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Research

The complete sequences and gene organisation of the mitochondrial genomes of the heterodont bivalves *Acanthocardia tuberculata* and *Hiatella arctica* – and the first record for a putative At*pase subunit 8* gene in marine bivalves

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Abstract

Background: Mitochondrial (mt) gene arrangement is highly variable among molluscs and especially among bivalves. Of the 30 complete molluscan mt-genomes published to date, only one is of a heterodont bivalve, although this is the most diverse taxon in terms of species numbers. We determined the complete sequence of the mitochondrial genomes of *Acanthocardia tuberculata and Hiatella arctica*, (Mollusca, Bivalvia, Heterodonta) and describe their gene contents and genome organisations to assess the variability of these features among the Bivalvia and their value for phylogenetic inference.

Results: The size of the mt-genome in *Acanthocardia tuberculata* is 16.104 basepairs (bp), and in *Hiatella arctica* 18.244 bp. The *Acanthocardia* mt-genome contains 12 of the typical protein coding genes, lacking the *Atpase subunit 8* (*atp8*) gene, as all published marine bivalves. In contrast, a complete *atp8* gene is present in *Hiatella arctica*. In addition, we found a putative truncated *atp8* gene when re-annotating the mt-genome of *Venerupis philippinarum*. Both mt-genomes reported here encode all genes on the same strand and have an additional *trnM*. In *Acanthocardia* several large non-coding regions are present. One of these contains 3.5 nearly identical copies of a 167 bp motive. In *Hiatella*, the 3' end of the *NADH dehydrogenase subunit (nad)6* gene is duplicated together with the adjacent non-coding region. The gene arrangement of *Hiatella* is markedly different from all other known molluscan mt-genomes, that of *Acanthocardia* shows few identities with the *Venerupis philippinarum*. Phylogenetic analyses on amino acid and nucleotide levels robustly support the Heterodonta and the sister group relationship of *Acanthocardia* and *Venerupis*. Monophyletic Bivalvia are resolved only by a Bayesian inference of the nucleotide data set. In all other analyses the two unionid species, being to only ones with genes located on both strands, do not group with the remaining bivalves.

Conclusion: The two mt-genomes reported here add to and underline the high variability of gene order and presence of duplications in bivalve and molluscan taxa. Some genomic traits like the loss of the *atp8* gene or the encoding of all genes on the same strand are homoplastic among the Bivalvia. These characters, gene order, and the nucleotide sequence data show considerable potential of resolving phylogenetic patterns at lower taxonomic levels.

Background

Metazoan mitochondrial genomes are typically conserved in gene content and length. They are usually circular, 14 to 20 kb long, and encode for 13 proteins of the respiratory chain [cytochrome *c oxidase subunits I-III* (*cox I – cox III*), apocytochrome b (*cytb*), *atpase subunits 6 and 8* (*atp6, atp8*), and *NADH dehydrogenase subunits 1–6 and 4L* (*nad 1–6, nad 4L*)] and 24 RNA genes of the translation system [small (S) and large (L) subunit ribosomal RNA (rrn) and 22 transfer RNAs] [1]. The high number of possible arrangements makes it very unlikely that identical gene orders arise by chance [2]. Such a complex character combined with a low frequency of gene rearrangements is highly valuable for reconstructing palaeozoic or even pre-Cambrian phylogenetic events. Examples for this situation are Vertebrata (over 540 species sequenced) and Arthropoda (over 100 species sequenced): both show few rearrangements within the phylum [3].

In contrast, only 30 complete mitochondrial genomes of Mollusca are published: ten Gastropoda, nine Bivalvia, one Polyplacophora, two Scaphopoda and eight Cephalopoda. However, even this small taxonomic sample reveals much greater variability of gene arrangements compared to vertebrates and arthropods and notable differences in rearrangement frequencies between phyla and also within the Mollusca [3]. Whereas the order of the protein coding and the rRNA (rrn) genes in the mt genomes of the polyplacophoran *Katharina tunicata*, the vetigastropod *Haliotis rubra* and the cephalopods *Octopus vulgaris and Octopus ocellatus* are identical and the apogastropod *Ilyanassa obsoleta* and the other cephalopods can be related to them, the euthyneuran gastropods, the scaphopods and the bivalves are highly rearranged.

An additional complication in the Bivalvia, termed doubly uniparental inheritance (DUI), is the existence of distinct male and female mitochondrial lineages [4-10]. It is not clear whether this mode of inheritance is characteristic for all bivalves, or if it contributes to the accelerated rearrangement rate in this group. There are yet more special features of molluscan mt genes. Hoffmann et al. [11] described an additional *trn*-Met in *Mytilus edulis; Katharina tunicata* has two additional tRNAs [12]. Some pulmonate gastropods have unusual tRNA *s* lacking the T-stem or the D-stem, similar to nematode mt tRNAs [13]. The *atp6* and *atp8* genes are separated in scaphopods [14,15] and most gastropods (only the prosobranch *Littorina saxatilis*, the vetigastropd *Haliotis rubra* and the apogastropod *Ilyanassa obsoleta* have adjacent *atp6* and *atp8*). The published heterodont and pteriomorph bivalve sequences lack the *atp8* gene altogether. This is unusual because the *atp6 – atp8* cluster is common to most animal mitochondrial genomes, often with overlapping reading frames [3]. It is, thus, not clear for which molluscan taxa and on which

systematic levels mitochondrial gene order data and genomic characters like those mentioned above are phylogenetically informative.

The phylogenetic relationships of the major taxa of the heterodont bivalves are only partly resolved. Molecular phylogenetic analyses [16,17] agreed on the exclusion of the Hiatellidae from the Myoida placing this taxon close to the base of the higher Heterodonta ("unnamed clade I" in [16] fig. 3.6). The latter clade also contains the Cardiidae and Veneridae. With the complete mitochondrial sequences of one species of each Hiatellidae, Carditidae and Veneridae available for the present study we are able to test the monophyly and sistergroup relationships of the higher heterodonts, *Acanthocardia* and *Venerupis*.

Results

Genome size, genes, base composition and codon usage

The size of the complete mt-genome of *Acanthocardia tuberculata* is 16.104 basepairs (bp) and has an overall A+T content of 59.6 %. All genes are on one strand (Fig. 1). The *Acanthocardia* genome features 1.751 non-coding bp. The largest non-coding region (Table 1), of 1.103 bp is located between *trn-Met* and *trn-His*. It contains a 599 bp fragment composed of 3.5 nearly identical copies of a 167 bp motive (Fig. 2). This repeat has an A+T content of 60 %. The other 23 non-coding regions range between 1 and 128 bp.

All but one (atp8) of the 37 typical mitochondrial genes are present, with an additional copy of the trn-Met (Fig. 3). The Acanthocardia mt-genome encodes for a total of 3.647 amino acids. The most frequent codon is TTT (Phe; n = 264), followed by TTA (Leu; n = 172). An A or T nucleotide is present at the third position in 2.269 codons (61.13%). Five of the 12 protein coding genes start with ATG or ATA (Table 2), six starts with the alternative start codon ATT (Isoleucine). The atp6 gene starts with a GTG codon. Eight genes are terminated by TAA and four by TAG. An incomplete stop codon is inferred from the alignment of the atp6 gene. The genes for trn-Gln and trn-Arg overlap by two 2 bp.

The mt-genome of *Hiatella arctica* is 18.204 bp in length and has an A+T content of 66.35 %. As in *Acanthocardia*, all genes are on the same strand (Fig. 1). The longest of the 30 non-coding regions (Tab 3) has 614 bp and is located between the genes for *trn-Ala* and *atp8*. The others range between 1 and 376 bp in length.*Hiatella* has two copies of a 121 bp motive (Fig. 4) starting in the 3' end of the *nad6* gene and extending into the non-coding region before the *tRNA-Trp* gene. The genes for *trn-Leu I* and *trn-LeuII* overlap by one nucleotide.
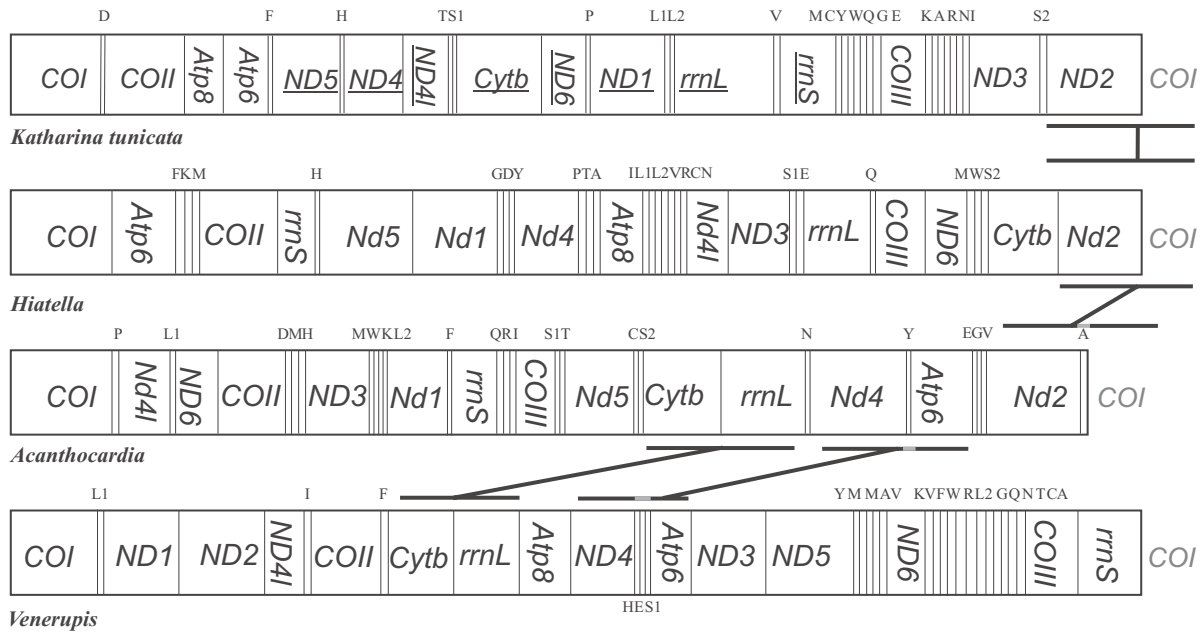
Figure 1
Gene order of *Katharina*, *Acanthocardia*, *Hiatella* and *Venerupis*. Linearized representation of the mitochondrial gene arrangement in *Acanthocardia tuberculata* and *Hiatella arctica*, in comparison with the near-plesiomorphic condition in the poly-placophoran *Katharina tunicata* and the third heterodont bivalve,*Venerupis philipinarium*. Genes encoded on the opposite strand are underlined. The bars indicate identical gene junctions.

The *Hiatella* mt-genome contains all 37 mitochondrial genes including *atp8* and a second copy of the *trn-Met* (Fig. 4). A total of 3.985 amino acids are encoded. As in *Acanthocardia*, the most frequent codons are TTT (Phe; n = 359) and TTA (Leu; n = 284). A or T are present in 2.873 third codon positions (72.09 %). Seven of the 13 protein coding genes start with ATA, the other six genes with ATG. The codon ATT terminates seven, and the codon ATG four protein coding genes (Tab 4). Truncated stop codons (T) are inferred for the *atp8* and the *coxII* genes.

Phylogenetic analysis of nucleotide and protein coding sequences
The concatenated amino acid alignment of 28 species (Tab 5) consists of 5.004 positions of which 3.085 are par-simony-informative. The corresponding nucleotide alignment including the rrnL sequences has 16.862 positions in total, 11.854 without 3rd codon positions, of which 7.130 are parsimony-informative. The Bayesian analyses resulted in almost fully resolved trees (Fig. 5) with total marginal -lnL of 156.458,59 for the amino acid data and 209.800,11 for the nucleotide data (arithmetic means). Most branches have posterior probabilities of 1.0. The

deeper nodes tend to be less supported. The parsimony analyses of both data sets yielded a single most parsimonious tree each (amino acid data: tree length 31.163, consistency index 0.5819, rescaled consistency index 0.2719; nucleotide data: tree length 49.859, consistency index 0.3440, rescaled consistency index 0.1366; trees not shown). All Bayesian and parsimony analyses recover the three heterodont species as a robust monophylum. *Acanthocardia* is the sister taxon of *Venerupis* with high to moderate support (fig 5). The Pteriomorpha are resolved as monophyletic from the nucleotide data in the parsimony analysis only. In most analyses the two unionid species are separated from the remaining bivalves and placed in a more basal position in the tree. Only the Bayesian tree of the nucleotide data resolves monophyletic Bivalvia, although with low support (posterior probability 0.76). This is also the only tree showing monophyletic Scaphopoda and Pulmonata. The unstable position of the vetigastropod *Haliotis*, near the base of the molluscan clade renders the Gastropoda diphyletic in all analyses. Cephalopoda are always robustly supported, and only the parsimony analysis of the amino acid data fails to resolve Mollusca as monophyletic. The exclusion of highly varia-

Figure 2 - Alignment of the large duplicated regions in *Acanthocardia tuberculata* and *Hiatella arctica.*

Tandem repeats in *Acanthocardia tuberculata* - Sequence position: 3389-3978

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3379 TCCCCCTTAG

3389 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG
   1 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG

3454 ACGCCAGAATTCGGAGCTTTTAACAATTTCCAGGCGATTCCAGTCAAATGCCGTTTTCCGATGAG
  66 ACGCCAGAATTCGGAGCTTTTAACAATTTCCAGGCGATTCCAGTCAAATGCCGTTTTCCGATGAG

3519 GGAGTTTTTTATCGTATTAGTAAGAACAAAAAAAAAAA
 131 GGAGTTTTTTATCGTATTAGTAAGAAC--AAAAAAAAA

3558 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG
   1 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG

3623 ACGCCAGAATTCGGAGCTTTTAACAATTTCCAGGCGATTCCAGTCAAATGCCGTTTTCCGATGAG
  66 ACGCCAGAATTCGGAGCTTTTAACAATTTCCAGGCGATTCCAGTCAAATGCCGTTTTCCGATGAG

3688 GGAGTTTTTTATCGTATTAGTAAGAACAAAAAAAAAA
     GGAGTTTTTTATCGTATTAGTAAGAACAAAAAAAAAA

3725 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG
   1 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG

3790 ACGCCAGAATTCGGAGCTTTTAACAATTTCCAGGCGATTCCAGTCAAATGCCGTTTTCCGATGAG
  66 ACGCCAGAATTCGGAGCTTTTAACAATTTCCAGGCGATTCCAGTCAAATGCCGTTTTCCGATGAG


3855 GGAGTTTTTTATCGTATTAGTAAGAACAAAAAAAAAA
 131 GGAGTTTTTTATCGTATTAGTAAGAACAAAAAAAAAA

3892 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG
   1 TAAGGTTTCTAAAGCATCTACCCCTCAATCGTAGCAAAAAAGTTGATTCAGGGTAAAATCAAGTG


3957 ACGCCAGAATTCGGAGCTTTTA
  66 ACGCCAGAATTCGGAGCTTTTA

3979 GATCTAGACA
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Tandem repeats in *Hiatella arctica* - Sequence position: 15349--15596

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15339 TTAGGATAAA

15349 AAAAAAAAACTAGATTTGGTTCACTAGAAGCTGGTGTGGTTATGTTATTAGGTTACTTGCTAGTGG
    1 AAAAAAAAACTAGATTTGGTTCACTAGAAGCTGGTGTGGTTATGTTATCAGGTTACTTGCTAGTAG


15414 TAACAGTATTTACTGTGGTCAGGTTGTGTAAAAAACTTTTTGGACGTTCTGTTGGG
   66 TAACACTATTTACTGTACTCAAGCTGTGTAAAAAACTCTTTACACGCTCCGTTGGG


15470 AAAAAAAAGTAGGTTTGGTTCACTAGAGGCT-GTAGTGGTTATGATT-TCAGGTTACTTGTTAGT
    1 AAAAAAAACTAGATTTGGTTCACTAGAAGCTGGT-GTGGTTATG-TTATCAGGTTACTTGCTAGT


15533 AGTAGCACTATTTATTGTAACT-AAGCTGTGT-AAAAAGTCTTTACACGCTCCGTTGGAG
   64 AGTAACACTATTTACTGT-ACTCAAGCTGTGTAAAAAAACTCTTTACACGCTCCGTTGG-G


15591 AAAAAA
    1 AAAAAA

15597 GTAAGCAGTA
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Figure 2
Alignment of the large duplicated regions in *Acanthocardia tuberculata* and *Hiatella arctica*.

Table 1: Non-coding regions and overlaps in the mitochondrial genome of *Acanthocardia tuberculata*

Position	between	between
Non-coding		
1594–1598	COI/tRNA-Pro	5
1663–1699	tRNA-Pro/ND4l	37
1982–2000	ND4l/tRNA-Leu	19
2528–2531	ND6/COII	4
3318–3326	tRNA-Asp/tRNA-MetI	9
3395–4497	tRNA-MetI/tRNA-His	1103
4559–4571	tRNA-HisI/ND3	13
4920–4927	ND3/tRNA-MetII	8
4992–4999	tRNA-MetII/tRNA-Trp	8
5064	tRNA-Trp/tRNA-Lys	1
5197–5226	tRNA-Leu/ND1	30
6139–6148	ND1/tRNA-Phe	10
7041–7070	12S rRNA/tRNA-Gln	30
8099–8104	COIII/tRNA-SerI	6
8174	tRNA-SerI/tRNA-Thr	1
8238–8293	tRNA-Thr/ND5	56
10005–10006	tRNA-Cys/tRNA-SerII	2
12521–12648	tRNA-Asn/ND4	128
13990–13994	ND4/tRNA-Tyr	5
14060–14064	tRNA-Tyr/Atp6	5
14777–14884	Atp6/tRNA-Glu	108
14944–14995	tRNA-Glu/tRNA-Gly	52
15067–15099	tRNA-Val/ND2	33
16027–16104	ND2/COI	78
Overlapping		
7132–7133	tRNA-Gln/tRNA-Arg	2

Table 2: Organisation of the mitochondrial genome of *Acanthocardia tuberculata*

Gene	Position	Strand	Start	Stop
COI	1–1593	+	ATA	TAG
tRNA-Pro	1599–1662	+		
ND4l	1700–1981	+	ATT	TAA
tRNA-LeuI	2001–2065	+		
ND6	2066–2527	+	ATT	TAA
COII	2532–3254	+	ATT	TAA
tRNA-Asp	3255–3317	+		
tRNA-MetI	3327–3394	+		
tRNA-His	4498–4558	+		
ND3	4572–4919	+	ATG	TAA
tRNA-MetII	4928–4991	+		
tRNA-Trp	5000–5063	+		
tRNA-Lys	5065–5132	+		
tRNA-LeuII	5133–5196	+		
ND1	5227–6138	+	ATG	TAG
tRNA-Phe	6149–6216	+		
12S rRNA	6217–7040	+		
tRNA-Gln	7071–7133	+		
tRNA-Arg	7132–7196	+		
tRNA-Ile	7197–7261	+		
COIII	7262–8098	+	ATG	TAA
tRNA-SerI	8105–8173	+		
tRNA-Thr	8175–8237	+		
ND5	8294–9940	+	ATT	TAA
tRNA-Cys	9941–10004	+		
tRNA-SerII	10007–10074	+		
Cytb	10075–11232	+	ATT	TAG
16S rRNA	11233–12455	+		
tRNA-Asn	12456–12520	+		
ND4	12649–13989	+	ATA	TAA
tRNA-Tyr	13995–14059	+		
Atp6	14065–14776	+	GTG	T incomplete
tRNA-Glu	14885–14943	+		
tRNA-Gly	14996–15007	+		
tRNA-Val	15008–15066	+		
ND2	15100–16026	+	ATT	TAA
tRNA-Ala	16029–16092	+		

ble alignment positions using GBLOCKS had no effect on the topologies of the trees and brought only minimal changes in branch support.

Discussion

Base frequencies, codon usage and amino acid frequencies in the mt genome of *Acanthocardia tuberculata* compare well with that of other bivalves. For instance, the A+T content of 59.6 % is similar to that *Inversidens* (57.2), *Lampsilis* (62.3), *Crassostrea gigas* (63.4%) and *Crassostrea virginica* (62.8%), *Placopecten* (55.7) and *Mytilus* (61.8%), but it is lower than that of *Venerupis* (69.7%) and *Hiatella arctica* (66.4).

The functional and selective significance of the duplicated regions in *Acanthocardia* is unclear. Tandem repeats are also present in other bivalve mitochondrial genomes: *Venerupis*, e.g., has four tandem repeats of 203 bp between the *nad2* and the *nad4l* genes [9]; *Placopecten* has seven repeats of a 79 bp motive between *trn- Asn* and *trn-Glu* and two repeats of 1.435 bp between *nad6* and *trn-Met* [18]. More unusual is the duplication in *Hiatella* starting 12 bases upstream of the 3' end of the *nad6* gene. Although the copies are 79 % identical the second repeat

has no open reading frame. It is likely that this non-functional copy of the coding part accumulated substitutions more rapidly due to relaxed selection and, thus, lost the reading frame. Nearly identical duplications of complete genes occur in mt-genomes of the cephalopods *Watasenia* and *Todarodes* [19,20].

Acanthocardia and *Hiatella* mt genomes encode 23 transfer RNA genes which can be folded in a typical secondary structure. Both genomes have an additional tRNA for Methionine. A second Methionine tRNAs is present in the bivalves *Mytilus edulis* [11], *Mytilus galloprovincialis* [7], *Crassostrea virginica* [21], *Placopecten magellanicus* [18] and *Venerupis phillipianarum* [9]. Overlaps of tRNA genes as observed in *Acanthocardia* and *Hiatella* are a common feature in mt-genomes [1].
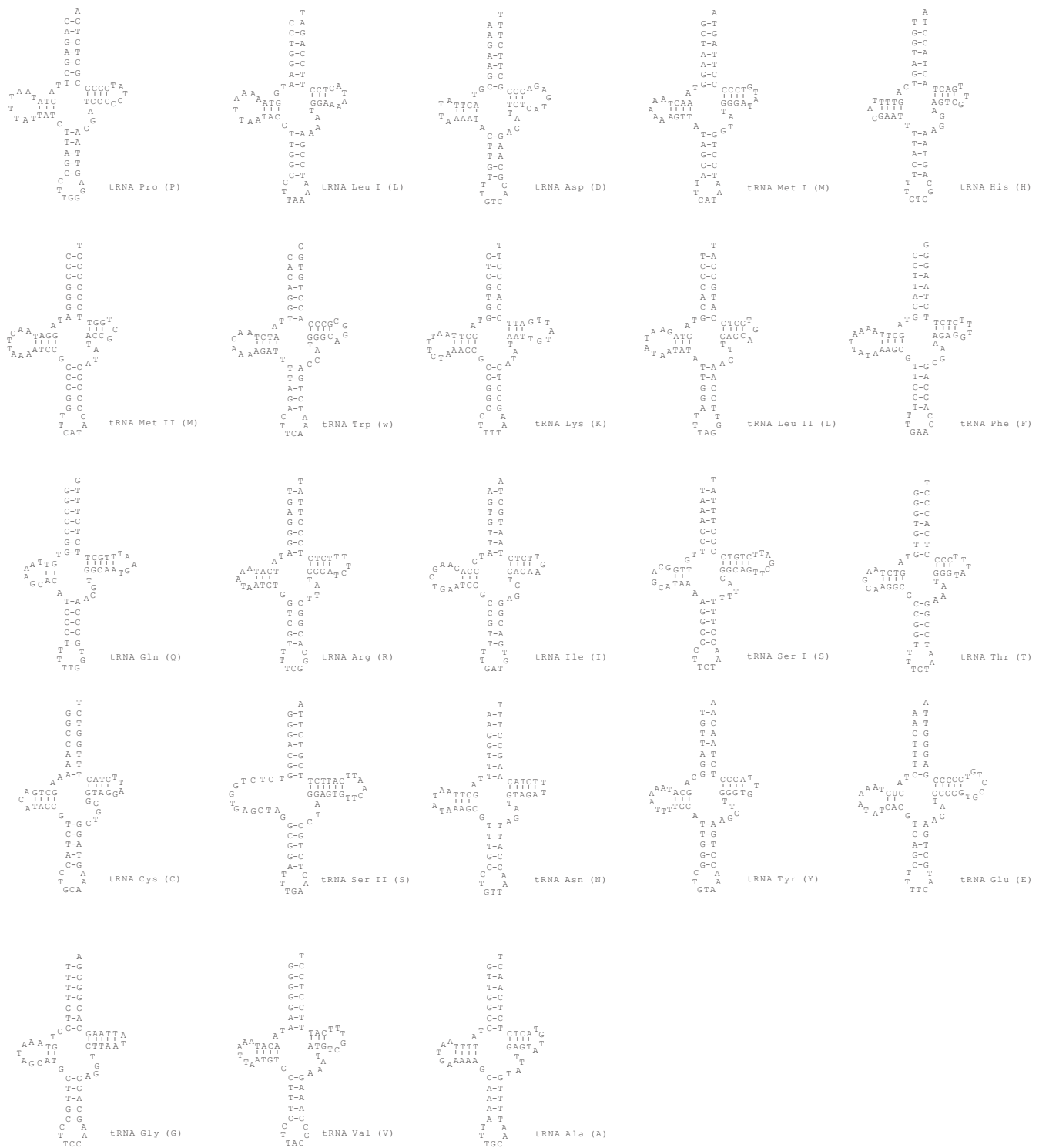
Figure 3
Cloverleaf structures of the 23 tRNA genes in the mitochondrial genome of *Acanthocardia tuberculata*.

The *atp6* gene of *Acanthocardia* lacks a Methione or Isoleucine at the putative 5' end and a complete stop codon. The first ATN codon is 48 bp downstream of the putative point of start as inferred from the alignment of the molluscan *atp6* genes. The assumed start codon is GTG as in the *nad 2* gene of polyplacophore *Katharina tunicata* [12]. Trun-
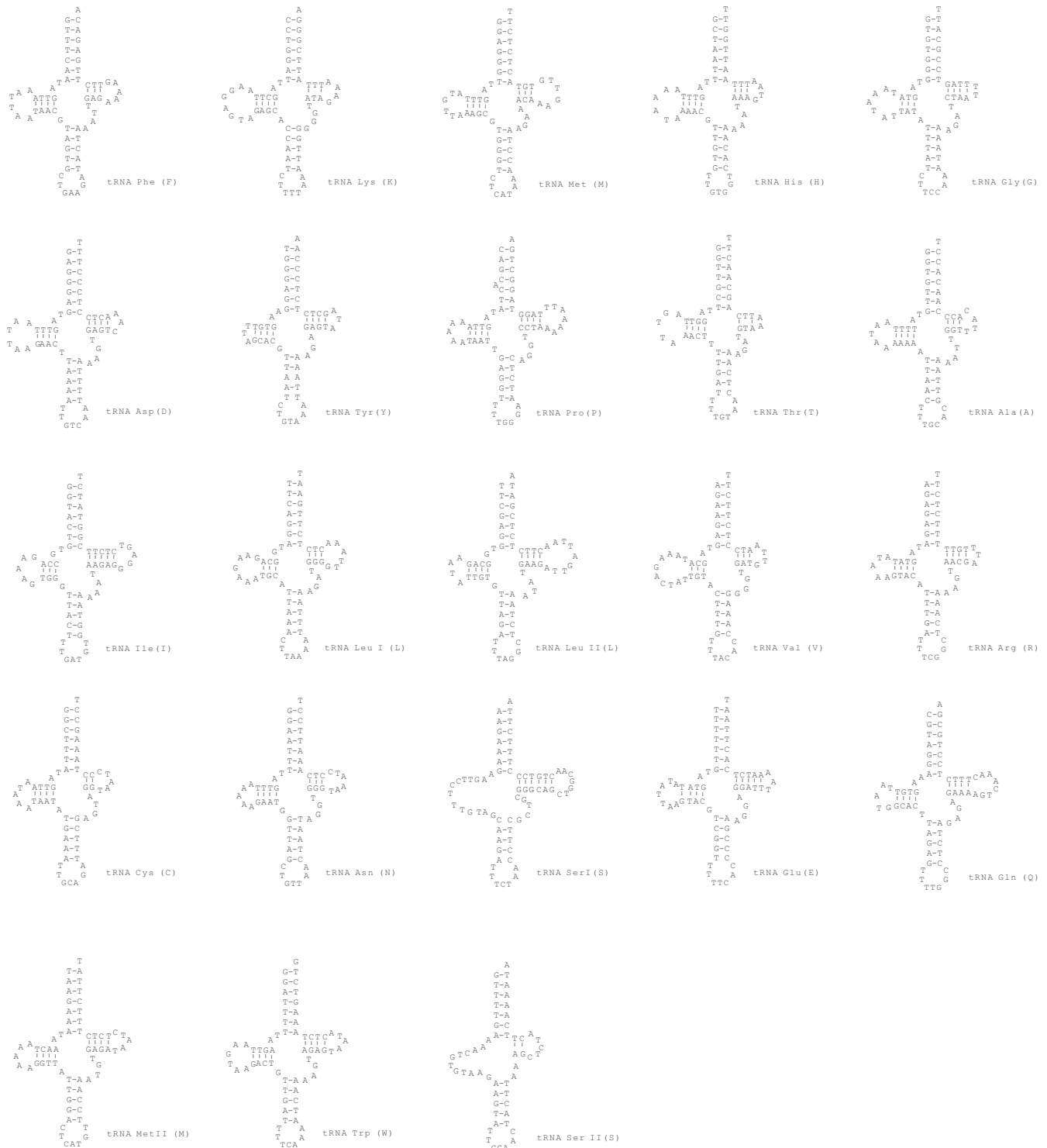
Figure 4
Cloverleaf structures of the 23 tRNA genes in the mitochondrial genome of *Hiatella arctica*.

Table 3: Non-coding regions and overlaps in the mitochondrial genome of *Hiatella arctica*

Position	between	Length
Non-coding		
1654–1819	COI/Atp6	166
2603–2609	Atp6/tRNA-Phe	7
2674–2678	tRNA-Phe/tRNA-Lys	5
2743	tRNA-Lys/tRNA-Met	1
2809	tRNA-Met/COII	1
3894–4270	COII/12S rRNA	376
5235–5294	tRNA-His/ND5	60
6963–6983	ND5/ND1	21
7929	ND1/tRNA-Gly	1
7992–7998	tRNA-Gly/tRNA-Asp	7
8063–8105	tRNA-Asp/tRNA-Tyr	43
8166	tRNA-Tyr/ND4	1
9529–9533	ND4-tRNA-Pro	5
9601–9621	tRNA-Pro/tRNA-Thr	21
9682–8690	tRNA-Thr/tRNA-Ala	9
9753–10366	tRNA-Ala/Atp8	614
10527–10730	Atp8/tRNA-Ile	204
10796–10800	tRNA-Ile/tRNA-LeuI	5
10932–10933	tRNA-Leu/tRNA-Val	2
11062	tRNA-Arg/tRNA-Cys	1
11124–11129	tRNA-Cys/tRNA-Asn	6
11094–11311	tRNA-Asn/Nd4l	218
11612–11672	Nd4l/Nd3	61
12030–12055	ND3/tRNA-SerI	26
13707–13924	tRNA-Gln/COIII	218
14753–14878	COIII/ND6	126
15482–15722	ND6/tRNAMetII	241
15855–15875	tRNA-Trp/tRNA-SerII	21
15931–16000	tRNA-SerII/Cytb	70
18197–18244	ND2/COI	48
Overlapping		
10864	tRNA-LeuI/tRNA-LeuII	1

Table 4: Organisation of the mitochondrial genome of *Hiatella arctica*

Gene	Position	Strand	Start	Stop
COI	1–1653	+	ATG	TAG
Atp6	1820–2602	+	ATG	TAA
tRNA-Phe	2610–2673	+		
tRNA-Lys	2679–2743	+		
tRNA-Met	2745–2808	+		
COII	2810–3893	+	ATA	T incomplete
12S rRNA	4271–5171	+		
tRNA-His	5172–5234	+		
ND5	5295–6962	+	ATA	TAA
ND1	6984–7928	+	ATA	TAA
tRNA-Gly	7930–7991	+		
tRNA-Asp	7999–8062	+		
tRNA-Tyr	8106–8165	+		
ND4	8167–9528	+	ATA	TAA
tRNA-Pro	9534–9600	+		
tRNA-Thr	9622–9681	+		
tRNA-Ala	9691–9752	+		
Atp8	10367–10526	+	ATG	T incomplete
tRNA-Ile	10731–10795	+		
tRNA-LeuI	10801–10864	+		
tRNA-LeuII	10864–10931	+		
tRNA-Val	10934–10998	+		
tRNA-Arg	10999–11061	+		
tRNA-Cys	11063–11123	+		
tRNA-Asn	11130–11193	+		
ND4l	11312–11611	+	ATG	TAG
ND3	11673–12029	+	ATA	TAG
tRNA-SerI	12056–12125	+		
tRNA-Glu	12126–12191	+		
16S rRNA	12192–13638	+		
tRNA-Gln	13639–13706	+		
COIII	13925–14752	+	ATA	TAA
ND6	14879–15481	+	ATG	TAG
tRNA-MetII	15723–15788	+		
tRNA-Trp	15789–15854	+		
tRNA-SerII	15876–15930	+		
Cytb	16001–17155	+	ATA	TAA
ND2	17156–18196	+	ATG	TAA

cated stop codons like in the *Acanthocardia atp6* and the *Hiatella co II* and the *atp8* genes require the inference of the ends of the genes from the alignment with other species. The completion of truncated stop codons by polyadenylation after transcript processing was described by Ojala [22].

Hiatella arctica is the first marine bivalve reported to have a complete *atp8* gene consisting of 53 amino acids. The alignment of this *atp8* gene (Fig. 6) shows a *Methionine* at the start and a truncated stop codon T. We also identified a putative *atp8* gene in the mt-genome of *Venerupis*, between the genes *rrnL* and *nad4* at positions 5.974 to 6.088. Although this region was annotated as part of the *rrnL* by the authors [9], it represents an open reading frame encoding for only 37 amino acid positions. It starts with *Leucine* instead of *Methionine*, but ends with a complete stop codon. The more conserved 5' region of the gene resembles other molluscan atp8 genes in amino acid sequence (Fig. 6) and in the hydrophilicity profile. The positively charged 3' region of the gene, which is known to vary greatly in length and composition [23,24], is reduced to a few residues in *Venerupis*. This is confirmed by the alignment of the amino acid sequence corresponding to the conserved *atp8* profiles in other metazoans [25]. It remains open, however, whether this gene is functional. Dreyer and Steiner [15] reported a comparably short *atp8* gene for the scaphopod *Siphonodentalium lobatum*. Serb and Lydeard [26] discuss a non functional version of the *atp8* gene in the freshwater mussel *Inversidens*, and Milbary and Gaffney [21] describe a potential remnant of the *atp8* gene in the eastern oyster *Crassostrea virginica*.

Many metazoan mt genomes have neighbouring *atp6* and *atp8* genes on the same strand. This arrangement is likely to be selected for, if the uncleaved transcripts are co-trans-
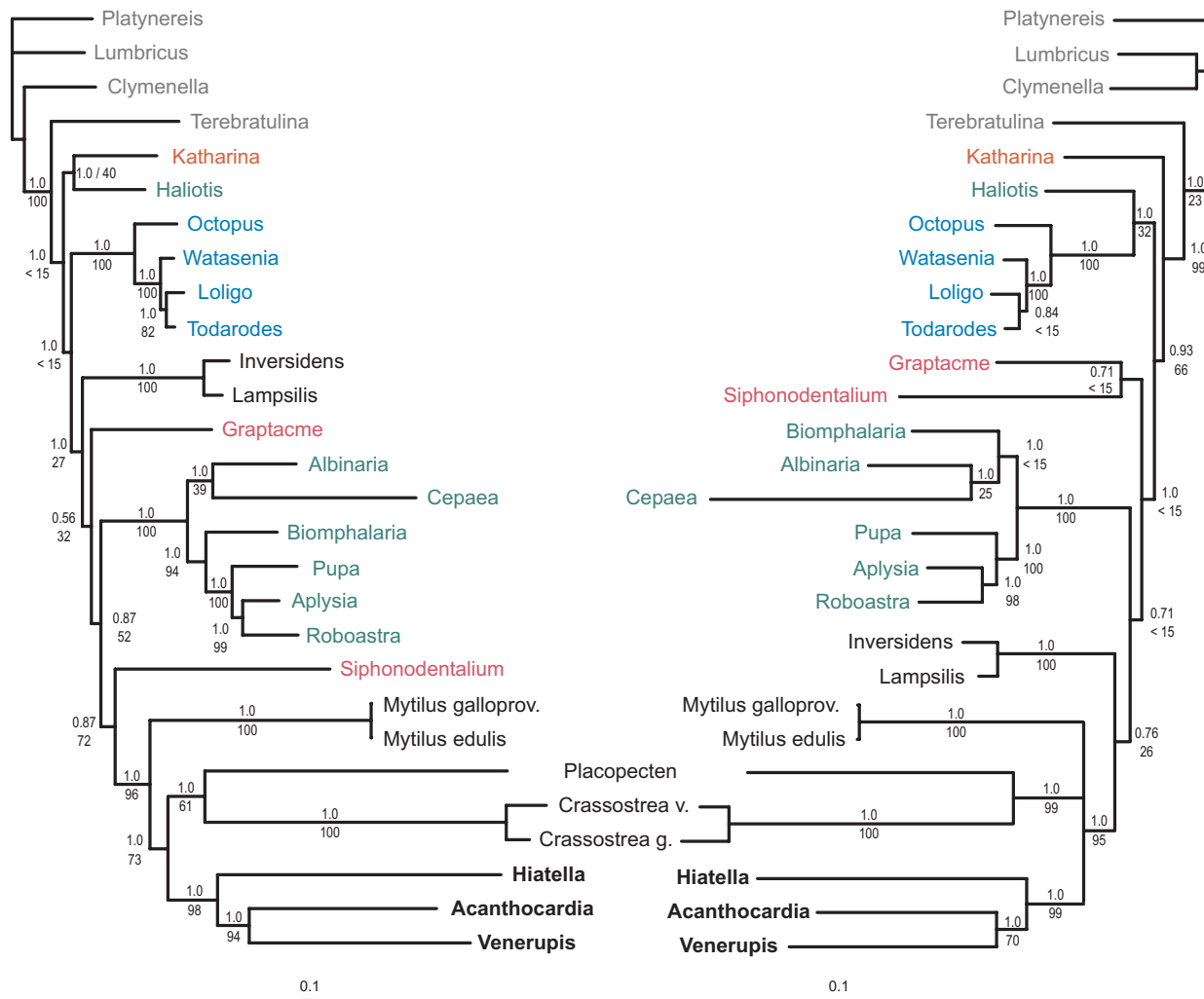
Figure 5
Phylogenetic analyses. Bayesian trees of the amino acid sequences of all protein coding genes (left) and nucleotide sequences of the protein coding genes and the *rrnL* gene (right). Bivalve species are in black font (Heterodonta in bold), Gastropoda in green, Cephalopoda in blue, Scaphopoda in magenta, Polyplacophora in red and the outgroup taxa in grey font. Posterior probabilities (above) and parsimony bootstrap values (below) are given for each branch.
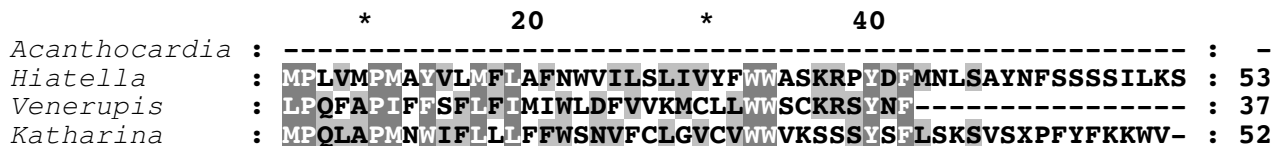

Figure 6
Alignment of the *atp8* genes of *Hiatella arctica, Venerupis philippinarum* and *Katharina tunicata.*

Table 5: List of taxa used in the phylogenetic analysis

Taxon	Classification	GenBank Accession number
Outgroup		
Terebratulina retusa	Brachiopoda	AJ245743
Lumbricus terrestris	Annelida, Clitellata	U24570
Platynereis dumerilii	Annelida, Polychaeta	AF178678
Clymenella torquata	Annelida, Polychaeta	AY741661
Polyplacphora		
Katharina tunicata	Mollusca, Polyplacophora, Neocoleida	U09810
Gastropoda		
Haliotis rubra	Mollusca; Gastropoda, Orthogastropoda, Vetigastropoda	AY588938
Aplysia californica	Mollusca; Gastropoda, Orthogastropoda, Apogastropoda	AY569552
Pupa strigosa	Mollusca; Gastropoda, Orthogastropoda, Apogastropoda	AB028237
Roboastra europaea	Mollusca; Gastropoda, Orthogastropoda, Apogastropoda	AY083457
Biomphalaria glabrata	Mollusca; Gastropoda, Pulmonata, Basammatophora	AY380531
Albinaria caerulea	Mollusca; Gastropoda, Pulmonata, Stylommatophora	X83390
Cepaea nemoralis	Mollusca; Gastropoda, Pulmonata, Stylommatophora	U223045
Scaphopoda		
Graptacme eborea	Mollusca, Scaphopoda, Dentaliida	AY484748
Siphonodentalium lobatum	Mollusca, Scaphopoda, Gadilida	AY342055
Cephalopoda		
Octopus vulgaris	Mollusca, Cephalopoda, Coleoidea, Neocoleoidea, Octopodiformes	AB158363
Loligo bleekeri	Mollusca, Cephalopoda, Coleoidea, Neocoleoidea, Decapodiformes	AB029616
Todarodes pacificus	Mollusca, Cephalopoda, Coleoidea, Neocoleoidea, Decapodiformes	AB158364
Watasenia scintillians	Mollusca, Cephalopoda, Coleoidea, Neocoleoidea, Decapodiformes	AB086202
Bivalvia		
Placopecten magellanicus	Mollusca, Bivalvia, Pteriomorphia, Pectinoida	DQ088274
Mytilus galloprovincialis	Mollusca, Bivalvia, Pteriomorphia, Mytiloida	AY497292
Mytilus edulis	Mollusca, Bivalvia, Pteriomorphia, Mytiloida	AY484747
Crassostrea gigas	Mollusca, Bivalvia, Pteriomorphia, Ostreoida	AF177226
Crassostrea virginica	Mollusca, Bivalvia, Pteriomorphia, Ostreoida	AY905542
Lampsilis ornata	Mollusca, Bivalvia, Palaeoheterodonta, Unionida	AY365191
Inversidens japanensis	Mollusca, Bivalvia, Palaeoheterodonta, Unionida	AB055625
Venerupis phillipinarium	Mollusca, Bivalvia, Heterochonchia, Veneroida	AB065375
Acanthocardia tuberculata	Mollusca, Bivalvia, Heterochonchia, Veneroida	DQ 632743 this study
Hiatella arctica	Mollusca, Bivalvia, Heterochonchia, Myoida	DQ 632742 this study

lated [25,2]. Several taxa lacking this gene arrangement in the mt genome, e.g. Plathyhelminthes, Nematoda, Annelida, Sipunculida, the brachipods *Laqueus* [27] and *Terebratalia* [28], and, among Mollusca, Bivalvia and Scaphopoda. Of these genomes, Plathyhelminthes, Nematoda except for *Trichinella*, and the pteriomorph bivalves lack *atp8* altogether. The disparate distribution of this feature clearly indicates that the loss of the *atp6 – atp8* coupling and the loss of *atp8* occurred several times independently in metazoan evolution. This is corroborated by finding truncated *atp8* genes separated from the *atp6* gene in the nematode *Trichinella* and in the scaphopod *Siphonodentalium*. It is possible that this situation represents an evolutionary stepping stone from the fully functional *atp6 – atp8* coupling, via decoupled but complete genes like in annelids and the scaphopod *Graptacme*, and the complete loss of *atp8*.

The location of all mt-genes on the same strand, as in *Acanthocardia* and *Hiatella*, is uncommon among Meta-

zoa, but is reported for several taxa [28] including all published marine bivalves. Only in the unioid freshwater bivalves *Lampsilis ornata* and *Inversidens japanensis* genes are located on both strands. Under the Heteroconchia concept postulating a sister group relationship of Unionida and Heterodonta, the "all-on-one-strand" situation either evolved independently in Heterodonta and Pteriomorph or was lost in the Unionida.

Comparing the gene arrangements of *Acanthocardia* and *Venerupis* no identities are apparent, if the tRNA genes are included. The tRNAs are more variable because the secondary structure allows them to translocate more frequently [12]. Even after excluding the tRNAs from the comparison the two mt-genomes show few identical gene junctions. These are limited to the block containing the *Cytb – rrnL – nad4 – atp6* genes in *Acanthocardia*, although this is interrupted by the putative *atp8* gene in *Venerupis*. This gene order may be inherited from the common ancestor of *Acanthocardia* and *Venerupis*, with the apomor-

Table 6: Amplification primers used in this study

Species	Primer	Sequence
Both species	HCO 2198[a]	TAAACTTCAGGGTGACCAAAAAATCA
	LCO 1490[a]	GGTCAACAAATCATAAAGATATTGG
	16S Fmwg[b]	CTCGCCTTTTAWCAAAAACAT
	16S Rmwg[b]	ACGCCGGTCTKAACTCAG
	12Sai[c]	AAACTAGGATTAGATACCCTATTAT
	12Sbi[c]	AAGAGCGACGGGCGATGTGT
Acanthocardia tuberculata	Ac16Flong	GAAGCTTAAACAGTGGGACTGTTCGTCC
	Ac16Rlong	CCTATAAGACAGCTATTCCATCGTCAAC
	AcHCOrev	CGGTGAATTCATAAGATTCCAATGCTACC
	AcLCOrev	GAGCATGGTTATGTAGGCATGAAGATG
Hiatella arctica	Hi12Flong	ACCTTCAATAGCTGATCTCTACCCCAGG
	Hi12Rlong	GCATCATATCCTGTAGGGGAACTTGGCC
	Hi16Flong	AAGCTACCACGGGATAACAGCGTG
	Hi16rlong	CACGTCAACCCCTTCTTCCTAGACTTC

[a]Folmer et al. [47]
[b]modified after Palumbi et al. [48]
[c]Simon et al. [49]

phic loss of *atp8* in *Acanthocardia*. The mt-genome of *Hiatella* appears almost completely rearranged. Only the neighbourhood of the *nad2* and *cox I* genes is present in other molluscs like *Katharina*, *Haliotis* and *Octopus* and may represent a plesiomorphic trait.

The mitochondrial genome sequence data confirm previous results [16,17] on the monophyly of Cardiidae and Veneridae relative to the Hiatellidae. Their common branch and the heterodont clade are robustly supported in all analyses. Similarly, the clade uniting Heterodonta and Pteriomorpha is well supported, although to the exclusion of the unionid branch. This is in accordance with the topology of Giribet and Distel [16] but contrasts that of Waller [29] and Steiner and Hammer [30] supporting the Heteroconchia clade (Unionida + Heterodonta). The Bivalvia clade is resolved by the Bayesian analysis of the nucleotide data only. This may indicate the higher potential of recovering correct topologies by this method or the superiority of nucleotide substitution models over amino acid substitution models or a combination of these factors. Note that the Bayesian nucleotide analysis also succeeds in resolving the pteriomorph, scaphopod, and pulmonate branches.

What could cause the unexpected position of the Unionida rendering the Heteroconchia diphyletic? In both amino acid and nucleotide-based trees the unionid species have conspicuously shorter branches compared to the other bivalves. Although the present data set is not large enough for statistical assessment, such obvious differences in substitution rates may cause phylogenetic analyses to find incorrect trees, as previously documented for Bivalvia [30]. In addition to lower substitution rates, different substitution patterns in the unionids may confound phylogenetic analyses. All bivalve mt-genomes have the genes encoded on the same strand, except for those of the unionids where three to four genes are encoded on the opposite strand. Due to the asymmetric replication process the strands show different substitution skews. Hassanin *et al.*[31] showed that skew differences may influence phylogenetic analyses.

The mitochondrial gene order in the Bivalvia is too divergent and the present taxon set too small to make use of this character set for phylogenetic inference at this point. In addition, the substitution models for phylogenetic inference presently do not take strand specific patterns into account. Similarly, gene rearrangement models are limited to one type of rearrangement only, either translocation or inversion. However, with a growing set of mt-genomes – their nucleotide and gene sequences – we are likely to enhance our understanding of patterns and modes of nucleotide substitutions and gene rearrangements. This will help to improve phylogenetic reconstructions by refining the models for these evolutionary processes. Improved taxon sampling and refined phylogenetic inference models are likely to resolve more open questions of bivalve phylogeny and evolution than with previously used markers.

Methods

Material, DNA isolation, PCR, sequencing

Hiatella arctica (Linné, 1767) and *Acanthocardia tuberculata* (Linné, 1758) were collected in the Adriatic Sea (Rovinj, Croatia) and frozen in liquid nitrogen. Total DNA was isolated with the DNeasy Tissue Kit (Qiagen).

Partial *cox I*, *rrnL* and *rrnS* genes were amplified (*Acanthocardia rrnL* and *cox I*, *Hiatella rrnS* and *rrnL*) using the primers HCO2198/LCO 1490, 16Sfmwg/16Srmwg and 12Sai/12Sbi (Table 6). The PCR was done on a Primus 96 advanced Gradient (Peqlab) in a 30 µl reaction containing 1,5 mM Mg Cl$_2$, each dNTP at 250 µM, each primer at 0,5 µM, 0,6 units Taq polymerase (Biotaq Red, Bioline) and the supplied buffer at 1× concentration. The PCR cycle conditions were: Initial denaturation step of 2 min at 94°C, 35 cycles of 30 sec denaturation at 94°C, 40 sec annealing at 48°C and 2 min (HCO2198/16Sbr) or 45 sec primer extension at 72°C followed by a final primer extension step at 72°C for 7 min. PCR products were purified with the E.Z.N.A Cycle-Pure Kit (Peqlab, Germany). PCR products were sequenced automatically with the amplification primers on an ABI-capillary-sequencer at Eurofins-Medigenomix GmbH (Martinsried, Germany). The sequences were used to design sets of long range primers (*12SrRNA, CO1, 16SrRNA* Primer see table 3) to amplify the whole mitochondrial Genome in three fragments with the TaKaRa LA Taq (Takara) on a Primus 96 advanced Gradient (Peqlab). The 50 µl reactions contained 5 µl of the supplied buffer 3 (including 22.5 mM MgCl$_2$), 25 pmol of each primer, 500 µM dNTPs, 5.25 units of Taq DNA polymerase, 0.3 µl Nonidet P40 and about 100 ng of total DNA. The PCR conditions for both fragments are: initial denaturation at 92°C for 2 min, 30 cycles of 15 sec denaturation at 92°C, 35 sec annealing at 63°C and 10 min primer extension at 72°C followed by a final primer extension step at 72°C for 10 min. The products were sequenced by primer walking.

Data analysis

Protein coding genes were analysed by the Open Reading Frame Finder [32] using the invertebrate mitochondrial code. Protein and rRNA genes were identified by their similarity to published gene sequences by BLAST searches [33]. The tRNA genes are usually too little conserved for BLAST hits. Some of them were identified by tRNA-scan SE Search Server [34] and DOGMA [35], others could only be reckognised by manually folding intergenic sequences to cloverleaf structures with anticodons. Codon usage analysis was performed by CodonW version 1.3 [36]. The whole sequence was tested for potentially tandem repeats by TANDEM REPEAT FINDER, Version 4.0 [37]. The hydrophobicity profiles for the *atp8* genes are generated using the general method of Kyte and Doolittle [38] with BIOEDIT version 7.0.5 [39].

Phylogenetic analysis

Deduced amino acid sequences were aligned with CLUSTAL X 1.83 [40] at default settings followed by manual correction. The nucleotide alignment was based on the amino acid alignment. All protein coding and the *rrnL* gene sequences of 24 molluscs (10 bivalves, 4 cephalopods, 2 scaphopods, 7 gastropods and 1 polyplacophore [tab 6]) were concatenated in a single Nexus file. Three annelids and one brachiopod served as outgroups. Separate analyses were run with all positions and with hypervariable positions excluded with GBLOCKS 0.91 [41]. We used PAUP* 4.0b10 [42] for equally weighted parsimony analyses with the heuristic search option and 50 random addition sequences with TBR branch swapping. Bootstrap support was assessed by 10.000 (amino acid data) or 20.000 replicates (nucleotide data) with three random addition sequences each. Bayesian inference was performed with MRBAYES 3.1 [43,44] on the Schrödinger II cluster of the Univ. Vienna computing facility under the Mtrev+Γ+I substitution model for the amino acid data set. The AIC criterion implemented in MODELTEST 3.06 [45] returned the GTR+Γ+I model as most appropriate for the nucleotide data set. We used separate and unlinked partitions for each gene and 2 × 4 chains of 5 × 10^5 generations, sampling every 100th tree. Burnin estimation by lnL and convergence diagnostics were used as implemented in the software. We excluded 3rd codon positions from the nucleotide analyses to reduce phylogenetic noise due to substitution saturation. Trees were visualized with TREEVIEW 1.6.6 [46].

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

HD carried out the genome sequencing and annotation, contributed to the phylogenetic analyses and drafted parts of the manuscript. GS designed the study, collected the animals, carried out the phylogenetic analyses, drafted parts of the manuscript and is responsible for the final editing.

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