiliation, is also discussed. Finally, our data indicated that Stromboidea diverged into two principal clades in the early Cretaceous while Strombidae diversified in the Paleocene, and lineage diversification within *A. gigas* took place in the Pleistocene.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AA           | Amino acid  |
| ATP genes    | Genes encoding ATP synthase subunits 6 and 8 |
| Bp           | Base pairs  |
| COX genes    | Genes encoding cytochrome C oxidase subunits I-II-III |
| CytB         | Cytochrome B |
| D-loop       | Control region |

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in the world with a siphonal length up to 30 cm³, and has high ecological, esthetic, and economic value. The economic value of A. gigas resides principally in the commercial and nutritional value of its meat. This mollusk is considered as the second most important fishery resource in the Caribbean after the spiny lobster (Panulirus argus). Due to overfishing and poaching, the species is listed as a vulnerable commercial species (Appendix II, CITES, 1992; in1). Populations continue to be decimated due to overexploitation and habitat loss. Many countries have implemented management strategies at the regional level, though an international synchronization of management and conservation practices is also in play to try to recover stock populations. A. gigas has been extensively studied to better understand its biology e.g.,4,7, ecology e.g.,8,9, population genetic structure e.g.,10,11, conservation status e.g.,12,13, and phylogenetic position e.g.,14,15.

The SPF Stromboidea (Rafinesque, 1815) belongs to the Hypsogastropoda clade within the higher taxonomic group Caenogastropoda16,17. The membership, taxonomy, and relationships within Hypsgastropoda, however, have been in considerable flux in the last two decades. Bouchet and Rocroi17 considered the Hypsogastropoda to be comprised of two groups: the Littorinimorpha (including Strombidae) and the Neogastropoda (Supplementary Table S1). More recently, Bouchet et al.18 reorganized the clade Hypsogastropoda. This revision also comprises two major groups, the superorder Latrogastropoda is home to the Neogastropoda and six SPF from the Littorinimorpha as “taxa of uncertain position” (Calyptraeoidea, Cypraeoidea, Ficoidea, Tonnoidea, Xenophoroidea, and of particular interest, Stromboidea), and the Non-Latrogastropoda13 which contain other Littorinimorpha SPF. Genetic and genomic studies proposed Tonnoida SPF as an early branching lineage within the Neogastropoda e.g.,12,13,16 supporting the Bouchet et al.18 classification. The phylogenetic position of Xenophoridae has been debated as a sister clade to Stromboidea16,19 or embedded within Stromboidea15.

Mitochondrial genomes have become popular in elucidating gastropod taxonomic controversies e.g.,20,21 and have proven particularly useful in the resolution of uncertainties in the Caenogastropoda e.g.,12,13,22. Ascertaining the phylogenetic position of family Strombidae has been particularly difficult; studies have considered them as belonging to Littorinimorpha15,16,24 or to Latrogastropoda e.g.,13,21,22. Identifying the closest relatives to Stromboidea has been debated as a sister clade to Xenophoroidea, and of particular interest, Stromboidea, and the Non-Latrogastropoda13 which contain other Littorinimorpha SPF. Genetic and genomic studies proposed Tonnoida SPF as an early branching lineage within the Neogastropoda clade e.g.,12,13,16.

Paleontological studies suggest the Stromboidea originated within the Cretaceous (and also Triassic or Jurassic)16,24,26. Fossils suggest that Strombidae probably diverged from Aporrhaidae in the late Cretaceous, initially with very low diversity followed by a rapid taxonomic radiation in the early Eocene27. Stromboidea became the most species-rich family of Stromboidea during the Cenozoic as it expanded its geographic range27. Many fossils are reported from the Eocene to Pliocene e.g.,24,28–30 with a possible radiation at mid-latitude areas in the early Eocene27.

Historically, population genetic studies have been based on a limited number of markers, for example microsatellites e.g.,10,31, which allow for interpretation of intraspecific population structure25. Also, some mitochondrial genes (e.g., COX1) have been extensively used for intra- or interspecific comparisons e.g.,33,34. Recently, the use of mitogenomes in investigations of intra- and interspecific variation has become the best tool available e.g.,14,32,35. It is useful to identify which of the 13 PCGs of the mitogenome have adequate polymorphisms to resolve population genetic and/or phylogenetic questions36. Within family Strombidae only one study35 has used mitogenomes to evaluate interspecific relationships (in Harpago and Lambis), and until now, no research has employed the complete mitochondrial genome to evaluate lineage diversity within Strombidae. Given the vulnerable conservation status of A. gigas, it is important to assess intraspecific variation and population divergence within the species to guide future management decisions. Our work can further identify polymorphic mitochondrial genes for focused population level studies.

We take advantage of the large number of mitogenomes in clade Hypsogastropoda available on GenBank, including the very recent Stromboidea mitogenomes published35,37, and the two mitogenomes of A. gigas to: (i) present and describe a completely annotated mitogenome of A. gigas from the Mexican Caribbean, (ii) quantify intraspecific variation between our newly generated A. gigas mitogenome with that of one from off the coast of Colombia, (iii) evaluate interspecific variation among eight Strombidae species, (iv) confirm the phylogenetic position of Stromboidea and its relationship with Xenophoridae, as well as the relationships between eight
Strombidiae species using 110 mitogenomes, and finally, (v) estimate divergence times throughout Hypsogastropoda, Stromboidea, Strombidiae and Aliger, respectively.

**Results and discussion**

**Mitogenome: structure and organization.** The complete mitochondrial genome of *Aliger gigas* from the Mexican Caribbean was sequenced, assembled, and deposited in GenBank (accession number MZ157283). The total length of the mitogenome is 15,460 bp which is consistent with other mitogenomes obtained from Strombidiae species: *Aliger gigas* 15,461 bp11, *Lambis lambis* 15,481 bp14, *Harpygochiragra* 15,460 bp14, *Tridens tenuis* 15,500 bp15, and *Laevistrombus canarium* 15,626 bp37. *Conomurex luhuanus*38 and *Strombus pugilis*15 have longer total lengths, 15,799 bp and 15,809 bp, respectively, due to the presence of a large, non-coding region (428 bp and 436 bp respectively) between the tRNA-Phe and the COX3 genes proposed as a candidate for D-loop38. No D-loop has been annotated in other Strombidiae species. *Ministombrus variabilis* presented a shorter mitogenome with 15,292 bp due to the lack of tRNA identification (only 18 resolved)35. Considering all other mitogenomes of Hypsogastropoda included in this study, only ten species (belonging to three families Conidae, Littorinidae, Cypraeidae) show annotations for D-loop.

The *A. gigas* mitogenome presented here contained 13 PCGs, two rRNAs (12S and 16S), and 22 tRNAs (Fig. 1 and Table 1). The length and gene organization of our mitogenome are similar to the first *A. gigas* mitogenome sequenced11 with the exception of the NAD5 gene. The NAD5 gene obtained in our mitogenome has 1,728 bp with a complete stop codon (TAA) which is consistent with the length and stop codon (TAA or TAG) of other Strombidiae species, while Márquez et al.11 obtained a NAD5 length of 1,753 bp with an incomplete stop codon (T–) which is not very common in Hypsogastropoda. Gene order in our mitogenome is similar to other Strombidiae species11,14,15,37,38. This is not surprising considering that gene organization is relatively stable throughout the Gastropoda. When rearrangements do occur in Gastropods, they principally occur in the tRNA12. An exception is the Vermetidae which present high levels of gene order rearrangement38. The total length of all genes in our mitogenomic sequence represents 97.8% of the length of mitogenome (equivalent to 15,117 bp with: PCGs = 11,259 bp; rRNAs = 2,367 bp; tRNAs = 1,491 bp), and all non-coding regions accounted for 343 bp. Most intergenic regions are very short (< 15 bp), but two larger intergenic regions were identified, one upstream of the COX3 gene (54 bp; between tRNA-Phe and COX3) and one downstream of the COX3 gene (41 bp; between COX3 and tRNA-Lys). Non-coding regions around the COX3 gene have been proposed as candidates for D-loop in other Gastropods40 and are characterized by AT-rich content41. High AT content (82%) was observed for the COX3 gene (54 bp; between tRNA-Phe and COX3) and one downstream of the COX3 gene (41 bp; between COX3 and tRNA-Lys). Three overlaps between adjacent genes were found in our *A. gigas* mitogenome (Table 1) as was identified for *T. dentatus*35. Other Strombidiae species have eight or six overlaps (S. pugilis and *M. variabilis* respectively35), four overlaps (*L. lambis* and *H. chiragra*14, and *L. canarium*37), or only one overlap (*C. luhuanus*38). Localization of overlaps into the mitogenome is relatively stable among Strombidiae species.

Nucleotide composition of our *A. gigas* mitogenome is AT-rich (65.8%) which is consistent with other Strombidiae species (Supplementary Table S2), and with the Hypsogastropoda species used in this study (from 60% as for *Dentropoma* sp. to 73% as for *Naticatopsis hebraeus*). The nine Strombidiae mitogenomes analyzed here show an important bias to T over A (AT skew < 0) and a small bias to G over C (GC skew > 0), though this pattern is less pronounced in *C. luhuanus* (note that *Ministombrus variabilis* has an incomplete genome; Supplementary Table S2). Including only PCGs or tRNAs, the nine Strombidiae genomes show a similar value of AT content (65–69%), while the two rRNAs have a lightly higher value (67–70%) (Supplementary Table S2). The AT skew is negative and large for the PCGs (from ~0.16 to ~0.20), but positive and less pronounced for rRNAs (~0.08) and tRNAs (~0.04). PCGs have little to no GC bias, with very low positive (GC skew < 0.03) or negative (GC skew = –0.01) values, while rRNAs and tRNAs show a disequilibrium in the use of G/C in favor of G (GC skew > 0) (Supplementary Table S2).

The heavy strand encodes for 13 PCGs, two rRNAs, and for 14 tRNAs (tRNA-Asp, tRNA-Val, tRNA-Leu1, tRNA-Leu2, tRNA-Pro, tRNA-Ser(TGA), tRNA-His, tRNA-Phe, KARNI complex, and tRNA-Ser(GCT)). The light strand encodes for eight tRNAs, the MYCWQGE complex and tRNA-Thr (which can be located on either strand depending on the species15). This genic organization between both strands is constant among Strombidiae species. Both strands on our mitogenome are AT-rich (heavy strand with 65.5% and light strand with 67.5%), but the heavy strand shows an important use of T over A (AT skew < 0) and a small bias to G over C (GC skew > 0), though this pattern is less pronounced in *C. luhuanus* (note that *Ministombrus variabilis* has an incomplete genome; Supplementary Table S2). Including only PCGs or tRNAs, the nine Strombidiae genomes show a similar value of AT content (65–69%), while the two rRNAs have a lightly higher value (67–70%) (Supplementary Table S2). The AT skew is negative and large for the PCGs (from ~0.16 to ~0.20), but positive and less pronounced for rRNAs (~0.08) and tRNAs (~0.04). PCGs have little to no GC bias, with very low positive (GC skew < 0.03) or negative (GC skew = –0.01) values, while rRNAs and tRNAs show a disequilibrium in the use of G/C in favor of G (GC skew > 0) (Supplementary Table S2).

The start and stop codons of the PCGs show variation among Strombidiae species (Supplementary Table S3). For our *A. gigas* mitogenome, 12 of the 13 PCGs initiate with ATG (NAD4 starts with GTG) which is the most common start codon in Strombidiae, and in gastropods in general12,14,15,22,38,44. The PCGs that present the highest start codon variation among Strombidiae are NAD4 followed by NAD2, NAD4L, CytB, and ATP6, which have been reported as variable in other species of gastropods (review in35). For our *A. gigas* mitogenome, the majority of PCGs end with a TAA stop codon and four ends with TAG (NAD1-NAD6-NAD4L-NAD3). These stop codons are the most common in gastropods12,14,15,22,38,44. Considering the Strombidiae species analyzed here, nine PCGs show variation among species (Supplementary Table S3).
The use of synonymous codons in the 13 PCGs varies among Strombidae species (Supplementary Fig. S1) and between the two A. gigas mitogenomes. Such a pattern could be considered non-random because some codons are used more than others\(^\text{13}\). The five most frequently used codons (with larger RSCU values) for L. lambis, H. chiragra, C. luhuanus, L. canarium, M. variabilis, and T. dentatus are Leu\(^2\) (UUA), Ser\(^2\) (UCU), Arg (CGA), Ala (GCU), and Pro (CCU) (codon order is species dependent) as reported for other gastropods\(^\text{13}\), while Strombus pugilis has Thr (ACU) instead of Arg (CGA). The two mitogenomes of Aliger gigas, however, present two different codons in the top 5 frequently used codons: Thr (ACU) and Val (GUU) (order is specimen dependent) instead of Arg and Pro. Leu (UUA), Ser (UCU), and Ala (GCU) are the most frequently used codons reported for gastropods\(^\text{13,45,46}\). The codons least frequently used (lower RSCU values) for both A. gigas specimens were Ser\(^2\) (UCG) and Thr (ACG), whereas the other Strombidae species presented variation: L. lambis [Arg (CGC) and Ser\(^2\) (UCG)], H. chiragra [Ala (GCG) and Leu\(^1\) (CUG)], C. luhuanus [Ala (GCG) and Thr (ACG)], T. dentatus [Leu\(^1\) (CUG) and Pro (CCG)], S. pugilis [Ala (GCG) and Arg (CGG)], and L. canarium [Ser\(^2\) (UCG) and Leu\(^1\) (CUG)]. Generally, these codons are reported less frequently in other gastropods as well\(^\text{13,45,46}\) except for Thr (ACG). Ministrombus variabilis is unique with Ala (GCG) and stop (UAG) codons as less frequently

**Figure 1.** Mitochondrial genome map of Aliger gigas (GenBank MZ157283). All 37 genes are represented outside of the circle (direction 5'→3') and to the inside (direction 3'→5') in order and relative size and including non-coding regions. Protein coding genes (blue), transfer RNAs (green) are identified using the three letters corresponding to their amino acid, and ribosomal RNAs (orange) are presented. Photo by: HBahena/ECOSUR.
used. As shown previously in other gastropods45,46, the codons rich in A and T are used more frequently in all mitogenomes analyzed here than codons with C or G content, and codons with A or T at the third position are even more utilized (RSCU from 0.89 to 2.62) than those with C or G (RSCU from 0.07 to 0.98).

### Table 1. Mitogenome profile of A. gigas. The 13 Protein coding genes (PCGs) in blue, the 22 transfer RNA (tRNA) in green, and the two ribosomal RNA (rRNA) in red. All tRNAs have the three and one letter code, numbers in brackets in length column represent the number of nucleotides separating two genes (+) or overlapping two genes (−) downstream from the gene where it is indicated.

| Name     | Complete name                          | Start | End  | Length (bp) | Strand | Anticodon | Start codon | End codon |
|----------|----------------------------------------|-------|------|-------------|--------|-----------|-------------|-----------|
| COX1     | Cytochrome C oxidase subunit I         | 1     | 1536 | 1536 (+19)  | +      | ATG (M)   | TAA         |
| COX2     | Cytochrome C oxidase subunit II        | 1556  | 2242 | 687 (-1)    | +      | ATG (M)   | TAA         |
| tRNA-Asp (D) | Aspartic acid transfer RNA             | 2241  | 2308 | 68          | +      | GTC       |
| ATP8     | ATP synthase subunit 8                 | 2309  | 2467 | 159 (+3)    | +      | ATG (M)   | TAA         |
| ATP6     | ATP synthase subunit 6                 | 2471  | 3166 | 696 (+35)   | +      | ATG (M)   | TAA         |
| tRNA-Met (M) | Methionine transfer RNA              | 3202  | 3269 | 68 (+14)    | -      | CAT       |
| tRNA-Tyr (Y) | Tyrosine transfer RNA          | 3284  | 3349 | 66 (+2)     | -      | GTA       |
| tRNA-Cys (C) | Cysteine transfer RNA           | 3352  | 3416 | 65 (+1)     | -      | GCA       |
| tRNA-Trp (W) | Tryptophane transfer RNA           | 3418  | 3484 | 67 (+3)     | -      | TCA       |
| tRNA-Gln (Q) | Glutamine transfer RNA         | 3486  | 3547 | 62 (+13)    | -      | TTG       |
| tRNA-Gly (G) | Glycine transfer RNA           | 3561  | 3627 | 67 (+2)     | -      | TCC       |
| tRNA-Glu (E) | Glutamic acid transfer RNA       | 3630  | 3698 | 69 (+1)     | -      | TTC       |
| 12S rRNA | 12S ribosomal RNA                   | 3700  | 4682 | 983 (+)     | +      |           |
| tRNA-Val (V) | Valine transfer RNA             | 4683  | 4749 | 67 (+)      | +      | TAC       |
| 16S rRNA | 16S ribosomal RNA                   | 4750  | 6133 | 1384 (+)    | +      |           |
| tRNA-Leu1 (L) | Leucine transfer RNA          | 6134  | 6202 | 69 (+1)     | +      | TAG       |
| tRNA-Leu2 (L) | Leucine transfer RNA          | 6204  | 6272 | 69 (+1)     | +      | TAA       |
| NAD1     | NADH dehydrogenase subunit 1        | 6274  | 7215 | 942 (+7)    | +      | ATG (M)   | TAG         |
| tRNA-Pro (P) | Proline transfer RNA           | 7223  | 7290 | 68 (+4)     | +      | TGG       |
| NAD6     | NADH dehydrogenase subunit 6       | 7295  | 8001 | 507 (+14)   | +      | ATG (M)   | TAA         |
| Cyb      | Cytochrome b                        | 7816  | 8955 | 1140 (+9)   | +      | ATG (M)   | TAA         |
| tRNA-Ser (S) | Serine transfer RNA          | 8965  | 9030 | 66 (+22)    | +      | TGA       |
| tRNA-Thr (T) | Threonine transfer RNA        | 9056  | 9124 | 69 (+9)     | -      | TGT       |
| NAD4L    | NADH dehydrogenase subunit 4L       | 9134  | 9430 | 297 (+7)    | +      | ATG (M)   | TAA         |
| NAD4     | NADH dehydrogenase subunit 4        | 9424  | 10797| 1374 (+9)   | +      | GTG (M)   | TAA         |
| tRNA-His (H) | Histidine transfer RNA       | 10807 | 10875| 69 (+)     | +      | GTG       |
| NAD5     | NADH dehydrogenase subunit 5       | 10876 | 12603| 1728 (+25)  | +      | ATG (M)   | TAA         |
| tRNA-Phe (F) | Phenylalanine transfer RNA      | 12629 | 12696| 68 (+54)    | +      | GAA       |
| COX3     | Cytochrome C oxidase subunit III    | 12751 | 13530| 780 (+41)   | +      | ATG (M)   | TAA         |
| tRNA-Lys (K) | Lysine transfer RNA           | 13572 | 13640| 69 (+33)    | +      | TTT       |
| tRNA-Ala (A) | Alanine transfer RNA           | 13672 | 13743| 70 (+13)    | +      | TGC       |
| tRNA-Arg (R) | Arginine transfer RNA         | 13757 | 13825| 69 (+14)    | +      | TCG       |
| tRNA-Asn (N) | Asparagine transfer RNA       | 13840 | 13908| 69 (+1)     | +      | GAT       |
| tRNA-Ile (I) | Isoleucine transfer RNA       | 13910 | 13978| 69 (+1)     | +      |           |
| NAD3     | NADH dehydrogenase subunit 3       | 13980 | 14333| 354 (+2)    | +      | ATG (M)   | TAA         |
| tRNA-Ser (S) | Serine transfer RNA           | 14332 | 14399| 68 (+)     | +      | GCT       |
| NAD2     | NADH dehydrogenase subunit 2       | 14400 | 15458| 1059 (+2)   | +      | ATG (M)   | TAA         |

**Intra- and interspecific variation in Strombidae.** The majority (82%; Supplementary Table S4) of SNPs among both A. gigas mitogenomes occur in the PCGs, which is similar to the number of SNPs observed at the intraspecific level in other marine species. A similar pattern emerges at the interspecific level, 80% of
SNPs occur in the PCGs among the eight Strombidae species. Few indels were identified for A. gigas, a pattern that has been previously demonstrated in other marine species32,47. Indels are more numerous at the interspecific level; but, the number of indel events remain very low in the PCGs which is further consistent with a previous study that suggests that indels largely decrease in coding regions49. All indels at the interspecific level are registered in the C. luhuanus mitogenome and are at the beginning or at the end of genes suggesting a possible bias in its annotation procedures.

At the nucleotide level, both A. gigas mitogenomes present high levels of divergence with a nucleotide diversity of 0.0074 considering the whole mitogenome (0.0084 for the PCGs concatenated; Supplementary Table S4). This level of divergence suggests genetic isolation between the Mexican and Colombian lineages of A. gigas. The wide geographic distribution of A. gigas could permit these levels of genetic divergence through population fragmentation and/or adaptation to local environments as previously suggested in an oyster mitogenome analysis (τ = 0.0068)32. Also, Galván-Tirado et al.48 identified similar genomic divergence between two individuals of A. gigas where darker color represent the lowest values of nucleotide divergence suggesting a closer relationship between species. Aliger gigas (A. gigas), Strombus pugilis (S. pugilis), Conomurex luhuanus (C. luhuanus), Lambis lambis (L. lambis), Harpago chiragra (H. chiragra), Tridentarius dentatus (T. dentatus), Ministrombus variabilis (M. variabilis), and Laevistrombus canarium (L. canarium).

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Table 2. Nucleotide divergence (k) for pairs of Strombidae species used in this study using complete mitogenomes (above diagonal, blue color) and the 13 PCGs (below diagonal, orange color). Colors broadly correspond to values of k, where darker color represent the lowest values of nucleotide divergence suggesting a closer relationship between species. (previously St. gigas (A. gigas), Strombus pugilis (S. pugilis), Conomurex luhuanus (C. luhuanus), Lambis lambis (L. lambis), Harpago chiragra (H. chiragra), Tridentarius dentatus (T. dentatus), Ministrombus variabilis (M. variabilis), and Laevistrombus canarium (L. canarium).

| Species | A. gigas | S. pugilis | C. luhuanus | L. lambis | H. chiragra | T. dentatus | M. variabilis | L. canarium |
|---------|----------|-----------|-------------|-----------|-------------|-------------|--------------|------------|
| A. gigas | 0.1577   | 0.1846    | 0.1853      | 0.1834    | 0.1793      | 0.1814      | 0.1739       |            |
| S. pugilis | 0.1675   | 0.1875    | 0.1801      | 0.1809    | 0.1801      | 0.1820      | 0.1770       |            |
| C. luhuanus | 0.1959   | 0.1953    | 0.1766      | 0.1715    | 0.1712      | 0.1731      | 0.1716       |            |
| L. lambis | 0.1920   | 0.1870    | 0.1871      | 0.1536    | 0.1705      | 0.1709      | 0.1628       |            |
| H. chiragra | 0.1933   | 0.1893    | 0.1829      | 0.1655    | 0.1665      | 0.1703      | 0.1603       |            |
| T. dentatus | 0.1868   | 0.1862    | 0.1820      | 0.1786    | 0.1753      | 0.1322      | 0.1214       |            |
| M. variabilis | 0.1871   | 0.1891    | 0.1838      | 0.1793    | 0.1789      | 0.1448      | 0.1180       |            |
| L. canarium | 0.1762   | 0.1801    | 0.1801      | 0.1691    | 0.1665      | 0.1304      | 0.1292       |            |

The intraspecific diversity at the PCG level (Fig. 2-A1) shows that ATP8 exhibits the highest variation between the two mitogenomes of A. gigas followed by COX3, NAD4, CytB, NAD2, and NAD6. NAD4L shows the lowest genetic variation. These results help evaluate which PCGs are best to resolve population genetic issues in the Strombidae. For example, COX1, which present an intermediate value of genetic variation (Fig. 2-A1), has been previously used to resolve population genetic structure in A. gigas34 but our data suggest that this gene is probably not the best to resolve such population level questions. Furthermore, the proportion of nonsynonymous substitutions (changes in nucleotides that lead to a change in AA) for the PCGs at the intraspecific level ranges from 0% (COX2, APT6, NAD6, NAD3, NAD4L) to almost 2% (ATP8) (Fig. 2-A2). These low values are consistently not the best to resolve such population level questions. Furthermore, the proportion of nonsynonymous substitutions for the PCGs at the intraspecific level are higher (>50%) for some genes from the NAD complex (NAD2, NAD6) and for ATP8 (Fig. 2-B2) and very low for COX1 gene. Such changes to AA in a particular gene can be informative about the impact of purifying or positive selection12. As shown for gastropods and other marine species, genes with higher AA substitutions, as seen here in the NAD complex/ATP8, are subjected to stronger positive selection32,47.
Phylogeny. Our genomic study highlights that the large Hypsogastropoda clade is a very complex taxonomic group for which many taxonomic representatives need to be included to elucidate relationships. We present the most complete phylogenomic understanding of the Hypsogastropoda to date including 17 superfamilies, 39 families, 85 genera, 109 species and 110 individuals (Fig. 3). Overall, relationships across the tree are very well supported.

SPFs of uncertain position. The increased sampling across the Hypsogastropoda allows us to provide some resolution for various taxonomic uncertainties. The Hypsogastropoda clade was initially divided into two major groups, Littorinimorpha and Neogastropoda17. Hypsogastropoda was recently revised by Bouchet et al.18 who suggested, instead, that the superorder Latrogastropoda included all Neogastropoda and six SPFs from Littorinimorpha (Calyptraeoidea, Cypraeoidea, Ficoidea, Stromboidea, Tonnoidea, and Xenophoroidea) of "uncertain position". All other Littorinimorpha SPFs were regrouped as Non-Latrogastropoda13. Of the six SPFs that Bouchet et al.18 moved from Littorinimorpha to Latrogastropoda but couldn't otherwise place, five have complete mitogenomes in GenBank (Cypraeoidea, Ficoidea, Stromboidea, Tonnoidea, and Xenophoroidea). The sixth SPF, Calyptraeoidea, has one incomplete mitogenome available (Calyptraea chinensis 8,530 pb, EU8271932). We decided not to include this mitogenome in our phylogeny as only five of the 13 PCGs were identified. Our results confirm, although with moderate support, that Stromboidea and Xenophoroidea SPFs belong to the Littorinimorpha as proposed originally17. Such a relationship has been supported previously by genetic, genomic, and morphological studies15,16,19,25,26,53. Other investigations have suggested instead that Stromboidea is sister to Cypraeoidea12,55 but with low support, or to Tonnoidea14,23. Alternative topologies clustering Stromboidea with SPF other than Xenophoroidea are probably due to the absence of representative Xenophoridae, highlighting the importance to include as many SPFs as possible in phylogenetic analyses.

Considering the Stromboidea clade, our results strongly support a monophyletic lineage including the Xenophoridae family (unique living family of Xenophoroidea and represented here by Xenophora and Onustus) confirming recent genomic work15 and, previous behavioral and morphological studies (reviewed in15). The well-supported Stromboidea clade is divided into two clades. Clade A suggests that members of family Xenophoridae are sister to representatives of Aporrhaidae + Struthiolariidae. Although this topology is not known to be supported by morphology15 such a topology has been similarly recovered15. Clade B is comprised of three groups, with one representative of Seraphidae (Terebellum) resolved as sister to members of Rostellariidae (Varicospira) + Strombidae. Recent morphological work15 supports these major clade assignments (Aporrhaidae + Struthiolariidae separate from the Seraphidae + Rostellariidae + Strombidae). Within Strombidae (in orange in Fig. 3), we obtained a topology identical to Irwin et al.15, though we further clarify the placement of one additional genus (Tridentarius). We identify two principal clades: Clade B1 strongly confirms the sister
Figure 3. Maximum likelihood phylogenetic tree based on the concatenated nucleotide alignment of the 13 PCGs of 110 Hypogastropoda mitogenomes. Numbers above branches indicate bootstrap values (branches without a number have a bootstrap of 100). Branch colors follow the classification of Bouchet & Rocroi (2005): Littorinimorpha (blue), Neogastropoda (red), outside both clades (black). Clades proposed by Bouchet et al. (2017) are highlighted: Latrogastropoda (red), Latrogastropoda “uncertain position” (dark blue), Non-Latrogastropoda (light blue). Names of SPF's labeled on branches, and species names are organized and colored by family. The # symbol signals specimen of *A. gigas* sequenced in this study. Three Heterobranchia species were used as outgroups (*Pupa strigosa*, *Aplysia californica*, and *Tyrannodoris europaea*). See Table S1 for sources.
relationship between *Strombus* and *Aliger* (*Aligerina* and *Strombina* clades in Fig. 3), and clade B2 is comprised of six genera. *Conomurex* is the earliest diverging lineage of this clade and sister to two clades (*Harpago + Lambis* and (*Tridentarius + (Laevistroumbus + Ministrombus*)). The clade formed by *L. lambis* and *H. chiragra* *(syn. L. chiragrata)* is determined by mitogenomes and is largely accepted in previous studies. Clade B1 and B2 represent biogeographically structured clades as previously noted. An Eastern Pacific/Atlantic clade to which *Aliger* *(syn. Strombus) gigas* and *Strombus pugilis* belong (clade B1), and an Indo-West Pacific clade with *Lambis, Harpago, Conomurex, Tridentarius, Laevistrongibobus* and *Ministrombus* (clade B2).

Though we clarify some relationships in Strombidae, as outlined above, and as confirmed by our measures of interspecific variation (Table 2), phylogenetic relationships between strombid genera remain controversial. For example, morphological and genetic studies in the two most species-rich genera, *Lambis* and *Strombus*, suggest different patterns. *Lambis* has been proposed as monophyletic in previous work nested within a paraphyletic *Strombus* while an older morphological study proposed *Lambis* as paraphyletic and *Strombus* as polyphyletic. The topology inside Strombidae obtained using mitogenomes (this study and14,15) has not been supported previously by morphology or genetic studies. Future work should aim to use mitogenomes with increased species sampling and including nuclear genes to explore relationships among these species rich and difficult to resolve genera.

The phylogenetic affinity of SPF *Cypreoida* remains unresolved in our study with respect to its membership in Littorinimorpha or Neogastropoda. Thus, we offer no resolution to its historically uncertain phylogenetic position in the Hypsogastropoda. Our genomic study resolved the phylogenetic position of *Tonnoidea* and *Ficoidea* SPF. The placement of *Tonnoidea* as an early branching lineage within the Neogastropoda clade is largely confirmed. The inclusion of the mitogenome of *Ficoidea* SPF *(Ficus variegatus)* confirms that *Tonnoidea* is sister to *Ficoidea* as recently suggested. Though some studies have considered *Tonnoidea* as the sister clade to the *Cancellariidae* (represented by *Bivetiella cancellata*;13,59), our results do not confirm this relationship. Further clarification of the position of *Ficoidea* within *Tonnoidea* highlights that *Raneliulidae* (now *Charoniidae* *(Charoniisp.))* and *Cymatiidae* *(Monoplex sp.*) is paraphyletic and supports previous studies. Our work confirms the ultimate *Tonnoidea* relationships based on genetic data where *Charoniidae* (here represented by *Charonia lampas*) are sister to *Cassidae* (here represented by *Galeodes echinophora*), which in turn are sister to *Bursidae* (here represented by *Buconaria rana* and *Lampasopsis rhodostoma*). This clade is in turn sister to *Cymatiidae* (here represented by *Monoplex parthenopeus*).

Non-Latrogastropoda. Within the Non-Latrogastropoda, the addition of the *Epitonium scalare* mitogenome suggests SPF *Epitonioidea* as sister to *Abyssochrysoidea* with high support. The clade (Epitonioidea + Abyssochrysoidea) is further resolved as sister to three Littorinimorpha SPF (Littorinoida, Naticoidea, and *Truncatellina*). SPF *Abyssochrysoidea* has been previously reported as sister to the majority of Littorinimorpha12,13. Foerdrilis et al.13 suggested that the relationship between the gene order rearrangement rate and the nuclear genome evolution has been associated with higher rates of nucleotide substitution and is observed as long-branches in phylogenetic trees.12,13

The SPF *Vermetoidea*, placed as sister clad to the rest of Caenogastropoda, is well-supported in our tree and has been largely demonstrated elsewhere e.g.,12,13,15,59. However, other phylogenetic work has suggested that *Vermetoidea* should be sister clad to a clad formed by the subclasses *Caenogastropoda* + *Neritimorpha* + *Vetigastropoda*, or that *Vermetoidea* is sister clad to *Abyssochrysoidea*.12 Mitochondrial gene rearrangement could explain the controversial phylogenetic position of *Vermetoidea*.15 Gene rearrangement has been associated with higher rates of nucleotide substitution and is observed as long-branches in phylogenetic trees.12,13,15,59. Foudi et al. suggested that the relationship between the gene order rearrangement rate and the nucleotide substitution rate might not apply for all Caenogastropoda. Mitochondrial gene order rearrangement is very common in many taxonomic groups but very few studies go on to explain the possible biological reasons for extensive rearrangement in mitochondrial genes. Lockridge and Boore suggested that selection at the organismal level might select for mitochondrial gene rearrangement. Vermetoidea represent a unique lifestyle and have long-branches in all phylogenetic studies. Vermetoidea present a unique life-style when compared to other Caenogastropoda considering that they are one of only two lineages that cement their shell directly to hard substrates and live a sessile life, the other group is the freshwater *Helicostoma*. We hypothesize that adaptive selection on the Vermetoidea lifestyle acted at both the organismal and cellular levels. More mitogenomic data as well as structural and functional genomic studies related to nuclear DNA will be necessary to understand the biological implications of mitochondrial gene rearrangement and to further clarify the phylogenetic position of the Vermetoidea.

**Latrogastropoda.** The backbone of the Latrogastropoda clade (highlighted in red on Fig. 3) places one species of Volutoidea, *Bivetiella cancellata*, as sister to the remainder of the group and subsequently identifies *Volutoidea* as not monophyletic. Furthermore, our genomic study confirms the inclusion of Ficoidea and Tonnoidea SPF in Latrogastropoda as suggested previously. *Bivetiella cancellata* (Cancellariidae, SPF Volutoidea) as sister to the rest of the clade has been proposed before (12,13), though sometimes in association with other SPF, for example with Calyptraeoida or Tonnoidea. Previously classified in Cancellarioidea SPF, the Cancellariidae was recently incorporated into Volutoidea SPF based on a recent phylogenetic analysis and used in the classification of Bouchet et al.14. Phylogenetic analyses suggested that Cancellarioidea SPF should be considered as separate SPF as also supported by our study. Turbinelloidea is unresolved in our phylogenomic tree though it was considered as sis-
ter to a clade formed by Olivoidea + Muricoidea + Babyloniidae (unassigned SPF) + Buccinoidea + Conoidea. Babyloniidae, an unassigned SPF, is sister to SPF Olivoidea with moderate support as previously proposed, though its relationship to other SPFs is uncertain on our tree. Yang et al. proposed Babyloniidae as sister to the Buccinoidea but didn’t include Olivoidea in their study. We increased the number of species of Buccinoidea (n = 16) included in a phylogenomic framework and we included two additional families (Melongenidae and Fasciolariidae) in our analysis. Buccinoidea families with more than one representative are monophyletic in our reconstruction, though phylogenetic relationships between several families is uncertain. Family Columbellidae (represented by Columbella adansoni) is supported as the earliest diverging lineage in the SPF as previously suggested, Melongenidae (Hemi fusus sp.) presents an unresolved situation in the clade, and Fasciolariidae (Fusinus sp.) is confirmed as sister clade to Buccinidae. Sister to the Buccinoidea is SPF Conoidea. Our tree considers a high number of specimens (n = 11; eight species and four families) representing SPF Conoidea. Our reconstruction highlights a complex situation for this SPF as recently suggested. Two distinct clades are observed and correspond to those previously identified; clade A includes three paralytic families (Turridae, Clavatulidae, and Pseudomelomitidae) as well as Drilliidae and Terebridae, and clade B includes all Conidae species that are sister to Raphitomidae (represented here by Typhlosyrinx sp.). These results emphasize and support the need to sequence more mitochondrial genomes to improve resolution within SPF Conoidea.

**Divergence times.** The reconstruction of a divergence time tree dates the Strombidae (in orange on Fig. 4) diversification to the Paleocene (63 Mya; 95% HPD: 47.6–78.0). Previous work similarly suggested that Strombidae originated at the end of the Cretaceous with a rapid diversification at early Cenozoic. In their genomic study, Jiang et al. identified the origin of Strombidae at late lower Cretaceous (112 Mya; interval: 44–206 Mya), however, Bandel suggested a much more recent origin of the Strombidae (Oligocene: 33.9–23.0 Mya). Within the Strombidae, our divergence time estimates suggest that Lambis and Harpago diverged around 33 Mya which is close to a previous estimate (~23 Mya). Jiang et al. suggested the divergence between genera to the Eocene. The earliest branching lineage of clade B2 (Fig. 3), C. luhuanus, is estimated to have diverged from the other species 55 Mya (late Eocene). Divergence between Strombus and Aliger (clade B1 in Fig. 3) was estimated at 40 Mya (Eocene) on our chronogram which coincides with the proposed pre-Miocene common ancestry between Aligerina and Strombina.

**Methods**

**Specimen collection, DNA extraction, and sequencing.** The individual of Aliger gigas used for this study comes from the Cozumel Island Protected Area of Fauna and Flora (Mexico) and was received in 2013 from the relevant authority of the park following a seizure of illegal catch. Sample tissue was preserved in 96% ethanol and maintained at 4°C until extraction. Total genomic DNA was extracted using the EZNA DNA purification kit (OMEGA Bio-Tek, Norcross, GA). DNA libraries were constructed by shearing the DNA on a BioRuptor Illumina TruSeq (Illumina, San Diego, CA) with compatible adapters and custom indices using Kapa BioSciences library preparation kits (KAPA BIOSYSTEMS, Woburn, MA). Library quality was checked, normalized, pooled, and run on an Illumina MiSeq (paired-end 250 reads, ILLUMINA, San Diego, CA) at the Georgia Genomics Facility (University of Georgia).

**Genome assembly and annotation.** The quality of sequence reads was evaluated using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and low-quality read ends (Phred score < 20) were removed manually in GENEIOUS 11.1.3 (http://www.geneious.com/). The A. gigas mitogenome was reconstructed by mapping reads to the Aliger gigas reference genome (NC024932) in GENEIOUS 11.1.3. The 13 PCGs were identified and annotated using MITOS and DOGMA, while the tRNA genes were identified using tRNAscan-SE 2.0 and ARWEN 1.2. The rRNA genes were identified and annotated by comparing the MITOS
Figure 4. Estimates of divergence time inferred from Bayesian analysis of the 13 PCGs of 110 Hypsgastropoda mitogenomes. Branch colors represent classification following Bouchet & Rocroi (2005): Littorinimorpha (blue), Neogastropoda (red), outside both clades (black). Branch lengths are proportional to time (in Mya). Node values represent posterior mean ages and green bars indicate the 95% HPD. Black asterisks indicate calibrated nodes (see methods). Outgroups have been trimmed. See Table S1 for sources.
Sequence analysis and genomic diversity. Analysis of nucleotide composition, including AT content, was assessed using GENEIOUS PRIME 2019.0.4; nucleotide skew (nucleotide bias) statistics were determined by AT skew (AT skew > 0 means A-rich and AT skew < 0 means T-rich) and GC skew (GC skew > 0 means G-rich and GC skew < 0 means C-rich). Nucleotide skew analyses were conducted for the nine Strombidae mitogenomes (Supplementary Table S1) considering the whole mitogenome, the 13 concatenated PCGs, the 22 concatenated tRNA, the two concatenated rRNA, and the heavy and light strands. The RSCU was determined using MEGA X for each of the 13 PCGs from the nine Strombidae mitogenomes.

Parameters of intra- and interspecific variation within Strombidae were assessed at three levels: whole mitogenomes, the 13 concatenated PCGs, and for each of the 13 PCGs. At each level, target sequences from each species were aligned using MAFFT 7.450 and the sequences from our A. gigas mitogenome was used as the reference. The total number of SNPs, indel sites, and indel events were determined using Dnasp 6.10.03 and manually checked in GENEIOUS PRIME. The number of synonymous and nonsynonymous substitutions were identified in GENEIOUS PRIME. Finally, nucleotide diversity (π) for each species pair, and nucleotide divergence (k), were determined using Dnasp 6.10.03 considering the whole mitogenome and the 13 concatenated PCGs.

Phylogenetic inference and divergence time analyses. Phylogenetic analyses were performed with a total of 110 complete or partial mitogenomes in clade Hypsogastropoda downloaded from GenBank (up to January 2021) including our A. gigas mitogenome, representing 109 species, 85 genera, 39 families, and 17 super-families (Supplementary Table S1). Three species belonging to the Heterobranchia clade were used as outgroups: Pupa strigosa Gould, 1859 (Acteonidae), Aplysia californica J.G. Cooper, 1863 (Aplysiidae), and Tyramnodoris europaea García-Gómez, 1985 (Polyaceridae). Phylogenetic analyses were performed with nucleotide sequences using the 13 concatenated PCGs. A saturation analysis was performed in DAME 7.2.43 and no saturation was observed at the node including all Hypsogastropoda (except SPF Vermetidae). The third codon position was retained in our dataset. Nucleotide sequences were aligned in MAFFT 7.450, and ambiguously aligned positions were removed with GBLOCKS 0.91b. The best-fit models of nucleotide substitution were evaluated using jModelTest 2.1.8.5 considering the Bayesian information criteria. Two models were tested (GTR + I + G and GTR + G) and the best fit model was selected for final analyses.

Phylogenetic relationships were inferred using the ML method and conducted with RAxML 8.2.11 implemented in GENEIOUS PRIME using the GTR + G nucleotide substitution model, and rapid bootstrapping using a rapid hill-climbing algorithm. Branch support was evaluated with 1000 bootstrap replicates. The majority consensus tree was constructed considering a 25% of burn-in. Range of branch support were defined for ML tree as follows: maximal for 100%, high for ≥70%, moderate for 50–69%, and poor for <50%.

Inference of divergence times using the 113 aligned mitogenomes (including outgroup) was performed in BEAST 2.6.3 on the public web server CIPRES Science Gateway v3.3 (http://www.phylo.org/index.php/) with the input file created in BEAUti 2.6.3. The best-fit model previously determined (GTR + G) was used under the uncorrelated lognormal relaxed clock and the Yule speciation evolutionary model. The ML phylogenetic reconstruction was used as a starting tree. Three independent runs were processed with a MCMC of 20 million generations sampling every 1,000 generations with 2 million pre-burn-in. LOGCOMBINER 2.5.2 was used to combine the log and tree files from the three independent runs generated in BEAST. TRACER 1.5 was used to evaluate the convergence of chains and confirm that the values of effective sample size (ESS) were above 200 for posterior and likelihood parameters as is recommended; finally, the first 15% of trees were discarded from the combined tree file and a maximum clade credibility tree with a posterior probability limit of 0.5 was obtained using TREEANNOTATOR 2.5.2. The posterior distribution of estimated divergence times was calculated using two calibration points based on fossils. The first calibration point was under a normal prior and set for the divergence of Nassariidae based on the oldest records of Buccinulittion 51.9 + /− 4.1 Mya. The second calibration point was the oldest fossil record for the Truncatellidae clade at 66.04 Mya (www.fossilworks.org). Finally, visualization of the ML and divergence time trees was performed using FigTree 1.4.9.

Data availability

The new mitochondrial genome of Aliger gigas is available at GenBank under the accession number MZ157283.

Received: 10 February 2021; Accepted: 24 May 2021
Published online: 07 June 2021

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Acknowledgements

We are grateful to Humberto Bahena-Basave (El Colegio de la Frontera Sur-Chetumal, Mexico) for the photograph of *A. gigas* presented in Fig 1, to Miguel A. Del Río-Portilla for his valuable advice on gene annotation, and to Onesimo de Dios de la Cruz (Universidad Juárez Autónoma de Tabasco, Mexico) for aiding in the jModelTest analysis. We thank two anonymous reviewers for their helpful comments that clarified and ameliorated the manuscript. Funds for this work are acknowledged from the “Centro de Investigaciones Biológicas del Noroeste S.C.” (CIBNOR) and the “Subsistema Nacional de Recursos Genéticos Acuáticos” of the “Secretaría de Ganadería, Agricultura, Desarrollo Rural, Pesca y Alimentación” (SAGARPA) (N° GO/04/05/2012-02) of Mexico to FIGDL. Thanks to “Consejo Nacional de Ciencia y Tecnología» (Conacyt) of Mexico for financial support of a sabbatical year to SMM (2017-2018, scholarship N°655716).

Author contributions

Conceptualized the idea: F.J.G.D.L., S.M.M.; Collected the sample: J.C.M.; Data generation and mitogenomic reconstruction: S.M.M.; Data analyses: S.M.M., J.J.M.N.; Prepared figures and tables: S.M.M., J.C.M.; Phylogenetic interpretation: S.M.M., M.M.H., F.J.G.D.L.; Coordinated project funding: F.J.G.D.L.; Wrote the first draft of the manuscript: S.M.M.; Substantially revised the manuscript: M.M.H.; Synthesized results and reviewed the manuscript: S.M.M., M.M.H., F.J.G.D.L., J.J.M.N., J.C.M.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-91224-0.

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