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University Postgraduate Interdisciplinary Doctoral Study
Molecular Biosciences

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Molecular markers in taxonomy of freshwater sponges and the Adriatic calcareous sponges

PhD Dissertation

Osijek, 2016

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište Josipa Jurja Strossmayera u Osijeku **Doktorska disertacija**
Sveučilište u Dubrovniku
Institut Ruđer Bošković
Poslijediplomski interdisciplinarni sveučilišni studij Molekularne bioznanosti

Znanstveno područje: Prirodne znanosti
Znanstveno polje: Biologija

Molekularni markeri u taksonomiji slatkovodnih spužvi i jadranskih spužvi vapnenjača

Mirna Halasz

Disertacija je izrađena u: Laboratorij za molekularnu genetiku, Institut Ruđer Bošković

Mentor/i: dr. sc. Helena Ćetković

Kratki sažetak doktorske disertacije:

Molekularna istraživanja doprinose brojnim izmjenama u taksonomiji pojedinih skupina životinja čiji se srodstveni odnosi temelje na morfološkim obilježjima. Mnoge slatkovodne spužve i spužve vapnenjače pogrešno su determinirane na temelju morfologije te su neophodne popratne molekularne analize. Analiza palindromskih elemenata u intergenskim regijama mitohondrijskih genoma slatkovodnih spužvi pokazuje da su porodice tih elemenata vjerojatno evolucijski povezane. Na osnovu molekularnih markera u kombinaciji s morfološkim obilježjima, u ovom je radu opisano 6 novih vrsta vapnenjača.

Broj stranica: 103
Broj slika: 38
Broj tablica: 19
Broj literaturnih navoda: 133
Prilog: 19 stranica, 3 slike, 2 tablice
Jezik izvornika: engleski
Ključne riječi: spužve, Jadransko more, molekularni markeri, taksonomija, filogenija

Datum obrane: 08.04.2016.

Stručno povjerenstvo za obranu:
1. dr. sc. Tatjana Bakran-Petricioli
2. dr. sc. Helena Ćetković
3. dr. sc. Vera Cesar
4. dr. sc. Andreja Ambriović Ristov (zamjena)

Disertacija je pohranjena u: Nacionalnoj i sveučilišnoj knjižnici Zagreb, Ul. Hrvatske bratske zajednice 4, Zagreb; Gradskoj i sveučilišnoj knjižnici Osijek, Europska avenija 24, Osijek; Sveučilištu Josipa Jurja Strossmayera u Osijeku, Trg sv. Trojstva 3, Osijek

BASIC DOCUMENTATION CARD

Josip Juraj Strossmayer University of Osijek **PhD thesis**
University of Dubrovnik
Ruđer Bošković Institute
University Postgraduate Interdisciplinary Doctoral Study of Molecular Biosciences

Scientific Area: Natural Sciences
Scientific Field: Biology

Molecular markers in taxonomy of freshwater sponges and Adriatic calcareous sponges

Mirna Halasz

Thesis performed at: Laboratory for Molecular Genetics, Ruđer Bošković Institute

Supervisor/s: dr. sc. Helena Ćetković

Short abstract:

Molecular studies impose many changes in the taxonomy of certain animal groups whose relations are based solely on morphological characters. Many freshwater and calcarean sponges have been wrongly identified at morphological level, hence concurrent molecular analyses are necessary. The analysis of repetitive elements in the intergenic regions of freshwater sponge mitochondrial genomes suggests that their families are probably evolutionary related. Based on molecular markers, together with morphological traits, 6 new calcarean species are described here.

Number of pages: 103
Number of figures: 38
Number of tables: 19
Number of references: 133
Appendix: 19 pages, 3 figures, 2 tables
Original in: english
Key words: Porifera, Adriatic Sea, molecular markers, taxonomy, phylogeny

Date of the thesis defense: 08.04.2016.

Reviewers:
1. Tatjana Bakran-Petricioli, PhD
2. Helena Ćetković, PhD
3. Vera Cesar, PhD
4. Andreja Ambriović Ristov, PhD (substitute)

Thesis deposited in: National and University Library in Zagreb, Ul. Hrvatske bratske zajednice 4, Zagreb; City and University Library of Osijek, Europska avenija 24, Osijek; Josip Juraj Strossmayer University of Osijek, Trg sv. Trojstva 3, Osijek

Research presented in this PhD thesis was performed at the Ruđer Bošković Institute, Division of Molecular Biology Laboratory for Molecular Genetics, under supervision of dr. sc. Helena Ćetković. This work was supported by the Croatian Ministry of Science, Education and Sports (MSES) grant 098-0982913-2874. Calcarean sponge samples were collected in collaboration with the Laboratory for Benthos of the Institute of Oceanography and Fisheries in Split (V. Nikolić). Morphological and electron microscopy analyses were performed at the Federal University of Rio de Janeiro – UFRJ as a part of collaboration with the Department of Zoology (M. Klautau and F. Azevedo), and partially at the Center for Marine Research of the Ruđer Bošković Institute in Rovinj (J. Godrijan, M. Pfannkuchen and D. Marić Pfannkuchen).

Acknowledgments

I thank my supervisor, Helena Ćetković, for giving me the opportunity to be a part of the LMG, for encouraging me to start my PhD, for all her support and freedom throughout my research.

I thank Dušica Vujaklija, Head of Laboratory, for her help and advices, especially during my first days of protein purification experiments.

I also thank my reviewers, Prof. Tatjana Bakran-Petricioli and Prof. Vera Cesar for their helpful advices and suggestions.

I thank all the other members of the LMG for helping, listening and solving many problems, and, most importantly, for having some of the best jokes ever. The biggest thanks goes to Bruna Pleše, who generously introduced me into her world of sponge mitochondria and has stayed by my side ever since. The other biggest thanks goes to Drago Perina, who without restraint shared all of his vast knowledge and never hesitated to share his great ideas and projects with me. Very big thanks also goes to Andreja Mikoč, who would always find time to answer my questions, explain methods and share her rich expertise and her solution supplies.

Many thanks go to the guys at CIR - Marko, Hrvoje, Filip, Igor, Mario, Perc – without whom it would be so much harder to finish this thesis. Thank you all for being there in any of those annoying moments of PC malfunctions. Thank you for not ever saying „Have you tried unplugging it and then plugging it back in?"

I thank Vedran, Bančo and Helena, who collected invaluable samples for this research.

I thank my collaborators from Rovinj – Jelena, Martin and Daniela – for bravely jumping into the calcarean world with me. I also thank the collaborators from Brasil – Michelle and Fernanda - who contributed significantly to this research and from who I have gained an enormous amount of knowledge.

I thank my parents for their never ending support through all the years of my education.

I thank Ivan for having faith in me, for his endless support and everything else.

I thank all my friends who are always by my side, no matter how annoying I may get, who will always cheer me up with their jokes and anegdotes. I feel safe knowing that I can always count on you.

Table of contents

1. Introduction

1.1. General introduction to sponges

Sponges (phylum Porifera) are among the simplest metazoan animals, exclusively aquatic, sessile, filter-feeding invertebrates distributed worldwide over marine and freshwater habitats. They are characterized by the possession of an aquiferous system comprising a complex network of channels and chambers [1,2]. The sponge body consists of an external layer of polygonal cells called pinacocytes and an internal layer that covers the surface of canals and chambers, lined by flagellated cells called choanocytes. Between these two layers is an area called mesohyl, which contains a number of different cell types, such as archaeocytes, sclerocytes and amebocytes (Figure 1). Mesohyl also comprises skeletal elements, which may consist only of spicules built from silicon dioxide or calcium carbonate, of the protein spongin, or of a combination of both. The spicules vary in shape and size; depending on the size, larger megascleres and smaller microscleres may be distinguished.

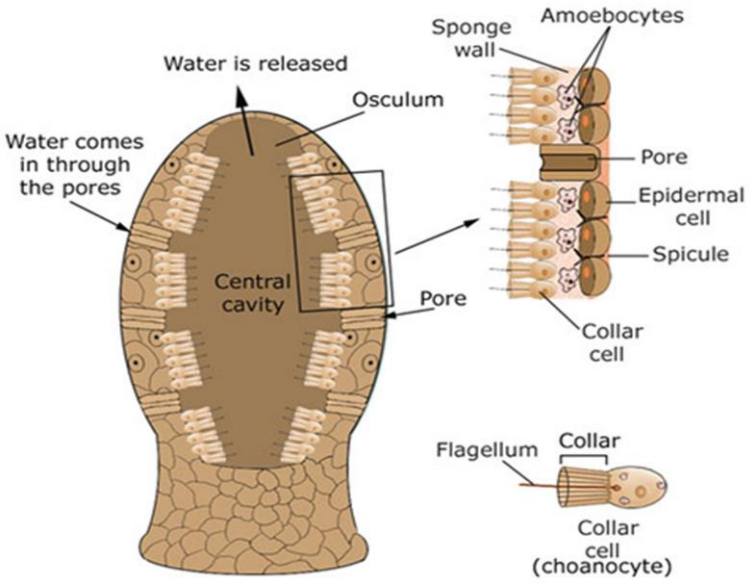


Figure 1. Schematic overview of an asconoid sponge structure. Taken from [129].

Sponges feed from water containing food particles and oxygen, which enters the sponge body through a small superficial openings called pores, where flagellated choanocytes create water current through more or less complex mesh of tubes and cavities, building a water canal system throughout the sponge's body wall.

A permanent and actively generated flow of water supplies the sponge with nutrients, oxygen and also removes any waste products [3,4]. Hoanocytes filter food particles from water and forward it to the mesohyl, where digestion takes place. Finally, water exits throughout a few openings, or sometimes only one larger opening called osculum. Depending on their level of complexity, five different types of aquiferous system can be distinguished in sponges: asconoid, syconoid, leuconoid, syllebid and solenoid (Figure 2).

If all internal cavities are lined with choanocytes, the aquiferous system is called homocoel. While if some parts of internal surfaces are lined with pinacocytes, the aquiferous system is termed heterocoel. Only asconoid type of organization is homocoel, as all its inner cavities are lined by choanocytes; all the other types are heterocoel.

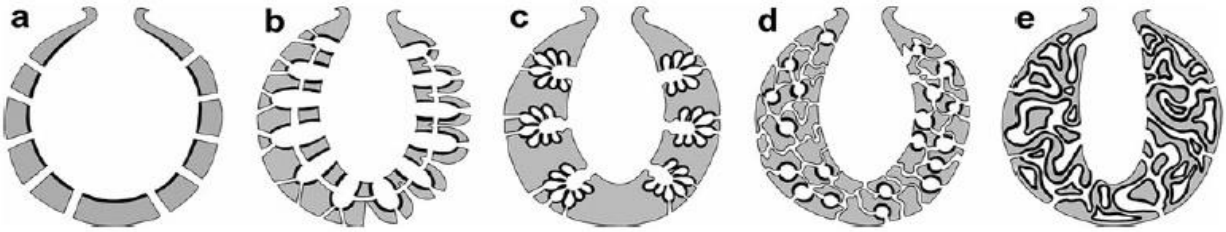


Figure 2. Five types of aquiferous systems in sponges. a Asconoid. b Syconoid. c Sylleibid. d Leuconoid. e Solenoid. Taken from [6].

Sponges reproduce either asexually or sexually and may be of separated sexes or facultative hermaphrodites. They take the sessile form as adults, following a dispersal through the mobile larvae phase. Unfavourable life conditions in some sponges may trigger the forming of reduction bodies called gemmules, out of which a new specimen can develop again after the environmental conditions become favourable. It is still not entirely understood whether gemmules represent a form of asexual reproduction or simply a resting life stage.

As one of the dominant benthic communities, sponges hold an important part in ecosystem functioning [5]. In this respect, delimitation of species boundaries is essential for accurate definition of the phylum's biodiversity. As the pharmaceutical industry frequently benefits from sponges production of bioactive compounds, it is also important to correctly assign analysed bioassays to a particular species. Therefore, the taxonomic definitions of species play a significant part of the sponge research.

1.2. Traditional sponge taxonomy

Traditional sponge taxonomy is almost solely based on their morphological characters. It is mainly focused on the degree of complexity of the aquiferous system and certain skeletal features, such as the presence, absence and the morphology of spicules. The type of aquiferous system is still one of the most important taxonomic features, especially for the determination of higher taxonomic orders (Figure 3).

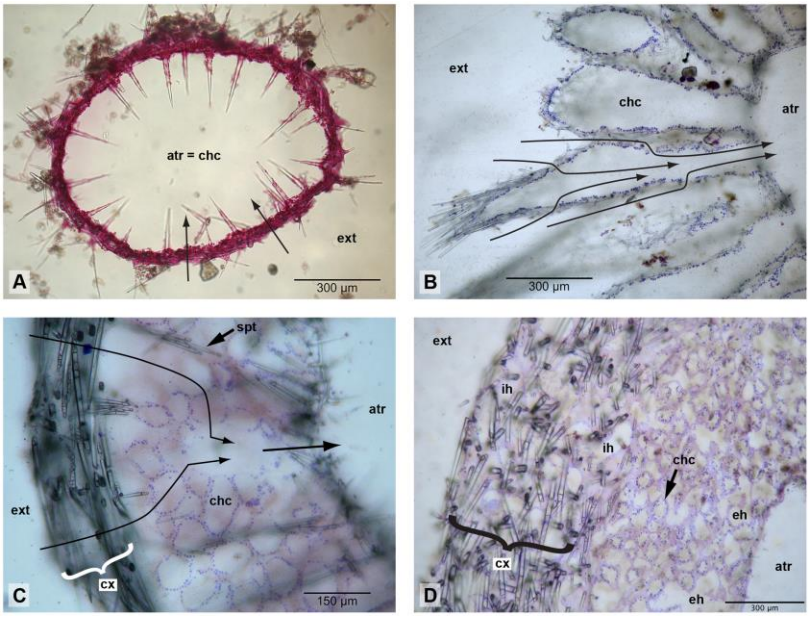


Figure 3. Different organizations of the aquiferous system in Calcarea. A-asconoid; B-syconoid; C-syllebid; D-leuconoid. The arrows show the direction of water flow in A, B and C. Atr=atrium, chc=choanocyte chambers, cx=cortex, eh=exhalant channel, ext=exterior of sponge, ih=inhalant channel, spt=spicule tract of modified triactines. Taken from [65].

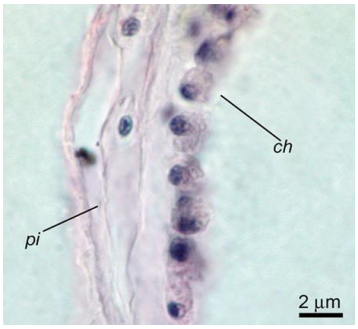


Figure 4. Solenoid aquiferous system. Pinacocytes (pi) of the cortical membrane and choanocytes (ch) of a tube. Taken from [6].

Out of five types of the aquiferous systems recognized in sponges, asconoid type of organization is considered as the simplest one. It comprises a single inner chamber called atrium, which is completely lined with choanocytes and opens through a single osculum (Figure 1). Syconoid type is a further step in development and consists of numerous radially arranged canals lined with choanocytes, which all open into an atrium lined with pinacocytes. Leuconoid type of aquiferous system represents an even more complex form, which is characterized by numerous chambers lined with choanocytes, that open through a larger number of oscula. Syllebid aquiferous system

represents a transient form between the syconoid and leuconoid types, where a few radially arranged chambers lined with choanocytes first open into a cavity lined with pinacocytes, which then opens into the atrium. Solenoid type (Figure 4) was so far described only for the genus *Leucascus* and is defined by the presence of anastomosed tubes lined with choanocytes and an atrium lined with pinacocytes [6]. Most sponges have syconoid or leuconoid grade of organization and only the Calcarea class encompasses all five types of aquiferous systems.

A traditional taxonomy relies on another crucially important feature, which considers spicule mineralogy, morphology and organization. Systematic and detailed microscopic analyses over the previous centuries have defined certain spicule patterns – their arrangement within the sponge body, as well as their shape, size and number – used for classification of sponges from the highest to the lower systematic categories. Particular varieties in spicule sizes and shapes of skeleton traditionally define certain orders, genera or species. Classes Demospongiae, Hexactinellida and Homoscleromorpha possess spicules made of silicon dioxide, while the fourth class, Calcarea, is somewhat special for possessing calcitic spicules (Figure 5).

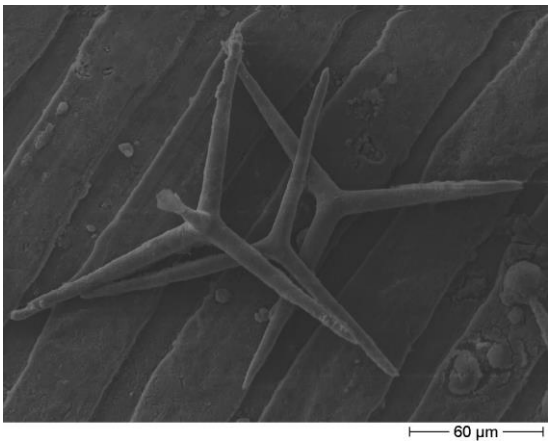


Figure 5. SEM photograph of calcareous triactines and a tetractine. Photo: J. Godrijan.

Demospongiae comprises the majority of all known sponge species, which are altogether characterized by a great diversity of spicule morphologies. Several examples of demosponges possess spongin skeletons without spicules - e.g. order Dyctioceratida Minchin, 1900, which also includes *Spongia officinalis*, the famous "bath sponge". Hexactinellida have characteristic six-rayed spicules with a triaxial symmetry, which are usually reduced to triactines, tetractines and diactines. Homoscleromorpha possess regular triactine spicules, which in some cases may be accompanied by

other spicule types. For Calcarea, the most characteristic spicules are triactines, to which sometimes diactines or tetractines may be added.

Sponges are generally characterized by simple morphology and phenotypic plasticity, which means that distinct specimens of the same species can obtain rather diverse morphology, depending on the certain environmental influences [7,8]. This is often the case with the overall colour, shape and texture of sponges, which exist in truly wide varieties of shapes, sizes and coloration. Therefore, the outer shape is rarely used as a classification criterion, since the same species can exhibit rather divergent morphotypes (Figure 6). However, spicule morphology, their number and size may be influenced by environmental factors as well, which can lead to erroneous classification due to undetected homoplasies.

Figure 6. Different habitus of the same sponge species. Two morphotypes of the freshwater sponge *Eunapius subterraneus* (top). Taken from [50]. Photo: I. Čukušić. Two morphotypes of the calcarean sponge *Clathrina clathrus* (bottom). Photo: V. Nikolić.

This is especially the case with taxa which comprise low diversity of morphological characters, where a number of reliable morphological characters required for classification is often scarce. Consequently, the sponge phylogeny has been highly controversial, followed by an extremely problematic taxonomy. Molecular data appear to be necessary for resolving these complex phylogenetic and taxonomic issues. The need for genetic evidence that corroborate the traditional taxonomic divisions based solely on morphological characters, has notably emerged within the last few years.

1.3. Molecular markers in sponge phylogenetic and taxonomic studies

Molecular approach to sponge systematics has opened new ways of verifying existing taxonomic hypotheses and enabled the correlation of genetic traits with the distinctive skeletal and morphological features. Integrative taxonomy combining molecular markers with morphological characters has been very helpful in solving the relationships within the most problematic of taxa, such as those with paucity of morphological characters, as well as in distinguishing the characters containing true phylogenetic signal from simple homoplasies. 18S rDNA was one of the first and most widely used markers in sponge systematics [9,10,11] and was shown adequate for higher-level phylogenies, but far too conserved for the taxonomic levels of orders and species. It was mostly used in combination with the 28S rDNA, which is shown to be somewhat less conserved [12] and suitable for resolving intra-ordinal sponge relationships [13]. Yet, some other studies have proven it more suited for higher-level phylogenies [14,15] and too conserved for inter-species studies. Internal transcribed spacers ITS1 and ITS2, which separate the 18S, 5.8S and 28S rDNA genes in the rDNA cistron, have also been extensively used molecular markers. Some arguments say that the possible intragenomic polymorphisms of ITS markers may potentially confound phylogenetic inferences [16]. Nevertheless, ITS1 and ITS2 have been successfully used in few phylogeographic studies [17,18] and were used to access the intra-specific differences between the *Leucetta* species [19]. These studies did not detect any intraindividual genomic variation in ITS sequences and have demonstrated that poriferan ITS sequence data provide enough information to resolve phylogeographical relationships. The ITS sequences have also been proved as good molecular markers for species separation [20,21]. The mitochondrial cytochrome *c* oxidase subunit 1 (COI) has been extensively used, as it was proven a good marker for higher-level phylogenies [22,23], although it has sometimes appeared to be too conserved for the lower level inter-species studies [24,25]. Compared to higher phyla, sponges possess a slow-evolving mitochondrial DNA [26,27], but when applied to Calcarea, it was shown that the mitochondrial sequence evolution rate seems to be five times higher than that of ITS [28]. Some other mitochondrial markers were used on a few occassions for phylogenetic studies, such as an intron from ATP synthetase beta subunit (ATPSbeta-iII) [29], NADH dehydrogenase subunit 1 (Nad1) [30], and the ATP synthase subunit 8 (Atp8) [31]. Considering the small size of sponge mitochondrial genomes in comparison to other bilaterians, the complete mitochondrial genomes are lately becoming more available for systematic studies and their potential role in elucidation of phylogenetic relationships between sponge taxa is being explored.

1.4. Sponge mitochondrial genomes as molecular markers

Apart from the nuclear DNA, organisms also possess an independent mitochondrial DNA (mtDNA) situated in the mitochondria. In phylogenetics, mtDNA has few advantages over the nuclear DNA. As it is inherited through the maternal line only, it facilitates the monitoring of changes along the line of evolution from an early start. It has a high level of variability and a high rate of mutation in animals when compared to the nuclear DNA [32]. Also, a high number of mitochondria per cell increases the possibility of successful DNA isolation from small or degraded biological samples.

The first complete sequence of sponge mitochondrial genome showed that the mitochondrial genomes of demosponges have very different organization comparing to other bilaterian animals [33]. The typical bilaterian mt genome is a compact molecule of 14–18 kbp that contains 13 protein-coding genes, 22 transfer RNA (tRNA) genes and 2 ribosomal RNA (rRNA) genes [34]. Sponges, as the first diverging animal phyla, have larger mt genomes (18–29 kbp) than typical bilaterians [35,36]. Demosponge mtDNA has a well conserved gene order and a compact organization [37], yet it harbors additional genes, including *atp9* (except *Amphimedon queenslandica*) [38], *trnI(cau)*, *trnR(ucu)*, multiple non-coding regions, a minimally derived genetic code and a bacteria-like rRNA and tRNA genes [33].

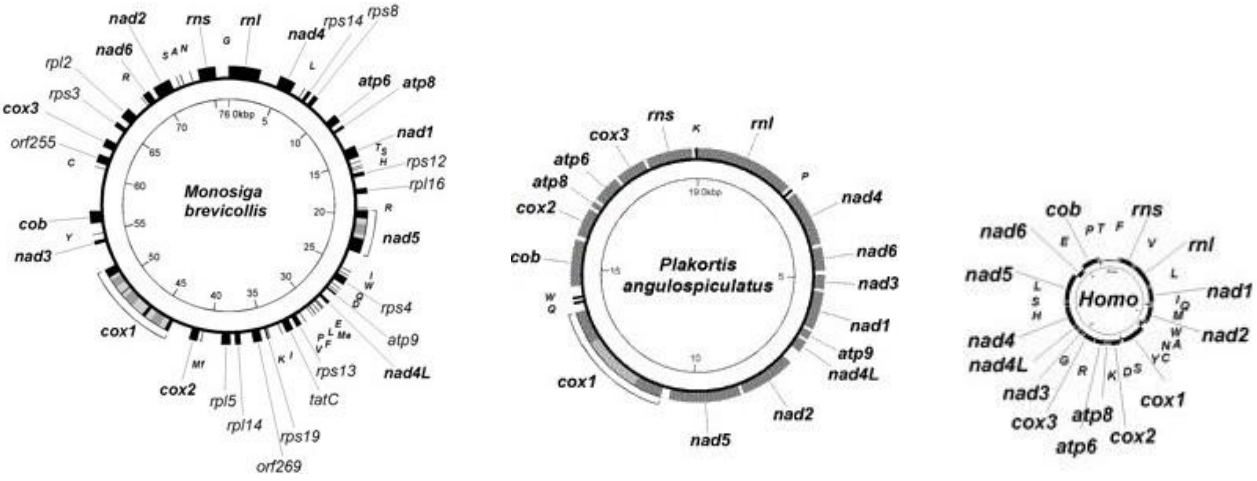


Figure 7. MtDNA organization in the choanoflagellate *Monosiga brevicollis*, the demosponge *Plakortis angulospiculatus* and bilaterian animals (represented by *Homo sapiens*). Abbreviations: *atp6*, *atp8-9* - subunits 6, 8, and 9 of adenosine triphosphatase synthase; *cob* - apocytochrome b; *cox1-3* - cytochrome c oxidase subunits 1–3; *nad1-6* and *nad4L* - NADH dehydrogenase subunits 1–6 and 4L; *rns* and *rnl* - small and large subunit rRNAs; *rps3–19* and *rpl2–16* - small and large subunit ribosomal proteins; *tatC* - twin-arginine translocase component C. tRNA genes are identified by the one-letter code for their corresponding amino acid. Adjusted from [33, 35].

For investigating animal relationships based on mitochondrial genome evolution, an intermediate size of sponge mitochondrial genome between the large 76 kbp choanoflagellate [39] and the typical bilaterian mitochondrial genome presents an important fact (Figure 7). When compared with the several-fold larger choanoflagellate genome, animal mt genomes show a reduction in the number of genes and a major reduction of intergenic regions (IGRs). More variations are found in non-bilaterian animals which harbor larger mt genomes [35]. Published mitochondrial genomes of marine sponges to date reveal moderate size variation of 16–26 kbp [37], with the notable exception of *Suberites domuncula* (>26 kbp) [40]. The larger size of mtDNA is mainly an outcome of larger non-coding regions that are abundant with palindromes and repetitive elements [35,36]. With respect to marine sponges, freshwater sponge mt genomes have proved to be among the largest, with *Lubomirskia baicalensis* comprising around 29 kbp, mainly because of the expansion of non-coding regions caused by proliferation of palindromes and repetitive elements [36]. Comparison of the mt genomes from four genera of the family Lubomirskiidae with the mt genome from *Ephydatia muelleri* (family Spongillidae) and the 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 [41] (Figure 8). As non-coding regions retained some ancestral features and are abundant with palindromes, the analysis of intergenic regions arguably provides a significant information that could improve phylogenetic classification at the taxonomic levels of order and/or genus.

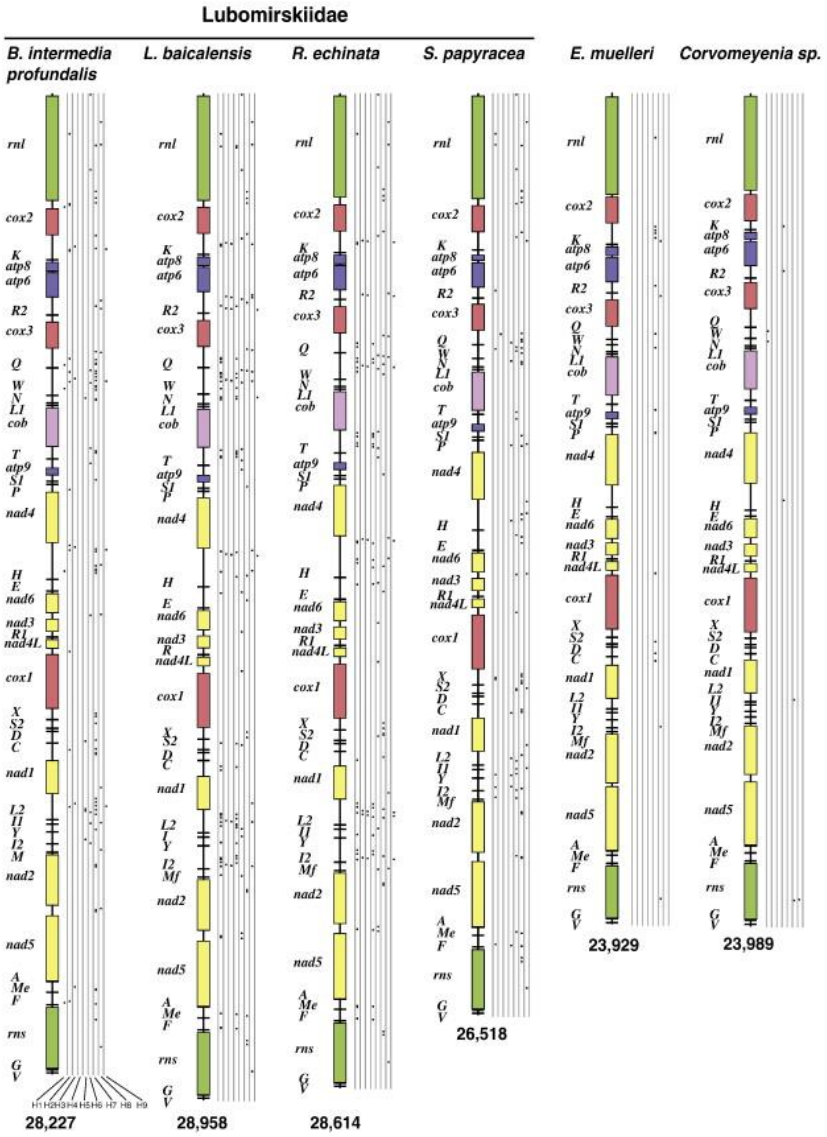


Figure 8. Linearized scaled maps of mitochondrial genomes of freshwater sponges. Heavy black line represents the nucleotide sequence of a genome. Color-coded rectangles represent protein and rRNA genes tRNA genes are marked with horizontal cross-lines and identified by the one-letter code for their corresponding amino acids. All genes have identical transcriptional polarity (top to bottom). Taken from [41].

1.5. Taxonomy of freshwater sponges

Sponges mainly inhabit marine ecosystems, but a small proportion of over 8,000 described species of sponges live in freshwater [42]. Freshwater sponges are currently divided into seven families within the cosmopolitan suborder Spongillina, which comprise 45 genera and one fossil family, Palaeospongillidae [43]. The suborder Spongillina is a group of extant freshwater demosponges

whose fossil record begins in the Cretaceous. Spongillidae constitute the largest family of freshwater sponges, comprising more than a half of the existing species of the suborder Spongillina [43]. Regardless of the intensive morphology and molecular biology-based research, the origin of freshwater sponges and their phylogenetic relationships are still unresolved and remain a matter of discussion. The lack of gemmules in various species, as one of the most reliable characters for freshwater sponge classification, as well as their simple morphology and a pronounced phenotypic plasticity, cause numerous problems in classification of Spongillina species. Several molecular analyses have supported the monophyly of freshwater sponges [10,11,44,45], but despite the increased research, the phylogenetic relationships among families are still unresolved. The analyses based on cox1 and 18S rDNA revealed the family Lubomirskiidae as polyphyletic, although with low support [e.g. 45,46], while ITS2 data again strongly supported the monophyly of Lubomirskiidae [48]. The family Spongillidae was found to be paraphyletic, particularly with respect to Lubomirskiidae [44,46,47]. Several of its genera, including the genus *Ephydatia*, were shown to be paraphyletic and that endemic sponge species might have originated from such cosmopolitan founder species [44,46,48,49]. Such an endemic species is *Eunapius subterraneus* Sket & Velikonja, 1984, the only stygobitic member of the suborder Spongillina known from only few caves near Ogulin, Croatia [50]. This endemic sponge is classified in the IUCN category EN (endangered species) as an organism at high risk of becoming extinct [51]. Phylogenetic study of *E. subterraneus* based on three molecular markers (18S rDNA, ITS2 and COI), showed that it does not group with *Eunapius* species, but rather with other freshwater sponge genera, thus raising the question of true taxonomical designation of this species [48]. 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) [45]. Although a later study synonimized 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), still remains unclear [45] (Figure 9).

Figure 9**:** Neighbour-joining (NJ) phylogenetic tree based on 18S rRNA sequences. Bootstrap values for NJ MP and ML are under the nodes, (top to bottom). The tree is rooted on *Aplysilla sulfurea* (Aplysillidae, Dendroceratida). Poec - Poecilosclerida, Hadr - Hadromerida, Halic - Halichondrida, Agel - Agelasida, Spir - Spirophorida, Astr - Astrophorida, Lith - 'Lithistid', Dendr - Dendroceratida, Petr - Petrosina (Haplosclerida), Spon – Spongillina (Haplosclerida), Hapl - Haplosclerina (Haplosclerida). Taken from [45].

1.6. Taxonomy of calcareous sponges

The class Calcarea Bowerbank, 1864 comprises over 690 exclusively marine species [52], currently divided into two subclasses: Calcinea Bidder, 1898 and Calcaronea Bidder, 1898. Unique features, such as skeleton made of calcium carbonate spicules, together with all five developmental stages of the aquiferous system – asconoid, syconoid, leuconoid, syllebid and solenoid – clearly distinguish calcareous sponges from the other three poriferan classes. Three basic spicule types can be

distinguished in Calcarea, depending on the number of actines: diactines, triactines, and tetractines. Only one species so far is known to possess pentactines [53]. Taxonomic methods used for the class Calcarea have long relied only on histological and morphological characters, especially the structure of the aquiferous system, types of calcareous spicules and the differences in their arrangement within the sponge body [54]. Later, it was suggested that the aquiferous system only should be taken into account [55]. Minchin [56] has divided Calcarea in two groups, later named Calcinea and Calcaronea [57] which were accepted [58,59] and used ever since. According to morphological and molecular data [60,61,62,63] both of them represent monophyletic groups, despite of their wide variability of forms. So far, only a few molecular studies within the subclass level [62,63,64,65] have been performed, revealing rather distinct outcomes in comparison with the traditional morphological classifications, thus raising a great deal of new questions. For instance, the most extensively analysed genus *Clathrina* is defined almost exclusively by negative characters [66] and the absence of many morphological characters and lack of molecular data induces a number of difficulties in the systematics of this genus. This was the reason why many species have been regarded cosmopolitan, which later showed to be the consequence of overconservative taxonomy that does not consider slight morphological differences. The number, size and form of the spicules itself have been efficiently used for the taxonomy of the order Clathrinida, even to the level of species [67,68], but many of those characters could have been interpreted as a consequence of a morphological plasticity [e.g. 69,70]. However, recent molecular analysis using 28S and ITS molecular markers on a larger number of taxa within the order revealed a strong phylogenetic signal in quite subtle morphological differences [71]. Further ITS analysis of species and genera of the order Clathrinida (Figure 10) redefined the genera *Ascandra* and *Clathrina* and described five more orders –*Ascaltis*, *Borojevia Ernstia*, *Arthuria* and *Brattegardia* - while the genus *Guancha* was shown not to be valid [72].

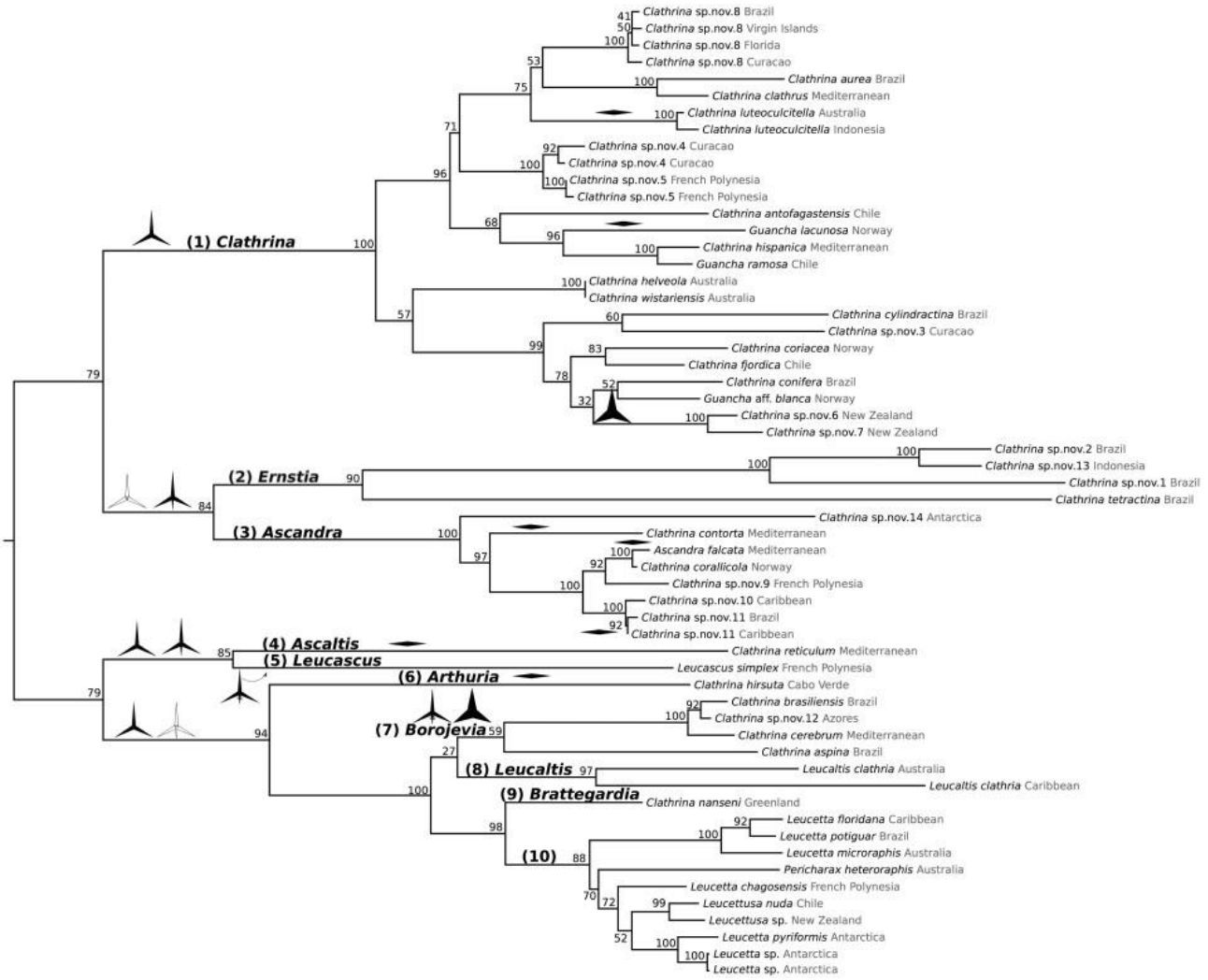


Figure 10**:** Maximum likelihood tree of the nuclear ITS marker for 50 clathrinid species from 12 genera. Black spicules represent the most abundant categories. Taken from [72].

Altogether, these findings show the major importance of DNA markers, which are especially helpful for boundaries elucidation at the lower taxonomic levels within calcineans. Unlike the freshwater sponges, the mitochondrial genome of calcareous sponges was determined recently and still remains poorly characterized [73]. Preliminary data indicate it as a highly unusual genome which exhibits a very high rate of sequence evolution, thus its use in the taxonomic inference analyses remains questionable and to be tested in the future.

1.7. Aims of the research

In recent years, integrative taxonomy combining molecular markers with the traditional taxonomy revealed a number of inconsistencies in taxonomic relationships within the freshwater and calcarean sponges, especially at the lower taxonomic levels of genera and species. Combining new molecular markers on a larger number of species may be helpful in correcting the errors of the current taxonomic divisions. First aim of this work was to test the potential of mitochondrial genomes as genetic markers in Spongillidae evolution, through the comparison of their length, gene content, and organization with known mitochondrial genomes of freshwater sponges. The second aim was to gain a better insight into the species-level relationships, achieved by using comparative analysis on mitochondrial genomes of two species belonging to the same genus, *Ephydatia* Lamouroux, 1816. The Adriatic Sea is considered the type locality for many of the first known species of calcareous sponges, since some of the first studies on the class Calcarea were done along the Dalmatian coast. Yet, these sponges are among least studied animals in the Adriatic and a vast number of their original descriptions are fragmentary, while many type specimens got lost over time. Hence, apart from the importance of species diversity records for this eco-region, another aim of this research was to establish new collections and descriptions of Adriatic calcareous sponges based on detailed morphological and molecular analyses.

2. Materials and methods

2.1. Materials

Table 1. Commercial kits

Kit	Manufacturer
E.Z.N.A.® Forensic DNA Kit	Omega bio-tek
G-spin™ Genomic DNA Extraction Kit (Cell/Tissue)	iNtRON Biotehnology
PCRquick-spin™ PCR Product Purification Kit	iNtRON Biotehnology
QIAprep Spin Miniprep Kit	Qiagen
DNeasy Blood & Tissue Kit	Qiagen
MEGA-spin™ Agarose Gel Extraction Kit	iNtRON Biotehnology
pGEM®-T Easy Vector Systems	Promega
ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit	Applied Biosystems

Table 2. Primers used for obtaining rDNA ribosomal sequences

Primer	Sequence (5'-3')	Source
18SFow	TCATTTAGAGGAAGTAAAAGTCG	[74]
5.8SRev	GCGTTCAAAGACTCGATGATTC	[74]
ITS2F	CGGCTCGTGCGTCGATGAAGAAC	[48]
ITS2R	CGCCGTTACTGGGGGAATCCCTGTTG	[48]
NL4F	GACCCGAAAGATGGTGAACTA	[75]
NL4R	ACCTTGGAGACCTGATGCG	[75]
CAL28SFw	GKCGGATCCGAAYGGACCG	This study
CAL28SRv	CCTCTAATCATTCGCTTTACC	This study

Table 3. Primers designed for obtaining mitochondrial genome sequences. LRN and LRJ are taken from [40].

Primer	Sequence (5'-3')
LRN	CGCCTGTTTATCAAAAACAT
LRJ	CTCCGGTTTGAACTCAGATCA
COX1F	AACATTCTTTGATCCTGCTGG
COX1R	TTGATCATCCCCTAACATTGACC
COX2F	CATCAATGRTATTGGTCYTAYGA
COX2R	ACAATNGGCATAAARGARTGATT

COX3F	TACTTATCAYCCTTAYCATTTAG
COX3R	AAACWACATCCACAAAATGTCAATATC
COBF	NGGCTTTTKCWTCYGTGG
COBR	GCAAATAAAAAATAYCACTCHG
ND1F	TTAACHTTAGCNGARCGAA
ND1R	AAAAATAAAGCAAAWGACA
ND2F	TAGCCGCATTAAAAAGAGACAGT
ND2R	AAAAAGTTAACCCCGCTACCA
ND3F	TATCTGGCGCTTCCTAT
ND3R	AACCCCCTTTTATCCACT
ND4F	TTTATGGGCDTCTTTTGATG
ND4R	ATTTAWATCTCTAGAAAAATAG
ND5F	AGGDACKAARGGGGCAGGTA
ND5R	ATACCCNACAAAAATACTHCC
12SF1	TGGAACTTTTATGTAGCGG
12SR1	GCTTGACACTTTGGGATTA
ATP6F3	TGCTCACAACAATCTATTTAGGGG
COX3R5	ATATATTACAGACCCCACCG
COX3F3	TGAGGCGGCGGCATGATATTG
EPHYMUCOB2	TTCCCAANCACACCCCTAATAAAG
EPHYCob1	TTGGGGGTCAACCGGTAGAAG
ATP9R	TCCTATTCCTGCTCCACTAC
COBF3'	CATACTGTTGTGTGCGATAGCG
ATP9F	CAGCTAAATTTGTAGGTGCTGGGGC
ND4R5'	ACCATCCACTGCAAAAAGAGCTGG
ND1F3'	TTCTATCTTAGCACCTACG
ND2R5'	TCTCCTGTTAATCCGTAC
ND2F3	ATCTGGGATATCTAATGGC
ND5R5	ATAACTTTATATAGGCGGCCG
ND5F_check	ATAGATAGAGGGGTCCTAG
12SR5	ACCGCTACATAAAAGTTCC
12SF3	TCCTTATGATCCTAATGTTTTGGGC

16SR_check	AAGATGGCTGGTTCCAAG
16SFor	AGAGGTGTAGCCGCTTCTAAGGGTTGGACT
COX2F3'	TGGTCAATGTTCAGAAATATGTGGTGC
ATP6R5'	CAATCAATCCCCTAAAAAGGC

Table 4. PCR reaction mixtures

	Reaction mixture (25 µl)	
1	DNA	100-150 ng
	Primers (10 mM)	0.8 µl each
	MgCl2 (25 mM)	3 µl
	dNTPs (10 mM each)	0.5 µl
	10×PCR buffer	2.5 µl
	Taq-DNA polymerase	0.2 µl
2	DNA	100-150 ng
	Primers (10 mM)	1 µl each
	MgCl2 (25 mM)	2,5 µl
	dNTPs (10 mM each)	4 µl
	La Taq buffer	2.5 µl
	LA Taq polymerase	0.2 µl
3	DNA	100-150 ng
	Primers (10 mM)	0.8 µl each
	ReadyMix™ Taq	12.5 µl

Table 5. PCR cycling parameters

PCR method	**Reaction mixture (25 µl)**	
Standard	initial denaturation 3 min/95 °C	
	denaturation 30 s/94 °C	30-35 cycles
	primer annealing 45 s/53-57 °C	
	extension 90 s/70 °C	
	final extension 10 min/72 °C	
Touch-down	initial denaturation 1 min/94° C	
	denaturation 30 s/94 °C	5 cycles
	primer annealing 45 s/59 °C	
	extension 2 min/72 °C	
	denaturation 30 s/94 °C	5 cycles
	primer annealing 45 s/57 °C	
	extension 2 min/72 °C	
	denaturation 30 s/94 °C	20 cycles
	primer annealing 45 s/55 °C	
	extension 2 min/72 °C	
	final extension 10 min/72 °C	

Table 6. Chemicals

Chemical	Manufacturer
Ethanol, absolute	Kemika; Gram-Mol
Ethanol, 96%	Kemika; Gram-Mol
EDTA	Kemika
NaAc	Kemika
HiDi Formamide	Applied Biosystems
Tris	Kemika
Mounting medium	Merck
Common bleach	-
Xylene	Sigma-Aldrich
Acid Fuchsin	Sigma-Aldrich
Paraffin wax	Sigma-Aldrich

2.2. Methods

2.2.1. Sampling

Sponge specimens analysed in this work were sampled through the year, from 2009-2012. Sponges were collected by snorkelling, scuba or cave diving and delivered to the laboratory stored in ethanol. The sequenced specimen of freshwater sponge *Eunapius subterraneus* was collected from near Ogulin (Croatia) and *Ephydatia fluviatilis* from the Tunnel Polje Jezero-Peračko blato (Croatia). Calcarean sponges were collected at 12 localities along the Croatian coastline (Supplementary figure S1). All specimens were fixed and preserved in 96% ethanol at 4°C prior to use.

2.2.2. DNA isolation

Total genomic DNA was isolated using commercially avaliable kits (Table 1). G-spin™ Genomic DNA Extraction Kit (iNtRON Biotehnology) or DNeasy Blood & Tissue Kit (Qiagen) were used for DNA isolation from freshwater sponges and specimens of calcareous sponges over a centimeter in size. Because a large number of calcareous specimens were only a few milimetres in size, and a part of each specimen was saved to be used in morphological analyses and museum deposition, it was

neccessary to use as less tissue as possible for DNA isolation. E.Z.N.A. Forensic DNA Kit (Omega bio-tek) was used for this purpose, as it produces a sufficient amount of DNA from very small amount of starting material. The extraction procedure followed the E.Z.N.A. forenzic DNA hair, nails and feathers protocol, or G-spin and DNeasy protocols for tissue. In order to get more concentrated DNA, the final elution step was adjusted to 4x50 µL eluates, instead the recommended 2x100 µL eluates. The same volume in the final elution step was used always, regardless of the used kit or the protocol instructions.

2.2.3. Polymerase chain reaction (PCR)

Selected DNA fragments were amplified using one of two different PCR methods – standard PCR or touch-down PCR. Standard method was used for amplifying ribosomal DNA markers of calcareous sponges. Touch-down PCR was used for amplification of fragments for which forward and reverse primers had very different annealing temperatures. This was the case for a number of mitochondrial DNA primers, as primers had to be placed in the specific positions to get the necessary overlapping DNA fragments. Because of this it was often impossible to adjust similar annealing temperatures for both pairs of primers. NetPrimer server [130] was used for primer design. Used primers are listed in Tables 2 and 3. All PCR reactions were performed on ice in 25 µL reaction volume. Various amounts of DNA were used in PCR reactions, of total volume from 0.5 to 2 µL. PCR reactions were performed using different reaction mixtures listed in Table 4. Different combinations of cycling parameters and reaction mixtures were adjusted according to the specific requirements (Table 5).

2.2.4. Agarose gel electrophoresis

The amplified DNA fragments were loaded onto 0.5% agarose gel and separated in the electric field, according to their molecular weight. Ethidium Bromide added into the 1x TAE (Tris–acetate, 1 mM EDTA, 20 mM Na–acetate, pH 8.3) loading buffer enabled visualization of DNA under the UV-light. MassRuler DNA Ladder (Fermentas) of known size was used to select fragments of specific sizes, which were cut out of gel and purified using MEGA-spin™ Agarose Gel Extraction Kit (iNtRON Biotehnology), according to the manufacturer's protocols.

2.2.5. DNA sequencing

Purified DNA fragments were sequenced using Sanger dideoxi sequencing method on the ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems), according to the standardized laboratory protocol. NaAc and EDTA were added to help the precipitation of short fragments. DNA was precipitated with 100% ethanol. HiDi formamide (Applied Biosystems) was added to each sample prior to denaturation at 95°C, to stabilize single strands of denaturated DNA chain. Sequencing was performed on four capillary system, using ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and basecalling was performed using standard sequencing software.

2.2.6. Cloning

If sequenced PCR products were of low quality or displayed mixed peaks caused by non-target DNA contamination such as bacteria or algae, those fragments were cloned. First, the desired fragments were amplified by PCR and purified using PCRquick-spin™ PCR Product Purification Kit (iNtRON Biotehnology), according to the manufacturer's protocol. PCR products were ligated with the pGEM-T Vector Kit (Promega) and cloned into XL1-Blue electrocompetent cells. Up to three clones were purfied using QIAprep Spin Miniprep Kit (Qiagen) and sequenced as described in the section 2.2.5.

2.2.7. TA cloning

TA cloning method is faster than standard cloning procedure, as it skips the restriction digestion step using the restriction enzymes. It is based on the complementary base hybridization of different DNA fragments, which in the presence of ligase become ligated together. PCR products were cloned into pGEM-T vector using pGEM®-T Easy Vector Systems kit (Promega). The insert was created by PCR using Taq DNA polymerase that lacks 3' to 5' proofreading activity and adds a single, 3'–adenine overhang to each end of the PCR product. Commercially obtained linearized pGEM-T vector has 3'-thymidine overhang and it can easily be ligated to PCR products after Taq polymerase adds 3'–adenine overhang to their ends. A-tailing reaction mixture typically contained 100 ng of PCR product, 1U Taq DNA polymerase (Fermentas), 1 x reaction buffer, 4 mM $MgSO_4$ and 0.2 mM dATP. Mixture was incubated 30 min at 72°C. PCR products were ligated using T4 DNA ligase. Reaction mixture containing 150 ng of PCR product, 50 ng of pGEM-T vector, 5U T4 DNA ligase was

incubated over-night at 4°C.

2.2.8. Transformation of bacterial cells

Escherichia coli XL1-Blue electrocompetent cells were prepared in the laboratory. Competent cells were transformed using "Electroporator 2510" (Eppendorf) and 2 mm electroporation cuvettets (BioRad). 20 µl of bacterial cell suspension was mixed with 2 µl of the ligation mixture and exposed to high voltage pulse (2500 V). High voltage increases the permeability of bacterial membrane, thereby allowing the introduction of plasmids into the cell. Transformed bacterial cells were regenerated for 50 minutes at 37 °C in 1 ml of LB medium and plated on LB agar containing ampicilin, IPTG and x-gal. The plates were incubated over-night at 37 °C.

2.2.9. Blue-white screening of transformants

The Blue-white screening method is based on the α-complementation of the β-galactosidase gene, which allows detection of cells transformed with the plasmid containing the desired insert. Bacterial colonies grown on LB plates display blue or white color, depending on the transformation efficiency (Figure 11). The non-functional β-galactosidase gene lacking part of its N-terminus can be complemented by a peptide formed of β-galactosidase residues. pGEM®-T Easy Vectors contain T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme beta-galactosidase. DNA ligated into the plasmid disrupts the α-peptide and therefore the complementation process, and no functional β-galactosidase can form. Insertional inactivation of the α-peptide allows direct identification of recombinant clones based on blue/white colour of colonies on plates. The presence of functional β-galactosidase can be detected by X-gal, which is cleaved by β-galactosidase, showing a blue pigment. Cells transformed with plasmid containing an insert therefore form white colonies, while cells transformed with plasmid without an insert form blue colonies.

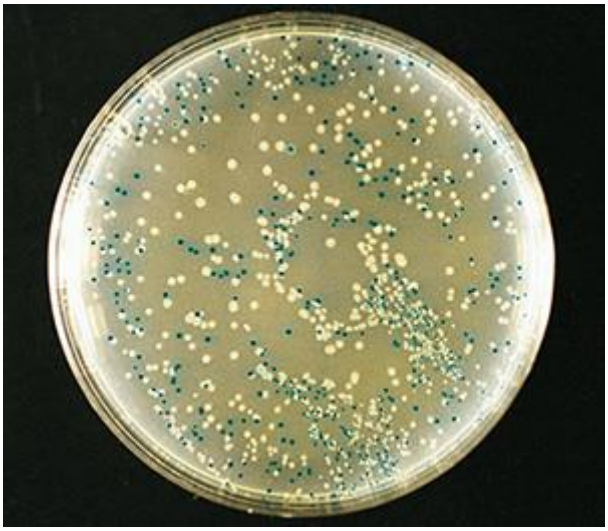


Figure 11. LB plate with blue and white screen. Taken from [131].

2.2.10. Isolation and purification of plasmids

Up to three transformed colonies (white ones) were inoculated in 4 ml of LB media containing ampicilin and incubated over-night at 37 °C in a shaking incubator. The next day, plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen) and sequenced as described in the section 2.2.5.

2.2.11. Sequence alignment and assembly

Forward and reverse sequences were assembled using Lasergene processing software (DNASTAR Inc., Madison, WI, USA) and checked manually for sequencing errors. The BLAST network service [132] was used for sequence homology searches. Selected sequences were used to obtain multiple alignments, taking into account the secondary structures of rDNA sequences. As paired sites of rRNA sequences do not display independent phylogenetic information, it is important to predict secondary structures which can direct the alignment. The multiple alignments were performed with the Q-INS-i option of the MAFFT program [76], using score matrix 200 PAM/k=2, gap penalty 1.53 and offset value 0. Amino acid sequences of mitochondrial protein-coding genes were concatenated and aligned under the default parameters in ClustalW 1.7 [77]. In the next step, the alignments were run through Gblocks v.0.91b server under less stringent parameters [78] to exclude poorly aligned regions from further analyses.

2.2.12. Mitochondrial genome assembly by primer walking

Forward and reverse sequences were assembled using Lasergene processing software (DNASTAR Inc., Madison, WI, USA) and manually checked. Sequence homology searches were done using BLAST network service [132]. A method of primer walking was used for assembly of the mitochondrial genome fragments obtained by PCR with specific primers spanning the desired region (Figure 12). Primers were synthesized in a way that the adjacent PCR products overlap each other. Primers designed for large ribosomal subunit [40] and *cox1* were used for obtaining the initial fragments. Degenerative primer sets were designed based on the multiple alignments with sequences available in GenBank and used for amplification of conserved regions of protein coding genes. Sequence data produced by primer walking from these fragments were added to the original gap-containing sequence based on sequence identity, until all gaps were filled. In this way, the whole circular molecule was obtained. Mitochondrial sequences are available in NCBI's GenBank under the accession numbers: GU086203 (*E. subterraneus*) and JN209966 (*E. fluviatilis*).

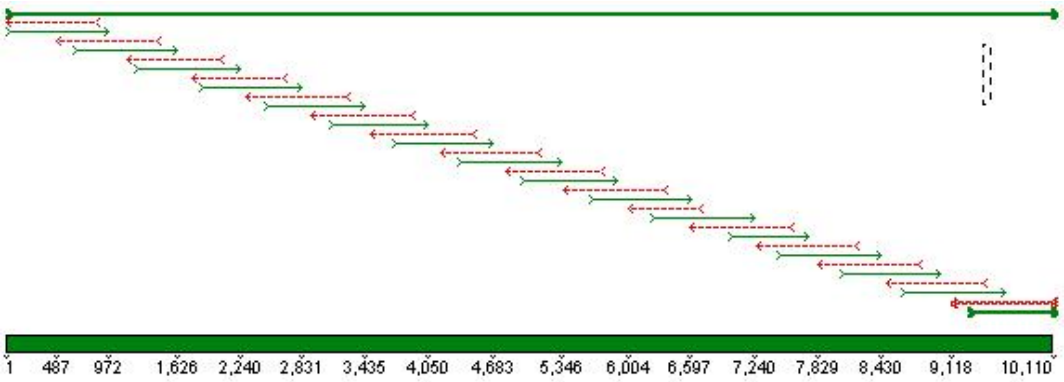


Figure 12. Assembly of DNA region using DNA fragments obtained with the primer walking method. Taken from [133].

2.2.13. Mitochondrial genome analysis

tRNA genes were identified by the tRNAscan-SE program [79] and secondary structures were drawn using CorelDraw12. Other genes were identified by BLAST homology searches from GenBank. The secondary structures of rRNA genes were manually folded based on the analogy with the published rRNA structures. The Palindrome program of the EMBOSS software package [80] was employed to search for closely spaced perfect inverted repeats using the following parameters: minimum length

of palindromic elements—7 nt; maximum length of palindromes—100 nt; maximum gap between repeat elements—10 nt. Secondary structures were predicted using mfold-server. All the secondary structures were drawn in CorelDraw12.

2.2.14. Phylogenetic reconstruction

Akaike information criterion (AIC) implemented in ProtTest v. 2.4 [81,82,83] was used to select the best-fit model of protein evolution, based on aligned amino acid sequences of *E. subterraneus* and *E. fluviatilis* mitochondrial protein-coding genes. Phylogenetic analysis on *E. fluviatilis* was performed under the AIC best-fit model (JTT+G+F), using maximum likelihood (ML) and maximum parsimony (MP) methods [84] in MEGA5 [85]. Support for the nodes in trees was estimated by bootstrapping (1,000 bootstrap replicates).

Phylogenetic analysis on *E. subterraneus* was performed under the same model, using ML in PhyML-aLRT program [82,86] and Bayesian MCMC analysis in MrBayes v. 3.1.2. [87]. Two parallel runs were applied, each with one hot and three cold Markov chains, for 1,500,000 generations. The sampling frequency was one in every hundred trees. The consensus tree was constructed based on the trees sampled after the burn-in. The convergence of Markov chains was checked through standard deviations of split frequencies and log-likelihood scores for each run. MP heuristic search was performed in PAUP v4.0b10 [88] on 1,000 random taxon addition replicates, using tree-bisection reconnection (TBR) algorithm. Support for the nodes in trees was estimated by bootstraping (1,000 bootstrap replicates in MP and 100 in ML) and by posteriror probabilities in MrBayes. Time of divergence of *E. subterraneus* from other freshwater species was estimated on Bayesian tree by relaxed-clock method with non-parametric rate smoothing (NPRS) in program r8s [89]. This method accounts for rate variation across lineages and provides more reliable estimates of divergence times on various timescales.

Maximum likelihood (ML) and Bayesian inference (BI) methods were also applied on calcarean rDNA multiple sequence alignments. AIC implemented in jModeltest 3.7 [82,90] was used to select the best-fit models of sequence evolution. The models were chosen for each dataset as follows: for 28S analysis, GTR+I+G and TrN+I+G models were chosen for Calcinea and Calcaronea, respectively; for ITS analysis, TrN+G model was chosen for both datasets. Phylogenetic analyses were performed in PhyML 3.0 [91], where datasets were analysed by ML method. Bootstrap tests of phylogeny were performed with 1000 replicates. Bayesian MCMC analyses were performed in MrBayes v. 3.1.2. [87], considering the same models for given datasets. Two parallel runs each comprising four Markov

chains were run for 1,000,000 generations with sampling frequency of one in every 100 trees. The consensus tree was constructed based on the trees sampled after burn-in of 100,000. Phylogenetic trees were generated separately for each dataset, rooted at midpoint and displayed in FigTree v.1.4.2 [134]. Obtained sequences were submitted to the GenBank, under the accession numbers KP739994-KP740035 and KT447551-KT447568 (Supplementary table S1).

2.2.15. Spicule preparations

Since the analysed sponges possess calcareous spicules which would dissolve in acid, sodium hypochlorite (common bleach) was used for spicule preparation. A small part of sponge cortex and choanosome was placed in a test tube containing 3 mL of bleach. After 1h, the spongin tissue was completely dissolved and spicules formed a pellet. After the careful pipeting of the supernatant, distilled water was added to wash the spicules. When spicules again formed a pellet, the distilled water was removed by pipeting and washing was repeated 3-4 times. In the end, the spicule pellet was washed with 95% ethanol 3 times to dehydrate spicules. After the last washing with ethanol, the spicules were left in approximately 0.5 mL of 95% ethanol. Spicules were transferred to a microscopic slide and after the ethanol completely evaporated, a few drops of the mounting medium were added and slide was covered with a coverslip.

2.2.16. Preparation of tissue slides

Sponges were cut longitudinally from osculum to base, using a shaving blade. Tangential sections of the surface and atrium of sponges were also taken. The sections were placed onto slides to dry. When sponges completely dried, a few drops of xylene and mounting medium was added and the slide was covered with a coverslip. Sponge part that will be used to prepare the sections of skelet was placed in a Petri dish containing Acid Fuchsin. After 20 minutes, Fuchsin was replaced with 93% ethanol for 10 minutes to remove the residual Fuchsin. Ethanol was then replaced with xylene and after 30 minutes this step was repeated. After xylene, sponges were placed in melted paraffin for 1 hour. Transversal cuts using microtome or razor blade were taken on parrafin blocks containing tissue pieces. The sections were cleaned from paraffin with xylene, placed on a slide in mounting medium and covered with a coverslip.

2.2.17. Electron microscopy analysis

Length and width at the base of each actine of the spicules were obtained using Axio Vision software V 4.8.1.0 (Zeiss GmbH, Germany). The results are presented in tabular form, featuring length (minimum [min], mean, standard deviation [sd] and maximum [max]), width (minimum [min], mean, standard deviation [sd] and maximum [max]) and sample size (n). Photomicrographs were taken with a digital camera mounted on a Zeiss Axioscop (at the UFRJ) or Axiovert 200 (at the CIM) microscope. Micrographs were taken on a Scanning Electron Microscope (SEM) (JEOL, JSM-6510) at the Biology Institute of the UFRJ.

2.2.18. Species deposition into sponge collections

The same specimen of each species was used for both, morphological and molecular analyses. Specimens are deposited in sponge collections of the Biology Institute at the Federal Institute of Rio de Janeiro in Brazil, the Natural History Museum in Rijeka, Croatia, and at the Ruđer Bošković Institute, Croatia. Specimen voucher numbers are listed in Supplementary table S1.

3. Results

3.1. Mitochondrial genomes of *Eunapius subterraneus* and *Ephydatia fluviatilis*

3.1.1. Genome organization

Both mitochondrial genomes are circular molecules, with size within the range of all previously determined mt genomes of freshwater sponges (23,929-28,958 bp). They contain the same 25 tRNA genes as found in other sponges, including 3 additional tRNA genes (*trnI2*(cau), *trnR2*(ucu), *trnMe*(cau)), 2 rRNA, and 14 protein coding genes, including the *atp9* gene (Figure 13).

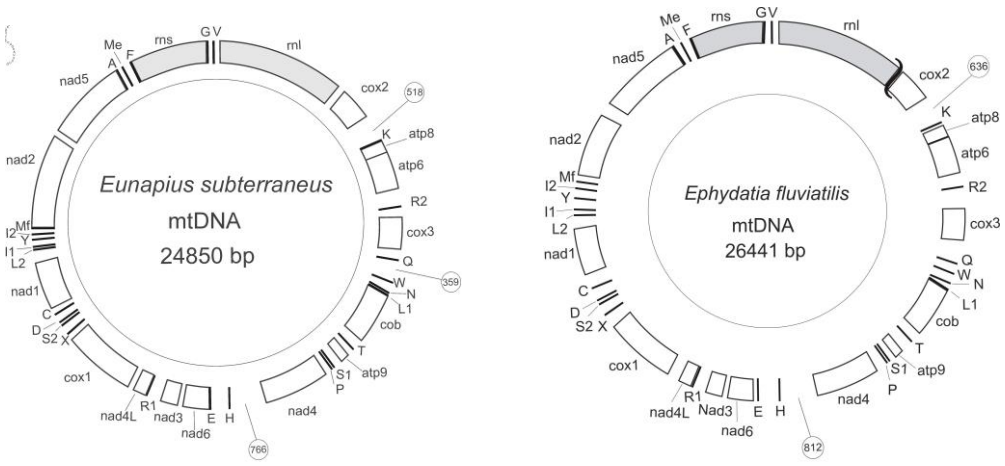


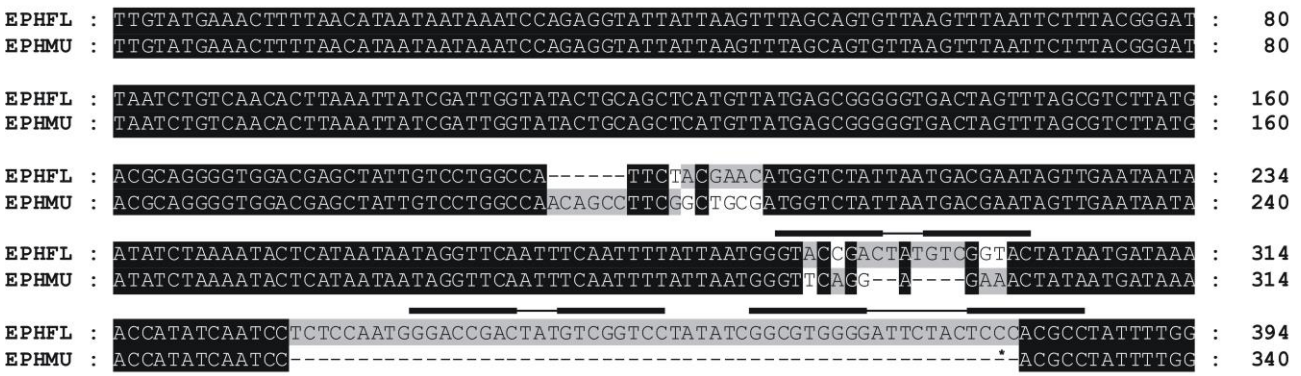
Figure 13. Genetic map of *Eunapius subterraneus* (left) and *Ephydatia fluviatilis* (right) mtDNA. Protein coding genes are in white, rRNA genes in grey and tRNA genes are in black and labelled by the one-letter code for their corresponding amino acid. The largest non-coding regions are indicated by circles with corresponding lengths. Sigmoidal curve indicates the lack of sequence data from the particular region of the genome. Abbreviations are the same as in Figure 8.

One gene boundary (*nad5*|*trnA*(ucg)-23 bp) was found, as well as tRNA-like structure *trnX*. Both features are well conserved within freshwater sponges. *TrnX* has a well-conserved primary structure with 95%/100% nucleotide identity with all thus far sequenced freshwater sponges. All genes in both mitochondrial genomes are positioned on the heavy strand and are transcribed clockwise. No introns or extra genes were found. Genes in all analysed species have the same transcriptional orientation and arrangements of protein coding genes, rRNA and tRNA. The A+T content of *E. subterraneus* mt-genome is 59.18 % and that of *E. fluviatilis* is 58.48 %, as has been observed for other freshwater sponges (56-72 %). All types of sequences in *E. subterraneus* showed positive GC-skew except the non-coding regions, as previously noted only for *E. muelleri* and *Aplysina fistularis* [37]. AT-skew was negative for all sequences except for the sense strand of rRNA genes. 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 the family Spongillidae [37,49]. The only inconsistency to the previously reported values was found within non-coding regions of *E. fluviatilis*, that showed positive GC-skew, 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 most probably contains multiple repeated sequences, therefore, it was problematic for sequencing.

3.1.2. Protein coding genes and codon usage

Protein coding genes in both *Ephydatia* species have 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 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*. *E. 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 (Figure 14) belonging to the H7 family in both *E. fluviatilis* and *E. subterraneus*. These palindromic elements were not found in *E. muelleri*.



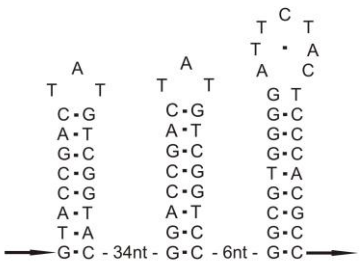


Figure 14. Alignment of *nad2* (NADH dehydrogenase subunit 2) gene from *Ephydatia fluviatilis* (EPHFL) and *Ephydatia muelleri* (EPHMU) (top); parts containing palindromic elements responsible for larger insertions in *E. fluviatilis* are indicated above the sequence. Triplet coding for amino acid proline (CCC) present only in *E. fluviatilis* and *Eunapius subterraneus* is marked with an asterisk (*). Secondary structure of palindromic elements in the inserted regions of *nad2* gene from *E. fluviatilis* (left).

Interestingly, in one of those palindromes, *E. subterraneus* and *E. fluviatilis* have an additional in-frame triplet (CCC) coding for the proline amino acid. A case like this has been reported previously in Rickettsiae [92], 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 [93]. Palindromic elements found in rRNA are in peripheral regions and those inserted within the coding regions are always in frame, which strongly indicates 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 [41]. Among the existing codons, those ending with A or T are preferred (38-40%), while those ending with C are least frequent. These data are consistent within all described mitochondrial genomes to date [37]. The ATG was the most frequent initiation codon, the GTG was inferred for *nad6*, and the TTG start codon was inferred for *nad2*. The stop codons were either TAA or TAG for all protein coding sequences. The CGC codon, absent in most demosponges, was found once. Interestingly, this codon was found in all thus far described freshwater sponges mt genomes at the same conserved position within the *nad5* gene [41].

3.1.3. RNA genes

tRNA genes have well conserved primary and secondary structure and D- and T-loops. Among individual tRNA genes of *E. subterraneus*, 17 showed 100% identity with *E. muelleri, E. fluviatilis* and *L. baicalensis* tRNAs, while the remaining 8 tRNAs (Gln, His, Leu1, Leu2, Phe, Pro, Thr and Val) varied in only 1 or 2 nucleotides. Moreover, all four mitochondrial genomes display an identical tRNA gene order. This is a surprisingly high similarity, knowing that tRNA gene order is highly variable among sponges [94].

Genes for small and large subunit ribosomal RNAs (*rns* and *rnl*) are arranged in the most common gene order, +*rns*+*trnG*+*trnV*+*rnl* [37]. 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 *E. subterraneus*. Since one region within the gene for the large subunit ribosomal RNA of *E. fluviatilis* was highly problematic for sequencing, which was potentially caused by additional stem-loop structures, only the rRNA genes of *E. subterraneus* were analysed in detail.

The 50 and 30 end nucleotides of *E. subterraneus* rRNAs were determined by similarity with *E. muelleri*, as well as secondary structure modeling, indicating the length of *rns* 1578 bp and *rnl* 2807 bp. rRNAs are abundant with repetitive elements (short dyads, dyad repeats and direct repeats). The longest direct repeat in *rnl* is 14 nucleotides long, in *rns* 24 nucleotides. Comparison between *E. subterraneus* and *E. muelleri* rRNAs revealed identical repetitive elements in *rns*, while in *rnl* of *E. subterraneus*, palindromes and inverted repeats were not found. Furthermore, one long (22 bp-GGAAGCTACGCTTCCATGCCGC) direct repeat found in *E. muelleri* was substituted with two shorter: 12 bp-GGGTTATAATGA and 14 bp-CGCTTCCATGCCGC repeats. Generally, in both, *rnl* and *rns*, repeats are identical and presumably genealogically related in these freshwater sponges. Their abundance is correlated with length. The primary sequences of *rnl* and *rns* are well conserved sharing a highest sequence identity with homologous genes in *E. muelleri* (94%/93%), *L. baicalensis* (87%/88%), *Topsentia ophiraphidites* (66%/63%), *Axinella corrugata* (62%/57%) and *Suberites domuncula* (55%/61%).

3.1.4. Intergenic regions

Eunapius subterraneus

Intergenic regions constitute 24.4% (6070 bp) of the mitochondrial genome of *E. subterraneus*, and are distributed among 38 segments with the largest of them downstream of *nad4* (766 bp). Furthermore, non-coding regions of *E. muelleri* and *E. subterraneus* share a considerable similarity which suggests a very recent split between the two taxa.

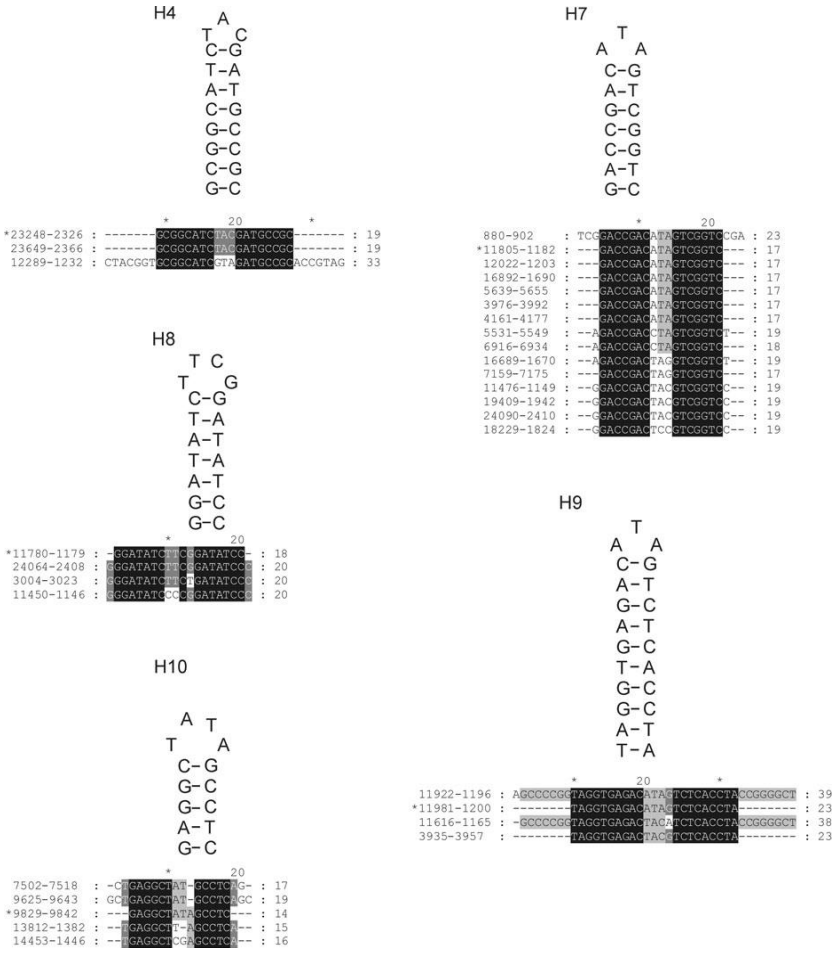


Figure 15. Secondary structures and corresponding alignments of palindromic repetitive elements in *Eunapius subterraneus*. Families present in *E. subterraneus* (H4-H10). The numbers in the alignment refer to their position in the mt genome. The sequence for which the secondary structure is given is marked with an asterisk (*).

Intergenic regions of *E. subterraneus* encompass various types of repeat motifs which are spread throughout the mt genome, including coding regions, rRNAs and tRNAs. Repetitive palindromic elements with potential to form hairpin structures were found in mtDNA of several sponges [36,95]. We identified more than 82 short repetitive elements in the whole mt genome of *E. subterraneus* that range in size from 7 to 18 bp. According to previous classification [36], these repetitive elements were subdivided into 5 distinct families (Figure 15). The most abundant families H7 and H8 are present in all analysed genomes. H9s reported to be specific for *E. muelleri* can be found in *E. subterraneus* as well. One new family, named H10 with a consensus sequence 5'-TGAGGCT-3' is species specific for *E. subterraneus*. GC-rich repetitive sequences are overrepresented in comparison with AT-rich counterparts. When the palindromic elements are folded into hairpin structures, the stem component of the hairpin varies from 5-10 bp in length, and the loop portion is usually 3-4 nt long.

Ephydatia fluviatilis

Intergenic regions (IGRs) in the 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. The palindromic element inserted in the trnS(gcu) and trnI(gau) is found at the same position in all available mt genomes of freshwater sponges. However, palindromic families between two species of the genus *Ephydatia* revealed a weak similarity in either their sequence or their distribution (Figure 16).

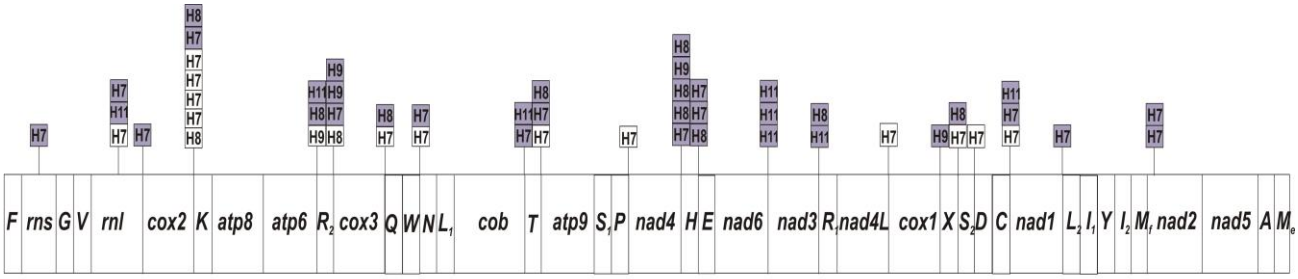


Figure 16. The distribution of repetitive hairpin-forming elements in intergenic regions of mitochondrial genomes of *Ephydatia fluviatilis* (dark gray) and *Ephydatia muelleri* (white). Modified according to [36].

The 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 H9s family is specific to Spongilidae. One new palindromic family named H11, with a consensus sequence 5'-TGGCACCG-3' was found only in *E. fluviatilis*. The distribution of families is reflected in the phylogenetic tree based on mitochondrial coding sequences (Figure 17). An identical topology was recovered when palindromic sequences were used for analysis, which indicates that these elements could provide useful information in species-level studies.

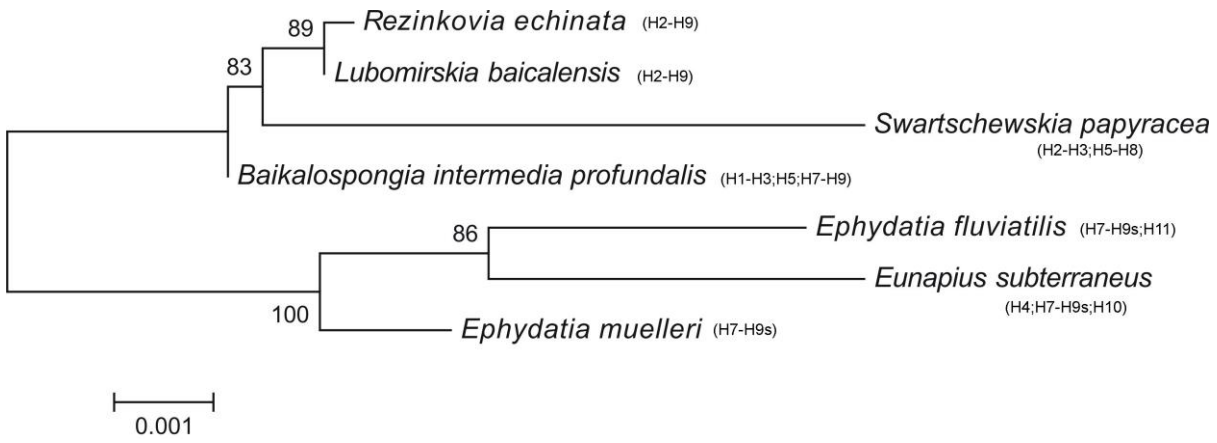


Figure 17. Phylogenetic relationships among freshwater sponges based on concatenated protein sequences of mitochondrial genes. Maximum likelihood tree obtained under the JTT+G+F model is shown. An identical topology was recovered in maximum parsimony analysis. Hairpin families are indicated in parentheses next to the species names.

3.2. Morphological analysis of the Adriatic calcarean sponges

3.2.1. Systematics of the Adriatic Calcinea Bidder, 1898

Systematic index

Class CALCAREA Bowerbank, 1864
Subclass CALCINEA Bidder, 1898
Order CLATHRINIDA Hartman, 1958

Family CLATHRINIDAE Minchin, 1900

Genus *Ascaltis* Haeckel, 1872

***Ascaltis reticulum* (Schmidt, 1862)**

(Figure 18; Table 7)

Type locality. Zara (Croatian: Zadar) and Sebenico (Croatian: Šibenik), Adriatic Sea.

Type specimen. BMNH 1896.9.15.13 (neotype proposed by Klautau and Valentine [67]). Banyuls-sur-Mer, Pyrenees, France. E.A. Minchin Collection.

Material examined. PMR-13739 = UFRJPOR 6870. Near the Island of Čiovo, Adriatic Sea, 43°28'58.5"N 16°21'25.6"E; 5 m deep. Collected by B. Pleše and V. Nikolić; 5 November 2010.

Colour. White in life and white in ethanol.

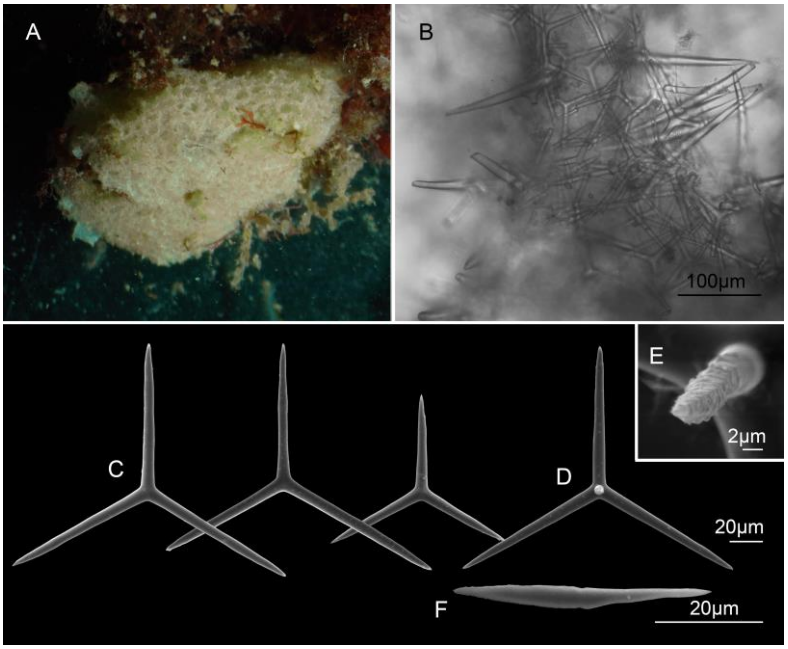


Figure 18. *Ascaltis reticulum* (PMR 13739 = UFRJPOR 6870). (A) Specimen *in situ*. (B) Section showing the perpendicular arrangement of diactines. (C) Regular triactines. (D) Regular tetractine. (E) Apical actine of a tetractine covered with short spines. (F) Diactine. Photo: F. Azevedo.

Description. Cormus is composed of regular and tightly anastomosed tubes. Water-collecting tubes are present (Figure 18A). As the specimen was fragmented, it was not possible to observe the pseudoatrium. The skeleton is composed of one category of triactines, one of tetractines and diactines. Diactines are organised in tufts of two to five spicules, perpendicularly disposed in the tubes (Figure 18B). Triactines are the most abundant spicules.

Spicules (Table 7).

Triactines: regular (equiangular and equiradiate). Actines are slightly conical to cylindrical with sharp tips (Figure 18C).

Tetractines: regular (equiangular and equiradiate). Actines are slightly conical to cylindrical with sharp tips (Figure 18D). The apical actine is very thin and shorter than the basal ones. It is cylindrical and blunt, covered with abundant tiny spines (Figure 18E).

Diactines: slightly curved. The tip that protrudes through the surface is lanceolated (Figure 18F). Trichoxeas are also present on the surface of the tubes.

Table 7. Spicules measurements of *Ascaltis reticulum* (PMR-13739 = UFRJPOR 6870).

		length (µm)				width (µm)				
		min	mean	sd	max	min	mean	sd	max	n
Diactine	-	60.0	106.3	26.0	142.5	3.8	4.9	0.5	6.3	20
Triactine	Basal	56.7	88.2	11.7	108.0	6.8	9.5	1.5	10.8	21
Tetractine	Basal	60.0	79.1	12.4	107.5	7.5	8.8	1.3	10.0	30
	Apical	27.5	41.9	9.7	62.5	2.5	3.2	0.6	3.8	17

Ecology. Specimens were collected on a vertical shaded hard limestone bottom.

Remarks. Klautau *et al*. [72] proposed to transfer this species to the genus *Ascaltis* based mainly on morphological, but also on molecular data. Although the type species of this genus (*A. lamarcki* Haeckel, 1870) was not included in the molecular dataset, *A. reticulum* did not group with any other of the included genera (Figure 36). Besides, morphologically it is more similar to *Ascaltis* than to other genera. Although the classification of *A. reticulum* in the genus *Ascaltis* must yet be verified regarding the type species, it was morphologically and molecularly proved that it can not be included in the genus *Clathrina*. According to the proposition of Klautau *et al*. [72], this species is denominated *A. reticulum*. This is the first time that spines were observed on the apical actine of the tetractines in *A. reticulum*. For this reason, the neotype of the species was examined and spines were detected there as well. They are abundant and very small. Also, a great variation in the size of the diactines was observed, which are much larger in the neotype (102.0 - 212.2 (±54.1) - 306.0 /14.3 (±5.1) µm).

Genus *Ascandra* Haeckel, 1872

Ascandra spalatensis sp. nov.

(Figure 19; Table 8)

Type locality. Zara (Croatian: Zadar), Adriatic Sea.

Type material. PMR-17806 = UFRJPOR 7540 (holotype / ethanol). Near Zadar, Adriatic Sea, 44°08'14.8"N, 15°12'38.2"E; 1 m deep. Collected by V. Nikolić; 13 February 2011.

Colour. White in ethanol.

Etymology. From the type locality.

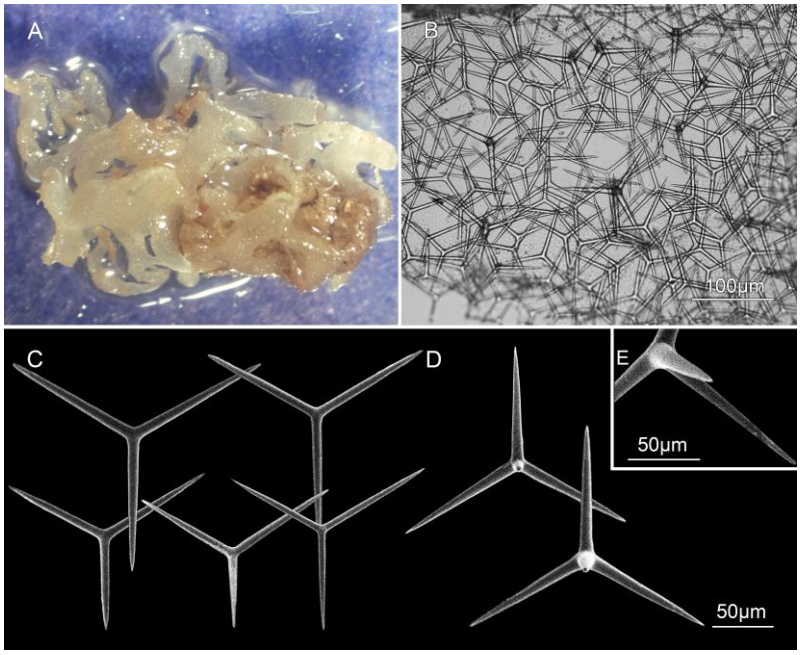


Figure 19. *Ascandra spalatensis* sp. nov. (Holotype - PMR 17806 =UFRJPOR 7540). (A) Specimen in ethanol. (B) Tangential section. (C) Triactines. (D) Tetractines. (E) Apical actine of a tetractine. Photo F. Azevedo.

Description. The sponge is small, but it is possible to recognize large and loosely anastomosed tubes, typical of *Ascandra* (Figure 19A). The skeleton is composed of triactines and few tetractines (Figure 19B).

Spicules (Table 8).

Triactines: regular (equiangular and equiradiate), but there are also subregular (sagittal) spicules. Actines are cylindrical to slightly conical with sharp tips (Figure 19C).

Tetractines: regular (equiangular and equiradiate) or subregular. Actines are strongly conical with sharp tips (Figure 19D). The apical actine is shorter than the basal ones, thick, conical, sharp and smooth (Figure 19E).

Table 8. Spicules measurements of the holotype of *Ascandra spalatensis* sp. nov. (PMR-17806 = UFRJPOR 7540).

		length (µm)				width (µm)				
Spicule	Actine	min	mean	sd	max	min	mean	sd	max	n
Triactine	Basal	43.2	90.5	17.2	113.4	6.8	8.0	0.8	9.5	20
Tetractine	Basal	51.3	99.4	16.9	135.0	8.1	12.0	1.6	14.9	21
	Apical	72.9	74.3	1.9	75.6	10.8	10.8	0	10.8	02

Ecology. Specimens were collected on a shaded vertical hard bottom.

Remarks. The genus *Ascandra* is so far comprised of 13 known species: *A. falcata* Haeckel, 1872; *A. ascandroides* (Borojević, 1971); *A. atlantica* (Thacker, 1908); *A. biscayae* (Borojević & Boury-Esnault, 1987); *A. brandtae* (Rapp *et al.*, 2013); *A. contorta* (Bowerbank, 1866); *A. corallicola*

(Rapp, 2006); *A. crewsi* Van Soest & De Voogd, 2015; *A. densa* Haeckel, 1872; *A. kakaban* Van Soest & De Voogd, 2015; *A. loculosa* (Dendy, 1891); *A. minchini* Borojević, 1966; and *A. sertularia* Haeckel, 1872. In 2013, the following diagnosis was proposed for *Ascandra* [72]:

"Calcinea with loosely anastomosed tubes. Tubes are free, at least in the apical region. The skeleton contains regular (equiangular and equiradiate) or sagittal triactines and tetractines. Tetractines are the main spicules, occurring at least in the same proportion as the triactines. They have very thin (needle-like) apical actines. Diactines may be added. Asconoid aquiferous system."

After the discovery of *A. spalatensis* sp. nov., an emendation to its diagnosis is proposed:

"Calcinea with loosely anastomosed tubes. Tubes are free, at least in the apical region. The skeleton contains regular (equiangular and equiradiate) or sagittal triactines and tetractines. The apical actine is very thin (needle-like) or very thick at the base. Diactines may be added. Asconoid aquiferous system."

The new species is a very typical *Ascandra*, with apically free, loosely anastomosed tubes. Its skeleton is very similar to that of *A. ascandroides*, composed of triactines and tetractines, the former being more abundant than the latter and the apical actine of the tetractines being very thick at the base. Both species, however, can be differentiated by the size of the spicules (*A. ascandroides* - triactines: 90-130(±20)-163 / 13(±2); small tetractines: 107.5-164.5(±35)-260 / 16.5(±2.8); large tetractines: 193.8-313.1(±63.2)-418.2 / 39.8(±8.2)). Moreover, *A. ascandroides* has two categories of tetractines and *A. spalatensis* sp. nov. has only one. This species is well nested within the *Ascandra* clade with high support values in both, Bayesian and ML analyses (Figure 36).

Genus *Borojevia* Klautau et al., 2013

***Borojevia cerebrum* (Haeckel, 1872)**

(Figure 20; Table 9)

Type locality. Lesina (Croatian: Island of Hvar), Adriatic Sea.

Type specimen. PMJ-Inv. Nr. Porif. 156 (syntype/ethanol). Haeckel Collection.

Material examined. PMR-17808; IRB-CLB33 = UFRJPOR 7539. Vrulja Cove, Adriatic Sea, 43°24'01.3"N, 16°53'10.9"E; 10 m deep. Collected by V. Nikolić; 24 August 2011.

Colour. White in life and white in ethanol.

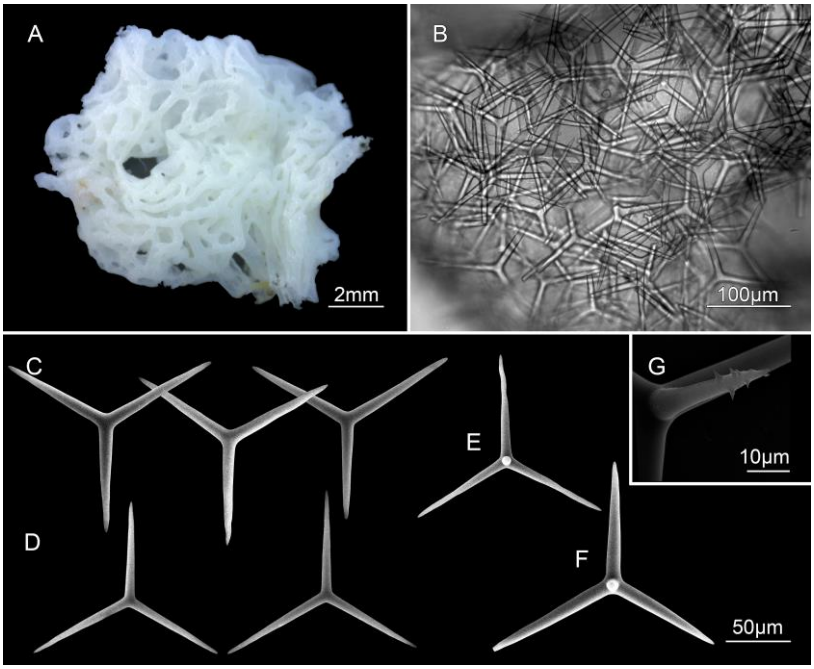


Figure 20. *Borojevia cerebrum* (IRB-CLB33=UFRJPOR 7539). (A) Specimen in ethanol. (B) Tangential section. (C) Tripods. (D) Triactines. (E) Small tetractine. (F) Large tetractine. (G) Apical actine of a tetractine ornamented with spines. Photo: F. Azevedo.

Description. Cormus is composed of regular and tightly anastomosed tubes (Figure 20A). Large water-collecting tubes are present. The skeleton consists of triactines, a few tetractines and tripods, which in fact are large triactines. It has no special organization (Figure 20B).

Spicules (Table 9).

Tripods: regular (equiangular and equiradiate). The tripods of analysed specimens are more similar to large triactines than to true tripods with an elevated centre. Actines are conical, straight, with sharp tips (Figure 20C).

Triactines: regular (equiangular and equiradiate). Actines are slightly conical to conical, straight, with sharp tips. Sometimes they are slightly undulated near the tips (Figure 20D).

Tetractines: regular (equiangular and equiradiate). Actines are slightly conical to conical, straight, with sharp tips. Sometimes they are slightly undulated near the tips. It is possible to recognize two types of tetractines: small (Figure 20E) and large (Figure 20F). Large tetractines are the same size as tripods. The apical actine of the tetractines is shorter than the basal ones, slightly conical, sharp and frequently curved only at the tip. It is ornamented with about six spines, which are large, conical and cover the last third of the apical actine. (Figure 20G).

Table 9. Spicules measurements of *Borojevia cerebrum* (IRB-CLB33 = UFRJPOR 7539).

		length (µm)				width (µm)				
Spicule	Actine	min	mean	sd	max	min	mean	sd	max	n
Tripod	Basal	72.9	91.8	9.9	108.0	8.1	11.2	1.5	13.5	20
Triactine	Basal	54.0	84.6	11.8	105.3	5.4	8.9	1.3	10.8	20
Tetractine	Basal	64.8	81.9	10.0	105.3	6.8	8.5	1.5	12.2	20
	Apical	35.1	46.8	8.4	64.8	5.4	5.4	0.0	5.4	20

Ecology. Specimens were collected on a semi-vertical hard limestone bottom.

Remarks. Similar to other borojevias, *B. cerebrum* has a thin, regular and tightly anastomosed tubes forming the cormus. The oscula are present at the end of water-collecting tubes. The skeleton is composed of tripods (with the characteristic elevated centre or similar to large triactines), triactines and tetractines. Individuals of *B. cerebrum* always have spines on the apical actine of their tetractines, however, in a same individual some tetractines may be smooth. In *B. cerebrum*, the spines are not very abundant; they are large and scattered only near the tip of the apical actine. The Adriatic and Mediterranean specimens of *B. cerebrum* formed a well supported clade (Figure 35) separated from the clade comprising *B. brasiliensis* (Solé-Cava, Klautau, Boury-Esnault, Borojević & Thorpe, 1991).

Borojevia cerebrum is the type species of the genus. Its type locality is Lesina (Island of Hvar) and it commonly occurs in the Mediterranean and the Adriatic Sea. The type specimen of *B. cerebrum* (PMJ-Inv. Nr. Porif. 156) is not well preserved [67], so this appeared as a great opportunity to re-describe the species from near its type locality. Analyses of other individuals of *B. cerebrum* from several sites in the Adriatic and Mediterranean Seas verify that the shape of tripods is very variable. It varies from characteristic shape of tripods, with stout actines and elevated centre, to only a large triactines. This kind of variability may be assigned to polymorphism or plasticity. Indeed, Haeckel [54] proposed two varieties of *B. cerebrum* (then known as *Ascaltis cerebrum*) based on the presence of either characteristic tripods or large triactines. He named the first variety *B. cerebrum* var. *gyrosa,* while the other one he considered *B. cerebrum* var. *decipiens*. Dendy & Row [58] elevated both varieties to the species category, without any further explanation. Considering that both varieties were proposed only to differentiate specimens with characteristic tripods from those with only large triactines, and since this morphological variation is here found inside individuals and among specimens placed within the same species, synonimizing of *B. gyrosa* and *B. decipiens* to *B. cerebrum* is now proposed.

***Borojevia croatica* sp. nov.**

(Figure 21; Table 10)

Type locality. Island of Čiovo, Adriatic Sea.

Type material. PMR-13740 = UFRJPOR 6864 (holotype / ethanol); PMR-13741 = UFRJPOR 6865 (paratype / ethanol). Near the Island of Čiovo, Adriatic Sea, 43°28'58.5"N, 16°21'25.6"E; 5 m deep. Collected by B. Pleše and V. Nikolić; 5 November 2010.

Colour. White in life and in ethanol.

Etymology. From the type locality.

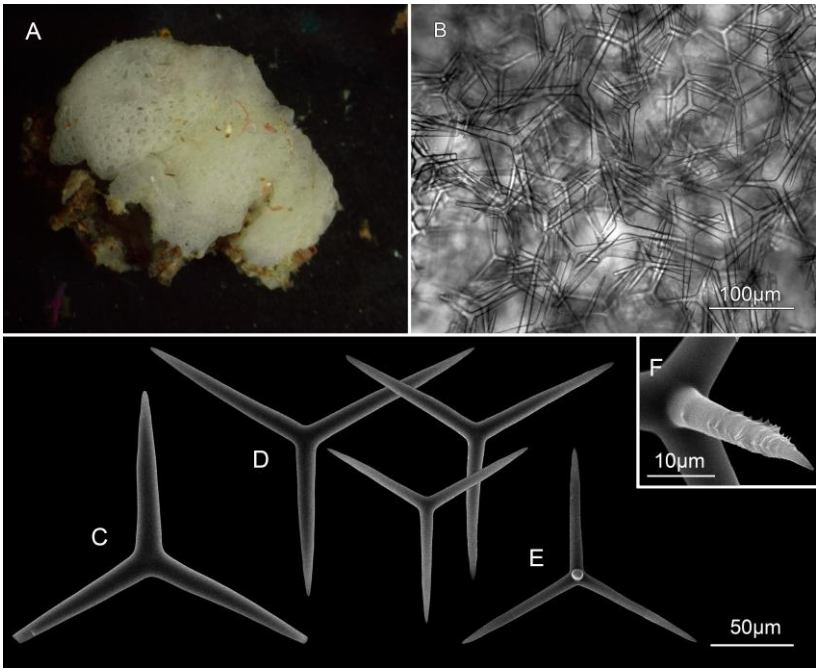


Figure 21. *Borojevia croatica* sp. nov. (Holotype - PMR 13740=UFRJPOR 6864). (A) Specimen *in situ*. (B) Tangential section. (C) Tripod. (D) Triactines. (E) Tetractine. (F) Apical actine of a tetractine ornamented with spines. Photo: F. Azevedo.

Description. Cormus is composed of regular and tightly anastomosed tubes (Figure 21A). Water-collecting tubes are present and form a single apical osculum. The skeleton is composed of tripods, triactines and rare tetractines. It has no special organization (Figure 21B).

Spicules (Table 10).

Tripods: regular (equiangular and equiradiate) or sagittal. Some have an elevated centre, but most appear like large regular triactines. Actines are conical, straight, with sharp tips (Figure 21C).

Triactines: regular (equiangular and equiradiate). Actines are conical, straight, with sharp tips (Figure 21D).

Tetractines: regular (equiangular and equiradiate). Actines are conical, straight, with sharp tips (Figure 21E). The apical actine has very short, abundant spines organized in parallel rows. The spines cover the first 2/3 of the apical actine (Figure 21F).

Table 10. Spicules measurements of *Borojevia croatica* sp. nov. Holotype (PMR-13740 = UFRJPOR 6864) and paratype (PMR-13741 = UFRJPOR 6865).

		length (µm)				width (µm)				
		min	mean	sd	max	min	mean	sd	max	n
Holotype										
Tripod	Basal	85.0	102.6	10.0	115.0	10.0	11.9	1.5	15.0	20
Triactine	Basal	57.5	66.6	6.7	82.5	7.5	7.5	0.0	7.5	20
Tetractine	Basal	60.0	70.0	6.3	77.5	7.5	8.3	1.2	11.3	10
	Apical	-	20	-	-	-	5.0	-	-	01
Paratype										
Tripod	Basal	50.0	78.8	19.1	115.0	7.5	10.8	1.8	15.0	13
Triactine	Basal	50.0	66.1	8.6	80.0	7.5	7.8	0.7	10.0	20
Tetractine	Basal	62.5	71.0	5.1	80.0	7.5	8.3	1.1	10.0	20

Ecology. Specimens were collected on a shaded, vertical, hard limestone bottom.

Remarks. The genus *Borojevia* is currently composed of five species: *B. aspina* (Klautau, Solé-Cava & Borojević, 1994), *B. brasiliensis*, *B. cerebrum*, *B. paracerebrum* (Austin, 1996) and *B. tetrapodifera* (Klautau & Valentine, 2003). All of them show a very well defined cormus, with regular and tightly anastomosed tubes and water-collecting tubes. The skeleton is always composed of tripods, triactines and tetractines with spines on the apical actines. Tetrapods may also be present (*B. tetrapodifera*). The sixth species of the genus, *B. croatica* sp. nov., is phylogenetically closer to *B. aspina* (Figure 35). Both species have short spines, however, *B. croatica* sp. nov. has numerous spines, while in *B. aspina* there are few. Given that *B. cerebrum* is also present in the Adriatic Sea, the best way to differentiate it from *B. croatica* sp. nov. is by the shape and location of spines. They are shorter, more abundant and distributed along most of the actine length in *B. croatica* sp. nov., and larger, fewer and scattered only near the tip of the apical actine in *B. cerebrum*.

Genus *Clathrina* Gray, 1867

***Clathrina blanca* (Miklucho-Maclay, 1868)**

(Figure 22; Table 11)

Type locality. NE Atlantic, Canary Islands, Lanzarote (Port del Arrecife)

Material examined. PMR-14307. Island of St. Giovanni (45° 2′ 46.63″N, 13° 37′ 21.96″E), 3 m depth; collected by M. Pfannkuchen; January 2011.

Colour. White in life and in ethanol.

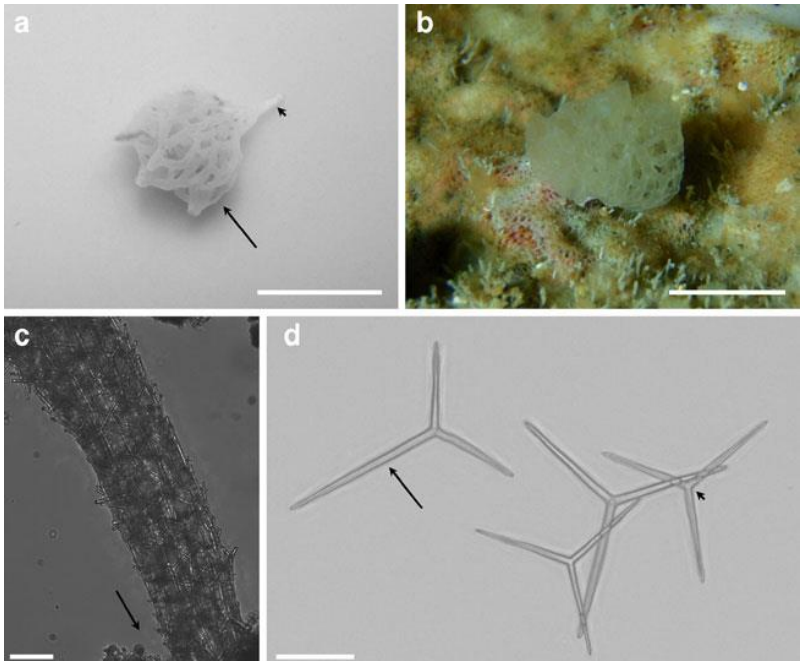


Figure 22. *Clathrina blanca*. a. Fixed specimen, lateral view. The cormus (arrow) is clearly distinguishable from the stalk (arrowhead). Scale bar=5 mm. b. Specimen *in situ*. Scale bar= 5 mm. c. Magnification of the stalk. The arrow points towards the attachment site. Scale bar= 100 µm. d. Parasagittal spicule (arrow) and regular triactines (arrowhead). Scale bar=50 µm. Photo: M. Pfannkuchen.

Description. The specimen is composed of a symmetrical, globular cormus and a clathroid body of irregular and loosely anastomosed tubes of equal size. (Figure 22a). A short thin peduncle is present, less than 4 mm long and less than a 1 mm wide. Water-collecting tubes were observed (Figure 22b). The skeleton of the tubes is thin and comprises regular and parasagittal triactines, which are tangentially oriented around the tubes. The peduncle is partially solid and its skeleton comprises only tangentially oriented parasagittal triactines with the unpaired, longer actine, basipetally oriented towards the attachment site (Figure 22c). Reproductive structures were not observed.

Spicules (Table 11).

Triactines: regular (equiangular and equiradiate) or parasagittal, with the unpaired actine being longer than the paired ones. Actines are cylindrical, straight or slightly undulated with rounded tips (Figure 22d).

TABLE 11. Spicules measurements of *Clathrina blanca* (PMR-14307).

		length (µm)				width (µm)				
Spicule		min	mean	sd	max	min	mean	sd	max	n
Triactine	Regular	51.2	67.6	7.2	81.5	4.7	5.5	0.5	6.9	33
	Parasagittal									
	Unpaired	54.7	91.5	17.1	135.2	4.5	5.3	0.4	6.0	33
	Paired	29.9	58.4	12.8	76.6	4.5	5.3	0.4	6.0	33

Ecology. The specimen was attached to the rocky substrate (limestone) of shallow cave ceilings.

Remarks. *Clathrina blanca* was originally described by Miklucho-Maclay [96] from Lanzarote (Canary Islands). He described the external shape of the specimens and mentioned the presence of triactines only, with a longer unpaired actine. Later, Haeckel [54] has given more details of the skeleton of specimens from Lanzarote, saying that the actines of the parasagittal triactines were cylindrical and measured: 50-70/3-4 (paired actines); 80-100/3-4 (unpaired actine). No morphological or molecular studies have been done to compare the variation among populations of *Clathrina blanca* from the Canary Islands and the Mediterranean and Adriatic Seas, and types of *C. blanca* are unknown. However, studies have shown a close similarity between the fauna of the Canary Islands and the Mediterranean Sea [97]. Therefore, considering this relatedness and the small difference between the morphology of the Adriatic specimens and *C. blanca* from the Canary Islands (there were differences only in the spicules thickness and in the partially solid peduncle in the Adriatic specimen), we identified it as *C. blanca*. Now for the first time, DNA sequence of this currently considered cosmopolitan species is available.

Clathrina clathrus (Schmidt, 1864)

(Figure 23, Table 12)

Type locality. Lesina, Adriatic Sea

Material examined. PMR-14308. Island of St. Giovanni (45° 2′ 46.63″N, 13° 37′ 21.96″E), 3 m depth; collected by M. Pfannkuchen; January 2011.

Colour. Yellow in life and in ethanol.

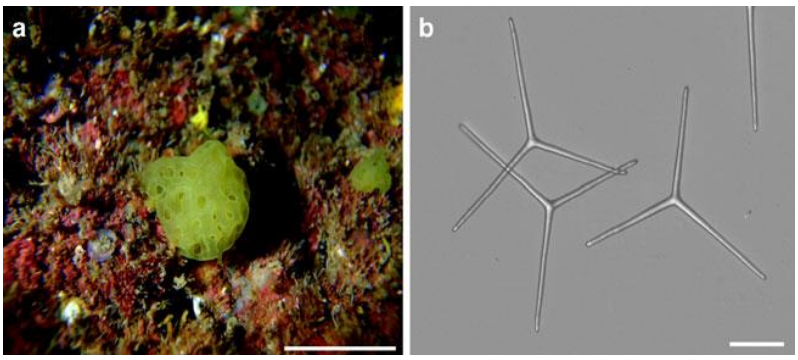


Figure 23. *Clathrina clathrus*. a. Specimen *in situ*. Scale bar=5 mm. b. Regular triactines. The actines are cylindrical, undulated at their distal part and with rounded tips. Scale bar=50 µm. Photo: M. Pfannkuchen.

Description. The clathroid cormus is composed of irregularly and loosely anastomosed tubes. Large superficial water-collecting tubes were observed, opening to a few large oscula (Figure 23a). The skeleton has no special organization, comprising only triactines. Reproductive structures were not observed.

Spicules (Table 12).

Triactines: Equiangular and equiradiate. Actines are cylindrical and undulated, with rounded tips (Figure 23b).

Table 12. Spicules measurements of *Clathrina clathrus* (PMR-14308).

		length (µm)				width (µm)				
Spicule	Actine	min	mean	sd	max	min	mean	sd	max	n
Triactine	Regular	77.9	91.1	4.9	100.7	6.9	7.2	0.2	7.7	33

Ecology. The specimen was found in a semi-dark cave attached to the rocky substrate (limestone) of shallow cave ceilings.

Remarks. *Clathrina clathrus* is a common species in the Mediterranean and Adriatic Seas. This species was considered to be cosmopolitan; however, genetic and morphological characters showed that Southwestern Atlantic yellow clathrinas identified as *C. clathrus* were a distinct species. This species was named *C. aurea* Solé-Cava et al., 1991 [67,98] and, since then, some other species of yellow clathrinas have been described (*C. chrysea* Borojević & Klautau, 2000, from New Caledonia; *C. luteoculcitella* Wörheide & Hooper, 1999, from Australia). As expected, morphological and molecular characters of the specimen studied were congruent with those of *C. clathrus* [67,71,72].

***Clathrina conifera* Klautau & Borojević, 2001**

(Figure 24; Table 13)

Type locality. Arraial do Cabo, Rio de Janeiro, Brazil.

Type specimen. BMNH 1999.9.16.19 (holotype / ethanol).

Material examined. PMR-13738 = UFRJPOR 6869. Near the Island of Lokrum, Adriatic Sea, 42°37'55.6"N, 18°06'49.4"E; 1-3 m deep. Collected by V. Nikolić; 8 Oct. 2010. PMR-17807; IRB-S2 = UFRJPOR 7541; IRB-S3 = UFRJPOR 7542. Near Dubrovnik, Adriatic Sea, 42°38'26.5"N, 18°06'14.2"E; 1 m deep. Collected by V. Nikolić; 24 September 2011.

Colour. White in life and white or brown in ethanol.

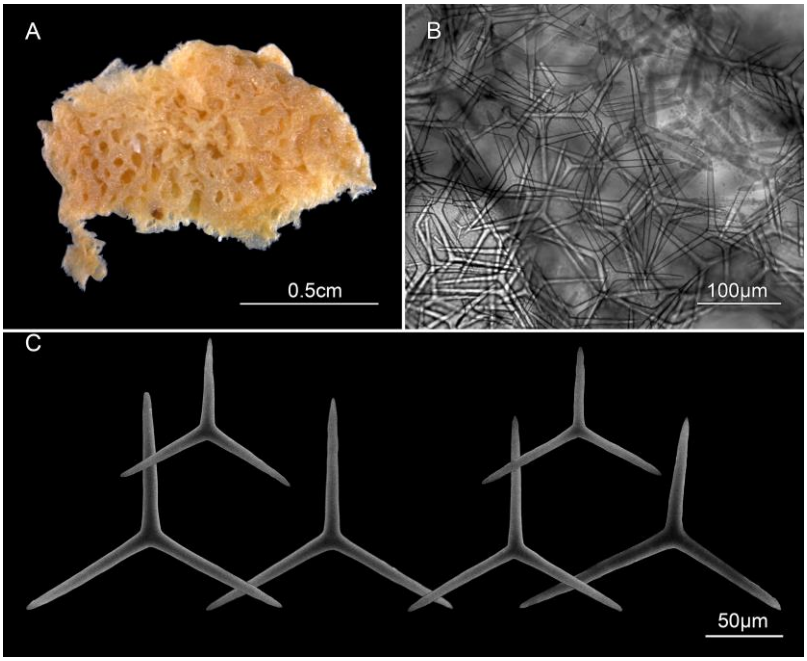


Figure 24. *Clathrina conifera* (PMR 13738=UFRJPOR 6869). (A) Specimen in ethanol. (B) Tangential section. (C) Triactines with variable sizes and shapes. Photo: F. Azevedo.

Description. Cormus is composed of irregular and loosely anastomosed tubes (Figure 24A). Water-collecting tubes are not present. The skeleton consists of triactines without organization (Figure 24B).

Spicules (Table 13).

Triactines: regular (equiangular and equiradiate). Their size is very variable. Actines are slightly conical to conical, straight, with blunt tips. Sometimes they are slightly undulated near the tip (Figure 24C).

Table 13. Spicules measurements (triactines) of *Clathrina conifera* (PMR-13738 = UFRJPOR 6869; IRB-S2 = UFRJPOR 7541; IRB-S3 = UFRJPOR 7542).

	length (µm)				width (µm)				
	min	mean	sd	max	min	mean	sd	max	n
PMR-13738 = UFRJPOR6869	57.5	88.5	11.8	122.5	7.5	10.2	1.6	15.0	30
IRB-S2 = UFRJPOR7541	59.4	86.3	9.6	97.2	8.1	9.5	1.4	10.8	20
IRB-S3 = UFRJPOR7542	64.8	82.5	8.9	102.6	6.8	8.8	1.3	10.8	20

Ecology. Specimens were collected on a semi-shaded, vertical hard limestone bottom under overhangs. They were often found in association with the macroalga *Corallina elongata* Ellis & Solander 1786.

Remarks. Until now, this species was considered endemic to Brazil [99,100,101 - as *C. primordialis* (Haeckel, 1872),102,103,104]. Originally, it was identified as *C. primordialis* [99,100]. However, considering the assumption that Mediterranean species would not be present along the Brazilian coast, it was regarded as a new species: *C. conifera*. This work has confirmed by morphological and molecular analyses (Figure 35) the occurrence of *C. conifera* in the Adriatic Sea and this species was compared with *C. primordialis*. *C. conifera* is shown to be a valid species, distinguished from *C. primordialis*. Although their external morphology is very similar, and both have only triactines, in *C. conifera* the actines are blunt and sometimes undulated, while they are sharp and straight in *C. primordialis*. Besides, the spicules of *C. conifera* have shorter and thinner actines compared to those of *C. primordialis*.

***Clathrina primordialis* (Haeckel, 1872)**

(Figure 25; Table 14)

Type locality. Lesina (Croatian: Island of Hvar), Adriatic Sea.

Type specimen. PMJ-Inv. Nr. Porif. 154 (lectotype / ethanol). E. Haeckel Collection.

Material examined. IRB-CLB3 = UFRJPOR 6863. Near the Island of Čiovo, Croatia, Adriatic Sea, 43°28'58.5"N, 16°21'25.6"E; 5 m deep. Collected by B. Pleše and V. Nikolić; 5 November 2010.

Colour. White in life and in ethanol.

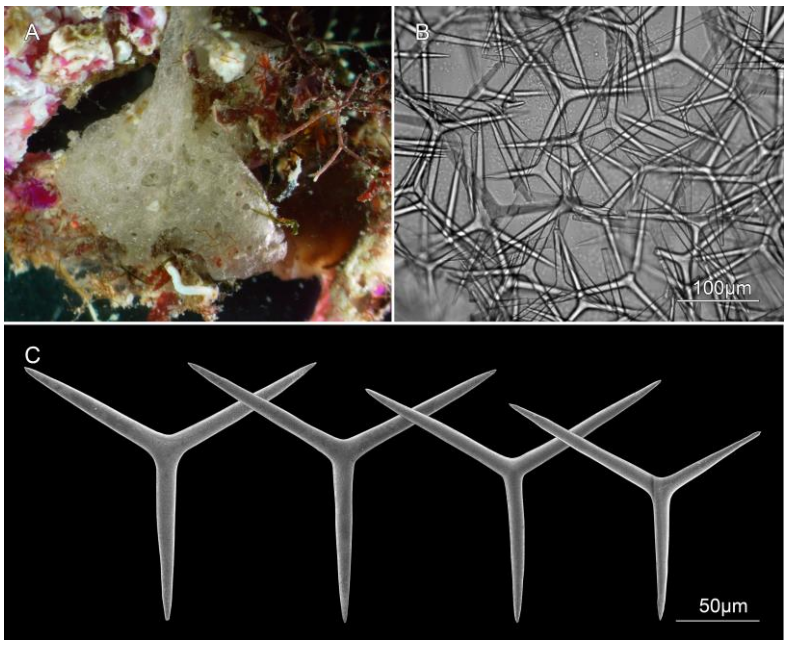


Figure 25. *Clathrina primordialis* (IRB-CLB3=UFRJPOR 6863). (A) Specimen *in situ*. (B) Tangential section. (C) Triactines with variable sizes and shapes. Photo: F. Azevedo.

Description. Cormus is formed of large and loosely anastomosed tubes. Water-collecting tubes are absent (Figure 25A). The skeleton is composed of one category of triactines (Figure 25B). The size of the spicules is very variable, therefore it is not possible to categorize them.

Spicules (Table 14).

Triactines: regular (equiangular and equiradiate). Actines are conical to slightly conical with sharp tips (Figure 25C).

Table 14. Spicules (triactines) measurements of *Clathrina primordialis*. IRB-CLB3 = UFRJPOR 6863 (present work); PMJ 154 (syntype); PMR-14305 (*C.* cf. *hondurensis* in Imešek et al., 2014); BMNH 1938.3.28.4 (holotype of *C. hondurensis*).

	length (µm)				width (µm)				
	min	mean	sd	max	min	mean	sd	max	n
IRB-CLB3 = UFRJPOR 6863	47.5	121.5	27.9	157.5	7.5	12.2	2.4	15.0	20
Haeckel, 1872	100.0	-	-	150.0	8.0	-	-	12.0	-
Syntype	97.5	134.0	16.3	157.5	10.0	13.0	2.2	17.5	30
PMR-14305	101.8	128.0	9.6	151.5	13.3	15.2	1.3	19.0	33
BMNH 1938.3.28.4	105.6	133.4	17.0	156.0	12.0	15.6	1.7	19.2	20

Ecology. Specimens were collected on a shaded, vertical, hard limestone bottom.

Remarks. Haeckel [54] assigned the name *Ascetta primordialis* to a group of different species whose skeleton comprised only triactines. Unfortunately, no holotype was elected. In 2003, the

genus *Clathrina* was revised by Klautau and Valentine [67] and two allegedly syntypes of *C. primordialis* were analysed, one from the Adriatic Sea (PMJ 154) and the other from Naples (ZMB 1306). Both specimens were clearly a different species and the authors suggested the specimen ZMB 1306 was the true *C. primordialis* because *C. primordialis* (originally *Prosycum primordiale* Haeckel, 1870) was first described from Naples. After re-analysing the slides and the catalogue from the ZMB, a different conclusion emerged. In the specimen's label and in the catalogue it is not noted that ZMB 1306 is a type of *C. primordialis*. Moreover, this specimen was on the label referred as *Leucosolenia primordialis* and not *Prosycum primordiale*, nor even *Ascetta primordialis*. Besides, the collection site was the Naples Zoological Station, which was founded only in 1874, after the description of this species [54,105]. Another clue was that the entry of this specimen in the ZMB was made by the curator of the collection (previously assistant lecturer) W. Weltner in 1889 (P. Bartsch pers. comm.), which reinforces the possibility that this specimen was collected after the description of *C. primordialis*. Consequently, the suggestion by Klautau and Valentine [67], that the specimen ZMB 1306 should be considered as a type specimen of *C. primordialis*, was equivocated. On the other hand, the label of the specimen PMJ 154 says it is a syntype of *C. primordialis*, which was collected by Haeckel from Lesina, Adriatic Sea. Therefore, the specimen PMJ 154 seems to be true type specimen of this species and it is proposed to become a lectotype of *C. primordialis*.
Considering the morphology of PMJ 154, the specimen IRB-CLB3/UFRJPOR 6863 is confirmed as *C. primordialis*. The similarities between *C. primordialis* and *C. hondurensis* opened the possibility to synonymize these species, but since DNA sequences of *C. hondurensis* from the type locality (Honduras) were not available to verify this, *C. hondurensis* is left as a valid species restricted to the Caribbean Sea, until further analyses are done. Additionally, the specimen ZMB1306 (from Naples), previously suggested as a type specimen of *C. primordialis*, seems to be *C. conifera*.

***Clathrina rubra* Sarà, 1958**

(Figure 26, Table 15)

Type locality. Bay of Naples (Grotta dei Misteri)

Material examined. PMR-14306. Beach north of the city of Rovinj (45° 6′ 40.08″N, 13° 36′ 41.84″E), 3 m depth; collected by M. Pfannkuchen; November 2010.

Colour. Red in life and brown in ethanol.

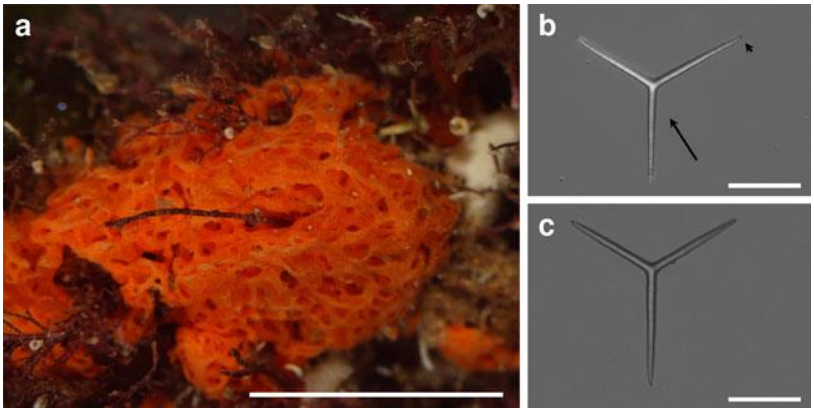


Figure 26. *Clathrina rubra*. a. Sponge in situ attached to the basal part of *Cystoseira crinita* thallus. Scale bar=1 cm. b. Regular triactine. Actine with cylindrical actine (arrow) and the typical wide, rounded tip (arrowhead). Scale bar=50 µm. c. Parasagittal triactine. Scale bar=50 µm. Photo: M. Pfannkuchen.

Description: Colour *in vivo* is bright red to orange. The specimen has a clathroid cormus, which is 2 cm long and up to 1 cm wide. It is composed of irregularly and loosely anastomosed tubes of equal size. Water-collecting tubes were not observed (Figure 26a). The skeleton comprises only triactines, which are tangentially oriented around the tubes and build an irregular mesh. Reproductive structures were not observed.

Spicules (Table 15).

Triactines: Equiangular and equiradiate (Figure 26b). Some parasagittal spicules can also be found (Figure 26c). Actines are straight and cylindrical, with blunt tips.

Table 15. Spicules measurements of *Clathrina rubra* (PMR-14306).

		length (µm)				width (µm)				
Spicule	Actine	min	mean	sd	max	min	mean	sd	max	n
Triactine	Regular	61.4	79.4	7.9	93.2	7.3	8.5	0.6	9.8	33

Ecology. The specimen was growing on the basal parts of a thallus of *Cystoseira crinita* Duby, 1830.

Remarks. This is the first time that *C. rubra* is being formally described. This species was originally mentioned as *C. coriacea* var. *rubra* by Sarà [106], in the work on sponge distribution in a cave in the Bay of Naples (Italy). In that paper, a formal description of the species was not given, but was only mentioned that *C. rubra* was orange/red and very similar to *C. coriacea*. In 1968, Borojević et al. [107], elevated this variety to the species category in a species list of sponges from the English Channel near Roscoff (France); in 2008, Pansini and Longo [108] mentioned it again in a species list of sponges from Italy. This is the first time that *C. rubra* was found in the Adriatic Sea. The specimen from the Adriatic Sea was compared to a slide of *C. rubra* prepared by Sarà. Although the

spicules on the slide are very corroded and seem to have more rounded tips than those of analysed specimen, it seems as *C. rubra*. *Clathrina rubra* is morphologically very similar to species of yellow clathrinas: *C. aurea*, *C. chrysea*, *C. clathrus* and *C. luteoculcitella* [71]. However, it is more similar to *C. aurea* and *C. clathrus* as these species also have cylindrical actines. Despite this similarity, *C. rubra* can be differentiated from *C. aurea* and *C. clathrus* by its colour (bright red or orange), the presence of parasagittal spicules and molecular differences.

3.2.2. Systematics of the Adriatic Calcaronea Bidder, 1898

Systematic index

Class Calcarea Bowerbank, 1864
Subclass CALCARONEA Bidder, 1898
Order LEUCOSOLENIDA Hartman, 1958

Family GRANTIIDAE Dendy, 1892

Genus *Leucandra* Haeckel, 1872

***Leucandra falakra* sp. nov.**
(Figures 27, 28; Table 16)
Type locality. Island of Blitvenica, Adriatic Sea.
Type material. PMR-13748 = UFRJPOR 8349 (holotype / ethanol); Near the Island of Blitvenica, Adriatic Sea, 43°37'31.96"N, 15°34'25.94"E; 5 m deep. Collected by V. Nikolić; 10 October 2012.
Colour. White in life and in ethanol.
Etymology. From the greek *falákra* (φαλάκρα), meaning bald, for the absence of diactines.

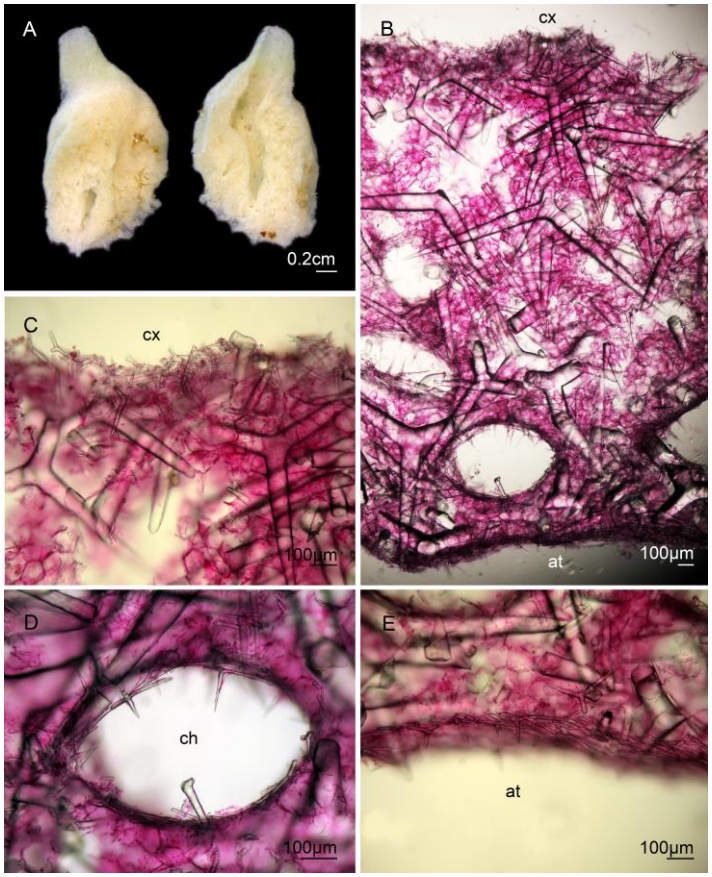


Figure 27. *Leucandra falakra* sp. nov. (Holotype - PMR-13748 = UFRJPOR 8349). (A) Specimen in ethanol. (B) Cross section. (C) Detail of the cortex. (D) Detail of a canal in the choanosome. (E) Atrial skeleton. Abbreviations: at - atrium; cx - cortex; ch - canal. Photo: F. Azevedo.

Description. The sponge is massive and presents a shape of vase with one apical osculum without crown. The atrium is central and large. The aquiferous system is leuconoid (Figure 27A). The sponge surface is smooth, but harsh. The cortical skeleton is composed of small, tangentially arranged triactines. The choanosomal skeleton has no organization (Figure 27B). It is composed of two categories of triactines (giant triactines and triactines larger than those of the cortex) (Figure 27C). There are also tetractines and some triactines surrounding the canals (Figure 27D). The atrial skeleton is smooth, composed mainly of triactines, with a few tetractines also present (Figure 27E).

Spicules (Table 16).

Cortical triactines: subregular to sagittal, equiradiate and small. Actines are cylindrical, blunt and curved (Figure 28A, B).

Choanosomal small triactines: subregular to sagittal. Actines are conical and sharp (Figure 28C, D).

Choanosomal giant triactines: subregular to sagittal, equiradiate. Actines are conical and sharp (Figure 28E, F).

Choanosomal tetractines: sagittal. They are present only surrounding the canals. Actines are

cylindrical, sharp and curved. The unpaired actine is somewhat shorter than the paired ones. The apical actine is straight, short, conical and sharp (Figure 28G, H).

Atrial triactine and tetractine: strongly sagittal. Triactines are the most abundant spicules (Figure 28I). Actines are cylindrical and blunt. The unpaired actine is shorter than the paired ones. The apical actine of the tetractines is conical, straight, sharp and short. Frequently they are longer and thicker than the apical actine of the choanosomal tetractines (Figure 28J).

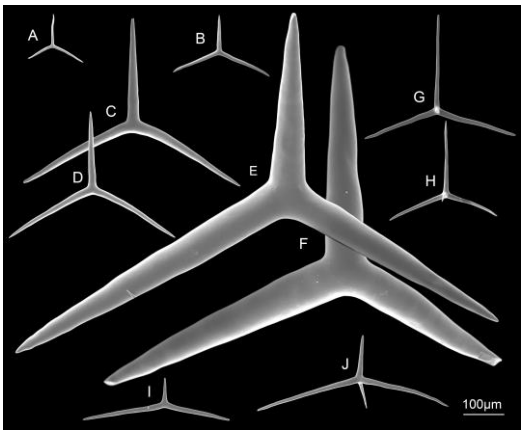


Figure 28. *Leucandra falakra* sp. nov. (Holotype - PMR-13748 = UFRJPOR 8349). (A, B) Cortical triactines. (C, D) Small choanosomal triactines. (E, F) Giant choanosomal triactines. (G, H) Tetractines of the canals. (I) Atrial triactine. (J) Atrial tetractine (scale = 100 µm). Photo: F. Azevedo.

TABLE 16. Spicules measurements of *Leucandra falakra* sp. nov. (PMR-13748=UFRJPOR 8349).

		length (µm)				width (µm)				
		min	mean	sd	max	min	mean	sd	max	n
Cortical triactine	Paired	94.5	136.4	24.0	180.9	8.1	11.1	1.9	13.5	20
	Unpaired	70.2	106.0	18.8	143.1	8.1	11.4	2.4	16.2	20
Cortical and choanosomal triactine		324.0	624.5	192.3	1047.6	48.6	81.5	20.6	118.8	23
Choanosomal triactine	Paired	162.0	214.2	39.8	288.9	13.5	18.3	4.0	27.0	20
	Unpaired	108.0	189.7	58.9	351.0	13.5	19.8	4.1	29.7	20
Tetractine (canals)	Paired	99.9	154.0	26.4	199.8	8.1	12.4	2.4	16.2	19
	Unpaired	45.9	143.0	56.5	288.9	9.5	12.4	1.9	16.2	19
	Apical	50.0	80.6	24.4	137.5	7.5	9.6	1.5	12.5	20
Atrial triactine	Paired	140.4	222.7	33.7	294.3	9.5	15.1	2.5	20.3	30
	Unpaired	78.3	111.2	24.4	159.3	8.1	12.3	1.7	16.2	30
Atrial tetractine	Paired	145.8	191.4	26.0	256.5	10.8	14.9	2.6	18.9	16
	Unpaired	59.4	92.0	22.1	126.9	10.8	13.1	1.7	16.2	16
	Apical	67.5	110.3	30.3	162.0	8.1	11.9	2.8	16.2	15

Ecology. Specimens were collected on a shaded, semi-vertical, hard limestone bottom.

Remarks. There are only three currently described species of *Leucandra* without diactines and with triactines being the main atrial spicules: *L. consolida* Tanita, 1943, *L. glabra* Hôzawa, 1940, and *L. okinoseana* Hôzawa, 1929, all three from Japan. *Leucandra falakra* sp. nov. can be differentiated from *L. consolida* by the presence of an oscular crown in the latter (although "feebly developed"), by the absence of tetractines in the choanosome, and by the absence of the large triactines in the cortex. Moreover, the size of some spicules is different (cortical triactines: 240-350/20-25 µm; choanosomal triactines - paired actines: 590-740/60-86 µm, unpaired actine: 550-720/60-86 µm; atrial triactines - paired actines: 220-270/15-18 µm, unpaired actine: 250-300/15-18 µm; atrial tetractines: same size of the atrial triactines but with an apical actine of 80/14 µm). *Leucandra glabra* has a different external morphology, with several oscula in a single individual. Besides, the size of some spicules is different (cortical triactines: 120-240/14-28 µm; small choanosomal triactines: 100-200/10-20 µm; large choanosomal triactines: 400-950/42-110 µm; choanosomal tetractines: similar to the small choanosomal triactines but with an apical actine of 80/10 µm; atrial triactines: 90-200/12-20 µm). *Leucandra okinoseana* can be differentiated from *L. falakra* sp. nov. by the presence of "small protuberances for attachment" in *L. okinoseana* and by the size of some spicules, which are larger in the Japanese species (cortical triactines - paired actines: 120-250/16-24 µm, unpaired actine: 150-350/14-16 µm; cortical and choanosomal large triactines: 400-1400/32-120 µm; tetractines of the canals - paired actines: 150-200/16-20 µm, unpaired actine: 120-570/12-16 µm , apical actine: 70-200/8-12 µm; atrial triactines - paired actines: 190-370/20-32 µm, unpaired actine: 70-270/16-24 µm; atrial tetractines - same size of the atrial triactines but with an apical actine of 50-110/8-16 µm).

***Leucandra spinifera* sp. nov.**

(Figures 29, 30; Table 17)

Type locality. Vrulja Cove, Adriatic Sea.

Type material. IRB-SG3 = UFRJPOR 8348 (holotype / ethanol); Vrulja Cove, Adriatic Sea, 43°24'01.3"N, 16°53'10.9"E; 1-3 m deep. Collected by Vedran Nikolić; 24 Aug. 2011; PMR-13742 = UFRJPOR 6861 (paratype / ethanol); Island of Čiovo, Adriatic Sea, 43°28'58.5"N 16°21'25.6"E; 5 m deep. Collected by B. Pleše and V. Nikolić; 6 November 2010.

Colour. White in life and in ethanol.

Etymology. From the Latin *spinifer*, meaning prickly, for the presence of numerous diactines.

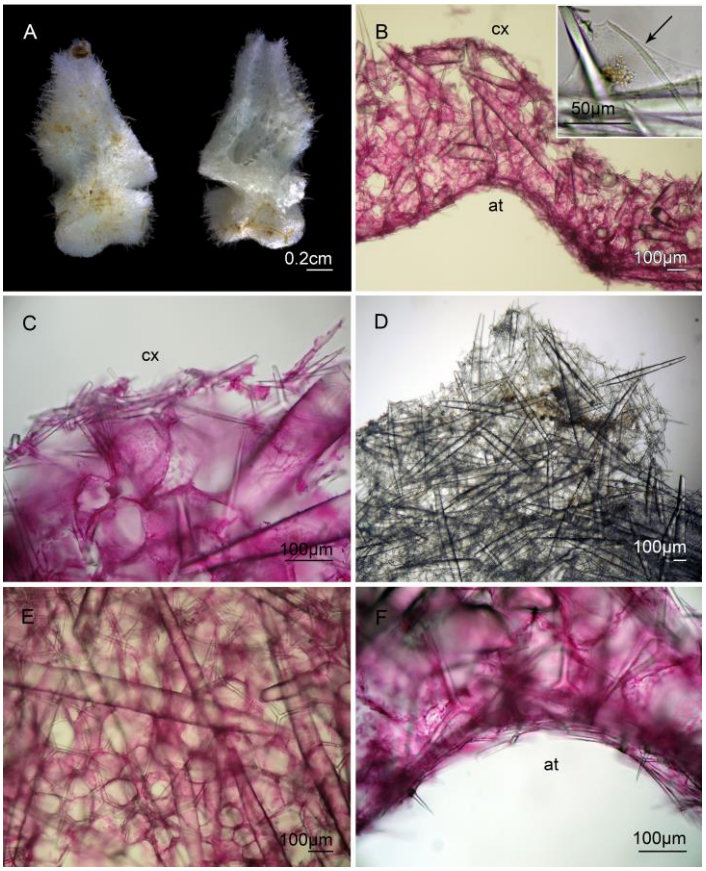


Figure 29. *Leucandra spinifera* sp. nov. (Holotype - IRB-SG3 = UFRJPOR 8348). (A) Specimen in ethanol. (B) Cross section. Detail: cortical microdiactine (arrow). (C) Detail of the cortex. (D) Tangential section of the cortex. (E) Choanosome. (F) Atrial skeleton. Abbreviations: at - atrium; cx - cortex. Photo: F. Azevedo.

Description. The body has a shape of a vase (0.8 x 0.4 cm), with a single apical osculum surrounded by a membrane and a crown of few, or even no trichoxeas (Figure 29A). The osculum is supported by sagittal tetractines, but a few triactines are also present. They are organized in parallel and point their apical actines towards the osculum. They become disorganized, smaller, thinner and less sagittal farther from the osculum. They are also substituted by triactines. Numerous diactines on the surface make it very hispid. The aquiferous system is leuconoid and the atrium is large (Figure 29A). The cortical skeleton is composed of tangential triactines, perpendicular giant diactines, microdiactines and rare trichoxeas (Figure 29B-E). The giant diactines frequently cross the entire choanosome (Figure 29B). The choanosomal skeleton has no organization. It is composed mainly of subregular triactines with curved paired actines. Tetractines are also present, but only surrounding canals. The atrial skeleton has triactines and few tetractines that project their apical actines into the atrium (Figure 29F). Microdiactines are also present in the atrium.

Spicules (Table 17).

Oscular triactines (very few) and tetractines (abundant): sagittal. Actines are cylindrical and blunt to sharp. The unpaired actine is thinner than the paired ones. The apical actine of the tetractines is conical, sharp, smooth and strongly curved towards the osculum.

Trichoxeas: very thin, long and straight. They are frequently broken. They are rare, but can be found in the cortex and atrium.

Diactines: almost fusiform. The tip that penetrates the choanosome is a little larger and more rounded (Figure 30A, B).

Microdiactines: fusiform (Figure 30C). They are present in the cortex and atrium. They frequently have microspines (Figure 30D), but smooth spicules are also present.

Cortical triactines: sagittal. Actines are slightly conical, with blunt tips. The unpaired actine is shorter than the paired ones, which are curved. One of the paired actines is frequently shorter than the other (Figure 30E, F).

Choanosomal triactines: subregular to sagittal. The paired actines are curved, consequently the unpaired angle is smaller than the paired angles. Actines are slightly conical with blunt tips. They are almost the same length (Figure 30G). These spicules are spread in the choanosome and surrounding the canals.

Choanosomal tetractines: sagittal. The paired actines are curved, consequently the unpaired angle is smaller than the paired angles. Actines are slightly conical with blunt tips. The apical actine is straight or curved, conical, smooth and sharp (Figure 30H, I). These spicules are present only surrounding the canals.

Atrial triactines and tetractines: triactines are much more abundant. These spicules are strongly sagittal. The paired actines are curved and much longer than the unpaired one. Actines are slightly conical and blunt (Figure 30AJ-L). The apical actine of the tetractines is straight or slightly curved near the end, conical, smooth and sharp (Figure 30M). These tetractines are very similar to the choanosomal ones.

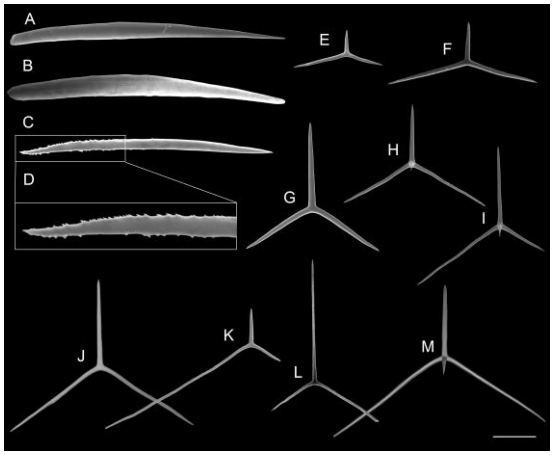


Figure 30. *Leucandra spinifera* sp. nov. (Holotype - IRB-SG3 = UFRJPOR 8348). (A, B) Cortical diactines (scale = 200 µm). (C) Microdiactine (scale = 20 µm). (D) Detail of the spines of a microdiactine (scale = 10 µm). (E, F) Cortical triactines. (G) Choanosomal triactine. (H, I) Choanosomal tetractines. (J-L) Atrial triactines. (M) Atrial tetractine (scale = 100 µm). Photo: F. Azevedo.

TABLE 17. Spicules measurements of *Leucandra spinifera* sp. nov. (IRB-SG3=UFRJPOR 8348).

		length (µm)				width (µm)				
		min	mean	sd	max	min	mean	sd	max	n
Diactine		430.0	866.5	217.6	1400.0	20.0	54.4	16.0	90.0	17
Microdiactine		70.0	100.4	29.9	180.0	2.5	4.2	1.2	7.5	20
Cortical triactine	Paired	110.0	189.5	45.2	260.0	7.5	12.9	2.8	17.5	20
	Unpaired	105.0	150.8	29.0	195.0	10.0	13.5	2.5	20.0	20
Choanosomal triactine	Paired	140.0	192.8	35.5	300.0	10.0	12.8	1.4	15.0	20
	Unpaired	115.0	188.8	36.6	260.0	10.0	14.4	1.6	17.5	20
Atrial triactine	Paired	230.0	305.3	69.8	500.0	7.5	7.9	0.9	10.0	14
	Unpaired	110.0	211.4	52.7	325.0	7.5	9.8	0.7	10.0	14
Atrial tetractine	Paired	165.0	276.8	63.3	362.5	7.5	8.4	1.1	10.0	15
	Unpaired	137.5	222.0	62.6	350.0	7.5	9.7	0.9	10.0	15
	Apical	32.5	42.5	16.8	67.5	5.0	6.9	1.6	8.8	04

Ecology. Specimens were collected on a cliff in a shaded area.

Remarks. This species is different from all other *Leucandra* species mainly by the composition of the skeleton. Particularly, by the presence of mainly triactines in the atrial skeleton, with very long and slender paired actines and few spiny microdiactines on the cortex. The most similar species is the Californian *L. heathi* Urban, 1906. However, this species has no tetractines, while *L. spinifera* sp. nov. has a few tetractines. Besides, microdiactines are not abundant in *L. spinifera* sp. nov., while in *L. heathi* they form a continuous palisade on the cortex.

We found 10 species of *Leucandra* recorded from the Mediterranean until now, and *L. spinifera* sp. nov. can be differentiated from all of them: *Leucandra aspera* (Schmidt, 1862) has larger

choanosomal and atrial spicules (diactines: 500-3000/10-80 μm; cortical triactines: 200/12 μm; choanosomal triactines and tetractines: basal - 120-250/10-15 μm, apical - 50-120/10-15 μm; atrial tetractines: paired - 180-300/15 μm, unpaired - 250-320/15 μm, apical - 100-150/15 μm), it has also more tetractines in the atrial skeleton and no microdiactines; *L. balearica* (Lackschewitz, 1886) has only tetractines in the atrium and its microdiactines are much smaller (12-24/1 μm); *L. globosa* (Sarà, 1951) has different microdiactines; *L. aspera* (Schmidt, 1862) has no microdiactines; *L. bolivari* Ferrer-Hernandez, 1916 has no diactines; *L. crambessa* Haeckel, 1872 has no microdiactines and has tetractines only in the atrium; *L. nausicaae* (Schuffner, 1877) has no diactines and the atrial skeleton comprises only tetractines; *L. riojai* Ferrez-Hernandez, 1918 has only tetractines in the atrium; *L. rodriguezii* (Lackschewitz, 1886) has shorter microdiactines (12-14/1 μm) which occur only in the atrium, while the atrium is also composed of only tetractines; *L. sulcata* Ferrer-Hernandez, 1918 has microdiactines of different shape, which are present abundantly only in the cortex, while the atrium is composed mainly of tetractines.

Family AMPHORISCIDAE Dendy, 1892

Genus *Paraleucilla* Dendy, 1892

***Paraleucilla dalmatica* sp. nov.**

(Figures 31, 32; Table 18)

Type locality. Island of Čiovo, Adriatic Sea

Type material. IRB-SD5 = UFRJPOR 8346 (holotype / ethanol); PMR-13747 (paratype / ethanol); Near the Island of Čiovo, Adriatic Sea, 43°29'02.0"N, 16°22'10.9"E; 5 m deep. Collected by B. Pleše and V. Nikolić; 5 November 2010.

Colour. Beige or light brown in life and white in ethanol.

Etymology. From the type locality.

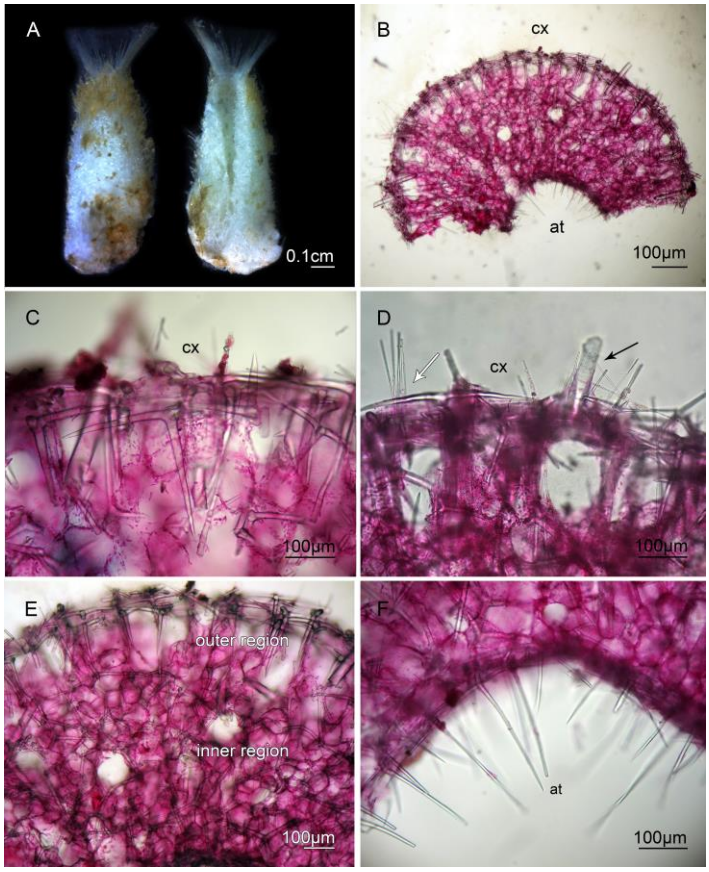


Figure 31. *Paraleucilla dalmatica* sp. nov. (Holotype - IRB-SD5 = UFRJPOR 8346). (A) Specimen in ethanol. (B) Cross section. (C) Cortex. (D) Detail of the cortex showing the tufts of diactines (white arrow - trichoxeas; black arrow - diactine). (E) Choanosome with the outer and inner regions. (F) Atrial skeleton. Abbreviations: at - atrium; cx - cortex. Photo: F. Azevedo.

Description. The body has a shape of a vase with a single apical osculum surrounded by a crown of trichoxeas (Figure 31A). Surface is very hispid. The aquiferous system is leuconoid (Figure 3B). The cortical skeleton is composed of the basal system of large tangential tetractines and few triactines (Figure 31C). Giant diactines cross the surface, penetrating deeply into the choanosome. They are present from the osculum to the base of the sponge. Among those giant diactines there are also very thin and long trichoxeas, organized in tufts, and very few microdiactines (Figure 31D). The choanosomal skeleton is characteristic of *Paraleucilla*, with an inarticulate region (outer region) and a zone without organization (inner region) (Figure 31E). The outer region is formed by the apical actine of the cortical tetractines, the unpaired actine of subatrial tetractines and very few triactines. The paired actines of these subatrial spicules are frequently curved, resembling a hook. The inner region is formed by scattered subatrial tetractines and very few triactines. The atrial skeleton is composed of tetractines only (Figure 31F). In some parts of the sponge the inarticulate skeleton seems not to exist and becomes more similar to *Leucandrilla*.

Spicules (Table 18).

Oscular triactines: strongly sagittal. Actines are conical and sharp. The unpaired actine is longer and thinner than the paired ones and basipetally directed.

Diactines: giant. They are present in the oscular crown and cortex. They are almost fusiform but slightly curved, with a thicker tip outside the sponge (Figure 32A). The size is very variable. Many diatoms are attached to the diactines surrounding the osculum.

Trichoxeas: present in the oscular crown and cortex. They are thin, straight and most of them are broken.

Microdiactines: very rare, fusiform or arrow-headed. Sometimes one of the tips has small spines while the other one is thicker (Figure 32B). They are present in the cortex.

Cortical tetractines: sagittal. Actines are conical with sharp tips. The apical actine is longer than the basal ones, conical, straight and sharp (Figure 32C, D).

Cortical triactines: there are very few, subregular to regular. Actines are slightly conical with sharp tips (Figure 32E).

Subatrial triactines and tetractines: the triactines are rare. Actines are conical and sharp. The unpaired actine is longer than the paired ones. The paired actines are frequently strongly curved. One of them is often shorter than the other. The apical actine of the tetractines is very short, thin, smooth and strongly curved (Figure 32F-K).

Atrial tetractines: sagittal. Actines are slightly conical and sharp. The apical actine is slightly conical, smooth, thinner than the basal ones and straight or only slightly curved (Figure 32L, M).

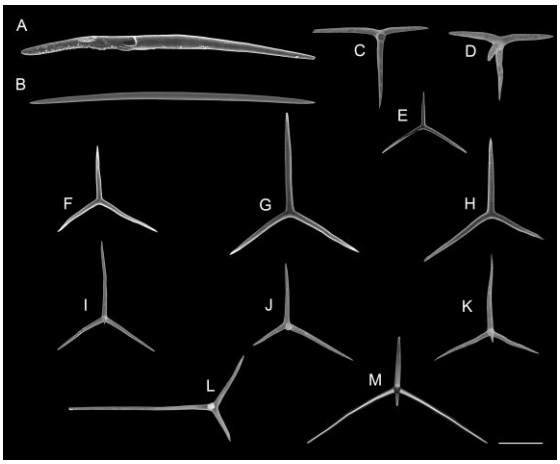


Figure 32. *Paraleucilla dalmatica* sp. nov. (Holotype - IRB-SD5 = UFRJPOR 8346). (A) Cortical diactine (scale = 50 µm). (B) Cortical microdiactine (scale = 20 µm). (C, D) Cortical tetractines. (E) Cortical triactine. (F-H) Subatrial triactines. (I-K) Subatrial tetractines. (L, M) Atrial tetractines. (scale = 100 µm). Photo: F. Azevedo.

TABLE 18. Spicules measurements of *Paraleucilla dalmatica* sp. nov. (IRB-SD5=UFRJPOR 8346).

		length (µm)				width (µm)				
		min	mean	sd	max	min	mean	sd	max	n
Diactine					1000.0	25.0			50.0	-
Trichoxea					>330.0	2.5			5.0	-
Microdiactine		57.5	95.0	23.5	142.5	2.5	2.5	0	2.5	15
Cortical triactine	Paired	85.0	142.8	35.8	190.0	5.0	12.4	4.3	20.0	20
	Unpaired	65.0	149.3	48.0	230.0	5.0	12.9	4.7	20.0	20
Cortical tetractine	Paired	120.0	159.1	19.2	195.0	10.0	13.4	2.0	17.5	16
	Apical	75.0	133.1	33.6	190.0	7.5	13.4	2.6	17.5	16
Subatrial tetractine and triactine	Paired	170.0	180.0	7.1	190.0	12.5	13.2	1.2	15.0	07
	Unpaired	155.0	205.8	26.7	245.0	10.0	12.7	1.1	15.0	15
	Apical	23.8	37.8	11.9	50.0	7.5	8.8	1.4	10.0	04
Atrial tetractine	Paired	105.0	157.9	32.0	197.5	5.0	10.5	3.0	17.5	21
	Unpaired	75.0	157.0	35.8	212.5	7.5	11.4	2.1	15.0	20
	Apical	57.5	115.7	55.4	245.0	5.0	7.3	0.6	7.5	25

Ecology. Specimens were collected on a cliff in a shaded area.

Remarks. Currently there are 11 known species of *Paraleucilla* and *P. magna* Klautau *et al*., 2004 was the only one recorded in the Mediterranean Sea, up to now. Both, external morphology and spicule composition are different in these two species. The most similar species to *P. dalmatica* sp. nov. are *P. perlucida* Azevedo & Klautau, 2007, from Brazil, and *P. princeps* (Row & Hôzawa, 1931), from Australia. Nonetheless, *P. dalmatica* sp. nov. can be differentiated from *P. perlucida* mainly by the absence of diactine I and trichoxea in the latter. *Paraleucilla princeps* also differs by the absence of diactine I and microdiactines. Therefore, *P. dalmatica* sp. nov. is the second species of *Paraleucilla* recorded from the Mediterranean Sea.

Family SYCETTIDAE Dendy, 1892

Genus *Sycon* Risso, 1826

Sycon ancora sp. nov.

(Figures 33, 34; Table 19)

Type locality. Island of Pag, Adriatic Sea

Type material. PMR 17809 = UFRJPOR 8345 (holotype / ethanol); Island of Pag, Adriatic Sea, 44°28'34.96"N, 15°02'39.74"E; 1 m deep. Collected by V. Nikolić; 14 Feb. 2011; IRB-SD12 = UFRJPOR 8347 (paratype / ethanol); near Split, Adriatic Sea, 43°30'27.57"N, 16°23'20.55?E; 5-10 m deep. Collected by V. Nikolić; 15 August 2011.

Colour. White in life and in ethanol.

Etymology. From the Latin *ancora*, meaning anchor, for the presence of anchor-like spicules for attachment.

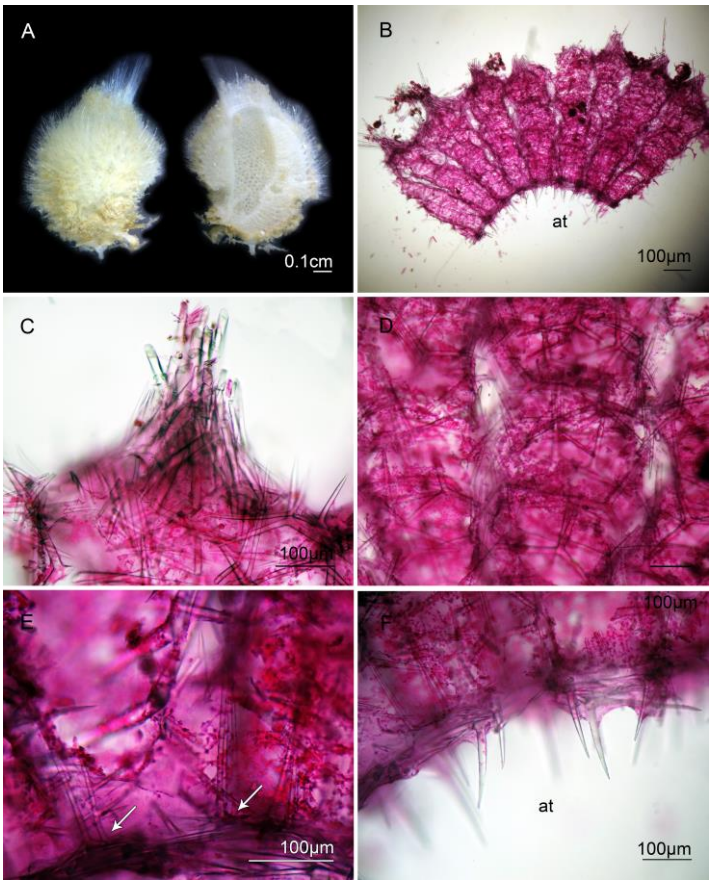


Figure 33. *Sycon ancora* sp. nov. (Holotype - PMR 17809 = UFRJPOR 8345). (A) Specimen in ethanol. (B) Cross section. (C) Detail of the distal cone. (D) Tubar and subatrial skeletons. (E) Atrial skeleton (white arrows: subatrial triactines). (F) Tangential section of the atrial skeleton. Abbreviation: at - atrium. Photo: F. Azevedo.

Description. The body has a shape of a vase (1,1 x 0.8 cm) with a single apical osculum surrounded by a crown of trichoxeas (Figure 33A) and diactines supported by sagittal tetractines. These tetractines are disposed in parallel to each other and their unpaired actines are basipetally directed. The unpaired actine is longer and thinner than the paired ones and the apical actine is curved towards the osculum aperture. The paired actines are slightly curved. There is no suboscular region. The aquiferous system is syconoid and the atrium is central. The radial tubes are coalescent (Figure 33B). Diactines and trichoxeas protrude through the distal cones, consequently, surface is very hispid. These diactines (ca. 10 to 15) penetrate only a little into the sponge surface (Figure 33C). The unpaired actine of some triactines also protrudes through the cones. The tubar skeleton

is articulated, but not as well organized as in most sycons (Figure 33D). It is composed of rows of sagittal triactines pointing their unpaired actines to the surface. These tubar triactines are larger than those of the distal cones and the paired actines are frequently curved. The subatrial skeleton is composed of sagittal triactines and tetractines (Figure 33E) with very thin actines. The unpaired actine is much longer than the paired ones and the longest ones are frequently localized among the choanocyte chambers. They point their unpaired actines towards the distal cones. Some of the subatrial triactines are similar to pseudosagittal spicules. The atrial skeleton is composed of two categories of tetractines tangentially organized (Figure 33E). They frequently have long unpaired and short paired actines. One of the paired actines is commonly shorter than the other, however, the three basal actines can have the same size (Figure 33F). When one of the paired actines is shorter than the other, it frequently penetrates an exhalant canal. The main difference between the two categories of atrial tetractines is in the apical actine. Tetractines with thinner apical actines project these actines mainly into the canals, while thicker and curved apical actines penetrate into the atrium (Figure 33E). Few anchor-like tetractines are present at the sponge base and project their basal actines into the substrate.

Spicules (Table 19).

Diactines: almost fusiform, but the tip outside the sponge is a little thicker (Figure 34A).

Trichoxeas: very thin, long and straight. They were always broken.

Anchor-like tetractines: the basal actines are very short and curved, while the apical one is very long. Frequently there are spines on the apical actine, but near the basal ones. They vary from four to seven, but seven spines are more common (Figure 34B).

Triactines of the cones: they are smaller than the tubar triactines. The unpaired actine protrudes through the cones and it is shorter than the paired ones, which are curved. Actines are slightly conical and sharp (Figure 34C, D).

Triactines of the tubes: subregular to sagittal. The unpaired actine is a little longer or of the same length as the paired ones. The paired actines are straight or slightly curved. Actines are slightly conical and sharp (Figure 34E-G).

Subatrial triactines and tetractines: the subatrial spicules are very thin. They are sagittal or, sometimes, similar to pseudosagittal spicules. Actines are slightly conical and sharp. The unpaired actine is longer than the paired ones (Figure 34H). The apical actine of the tetractines is conical, sharp, smooth, shorter than the basal ones and curved towards the atrium direction.

Atrial tetractines and triactines: there are two categories of atrial tetractines and the triactines are very rare. They are sagittal or subregular. The unpaired actine is frequently longer than the paired

ones (Figure 34I). It is also common to find one of the paired actines shorter than the other (63.5-109.3(±64.7)-155.0/10-11.3(±1.8)-12.5 µm (n=2); Figure 34J). This shorter paired actine is frequently projected inside the exhalant canal. Actines are cylindrical and sharp. Sometimes, the tip of the unpaired actine is thicker (Figure 34K). The main difference between the two categories of tetractines is in the shape and size of the apical actines, which are straight and thinner in one and curved and thicker in the other.

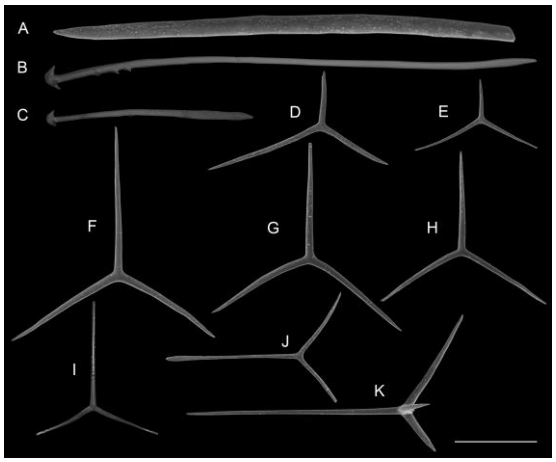


Figure 34. *Sycon ancora* sp. nov. (Holotype - PMR 17809 = UFRJPOR 8345). (A) Cortical diactine. (B, C) Anchor-like tetractines. (D, E) Triactines of the cones. (F-H) Tubar triactines. (I) Subatrial triactine. (J) Atrial triactine. (K) Atrial tetractine (scale = 100 µm). Photo: F. Azevedo.

TABLE 19. Spicules measurements of *Sycon ancora* sp. nov. (PMR 17809=UFRJPOR 8345).

		length (µm)				width (µm)				
		min	mean	sd	max	min	mean	sd	max	n
Diactine		378.0	537.8	180.1	800.0	10.8	16.1	4.5	20	06
Diactine anchor-like		-	-	-	>1000	25.0			37.5	02
Triactine (distal cone)	Paired	59.4	112.3	27.2	148.5	5.4	6.9	1.4	10.8	20
	Unpaired	54.0	78.6	18.7	124.2	5.4	7.0	1.5	10.8	20
Tubar triactine	Paired	116.1	168.2	25.8	216.0	8.1	13.0	2.7	16.2	20
	Unpaired	143.1	188.1	29.6	259.2	8.1	12.4	2.8	18.9	20
Subatrial triactine and tetractine	Paired	67.5	97.9	41.6	159.3	4.1	5.4	1.1	6.8	04
	Unpaired	108.0	212.4	36.5	264.6	4.1	6.0	1.5	8.1	21
Atrial tetractine I	Paired	94.5	153.5	31.7	202.5	7.6	10.8	2.1	16.2	17
	Unpaired	55.1	219.4	75.8	332.1	7.6	10.7	1.5	13.5	17
	Apical	97.5	123.8	21.9	177.5	8.8	11.4	1.4	12.5	20
Atrial tetractine II	Paired	-	162.5	-	-	-	6.3	-	-	01
	Unpaired	-	137.5	-	-	-	6.3	-	-	01
	Apical	50.0	77.1	14.5	112.5	5.0	5.6	1.0	7.5	20

Ecology. Specimens were collected on a semi-vertical hard limestone bottom, among *Cystoseira* macroalgae.

Remarks. Currently there are 12 accepted species of *Sycon* in the Mediterranean Sea, 10 of which are already reported for the Adriatic. The descibed specimens were compared to all known species of *Sycon* and even more carefully to the Mediterranean ones, yet a perfect match could not be found. The main characteristic discerning *Sycon ancora* sp. nov. from other species is the shape of the atrial triactines and the presence of anchor-like tetractines at the base. If we exclude these characteristics, this species would be most comparable to *S. raphanus*, however, there are several important differences. *Sycon raphanus* Schmidt, 1862 was originally described from the Adriatic Sea. Unfortunately, the description was not detailed enough. According to Schmidt [102], *S. raphanus* has a bulb shape and a peduncle. He even considered these characteristics to distinguish *S. raphanus* from *S. ciliatum*, a species from the English Channel which he believed to be present in the Adriatic Sea. Haeckel [54] disagreed with the possibility of *S. ciliatum* occuring in the Mediterranean Sea and considered that all specimens called *S. ciliatum* were in fact *S. raphanus*. He also mentioned that he analysed all the specimens from Schmidt's collection identified as *S. raphanus* and found a potpourri of species, including *Leucandra aspera*, *Sycon humboldti*, *Sycon setosum* and "the real *S. raphanus*". Therefore, he made a detailed description of this species which is since then considered as the official description of *S. raphanus*. According to his description, *S. raphanus* is morphologically very variable, being solitary or not and having or lacking peduncle. The skeleton is composed of tufts of 5-10 cylindrical diactines (var. *tergestina*) to 20-50 diactines (var. *procumbens*) and the size of the diactines vary from 400-800/20-30 µm up to 1000-2000/20-40 µm, rarely attaining 3000 µm. Analysed specimens of *S. ancora* sp. nov. have tufts of 10-15 diactines measuring 378->1500/10.8-18.9 µm. *Sycon raphanus* has triactines with curved paired actines in the distal cones and in the tubar skeleton. The tubar triactines are 100-180/10-12 µm (paired) and 150-250/10-12 µm (unpaired), which are thinner than in *S. ancora* sp. nov. The subatrial skeleton of *S. raphanus* has triactines (paired: 100-180/5-8 µm; unpaired: 150-250/5-8 µm), while *S. ancora* sp. nov. has triactines and tetractines. The atrial skeleton of *S. raphanus* shows subregular to regular (rarely sagittal) triactines and tetractines (basal: 150-250/8-10 µm; apical: 60-120 µm), while our species has tetractines with two types of apical actines (there is a variation in the thickness and position), a long unpaired actine and paired actines with different sizes. Haeckel [54] mentioned the presence of triactines supporting the oscular crown, while *S. ancora* sp. nov. has only tetractines. Although a revision of the entire genus *Sycon* is urgently needed, the characteristics found in the Adriatic specimens strongly indicate the presence of new species.

3.3. Molecular analysis of the Adriatic calcareous sponges

The number of sites used for the final alignments (gaps included) were as follows: 513 for ITS Calcinea, 1434 for 28S Calcinea, 734 for ITS Calcaronea and 846 for 28S Calcaronea. Both markers revealed the same tree topology in both analyses (but see Figure 38), yet the Bayesian analysis rendered much better support values than ML in all cases. However, the Adriatic species nested within the respective genera with high bootstrap support (BS) and posterior probability (PP) values, thereby confirming the results of the morphological analysis (Figures 35-38).

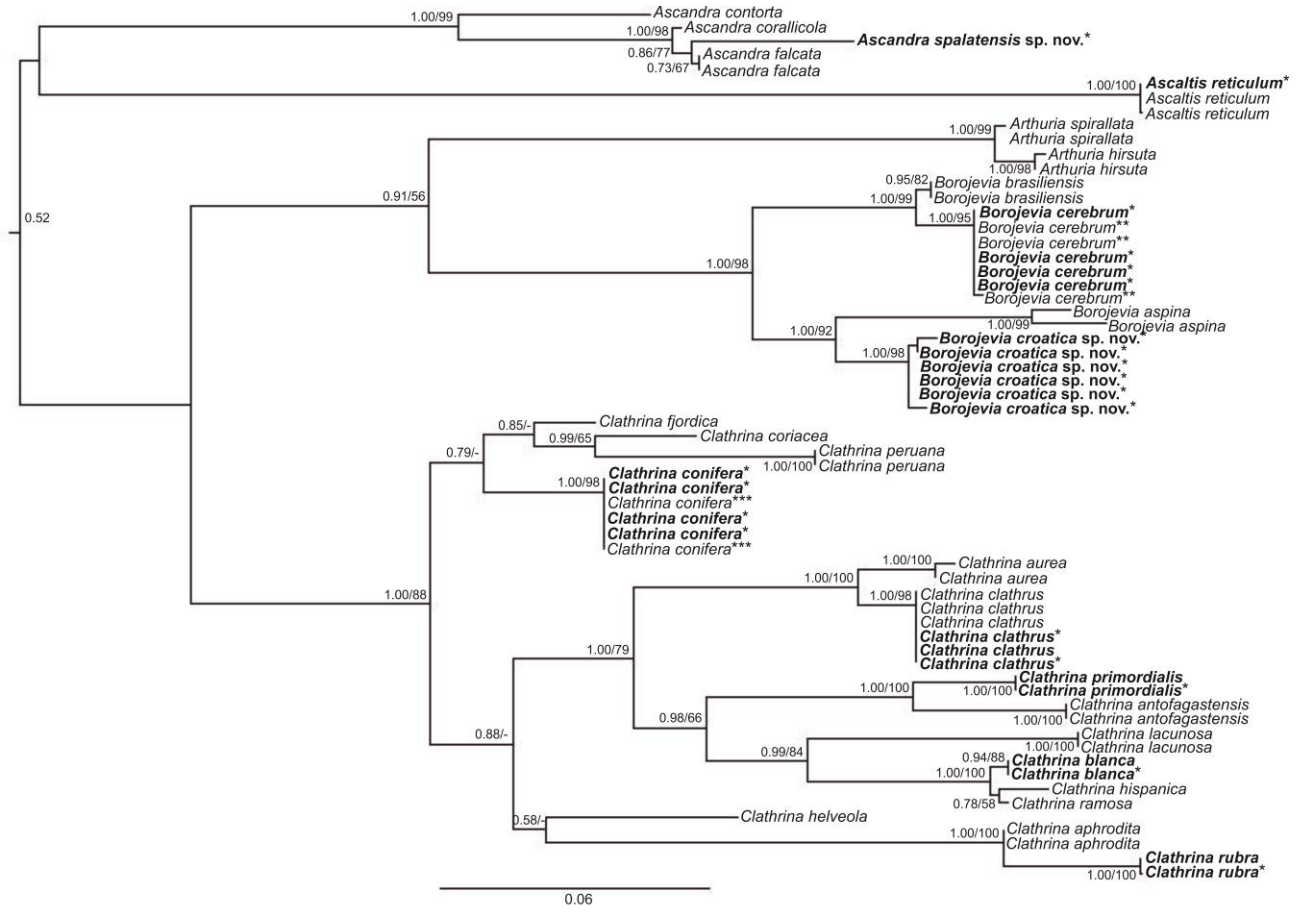


Figure 35. Maximum likelihood (ML) tree based on ITS1-5.8S-ITS2 rDNA sequences of Calcinea. Bayesian posterior probabilities (PP) and bootstrap values (BS) are given near the branches (PP/BS; when >0.50). Adriatic species are written in bold; *Adriatic species obtained in this study; **Mediterranean specimens of *Borojevia cerebrum*; ***Brazilian specimens of *Clathrina conifera*.

Once more the presence of diactines did not show any phylogenetic signal [71,72]. Furthermore, we found former guanchas with only triactines reunited in a monophyletic clade in ITS analysis, with high support values inside the *Clathrina* group (0.99 PP and 0.84 BS; Fig. 35). In 28S calcinean tree (Figure 36) we recovered a clade where *Levinella* represents a sister species to *Ascandra* with high support values (1.00 PP and 0.99 BS), which confirms the results of Voigt and collaborators [65].

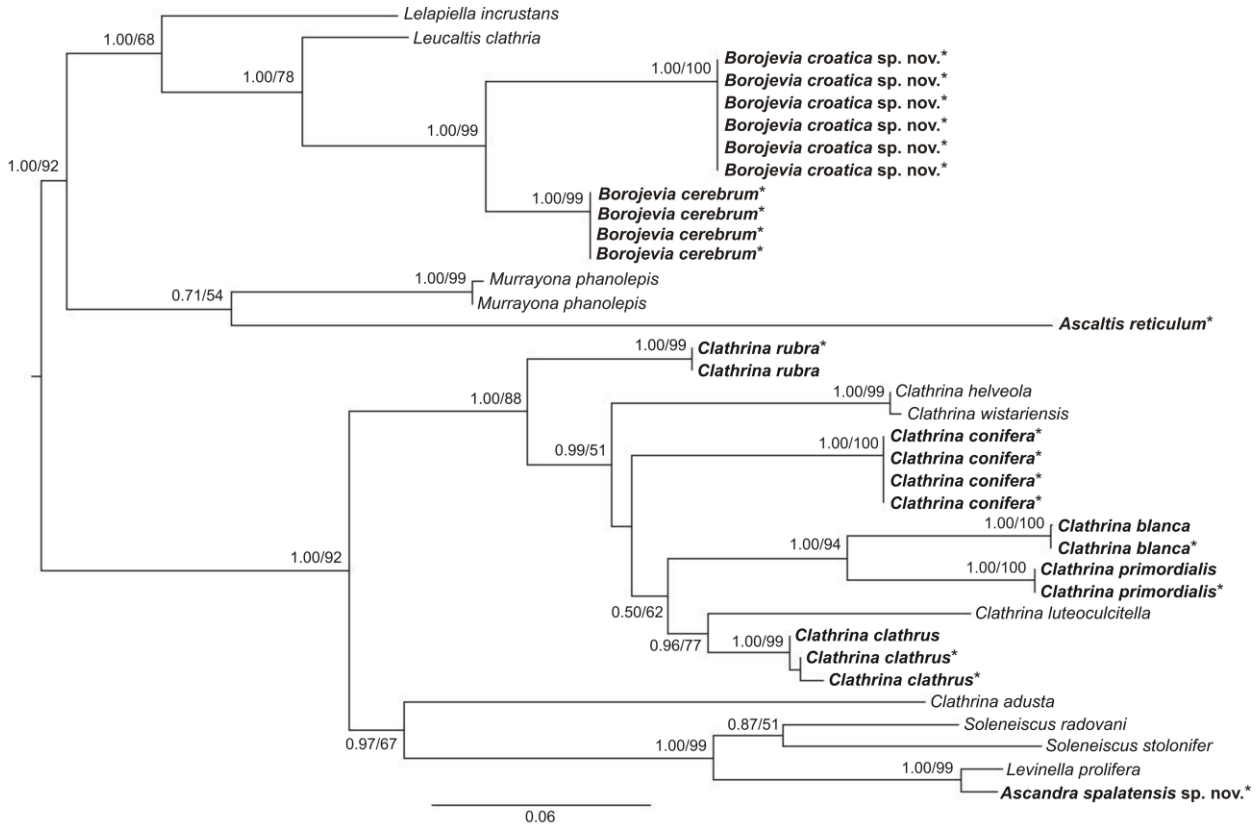


Figure 36. Maximum likelihood tree (ML) based on partial 28S rDNA sequences of Calcinea. Bayesian posterior probabilities (PP) and bootstrap values (BS) are given near the branches (PP/BS; when >0.50). Adriatic species are written in bold. Adriatic species obtained in this study are marked with an asterisk.

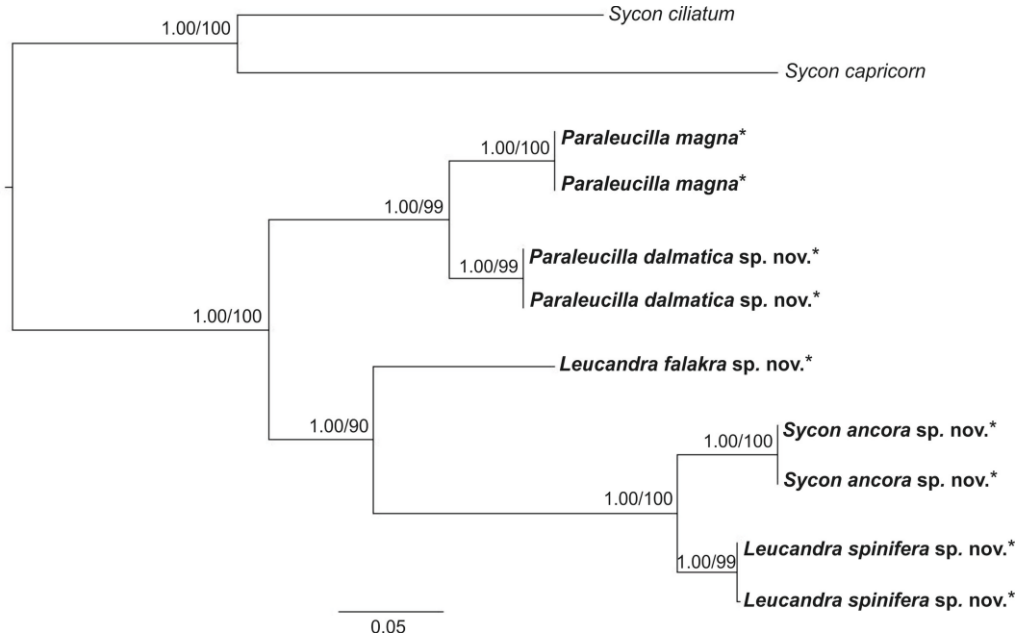


Figure 37. Maximum likelihood tree based on ITS1-5.8S-ITS2 rDNA sequences of Calcaronea. Bayesian posterior probabilities (PP) and bootstrap values (BS) are given near the branches (PP/BS; when >0.50). Adriatic species are written in bold. Adriatic species obtained in this study are marked with an asterisk.

We have also recovered a clade comprising genera *Murrayona* and *Ascaltis* in both analyses, however, the support values were less good (0.71 PP and 0.54 BS). The molecular analyses also confirmed the presence of *P. magna* in the Adriatic Sea (Figures 37 and 38). Besides, we recovered a calcaronean clade with high support (1.00 PP and 100 BS in ITS analysis; 0.95 PP and 64 BS in 28S analysis) formed only by *Paraleucilla* species. The genus *Paraleucilla* formed a high supported clade with *Leucandra nicolae*, while *Leucandra spinifera* sp. nov. is a sister species of *L. aspera* (Fig. 38). *Sycon ancora* sp. nov. represents a sister species of *S. raphanus* (Fig. 37). We have confirmed the paraphyly of the genera *Sycon* and *Leucandra* [65].

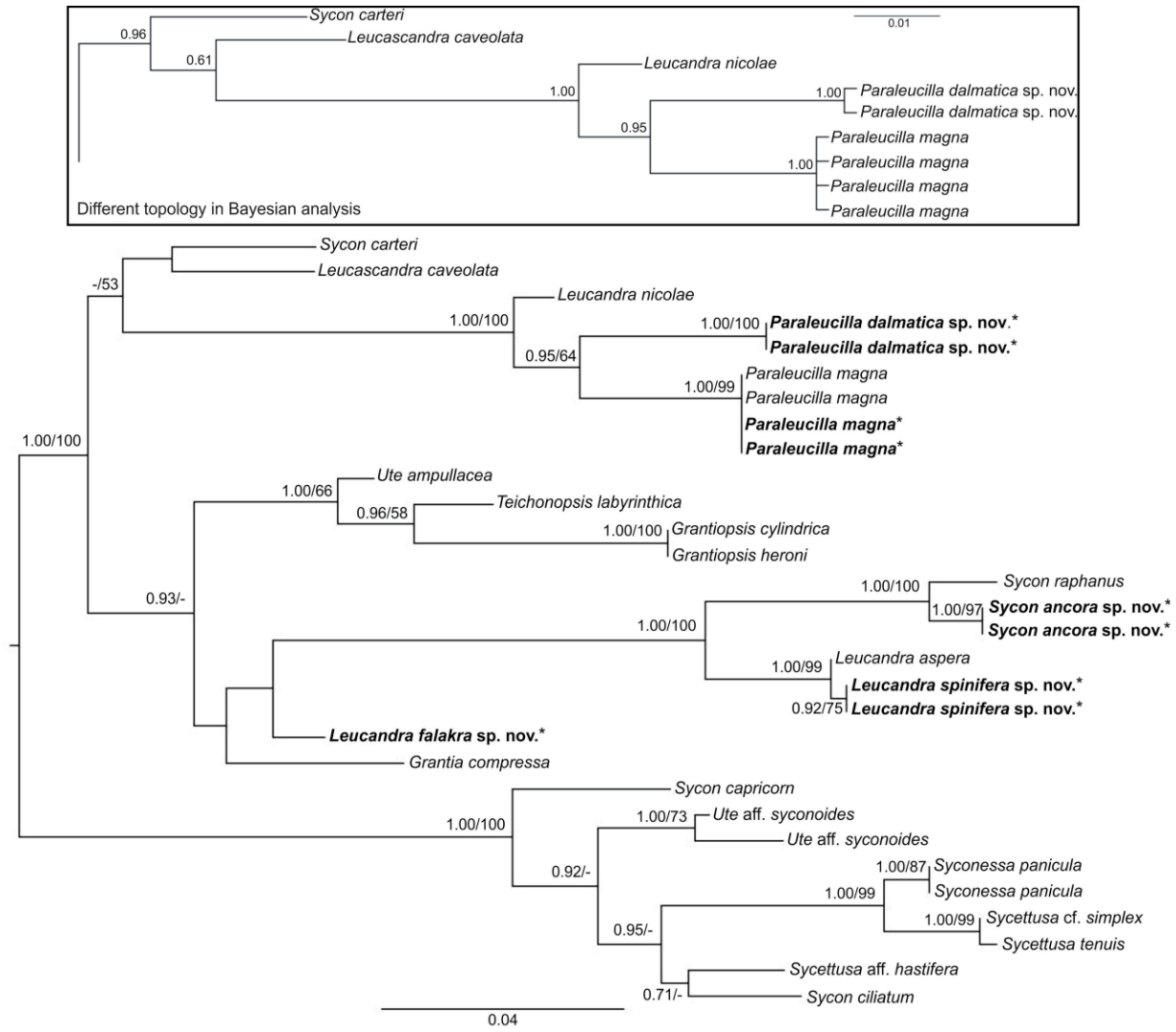


Figure 38. Maximum likelihood tree based on partial 28S rDNA sequences of Calcaronea. Bayesian posterior probabilities (PP) and bootstrap values (BS) are given near the branches (PP/BS; when >0.50). Adriatic species are written in bold. Adriatic species obtained in this study are marked with an asterisk. The detached tree shows the only difference in the topology of the ML and Bayesian analysis.

3.4. Diversity of calcarean sponge species

Considering previous data, together with the present results based on morphological and molecular analyses, a total of 13 species of Calcinea and 26 of Calcaronea is recorded in the Adriatic Sea until now (Supplementary table S2). Taking into account species richness by sectors (Supplementary figure S2), the richest sector is the Central Adriatic, where 38 species were found, followed by the Northern Adriatic with 18 species, and the Southern Adriatic with 4 species. Most of the species present in the Adriatic Sea are present in other Mediterranean areas. Altogether 6 here recorded

species, two calcinean and four calcaronean, are new for science and provisionally endemic for the Adriatic.

Apart from the here described specimens, few specimens of *C. blanca* (PMR-13744) were recorded near Selce (45°09'07.8"N, 14°43'15.0"E), about 1 m deep and of *C. rubra* (IRB-CLC2) near the Island of Čiovo (43°28'58.5"N, 16°21'25.6"E), about 5 m deep on a shaded hard bottom. They were quite abundant through August and November 2010., always only a few milimeters in size and often found on bryozoans. *C. clathrus* was found in numerous locations along the Adriatic coast (e.g. Prapratno Cove, 42°48'36.8"N, 17°40'38.4"E; near the Island of Čiovo, 43°28'58.5"N, 16°21'25.6"E) and the cryptogenic species *Paraleucilla magna* was found in large numbers on aquaculture installations in Grška Cove near the Island of Brač (43°17'23.1"N 16°29'01.4"E) and in the Port of Ploče (43°02'38.49"N, 17°25'32.20"E).

4. Discussion

4.1. Molecular taxonomy of the endemic freshwater sponges

Even though the presence of gemmules and their morphological traits were the main diagnostic characters in the classification at the family, genus and species level [43], molecular studies proved that those are not as informative as previously thought. For example, reduction or loss of gemmules occurred independently several times in the families (Lubomirskiidae, Malawispongiidae and Metschnikowiidae), as well as at lower taxonomical levels, e.g. *E. cooperensis* in contrast to all other *Ephydatia* species [43,44]. This indicates that gemmular traits may not depict with enough accuracy neither the taxonomic position nor phylogenetic relationships within freshwater sponges. The taxonomic position of freshwater stygobiotic sponge *Eunapius subterraneus* was also based on morphological characters, although it displays some important morphological differences from the typical *Eunapius*, namely the reduction of gemmules. These differences could have developed as a consequence of adaptations to an underground habitat, but they might likewise have some deeper taxonomical meaning. To reevaluate the usefulness of morphological characters in phylogenetic studies of *E. subterraneus*, characterization at the molecular level was employed using two ribosomal (18s rDNA and ITS2) and one mitochondrial (COI) marker [48]. It uncovered the proper taxonomic position of this species, which does not belong to the *Eunapius* order, but is placed within the rather unresolved clade comprising a few Spongillidae and all Lubomirskiidae genera, with complete exclusion of all other *Eunapius* species. However, morphological characters used in current taxonomical practice are in collision with this finding suggesting the need for thorough revision of the entire suborder. The analysis of the mitochondrial genome of *E. subterraneus* in combination with the work of Harcet et al. indicates that *E. subterraneus* is closer to the genus *Ephydatia* than to other *Eunapius* species. Phylogenetic analysis additionally highlighted these results (Supplementary figure S3), where short terminal branches of three sponge species suggest their recent diversification from a common ancestor. This is consistent with their quite recent divergence time – the origin of endemic Baikal sponge is estimated to be around 3–10 MYA [36], while the estimated split between endemic *E. subterraneus* and cosmopolitan *E. muelleri* is around 7 MYA. These results support the hypothesis that endemic freshwater sponge species might have originated from a cosmopolitan founder species such as *Ephydatia* [46], but the question remains whether this phenomenon is common to other endemic sponges. *L. baicalensis* and *E. subterraneus* have several things in common. Both are endemic sponges living in similar type of habitats which is characterized by constant conditions with little fluctuations during the year. Smaller amount of gemmules or their complete absence is caused by stable ecological conditions [48]. Furthermore, *L. baicalensis* is

restricted to the ancient Lake Baikal (Siberia) and *E. subterraneus* is found only in the caves of Croatian Karst [50]. We tried to elucidate these relationships in more detail by comparing the mitochondrial genomes of the three freshwater species. Since the lack of mt genomes of closely related species prevented a more accurate comparison, in addition we compared two species of the same genus.

4.2. Mitochondrial genomes in taxonomy of freshwater sponges

As the morpho-taxonomic characteristics of freshwater sponges appear unsuitable for clear separation of either specific species or higher taxonomic levels, the molecular biological approach here appears to be crucial. However, molecular data based on different molecular markers did not provide sufficient information for setting up adequate markers that could be generally used to elucidate the evolutionary course between the taxa within the suborder Spongillina. After we compared mitochondrial genomes from three freshwater sponges – *Eunapius subterraneus, Ephydatia muelleri and Lubomirskia baicalensis* – at first, we have reached the same conclusion, because the gene sequence similarity and genome organization strongly indicated that mitochondrial DNA does not provide enough phylogenetic information on the genera or species level. In an on-going approach to unravel disputable class-level relationships among sponges, a comparative analysis of mitochondrial genomes (mtDNA) is used rather often. Initially, the focus was set on finding analogies between higher poriferan taxa, where species were generally chosen randomly. After Wang and Lavrov [37] determined mitochondrial DNA from 17 demosponge species, they uncovered an extensive mitochondrial genomic diversity within the Demospongiae, especially considering their size, gene order, gene content and the rates of sequence evolution. This was recorded previously, where mitochondrial genomes of most related species available for comparison at the time, *Suberites domuncula* [40] and *Tethya actinia* [33] belonging to the same order, displayed a surprisingly low level of mtDNA primary sequence conservation: 55% for the whole mtDNA, 47–83% for coding regions, 62–91% for tRNAs and 12% for non-coding regions. However, our comparison between three freshwater sponge mtDNA sequences showed identity of 75–84, 91–100, 98–100, and 34–53%, respectively. Furthermore, *atp8* and *nad6*, the least conserved genes, showed high identity (more than 94%) between three freshwater sponges, while nucleotide analysis of the same genes between *S. domuncula* and *T. actinia* showed 57 and 73% identity. It was obvious that freshwater sponges show a remarkable similarity of mtDNA genomes in the view of identical gene content, gene arrangement and sequence conservation, which apparently bears no

phylogenetic information. Yet, the size of three mt genomes was obviously unequal due to the variation in size of the intergenic regions. Hence, we decided to pay more attention to the differences in the intergenic non-coding regions.

4.3. Mitochondrial intergenic non-coding regions as a source of diversity

Considering a high degree of conservation of mtDNA among analysed freshwater sponges, non-coding regions seem to provide additional valuable information. So far, with a few exceptions, freshwater sponges are known to possess larger non-coding regions and have displayed a higher abundance of open reading frames (ORFs) in comparison to other mt genomes, although the putative ORFs from *E. subterraneus* show no significant similarity with any of the known proteins. For instance, 184 ORFs of the yeast *Saccharomyces cerevisiae* are related to sequences in other eukaryotes, suggesting the evolutionary conservation of structure and perhaps function in key cellular processes [110]. Interestingly, some demosponge protein-coding genes, such as *atp9*, are absent in all other metazoan phyla. However, their presence in some demosponges as well as choanoflagellates and fungi [35] suggests that this is probably an ancient premetazoan feature. ORFs without any significant similarity to any known protein have been found in the Cnidaria and Porifera [37,111,112]. Due to the highly divergent sequences of ORFs, it is difficult to pin point their origin and nature. In this light, the intergenic regions (IGRs) abundant with palindromic elements and repetitive hairpin-forming elements provide a source of higher diversity and possibly better phylogenetic signal. The potential importance of repetitive hairpin elements in mtDNA evolution was suggested, but their origin and function is still unknown [36]. It was postulated that the proliferation of repetitive elements often results in expanded intergenic regions. Previous research demonstrated positive correlation between the abundance of repetitive elements and the length of non-coding regions, and consequently total genome size, in sponge mitochondria [36]. Namely, *L. baicalensis* possesses the largest number of these elements which tend to show a rapid proliferation, therefore has the longest intergenic regions. We found that repetitive sequences in the mtDNA of *E. subterraneus* share primary sequence similarity with those in *E. muelleri*, *E. fluviatilis* and Baikal sponges only for H7–8 family. In addition, we noticed that every species has its own characteristic repetitive elements which are scattered through the whole mt genome.

4.4. Taxonomic importance of repetitive elements in mitochondrial intergenic regions

Although there are numerous repetitive elements in the mt genomes of *E. subterraneus*, *E. muelleri* and *E. fluviatilis*, we found no evidence of their rapid proliferation, as described for *L. baicalensis*. Many questions regarding these repetitive elements, such as their function and evolution, are still controversial. The absence of sequence similarity between repetitive elements suggests that evolution of these elements occurred independently after the divergence of different phyla, leading to their variations [40,113]. Several studies have reported the involvement of palindromic elements in biological processes [114], DNA replication and gene regulation [115,116]. Moreover, these elements were found in high concentration close to the replication origins in viruses [114]. So far, mtDNA control regions including the origin of replication are still unknown in sponges. However, in sponge *Aphrocallistes vastus* [117] putative control region abundant with repetitive elements was determined. Therefore, palindromic elements could be part or control regions of mtDNA. Another hypothesis is that small palindromic sequences are mobile elements [36,118], as reported for algae *Volvox carteri* [119]. Palindromic elements are spread through the whole mtDNA including the protein coding regions where they could influence molecular evolution of proteins [93]. Recent studies suggest that repetitive elements present in mt genomes indicate either an early origin or multiple independent invasions, while the fact that these palindromic elements form secondary structures could be functionally important [95]. Considering the diversity of these repetitive elements in freshwater sponges, their sparse distribution, short evolutionary split for Spongillidae and the fact that most of the repetitive families are species specific, it is unlikely that they were present in an ancestral mt genome. Therefore, insights into mt IGRs may play an important role in the evolution of the metazoan mt genome, although more mtDNA data of freshwater sponges are necessary before these events could be adequately understood.

4.5. Mitochondrial palindromic elements in the taxonomy of Spongillidae

It was already pointed out that the uniformity of mt genomes of freshwater sponges has been shattered by frequent insertions of palindromic elements during evolution [36,49]. Their accumulation through time likely triggered evolutionary events responsible for the observed differences between the compared mt genomes. The comparison of two species from the same genus suggests that repetitive elements present in freshwater sponges are evolutionary related and were probably present in a common ancestor (H7 and H8 families). When we compared

Spongillidae species with the newly sequenced mitochondrial genomes from four Lubomirskiidae, it became obvious that certain families of palindromic elements were recently introduced into the genome of individual species, while *E. muelleri* lacks all but the H7 and H8 families. The phylogenetic analysis on concatenated mitochondrial gene sequences 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), but also with the endemic stygobiotic sponge *Eunapius subterraneus* and the endemic family Lubomirskiidae. This also makes sense in terms of the colonization of freshwater habitats, which was most probably achieved by a single cosmopolitan founder species, such as *Ephydatia muelleri*. It was later followed by diversification into a number of different genera and species which subsequently colonized more steady habitats, such as caves and lakes. Although mitochondrial genomes of freshwater sponges showed remarkable uniformity despite differences in their 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. 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. These elements lacking obvious cellular function could introduce genetic variability and potentially be used in order to address unresolved taxonomic questions among freshwater sponges. Nevertheless, more data from closely related taxa are needed to test this hypothesis and more specimens per species must be evaluated.
From a series of molecular analyses completed in the last decade, a pattern in the evolution of freshwater sponges emerges. 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. Whatever their origin and function might be, these sequences do have an effect on genome plasticity, and could be important in regard to mitochondrial evolution.

4.6. Integrative taxonomy of calcareous sponges

Since Haeckel [54], the most important taxonomic characters of Calcarea were the aquiferous system and the organization of skeletal elements. Using these characters, the taxonomic position of numerous taxa within the class has gone through considerable changes over the past few decades. The integrative taxonomy uses both, morphological and molecular data, to evaluate taxa. DNA

taxonomy based on the molecular markers introduced within the last couple of years, has shown to be necessary for resolving some taxonomic dilemmas, as well as for elucidation of phylogenetic signals in the certain morphological features. Only after the molecular analyses were applied on a higher number of calcinean species, a strong foundation for clear and precise systematics changes on different taxonomic levels could be achieved [71,72]. Therefore, expanding the number of analysed specimens seems to be important for defining phylogenetically meaningful genera and species-specific morphological features.

4.7. Diversity of the Adriatic calcarean sponge species

Since some of the first studies on the class Calcarea were mainly done along the Dalmatian coast by Schmidt and Haeckel in the 19th century [54,105,109,120], the knowledge of current species diversity and distribution certainly awakes taxonomic interest. Several calcarean species are currently known in the Mediterranean, included the Adriatic [108], but literature data on these Adriatic species is very scarce or difficult to access. In combination with previous results, a total of 39 species of calcareous sponges is so far recorded in the Adriatic Sea (Supplementary table S2). In this species list the registers of *Clathrina coriacea* (Montagu, 1814), *Sycon ciliatum* (Fabricius, 1780), and *S. proboscideum* (Haeckel, 1870) were not included. Analysis of the descriptions of *C. coriacea* from the Adriatic Sea indicates that this species is most probably *C. conifera* or *C. primordialis*. *Sycon ciliatum* seems to be restricted to the North Atlantic and was probably mistaken for *S. raphanus* [54]. *Sycon proboscideum* is a species known from the Red Sea and its occurrence in the Adriatic Sea was mentioned only by Breitfuss [121]. He suggested that some specimens previously identified as *S. raphanus* could be in fact *S. proboscideum*, but did not give a description or any further clues. Therefore, the occurrence of this species in the Adriatic Sea is somewhat doubtful [122,123]. The results presume *Sycon* as the most diverse genus with eight species, followed by *Clathrina* with six species. However, it is important to keep in mind that *Sycon* is not a monophyletic genus. It is very difficult to identify *Sycon* species with certainty, as most of the species have similar spicule composition - diactines, trichoxeas and triactines in the distal cones, tubar triactines, subatrial triactines and tetractines, and atrial tetractines. Up to date there are no studies on intraspecific morphological variability of *Sycon*. In addition, most species were poorly described and insufficiently analysed at the molecular level, which also applies to the calcaronean genera *Paraleucilla*, *Leucandrilla*, *Leucandra* and *Leucilla.* Molecular phylogenetic study including as many species as possible would be very desirable to draw the boundaries among these genera.

4.8. Taxonomic position of the Adriatic calcarean sponges revealed by molecular markers

The molecular analyses have revealed some interesting taxonomic traits. On the genera level, the monophyletic clade of former genus *Guancha* indicates that the development of a peduncle and of parasagittal spicules probably appeared only once in the evolution of *Clathrina*. *Clathrina hispanica* was nested within this group, although in the original description of this species neither peduncle nor parasagittal spicules have been mentioned [67]. The type specimen of this species is fragmented, resulting in the impossibility of confirming if a peduncle was present or not, however, we re-analysed the slides of the holotype and found some parasagittal spicules. In our tree it is possible to see that *C. blanca* is not only morphologically but also molecularly close to the Chilean species *Clathrina ramosa* Azevedo et al., 2009. In fact, the only morphological difference between them is the external appearance of the clathroid body, which is globular in *C. blanca* and coarsely branched in *C. ramosa*. As yellow clathrinas are phylogenetically very close [71,72], we expected that the red *C. rubra* would group with them in the phylogenetic tree. However, it was well separated from those species, which suggests that the red/orange colour appeared independently of the yellow colour. Another interesting result indicated the close relation among *Ascandra*, *Soleneiscus* and *Levinella* revealed in the 28S analysis. Voigt *et al.* [65] have shown that the genus *Ascandra* is closely related to *Soleneiscus* and *Levinella*, which is now being confirmed. This implies that in the future genera *Levinella* and *Soleneiscus* might be synonymised to the genus *Ascandra*, nonetheless more detailed molecular and morphological analyses on a larger number of specimens and species are needed to confirm this action. On the species level, a molecularly confirmed presence of *Clathrina conifera* in the Adriatic raised a doubt of the former identification of the *C. primordialis* syntype, allowing the selection of a true lectotype of this species. Additionally, the re-description of *Borojevia cerebrum* from near its type locality (Lesina - Island of Hvar) based on its molecular analysis, confirmed the presence of this species in the Mediterranean Sea (Figure 35). Observing the morphological variations within a single, molecularly verified species, enabled the synonymization of two "cerebrum" varieties. Step by step, the "cerebrum complex" is being solved.

4.9. Calcarean species first time recorded in the Adriatic

The results have confirmed the presence of few species known from the Atlantic. It was unexpected to find *Clathrina conifera* in the Adriatic Sea, as this species was first described along the Brazilian

coast and was considered endemic [101]. This finding raises the question whether this species was ever truly endemic to Brazil. Since Adriatic calcarean sponges are vastly unexplored and *C. conifera* is part of the *C. primordialis* species complex, it is possible that it has been recorded previously as *C. primordialis* (or *C. coriacea*). Indeed, the specimen from Naples (ZMB 1306) previously identified as *C. primordialis* [67] is most likely *C. conifera*, although a molecular confirmation would be desirable. If this is true, the presence of *C. conifera* in the Adriatic Sea would be at least as old as the beginning of the 20th century. Unfortunately, it is not possible to know whether this is the result of anthropogenic introduction or a natural distribution. In 2010, a specimen of *Clathrina conifera* was observed for the first time in the Southern Adriatic, near the Island of Lokrum, and a year later, more than 20 specimens were recorded near the city of Dubrovnik. As both locations are close to the area in Dubrovnik frequently visited by cruise ships, it is possible that this species has been introduced from the Brazilian coast into the Adriatic. However, if this species arrived by anthropogenic means, we cannot state whether it arrived from the Western Atlantic to the Adriatic or vice-versa. It is also important to mention that the presence of *Paraleucilla magna* has been confirmed earlier in the Southern Adriatic [124], while this work molecularly confirmed the presence of *P. magna* at the same location near the Port of Ploče, as well as at another location near the Island of Brač (Supplementary table S2). This species was first recorded in Brazil in the 1980's, but its origin is still unknown. It seems to have been introduced by anthropogenic means into the Mediterranean [125] and to be spreading into the Eastern Mediterranean, including the Adriatic Sea.

4.10. Calcarean species new for science and currently endemic for the Adriatic

The results have also confirmed six species endemic of the Adriatic and new for science. The first records of *Borojevia croatica* sp. nov. and *Ascandra spalatensis* sp. nov. expands the number of species of these genera. The genus *Ascandra* until now comprised nine species, only two of which (*Ascandra falcata* and *Ascandra corallicola)* were recently analysed at the molecular level [72]. The new species molecularly fits to the genus *Ascandra*, but considering the diagnosis given by [72], it shows some different morphological features. New species records provide more accurate and detailed descriptions and strongly depend on a greater number of analysed specimens. This is especially important for most of the calcaronean genera, especially the genus *Sycon*. As already pointed out, calcaronean species are very hard to delimit based on morphological characters and this group has thus far been considerably less investigated than calcineans. From this point of view, the record of four calcaronean species completely new for science seems rather expected, especially

in the Adriatic. New calcaronean species are welcome to facilitate more thorough revision of their systematics and link the molecular traits to the phylogenetically important morphological traits.

4.11. Taxonomic importance of the Adriatic calcareous sponges

The Adriatic Sea is considered a biodiversity hotspot [126] of major ecological importance which holds a high number of vulnerable or endangered species. Although most of the marine research focuses on commercially exploitable vertebrates such as mammals or fishes, it is important to raise the notion that less investigated invertebrates are as much important for retaining a balanced ecosystem. Sponges represent one of the major invertebrate groups inhabiting hard-bottom benthic environment, which has numerous roles in marine ecosystem maintenance [5]. Still, it remains one of the least investigated groups on the molecular level. Conservative systematics relying on the paucity and plasticity of morphological traits is one of the main causes of phylum's biodiversity underestimation. This is especially true for the calcarean sponges, known to be taxonomically difficult and offering very few morphological characters for reliable phylogenetic reconstruction. There is no recent systematic survey on the diversity, number and distribution of the Adriatic calcareous sponges. With the increasing growth of economic activities in the marine domain, negative impacts on biodiversity will be even more present along the Croatian coastline. As many benthic species are strongly dependent on their habitats, its degradation has a huge effect on marine biodiversity [127,128]. This affects calcarean sponges, which are also more vulnerable to seawater acidification. These sponges largely inhabit cryptic habitats, such as semi-shaded caves or crevices and there is a real danger that without adequate protection, many species will be destroyed even before they are discovered. Likewise, very little is known about introduced calcareous sponges, their potential of spreading and their impact on the native benthic communities. With the increasing knowledge about marine biodiversity in general, the value of benthic habitats and their communities will rise and make them eligible for more efficient protection. Therefore, a larger number of detailed identifications and complete descriptions need to be done. It also bears a significant weight in reviving museum collections, which would be of a great help for systematics research of calcareous sponges in the future.

5. Conclusions

Analysis of the mitochondrial genome of *E. subterraneus* supports the hypothesis that endemic freshwater sponge species might have originated from a cosmopolitan founder species such as *Ephydatia*. This may be common to all other endemic sponges, including Lubomirskiidae.

Regardless of the presence of numerous repetitive elements in the mitochondrial genomes of *Eunapius* subterraneus, *Ephydatia muelleri* and *Ephydatia fluviatilis*, we found no evidence of their rapid proliferation, as described for *Lubomirskia baicalensis*.

Repetitive palindromic sequences in the mitochondrial DNA of *E. subterraneus*, *E. muelleri*, *E. fluviatilis* and Lubomirskiidae share primary sequence similarity only for H7–8 family.

The comparison of two species from the same genus suggests that repetitive elements present in freshwater sponges are evolutionary related and that H7 and H8 families were probably present in a common ancestor.

The new palindromic families are species-specific and were possibly inserted into mitochondrial genomes after the divergence of 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.

These sequences affect genome plasticity and could be important in regard to mitochondrial evolution. To test this hypothesis, we need more data from closely related taxa and more specimens per species must be checked.

Analysis of the intergenic regions provides significant information for improving phylogenetic determination at the taxonomic level of order and/or genus. Repetitive palindromic elements introduce genetic variability and may be used to address unresolved taxonomic questions.

Total of 38 species of calcareous sponges is so far recorded in the Adriatic Sea. *Sycon* seems to be the most diverse genus with eight species, followed by *Clathrina* with six species. It is important to have in mind that *Sycon* is not monophyletic.

Clathrina coriacea from the Adriatic Sea seems to be *C. conifera* or *C. primordialis*. *Sycon ciliatum* seems to be restricted to the North Atlantic and probably was mistaken for *S. raphanus*. *Sycon proboscideum* is a species known from the Red Sea and its occurrence in the Adriatic Sea was

mentioned only once. The occurrence of these species in the Adriatic Sea is somewhat doubtful.

Six species new for science are provisionally endemic of the Adriatic: *Ascandra spalatensis* sp. nov., *Borojevia* croatica sp. nov., *Leucandra falakra* sp. nov., *Leucandra spinifera* sp. nov., *Paraleucilla dalmatica* sp. nov. and *Sycon ancora* sp. nov.

Two species known from the Atlantic, *Clathrina conifera* and *Paraleucilla magna*, are molecularly confirmed to exist in the Adriatic.

Molecularly confirmed presence of *Clathrina conifera* in the Adriatic raised a doubt of the former identification of the *C. primordialis* syntype, allowing the selection of a true lectotype of *C. primordialis*.

The re-description of *Borojevia cerebrum* from near its type locality based on molecular analysis, confirmed the presence of this species in the Mediterranean Sea. Observing the morphological variations within a single, molecularly verified species, enabled synonimizing the two "*cerebrum*" varieties: *B. cerebrum* var. *gyrosa and B. cerebrum* var. *decipiens*.

Clathrina rubra was first recorded in the Adriatic in 1958 by Sarà, but its formal description was not presented until now. The first formal description of *C. rubra* is given, supported by molecular analyses.

The increased species number of the abandoned genus *Guancha* formed a monophyletic clade, indicating that the development of a peduncle and of parasagittal spicules appeared only once in the evolution of *Clathrina*. New species records provide more accurate and detailed descriptions and strongly depend on a greater number of analysed specimens.

Calcarean sponge fauna of the Adriatic Sea is still underestimated and needs further systematic surveys, including molecular markers. Knowledge of marine biodiversity increases the value of benthic communities and makes them eligible for a more efficient protection.

More of detailed identifications and complete descriptions are significant in reviving museum collections, which would be of a great help for systematics research of calcareous sponges in the future.

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7. Summary

Morphological characters used by traditional taxonomy have been often shown having no phylogenetic signal. Without the molecular confirmation, it is hard or even impossible to deduce phylogenetically important morphological characters, or whether certain characters have resulted from convergent evolution. Integrative taxonomy including molecular markers opened a new way to test the existing taxonomic hypotheses based on morphology alone and enabled the correlation of genetic traits with distinctive morphological features. Combining molecular markers with traditional taxonomy revealed a number of inconsistencies in taxonomic relationships within freshwater and calcarean sponges, notably on the lower taxonomic levels of genera and species. Mitochondrial DNA of freshwater sponges, especially large intergenic regions, represents a target for the insertion of repetitive hairpin-forming elements. These elements are responsible for large mitochondrial genome size differences observed even among closely related sponge taxa. Comparing mitochondrial genome sequences from closely related species, *Ephydatia muelleri* and *Ephydatia fluviatilis*, showed that palindromic elements are widespread through whole mitochondrial genomes of both species, including protein coding genes, thereby introducing genetic variability into the genomes. Comparison to the mitochondrial genomes of Lubomirskiidae revealed that repetitive hairpin-forming elements share the sequence similarity only for H7 and H8 palindromic families. These families were probably present in a common ancestor of freshwater sponges. The other species-specific palindromic families were then introduced after the divergence of species, rather than being subsequently lost in all other species. These sequences introduce genetic variability across the mitochondrial genomes and are possibly evolutionary related. The phylogenetic analysis on mitochondrial protein coding genes from *E. subterraneus* confirmed its close relationship with *E. muelleri* and *L. baicalensis*. Another phylogenetic analysis on all available mitochondrial genomes from freshwater sponges indicates *E. muelleri* as evolutionary older species in comparison to *E. fluviatilis*, but also to the endemic *E. subterraneus* and Lubomirskiidae. Most probably a single cosmopolitan founder species, such as *Ephydatia muelleri*, has initiated the colonization process of freshwater habitats and later evolved into different species which subsequently colonized more stable habitats.

Some of the first calcarean sponge studies were performed in the Adriatic Sea, which makes it the type locality for many of the first known species. Still, the species identifications are frequently doubtful, as the descriptions are fragmentary or simply lost. The taxonomic methods for the class Calcarea have long relied only on histological and morphological characters. The lack of reliable morphological characters contributes to difficulties in observing their distribution and collection of new samples. Analyses of the Adriatic calcarean sponge diversity based on morphological and

molecular characters revealed six species new to science and provisionally endemic of the Adriatic (*Ascandra spalatensis* sp. nov., *Borojevia croatica* sp. nov., *Leucandra falakra* sp. nov., *L. spinifera* sp. nov., *Paraleucilla dalmatica* sp. nov. and *Sycon ancora* sp. nov.), one species previously known only from the Southwestern Atlantic (*Clathrina conifera*), and six species already known from the Adriatic Sea (*Ascaltis reticulum*, *Borojevia cerebrum*, *Clathrina primordialis, C. blanca*, *C. clathrus* and *C. rubra*). The presence of the alien species *Paraleucilla magna* in the Adriatic is confirmed. An emendation was added to the description of the genus *Ascaltis,* a lectotype of *Borojevia cerebrum* proposed, and *B. gyrosa* and *B. decipiens* synonymized to *B. cerebrum*. The species checklist of all calcarean species previously and currently known from the Adriatic Sea now counts a total of 39 species. The Central Adriatic seems to be the richest calcarean sponge fauna sector, but biodiversity of this class seems to be strongly underestimated in the whole Adriatic Sea and needs further systematic surveys.

8. Sažetak

Tradicionalna taksonomija spužvi dugo se oslanjala isključivo na morfološka obilježja. Najvažnija morfološka obilježja su tip građe te veličina, oblik i građa skeletnih tvorbi – spikula. Postoji pet tipova građe spužvi – askon, sikon, leukon, silebid i solenoid, te velik broj različitih oblika, veličina i tipova spikula. Na temelju morfoloških karakteristika, spužve su dugo bile podijeljene u četiri razreda: Demospongiae (kremenorožnjače), Hexactinellida (staklače) i Calcarea (vapnenjače). Nedavno je na temelju molekularnih analiza, iz razreda Demospongiae izdvojen četvrti razred, Homoscleromorpha. Uvođenjem molekularnih markera u taksonomiju spužvi, otkrivene su brojne nepravilno determinirane vrste, osobito na taksonomskim razinama roda i vrste. Bez molekularnih analiza, teško je odrediti koja od morfoloških obilježja imaju genetičku podlogu, a koja od njih su posljedica konvergentne evolucije. Integrativna taksonomija pomoću molekularnih markera omogućila je nove načine testiranja postojećih taksonomskih hipoteza utemeljenih na morfologiji, kao i povezivanje molekularnih rezultata s filogenetski značajnim morfološkim karakteristikama. U mitohondrijskoj DNA slatkovodnih spužvi, osobito u intergenskim regijama, česta je pojava ugradnja kratkih ponavljajućih palindromskih elemenata. Oni su odgovorni za vidljive razlike u veličinama mitohondrijskih genoma unutar srodnih skupina spužvi. Usporedbom mitohondrijskih genoma srodnih vrsta slatkovodnih spužvi *Ephydatia muelleri* i *Ephydatia fluviatilis* otkriveno je da su palindromski elementi prošireni po čitavim mitohondrijskim genomima kod obje vrste, čak i unutar gena, te time uvode genetsku varijabilnost između vrsta. Usporedba mitohondrijskih genoma Spongilidae i Lubomirskiidae pokazuje da su samo kratki ponavljajući elementi koji se ubrajaju u palindromske obitelji H7 i H8, prisutni u obje skupine. Stoga ti elementi vjerojatno potječu od zajedničkog pretka slatkovodnih spužvi. Ostale palindromske obitelji specifične su za pojedine vrste, pa je vjerojatnije da su se one pojavile nakon odvajanja tih vrsta. Ti su ponavljajući sljedovi najvjerojatnije evolucijski značajni, obzirom da unose genetičku varijabilnost u mitohondrijske genome. Filogenetska analiza mitohondrijskih gena iz spužve *E. subterraneus* pokazala je da je ta vrsta taksonomski bliska vrstama *E. muelleri* and *L. baicalensis*, dok filogenetska analiza svih dostupnih mitohondrijskih genoma slatkovodnih spužvi ukazuje na to da je *E. muelleri* evolucijski starija od *E. fluviatilis*, ali i od endemskih vrsta *E. subterraneus* i porodice Lubomirskiidae. Iz toga proizlazi zaključak kako je kozmopolitska vrsta poput vrste *Ephydatia muelleri* najvjerojatnije prva naselila slatkovodna staništa te je naknadno evoluirala u različite vrste koje su zatim naseljavale stabilnija staništa.

Jadransko more predstavlja tipski lokalitet za velik broj spužvi vapnenjača, obzirom da iz njega potječu prva istraživanja tih spužvi. No, postojeći opisi vrsta vrlo su upitni jer su mnoge vrste opisane nepotpuno, a brojni opisi tijekom vremena oštećeni ili izgubljeni. Taksonomija vapnenjača

dugo se oslanjala isključivo na histološka i morfološka obilježja. Nedostatak pouzdanih morfoloških obilježja uzrokuje poteškoće pri istraživanju rasprostranjenosti ovih spužvi. U ovom je radu, na osnovu molekularnih i morfoloških analiza jadranskih vapnenjača, opisano šest vrsta novih za znanost, a trenutno i endemskih za Jadransko more (*Ascandra spalatensis* sp. nov., *Borojevia croatica* sp. nov., *Leucandra falakra* sp. nov., *L. spinifera* sp. nov., *Paraleucilla dalmatica* sp. nov. i *Sycon ancora* sp. nov.). Zabilježena je i jedna vrsta do sad poznata jedino iz Atlantskog Oceana (*Clathrina conifera*) te šest vrsta otprije poznatih u Jadranskome moru (*Ascaltis reticulum*, *Borojevia cerebrum*, *Clathrina primordialis*, *C. blanca*, *C. clathrus* i *C. rubra*). Molekularnim analizama potvrđena je i prisutnost strane invazivne vrste *Paraleucilla magna* u Jadranskom moru. Nadopunjen je opis roda *Ascaltis*, potvrđen lektotip vrste *Borojevia cerebrum*, a varijeteti *B. gyrosa* i *B. decipiens* izjednačeni su s vrstom *B. cerebrum*. Popis poznatih vrsta vapnenjača u Jadranskom moru sada sadrži ukupno 39 vrsta. Središnji Jadran pokazao se područjem najveće raznolikosti, ali bioraznolikost ove skupine u cjelokupnom Jadranu izuzetno je slabo istražena te zahtijeva što veći broj sistematskih istraživanja. Značaj i vrijednost bentičkih staništa raste s novim spoznajama o brojnosti i rasprostranjenosti životnih zajednica. Stoga je važno identificirati i opisati što veći broj vrsta kako bi mogle dobiti potrebnu zaštitu. Detaljni opisi vrsta također su značajni i za obnovu postojećih muzejskih zbirki, što će biti od velike pomoći u budućim taksonomskim i sistematskim istraživanjima spužvi vapnenjača.

9. Abbreviations

18S	18 Svedberg units; component of the small eukaryotic ribosomal subunit
28S	28 Svedberg units: component of the large eukaryotic ribosomal subunit
5.8S	5.8 Svedberg units: component of the large eukaryotic ribosomal subunit
AIC	Akaike information criterion
ATP 6/8/9	ATP synthase subunit 6/8/9 of F0 adenosine triphosphatase (ATP) synthase
ATPSbeta-iII	ATP synthetase beta subunit gene
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
BMNH	British Museum of National History
bp	Base pairs
BS	Bootstrap support
COB	Apocytochrome B
COX 1-3	Cytochrome *c* oxidase subunits 1-3
dATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMBOSS	European Molecular Biology Open Software Suite
EN	Endangered
Gln	Glutamine
GTR+I+G	General time reversible+proportion of invariable sites+gamma distribution
HiDi	Highly deionized
His	Histidine
I1-trnI(gau)	Isoleucine tRNA gene
I2-trnI(cau)	Isoleucine tRNA gene
IGR	Intergenic region
Inv.Nr.Porif.	Inventory number Porifera
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRB	Institut Ruđer Bošković
ITS1/2	Internal transcribed spacer 1/2
IUCN	International Union for Conservation of Nature
JTT+G+F	Jones-Taylor-Thornton+gamma distribution+amino acid frequencies
kbp	Kilobase pairs

L1-trnL(uag)	Leucine tRNA gene
L2-trnL(uaa)	Leucine tRNA gene
LB	Luria (lysogeny) broth
Leu1	Leucine 1
Leu2	Leucine 2
MCMC	Markov chain Monte Carlo
Me/Mf	Genes for inferred elongator and initiator tRNA(Met)
MgSO4	Magnesium sulfate
ML	Maximum likelihood
MNRJ	Museu Nacional do Rio de Janeiro, Brazil
MP	Maximum parsimony
mt	Mitochondrial
mtDNA	Mitochondrial DNA
NaAc	Sodium acetate
NAD1-6	NADH dehydrogenase subunit 1-6
NAD4L	NADH dehydrogenase subunit 4L
NCBI	National Center for Biotechnology Information
NJ	Neighbour joining
NPRS	Non-parametric rate smoothing
nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase chain reaction
Phe	Phenylalanine
PMJ	Phyletisches museum Jena
PMR	Prirodoslovni muzej Rijeka
PP	Posterior probability
Pro	Proline
R1-trnR(ucg)	Arginine tRNA gene
R2-trnR(ucu)	Arginine tRNA gene
rDNA	Ribosomal DNA
rnl	Large subunit rRNA
rns	Small subunit rRNA
rpl2–16	Large subunit ribosomal proteins

rps3–19	Small subunit ribosomal proteins
tatC	Twin-arginine translocase component C
tRNA	Transfer RNA
tRNAx	tRNA-like structure
S1- trnS(ucu)	Serine tRNA gene
S2- trnS(uga)	Serine tRNA gene
sd	Standard deviation
sp.	Unknown species
sp. nov.	Species nova
TAE	Tris-acetate-EDTA
TBR	Tree bisection and reconnection
Thr	Threonine
Trn+G	Tamura-Nei + gamma distribution
TrN+I+G	Tamura-Nei + proportion of invariable sites + gamma distribution
UFRJ	Universidade Federal do Rio de Janeiro
UFRJPOR	Universidade Federal do Rio de Janeiro, Porifera collection
Val	Valine
ZMB	Zoologisches Museum Berlin

10. Supplement

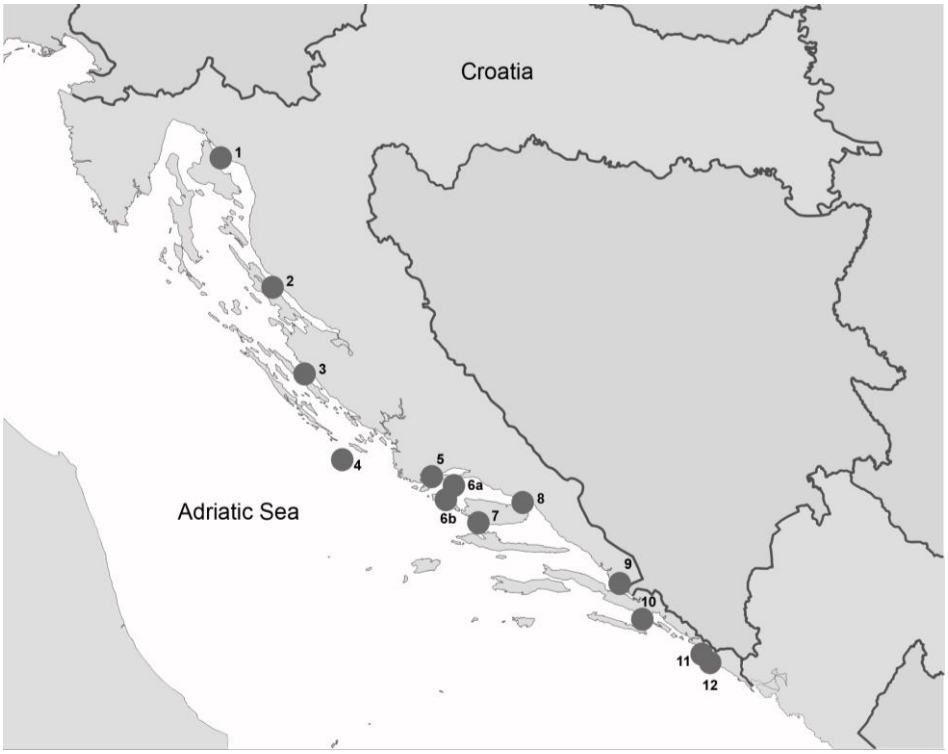

Figure S1. Map of the Croatian coast. Studied locations along the coast are marked with gray circles and numbers. 1) near Selce; 2) Island of Pag; 3) near Zadar; 4) Island of Blitvenica; 5) near Split; 6a) and 6b) Island of Čiovo; 7) Island of Brač; 8) Vrulja Cove; 9) Port of Ploče; 10) Prapratno Cove; 11) near Dubrovnik; 12) Island of Lokrum.

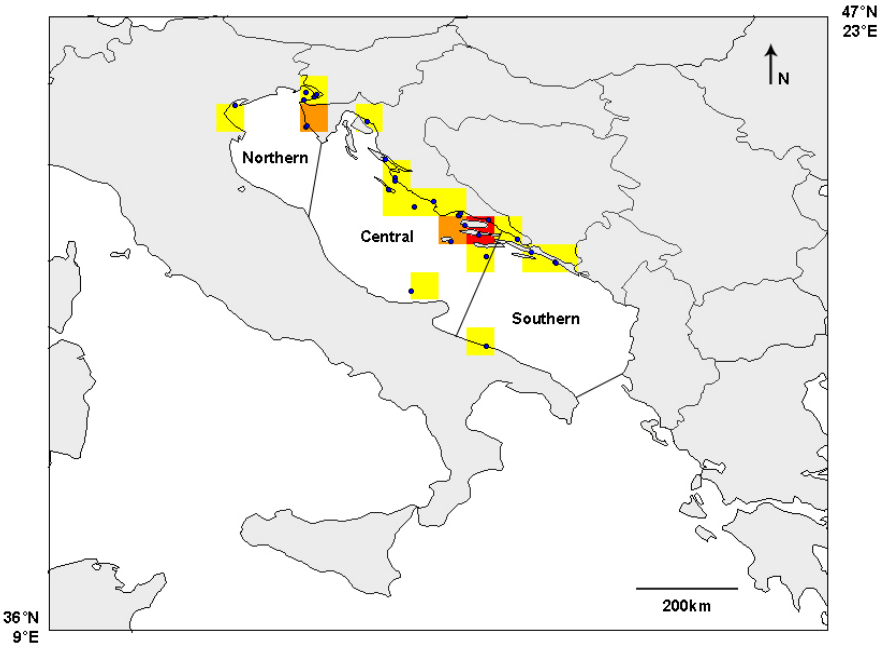

Figure S2. Species richness in the Adriatic Sea divided by sectors. Yellow: 1-9 species; orange: 10-17 species; red: 18-26 species.

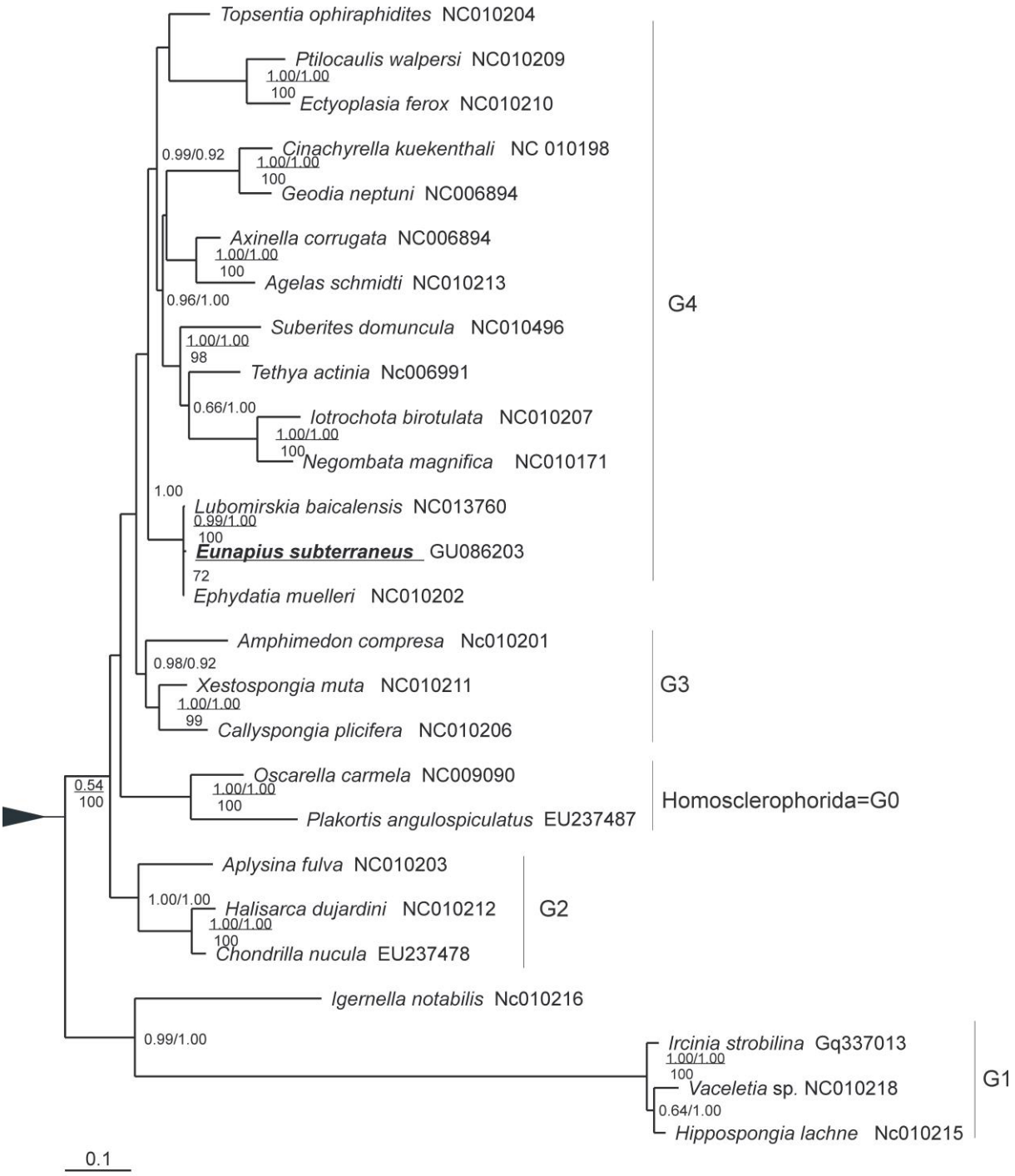

Figure S3. Phylogenetic position of *Eunapius subterraneus* based on concatenated protein sequences of mitochondrial genes—PhyML-aLRT tree with SH-like branch support/Bayesian PP support (above nodes) and MP bootstrap support (below nodes). Five major clades within Demospongiae (G0–G4) are indicated.

Table S1. Specimens included in the analyses with collection sites, voucher numbers and GenBank accession numbers.

Species	Collection site	Voucher number	GenBank accession number	
CALCINEA			**ITS**	**28S**
Arthuria hirsuta	Cabo Verde	ZMAPOR07061	KC843431	-
Arthuria hirsuta	Cabo Verde	ZMAPOR 07103	KC985143	-
Arthuria spirallata	Peru	MNRJ 13652	KC985140	
Arthuria spirallata	Peru	MNRJ 11414	KC985142	
Ascaltis reticulum	Mediterranean Sea	UFRJPOR6258	HQ588973	-
Ascaltis reticulum	Mediterranean Sea	UFRJPOR6260	HQ588977	-
Ascaltis reticulum	Adriatic Sea	PMR-13739=UFRJPOR 6870	KP740022	KP739
Ascandra contorta	Mediterranean Sea	UFRJPOR 6327	HQ588970	-
Ascandra corallicola	Norway	UFRJPOR 6329	HQ588994	-
Ascandra falcata	Mediterranean Sea	UFRJPOR 5856	HQ588962	-
Ascandra falcata	Mediterranean Sea	UFRJPOR 6320	HQ588963	-
Ascandra spalatensis sp. nov.	Adriatic Sea	PMR-17806=UFRJPOR 7540	KP740024	KP740003
Borojevia aspina	Brazil	UFRJPOR 5211	HQ588969	-
Borojevia aspina	Brazil	UFRJPOR 5245	HQ588998	-
Borojevia brasiliensis	Brazil	UFRJPOR 5214	HQ588978	-
Borojevia brasiliensis	Brazil	UFRJPOR 5230	HQ588999	-
Borojevia cerebrum	Mediterranean Sea	UFRJPOR 6322	HQ588964	-
Borojevia cerebrum	Mediterranean Sea	UFRJPOR 6323	HQ588971	-
Borojevia cerebrum	Mediterranean Sea	UFRJPOR 6324	HQ588975	-
Borojevia cerebrum	Adriatic Sea	IRB-CLB26	KP740029	KP740008
Borojevia cerebrum	Adriatic Sea	IRB-CLB32	KP740031	KP740010
Borojevia cerebrum	Adriatic Sea	PMR-17808	KP740030	KP740009
Borojevia cerebrum	Adriatic Sea	IRB-CLB33= UFRJPOR 7539	KP740032	KP740011
Borojevia cerebrum	Adriatic Sea	PMR-13740=UFRJPOR 6864	KP740020	KP739995
Borojevia croatica sp. nov.	Adriatic Sea	PMR-13741=UFRJPOR 6865	KP740021	KP739997
Borojevia croatica sp. nov.	Adriatic Sea	IRB-CLB6	KP740023	KP740002
Borojevia croatica sp. nov.	Adriatic Sea	IRB-CLB17	KP740026	KP740005
Borojevia croatica sp. nov.	Adriatic Sea	IRB-CLB18	KP740027	KP740006
Borojevia croatica sp. nov.	Adriatic Sea	IRB-CLB19	KP740028	KP740007
Clathrina adusta	GBR, Wistari Reef	QM G313665	-	JQ272288
Clathrina aphrodita	Peru	MNRJ 14180	KC985137	-
Clathrina aphrodita	Peru	MNRJ 12994	KC985138	-
Clathrina aurea	Brazil	MNRJ 8998	HQ588968	-
Clathrina aurea	Brazil	MNRJ 8990	HQ588958	-
Clathrina antofagastensis	Chile	MNRJ 9289	HQ588985	-
Clathrina antofagastensis	Peru	MNRJ 11294	KF002722	-
Clathrina blanca	Adriatic Sea	PMR-14307	KC479087	KC479085
Clathrina blanca	Adriatic Sea	PMR-13744	KP740017	KP740000
Clathrina clathrus	Mediterranean Sea	UFRJPOR 6315	HQ588974	-
Clathrina clathrus	Mediterranean Sea	UFRJPOR 6325	HQ588965	-
Clathrina clathrus	Mediterranean Sea	UFRJPOR 6326	HQ588972	-

Clathrina clathrus	Adriatic Sea	IRB-CLB12	KP740025	KP740004
Clathrina clathrus	Adriatic Sea	PMR-14308	KC479089	KC479083
Clathrina clathrus	Adriatic Sea	PMR-13745	KP740015	KP740001
Clathrina conifera	Brazil	MNRJ 8997	HQ588957	-
Clathrina conifera	Brazil	MNRJ 8991	HQ588959	-
Clathrina conifera	Adriatic Sea	PMR-13738=UFRJPOR 6869	KP740019	KP739994
Clathrina conifera	Adriatic Sea	PMR-17807	KP740033	KP740012
Clathrina conifera	Adriatic Sea	IRB-S2= UFRJPOR 7541	KP740034	KP740013
Clathrina conifera	Adriatic Sea	IRB-S3= UFRJPOR 7542	KP740035	KP740014
Clathrina coriacea	Norway	UFRJPOR 6330	HQ588986	-
Clathrina fjordica	Chile	MNRJ 8143	HQ588984	-
Clathrina helveola	Australia	QMG313680	HQ588988	AM180987.1
Clathrina hispanica	Mediterranean Sea	UFRJPOR6305	KC843432	-
Clathrina lacunosa	Norway	UFRJPOR 6334	HQ588991	-
Clathrina lacunosa	Norway	UFRJPOR 6335	HQ588992	-
Clathrina luteoculcitella	Australia	QMG313684	-	AM180988.1
Clathrina peruana	Peru	MNRJ 13144	KC985134	-
Clathrina peruana	Peru	MNRJ 12839	KC985135	-
Clathrina primordialis	Adriatic Sea	PMR-14305	KC479086	KC479084
Clathrina primordialis	Adriatic Sea	IRB-CLB3= UFRJPOR 6863	KP740016	KP739996
Clathrina ramosa	Chile	MNRJ 10313	HQ588990	-
Clathrina rubra	Adriatic Sea	PMR-14306	KC479088	KC479082
Clathrina rubra	Adriatic Sea	IRB-CLC2	KP740018	KP739999
Clathrina wistariensis	Australia	QMG313663	-	AM180990
Lelapiella incrustans	Vanuatu	QM G313914	-	JQ272306
Leucaltis clathria	GBR, DJ's reef	QM G316022	-	JQ272302
Levinella prolifera	GBR, Hook Reef	QM G313818	-	JQ272292
Murrayona phanolepis	Coral Sea, Osprey Reef	QM G313992	-	JQ272304
Murrayona phanolepis	Coral Sea, Bougainville Reef	QM G316290	-	AM180998
Soleneiscus radovani	GBR, Wistari Reef	QM G313661	-	JQ272289
Soleneiscus stolonifer	GBR, Wistari Reef	QM G313668	-	JQ272290
CALCARONEA			**ITS**	**28S**
Grantia compressa	-	-	-	AY563538
Grantiopsis cylindrica	GBR, Lizard Island	GW 973	-	JQ272263
Grantiopsis heroni	GBR, Wistari Reef	QM G313670	-	JQ272261
Leucandra aspera	-	-	-	AY563535
Leucandra falakra sp. nov.	Adriatic Sea	PMR-13748=UFRJPOR 8349	KT447551	KT447560
Leucandra nicolae	-	-	-	JQ272268
Leucandra spinifera sp. nov.	Adriatic Sea	PMR-13742=UFRJPOR 6861	KT447552	KT447562
Leucandra spinifera sp. nov.	Adriatic Sea	IRB-SG3=UFRJPOR 8348	KT447553	KT447561
Leucascandra caveolata	GBR	QM G316057	-	JQ272259
Paraleucilla dalmatica sp. nov.	Adriatic Sea	PMR-13747	KT447556	KT447565
Paraleucilla dalmatica sp. nov.	Adriatic Sea	IRB-SD5=UFRJPOR 8346	KT447557	KT447566
Paraleucilla magna	Brazil	GW 824	-	JQ272267

Paraleucilla magna	South Atlantic	-	-	AM181005
Paraleucilla magna	Adriatic Sea	PMR-13743	KT447554	KT447563
Paraleucilla magna	Adriatic Sea	IRB-P14	KT447555	KT447564
Sycettusa aff. *hastifera*	Red Sea	GW 893	-	JQ272282
Sycettusa cf. *simplex*	Western Indian Ocean	ZMA POR11566	-	JQ272279
Sycettusa tenuis	GBR, Heron Reef	QM G313685	-	JQ272281
Sycon ancora sp. nov.	Adriatic Sea	PMR-17809=UFRJPOR 8345	KT447558	KT447567
Sycon ancora sp. nov.	Adriatic Sea	IRB-SD12=UFRJPOR 8347	KT447559	KT447568
Sycon capricorn	-	QM G316025	AJ633889	-
Sycon capricorn	GBR, Ribbon Reef	QM G316187	-	JQ272272
Sycon carteri	Australia	SAM PS 0142	-	JQ272260
Sycon ciliatum	-	-	AJ627187	AY563532
Sycon raphanus	-	-	-	AY563537
Syconessa panicula	GBR, Wistari Reef	QM G313671	-	JQ272276
Syconessa panicula	GBR, Wistari Reef	QM G313672	-	AM181007
Teichonopsis labyrinthica	Australia	SAM PS 0228	-	JQ272264
Ute ampullacea	GBR, Wistari Reef	QM G313669	-	JQ272266
Ute aff. *syconoides*	GBR, Yonge Reef	QM G313694	-	JQ272271
Ute aff. *syconoides*	Tasmania	QM G323233	-	JQ272269

Table S2. Calcarean species reported from the Adriatic Sea and their distribution. *Type locality. **Probably *Clathrina conifera* or *C. primordialis*.

Calcinea	Longitude	Latitude	Locality	Source
Ascaltis reticulum (Schmidt, 1862)	15.22	44.10	Zadar = Zara*	Schmidt, 1862
Ascaltis reticulum (Schmidt, 1862)	15.92	43.73	Šibenik = Sebenico*	Schmidt, 1862
Ascaltis reticulum (Schmidt, 1862)	16.73	43.13	Hvar = Lesina	Schmidt, 1862 (according to Haeckel, 1872); Heller, 1864 (*apud* Haeckel, 1872); Haeckel, 1872
Ascaltis reticulum (Schmidt, 1862)	13.63	45.08	Rovinj	Longo & Pronzato, 2011
Ascaltis reticulum (Schmidt, 1862)	16.36	43.48	Island of Čiovo	Present work
Ascandra contorta Bowerbank, 1866 (*sensu* Minchin, 1905)	15.5	42.12	Tremiti Island	Lendenfeld, 1891 (as *Ascetta spinosa*); Sarà, 1961
Ascandra contorta Bowerbank, 1866 (*sensu* Minchin, 1905)	13.80	45.63	Trieste	Lendenfeld, 1891 (as *Ascetta spinosa*)
Ascandra contorta Bowerbank, 1866 (*sensu* Minchin, 1905)	13.77	45.60	Muggio	Lendenfeld, 1891 (as *Ascetta spinosa*)
Ascandra contorta Bowerbank, 1866 (*sensu* Minchin, 1905)	16.22	43.01	Island of Vis = Lissa	Lendenfeld, 1891 (as *Ascetta spinosa*)
Ascandra contorta Bowerbank, 1866 (*sensu* Minchin, 1905)	16.73	43.13	Hvar = Lesina	Lendenfeld, 1891 (as *Homandra falcata*)
Ascandra falcata Haeckel, 1872	13.63	45.08	Rovinj	Longo & Pronzato, 2011
Ascandra falcata Haeckel, 1872	13.80	45.63	Trieste	Lendenfeld, 1891
Ascandra falcata Haeckel,	16.73	43.13	Hvar = Lesina*	Haeckel, 1872; Lendenfeld,

1872				1891 (as *Homandra falcata*)
Ascandra falcata Haeckel, 1872	15.50	42.12	Tremiti Island	Sarà, 1961; Longo & Pronzato, 2011
Ascandra spalatensis sp. nov.	15.21	44.14	Zadar = Zara*	Present work
Borojevia cerebrum (Haeckel, 1872)	16.73	43.13	Hvar = Lesina*	Haeckel, 1872; Lendenfeld, 1891; Imešek *et al*., 2014
Borojevia cerebrum (Haeckel, 1872)	16.89	43.40	Vrulja Cove	Present work
Borojevia cerebrum (Haeckel, 1872)	13.63	45.08	Rovinj	Lendenfeld, 1891
Borojevia croatica sp. nov.	16.37	43.48	Island of Čiovo*	Present work
Clathrina blanca (Miklucho-Maclay, 1868)	13.62	45.05	Island of St. Giovanni (near Rovinj)	Imešek *et al*., 2014
Clathrina blanca (Miklucho-Maclay, 1868)	13.63	45.08	Rovinj	Longo & Pronzato, 2011
Clathrina blanca (Miklucho-Maclay, 1868)	16.73	43.13	Lesina=Hvar	Lendenfeld, 1891
Clathrina blanca (Miklucho-Maclay, 1868)	14.72	45.15	Selce	Imešek *et al*., 2014
Clathrina blanca (Miklucho-Maclay, 1868)	16.37	43.48	Island of Ciovo	Imešek *et al*., 2014
Clathrina lacunosa (Johnston, 1842)	13.63	45.08	Rovinj	Lendenfeld, 1891 (as *Ascandra angulata*)
Clathrina lacunosa (Johnston, 1842)	16.73	43.13	Hvar = Lesina	Lendenfeld, 1891 (as *Ascandra angulata*)
Clathrina conifera Klautau & Borojević, 2001	18.1	42.64	Dubrovnik	Present work
Clathrina conifera Klautau & Borojević, 2001	18.11	42.63	Island of Lokrum	Present work
Clathrina clathrus (Schmidt, 1864)	15.92	43.73	Šibenik = Sebenico*	Schmidt, 1864
Clathrina clathrus (Schmidt, 1864)	16.73	43.13	Hvar = Lesina	Schmidt (according to Haeckel, 1872); Heller, 1864 (*apud* Haeckel, 1872); Haeckel, 1872
Clathrina clathrus (Schmidt, 1864)	16.22	43.01	Island of Vis = Lissa*	Heller, 1864 (*apud* Haeckel, 1872)
Clathrina clathrus (Schmidt, 1864)	13.63	45.08	Rovinj	Imešek *et al*., 2014
Clathrina clathrus (Schmidt, 1864)	13.62	45.05	Island of St. Giovanni (near Rovinj)	Present work
Clathrina clathrus (Schmidt, 1864)	16.22	43.01	Island of Vis = Lissa*	Present work
Clathrina clathrus (Schmidt, 1864)	17.68	42.81	Prapratno Cove	Present work
Clathrina clathrus (Schmidt, 1864)	16.36	43.48	Island of Čiovo	Present work
Clathrina coriacea (Montagu, 1814)**	13.63	45.08	Rovinj	Longo & Pronzato, 2011
Clathrina coriacea (Montagu, 1814)**	15.5	42.12	Tremiti Island	Sarà, 1961; Longo & Pronzato, 2011
Clathrina coriacea (Montagu, 1814)**	16.87	41.13	Bari	Longo & Pronzato, 2011
Clathrina primordialis (Haeckel, 1872)	16.73	43.13	Hvar = Lesina*	Schmidt (according to Haeckel, 1872); Haeckel, 1872; Heller, 1864 (*apud* Haeckel, 1872); Lendenfeld, 1891
Clathrina primordialis (Haeckel, 1872)	13.80	45.63	Trieste	Lendenfeld, 1891

Clathrina primordialis (Haeckel, 1872)	13.77	45.60	Muggio	Lendenfeld, 1891
Clathrina primordialis (Haeckel, 1872)	15.22	44.10	Zadar = Zara	Schmidt (according to Haeckel, 1872); Lendenfeld, 1891
Clathrina primordialis (Haeckel, 1872)	15.92	43.73	Šibenik = Sebenico	Schmidt (according to Haeckel, 1872); Lendenfeld, 1891
Clathrina primordialis (Haeckel, 1872)	16.87	42.73	Lastovo = Lagosta	Schmidt (according to Haeckel, 1872); Lendenfeld, 1891
Clathrina primordialis (Haeckel, 1872)	16.22	43.01	Island of Vis = Lissa	Lendenfeld, 1891
Clathrina primordialis (Haeckel, 1872)	13.63	45.08	Rovinj	Lendenfeld, 1891; Imešek *et al*., 2014 as *Clathrina* cf. *hondurensis*)
Clathrina primordialis (Haeckel, 1872)	16.36	43.48	Island of Čiovo	Present work
Clathrina rubra Sarà, 1958	13.63	45.08	Rovinj	Imešek *et al*., 2014
Clathrina rubra Sarà, 1958	16.36	43.48	Island of Čiovo	Present work
Leucetta solida (Schmidt, 1862)	15.92	43.73	Šibenik = Sebenico*	Schmidt, 1862; Lendenfeld, 1891
Leucetta solida (Schmidt, 1862)	16.87	42.73	Lastovo = Lagosta	Schmidt (according to Haeckel, 1872); Lendenfeld, 1891
Leucetta solida (Schmidt, 1862)	16.73	43.13	Hvar = Lesina	Schmidt (according to Haeckel, 1872); Haeckel, 1872; Lendenfeld, 1891
Leucetta solida (Schmidt, 1862)	16.87	42.73	Lastovo = Lagosta (Zaklopatica = Porto Chiave)*	Schmidt, 1864
Leucetta solida (Schmidt, 1862)	15.5	42.12	Tremiti Island	Sarà, 1961; Longo & Pronzato, 2011
Leucetta solida (Schmidt, 1862)	16.87	41.13	Bari	Longo & Pronzato, 2011

Calcaronea	**Longitude**	**Latitude**	**Locality**	**Source**
Amphoriscus chrysalis (Schmidt, 1864)	16.73	43.13	Hvar = Lesina*	Schmidt, 1864; Haeckel, 1872
Amphoriscus chrysalis (Schmidt, 1864)	16.22	43.01	Island of Vis = Lissa*	Schmidt, 1864
Amphoriscus cylindrus (Haeckel, 1872)	16.73	43.13	Hvar = Lesina*	Haeckel, 1872; Lendenfeld, 1891
Amphoriscus gregori (Lendenfeld, 1891)	16.73	43.13	Hvar = Lesina*	Lendenfeld, 1891
Aphroceras corticata (Lendenfeld, 1891)	16.73	43.13	Hvar = Lesina*	Lendenfeld, 1891
Grantia capillosa (Schmidt, 1862)	15.92	43.73	Šibenik = Sebenico*	Schmidt, 1862
Grantia capillosa (Schmidt, 1862)	13.63	45.08	Rovinj	Lendenfeld, 1891
Grantia capillosa (Schmidt, 1862)	13.77	45.60	Muggio	Lendenfeld, 1891
Grantia capillosa (Schmidt, 1862)	13.57	45.53	Pirano	Lendenfeld, 1891
Grantia capillosa (Schmidt, 1862)	16.73	43.13	Hvar = Lesina	Schmidt, 1864; Haeckel, 1872; Lendenfeld, 1891
Grantia capillosa (Schmidt, 1862)	15.92	43.73	Šibenik = Sebenico	Lendenfeld, 1891
Leucandra aspera (Schmidt,	15.22	44.10	Zadar = Zara*	Schmidt, 1862; Lendenfeld,

1862)				1891
Leucandra aspera (Schmidt, 1862)	15.50	42.12	Tremiti Island	Sarà, 1961; Longo & Pronzato, 2011
Leucandra aspera (Schmidt, 1862)	16.22	43.01	Island of Vis = Lissa*	Heller, 1864 (*apud* Haeckel, 1872); Lendenfeld, 1891
Leucandra aspera (Schmidt, 1862)	15.92	43.73	Šibenik = Sebenico	Schmidt, 1862; Lendenfeld, 1891
Leucandra aspera (Schmidt, 1862)	16.87	42.73	Lastovo = Lagosta	Schmidt, 1862; Lendenfeld, 1891
Leucandra aspera (Schmidt, 1862)	16.73	43.13	Hvar = Lesina	Schmidt, 1862; Haeckel, 1872; Lendenfeld, 1891
Leucandra falakra sp. nov.	15.57	43.63	Blitvenica	Present work
Leucandra spinifera sp. nov.	16.36	43.48	Island of Čiovo	Present work
Leucandra spinifera sp. nov.	16.89	43.40	Vrulja Cove	Present work
Leucosolenia goethei Haeckel, 1870	13.63	45.08	Rovinj	Lendenfeld, 1891
Leucosolenia variabilis Haeckel, 1870	13.63	45.08	Rovinj	Longo & Pronzato, 2011
Paraleucilla dalmatica sp. nov.	16.36	43.48	Island of Čiovo	Present work
Paraleucilla magna Klautau, Monteiro & Borojević, 2004	17.43	43.05	Port of Ploče	Cvitković *et al.*, 2013
Paraleucilla magna Klautau, Monteiro & Borojević, 2004	16.39	43.51	Port of Ploče	Present work
Paraleucilla magna Klautau, Monteiro & Borojević, 2004	16.48	43.29	Island of Brač	Present work
Polejaevia telum (Lendenfeld, 1891)	16.73	43.13	Hvar = Lesina	Lendenfeld, 1891
Sycantha tenella Lendenfeld, 1891	13.8	45.63	Trieste	Lendenfeld, 1891
Sycetta conifera (Haeckel, 1872)	16.73	43.13	Hvar = Lesina	Haeckel, 1872; Lendenfeld, 1891
Sycon ancora sp. nov.	15.04	44.48	Island of Pag	Present work
Sycon ancora sp. nov.	16.39	43.51	Split	Present work
Sycon elegans (Bowerbank, 1845)	15.50	42.12	Tremiti Island	Sarà, 1961
Sycon helleri (Lendenfeld, 1891)	16.73	43.13	Hvar = Lesina	Lendenfeld, 1891
Sycon humboldti Risso, 1826	42.75	16.87	Lastovo = Lagosta	Schmidt, 1862
Sycon humboldti Risso, 1826	16.22	43.01	Island of Vis = Lissa	Heller, 1864 (*apud* Haeckel, 1872); Schmidt, 1862
Sycon humboldti Risso, 1826	16.73	43.13	Hvar = Lesina	Schmidt, 1862; Haeckel, 1872
Sycon humboldti Risso, 1826	12.34	45.44	Venice	Martens, 1824 (*apud* Haeckel, 1872)
Sycon humboldti Risso, 1826	15.10	43.93	Dugi otok = Isola Grossa	Martens, 1824 (*apud* Haeckel, 1872)
Sycon quadrangulatum (Schmidt, 1868)	13.61	45.68	Dalmatia, Gulf of Trieste	Schmidt, 1868
Sycon quadrangulatum (Schmidt, 1868)	16.73	43.13	Hvar = Lesina	Haeckel, 1872
Sycon quadrangulatum (Schmidt, 1868)	16.22	43.01	Island of Vis = Lissa	Heller, 1864 (*apud* Haeckel, 1872)
Sycon raphanus Schmidt, 1862	13.63	45.08	Rovinj	Longo & Pronzato, 2011

Sycon raphanus Schmidt, 1862	16.73	43.13	Hvar = Lesina	Schmidt, 1862; Haeckel, 1872
Sycon raphanus Schmidt, 1862	16.87	41.13	Bari	Longo & Pronzato, 2011
Sycon raphanus Schmidt, 1862	13.8	45.63	Trieste	Lieberkühn, 1859 (*apud* Haeckel, 1872); Schmidt, 1862 (as *S. ciliatum*); Haeckel, 1872
Sycon raphanus Schmidt, 1862	15.22	44.10	Zadar = Zara	Schmidt, 1862
Sycon raphanus Schmidt, 1862	15.92	43.73	Šibenik = Sebenico	Schmidt, 1862
Sycon schmidt (Haeckel, 1872)	42.75	16.87	Lastovo = Lagosta	Schmidt, 1862 (*apud* Haeckel, 1872); Haeckel, 1872
Sycon schmidt (Haeckel, 1872)	16.73	43.13	Hvar = Lesina*	Haeckel, 1872
Sycon setosum Schmidt, 1862	16.73	43.13	Hvar = Lesina	Heller, 1864 (*apud* Haeckel, 1872); Haeckel, 1872
Sycon setosum Schmidt, 1862	16.22	43.01	Island of Vis = Lissa	Heller, 1864 (*apud* Haeckel, 1872)
Sycon setosum Schmidt, 1862	13.63	45.08	Rovinj	Longo & Pronzato, 2011
Sycon tuba (Lendenfeld, 1891)	13.8	45.63	Trieste*	Lendenfeld, 1891
Sycyssa huxleyi Haeckel, 1872	16.73	43.13	Hvar = Lesina*	Haeckel, 1872
Ute glabra Schmidt, 1864	16.73	43.13	Hvar = Lesina	Haeckel, 1872; Lendenfeld, 1891
Ute glabra Schmidt, 1864	16.22	43.01	Island of Vis = Lissa	Heller, 1864 (*apud* Haeckel, 1872)
Ute glabra Schmidt, 1864	16.87	42.73	Lastovo = Lagosta (Zaklopatica = Porto Chiave)*	Schmidt, 1864

11. Curriculum vitae

EDUCATION

Date	2007-present
Place	Osijek, Croatia
Institution	University in Osijek, Interdisciplinary doctoral study of Molecular Biosciences

Date	2006
Place	Zagreb, Croatia
Institution	Faculty of Natural Sciences and Mathematics, University of Zagreb, Department of Biology, undergraduate study

WORKING EXPERIENCE

Date (from – until)	2008-present
Institution	Ruđer Bošković Institute
Position	Expert associate
Date (from – until)	2006-2007
Institution	Neodidacta publisher
Position	Textbook author

PROFESSIONAL TRAINING

Year	2013
Place	Heidelberg, Germany
Institution	EMBL Genomic Core Facility
Subject and skills covered	Training in high-throughput sequencing and library preparation

Year	2013
Place	Zagreb, Croatia
Institution	School of Medicine, University of Zagreb
Subject and skills covered	Illumina Next Generation Sequencing seminar

Year	2011
Place	Zagreb, Croatia
Institution	Faculty of Natural Sciences and Mathematics, University of Zagreb
Subject and skills covered	Introduction to Bioinformatics-practical course and EBI Bioinformatics Roadshow

Year	2010
Place	Split, Croatia
Institution	Institute of Oceanography and Fisheries
Subject and skills covered	Phylogenetic Reconstruction Workshop

PROJECT COLLABORATIONS

2015–present, Croatian Science foundation project "Elucidating animal development, differentiation and evolution through basal metazoan genomics" (project leader: dr.sc. K. Vlahoviček)

2013-present, UKF project "Protein ADP-ribosylation in a model prokaryote *Streptomyces coelicolor* and human" (project leader: dr.sc. I. Ahel)

2007-2013, "Genes and genomes: structure, function and evolution", Ministry of Science, Technology and Sports, Republic of Croatia (project leader: dr.sc. H. Ćetković)

PUBLICATIONS

Klautau M[*a], **Imešek M**[*a], Azevedo F, Pleše B, Nikolić V, Ćetković H (2016) Adriatic calcarean sponges (Porifera, Calcarea) with description of six new species and richness analysis. *European Journal of Taxonomy* 178; 1-52. (a- equally contributed)

Perina D, Korolija M, Popović Hadžija M, Grbeša I, Belužić R, **Imešek M**, Morrow C, Marjanović Posavec M, Bakran-Petricioli T, Mikoč A, Ćetković H (2015) Functional and Structural Characterization of FAU Gene/Protein from Marine Sponge *Suberites domuncula*. *Marine drugs* 13, 7; 4179-4196.

Imešek M[*], Pleše B, Pfannkuchen M, Godrijan J, Marić Pfannkuchen D, Klautau M, Ćetković H (2014) Integrative taxonomy of four *Clathrina* species of the Adriatic Sea, with the first formal description of *Clathrina rubra* Sarà, 1958. *Organisms Diversity and Evolution* 14, 1; 1-9.

Imešek M, Pleše B, Lukić-Bilela L, Lelo S, Ćetković, H (2013) Mitochondrial genomes of the genus *Ephydatia* Lamouroux, 1816: can palindromic elements be used in species-level studies? *Organisms diversity & evolution* 13, 2; 127-134.

Godrijan J, Marić D, **Imešek M**, Janeković I, Schweikert M, Pfannkuchen M (2012) Diversity, occurrence, and habitats of the diatom genus *Bacteriastrum* (Bacillariophyta) in the northern Adriatic Sea, with the description of *B. jadranum* sp. nov.. *Botanica marina* 55, 4; 415-426.

Perina D, Mikoč A, Harcet M, **Imešek M**, Sladojević D, Brcko A, Ćetković H. (2012) Characterization of Bruton's Tyrosine Kinase Gene and Protein from Marine Sponge *Suberites domuncula*. *Croatica chemica acta* 85, 2; 223-229.

Perina D, Korolija M, Mikoč A, Roller M, Pleše B, **Imešek M**, Morrow C, Batel R, Ćetković H (2012) Structural and functional characterization of ribosomal protein gene introns in sponges. PLoS ONE 7(8):e42523.

Pleše B, Lukić-Bilela L, Bruvo-Mađarić B, Harcet M., **Imešek M**, Bilandžija H, Ćetković H (2011) The mitochondrial genome of stygobitic sponge *Eunapius subterraneus*: mtDNA is highly conserved in freshwater sponges. *Hydrobiologia* doi:10.1007/s10750-011-0789-y.

Vlašić I, Ivančić-Baće I, **Imešek M**, Mihaljević B, Brčić-Kostić K (2008) RecJ nuclease is required for SOS induction after introduction of a double-strand break in a RecA loading deficient recB mutant of *Escherichia coli*. *Biochimie* 90, 9; 1347-1355.

HOME AND INTERNATIONAL CONFERENCES

2015 12th Croatian Biological Congress (Sv. Martin na Muri, Croatia) Perina, Dragutin; Korolija, Marina; Popović Hadžija, Marijana; Grbeša, Ivana; Belužić, Robert; **Imešek, Mirna**; Morrow, Christine; Marjanović Posavec, Melanija; Bakran-Petricioli, Tatjana; Mikoč, Andreja; Ćetković, Helena "Functional and Structural Characterization of FAU Gene/Protein from Marine Sponge *Suberites domuncula*"

2014 HDBMB 2014 (Zadar, Croatia), poster presentation: Lalić, Jasna; Mikoč, Andreja; Perina, Dragutin; Sabljić, Igor; Pleše, Bruna; **Imešek, Mirna**; Ćetković, Helena; Luić, Marija; Žaja, Roko; Ahel, Ivan "Macrodomain protein from bacterium *Streptomyces coelicolor*"

2013 9th World Sponge Conference (Freemantle, Australia), poster presentation: Roller, Maša; **Imešek, Mirna**; Franke, Vedran; Harcet, Matija; Wörheide, Gert; Hill, April; Ćetković, Helena, Leys, Sally; Vlahoviček, Kristijan "Sequencing of the *Ephydatia muelleri* genome: preliminary results"

2013 9th World Sponge Conference (Freemantle, Australia), poster presentation: **Imešek, Mirna**; Klautau, Michelle; Pleše, Bruna; Nikolić, Vedran; Roller, Maša; Ćetković, Helena "Calcareous sponges from the Adriatic Sea" 2012 FEBS3+Meeting From molecules to life and back (Opatija, Croatia), poster presentation: **Imešek, Mirna**; Pleše Bruna; Klautau, Michelle; Ćetković, Helena "Taxonomy and diversity of the calcareous sponges in the Adriatic Sea"

2011 46th EMBS European Marine Biology Symposium (Rovinj, Croatia), poster presentation: **Imešek, Mirna**; Pleše, Bruna; Pfannkuchen, Martin; Godrijan, Jelena; Marić, Daniela; Ćetković, Helena "Sponges of the genus *Clathrina* from the northeastern Adriatic"

2010 20th International Conference on Subterranean Biology (Postojna, Slovenia), oral presentation: Lukić-Bilela, Lada; Pleše, Bruna; Bruvo-Mađarić, Branka; **Imešek, Mirna**; Bilandžija, Helena; Ćetković, Helena "The mitochondrial genome analysis of unique cave dweller sponge *Eunapius subterraneus* Sket & Velikonja, 1984"

2010 SMBE 2010, Annual meeting of the Society for Molecular Biology and Evolution (Lyon, France), poster presentation: Pleše, Bruna; Harcet, Matija; Bilandžija, Helena; Bruvo-Mađarić, Branka; **Imešek, Mirna**; Lukić-Bilela, Lada; Ćetković, Helena "Insights into evolution of freshwater sponges: molecular life of stygobitic sponge *Eunapius subterraneus*"

2008 50 Years of Molecular Biology in Croatia (Zagreb, Croatia), poster presentation: Mikoč, Andreja; Ahel, Ivan; **Imešek, Mirna**; Ćetković, Helena "Protein RecX from bacteria *Streptomyces rimosus.*"

2008 50 Years of Molecular Biology in Croatia (Zagreb, Croatia), oral presentation: Vlašić, Ignacija; Ivančić-Baće, Ivana; **Imešek, Mirna**; Mihaljević, Boris; Brčić-Kostić, Krunoslav "The role of recJ nuclease in the induction of the SOS response after introduction of the double-strand breaks in *Escherichia coli recB1080* mutant strain"

2006 Ninth international summer school on Biophysics, (Zagreb, Croatia), poster presentation: Ivančić-Baće, Ivana; Vlašić, Ignacija; Mihaljević, Boris; **Imešek, Mirna**; Salaj-Šmic, Erika; Brčić-Kostić, Krunoslav "The SOS response signalling mechanism: possible involvement of RecA loading activity"

2005 Second Congress of Croatian Geneticists with International Participation, (Zagreb, Croatia), poster presentation: Ivančić-Baće, Ivana; Vlašić, Ignacija; Mihaljević, Boris; **Imešek, Mirna**; Salaj-Šmic, Erika; Brčić-Kostić, Krunoslav "The SOS response signalling mechanism: possible involvement of RecA loading activity"

2004 HDBMB 2004, (Zagreb, Croatia), poster presentation: Ivančić-Baće, Ivana; Vlašić, Ignacija; Mihaljević, Boris; **Imešek, Mirna**; Salaj-Šmic, Erika; Brčić-Kostić, Krunoslav "The SOS response signaling mechanism: possible involvement of RecA loading activity"