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Short Communication

The complete mitochondrial genome of the demosponge *Negombata magnifica* (Poecilosclerida)

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1. Introduction

Great uniformity exists among mitochondrial genomes of bilaterian animals (e.g., chordates, mollusks, arthropods). The typical bilaterian mitochondrial genome (mt-genome) is a compact molecule of 13–16 kilobase pairs (kbp), containing 13 protein-coding genes, 22 transfer RNA (tRNA) genes, and 2 ribosomal RNA (rRNA) genes. Sponges are among the first diverging animal phyla. They have larger (18–25 kbp) mt-genomes than the typical bilaterian mt-genome and usually contain an extra protein-coding gene, the *atp9* gene (reviewed in Lavrov, 2007). Because sponges exhibit an interesting intermediate state of genome size between the large 76 kbp choanoflagellates mt-genome (Burger et al., 2003) and the typical bilaterian mt-genome, studying their mitochondrial evolution is important for understanding such processes as mitochondrial to nucleus gene transfer and evolution of early metazoans (reviewed in Lavrov, 2007). One remarkable characteristic of sponge mt-genome is the variable number of tRNA genes among sponges. Three processes are responsible for this variability: tRNA loss, reported in glass sponges (Haen et al., 2007); tRNA gene duplication, reported in demosponges (Wang and Lavrov, 2007); and tRNA gene recruitment, also reported in demosponges (Lavrov and Lang, 2005). In the latter, a tRNA gene is duplicated, and one of the duplicates undergoes substitutions that alter the anticodon and the acceptor site (the amino acid binding site), resulting in a tRNA that recognizes a different codon and binds the corresponding amino acid. Such a process was reported in the mt-genome of the sponge *Axinella corrugata* and it was suggested that this phenomenon is general and can account for the generation of tRNA variability (Lavrov and Lang, 2005). However, the hypothesis that such a process is common in sponge mitochondria evolution currently relies on very few supporting data since tRNA recruitment was only shown in one out of the seven sponge mt-genomes publicly available.

Here we present the complete mt-genome of the sponge *Negombata magnifica*. It is the first sequenced representative of the order Poecilosclerida, which is the most specious and the most diverse in morphological characteristics among Porifera (Hooper and Van Soest, 2002a).

2. Material and methods

2.1. Mitochondria isolation

A specimen of *N. magnifica,* a common sponge in the Red Sea (Ilan, 1995), was collected by SCUBA diving at a depth of 12 m in front of the Interuniversity Institute for Marine Sciences (Eilat, Israel). The sample was lyophilized and kept at −20 °C. Sponge cells were later separated from spongin and skeleton elements by placing 0.7 g of lyophilized sponge tissue in ice-cold calcium magnesium-free artificial sea water (NaCl 26.22 g/l, KCl 0.67 g/l, Na_2SO_4 4.62 g/l, $NaHCO_3$ 0.21 g/l, Na_2-EDTA 0.37 g/l at pH 8.0) and squeezing it through gauze. The solution containing the sponge cells was centrifuged at 1000*g* and 4 °C for 10 min. To disrupt the cells, the pellet obtained was homogenized in a glass—Teflon Potter-Elvehjem homogenizer using 4 ml of ice-cold homogenization buffer (TEK buffer: 0.25 M sucrose, 10 mM Tris–HCl, pH 7.6, 1 mM EDTA, 0.24 M KCl). To avoid the amplification of nuclear DNA sequences of mitochondrial origin, mitochondria were then separated from cell debris and nuclei

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using a sucrose gradient, following Meisinger et al. (2000). Finally, the mtDNA was extracted following Arnason et al. (1991).

2.2. DNA sequencing and gene identification

Primers were designed to match conserved regions of animal and choanoflagellate genomes in the *cox1, cox3, nad5*, and *rnl* genes (Appendix A, Table S1) Partial sequences of these genes were obtained, and based on these sequences, specific primers were designed in the *nad5* and *cox3* genes:

ND5d3 5′-GGTTGAGAAGGAGTGGGCTTATGTTCTTA-3′;

ND5r3 5′-CACCCCTACTGTAGCCGCAAAAAGAGC-3′;

COXIIId2 5′-CTTTAATTGGGGCTTGTGGGGCATTTTTTA-3′.

COXIIIr2 5′-CCACTAATTATTCCATGATGCGCCCAAGTTAC-3′;

The complete genome was then amplified in two overlapping fragments (ND5d3/COXIIIr2: ~9.5 kbp; COXIIId2/ND5r3: ~12 kbp). These two fragments were then reamplified into six smaller overlapping fragments using specific primers (Appendix A, Table S2). The six fragments were directly sequenced on both strands using primer walking. Sequence reads were assembled with the program Chromas Pro (Technelysium) and were verified manually.

Protein-coding genes were identified using the ORF-Finder tool of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/). The tRNAscan-SE 1.23 program was used to identify and infer the secondary structures of tRNA genes (Lowe and Eddy, 1997). Manual sequence alignment with other sponge sequences was used to identify rRNA genes. Compositional asymmetry was computed following (Perna and Kocher, 1995). The complete mitochondrial sequence of *N. magnifica* has been submitted to EMBL under accession number: AM420314.

2.3. Phylogenetic analysis of tRNA sequences

As tRNA genes show a high evolutionary rate, their automated alignment using multiple-sequence alignment programs such as ClustalX (Thompson et al., 1997) or MAFFT (Katoh et al., 2005) is not reliable. Hence, tRNAs from *N. magnifica*, and from previously sequenced demosponge mt-genomes (*Axinella corrugata* NC_006894, *Geodia neptuni* NC_006990, *Tethya actinia* NC_006991, *Oscarella carmela* NC_009090, and *Amphimedon queenslandica* NC_008944), were manually aligned taking into account their predicted secondary structure. Following Lavrov and Lang (2005), nucleotides of the anticodons and variable length portions of the extra loop were excluded from the analysis. The tRNA data set included 143 sequences and 68 characters, 62 of which were parsimony informative and two invariant. A neighbor-joining tree based on uncorrected pairwise distances (i.e., *p*-distances) between each pair of tRNA genes was reconstructed using PAUP* 4.0b10 program (Swofford, 2003). Bootstrap percentages (BP) based on 1000 replicates were computed to evaluate branch supports.

2.4. Phylogenetic analysis of protein-coding genes

Amino acid sequences of each mitochondrial protein-coding gene of *N. magnifica* (not including *atp9*) and seven other sponge species were aligned twice using two different software, MAFFT v. 6.240 (Katoh et al., 2005) with L-INS-i iterative refinement method, and ProbCons v. 1.12 (Do et al., 2005) with default parameters. For each gene, the two alignments were compared with the SOAP v. 1.2a4 program (Loytynoja and Milinkovitch, 2001) and only positions that were identically aligned by these two programs were kept for the phylogenetic analyses. Additionally, positions in which gaps are present in more than 25% of the taxa were manually removed. The protein-coding gene data set included 3619 characters, 1386 of which were parsimony informative and 1356 invariant.

The concatenated alignment of all genes was analyzed using Maximum Likelihood (ML) and Bayesian analyses. For the ML analysis, the best three models of sequence evolution were identified with the ProtTest 1.3 program (Abascal et al., 2005). For the Bayesian analysis we used the CAT model (Lartillot and Philippe, 2006). Two programs were used for ML tree reconstructions, the Leaphy 1.0 program (Whelan, 2007) under the JTT+F+Γ_4 model of sequence evolution and the TREEFINDER program version of May, 2007 (Jobb et al., 2004) under the mtART+F+Γ_4 and cpREV+F+Γ_4 models. The α-parameter was estimated using ML. Branch statistical support was obtained after 100 bootstrap replicates. Bayesian analysis under the CAT+Γ_4 model was performed using the program Phylobayes 2.1c (Lartillot and Philippe, 2006). Three chains were run for 80,000 cycles and trees were sampled every 100th cycle after the first 5000 cycles (the maximum difference in bipartitions between the three runs was 0.055).

3. Results and discussion

3.1. Genome organization and gene arrangement

The map of the complete nucleotide sequence of the mt-genome of *N. magnifica* is presented in Fig. 1A. The genome is 20,088 bp long, resembling those of other demosponges both in size and in composition (Erpenbeck et al., 2007; Lavrov et al., 2005; Lavrov and Lang, 2005; Wang and Lavrov, 2007). It contains all coding genes found in typical bilaterian mtDNA genomes: cytochrome oxidase subunits (*cox1, cox2*, and *cox 3*), apocytochrome *b* (*cob*), reduced nicotinamide adenine dinucleotide ubiquinone oxireductase subunits (*nad1, nad2, nad3, nad4, nad4L, nad5*, and *nad6*), and ATP synthase subunits (*atp6* and *atp8*). Similar to other sponge mt-genomes except

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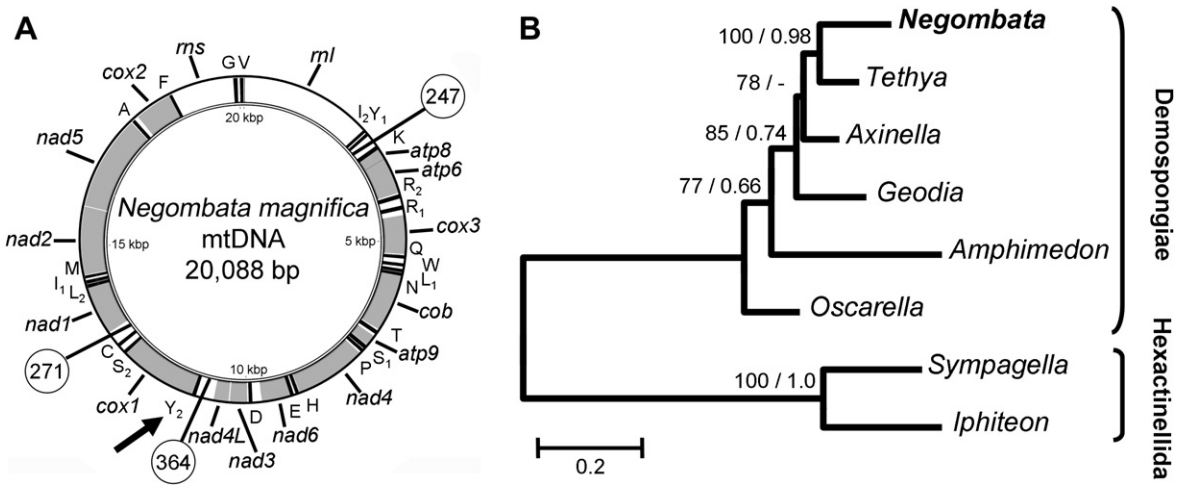

Fig. 1. (A) Genetic map of *Negombata magnifica* mtDNA. Protein-coding genes are in grey, rRNA genes are in white, and tRNA genes are in black and are labeled by the one-letter code for their corresponding amino acid. The lengths in nucleotide of the three largest none-coding regions are indicated by circles. The position of the *trnY*$_2$*(aua)* is indicated by an arrow. (B) ML tree under the JTT+Γ_4+F based on concatenated aligned mitochondrial protein sequences of sponges. For each node the ML bootstrap percentage (BP) and the Bayesian posterior probabilities (PP) under the CAT model are given at the left and right of the slash, respectively. A dash indicates that this node was not recovered in the Bayesian tree. Glass sponge sequences (Hexactinellida) were used as outgroup.

Amphimedon queenslandica (Erpenbeck et al., 2007), it also includes the *atp9* gene. This gene is absent in other metazoan phyla, but present in the metazoan outgroups choanoflagellates and fungi (reviewed in Lavrov, 2007). The genome also contains the genes for the small and large ribosomal RNAs (*rns* and *rnl*). Interestingly, the genome contains 25 tRNA sequences, only 24 of which have been found in other sponges. An extra tyrosine tRNA is thus a characteristic of the *N. magnifica* mt-genome (see Discussion below). All the genes are positioned on the heavy strand and are transcribed clockwise. No intron or extra genes (except *atp9*) were found, although it has been shown that introns are present in the *cox1* gene of *Tetilla* and *Plakotrix* (Lavrov, 2007; Rot et al., 2006) and that the genome of *Oscarella* contains a putative gene for the C subunit of the twin-arginine translocase (Wang and Lavrov, 2007).

The *N. magnifica* mt-genome is compact: its coding regions constitute 89.8% of the genome, while the intergenic regions constitute the remaining 10.2%. This compactness is further emphasized by the presence of four pairs of overlapping genes: (1) *atp8/atp6* (7 bp overlap); (2) *trnE(ttc)/nad6* (3 bp); (3) *cox1/trnS(tga)* (10 bp); (4) *cox2/trnF(gaa)* (10 bp). The intergenic regions, which comprise 2065 bp, are divided into 34 segments with lengths between 1 and 364 bp. Seven of these regions are longer than 100 bp. A BLAST analysis revealed that the non-coding areas of *N. magnifica* mt-genome do not share significant similarities to any known sequences, including non-coding areas of other sequenced poriferan mt-genomes, probably reflecting the lack of strong purifying selection in these regions.

The gene arrangement of protein-coding genes and rRNA is similar in all sequenced demosponge mt-genomes, with the exception of *O. Carmella,* which shows evidence of several rearrangements relative to *A. corrugata* and *G. neptuni* as described in Wang and Lavrov (2007). *A. corrugata* and *G. neptuni* have an identical gene arrangement: *rnl, cox2, atp8, atp6, cox3, cob, atp9, nad4, nad6, nad3, nad4L, cox1, nad1, nad2, nad5, and rns*. *T. actinia* differs from this arrangement by a single modification: *nad6* is located downstream to *rnl* and upstream to *cox2*. *A. queenslandica* differs from *A. corrugata* and *G. neptuni* by a single translocation: the part of the genome including *nad4L, cox1, nad1, nad2,* and *nad5* was translocated to a position downstream to *rnl* and upstream to *cox2*. Similarly, *N. magnifica* differs from *A. corrugata* and *G. neptuni* by a single rearrangement: *cox2* is downstream to *nad5* and upstream to *rns*. It is worth noting that all rearrangements are located near the *rnl* gene (except for *Oscarella* where there is more than one translocation). We thus speculate that in demosponges the region downstream to this gene is more prone to gene rearrangements.

In contrast to rRNA and protein-coding genes, tRNA order is highly variable among sponges. The rapid rate of tRNA rearrangements and the presence of the tRNA recruitments (see below) make it difficult to reliably reconstruct the sequence of events for the rearrangements of tRNA genes.

3.2. Base composition and compositional asymmetry

The A+T content of the *N. magnifica* mt-genome is 67.2%. This value is within the range of other demosponge genomes. The nucleotide composition of the coding strand is 37.5% T, 10.4% C, 29.7% A, and 22.4% G. The two DNA strands thus have different nucleotide compositions. The heavy strand of the *N. magnifica* has a positive GC

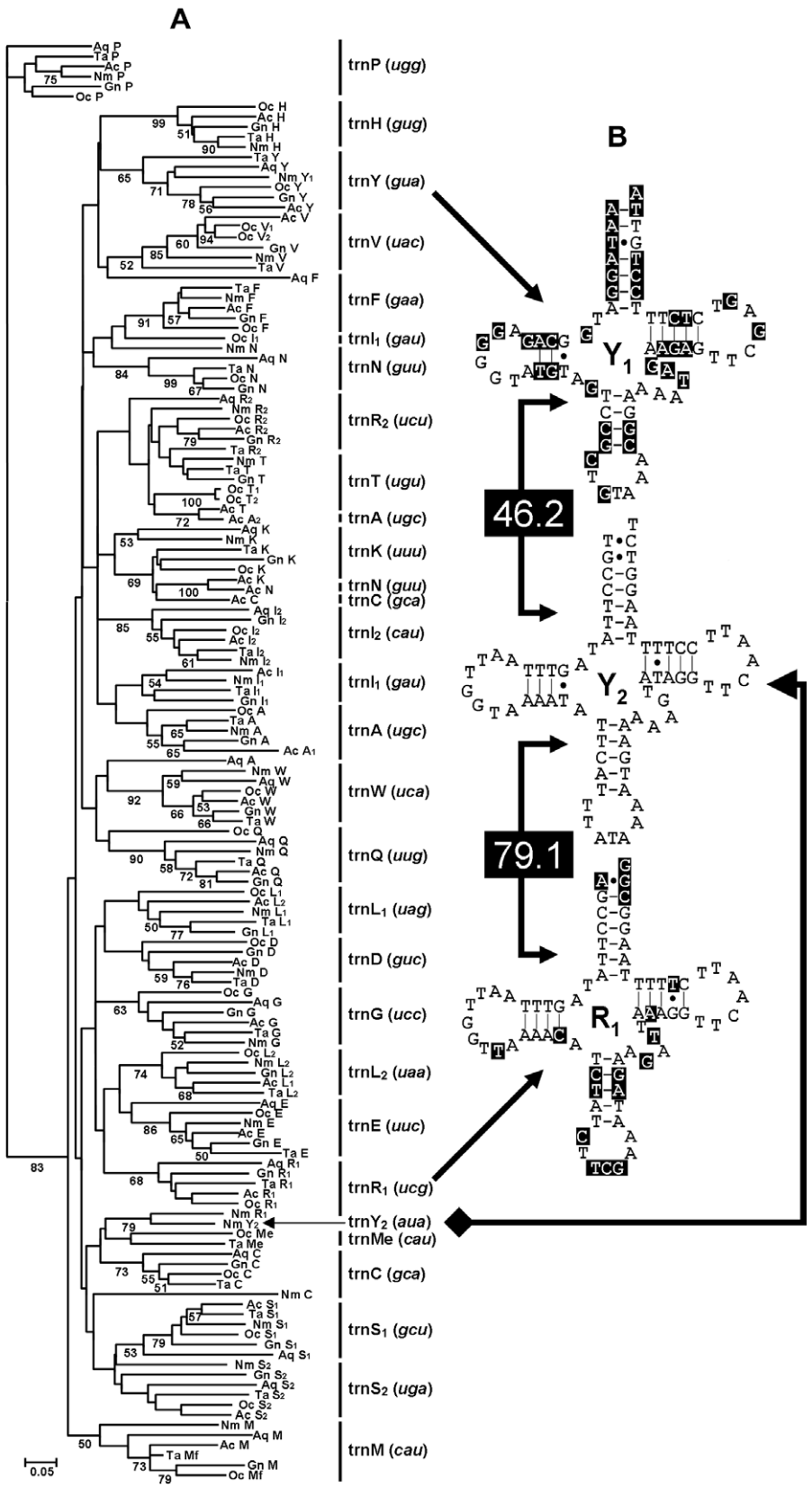

Fig. 2. (A) Neighbor-joining tree based on *p*-distances among mitochondrial tRNA genes from *Negombata magnifica* (Nm), *Axinella corrugata* (Ac), *Geodya neptuni* (Gn), *Tethya actinia* (Ta), *Amphimedon queenslandica* (Aq), and *Oscarella carmela* (Oc). The arrow points to *trnY*$_2$*(aua),* which is inferred to have evolved via gene recruitment. Bootstrap support values above 50% are indicated below the corresponding branches. (B) Predicted secondary structure of *Negombata magnifica trnR*$_1$*(ucg), trnY*$_2$*(aua),* and *trnY*$_1$*(gua)* genes. Percentage of pairwise sequence identity between tRNA genes is indicated inside the arrows. Highlighted bases on *trnR*$_1$*(ucg)* and *trnY*$_1$*(gua)* denote nucleotide substitutions when compared to *trnY*$_2$*(aua)*.

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skew (0.37) and a negative AT skew (−0.12). The GC skew is similar between protein-coding genes and rRNA genes (0.36 and 0.33, respectively), but is lower in the tRNA genes (0.2), and higher in the third codon position (0.75) and in intergenic regions (0.61). This pattern is common to all other Porifera and Cnidaria mt-genomes sequenced except Hexactinellida (Haen et al., 2007).

3.3. tRNA gene recruitment

There are two possible explanations for the presence of an additional *trnY$_2$(aua)* in the mt-genome of *N. magnifica*. It could have originated from either a duplication of *trnY$_1$(gua)* or from a gene recruitment. In order to identify the origin of the additional *trnY$_2$(aua)*, a phylogenetic tree of all mitochondrial tRNAs of *N. magnifica* and five other demosponges was reconstructed (Fig. 2A). We found that one tyrosine tRNA is most closely related to the arginine *trnR$_1$(ucg)* of *N. magnifica*. The grouping of *N. magnifica* *trnY$_2$(aua)* and *trnR$_1$(ucg)* is highly supported (BP = 80; Fig. 2A). Moreover, *N. magnifica* *trnY$_2$(aua)* and *trnR$_1$(ucg)* share 79.1% sequence identity, which is much higher than between *trnY$_2$(aua)* and any other tRNA gene of Porifera (34.3–68.6%). In particular, *trnY$_2$(aua)* exhibits only 46.2% sequence identity with *trnY$_1$(gua)* (Fig. 2B). These results suggest that *trnY$_2$(aua)* evolved by a gene recruitment and not by duplication of *trnY$_1$(gua)*. This is the second time that tRNA recruitment has been reported in sponge mt-genome (Lavrov and Lang, 2005). More sequences are needed to determine the prevalence of this phenomenon in sponges.

3.4. Molecular phylogeny

All phylogenetic analyses (ML and Bayesian) resulted in similar trees (Fig. 1B, Appendix A, Figure S1A–C) which strongly support a sister clade relationships of Poecilosclerida (represented by *Negombata magnifica*) and Hadromerida (*Tethya actinia*) (BP = 100, PP = 0.98). This result contradicts the traditional view, in which these orders are placed within different subclasses (Hooper and Van Soest, 2002b). The traditional phylogenetic tree based on morphological characters groups the order Poecilosclerida (*N. magnifica*) with the orders Halichondrida (*A. corrugata*) and Haplosclerida (*Amphimedon queenslandica*) in the subclass Ceractinomorpha, while the orders Hadromerida (*T. actinia*) and Astrophorida (*G. neptuni*) are grouped together in the subclass Tetractinomorpha. Our results showing the paraphyly of sponge subclasses are in agreement with molecular works based on nuclear rRNA and *cox1* genes (Borchiellini et al., 2004; Nichols, 2005). Nevertheless, additional taxon sampling is needed to confirm the sister clade relationship of Poecilosclerida and Hadromerida.

In agreement with Wang and Lavrov (2007) and Borchiellini et al. (2004), the Homoscleromorpha (*Oscarella carmela*) appears to be the most divergent demosponge (BP = 77–86, PP = 0.66). The next diverging sponge order is the Haplosclerida (BP = 85–97, PP 0.74). This result has also been supported by phylogenetic reconstructions based on 18S rRNA sequences (Borchiellini et al., 2004) and concatenated mitochondrial protein sequences (Erpenbeck et al., 2007). Finally, the position of Halichondrida as the sister clade of Poecilosclerida + Hadromerida is supported by ML analyses (BP = 88–73). However, this node was not recovered in the Bayesian analysis (Appendix A, Figure S1C). Additional sampling among sponge orders seems necessary to solve this relationship.

4. Conclusions

The complete nucleotide sequence of the mt-genome of the demosponge *N. magnifica* was sequenced. Our results suggest that *trnY$_2$(aua)* evolved via tRNA gene recruitment and support a close relationship between Poecilosclerida and Hadromerida. Larger sponge sampling is needed to increase resolution of sponge phylogeny and to determine the prevalence of tRNA gene recruitment in sponges.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2007.12.004.

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