

Hypervariability of Ascidian Mitochondrial Gene Order: Exposing the Myth of Deuterostome Organelle Genome Stability

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Abstract

The few sequenced mitochondrial (mt) genomes of the class Ascidiacea (Chordata, Tunicata), mostly belonging to congeneric species of the Phlebobranchia order, show extraordinary gene order rearrangements. In order to assess if this hypervariability in gene order is a general feature of Ascidiacea, we report here the gene arrangement of five ascidians belonging to the Aplousobranchia and Stolidobranchia orders. Our data show that Ascidiacea are characterized by: 1) extensive gene order rearrangements both within and between the three major lineages; 2) lack of significant similarities to the gene order of other deuterostomes; and 3) an extent of rearrangements comparable with that of Mollusca (especially the Gastropoda, Bivalvia, and Scaphopoda classes), a phylum with highly rearranged mtDNAs. The only conserved feature is the location of all genes on the same strand, which suggests that selective constraints are related to the mt transcription. Finally, a higher mobility of the tRNA genes is undetectable because of saturation effect, and only the partially conserved *cox2-cob* gene block seems to retain some phylogenetic signals.

Key words: gene order, mitochondrial genome, rearrangements, ascidians.

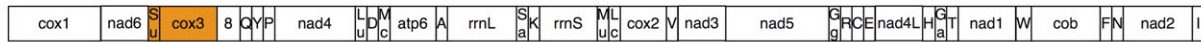
Variations of the mitochondrial (mt) gene order in Metazoa are generally considered rare events not following a clocklike behavior. Indeed, rearrangements are infrequent, rarely subjected to reversion or convergent evolution (Boore 1999; Rokas and Holland 2000; Boore 2006), and in some taxa concern mostly the tRNA genes (Boore 1999; Gissi et al. 2008). The slow pace of gene rearrangements holds especially for deuterostomes, which show few gene order rearrangements, except in Tunicata (Chordata) (Gissi et al. 2008). However, at present, the completely sequenced mtDNAs of tunicates include only one thaliacean (*Doliolum nationalis*) (Yokobori et al. 2005) and six ascidians whose taxonomic range is very narrow, as it comprises five closely related species of the Phlebobranchia order (i.e., three species of the *Ciona* genus and two species of *Phallusia*), one representative of the order Stolidobranchia (i.e., *Halocynthia roretzi*) and no species of the order Aplousobranchia (Yokobori et al. 1999, 2003; Gissi et al. 2004; Iannelli, Griggio, et al. 2007; Iannelli, Pesole, et al. 2007). This small and taxonomically biased data set indicates that no mt gene block is shared by the few tunicate mtDNAs and other deuterostomes (Iannelli, Griggio, et al. 2007) and that gene order rearrangements have occurred frequently within a clade of closely related phlebobranchian taxa. However, this data set is clearly insufficient to define: 1) the extent of gene order variability in the class

Ascidiacea, and 2) the existence of significant gene order similarity between all ascidians and remaining deuterostomes. Indeed, the current data cannot exclude the existence of ascidian lineages having a mt gene order conserved within the class and/or rather similar to that of other deuterostomes.

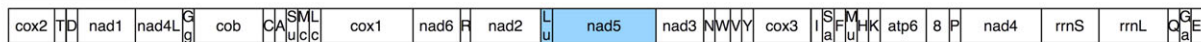
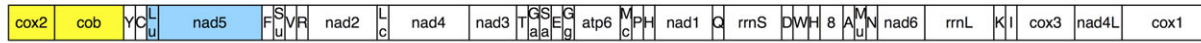
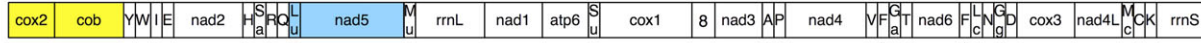
Here, we report the mt gene arrangement of three aplousobranch and two stolidobranch ascidians, and we show that the class Ascidiacea is characterized by: 1) extensive rearrangements both within and between the major lineages; 2) absence of significant gene order similarities to other deuterostomes; and 3) a degree of rearrangements comparable with that observed in Mollusca, one of the highly rearranged metazoan groups.

We have increased the ascidian data set by sequencing the entire mtDNA of five ascidians (underlined species in fig. 1, see Supplementary Material online for the amplification and annotation strategy), each belonging to a different family of the two underrepresented ascidian orders, thus obtaining a final data set consisting of at least three species for each order. In comparative analyses, ascidians have been analyzed alone or together with thaliaceans (hence named Asc + Tha), as several molecular data support a sister relationship of Thaliacea to Phlebobranchia (Swalla et al. 2000; Yokobori et al. 2005; Zeng and Swalla 2005) or to Enterogona (i.e., Phlebobranchia + Aplousobranchia) (Yokobori et al.

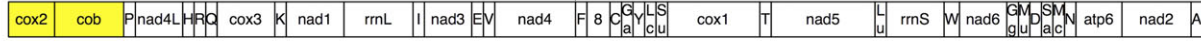
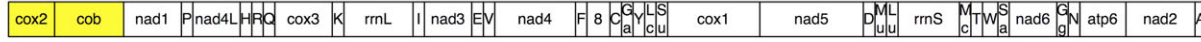
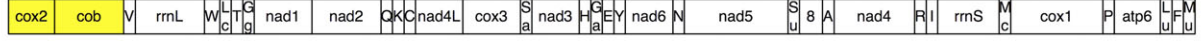
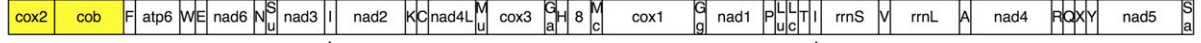
Aplousobranchia

Clavelina lepadiformis (Polycitoridae)*Diplosoma listerianum* (Didemnidae)*Aplidium conicum* (Polyclinidae)

Stolidobranchia

Styela plicata (Styelidae)*Microcosmus sulcatus* (Pyuridae)*Halocynthia roretzi* (Pyuridae)

Phlebobranchia

Ciona intestinalis spA (Cionidae)*Ciona savignyi* (Cionidae)*Phallusia mammillata* (Asciidiidae)*Phallusia fumigata* (Asciidiidae)

Thaliacea

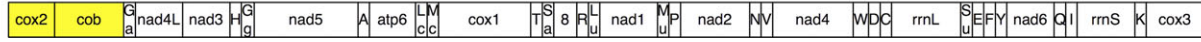
Doliolum nationalis

Fig. 1. Mitochondrial gene arrangement of 11 ascidians and 1 thaliacean, with taxonomic order and family they belong to. An arrow indicates the different position of *trnC* in *Ciona intestinalis* spB compared with spA. Underlined names indicate mtDNAs sequenced in this study. Gene block color code—yellow: conservation in most tunicates; orange: conservation within Aplousobranchia; blue: conservation within Stolidobranchia. tRNA genes in multiple copies are indicated with black and white triangles, corresponding to orthologous and paralogous genes, respectively (in *Halocynthia roretzi* the two almost identical *trnF* genes are both indicated as orthologs). All genes are transcribed from the same strand. Gene abbreviations: 8, atp6: subunits 8 and 6 of the F0 ATPase; cox1–3: cytochrome c oxidase subunits 1–3; cob: cytochrome b; nad1–6 and nad4L: NADH dehydrogenase subunits 1–6 and 4L; rrnS and rrnL: small and large subunit rRNAs. tRNA genes are indicated by the one-letter code of the transported amino acid, except for: Ga, Gly(AGR); Gg, Gly(GGN); Lu, Leu(UUR); Lc, Leu(CUN); Mc, Met(CAU); Mu, Met(UAU); Sa, Ser(AGY); and Su, Ser(UCN).

2006; Tsagkogeorga et al. 2009), with the paraphyly of Ascidiacea.

Figure 1 shows that each ascidian species has its own, unique mt gene order, and no gene block is conserved among all ascidians. Only the gene pair *cox2*–*cob* (yellow box in fig. 1) is present in 7 of the 11 ascidians (including *Ciona intestinalis* spB), that is, in all Phlebobranchia and most Stolidobranchia (except for *Styela plicata*) as well as in the thaliacean *D. nationalis*. In all these species, the *cox2* and *cob* genes have a short frame overlap ranging

from 11 to 29 bp and share only few variable amino acid positions. Moreover, these two genes are transcribed as a single mature bicistronic mRNA in *C. intestinalis* spA and in *H. roretzi* (Gissi and Pesole 2003), thus suggesting that the *cox2*–*cob* conservation is due to transcriptional constraints. Interestingly, the peculiar taxonomic distribution of the *cox2*–*cob* gene pair (yellow box in fig. 1) supports a close relationship of Thaliacea to Phlebobranchia + Pyuridae but not to Aplousobranchia, confirming the paraphyletic nature of Ascidiacea and leaving unsolved the position of

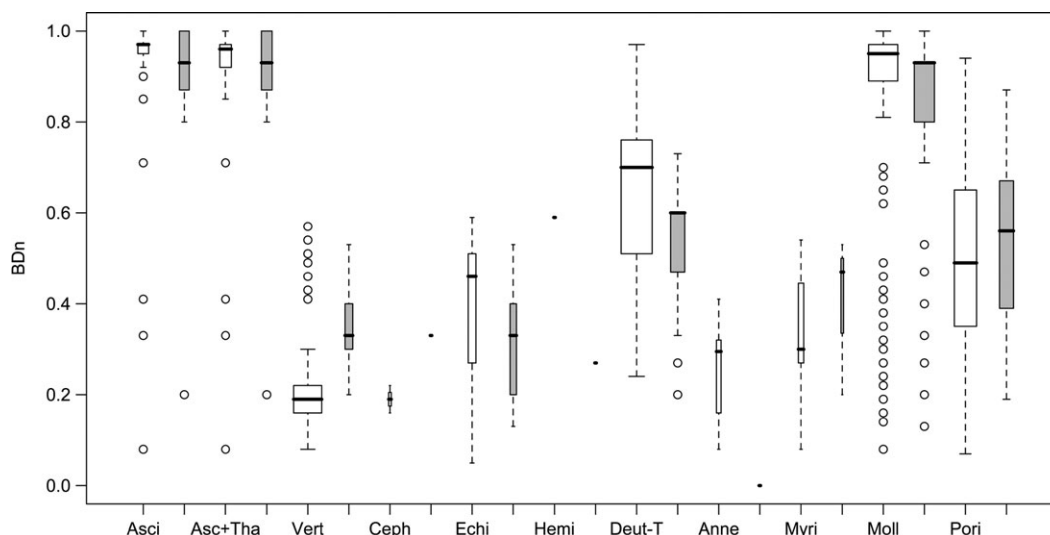


Fig. 2. Box plot of the normalized breakpoint distances (BDn) calculated for the “ALL” and “No-trn” gene data sets (in white and gray boxes, respectively). Middle line: median value; box upper and lower edges: interquartile range, including 50% of the observations; circles: outliers. Whisker lines extend for 1.5 times the interquartile range. The width of each box is proportional to the number of different gene orders of that group. The box is a single dot in groups where only one comparison is available. Asc: Ascidiacea; Asc + Tha: Ascidiacea + Thaliacea; Vert: Vertebrata; Ceph: Cephalochordata; Echi: Echinodermata; Hemi: Hemichordata; Deut-T: comparisons among major deuterostome groups, excluding Tunicata; Pori: Porifera; Anne: Annelida; Myri: Myriapoda; and Moll: Mollusca.

Thaliacea. Interestingly, because the *cox2-cob* gene pair has not been found in non-tunicate mtDNAs, it could be a derived feature of Thaliacea, Phlebobranchia, and Pyuridae. Thus, in spite of the extensive rearrangements, this conserved gene block seems to retain some phylogenetic signals and could be helpful to solve phylogenetic issues.

The gene arrangement of ascidians is highly variable even at intra-order level. Indeed, only a single gene pair is shared by all species of a given taxonomic order (see color boxes in fig. 1). Unlike *cox2-cob*, the other conserved gene pairs do not show gene overlaps; thus, there are no clues on the existence of physical constraints forcing these genes to remain adjacent. Moreover, the gene order is slightly conserved even in the family Pyuridae (Stolidobranchia), where only three small gene blocks are shared by the two species *Microcosmus sulcatus* and *H. roretzi* (i.e., *trnL(UUR)-nad5*, *cox3-nad4L*, and *cox2-cob-trnY*).

Excluding the tRNA genes, there are no additional or largest gene blocks conserved in all tunicates or in low-rank ascidian groups.

The only feature fully conserved in Tunicata consists in the location of all genes on the same strand, that is, despite the extensive rearrangements of these genomes, the transcriptional polarity is preserved for all genes. This trait could be due to strong selective constraints related to the mt-transcriptional process, such as the presence of unidirectional promoter(s) counter-selecting all rearrangements modifying the coding strand of a gene; however, the lack of experimental data on mt transcription in ascidians prevents verification of specific hypotheses.

To better evaluate the extent of ascidian rearrangements, we compared the gene order variability of Ascidiacea and

Asc + Tha with that of other metazoans, in particular to that of:

1. each major deuterostome group (i.e., phylum/subphylum/class);
2. the entire deuterostome taxon, excluding Tunicata (the Deut-T data set of fig. 2, consisting only of comparisons between the major deuterostome groups, except for Tunicata);
3. four non-deuterostome lineages (i.e., Annelida, Myriapoda, Mollusca, and Porifera) already known to have highly rearranged mtDNAs (Gissi et al. 2008);
4. several taxonomic classes belonging to the above-mentioned major metazoan lineages (this last data set allows comparisons among equivalent taxonomic levels).

The extent of gene order rearrangements within each of these taxa was quantified by calculating for each pair of different gene order the normalized breakpoint distance (BDn), which ranges from 0 to 1 (see Supplementary Material online). We analyzed two gene data sets, one including all mt genes (ALL) and the other without tRNA genes (No-trn), with the aim to verify the existence of a high mobility of the tRNA genes.

Figures 2 and 3 show the box plot of the BDn distribution for each analyzed major lineage and class, respectively: white and gray colors indicate the ALL and No-trn data sets, respectively, and the width of each box is proportional to the number of different gene orders.

The BDn distribution is almost identical for Ascidiacea and Asc + Tha in both the ALL and the No-trn data sets (fig. 2), indicating that the only available thaliacen mtDNA did not alter the distribution of BDn noticeably.

With respect to the ALL data set (white boxes in fig. 2), the BDn distribution of Ascidiacea and Asc + Tha is very

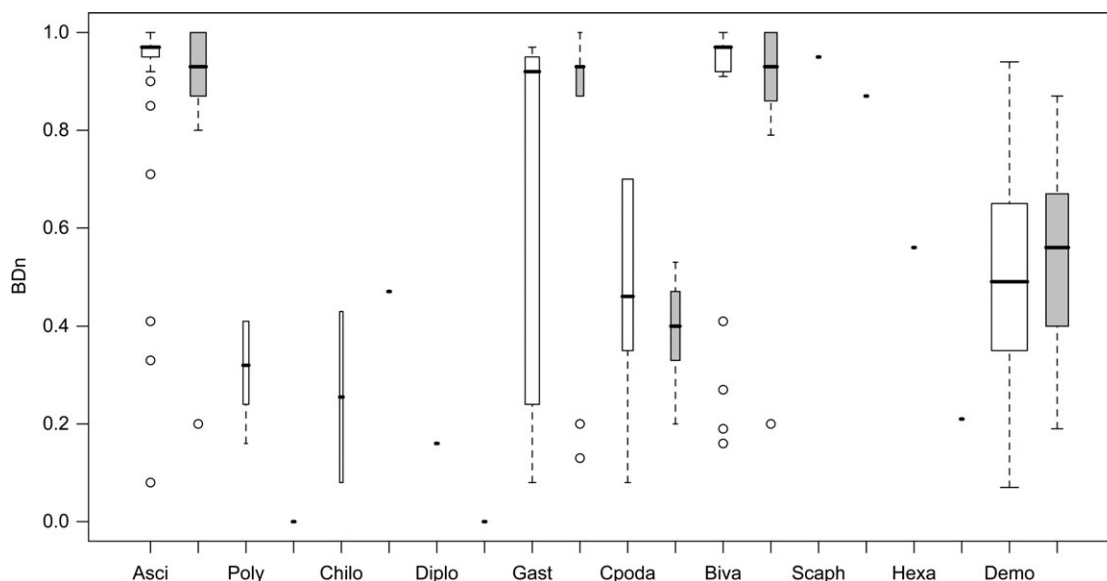


Fig. 3. Box plot of the normalized breakpoint distances (BDn) calculated for the ALL and No-trn gene data sets (in white and gray boxes, respectively) for several taxonomic classes. See legend details in figure 2. Asci: Ascidiacea; Poly: Polychaeta (Annelida); Chilo: Chilopoda (Myriapoda); Diplo: Diplopoda (Myriapoda); Gast: Gastropoda (Mollusca); Cpoda: Cephalopoda (Mollusca); Biva: Bivalvia (Mollusca); Scaph: Scaphopoda (Mollusca); Hexa: Hexactinellida (Porifera); and Demo: Demospongia (Porifera).

different in terms of both median and shape from that of other deuterostome and non-deuterostome taxa. The only taxon resembling ascidians in this context was Mollusca. In fact, in ascidians and Asc + Tha, the BDn median is almost 1, implying almost random rearrangements, and the interquartile range is very small, whereas in all remaining groups, except for Mollusca, the median is lower than 0.6, and the interquartile range is large (only in Hemichordata and Cephalochordata is the interquartile range almost null, because of the presence of only two and three different gene orders). The median and interquartile BDn values of Ascidiacea and Asc + Tha are even higher than those among the major deuterostome groups excluding Tunicata (Deut-T in fig. 2), indicating that the gene order of a given deuterostome group can be traced back to that of a different major group, whereas the gene order of a given ascidian species is almost a random permutation of that of other ascidians and even of other deuterostomes. Only the BDn distribution of the phylum Mollusca is comparable with that observed for the class Ascidiacea and for Asc + Tha, as in these taxa the median is higher than 0.95, and there are a number of outliers corresponding to congeneric pairs or to comparisons within a major lineage (i.e., intra-order pairs in ascidians; intra-class pairs in molluscs, plus some comparisons between Polyplacophora, Gastropoda, and Cephalopoda; the outlier percentage is 13% and 17% of the whole number of pairwise comparisons in Ascidiacea and Mollusca, respectively).

Figure 3 compares the BDn distribution of the class Ascidiacea only with that of other non-deuterostome classes. As already seen in figure 2, it is evident that the ascidian BDn distribution is completely different from that of any other deuterostome class (the deuterostomes shown in fig. 2 belong only to taxonomic levels higher than classes, and

all have a BDn distribution different from ascidians). Although some groups includes only few comparisons (see Supplementary Materials online), figure 3 shows that only the BDn distributions of the classes Gastropoda, Bivalvia, and Scaphopoda (Mollusca) resemble to that of Ascidiacea, as they all have a median value close to the maximum (≥ 0.92 in both the ALL and No-trn data sets). On the contrary, the shape of BDn distribution is very variable among classes and a similarity can be observed only between Ascidiacea and Bivalvia (both gene data sets) or Gastropoda (only in the No-trn data set). In conclusion, these data indicate that the gene order variability is lineage specific and not related to the taxonomic level (i.e., phylum, subphylum, or class), in fact the same taxonomic rank can exhibit very different extents of gene order rearrangements in different lineages.

Excluding the tRNA genes (gray boxes in figs. 2 and 3), only Ascidiacea and Mollusca (especially Gastropoda, Bivalvia, and Scaphopoda) retain a median BDn close to the maximum value and almost the same number of different gene order (supplementary table S3, Supplementary Materials online). In addition, in Ascidiacea and Bivalvia most outliers corresponding to congeneric pairs are lost in the No-trn compared with the ALL data set, as the gene order becomes identical within the same genus when tRNAs are excluded. In contrast, other taxa show a remarkable decrease of the median value and/or of the number of different gene orders, sometimes associated with an apparent median increase (see box width in figs. 2 and 3 and supplementary table S3, Supplementary Materials online: the increase of the median in Vertebrata, Porifera, Myriapoda, Chilopoda, and Demospongiae is due to the strong reduction of the number of different gene orders in the No-trn data set). These results indicate a high mobility of the

tRNA genes in most taxa, which is undetectable in Ascidiaceae and Bivalvia due to saturation. The saturation is supported by the observation that for most congeneric pairs the BDN decreases or becomes equal to zero in the No-trn compared with the ALL data set. Given that in the available ascidian congeneric pairs, all belonging to Phlebobranchia, the gene order is quite similar in the *Ciona* genus and very different in *Phallusia* (Gissi et al. 2004; Iannelli, Griggio, et al. 2007; Iannelli, Pesole, et al. 2007), the analysis of additional congeneric ascidian, sampled in all major groups, will help to accurately quantify the tRNA mobility and shed light on the rearrangement saturation.

In conclusion, in Ascidiaceae the mt gene order rearrangements are very extensive in all major lineages and drastically differ from the conservative rearrangements observed both within and between other deuterostome groups. Our data confirm that the trend toward stabilization of the deuterostome mt gene order is particularly evident in Vertebrata and Cephalochordata, whereas it is absent in Ascidiaceae (plus Thaliacea) and potentially also in Hemichordata, where the BDN found in the only available species pair (two enteropneusts, BDN of 0.6) suggests the presence of substantial rearrangements even in this phylum. Compared with metazoans with highly rearranged mtDNAs, the gene order variability of Ascidiaceae is extraordinary and comparable only with that of the phylum Mollusca and those of the three classes Gastropoda, Bivalvia, and Scaphopoda.

All these observations indicate that there is a need to further investigate the extent and mechanisms of mt gene order variation in the class Ascidiaceae and in the entire subphylum of Tunicata, which are candidates to be the metazoan groups with the most highly rearranged mtDNAs.

Supplementary Material

Detailed Materials and Methods and **supplementary tables S1–S3** are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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yses, and wrote the paper; G.P. contributed to study design and manuscript preparation; F.I., V.G., and F.G. sequenced and annotated the mtDNAs; F.M. provided and identified the biological samples.

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