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Mitochondrial genomes of the genus *Ephydatia* Lamouroux, 1816: can palindromic elements be used in species-level studies?

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Abstract Poriferan mitochondrial DNA (mtDNA), especially large intergenic regions, is a target for the insertion of repetitive hairpin-forming elements. These elements are responsible for the large mt genome size differences observed even among closely related sponge taxa. In this study, we present the new, nearly complete, mt genome sequence of *Ephydatia fluviatilis* and compare it with previously published mt genomes of freshwater sponges. Special emphasis was placed on comparison with the closely related species *Ephydatia muelleri*, thereby comparing the only two species of the genus *Ephydatia* on the western Balkan Peninsula. In particular, we analyzed repetitive palindromic elements within the mitochondrial intergenic regions. The genomic distribution of these repetitive elements was analyzed and their potential role in the evolution of mt genomes discussed. We show here that palindromic elements are widespread through the whole mt genome, including the protein coding genes, thus introducing genetic variability into mt genomes.

Keywords Porifera · Freshwater sponges · Mitochondria · Repetitive DNA · Ephydatia fluviatilis

Introduction

In an ongoing effort to resolve the class-level phylogeny of Porifera, numerous mitochondrial (mt) genomes of sponges have been obtained and analyzed. It has been shown that mt evolution of these non-bilaterian Metazoa is important for comprehension of the mitochondrial to nucleus gene transfer and evolution of early metazoans (Lavrov 2007). While animal mt genomes, when compared with the several-fold larger choanoflagellate genome, show a reduction in the number of genes and major reduction of intergenic regions (IGRs), in non-bilaterian animals, which harbor larger mt genomes, greater variations are found (Lavrov 2007). With the notable exceptions of *Suberites domuncula* (>26 kbp) (Lukić-Bilela et al. 2008) and *Axinella corrugata* (>25 kbp) (Lavrov et al. 2005), mt genomes of marine sponges published to date reveal moderate size variation (16–20 kbp). With respect to marine sponges, freshwater sponge mt genomes have proved to be among the largest, with *Lubomirskia baicalensis* comprising around 29 kbp, which is mainly the result of the expansion of non-coding regions caused by proliferation of palindromes and repetitive elements (Lavrov 2010). Comparison of the mt genomes out four genera of freshwater sponges from the family Lubomirskiidae with the mt genome from *Ephydatia muelleri* (family Spongillidae) and more distantly related *Corvomeyenia sp.* (family Metaniidae) revealed an expansion of non-coding DNA in Lubomirskiidae, as the result of proliferation of short inverted repeats (Lavrov et al. 2012).

One type of proliferating element that influences genome compactness in mt genomes are short palindromic repeats with the ability to fold into hairpin structures. It has been shown that the repetitive hairpin-forming elements that invade sponge mitochondrial genomes are responsible for the large genome size differences, even among closely related taxa (Erpenbeck et al. 2009). Multiple repetitive hairpin forming elements have been found in several demosponge mitochondrial genomes, with some freshwater sponges (e.g., *Ephydatia muelleri*, *Eunapius subterraneus*, *Lubomirskia baicalensis*, *Rezinkovia echinata*,

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Swartschewskia papyracea, and Baikalospongia intermedia profundalis) being found where repeats have spread through the whole mt genome, including protein coding genes (Wang and Lavrov 2008; Lavrov 2010; Pleše et al. 2011; Lavrov et al. 2012).

Without an obvious cellular function, these elements were probably present, up to some number, in ancestral genomes of Porifera that were richer in IGRs, and therefore occurred early in mt genome evolution (Erpenbeck et al. 2009). Similar palindromic repetitive sequences are found in the mt genomes of various organisms, for example, in the fungi *Saccharomyces cerevisiae* (Weiller et al. 1989) and *Allomyces macrogynus* (Paquin et al. 2000), alga *Volvox carteri* (Smith and Lee 2009) and plant *Oryza sativa* (Nakazono et al. 1994). Although their origin and function remain uncertain, they represent an untapped source of information and may have played an important role in mt genome evolution. Some studies indicate their potential role as control regions (Erpenbeck et al. 2007; Rosengarten et al. 2008), with involvement in RNA processing or even recombination and mtDNA reorganization (Erpenbeck et al. 2009). Such sequences have been suggested to be one of the causes of mt DNA rearrangements and molecular evolution of protein sequences. Palindromic repetitive sequences were found inserted in frame within many protein coding sequences of Rickettsiae, the closest extant relative of mitochondria, showing their possible role in the creation of new protein sequences (Ogata et al. 2000). It has also been suggested that they are mobile elements that can move throughout the mt genome in yeast (Wenzlau and Perlman 1990) and fungus *Podospora* (Koll et al. 1996) or they can move between compartments in *Volvox carteri* (Smith and Lee 2009). Valuable results concerning the nature of such motifs arose from the comparison of mtDNA of closely related species (Lang et al. 1998; Nedelcu and Lee 1998; Aono et al. 2002; Lavrov et al. 2012). Although it is still not clear whether they have any taxonomic preferences, it is possible that these proliferating inverted repeats may act as an evolutionary force of mtDNA and therefore represent a useful marker in phylogenetic studies.

Freshwater sponges are currently divided into seven families within the suborder Spongillina, which comprise 42 genera and one fossil family, Palaeospongillidae (Manconi and Pronzato 2002). Despite increased research, the phylogenetic relationships among families is still unresolved. It has been suggested that the genus *Ephydatia* is paraphyletic and that endemic sponge species might have originated from such cosmopolitan founder species (Addis and Peterson 2005; Meixner et al. 2007; Harcet et al. 2010; Pleše et al. 2011). Comprehensive phylogenetic analysis based on 18S rDNA separated two closely related freshwater sponges *Ephydatia fluviatilis* Linnaeus, 1759 and *Ephydatia muelleri* Lieberkuhn, 1855 with *Clypeatula cooperensis* Addis & Peterson, 2000 (Spongillidae) and *Baikalospongia*

bacillifera Dybowsky, 1880 (Lubomirskiidae) (Addis and Peterson 2005). Although a recent study synonymized species *Clypeatula cooperensis* with *Ephydatia fluviatilis*, a close relationship among *Baikalospongia bacillifera*, *Ephydatia muelleri* and *Ephydatia fluviatilis*, as well as *Swartschewskia papyracea* Dybowsky, 1880 (Lubomirskiidae), remains unclear (Itskovich et al. 2007).

In this study, we introduced nearly complete mtDNA from *Ephydatia fluviatilis* and compared it to the currently available freshwater sponge mt genomes. Palindromic elements, their structure, distribution and organization within the mt genome were analyzed. Within this context, comparative analysis of mt genomes with special emphasis on two species belonging to the same genus, *E. fluviatilis* and *E. muelleri*—the only species of the genus *Ephydatia* Lamouroux, 1816 at the western Balkan Peninsula (Manconi and Pronzato 2002)—gave us the opportunity to gain a better insight into the species-level relationships between freshwater sponges.

Materials and methods

Specimen collection, DNA isolation, mtDNA amplification, cloning and sequencing

Specimens of *Ephydatia fluviatilis* were collected from a tunnel between polje Jezero and Peračko blato (Croatia).

Material was kept in 70 % EtOH at 4 °C until use. Total DNA was extracted from 0.3 g tissue using G-spin Genomic DNA Extraction Kit (iNtRON, http://www.techdragon. com.hk), according to manufacturer's protocol.

Primer sets for PCR and sequencing were designed in our laboratory, based on the multiple alignments with sequences available in GenBank and were used for amplification of the conserved regions of protein coding genes *nad1*, *nad2*, *nad3*, *nad4*, *nad5*, *rnl*, *cox2*, *cox3* and *cob* (Table 1). Primer walking was applied to obtain the nearly complete mtDNA. Touchdown PCR reactions were performed with following conditions: 1 min/94 °C, 5 cycles (30 s/94 °C, 45 s/59 °C, 1–2 min/72 °C), 5 cycles (30 s/94 °C, 45 s/57 °C, 1–2 min/72 °C), 20 cycles (30 s/94 °C, 45 s/55 °C 1–2 min/ 72 °C) and final elongation 10 min/72 °C. Reaction mixtures contained 2.5 µl 10x PCR Buffer, 3 µl MgCl$_2$ (25 mM), 1 µl of each primer (10 mM), 0.5 µl dNTPs (10 mM each), 0.2 µl *Taq* DNA polymerase and 100–150 ng template. Problematic regions were amplified with TaKaRa LA Taq^{TM} DNA polymerase with GC Buffer (Takara Bio, Tokyo, Japan), according to manufacturer's protocol. Mitochondrial DNA was sequenced directly by primer walking using the ABI PRISM 3100 automatic sequencer and ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Problematic regions

Table 1 PCR primers used for mtDNA amplification

Primer name	Primer sequence (5′→3′)
ND1F	TTAACHTTAGCNGARCGAA
ND1R	AAAAATAAAGCAAAWGACA
ND2F	TAGCCGCATTAAAAAGAGACAGT
ND2R	AAAAAGTTAACCCCGCTACCA
ND3F	TATCTGGCGCTTCCTAT
ND3R	AACCCCCTTTTATCCACT
ND4F	TTTATGGGCDTCTTTTGATG
ND4R	ATTTAWATCTCTAGAAAAATAG
ND5F	AGGDACKAARGGGGCAGGTA
ND5R	ATACCCNACAAAAATACTHCC
LRN	CGCCTGTTTATCAAAAACAT
LRJ	CTCCGGTTTGAACTCAGATCA
COX2F	CATCAATGRTATTGGTCYTAYGA
COX2R	ACAATNGGCATAAARGARTGATT
COX3F	TACTTATCAYCCTTAYCATTTAG
COX3R	AAACWACATCCACAAAATGTCAATATC
COBF	NGGCTTTTKCWTCYGTGG
COBR	GCAAATAAAAAATAYCACTCHG

containing multiple palindromic elements were ligated with pGEM-T Vector Kit (Promega, Madison, WI) and cloned into *Escherichia coli* XL1-Blue competent cells. Up to three clones were sequenced. The nearly complete mitochondrial genome of *Ephydatia fluviatilis* has been deposited with the GenBank database (GenBank ID: JN209966).

Sequence analysis and gene identification

Sequencing reads were assembled using Lasergene processing software (DNASTAR, Madison, WI) and checked manually for sequencing errors. tRNA genes were identified by tRNAscan-SE program (Lowe and Eddy 1997). Other genes were identified by homology searches in GenBank using BLAST network service (Benson et al. 2003). The Palindrome program from the EMBOSS software package (Rice et al. 2000) was used to search for closely spaced perfect inverted repeats with parameters as follows: minimum length of palindromic elements, 7 nt; maximum length of palindromes, 100 nt; maximum gap between repeat elements, 10 nt. Secondary structures of the palindromes were predicted with mfold-server server http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form and manually drawn with CorelDraw12.

Phylogenetic analysis

Concatenated amino acid sequences of mitochondrial protein-coding genes were aligned under the default

parameters in ClustalW 1.7 (Thompson et al. 1994). Ambiguously aligned regions were determined and excluded from further analyses using the program Gblocks 0.91b (Castresana 2000). Aligned sequences were imported into MEGA version 5 (Tamura et al. 2011), where datasets were analyzed. Akaike information criterion (AIC) implemented in ProtTest v. 2.4 (Drummond and Strimmer 2001; Guindon and Gascuel 2003; Abascal et al. 2005) was used to select the best-fit model of protein evolution.

Phylogenetic analyses were performed under the AIC best-fit model (JTT+G+F) by maximum likelihood (ML) and maximum parsimony (MP) (Nei and Kumar 2000). Support for the nodes in trees was estimated by bootstrapping (1,000 bootstrap replicates in ML and MP).

Results and discussion

Mitochondrial genome organization

The sequenced portion of the mitochondrial genome of *Ephydatia fluviatilis* is 26,441 bp long, and within the range of all previously determined mt genomes of freshwater sponges (23,929–28,958 bp). Like most demosponges it contains 14 protein coding genes, including *atp9* (ATP synthase subunit 9), 2 rRNA genes and 25 tRNA genes (Fig. 1). One gene boundary (nad5/trnA(ucg)-23 bp) was
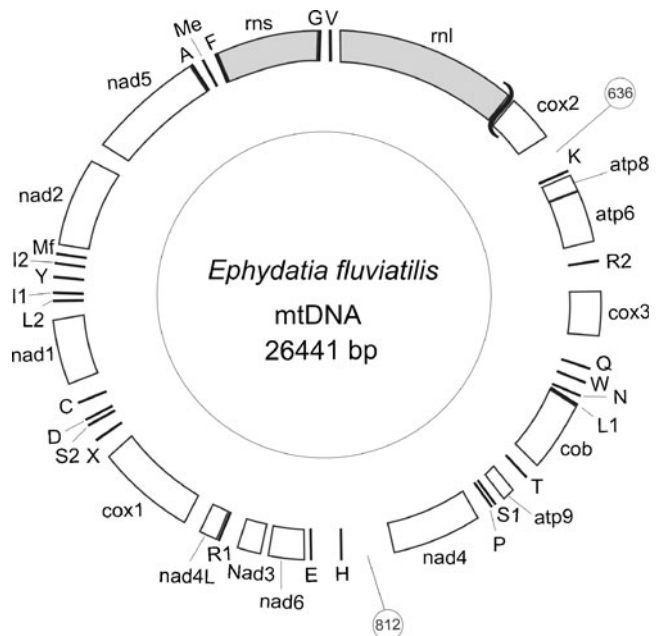

Fig. 1 Genetic map of *Ephydatia fluviatilis* mtDNA. Protein coding genes are in *white*, rRNA genes are in *grey* and tRNA genes are in *black* and are labelled by the one-letter code for their corresponding amino acid. The largest non-coding regions are indicated by *circles* with corresponding lengths. *Sigmoidal curve* indicates the lack of sequence data from the region

found, as well as tRNA-like structure *trnX*. Similar sequences in the same genomic location were reported in the mt genomes of Lake Baikal sponges (Lavrov et al. 2012), *Eunapius subterraneus* (Pleše et al. 2011) and *Xestospongia muta* (Wang and Lavrov 2008). Both these features are well conserved within freshwater sponges. Genes in all species analyzed have the same transcriptional orientation and arrangements of protein coding genes, rRNA and tRNA.

The A+T content of *E. fluviatilis* mtDNA is 58.48 %, as previously observed for other freshwater sponges (56–72 %). The coding strand of *E. fluviatilis* mtDNA displayed negative AT-skew and positive GC-skew in all types of sequences except rRNA genes, which had positive AT-skew, as previously reported for family Spongillidae (Wang and Lavrov 2008; Pleše et al. 2011). The only inconsistency to the previously reported values was found in non-coding regions that showed positive GC-skew, which is uncommon for freshwater sponges. However, the omission of part of the IGR region between *rnl* and *cox2* from the analysis due to sequencing problems affected the actual GC ratio of non-coding regions and likely caused this discrepancy. This non-coding region probably contains multiple repeated sequences and was problematic for sequencing. Differences in *rns* and *rnl* are mainly found in variable regions and could contribute to the presence of palindromic

repetitive elements. Therefore, both rRNAs in *E. fluviatilis* are larger when compared to *E. muelleri* and *Eunapius subterraneus*.

Protein coding genes and codon usage

As is the case with all freshwater sponges, all protein coding genes in the mt genome of *E. fluviatilis* showed positive GC- and negative AT-skew except *atp8*, which differs from this model and has negative GC- and positive AT-skew (–0.067 and 0.018, respectively). GC-skew is strongly positive at the first codon position (0.4), negative at the second (–0.17) and positive at the third (0.26). AT-skew is weakly negative (–0.06) at the first position, strongly negative at the second (–0.37) and negative at the third (–0.025). Synonymous codons ending with A or T are preferred, while those ending with C are the least frequent. The CGC codon, absent in most demosponges, was used once. Interestingly, this codon was found in all so far described freshwater sponges at the same well conserved position within the *nad5* gene.

Protein coding genes in both *Ephydatia* species had well conserved primary structure with high identity between 92 % and 98 %, although five genes vary in size from 24 to 54 bp. Two deletions of 27 bp and 21 bp, respectively, were found in the *cox2* and *nad6* genes in *E. fluviatilis*. Notably, this 27 bp deletion, absent in other freshwater

Fig. 2 a Alignment of *nad2* (NADH dehydrogenase subunit 2) gene from *Ephydatia fluviatilis* (EF), *Eunapius subterraneus* (ES), *Ephydatia muelleri* (EM), *Lubomirskia baicalensis* (LB), *Baikalospongia intermedia profundalis* (BIP), *Rezinkovia echinata* (RE) and *Swartschewskia papyracea* (SP), respectively ; parts containing palindromic elements responsible for larger insertions in *E. fluviatilis* are indicated above the sequence. Triplet coding for amino acid proline (CCC) that is present only in *E. fluviatilis* and *Eunapius subterraneus* is marked with *asterisks* (*). **b** Secondary structure of palindromic elements in the inserted regions of *nad2* gene from *E. fluviatilis*

sponges, was also found in the *cox2* gene of *Eunapius subterraneus*, while *nad6* gene of *E. subterraneus* has two shorter deletions at the position of the 21-bp deletion in *E. fluviatilis*. *Eunapius subterraneus* and *E. fluviatilis* also have one in-frame insertion in *nad6* downstream of the deletions mentioned. Protein domain *nad2* is disrupted by the in-frame insertion of palindromic elements (Fig. 2) belonging to the H7 family in both *E. fluviatilis* and *Eunapius*
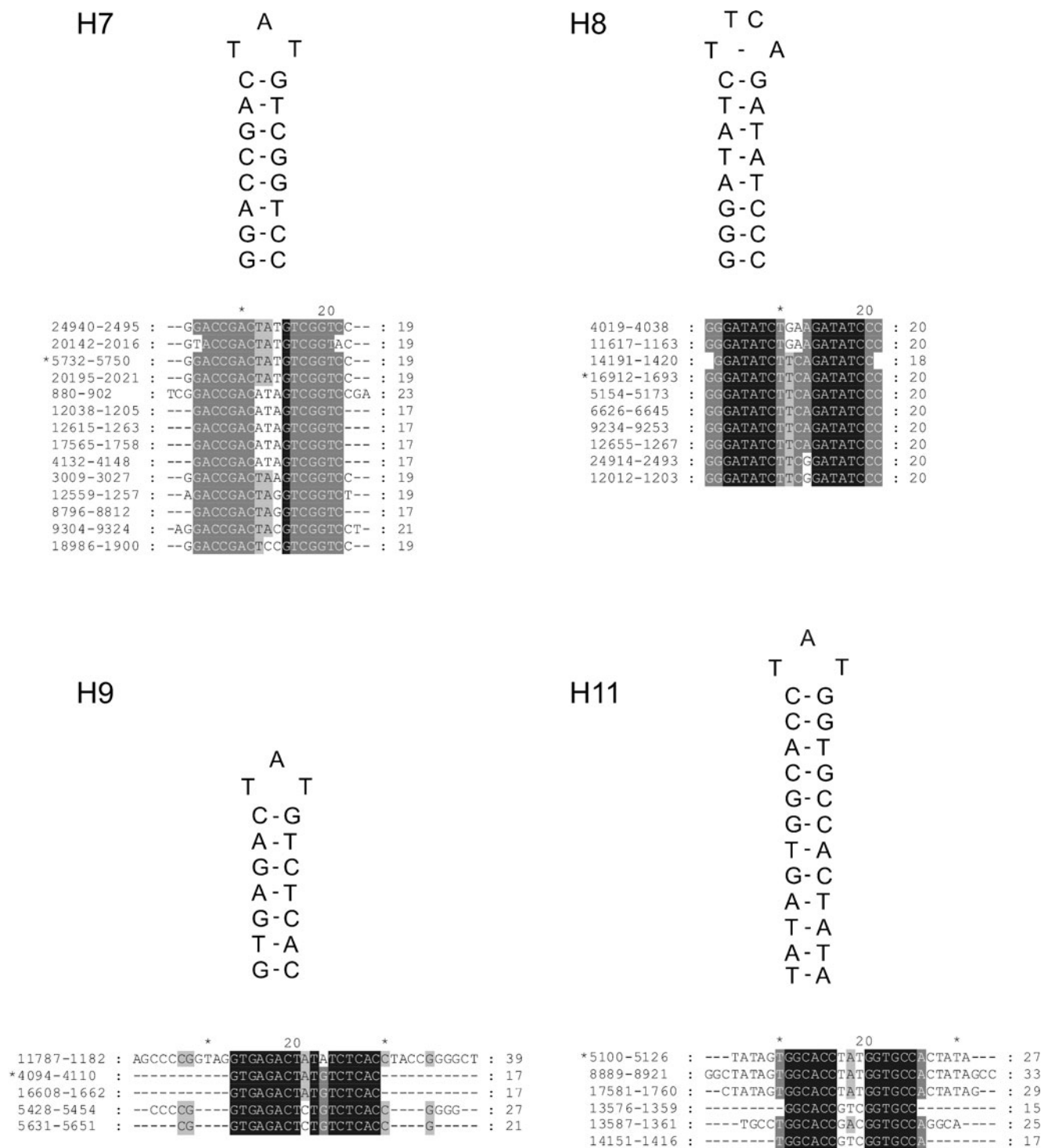
Fig. 3 Secondary structures and corresponding alignments of palindromic repetitive elements in *Ephydatia fluviatilis* divided into four distinct families (H7, H8, H9s and H11). The *numbers* in the alignment refer to their position in the mt genome. Sequences for which the secondary structures are given are marked with an *asterisk* (*)
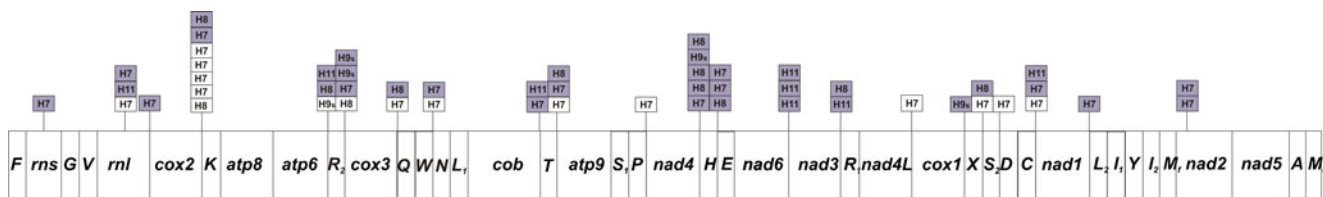
Fig. 4 The distribution of repetitive hairpin-forming elements in intergenic regions of mitochondrial genomes of *Ephydatia fluviatilis* (*dark gray*) and *Ephydatia muelleri* (*white*), (modified according to Lavrov 2010)

subterraneus, as described in the next section. Those palindromic elements were not found in *E. muelleri*. Interestingly, in one of those palindromes, *Eunapius subterraneus* and *E. fluviatilis* have an additional in-frame triplet (CCC) coding for the amino acid proline. A case like this has been reported previously in Rickettsiae (Ogata et al. 2000), suggesting the potential role of these elements in the creation of new protein sequences. Due to the fact that they are always inserted in frame with the protein coding genes, they do not cause frameshifts; hence, they are probably not spliced out of the transcripts, which indicates their possible contribution to the molecular evolution of protein sequences and mitochondrial rearrangements (Aono et al. 2002). Palindromic elements found in rRNA are in peripheral regions and those inserted within the coding regions are always in frame, which indicates strongly that they are not spliced out at the RNA level. Analysis of mt coding sequences of the family Lubomirskiidae revealed no insertion-deletion events within the group (Lavrov et al. 2012).

Intergenic regions in mitochondrial genomes

Intergenic regions (IGRs) in mtDNA of *E. fluviatilis* comprise approximately 7,860 bp (29.04 %), scattered among 39 segments, some being several hundred base pairs long. In comparison to *E. muelleri*, IGR is 6.84 % larger, due mostly to the presence of palindromic repeats, and within the range

of previously observed values for freshwater sponges (22.2–33.8 %). The A+T content of *E. fluviatilis* IGR is 45.36 % which is slightly higher in comparison to *E. muelleri* (42.37 %) but still within the range of values obtained for freshwater sponges (42.7–48.5 %).

Mitochondrial DNA of *E. fluviatilis* encompasses various types of repeat motifs (up to 80 bp in length), which are spread throughout the mt genome: around 700 direct repeats (10–16 bp long), almost 2,000 inverted repeats (the longest is 48 bp) and 98 palindromes have been identified. Of these repetitive elements, 35 have been assigned to three distinct known families named H7, H8, and H9, and one new family, H11 (Fig. 3). We have investigated the proliferation and distribution of repetitive hairpin elements (RHE) in intergenic regions as well as in protein coding genes, rRNA and tRNA genes. In *rnl* genes of both *Ephydatia* species (as reported previously for freshwater sponges) several palindromes are found, one of them belonging to the H7 family, whereas *E. fluviatilis* possesses an additional palindrome from the H11 family. Small rRNA subunits (*rns*) in *E. fluviatilis*, *Eunapius subterraneus* and *L. baicalensis* possess a palindrome from the H7 family (with consensus sequence 5′-GACCGAC-3′), which is absent in *E. muelleri*. Taking into account all palindromes (not only those exclusively belonging to families), 30 % of the total amount of palindromes found are in the same position and are well conserved in both *Ephydatia* species. An interesting feature
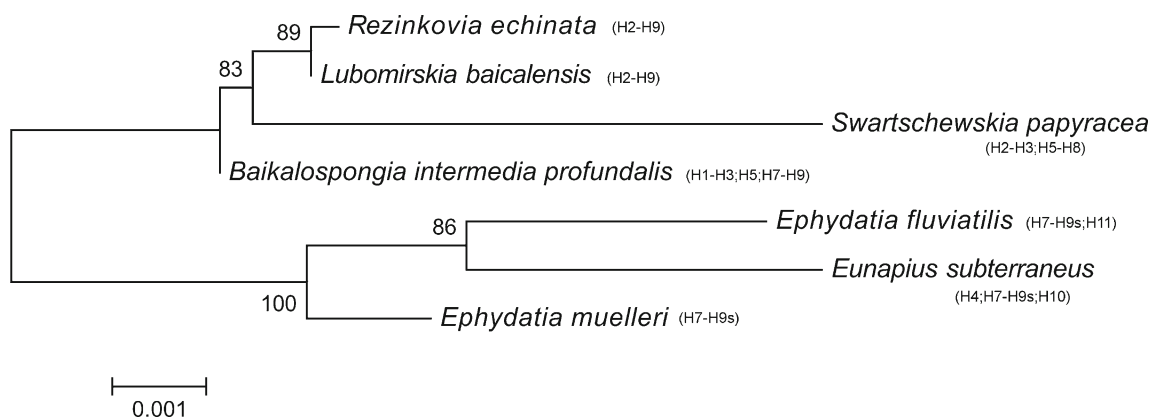
Fig. 5 Phylogenetic relationships among freshwater sponges based on concatenated protein sequences of mitochondrial genes. Maximum likelihood tree obtained under the JTT+G+F model is shown. An identical topology was recovered in maximum parsimony analysis. Hairpin families are indicated in the tree

is the presence of the palindromic element inserted in the *trnS*(gcu) and *trnI*(gau) at the same position in all available mt genomes of freshwater sponges.

Palindromic families between two species of the genus *Ephydatia* revealed small similarity in either their sequence, or their distribution (Fig. 4). However, comparison of the distribution of families among freshwater sponges revealed that H7 and H8 families are common to the mtDNA of all freshwater sponges sequenced to date. In addition, the H9 family is specific to Spongilidae. A new palindromic family named H11, with a consensus sequence 5′-TGGCACCG -3′ was found in *E. fluviatilis*. The distribution of families is reflected in the phylogenetic tree based on mitochondrial coding sequences (Fig. 5). An identical topology was recovered when palindromic sequences were used for analysis (data not shown), which indicates that these elements could provide useful information in species-level studies.

This study suggests that repetitive elements present in freshwater sponges are evolutionary related and were probably present in a common ancestor (H7 and H8 families). The uniformity of mt genomes of freshwater sponges has been shattered by frequent insertions of palindromic elements during evolution (Lavrov 2010; Pleše et al. 2011). Their accumulation through time likely triggered evolutionary events responsible for the observed differences between the two mt genomes. The new families are species-specific and were possibly inserted into mt genomes after divergence of the species, rather than being deleted in all other freshwater sponges. It is more likely to assume that they appeared independently in a few sponge lineages, than to assume that they were lost in most sponge genomes.

Many questions regarding these repetitive elements, such as their function and evolution, are still controversial. Although, as far as we know, this problem has not yet been addressed experimentally, the fact that these palindromic elements form secondary structures is functionally important (Erpenbeck et al. 2009). The presence of palindromic elements in mt genomes has been described in various species. They are suggested to be mobile elements (Koll et al. 1996), causes of mtDNA rearrangements, or involved in replication of mtDNA (Lavrov 2010). Whatever their origin and function might be, these sequences do have an effect on genome plasticity, and could be important with regards to mitochondrial evolution.

The morpho-taxonomic characteristics of freshwater sponges are not adequate for clear resolution of either specific species or higher taxonomic levels. The molecular-biological approach is crucial; however, molecular data (mtDNA, different markers, etc.) is not providing sufficient data for establishing acceptable markers that could be used to elucidate evolutionary flow and phylogeny between the taxa within the suborder Spongillina.

Until now, the focus was set on analogy between higher poriferan taxonomic categories where the species were chosen randomly. Mitochondrial DNA comparison of closely related species from western Balkan Peninsula, inhabited exclusively with these two sympatric taxa (Manconi and Pronzato 2002), enables speculation of an ancestor species. The lack of mt genomes from other freshwater sponges prevents complete clarification of phylogenetic relationships within this suborder.

Nevertheless, our analysis indicates *E. muelleri* as being an older species in evolutionary terms in comparison with *E. fluviatilis* (the only two *Ephydatia* species on the Balkan Peninsula). Variety of morphological characters, fostered with speciation as a result of adaptation to specific habitats, in the case of endemic species like *Eunapius subterraneus* (Croatian caves) and Baikal sponges from different families (first Spongillidae and second Lubomirskiidae), makes phylogeny of freshwater sponge taxa among different categories (species, genus, family) very difficult.

Although mitochondrial genomes of freshwater sponges showed remarkable uniformity despite differences in primary biology, especially the absence of gemmules as a main morphological character, intergenic regions and presence/absence of palindrome families are indicated as taxonomic sources. However, more specimens per species must be checked. We suggest further mt genome comparison among all members of the phylogenetically very difficult family Spongillidae, in order to reconstruct accurate evolutionary events and reveal the origins and relationships of particular species.

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