A comparative study of nemertean complete mitochondrial genomes, including two new ones for *Nectonemertes cf. mirabilis* and *Zygeupolia rubens*, may elucidate the fundamental pattern for the phylum Nemertea

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Abstract

**Background:** The mitochondrial genome is important for studying genome evolution as well as reconstructing the phylogeny of organisms. Complete mitochondrial genome sequences have been reported for more than 2200 metazoans, mainly vertebrates and arthropods. To date, from a total of about 1275 described nemertean species, only three complete and two partial mitochondrial DNA sequences from nemerteans have been published. Here, we report the entire mitochondrial genomes for two more nemertean species: *Nectonemertes cf. mirabilis* and *Zygeupolia rubens*.

**Results:** The sizes of the entire mitochondrial genomes are 15365 bp for *N. cf. mirabilis* and 15513 bp for *Z. rubens*. Each circular genome contains 37 genes and an AT-rich non-coding region, and overall nucleotide composition is AT-rich. In both species, there is significant strand asymmetry in the distribution of nucleotides, with the coding strand being richer in T than A and in G than C. The AT-rich non-coding regions of the two genomes have some repeat sequences and stem-loop structures, both of which may be associated with the initiation of replication or transcription. The 22 tRNAs show variable substitution patterns in nemerteans, with higher sequence conservation in genes located on the H strand. Gene arrangement of *N. cf. mirabilis* is identical to that of *Paranemertes cf. peregrina*, both of which are Hoplonemertea, while that of *Z. rubens* is the same as in *Lineus viridis*, both of which are Heteronemertea. Comparison of the gene arrangements and phylogenomic analysis based on concatenated nucleotide sequences of the 12 mitochondrial protein-coding genes revealed that species with closer relationships share more identical gene blocks.

**Conclusion:** The two new mitochondrial genomes share many features, including gene contents, with other known nemertean mitochondrial genomes. The tRNA families display a composite substitution pathway. Gene order comparison to the proposed ground pattern of Bilateria and some lophotrochozoans suggests that the nemertean ancestral mitochondrial gene order most closely resembles the heteronemertean type. Phylogenetic analysis proposes a sister-group relationship between Hetero- and Hoplonemertea, which supports one of two recent alternative hypotheses of nemertean phylogeny.

**Keywords:** MtDNA, Nemertea, *Nectonemertes mirabilis*, *Zygeupolia rubens*, Phylogeny, Gene rearrangement

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Background

Knowledge of mitochondrial genomes is important for many scientific disciplines [1,2] and the relative arrangement of mitochondrial genes has been effective for studying phylogenetic relationships [3,4]. However, current knowledge of mtDNAs is uneven, and sequences available in GenBank are predominantly from vertebrate taxa. There are about 1275 described species [5] of nemerteans (ribbon worms, phylum Nemertea); these are mainly marine but terrestrial and freshwater species also are known. To date, complete mitochondrial genomes have been published for only three species in the phylum, Cephalothrix hongkongiensis (Palaeonemertea) [reported as Cephalothrix simula in [6]], Lineus viridis (Heteronemertea) [7], and Paranemertes cf. peregrina (Hoplonemertea) [8]. Nearly complete sequences exist for the palaeonemerteans Cephalothrix sp. [8] and Cephalothrix rufifrons [9]. Thus, current genomic knowledge of nemerteans is scant and taxon diversity is poorly sampled. In this study, we sequenced the complete mitochondrial genomes of two nemertean species, Nectonemertes cf. mirabilis (Hoplonemertea: Polystilifera) and Zygeupolia rubens (Heteronemertea). Mitochondrial gene arrangements, structures, and compositions, as well as translation and initiation codons and codon usage patterns, were compared with complete mtDNA sequences of other nemerteans. In addition, we compare gene order among Lophotrochozoa and we use the nucleotide sequences to analyze phylogenetic relationship among the included nemerteans.

Results and discussion

Genome organization and structure

Genome composition and gene arrangement of Nectonemertes cf. mirabilis and Zygeupolia rubens are summarized in Figure 1 and Table 1. The mitochondrial genomes of N. cf. mirabilis and Z. rubens are circular DNA molecules of 15365 bp and 15513 bp, respectively. Lengths of the two nemertean mitochondrial genomes are within the range of previously sequenced nemertean mtDNAs - 14558 bp in Paranemertes cf. peregrina to 16296 bp in Cephalothrix hongkongiensis [6]. Both of the newly sequenced mitochondrial genomes contain 37 genes, including 13 protein-coding genes, two ribosomal RNAs, and 22 transfer RNAs. All genes except trnP and trnT are encoded on the same strand (Figure 1).

For both species, protein-coding genes nad4L and nad4 share an overlap, by seven nucleotides, and nad6 overlaps cob by eight nucleotides in Z. rubens (Figure 1, Table 1). Such overlaps are common to all known mtDNA genomes of nemerteans [6,8], and are found in many metazoan mtDNAs [10].

Protein-coding genes

Thirteen protein-coding genes (cox1-cox3, nad1-nad6, nad4L, cob, atp6, and atp8) were identified. Mitochondrial genomes often use a variety of nonstandard initiation codons [11]. Except for nad4 (GTG), nad5 (GTG), atp8 (GTG) and atp6 (GGT) in N. cf. mirabilis, and nad1 (GTG) and nad2 (GTG) in Z. rubens, the protein-coding genes of both species begin with ATG. The majority of genes in both species contain the full termination codon TAA or TAG, but some end with T (atp8 in N. cf. mirabilis, and nad5, cob2 and nad1 in Z. rubens). Such abbreviated stop codons are common among animal mitochondrial genes. In Z. rubens, the incomplete stop codons are immediately followed by the downstream tRNA gene (Figure 1, Table 1), whose secondary structure has been suggested to act as a signal for the cleavage of the polycistronic primary transcript [12,13]. However, there also are direct junctions pairing ten protein-coding genes in N. cf. mirabilis (nad6/cob, nad4L/nad4, nad3/cob1, nad2/cob2, and atp8/atp6) and eight in Z. rubens (nad6/cob, nad4L/nad4, nad2/cob1 and atp8/atp6) (Figure 1, Table 1). Here, cleavage signals other than secondary structure of a tRNA gene may initiate processing of the polycistronic primary transcript [14]. For two protein-coding genes (nad6 and nad2) in both nemertean species and nad3 in N. cf. mirabilis, stem-loop structures were inferred to be at the 3' end and abutting the 5' end of the neighboring protein-coding gene, and may signal cleavage of the immature mRNA [15,16].

Transfer RNA and ribosomal RNA genes

Both of the mitochondrial genomes encoded 22 tRNA genes found in other nemertean mtDNAs, which is
tRNA cloverleaf structures generally contained 7 bp in the aminoacyl stem, 2 to 5 bp in the TψC stem, 5 bp in the anticodon stem, and 0 to 4 bp in the dihydrouridine (DHU) stem. Some tRNAs showed DHU-loop replacement (e.g., trnS1 of N. cf. mirabilis), as also found in L. c. mirabilis and to 6 1 (trnK) to 7 2 (trnS2) nucleotides in Z. rubens (Table 2); most were folded into a typical cloverleaf secondary structure (Figures 2, 3). The postulated tRNA cloverleaf structures generally contained 7 bp in the aminoacyl stem, 2 to 5 bp in the TψC stem, 5 bp in the anticodon stem, and 0 to 4 bp in the dihydrouridine (DHU) stem. Some tRNAs showed DHU-loop replacement (e.g., trnS1 of N. cf. mirabilis), as also found in L. c. mirabilis and to 6 1 (trnK) to 7 2 (trnS2) nucleotides in Z. rubens (Table 2); most were folded into a typical cloverleaf secondary structure (Figures 2, 3). The postulated
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Table 1 Location of genes in the mitochondrial genomes of Nectonemertes cf. mirabilis and Zygeupolia rubens

| Genes | From 5’to 3’ | Size (bp) | 3’ spacer | Genes | From 5’to 3’ | Size (bp) | 3’ spacer |
|-------|--------------|-----------|------------|-------|--------------|-----------|------------|
| trnY  | 1-62 | 62 | 0 | tmY | 1-64 | 64 | 0 |
| trnP^b | 125-63 | 63 | 2 | tmP^b | 131-65 | 67 | 3 |
| nad6  | 128-583 | 456 | 21 | nad6 | 135-599 | 465 | -8 |
| cob   | 605-1741 | 1137 | 9 | cob | 592-1728 | 1137 | -1 |
| trnS1 | 1751-1811 | 61 | -1 | trnS1 | 1728-1798 | 71 | -1 |
| tmT^b | 1876-1811 | 66 | 2 | tmT^b | 1861-1798 | 64 | 2 |
| nad4L | 1879-2181 | 303 | -7 | nad4L | 1834-2169 | 306 | -7 |
| nad4  | 2175-3536 | 1362 | 6 | nad4 | 2163-3509 | 1347 | 1 |
| tmH   | 3543-3602 | 60 | 0 | tmH | 3511-3574 | 64 | 2 |
| nadS  | 3603-3548 | 1746 | -1 | nadS | 3577-5308 | 1732 | 0 |
| tmE   | 5348-5410 | 63 | 1 | tmE | 5309-5372 | 64 | 1 |
| trnP^b | 5412-5474 | 63 | 2 | tmP^b | 5374-5438 | 65 | 2 |
| tmN   | 6000-6662 | 63 | 2 | tmN | 6552-6616 | 65 | 0 |
| tmL   | 6605-6730 | 66 | 1 | tmL | 6617-6681 | 65 | 1 |
| nad3  | 6732-7085 | 354 | 5 | nad3 | 6683-7039 | 357 | 0 |
| cox1  | 7091-8626 | 1536 | 12 | AT-rich | 7040-7877 | 838 | 0 |
| trnW  | 8639-8703 | 65 | 0 | trnS2(AGN) | 7878-7949 | 72 | 0 |
| AT-rich | 8704-9405 | 702 | 0 | nad2 | 7950-9857 | 1008 | 3 |
| trnS2 (AGN) | 9406-9473 | 68 | -1 | cox1 | 8961-10493 | 1533 | 0 |
| nad2  | 9473-10480 | 1008 | 5 | tmW | 10494-10558 | 65 | 3 |
| cox2  | 10486-11166 | 681 | 14 | cox2 | 10562-11246 | 685 | 0 |
| trnD  | 11181-11245 | 65 | 0 | trnD | 11247-11312 | 66 | 0 |
| atp8  | 11246-11402 | 157 | 40 | atp8 | 11313-11471 | 159 | 5 |
| atp6  | 11443-12132 | 700 | 5 | atp6 | 11477-12169 | 693 | 1 |
| trnC  | 12138-12198 | 61 | 0 | trnC | 12171-12232 | 62 | 0 |
| trnM  | 12199-12263 | 65 | 0 | trnM | 12233-12296 | 64 | 0 |
| rrs   | 12264-13068 | 805 | 0 | rrs | 12297-13132 | 836 | 0 |
| trnV  | 13069-13130 | 62 | 0 | trnV | 13133-13200 | 68 | 0 |
| rml   | 13311-14308 | 1178 | 0 | rml | 13201-14448 | 1248 | 0 |
| trnL1(CUN) | 14309-14372 | 64 | 1 | trnL1(CUN) | 14449-14515 | 67 | 0 |
| trnL2(UUR) | 14374-14435 | 62 | 2 | trnL2(UUR) | 14516-14582 | 67 | 0 |
| nadA  | 14438-15361 | 924 | 4 | nadA | 14583-15513 | 931 | 0 |

*Negative numbers indicate that genes were overlapping
bGenes coding in L strand

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viridis and P. cf. peregrina. In general, the lack of a DHU arm in two serine tRNAs is a common condition in metazoan mtDNAs [17]. The presence of such aberrant tRNA genes in mitochondrial genomes could be due to modification of tRNA secondary structure by replication slippage [18], or selection for mitochondrial genome minimization [19].

The mtDNAs of nemerteans investigated to date all have 20 tRNAs on the L strand and 2 tRNAs on the H strand ([6-9]). Secondary structures of nemertean tRNAs are presented and compared in Figures 2 and 3 (pattern follows [20]). Table 3 presents the tRNA lengths and the percent of identical nucleotides (%INUC) for the six nemerteans.

Table 2 Base composition of the mtDNA in six nemerteans

| Species              | Total nt | T   | C   | A   | G   | A + T | AT skew | GC skew | References |
|----------------------|----------|-----|-----|-----|-----|-------|---------|---------|------------|
| Cephalothrix hongkongiensis | 16296    | 47.4| 10.2| 27.5| 14.9| 74.9  | -0.266  | 0.187   | [6]        |
| Cephalothrix sp.      | 15800    | 47.9| 10.0| 27.8| 14.3| 75.7  | -0.266  | 0.178   | [8]        |
| Pananemertes cf. peregrina | 14558    | 47.5| 10.0| 22.8| 19.7| 70.3  | -0.351  | 0.322   | [8]        |
| Nectonemertes cf. mirabilis | 15365    | 48.5| 10.5| 21.8| 19.2| 70.3  | -0.380  | 0.293   | Present study |
| Lineus viridis        | 15388    | 44.4| 11.9| 21.3| 22.4| 65.7  | -0.352  | 0.306   | [7]        |
| Zygeupolia rubens     | 15513    | 45.0| 9.8 | 21.0| 24.2| 66.0  | -0.364  | 0.424   | Present study |

Conservation was positively H strand-biased, but no other pattern could be identified with respect to location of tRNAs along the genome. Two of the three most conserved tRNAs, trnC and trnM, are adjoining, while the third, trnG, adjoins the moderately conserved trnE and is relatively close to the three least conserved genes, trnA, trnN and trnR (Figure 1, Table 1). As observed by others (e.g., [20]), there was no self-evident link between abundance of codon families and the level of tRNA conservation, with the most abundant codon families (Leu2, Ile and Phe) not having the highest %INUCs (see below).

A few mismatched nucleotide pairs (e.g., G-A, A-A, T-C, T-T) were found in the acceptor and/or the discriminator arms, without regard to the overall level of conservation of the tRNAs. As recently pointed out by Negrisolo et al. [20] for arthropods, metazoan mtDNAs commonly have such mismatches. It has been suggested that these may be corrected via RNA-editing mechanisms (e.g., [17]) or they may represent unusual pairings [21].

Among the most conserved tRNAs in nemerteans, as in insects (e.g., [20]), nucleotide substitutions are mostly confined to TΨC and DHU loops and extra arms (Figures 2, 3), with 2-7 fully compensatory base changes (cbc; e.g., G-C vs. A-T) or hemi-cbcs (e.g., T-A vs. T-G) on acceptor and anticodon stems (see [20,22]). As in insects [20], the number of cbcs and hemi-cbcs increased in stems as overall variation increased, especially in the TΨC stem.

As found in insects, cbcs and hemi-cbcs characterized either single species or taxa at a higher taxonomic rank. An example of the first type is the A-T pair found in the trnC acceptor arm of P. cf. peregrina, which was mirrored by G-C in all other nemerteans (Figure 2). Few substitutions were present among C. hongkongiensis and Cephalothrix sp. (Figures 2, 3). An example of a full cbc characterizing a unique family is the A-T pair found in the acceptor stem of trnL1s of family Lineidae (L. viridis and Z. rubens), while other taxa exhibited the G-C pair (Figure 2). Similarly, a full cbc in the anticodon stem of trnG of two hoplonemerteans characterizes another high-taxonomic rank (Figure 2). Figures 2 and 3 depict several more examples. This points to the potential phylogenetic value of tRNA sequences, as demonstrated for other animal groups (e.g., [20,23]), especially when secondary structures are taken into account [20]. While encouraging, clearly we need substantially more nemertean mitochondrial genomes to test this assertion for nemerteans.

The anticodon usage of N. cf. mirabilis and Z. rubens was congruent with the corresponding tRNA genes of other nemerteans, with one exception. The anticodon of the trnS2 (AGN) gene in N. cf. mirabilis, P. cf. peregrina and three Cephalothrix species is GCT, but it is TCT in L. viridis and Z. rubens. Cameron et al. [24] found that anticodon changes in trnS2 (AGN) (GCT→TCT) must have occurred in the common ancestor of the insect clade Ischnocera, which was consistent with its phylogeny of lice. Similarly, this may constitute a kind of “rare genomic change” [25] in nemerteans and be a synapomorphy of Lineidae.
As in all other metazoan mtDNAs sequenced to date, N. cf. mirabilis and Z. rubens mtDNAs contain genes for both small and large ribosomal subunit RNAs (rrnS and rrnL). Both genes are encoded by the same strand and are separated by trnV, as in many other metazoans. For N. cf. mirabilis and Z. rubens, respectively, the lengths of rrnL/rrnS are 1178/805 bp and 1248/836 bp, and the A + T contents are 75.5/72.4% and 70.9/70.5%.

Figure 2 Secondary structure of tRNA families (trnA-trnL) in nemertean mtDNAs. The nucleotide substitution pattern for each tRNA family was modeled using as reference the structure determined for Nectonemertes cf. mirabilis.
Base composition and codon usage

The mtDNA of many invertebrates is characterized by a composition bias showing high values of A% and T% over G% and C%. The overall A + T content of \textit{N. c. mirabilis} and \textit{Z. rubens} (70.3% and 66.0%, respectively) is consistent with those observed in other nemertean mitochondrial genomes. Though sample size for nemerteans is small, the A + T values appear to be linked in

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**Figure 3** Secondary structure of tRNA families (trnL2-trnV) in nemertean mtDNAs. The nucleotide substitution pattern for each tRNA family was modeled using as reference the structure determined for \textit{Nectonemertes c. mirabilis}.

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less (e.g., genus - e.g., *Cephalothrix* sp./*C. hongkongiensis*), as well as in more inclusive taxa (e.g., order - e.g., *P. cf. peregrina*/*N. cf. mirabilis*; *L. viridis/Z. rubens*) (Table 2). This might indicate a phylogenetic signal in nemerteans.

Another feature of metazoan mtDNAs is asymmetry in nucleotide composition between the two strands, with one being rich in A and C, and the other being rich in T and G [26]. This asymmetry also is evident in the two nemertean mtDNA genomes here, with the genes encoded on the coding strand showing a strong bias toward T over A and toward G over C, as seen in the four other nemerteans, which have similar skewnesses (Table 2; Figure 4). This situation is common for mitochondrial genomes [26] and may be due to the presence of asymmetric patterns of mutational changes between strands [27,28], and has been related with nucleotide deamination of DNA while transiently single-stranded during replication (this is not without controversy [29]) and/or transcription [30]. The relative importance of the two contributing processes (i.e., transcription vs. replication) remains to be assessed.

We follow the pattern of [2] for displaying codon family abundance and relative synonymous codon usage (RSCU) for available nemertean protein-coding genes (Figures 5 and 6). To avoid bias due to incomplete stop codons, all stop codons are excluded from the analysis. The six nemertean mtDNAs use similar total numbers

Table 3 Summary of multiple alignments of tRNA genes in nemertean mtDNAs

| ALN   | amino acid | alignment length | identical positions | %INUC |
|-------|------------|-----------------|---------------------|-------|
| trnA  | Alanine    | 72              | 21                  | 29.17 |
| trnC  | Cysteine   | 66              | 39                  | 59.09 |
| trnD  | Aspartate  | 66              | 39                  | 59.09 |
| trnE  | Glutamate  | 65              | 27                  | 41.54 |
| trnF  | Phenylalanine | 68        | 22                  | 32.35 |
| trnG  | Glycine    | 67              | 39                  | 58.21 |
| trnH  | Histidine  | 67              | 25                  | 37.31 |
| trnI  | Isoleucine | 72              | 28                  | 38.89 |
| trnK  | Lysine     | 73              | 23                  | 31.51 |
| trnL1 | Leucine (CUN) | 69        | 21                  | 30.43 |
| trnL2 | Leucine (UUR) | 68        | 28                  | 41.18 |
| trnM  | Methionine | 66              | 38                  | 57.58 |
| trnN  | Asparagine | 69              | 20                  | 28.99 |
| trnP  | Proline    | 67              | 24                  | 35.82 |
| trnQ  | Glutamine  | 70              | 28                  | 40.00 |
| trnR  | Arginine   | 67              | 16                  | 23.88 |
| trnS1 | Serine (UCN) | 71        | 23                  | 32.39 |
| trnS2 | Serine (AGN) | 73        | 20                  | 28.99 |
| trnT  | Threonine  | 71              | 23                  | 32.39 |
| trnV  | Valine     | 69              | 33                  | 47.83 |
| trnW  | Tryptophan | 70              | 27                  | 38.57 |
| trnY  | Tyrosine   | 68              | 32                  | 47.06 |

ALN, alignment name; %INUC, percent of identical nucleotides

*genes on the L strand
of non-stop codons (CDs), ranging from 3662 in *P. cf. peregrina* to 3707 in *L. viridis*. The codon families reveal a consistent pattern among the six nemertean species: the families with at least 50 CDs per thousand CDs (Leu1, Ile, Phe, Gly, Val) encompass an average 48.78% ± 1.33% of all CDs (Figure 5), with CDs rich in A + T favored over synonymous CDs of lower A + T content (Figure 6). For instance, the TTA codon accounts for a
large majority of CDs in the Leu1 family. Whereas representation of the Leu1 (average = 77.3 ± 7.3%) and Leu2 (average = 22.7 ± 7.3%) codon families in nemertean protein-coding genes differs greatly, that of Ser1 (average = 60.8 ± 7.3%) and Ser2 (average = 39.2 ± 7.3%) is less extreme.

The invertebrate mitochondrial genome codes for 62 amino-acid codons [10]. As pointed out for Lepidoptera...
[2], the total number of codons used seems to be linked to the A + T content, which is the case among the six nemertean genomes analyzed. Thus, Cephalothrix sp. mtDNA has the highest (A + T)% content (see Table 2) and uses only 58 codons, never using the four codons rich in G + C (TGG, CGC, ACG, CGC) (Figure 6). Lineus viridis mtDNA uses all 62 codons and has the lowest A + T% among known nemertean genomes.

The abundance of the four amino acid residues - Leu, Ile, Phe and Ser - is typical for invertebrate membrane proteins [2,31], and they account here for more than 46.70% (average A + T = 50.14 ± 2.70%) of residues comprising the 13 mitochondrial proteins. The Leu and Ile amino acids share hydrophobic lateral chains.

Two- and four-fold degenerate codon usage was similarly biased, with A/T favored over G/C in the third position (Figure 6) and in agreement with the AT-bias of protein-coding genes. Since the nemertean mitochondrial genome is AT-rich (Table 2), it can be expected that codons ending in A or T will predominate. From the overall RSCU values, it could be assumed that compositional constraints are the factor in shaping variation in codon usage among the genes in these mitochondrial genomes.

Non-coding regions
Metazoan mtDNAs usually have lengthy non-coding regions varying in size from ~100 bp to > 20 kbp [32,33]. The mtDNAs of N. cf. mirabilis and Z. rubens contain a large number of unassigned nucleotides. There are 23 non-coding regions, with up to 855 nts, found throughout the N. cf. mirabilis mitochondrial genome. The AT-rich region located between the nad3 and trnS2 genes accounts for 838 nts and its AT content is 81.5%, which is higher than the remainder of the genome. Zygeupolia rubens has up to 879 non-coding nts distributed in 15 regions. The AT-rich region located between trnW and trnS2 genes is 702 nts and has an AT content of 74.9%, which also is higher than the remainder of the genome.

In most metazoan mtDNAs, the largest non-coding region is thought to contain signals for replication and transcription, and is thus referred to as the control region [11]. The non-coding region has an increased AT composition, a characteristic typically used to identify origins of replication [10]. As in mtDNA genomes of other nemerteans, the AT-rich regions of N. cf. mirabilis and Z. rubens mtDNAs have the potential to form secondary structures such as stems and loops (Figure 7), which are thought to play an important role in the early stages of the replication and transcription process [34,35]. Additionally, the AT-rich region in mtDNA of N. cf. mirabilis contains the tandemly repeated sequences (AAAAATATAAGATTTTTCAATTTCCAAAAATATAAT)_3, (TTTTG)_10, (TTTTTC)_7, and several (A)_n and (T)_n homopolymer tracts. In mtDNAs of Z. rubens, we found the tandemly repeated sequences (GGGGGGGGGGTGATGTTATGTGTTTTACTACAATCTTTAGAAAAATATAAA)_2, (TTTTTTTGT)_10, and (TTTTTTTTTA)_6. Similar tandem repeat units within the largest non-coding regions also were found in the nemerteans Cephalothrix sp. [8], and C. hongkongiensis [6]. Tandem repeats are common within the control region of animal mtDNAs [34] and might be associated with regulatory mechanisms and recombination hot spots, and they might be the result of replication slippage events [36]. The high AT content and the predicted secondary structures of the AT-rich non-coding region of the N. cf. mirabilis and Z. rubens mtDNAs suggest that this region most likely contains the control region, though the control region in invertebrates, unlike that of vertebrates, is not well characterized and lacks discrete and conserved sequence blocks used in identification [37]. The nemertean mtDNA sequences examined here had multiple non-coding regions throughout their genomes. However, the location of the largest non-coding region is not conserved, and there is no obvious conservation of size (e.g., [6,8]), nucleotide identities or potential secondary structures for the nemertean non-coding regions.

Gene order comparison
Gene arrangements of the animal mitochondrial genome usually remain stable over long periods of evolutionary time, especially for protein-coding genes [10]. With some exceptions, mitochondrial gene order is relatively stable within major groups, and more variable between
and hoplonemerteans share the adjacency selected species. Based on both gene sets, the heteronemerteans share the adjacency pattern of Bilateria and the other species except U. caupo and atp8/atp6 adjacency pattern.

Comparison with the putative bilaterian ground pattern of Bilateria and with the selected species (Figure 8; Additional file 1: Figure S1). The same adjacencies may be a common plesiomorphic feature of Lophotrochozoa, such as Mollusca, Brachiopoda, and also Arthropoda mitochondrial genomes (e.g., [10]; [44]). However, neither of the latter two adjacencies is present in two Cephalothrix species, nor in the brizoan Flustrellidra hispida [45].

In addition to visual comparison of genome maps, we analyzed gene order data with CREx [38], determining the number of common intervals. As shown in Table 4, the nemerteans share the highest number of common intervals (1124) with Bilateria and with the selected species except U. caupo and other species except U. caupo. The adjacency H/nad5 is shared by nemerteans and the selected species. Based on both gene sets, the heteronemerteans share the adjacency nad6/cob with K. tunicata [14], P. psammophila [41], P. excavatus [42], U. caupo [43], and S. nudus [44] and they share the adjacency atp8/atp6 with T. retusa, K. tunicata and the putative ground pattern of Bilateria (Figure 8; Additional file 1: Figure S1). These adjacencies may be a common plesiomorphic feature of Lophotrochozoa, such as Mollusca, Brachiopoda, and also Arthropoda mitochondrial genomes (e.g., [10]; [44]). However, neither of the latter two adjacencies is present in two Cephalothrix species, nor in the brizoan Flustrellidra hispida [45].

Figure 8 shows tRNA genes change relative position much faster than the protein-coding and rRNA genes, as reported from previous studies (e.g., [46,47]).

Excluding tRNAs, the gene order of heteronemerteans is identical to that of T. retusa [40] and some gastropods, e.g., Conus textile [48], Ilyanassa obsoleta [49], Thais clavigera [37] and Lophiotoma cerithiformis [50]. Other molluscs, like the polyclacophoran K. tunicata [14], the gastropod Haliotis rubra [51] and the cephalopod Octopus vulgaris [52] show a similar gene order, but are distinguished by a large inversion of much higher complexity that is not significant, nemerteans and T. retusa, K. tunicata, and P. excavatus yield the highest numbers (18-20) in comparison with the putative bilaterian ground pattern.

Phylogenetic analysis
We performed a phylogenetic analysis based on nucleotide sequences of protein-coding genes to better understand relationships within the Nemertea. The phylogenetic tree in Figure 9, reconstructed by maximum likelihood and Bayesian analyses, indicates that similar gene arrangements reflect close phylogenetic affinity. This supports previous hypotheses that Hoplonemertea and Heteronemertea are sister taxa (e.g., [53-55]). However, a phylogenetic analysis based on amino acid sequences (data not shown) suggests Hoplonemertea as sister group to Palaeonemertea. This contradicts many but not all previous analyses (e.g., [55]). We consider it unsupported by our data, given the low Bayesian posterior probability (0.61) for this clade. This suggests, however, that amino acid sequence data deserve continued attention in future analyses with new, additional data.

Conclusion
To date, complete or nearly complete mtDNA sequences have been determined for seven nemerteans, a very small sampling compared to those available for vertebrates or
Figure 8 Mitochondrial gene order (all 37 genes) of Nemertea and selected lophotrochozoan species and the putative bilaterian ground pattern (according to [39]). Gene segments are not drawn to scale. All genes are transcribed from left to right except those in gray, which are transcribed from right to left. Unsequenced regions are in black. The adjacencies nad6/cob and atp8/atp6 are underlined. Previous gene orders from the following references: Cephalothrix [6,8], Lineus [7], Paranemertes [8], Terebratulina [40], Katharina [14], Phoronis [41], Perionyx [42], Urechis [43], Sipunculus [44].
arthropods. The two new mtDNA genomes, for *Nectonemertes cf. mirabilis* and *Zygeupolia rubens*, share substantial similarity with those of other nemertean mitochondrial genomes, and gene content and A + T richness is similar to those common for animal mtDNAs.

There is strong skew in the distribution of nucleotides between the two strands.

The evolution of nemertean tRNAs seems to have been variable both in terms of sequence conservation and nucleotide substitution processes. The presence of full and hemi-cbcs characterizing taxa at different taxonomic levels may indicate the potential phylogenetic value of tRNA sequences.

Nemertean mtDNAs are punctuated by non-coding portions highly variable in size. Among them, the AT-rich non-coding region, which appears to be a fast-evolving genomic region characterized by short to medium-size repeated motifs/AT-rich patterns, may be associated with the initiation of replication or transcription.

Phylogenetic analysis supports the close phylogenetic affinities in nemerteans one might infer from similarities in gene arrangements, with Hetero- and Hoplonemerteans as sister-clades. Gene order analysis suggests that the heteronemertean pattern most closely resembles the likely ancestral condition among nemerteans, which is counterintuitive in light of the phylogenetic analysis. Confidence that we understand evolution of the nemertean mitochondrial genome is likely to require investigating many more nemertean mtDNAs, especially a full representation of palaeonemertean diversity.

**Methods**

**DNA extraction, PCR and sequencing**

Specimens were collected off Point Conception, California (*Nectonemertes cf. mirabilis*) and at Fort Pierce, Florida (*Zygeupolia rubens*), USA. We use the “cf.” qualifier to confer reasonable caution that the Pacific worm traditionally designated *N. mirabilis* (see [56]) is conspecific with its presumed cognate originally described from the North Atlantic Ocean. Samples were frozen in liquid nitrogen and preserved in RNAlater. Total DNA was extracted from a single individual specimen using the DNeasy Tissue Kit following the manufacturer's protocol (Qiagen, Valencia, CA, USA). PCR primers used for amplification are listed in Table 5. Preliminary nemertean-specific primers (N12SF, N16SR, NCOX2R) were designed based on sequence alignment of four mitochondrial genome sequences (*Cephalothrix honkgiensis, Cephalothrix sp.*, *Lineus viridis*, and *Paranemertes cf. peregrina*) retrieved from Genbank. For both species, the partial regions *rrnS-rrnL* and *rrnL-cob* lack several tRNAs.

**Table 4** Pairwise common interval distance matrix of mitochondrial gene orders of nemerteans, the putative bilaterian ground pattern and six other lophotrochozoans *

| Common interval | B   | P   | H   | H   | Tr  | Kt  | Uc  | Sn  | Pe  | Pp  |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Bilaterian ground pattern (B) | 204 | 1326 | 18  | 20  | 20  | 18  | 20  | 12  | 14  | 20  |
| Palaeonemertean (P) | 44  | 204 | 1326 | 108 | 112 | 40  | 154 | 28  | 42  | 38  | 142|
| Heteronemertean (H) | 52  | 86  | 204 | 1326 | 1124 | 68  | 184 | 18  | 64  | 56  | 230|
| Hoplonemertean (H*) | 44  | 72  | 178 | 204 | 1326 | 84  | 212 | 18  | 68  | 66  | 254|
| Terebratulina retusa (Tr) | 52  | 86  | 204 | 1326 | 128 | 20  | 74  | 82  | 110 |
| Katharina tunicata (Kt) | 48  | 56  | 106 | 94  | 106 | 204 | 1326 | 20  | 62  | 64  | 266|
| Urechis caupo (Uc) | 16  | 8   | 14  | 8   | 14  | 34  | 204 | 1326 | 54  | 144 | 22 |
| Spiculorus nudus (Sn) | 34  | 12  | 22  | 16  | 22  | 26  | 26  | 204 | 1326 | 158 | 38 |
| Perionyx excavatus (Pe)* | 28  | 24  | 40  | 32  | 40  | 48  | 44  | 60  | 204 | 1254 | 44 |
| Phoronis psammophila (Pp)* | 40  | 48  | 84  | 76  | 84  | 98  | 22  | 24  | 38  | 204 | 864*|

*Bold numbers represent pairwise common interval distances between mitochondrial gene orders (37 genes in total), while italic numbers represent pairwise common interval distances between mt gene orders without tRNAs (15 genes in total)

*a lacks tRNA

*b lacks several tRNAs

![Figure 9 Best tree from the Maximum Likelihood analysis with 5921 nt (first and second codon positions) of protein-coding genes. Node support is indicated above (Bayesian posterior probabilities) and below (maximum likelihood bootstrap values) each branch. A Bayesian analysis resulted in the same species topology.](image-url)
were amplified first. For *N. cf. mirabilis*, partial fragments of *cox1* and *cox3* genes were amplified using universal PCR primers LCO-2198/HCO-1490, *cox3F/cox3R* ([59]; [9]). These sequences were used to design specific primers to amplify the remaining mitochondrial genome fragments (*cob-cox3, cox3-cox1* and *cox1-rrnS*). For *Z. rubens*, the fragment of *cox1-cox2* was amplified using the universal primer LCO-2198 [59] combined with the specific primer NCOX2R. Based on sequences obtained, specific primers were designed to amplify the regions *cox2-rrnS, cob-cox3* and *cox3-cox1*. Conventional PCR and long PCR, cloning, and sequencing were performed as described in Chen et al. [6,8].

### Sequence assemblage and annotation

All sequences were checked and assembled by visual inspection using the program SeqMan (DNA star, Madison, WI, USA). Protein-coding genes and ribosomal RNA genes were identified by their similarity to previously reported mitochondrial genomes of *Cephalothrix*

| Primer name | Sequence (5’ → 3’) | References |
|-------------|--------------------|------------|
| Universal   |                    |            |
| rrnS-rrnL   | TGTGCCAGCTTCGCCGTTATAC | Present study |
| N12SF      | ACGCTGTTATCCTATGTA | Present study |
| N16SR      | GCRTAWGCRAAWARRATAYCAYTCCWGG | [58] |
| mt-cob     |                   |            |
| 16SarL     | CGCCTGTATTCAAAAACAT | [57] |
| CytB R     |                   |            |

**Nectonemerteis cf. mirabilis**

| Primer name | Sequence (5’ → 3’) | References |
|-------------|--------------------|------------|
| cox1        |                    |            |
| LCO-1490    | GGTCAAAACATTAAAAAGATAT | [59] |
| HCO-2198    | TAAACTTCAGGGTGAACAAAATCA | [59] |
| cox3        |                    |            |
| cox3F       | TCGGWTCAGGWWAATAATTATTTATT | [8] |
| cox3R       | ACCAAGCGAGCCTCCTAAAAAC | [8] |
| cob-cox3    |                    |            |
| Nm cobF     | TCGGTGTTAATGCTACTTGT | Present study |
| Nm COX3R    | ACCAAGCGAACAATAAGC | Present study |
| cox3-cox1   |                    |            |
| Nm COX3F    | TGGTGGCTCTCTGTGTAGT | Present study |
| Nm COX1R    | GAGCCTTCTTCAAACACACA | Present study |
| cox1-rrnS   |                    |            |
| NmCOX1F     | AATCTGTCTGGETGGTGGCACCCTGCTGA | Present study |
| Nm12SR      | GACTCCCCTGAAAGGACATAAAACCA | Present study |

**Zygeupolia rubens**

| Primer name | Sequence (5’ → 3’) | References |
|-------------|--------------------|------------|
| cob-cox3    |                    |            |
| ZrCOB F     | CTGGTGGTGTGGTTGGTT | Present study |
| ZrCOX3R     | GTTGAAACCATAATCCCAT | Present study |
| cox3-cox1   |                    |            |
| cox3F       | TGGTGGCTCTCTGTGTAGT | Present study |
| ZrCOX1R     | GAGCCTCCTTCAAACACACA | Present study |
| cox1-rrnS   |                    |            |
| ZrCOX2F     | TITGGCTTTTACCTTCTTGTG | Present study |
| Zr12SR      | AAATAAGACACCCCGAAGT | Present study |
hongkongiensis (GenBank #NC_012821), C. rufifrons (EF140788), Cephalothrix sp. (NC_014869), Lineus viridis (NC_012889), and Paranemertes cf. peregrina (NC_014865). Most tRNAs were identified by using invertebrate mitochondrial codon predictors with tRNAscan-SE 1.21 [60]. The remaining tRNA genes were found by inspecting sequences for tRNA-like secondary structures and anticodons. Multiple alignments of tRNA genes were produced, and the percent of identical nucleotides (%INUC) was calculated for six nemertean tRNA sequences. Secondary structures within the non-coding fragments were visualized by using RnaViz 2.0 [61], and the mitochondrial genome was visualized using CGView [62].

Genomics analysis
Nucleotide composition and Relative Synonymous Codon Usage (RSCU) values were analyzed with MEGA 4.0 [63]. AT- and GC-skew were determined by using the formulation of [26].

Gene order comparisons
Gene orders were compared between all available nemerteans (see above), the putative bilaterian ground pattern [39], Terebratulina retusa [40], Katharina tunicata [14], Phoronis psammophila [41], Perionyx excavatus [42], Urechis caupo [43] and Sipunculus nudus [44]. The gene orders were compared with two different gene sets: “all genes” included all 37 mitochondrial genes, whereas “non-tRNA genes” included only the two ribosomal genes and the 13 protein-coding genes.

The differences between gene orders were analysed using common intervals [38], breakpoints [64] and reversal distances [65] implemented in the CREx tool [38].

Phylogenetic analysis
The currently available near-complete and complete mitochondrial nemerteans genome data (Cephalothrix sp., C. hongkongiensis, L. viridis, and P. cf. peregrina, but not the partial genome sequence of C. rufifrons) were combined with sequences from this study for phylogenomic analyses. The nucleic acids for all 12 protein-coding genes (except atp8, which is shortest and least conserved between the taxa) were aligned using Clustal X [66] with the default settings. Ambiguously aligned portions of both alignments were excluded using Gblocks version 0.91b [67] with default block parameters. We also excluded third codon positions because of the fast substitution rate. The total number of nucleotides used for the phylogenetic analysis was 5921.

Based on previous studies of metazoan relationships (e.g., [68-73]), the following six species were selected as outgroups: a mollusc (Katharina tunicata), a brachiopod (Terebratula retusa), a phoronid (Phoronis psammophila), and three annelids (Perionyx excavatus, Sipunculus nudus and Urechis caupo).

Phylogenetic trees were estimated under maximum likelihood (ML) and Bayesian inference (BI). ML analysis on the combined nucleotide dataset alignments was performed in RAxML 7.2.7 [74,75] available on the CIPRES web portal [76] with the GTRGAMMA substitution model. Support was estimated by performing 1000 bootstrap replicates. BI analysis was performed with MrBayes version 3.0b4 [77,78], using GTR + I + G model, a best-fit model selected by MrModeltest v2.2 [79] following the Akaike information criterion (AIC). The Monte Carlo Markov chain (MCMC) length was 1,000,000 generations and sampled every 100 generations. The first 2500 trees from each run were discarded as a burn-in.

Amino acid sequences were aligned with both Clustal X [66] and MAFFT using the G-INS-i strategy [80]. BI analyses were performed with MrBayes version 3.0b4 [77,78] with the mtRev + I + G model, selected by Repeat 10.2 [81] as optimal. We also implemented the CAT + GTR model in PhyloBayes 3 [82]. The ML analysis was carried out with RAxML 7.2.7 [74,75] with CAT model.

The mitochondrial genome sequences of N. cf. mirabilis and Z. rubens are deposited in GenBank under the accession numbers HQ997772 and HQ997773.

Additional material

Additional file 1: Figure S1. Mitochondrial gene order (protein-coding genes and tRNAs only) of Nemertea and selected lophotrochozoans species and the putative bilaterian ground pattern (according to [39]). Gene segments are not drawn to scale. All genes are transcribed from left-to-right except those in gray, which are transcribed from right to left. The adjacencies nad6/cob and atp8/cob are underlined. The translocation of nad2 in the heteronemerteans and hoplonemerteans is highlighted by *. Gene orders according to the following references: Cephalothrix (68), Lineus (7), Paranemertes (8), Terebratulina (40), Katharina (14), Phoronis (41), Perionyx (42), Urechis (43), Sipunculus (44).

Additional file 2: Table S1. Pairwise breakpoint distance matrix of mitochondrial gene orders of nemerteans, the bilaterian ground pattern and six other lophotrochozoans*

Additional file 3: Table S2. Pairwise reversal distance matrix of mitochondrial gene orders of nemerteans, the bilaterian ground pattern and six other lophotrochozoans*

Abbreviations
atp6 and atp8: ATP synthase subunits 6 and 8; cob: cytochrome b; cox1-3: subunits I-III of cytochrome c oxidase; nad1-6 and nad4L. NADH dehydrogenase subunits 1-6 and 4 L; mll and rrnS: the large and small subunits of ribosomal RNA; tmk: genes encoding for transfer RNA molecules with corresponding amino acids denoted by the one-letter code and codon indicated in parentheses (xxx) when necessary; DHU: dihydrouridine loop; MDNA: mitochondrial DNA; NC: non-coding region; PCR: polymerase chain reaction; Kb: kilobase; bp: base pair; nt: nucleotide; nucleotide symbol combination: R = A/G, Y = C/T, W = A/T, K = G/T; N = A/G/C/T.
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Authors’ contributions
HXC performed the majority of the molecular experiments and analyzed the data, and drafted the manuscript. SCS supervised the research. PS contributed to the analysis of the data. WCR performed part of the study, and provided technical assistance. JLN collected specimens, conceived, designed the research plan and did significant revisions of the manuscript draft. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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