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The molecular evolution of the cave animals

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Molekularna evolucija špiljskih životinja

Helena Bilandžija

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Kratki sažetak doktorskog rada:
Albinizam, najčešća prilagodba na život u podzemlju, evoluirao je promjenom na istom koraku sinteze melanina kod različitih špiljskih životinja što ukazuje na konvergentne i paralelne molekularne mehanizme adaptacija na život u špiljama. Posljedica istih adaptacija jest slična morfologija i molekularne su metode nužne za razumijevanje filogenetskih odnosa špiljskih životinja, a nude i uvid u zagonetku velike bioraznolikosti podzemlja Dinarida.

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The molecular evolution of the cave animals

Helena Bilandžija

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Short abstract:
Albinism, the most common adaptation to subterranean lifestyle, has evolved by a change at the same step of melanin synthesis in diverse cave animals revealing that molecular convergence underlies the adaptations to life in caves. Identical adaptations lead to similar morphologies and molecular tools are necessary for understanding the phylogenetic relationships of cave animals and also offer insights into the causes of rich biodiversity in Dinaric caves.

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1. INTRODUCTION

The karts landscape and subterranean biodiversity of the Dinaric Mountain chain

Dinarides are 650 km long and 150 kilometers wide mountain chain that spreads along the eastern Adriatic coast. Dinarides include the small easternmost piece of Italy, big parts of Slovenia, Croatia, Bosnia and Herzegovina, Montenegro and a small part of Albania, Kosovo and Serbia. It is almost entirely comprised of limestone rocks. The intensively karstified landscape of Dinarides is known as the classical karst and international names of various karstic features originate from local languages. The process of karstification, i.e. dissolution of carbonate rocks by the water, produced numerous karstic forms and features: karst poljes, sinking rivers, dolines, uvalas, kamenitzas, karren, vruljas, but also a vast underground system of voids, fissures and chambers, only partly accessible to humans through caves and pits [1].

The specificity of Dinaric Karst extends beyond its geology. Namely, the Dinaric karst contains the richest cave fauna of the world [2–4]. This richness can be seen in the number of obligatory cave species, abundance of their populations and also in peculiar faunistical elements. The number of obligatory cave species in the Dinarides is not known since a comprehensive list was never made. Also, the subterranean fauna is seriously unexplored and new species are being discovered on a regular basis. The only approximation of number of Dinaric cave species comes from the number of species described from caves in the Dinaric Karst which is 1200 (Bedek et al, unpublished data). In comparison, there is 5000 obligate subterranean taxa described from the whole Europe, however many epikarst and other non-cave taxa are included in that list [5]. Uniquely, the subterranean fauna of the Dinarides encompasses a few peculiar representatives unknown from any other regions. Among others, there are two subspecies of sponges *Eunapius subterraneus subterraneus* Sket & Velikonja 1984 and *Eunapius subterraneus mollisparspanis* Sket & Velikonja 1984; hydrozoan *Velkovrhia enigmatica* Matjašić & Sket 1971, bivalve *Congeria kusceri* Bole 1962, polychaete *Marifugia cavatica*, Absolon & Hrabe 1930 and the only European cave vertebrate – the

olm *Proteus anguinus* Laurenti, 1768 [6]. What are the causes for such a great biodiversity in the underground of the Dinaric karst and why a single sponge, bivalve and a polychaete have successfully colonized caves only here are questions that have puzzled cave biologists for decades.

The characteristics of subterranean habitats

The underground habitats are rather uniform throughout the world and their most conspicuous feature is the lack of light. Microclimate parameters in the caves are fairly constant and day/night as well as annual cyclical fluctuations are lost in the majority of cases. The temperature is roughly the same as the annual average temperature of the region where the cave is situated and the air humidity is very high, close to or exactly 100% saturation. In contrast, the aquatic cave environment is much less constant and greatly influenced by the surface changes. Many caves get periodically flooded and during low water level periods the water can completely evaporate changing its quality, oxygen content and the accessibility of nutrients [7,8].

Direct consequence of the lack of light is the lack of photoautotrophs which form the base of food webs in almost all other habitats. Therefore, the starting point of the food web in underground habitats is disrupted (Figure 1) and the accompanied scarcity of food is what makes caves extreme environments. What is the foundation of food webs in the caves? One alternative is the chemoautotrophs, but to our knowledge this is restricted to just a few very special cases such as Movile cave in Romania [9]. It is possible that this form of ecosystem is much more widespread [10] but largely unknown due to being inaccessible to humans. More common and prevalent situation is that the origin of organic matter is in the surface habitats from where it gets transported to underground in different ways. Nutrients can be transported by percolating water or periodical flooding. In the terrestrial habitats, cave and pit entrances are sites of major organic matter input in the form of fallen leaves, logs or

strayed animals. Also, there are cave inhabitants that are not entirely bound to caves. For example, cave insects from the order Orthoptera feed on the surface. Other animals such as bats use caves just occasionally, for overwintering or raising the young. These animals play an important role of bringing organic matter in the subterranean environments and, in fact, the bat or bird guano is one of the most productive cave habitats [11,12].

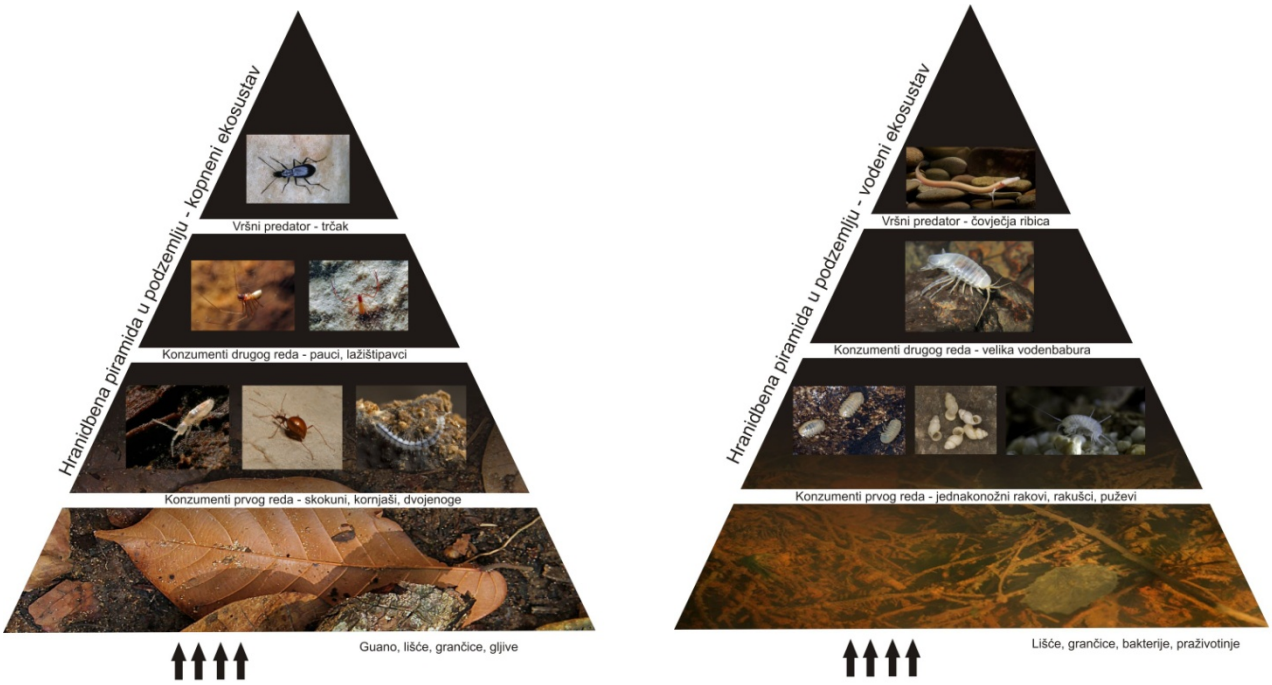

Figure 1. The food pyramids in terrestrial (left) and aquatic (right) underground habitats. Drawn by Petra Bregović, used with permission.

The adaptations of cave fauna

In order to survive in a demanding cave environment, animals had to develop a series of morphological, physiological and ethological modifications collectively called troglomorphic adaptations (Figure 2). Regressive adaptations involve traits that are lost

or regressed in cave dwellers in comparison to surface relatives. The loss of eyes and pigments has evolved in all animal groups that have representatives in the caves, thinning of the cuticle and loss of wings in several groups of terrestrial arthropods and scale and swimming bladder are reduced in fish. Constructive or progressive adaptations are enlarged or modified traits in cave dwellers. The expansion and overdevelopment of non-visual sensory systems as well as elongation of body appendages is present universally in all taxonomic groups inhabiting caves. Some groups such as Collembola and Arachnids have evolved gigantism.

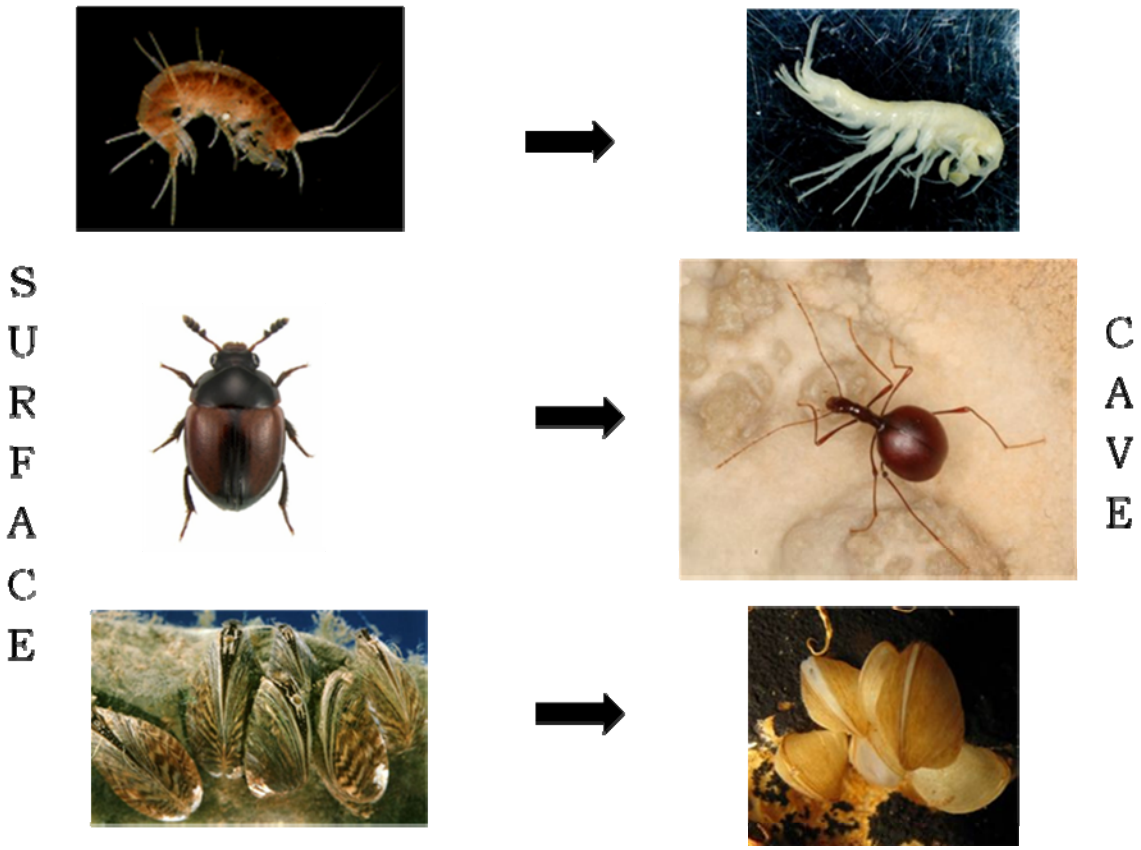

Figure 2. Ancestors of all cave animals were once surface-dwellers. During colonization of the subsurface they acquired numerous adaptations including loss of eyes and pigments, elongation of appendages etc.

Physiological adaptations often seen in cave inhabitants are lowered metabolism, increased starvation resistance and fat deposition, longevity and change from r to K life history traits including reduction in the number of offspring, increase of the size of eggs and their prolonged incubation, extended parental care, slower growth and prolonged first reproduction age. Of the ethological traits, changes in the foraging and feeding behaviors, reduced activity, aggression and excitement as well as the loss of schooling behavior have been registered in some cave inhabitants so far [13–15].

The melanin synthesis pathway

The melanin synthesis pathway (Figure 3) is rather simple, relatively well known and to a great extent conserved throughout the animal kingdom [16]. It starts from the essential amino acid L-tyrosine. In vertebrates the enzyme tyrosinase converts L-tyrosine into L-DOPA during the first step of melanin synthesis and subsequently L-DOPA into L-DOPA-quinone. Through a series of reactions catalyzed by various enzymes (DCT, TYRP1, TYRP2) melanin is formed and deposited in specialized organelles called melanosomes. Several transporter proteins localized within the melanosomal membrane are also essential for the melanin synthesis (OCA2, MATP or SLC24a5) [17].

In insects, the melanin synthesis pathway is slightly different. The first step, conversion of L-tyrosine to L-DOPA, is catalyzed by the enzyme Tyrosine hydroxylase (TH). The pathway subsequently splits into two branches, one, whose product is DOPA-melanin, resembles the vertebrate pathway except that the enzymes are different. The other branch starts with conversion of L-DOPA to dopamine by Dopa decarboxylase (DDC) and results in dopamine-melanin [18,19].

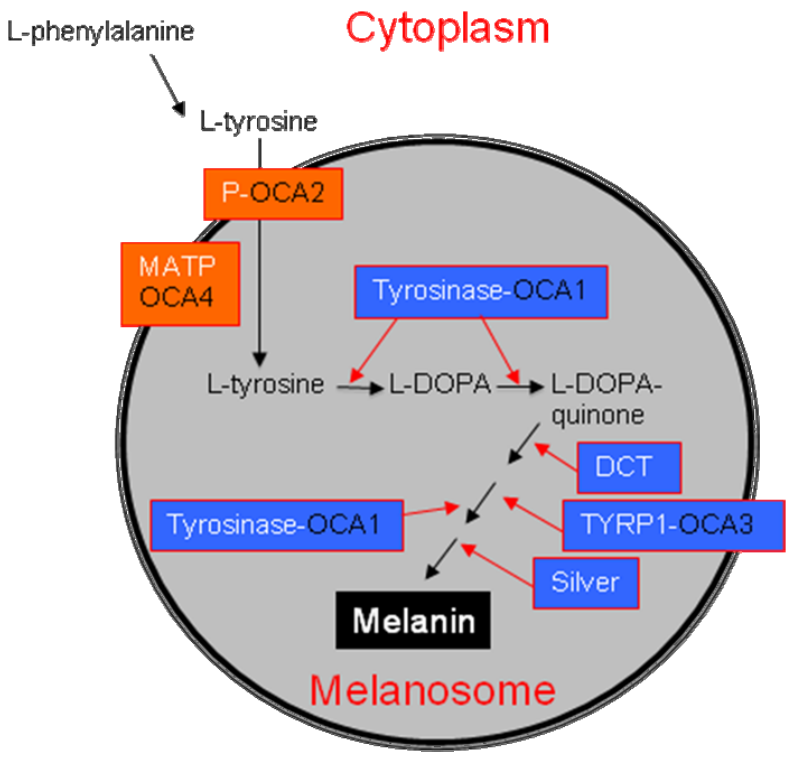

Figure 3. The melanin synthesis pathway begins after transport of L-tyrosine into the melanosome (gray sphere) and involves several enzymes (blue boxes) and other gene products (orange boxes), the latter functioning before the conversion of L-tyrosine to L-DOPA. There are several types of oculocutaneous albinism in humans (OCA1-OCA4). Each type affects different part of the melanin synthesis pathway, and is denoted next to its corresponding gene product.

The loss of melanin pigment in cave animals

Albinism, the reduction or loss of melanin pigment, is one of the most prominent cave adaptations and has evolved repeatedly in all the groups that have successfully colonized subterranean habitats. The function of melanin in protection against damaging UV irradiation is not needed in the ever-dark caves. Also, all the melanin functions related to visual signals and behaviors such as protection from the predators or attraction of a mate are useless in the conditions of constant darkness. Therefore,

natural selection that eliminates albinos accounting for their rare appearance in surface habitats can be relaxed in caves.

Despite the widespread occurrence of albinism in the cave adapted animals, the molecular mechanism underlying the loss of melanin is known for just one cave species, the Mexican tetra *Astyanax mexicanus* (De Filippi, 1853). Using QTL analyses Protas et al. [20] identified a mutation in a single gene, *Pink-eyed dilution* (*P* or *oca2*) to be responsible for the albino phenotype. The gene is coding for a 12 trans-membrane transporter whose function is still dubious. It is thought it may be involved in L-tyrosine transport [21], control of melanosomal pH [22] or tyrosinase processing [23]. Interestingly, fish from several different cave populations have different loss of function deletions in *oca2* meaning that their albino phenotype evolved independently and in parallel [20].

Just one other investigation of molecular basis for the albinism in cave animals was preformed on the cave isopod *Asellus aquaticus* (Linnaeus, 1758). QTL analysis identified that albinism in this animal is a recessive trait caused by mutations in either one or two gene loci [24].

The use of molecular tools in cave biodiversity research

The molecular studies of various cave taxa have identified many unexpected patterns and are often in contrast with the systematic positions of these animals as conceived at the time. A few examples that illustrate this problem come from various Dinaric cave taxa. The phylogenetic study of *Proteus anguinus* [25] as well as *Troglocaris* Dormitzer, 1853 and *Niphargus* Schiödte 1849, identified several cryptic phyletic lineages in each animal. The phylogenetic study of cave shrimp identified two deeply divided lineages in the Dinaric Karst, one even being more closely related to Caucasus cave shrimps then to the other lineage from the Dinarides [26]. The phylogeographical studies of *Asellus aquaticus* revealed unexpected relationships among cave and surface morphs from

different basins. The hydrology shapes and drives the speciation of different waterlouse lineages and the same morphologies seen in cave forms from different basins were all acquired in parallel [27]. Molecular phylogeny of genus *Niphargus*, the most specious genus of all Dinaric cave taxa, identified a complete misconception of traditional systematic based on morphology [28]. Namely, species that have the same or similar morphologies do not form a single clade but are scattered around the phylogenetic tree. This is true for all morphological types identified by the classical taxonomy and means that all these morphologies have evolved independently and in parallel. The molecular tools are being increasingly used in the studies of subterranean biodiversity and they are often essential in correctly assessing phylogenetic positions and relationships of many cave taxa.

Model organisms

Several different model organisms were used in this study. The cave sponge, *Eunapius subterraneus* (Figure 4) is the only subterranean sponge in the world. It is a stenoendemic species known from caves and springs of the Mrežnica and Dobra basins in the wider region of town Ogulin [29,30]. Recently it was discovered in the sump at -1400 m below ground at the bottom of Lukina jama-Trojama system in Lika basin. The cave bivalve, *Congeria kusceri* (Figure 5), only cave adapted bivalve in the world [31], is known from altogether 15 localities and exhibits a holodinaric and disjunctive distribution. It inhabits 3 localities in the Lika river basin in Lika region of Croatia, one locality in Kupa river basin in eastern Slovenia, 3 localities in the Sana river Basin in north-western Bosnia and the remaining 8 localities are from the Neretva river basin in southern Croatia and south-eastern Herzegovina. Both of these animals are sessile filter-feeders which is a mode of life found very rarely in caves.

Cixiidae are an insect family distributed worldwide. Their nymphs live underground and feed on leaves and roots. After metamorphosis, winged adults emerge and feed on

leaves of various herbs and trees [32]. There are several cave adapted species, most famous being *Oliarus polyphemus*, Fennah 1973 from the Hawaiian islands in USA. It has been postulated that this species has colonized caves via adaptive shift [33]. In the caves it feeds on the roots of plants that penetrate through the cave ceilings. In Dinarides there are no known cave adapted species but we have found a troglomorphic species (Figure 6) during this research which is currently under taxonomical evaluation and description.

Figure 4. (left) The cave sponge *Eunapius subterraneus* surrounded by *Marifugia cavatica* tubes and gravel. Photo: M. Lukić

Figure 5. (bottom, left) The cave bivalve *Congeria kusceri* surrounded and party overgrown by *Marifugia cavatica.* Photo: B. Jalžić

Figure 6. (below) The un-described species of cixiids from a cave on the Island of Mljet. Photo: H. Bilandžija

The aims of the research

During the process of cave colonization animals had to develop a suite of troglomorphic adaptations that enabled them to survive the inhospitability of subterranean environment. One aim is to explore the molecular basis of loss of melanin pigmentation and gain an understanding of the evolutionary mechanisms that resulted in convergent occurrence of this regressive trait in all cave dwelling animals, from sponges to vertebrates. Due to strong and similar selective forces that act in the underground and result in similar morphologies of cave inhabitants across phyla, molecular tools are necessary to resolve the systematic position and true relationships in cave animals. Second aim is to determine the phylogenetic positions of cave sponges and bivalves, two animals that are single cave dwelling representatives of their phylum and class, respectively. Finally, great biodiversity of the Dinaric Karst is the result of intense colonization. Third aim is to uncover the timeframe as well as sources and causes of Dinaric underground colonization. This can be done by molecular dating of fossil rich groups such as bivalves. Additionally, by comparing the phylogeographical patterns of cave sponges, bivalves and other subterranean aquatic animals the aim is to uncover processes underlying current biogeographical patterns of the subterranean biodiversity of Dinaric karst.

2. MANUSCRIPTS

Evolution of albinism in cave planthoppers by a convergent defect in the first step of melanin biosynthesis

Helena Bilandžija, Helena Ćetković and William R. Jeffery

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SUMMARY

Albinism, the reduction or loss of melanin pigment, is found in many diverse cave-dwelling animals. The mechanisms responsible for loss of melanin pigment are poorly understood. In this study we use a melanogenic substrate assay to determine the position where melanin synthesis is blocked in independently evolved cave planthoppers from Hawaii and Croatia. In this assay, substrates of enzymes responsible for melanin biosynthesis are added to fixed specimens in vitro and their ability to rescue black melanin pigmentation is determined. L-tyrosine, the first substrate in the pathway, did not produce melanin pigment, whereas L-DOPA, the second substrate, restored black pigment. Substrates in combination with enzyme inhibitors were used to test the possibility of additional downstream defects in the pathway. The results showed that downstream reactions leading from L-DOPA and dopamine to DOPA-melanin and dopamine-melanin, the two types of insect melanin, are functional. It is concluded that albinism is caused by a defect in the first step of the melanin synthesis pathway in cave-adapted planthoppers from widely separated parts of the world. However, Western blots indicated that tyrosine hydroxylase (TH), the only enzyme shown to operate at the first step in insects, is present in Hawaiian cave planthoppers. Thus, an unknown factor(s) operating at this step may be important in the evolution of planthopper albinism. In the cavefish *Astyanax mexicanus*, a genetic defect has also been described at the first step of melanin synthesis suggesting convergent evolution of albinism in both cave-adapted insects and teleosts.

INTRODUCTION

Cave-dwelling animals have evolved a suite of regressive phenotypes highlighted by the degeneration of eyes and pigmentation (Porter and Crandall 2003; Culver and Pipan 2009; Juan et al. 2010). Pigmentation normally protects organisms from the harmful effects of UV irradiation, reduces the extent of predation through camouflage and mimicry, and facilitates sexual reproduction by mediating colorful behavioral displays (Protas and Patel 2008). In the darkness of caves, however, these functions are subjected to relaxed selection and pigmentation can disappear without deleterious consequences on fitness. The loss of melanin pigment, which occurs in a wide range of different cave animals (including flatworms, mollusks, crustaceans, insects, and vertebrates), is known as albinism. In addition to cave dwellers, albinism is frequently encountered in diverse animals inhabiting other lightless environments, such as the deep sea, the soil, and in parasites living within the body of their hosts. Albinism is also found in animals living in lighted environments, but it is present at low frequencies in natural populations probably because of deleterious effects on fitness. In all of these situations, the developmental and evolutionary mechanisms underlying albinism are poorly understood.

Melanin is produced in a biochemical pathway whose basic features are conserved across a broad range of different organisms (Riley 1997; Vavricka et al. 2010). This pathway is best characterized in vertebrate melanosomes, the organelles responsible for melanin production (Fig. 1A). The first step in the pathway is the conversion of L-tyrosine into L-3,4-Dihydroxy-L-phenylalanine (L-DOPA), which is subsequently converted through L-DOPAquinone, L-DOPAchrome and a few other intermediates to melanin. The first two steps from L-tyrosine through L-DOPA to L-DOPAquinone are catalyzed by the multifunctional enzyme tyrosinase and the rest by several different enzymes, including the tyrosinase-related proteins and tyrosinase itself.

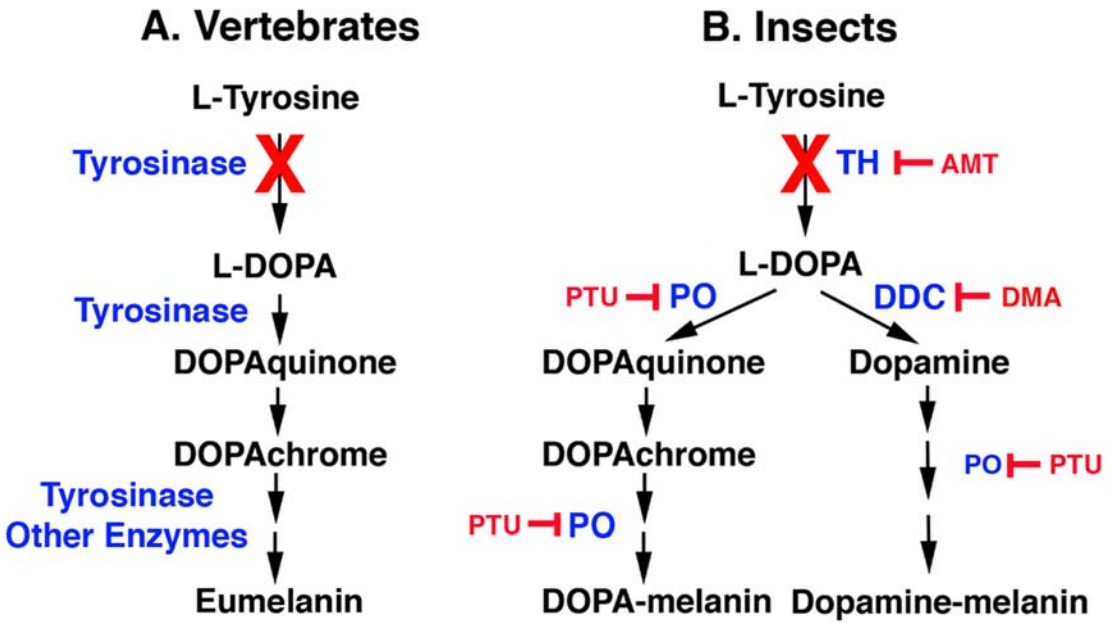

Fig. 1. Melanin synthesis pathways compared in vertebrates and insects. (A) The generalized vertebrate melanin synthesis pathway emphasizing the early steps in which tyrosinase successively converts L-tyrosine to L-DOPA and DOPAquinone and subsequent reactions produce eumelanin. (B) A simplified insect melanin synthesis pathway in which TH converts L-tyrosine to L-DOPA and two subsequent branches in which PO is involved in the conversion of L-DOPA to produce DOPA-melanin and DDC is involved in the conversion of L-DOPA to dopamine to produce dopamine-melanin, both through a series of reactions. Enzymes are shown in blue. Enzyme inhibitors are shown in red. Red Xs illustrate the defective steps in the cavefish and planthopper pathways. Abbreviations are explained in the text. Multiple arrows are not representative of the number of steps in the pathways. The consensus insect melanin synthesis pathway (B) is drawn according to True (2003).

One of the types of human albinisms is known as oculocutaneous albinism (OCA) because of the absence of melanin in both the eyes and skin. Four types of OCA are known: OCA-1 is caused by mutations in the *tyrosinase* gene, OCA-2 and OCA-4 by mutations in the *oca2* and *mapt* genes, respectively, which function upstream of tyrosinase at the beginning of the pathway, and OCA-3 by mutations in the *tyrosinase-related protein-1* gene, which functions downstream of tyrosinase (Oeting and King 1999). The presence of multiple human OCAs implies that the melanin synthesis is vulnerable to change throughout the biosynthetic pathway.

Little is known about what steps of the melanin synthesis pathway have been changed during the evolution of albinism in cave animals with the notable exception of the cavefish *Astyanax mexicanus* (Jeffery 2006). In this species, the first step in melanin production, the conversion of L-tyrosine into L-DOPA, is affected due to loss-of-function mutations in the *oca2* gene (Protas et al. 2006). Therefore, according to the human nomenclature, cavefish would be classified as OCA-2 albinos. The precise function of OCA2 protein is currently unclear but it could possibly control the availability of L-tyrosine for conversion to L-DOPA by tyrosinase. Consistent with its disruption at the first step of the pathway, cavefish melanin pigmentation can be rescued by providing exogenous L-DOPA but not L-tyrosine to specimens in vitro (McCauley et al. 2004). Furthermore, the first step of the pathway has been targeted repeatedly by different *oca2* mutations in several independently evolved *Astyanax* cavefish lineages (McCauley et al. 2004; Protas et al. 2006), suggesting that albinism has evolved multiple times by convergence. The evolution of pigmentation has also been studied in another albino cave animal, the crustacean *Asellus aquaticus* (Protas et al. 2011). In this species, albinism is inherited as a recessive trait caused either by mutation in a single gene locus or in two different gene loci.

The details of the insect melanin synthesis pathway are not as well resolved as in vertebrates and differ from them in several fundamental aspects (Fig. 1B; True 2003). First, tyrosine hydroxylase (TH), rather than tyrosinase, is responsible for the conversion of L-tyrosine to L-DOPA. Second, phenol oxidase (PO) catalyzes the conversion of LDOPA to DOPAquinone and some of the downstream reactions in the pathway. Third, there are two types of insect melanins: L-DOPA-melanin and dopamine-melanin, which are produced by separate branches of the pathway beginning at the level of L-DOPA. In one branch, L-DOPA is converted to L-DOPAquinone by PO, and downstream reactions produce DOPA-melanin. In the other branch, L-DOPA is converted to dopamine by DOPAdecarboxylase (DDC), and subsequent reactions also involving PO activity produce dopamine-melanin. DOPA-melanin and dopamine-melanin

are secreted into the hemolymph or deposited into the cuticle by epidermal cells, where they are responsible for dark pigmentation. In addition to pigmentation, melanin and the enzymes participating in its production have been implicated in cell-mediated immunity (Marmaras and Lampropoulou 2009) and hardening of the cuticle during molting (Sugumaran 1988; Hopkins and Kramer 1992; Gorman and Arakane 2010).

Similar to some vertebrates, insects have become adapted to life in caves, resulting in the reduction or loss of eyes, reduced wings, and albinism in many species (Barr 1968). However, the mechanisms underlying albinism in cave-adapted insects are unknown. In order to shed light on these mechanisms and examine their possible convergence, we have studied melanin synthesis in two independently evolved cixiid planthopper species from lava tubes in Hawaii (Howarth 1972; Fennah 1973) and limestone caves in Croatia. Surface dwelling cixiid planthoppers spend their immature stages as juveniles associated with plant roots in the soil. After the final molt, adults emerge, migrate to the surface, and feed on the aerial portions of the host plant(s). Mature adults of cave adapted Hawaiian planthoppers and possibly the Croatian species as well have undergone an adaptive shift in which they remain associated with roots in caves rather than migrate to the surface (Howarth 1987). The present study describes the convergent evolution of albinism in Hawaiian and Croatian cave planthoppers and compares its underlying causes with those in a distantly related organism, *Astyanax* cavefish.

MATERIALS AND METHODS

Animals

Late instars and adults of the Hawaiian cixiid planthopper *Oliarus polyphemus* were collected by aspiration from *Metrosiderus polymorpha* roots and lava tube walls in Kaumana Cave near Hilo, HI, USA. Animals were maintained in humidified plastic chambers containing roots as food until being assayed. Fourth and fifth instars of an undescribed Croatian cixiid species were collected from roots and under rocks in Male ponte jama and Špilja kod Nerezinog dola on the island of Mljet, Croatia and were maintained in humidified chambers containing coniferous needles prior to the assay. Immature developmental stages of both cave species were classified according to Sforza et al. (1999). Surface-dwelling planthoppers were collected in central Dalmatia, Croatia, *Drosophila melanogaster* (Oregon R strain) was a gift of Dr. Leslie Pick, University of Maryland, and *A. mexicanus* was obtained from the Jeffery Laboratory, University of Maryland.

Melanogenic substrate assays

Melanogenic substrate assays were adapted from those previously developed for the cavefish *A. mexicanus* (McCauley et al. 2004) and several different insect species (Jones and Sinclair 1958; Nijhout 1980; Hiruma et al. 1985; Walter et al. 1996; Fatahashi and Fugiwara 2005). Assays were either conducted in the field immediately after animal collection or after living or fixed specimens were brought to the laboratory. Animals were fixed in 5% formalin dissolved in phosphate buffered saline (PBS) for 1 hr at room temperature. The fixative was removed by five washes in PBS before adding the substrates. The specimens were subsequently immersed in various substrates or combinations of substrates and inhibitors: 0.1% L-tyrosine, 0.1% L-DOPA, 0.1% dopamine hydrochloride, a mixture of 0.1% L-DOPA or 0.1% dopamine and 0.1%

phenylthiourea (PTU), or 1 mM alpha-methyl-DL-tyrosine (AMT) or 1 mM 3-(3, 4-Dihydroxyphenyl)-2-methyl-L-alanine sesquihydrate (DOPA methyl-L-alanine). The substrates and substrate-inhibitor mixtures were dissolved in phosphate buffer (pH 7.4). All substrates and inhibitors were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Assays containing dopamine were conducted in the absence of light. Control specimens were immersed in buffered water (pH 7.4) instead of substrates. The assays were terminated after pigment deposition occurred (2–5 days) by washing specimens in PBS and post fixation in 5% formalin or 4% paraformaldehyde. Some specimens were embedded in polyester wax and serial sectioned at 10 μm.

Protein extraction and Western blots

Protein extracts were prepared by homogenizing animals in RIPA buffer containing a protease inhibitor cocktail (Sigma- Aldrich). The homogenates were cleared by centrifugation, and the supernatants were stored at −20˚C. Protein extracts were subjected to electrophoresis through 10% SDS-polyacrylamide (SDS/PAGE) gels containing prestained protein markers and transferred to PVDF filters (Bio-Rad Laboratories, Hercules, CA) by standard methods (Harlow and Lane 1988). The filters were incubated with 5% nonfat dry milk in TBST buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.1% Tween-20) to block nonspecific binding, then washed three times for 5 min in TBST, incubated overnight in a 1:100 dilution of mouse TH monoclonal antibody (clone LNC1, Millipore, Billerica, MA, USA) in TBST at 4˚C, and finally incubated for 1 hr at room temperature in a 1:25,000 dilution of HRP-conjugated anti-mouse IgG secondary antibody. After three washes for 5 min with TBST, the signals were visualized with Chemiluminescence Luminol (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used according to the supplier's specifications. Images of X-ray films were taken with a digital camera.

RESULTS

Albinism in cave-adapted planthoppers

Surface-dwelling planthoppers are darkly pigmented due to melanin presence throughout their bodies, including in the eyes and wings (Fig. 2Aand B). The extent of pigment regression in the Hawaiian and Croatian planthopper species was determined by microscopy. All of the developmental stages tested, late instars and adults of the Hawaiian species and late instars of the Croatian species, lacked black pigmentation externally, although they showed yellow-tan pigmentation on the dorsal cuticle of the head, thorax, abdomen, and vestigial wings (when present) (Fig. 2B inset, C–H). Serial sectioning showed that there was also no internal melanin pigmentation in either species (data not shown). From this, we conclude that the Hawaiian and Croatian cave planthopper species are albinos lacking any black pigmentation.

L-DOPA produces melanin pigmentation in albino planthoppers

The melanogenic substrate assay was used to determine the position of the defect(s) in melanin synthesis leading to albinism in the Hawaiian and Croatian cave planthoppers. In this assay, different substrates were added to lightly fixed and permeabilized specimens, and the appearance of black pigmentation was determined by microscopy. When late instars and adult planthoppers were incubated in L-DOPA black pigment was formed throughout the body, including the dorsal cuticle, abdomen, legs, the shafts, and surrounding areas of sensory bristles and hairs on the body and wings (when present), and the antennal bases in the head (Fig. 3A–C; Table 1). Controls exposed to the same conditions without L-DOPA did not form black pigment (Table 1). Additional controls were done to determine whether black pigment deposition was due to an enzymatic reaction. First, fixed animals were heated to 65°C prior to the assay to denature proteins and destroy enzyme activity. High temperature abolished the ability

of L-DOPA to rescue pigmentation (Table 1). Second, specimens were incubated with a mixture of L-DOPA and the PO inhibitor PTU. The accumulation of black pigment was eliminated or substantially reduced by PTU (Fig. 3D–F; Table 1). In contrast, incubation of specimens with L-DOPA and the TH inhibitor ADT did not affect black pigment formation (Table 1), consistent with TH function at the step before L-DOPA in the pathway. These results support the conclusion that pigment synthesis was due to an enzymatic reaction.

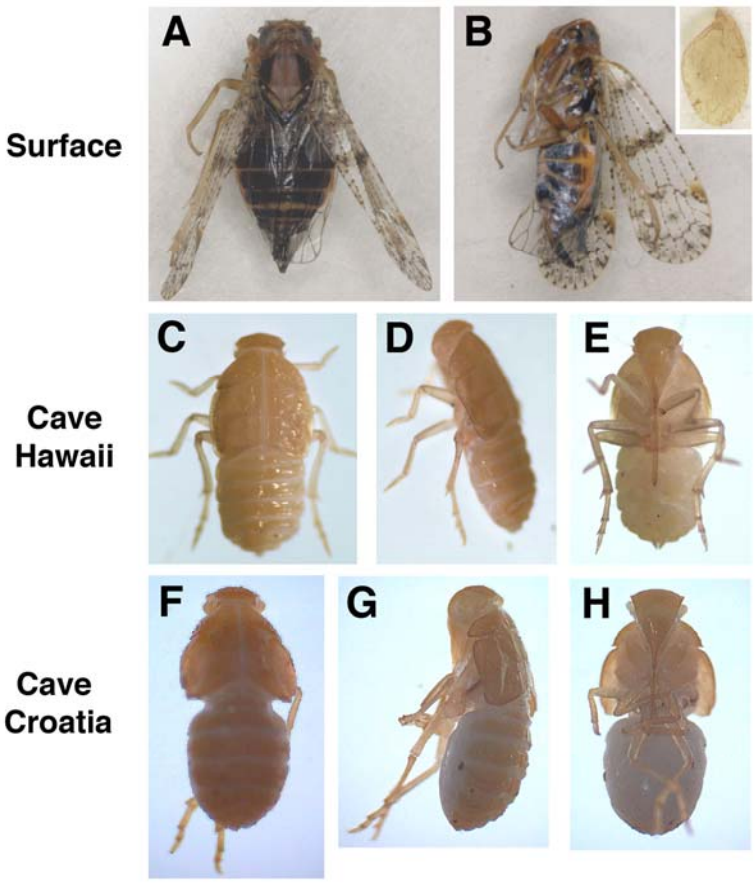

Fig. 2. Pigmentation and albinism in planthoppers. (A, B). A surface-dwelling cixiid planthopper viewed from the dorsal (A) and lateral (B) sides showing melanin pigmentation in the body and wings. Inset in (B) shows the vestigial wing of an albino Hawaiian cave planthopper at the same magnification as the surface planthopper. (C–H). Albino fifth instar Hawaiian (C–E) and Croatian (F–H) cave planthoppers viewed from the dorsal (C, F), lateral (D, G), and ventral (E, H) sides.

Based on the ability of L-DOPA to produce black pigmentation, we conclude that PO and all of the downstream enzymes are present and potentially functional in at least one of the two pathways responsible for melanin production (see below). In addition, these data suggest that the defect in melanin synthesis is upstream of L-DOPA in albino planthoppers of both species, possibly at the step in which L-tyrosine is converted to L-DOPA.

L-tyrosine does not produce melanin pigmentation in albino planthoppers

Hawaiian and Croatian planthoppers were incubated in L-tyrosine to determine whether the melanin synthesis pathway is disrupted at the step between L-tyrosine and L-DOPA. The rationale for conducting these experiments was as follows. Considering the demonstration that L-DOPA incubation produces melanin pigmentation (see above), if L-tyrosine incubation produced melanin production, then the defect would be located upstream of L-tyrosine, whereas if L-tyrosine failed to rescue pigmentation, then the defect would be located between L-tyrosine and L-DOPA. All of the developmental stages we tested in both planthopper species failed to produce black pigment during L-tyrosine incubation (Fig. 3G–I;Table 1).When the same specimens were subsequently incubated in L-DOPA black pigment was formed (Table 1), showing that they were able to produce pigment when incubated with an appropriate substrate. These results indicate that the Hawaiian and Croatian planthopper species are unable to use L-tyrosine to make black pigment. Therefore, it is concluded that melanin synthesis is probably blocked at the step between L-tyrosine and L-DOPA in cave planthoppers.

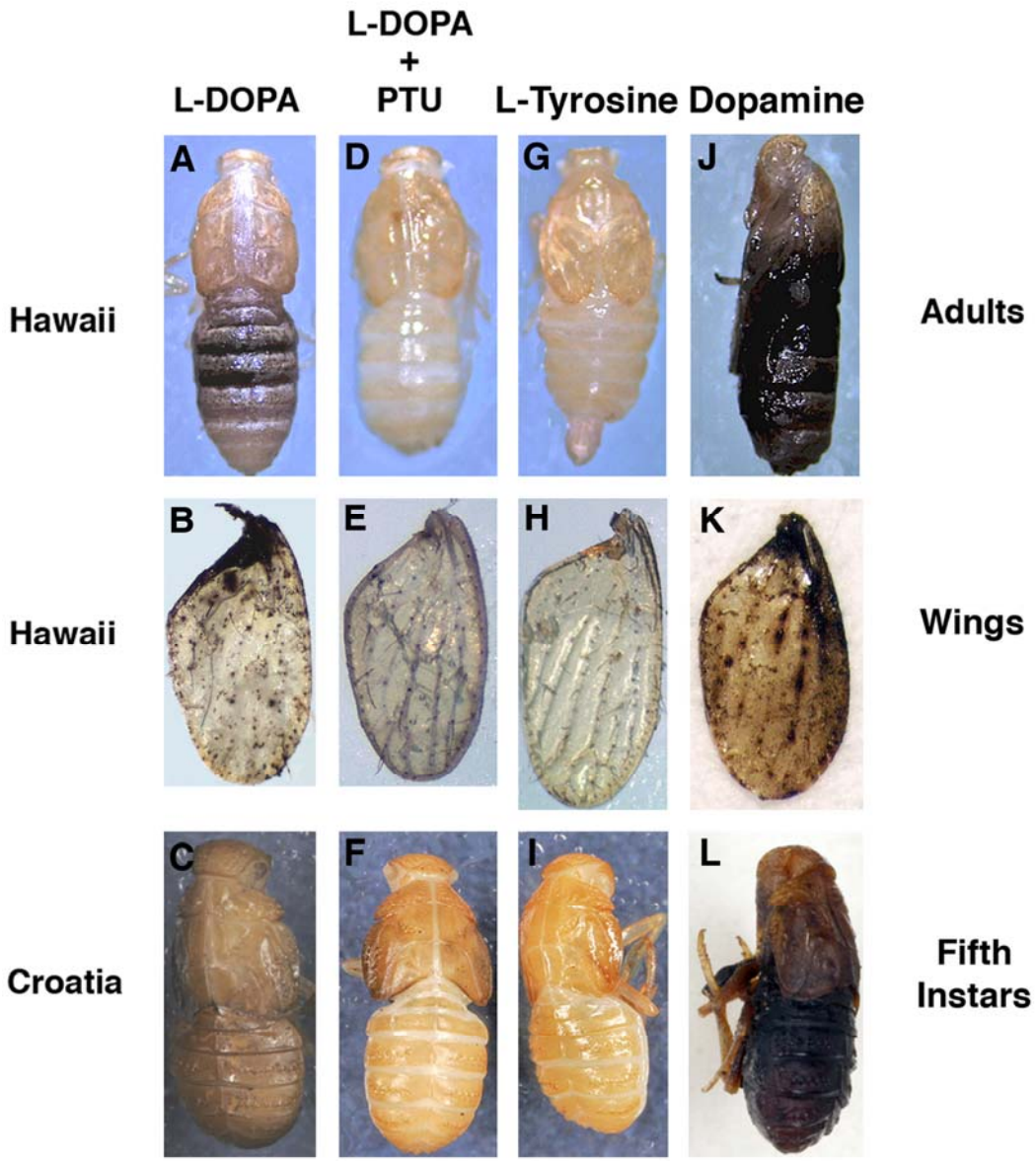

Fig. 3. Melanogenic substrate assays in cave planthoppers after incubation in L-DOPA, L-DOPA and PTU, L-tyrosine, or dopamine. (A–C). Adult Hawaiian cave planthopper (A, B) and fifth instar Croatian (C) cave planthopper showing production of black pigment after incubation in L-DOPA. (D–F). Adult Hawaiian cave planthopper (D, E) and fifth instar Croatian cave planthopper (F) lacking black pigment after incubation in L-DOPA and the PO inhibitor PTU. (G–I). Adult Hawaiian cave planthopper (G, H) and fifth instar Croatian cave planthopper (I) showing no black pigment deposition after incubation in L-tyrosine. (J–L). Adult Hawaiian cave planthopper (J, K) and fifth instar Croatian (L) cave planthopper showing production of black pigment after incubation in dopamine. (B, E, H, K). Wings of Hawaiian cave planthoppers dissected from the bodies after completion of the assays.

Downstream steps in the melanin synthesis pathway are not affected in albino planthoppers

The insect melanin synthesis pathway branches in two directions from the point of L-DOPA production: one branch leads to L-DOPA-melanin and the other to dopamine-melanin (Fig. 1B). As both branches produce dark pigment, our conclusion that melanin synthesis is blocked at the first step does not preclude the possibility of additional defects further downstream from the bifurcation in one of the two branches. The possibility of such downstream defects was examined in two experiments. First, dopamine, the key substrate in the dopamine-melanin branch (Fig. 1B), was used as the substrate, and all treated specimens of both albino planthopper species were able to produce black pigmentation (Fig. 3J–L; Table 1). The ability of dopamine to produce melanin was blocked by the PO inhibitor PTU (Table 1), confirming PO function downstream in the dopamine-melanin branch (Fig. 1B). Second, to examine the functionality of the DOPA-melanin branch pathway, L-DOPA was used as a substrate in combination with the DDC inhibitor DOPA-methyl-L-alanine (DMA). Black pigment was deposited in all specimens in this experiment (Table 1). Because DMA blocks the dopamine-melanin branch of the pathway, this shows that the DOPA-melanin pathway is functional (Table 1). The results demonstrate that the branch pathways leading from dopamine to dopamine-melanin and from L-DOPA to DOPA-melanin have no lesions and can be functional in both species of cave-adapted planthoppers.

Tyrosinase hydroxylase is present in an albino planthopper

TH is the only enzyme known to function between L-tyrosine and L-DOPA in the insect melanin biosynthesis pathway (True 2003). To determine whether TH is present, we subjected protein extracts of Hawaiian cave planthoppers to SDS/PAGE and Western blot analysis with a cross-reacting monoclonal TH antibody (Fig. 4A). As controls, the Western blots also contained *D. melanogaster* (Fig. 4B) and *A. mexicanus* (Fig. 4C)

protein extracts. A protein band of the expected molecular mass (58–62 kDa; Stathakis et al. 1999; Gorman and Arakane 2010) was detected in each species. We conclude that TH is present in the Hawaiian cave planthopper species.

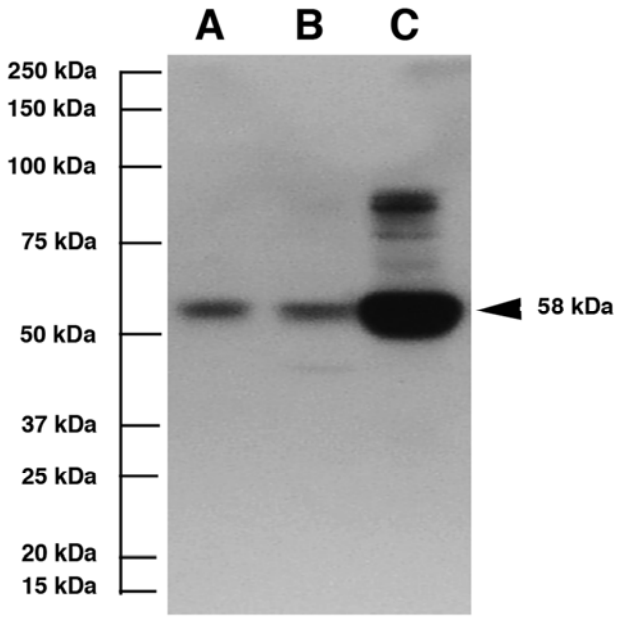

Fig. 4. Western blot of proteins extracted from whole adult animals probed with TH antiserum showing immunoreactive bands of 58–62 kDa in (A) the Hawaiian cave planthopper, (B) *Drosophila melanogaster* and (C) *Astyanax mexicanus*. The molecular mass scale is indicated on the left and 58-kDa band representing *A. mexicanus* TH on the right.

DISCUSSION

The results of this study suggest that independently evolved albino planthoppers living in lava or limestone caves are defective in the first step of the melanin synthesis pathway, the conversion of L-tyrosine to L-DOPA. The evidence for this conclusion is that administration of L-DOPA, the second substrate in the pathway, but not L-tyrosine, the first substrate, can rescue pigmentation in both planthopper species. If the

planthopper pathway was functional at the first step, exogenously supplied L-tyrosine should have been capable of normal conversion to L-DOPA, as shown in in vitro studies of pigmented insect species (Owen and Bouquillon 1992; Fatahashi and Fugiwara 2005). Blockage upstream of L-tyrosine in the pathway beginning with L-phenylalanine is unlikely because of expected effects on protein synthesis. Furthermore, the L-tyrosine to L-DOPA step is apparently the only lesion in the albino planthopper melanin synthesis pathway because the parallel branches from L-DOPA to DOPA-melanin and dopamine to dopamine-melanin can be functional when animals are provided with appropriate substrates. Accordingly, all of the enzymes and other factors downstream of L-DOPA are likely to be conserved and potentially functional in cave planthoppers. The first step of the pathway is also the single lesion in melanin production in independently evolved populations of the cavefish *A. mexicanus* (McCauley et al. 2004; Protas et al. 2006). We conclude that a critical change(s) resulting in the evolution of albinism has occurred at the beginning of melanin synthesis in divergent lineages of cave-adapted insects and vertebrates.

Based on human albinisms (Oeting and King 1999), defective melanin pigmentation can be caused by mutations in the tyrosinase gene, which encodes the rate-limiting enzyme in the pathway, and in genes that function upstream or downstream of tyrosinase. If defects throughout the pathway can lead to albinism, then why is the critical lesion restricted to the first step in both cave-adapted insects and vertebrates? There are several possible explanations. First, the gene(s) operating at the first step may be more susceptible to mutation, perhaps due to the large size or genomic location. For example, the *oca2* gene, which is responsible for cavefish albinism, covers about 345 KB in a region of frequent chromosomal rearrangements in humans (Yi et al. 2003). Although *oca2* genes have only been studied in vertebrates, a similarly large gene involved in L-tyrosine metabolism or processing could be a mutational hotspot in planthoppers as well. Second, this step may be the only place where mutations are allowed because changes in other genes, such as those encoding enzymes involved in

innate immunity (see below), would be lethal. Third, blocking the pathway at its beginning could enhance the pool of available L-tyrosine for other metabolic functions that are adaptive for survival in the cave environment. For example, L-tyrosine is also the starting point for synthesis of catecholamines, which have many different physiological and behavioral functions. Fourth, assuming that pigmentation loss is adaptive for life in caves, blocking the initial step of the pathway may be the simplest way to arrest pigmentation because it is the rate-limiting step and it also appears to be a developmental control point in pigmented species. Prior to the formation of a pigmented cuticle during molting, incubation with L-DOPA can cause premature melanin formation in non albino insects (Nijhout 1980; Hiruma et al. 1985; Walter et al. 1996), showing that L-DOPA is a limiting factor during the normal development of pigmentation. The temporal extension of this developmental control point at later stages of the life cycle would be a convenient way to evolve permanent albinism. None of the explanations provided above are mutually exclusive, and several of them could potentially act in concert to repeatedly target the first step of melanin synthesis for evolutionary change.

Regressive evolution in cave animals has often been attributed to the benefits of conserving energy under conditions of food limitation in cave environments, which lack primary productivity (Culver and Pipan 2009). This explanation seems consistent with blocking melanin synthesis at its initial step because it would prevent potentially useless and energy consuming downstream reactions. However, cave planthoppers feed on roots that penetrate caves from the surface foliage (Howarth 1972, 1987) and are probably not food limited in the sense of most other cave animals. Therefore, energy conservation is not a likely driving force for blocking melanin synthesis at its first step in cave planthoppers. In general, cave planthoppers can provide unique insights into the relationship between energy conservation and regressive evolution, including the reduction of eyes, pigmentation, and wings, because they are not subjected to food limitation as are other animals living in caves.

Table 1. The effects of substrates, various treatments, and enzyme inhibitors on rescue of black pigmentation in Hawaiian and Croatian cave planthoppers

Substrate	Treatments			Enzyme affected	Pigment deposition (+or- / N)	
	Pre	Co	Post		Hawaiian	Croatian
None	—	—	—	—	(−/2)	(−/3)
L-tyrosine	—	—	—	—	(−/6)	(−/5)
L-tyrosine	—	—	L-DOPA	—	(+/2)	(+/2)
L-DOPA	—	—	—	—	(+/8)	(+/8)
L-DOPA	65◦C	—	—	all	(−/2)	(−/2)
L-DOPA	—	PTU	—	PO	(−/3)	(−/3)
L-DOPA	—	AMT	—	TH	(+/2)	(+/1)
L-DOPA	—	DMA	—	DDC	(+/1)	(+/3)
Dopamine	—	—	—	—	(+/4)	(+/5)
Dopamine	—	PTU	—	PO	(−/3)	(−/3)

N: number of specimens. Other abbreviations are explained in the text.

The insect melanin synthesis pathway has two functions in addition to pigment development. First, the pathway is coupled to cuticle formation during molting (Sugumaran 1988; Hopkins and Kramer 1992). Cave planthoppers have a cuticle, but it is completely lacking in melanin. Therefore, it seems likely that there has been a dissociation of pigmentation and cuticle formation during the evolution of albinism. In support of this idea, cuticle development and melanin production are also known to be independent processes in an albino locust strain (Malek 1957; Jones and Sinclair 1958), and inhibition of the melanin pathway by PTU injection can block black pigmentation but not cuticle development during pupation in blowfly larvae (Dennell 1958). Thus, the normal linkage between melanin and cuticle formation may not be obligatory and can be uncoupled in both experimental and evolutionary situations. Second, melanin

synthesis has been implicated in innate immunity due to a requirement for some of its enzymes (e.g., PO) in this process, and melanin itself can serve as a structural element to encapsulate invading organisms. Our demonstration that L-DOPA substrate can drive melanin synthesis in cave planthoppers implies that all downstream enzymes that function in both pigmentation and immunity are present and functional. As for the role of melanin itself in an effective immune response, it has been shown that it may not always be required. For example, darkening of encapsulated parasitoid egg infections in *Drosophila* is due to cell death and not due to extra-cellular melanin deposition (Russo et al. 1996). Therefore, cave planthoppers probably have a normal innate immune system despite the absence of melanin.

What is the critical molecule(s) involved in melanin synthesis that is defective in cave planthoppers? According to the consensus pathway for insects (True 2003), the only enzyme known to be involved in the first step of melanin synthesis is TH, which converts L-tyrosine to L-DOPA. Mutations in the *pale* (TH) gene cause pigment reduction in *Drosophila* (Neckameyer and White 1993), and *pale* is a candidate gene associated with a pigment loss QTL in *A. aquaticus* (Protas et al. 2011). However, TH is also required for cuticle development, neural functions, and immunity, and its absence would be expected to be lethal (Gorman et al. 2007; Gorman and Arakane 2010). This reasoning is consistent with our demonstration of a TH immunoreactive protein in Hawaiian cave planthoppers. Although cave planthopper TH could be mutated without a detectable change in protein size, the results open the possibility that an unknown factor functioning at the first step of the pathway could be responsible for cave planthopper albinism. This factor could modulate TH activity or make L-tyrosine available for conversion into L-DOPA by TH to initiate melanin production. If the latter, then this situation would be analogous to that in cavefish where mutations in the *oca2* gene, which has been postulated to be involved in L-tyrosine transport (Toyofuku et al. 2002), may cause albinism by limiting L-tyrosine availability (Protas et al. 2006). Thus, cave planthoppers and cavefish might converge not only in the position in which

melanin synthesis is blocked but also in the physiological and molecular mechanisms underlying albinism.

Animals in phyla ranging from Porifera to Chordata have become adapted to life in caves and consequently have reduced or lost melanin pigmentation. In addition, other animals living in lightless conditions such as the deep sea, soils, and parasites in the bodies of their hosts have evolved albinism. Here, we have shown that albinism has appeared in two independently evolved cave planthoppers by a convergent defect in the first step in melanin synthesis, the same place in melanogenesis in which a lesion is found in independently evolved cavefish populations, indicating repeated evolutionary targeting of the first step of the pathway. To fully understand the convergence of albinism, it will be important to determine the position of the defect in melanin formation in a wider array of albino cave animals and in animals living in other lightless environments.

Acknowledgments

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Taxonomic position of *Eunapius subterraneus* (Porifera, Spongillidae) inferred from molecular data –A revised classification needed?

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MOLECULAR PHYLOGENETICS AND EVOLUTION

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Abstract

The freshwater sponge *Eunapius subterraneus* was described in 1984 on the basis of its morphology and unique ecological features. It inhabits caves in the Ogulin karst area as the only known stygobitic sponge, and an endangered karst species. We used three genetic markers with different evolutionary rates in phylogenetic analyses of *E. subterraneus*. All of the markers exclude this sponge from the genus *Eunapius*. Based on our results, we emphasize the need for revision of the taxonomic classification of *E. subterraneus* as well as the need for a thorough re-evaluation of freshwater sponge systematics.

1. Introduction

The Dinarides are a hotspot of subterranean biodiversity with some famous and peculiar animals inhabiting its underground. Apart from the olm (*Proteus anguinus*, Laurenti, 1768) which was the first subterranean animal described, particularly interesting are the unique troglobitic representatives of Temnocephalida (Turbellaria), Hydrozoa (Cnidaria), Dreissenidae (Bivalvia), Serpulidae (Polychaeta) and Spongillidae (Porifera) (Sket, 1986).

Although there are records of freshwater sponges inhabiting underground habitats, all of them are considered stygoxenes (Vacelet, 1994). The only stygobitic sponge species is *Eunapius subterraneus* (Sket and Velikonja, 1984), an endemic member of the suborder Spongillina (freshwater sponges) known from merely six caves near Ogulin, Croatia (Bilandžija et al., 2007). The body of this sponge is very fragile and white in color. Its habitus varies according to growth phases (Sket and Velikonja, 1986) as well as among different cave populations (Bilandžija et al., 2007). It lacks microscleres and has amphioxeal megascleres covered with spines (Fig. S1C, Supplementary data). Gemmules are subspherical in low number adhered to the substratum. Gemmuloscleres from strongyles to oxeas are tangentially arranged and embedded into a very poorly developed pneumatic coat (Fig. S1B, Supplementary data). Foramina are in different numbers without tubes. Although most of its morphological characteristics place this species in the genus *Eunapius*, there is a difference in the gemmular structure which is one of the most important morphological characteristics in the current taxonomy of freshwater sponges. A small number of gemmules, a poorly developed pneumatic coat and the absence of foramina tubes was assigned to reductions in gemmular formation caused by adaptation to special habitat (Sket and Velikonja, 1986). *E. subterraneus* is listed in the Red book of threatened species in the IUCN category EN (endangered) as an organism which is at risk of becoming extinct (Tvrtković et al., 2004).

Sponges in general are characterized by simple morphology and even more by phenotypical plasticity which is often influenced by environmental factors, which leads to the lack of determination criteria. The most important diagnostic features for classification of freshwater sponges are the presence and shape of their microscleres and the structure of their gemmules and gemmuloscleres. However, in three out of six extant freshwater sponge families (Lubomirskiidae, Malawispongiidae, Metschnikowiidae), such important morphological diagnostic features as microscleres, gemmules, and, accordingly, gemmuloscleres are not present (Manconi and Pronzato, 2002). Therefore, development and use of molecular markers are especially important

tools for investigating the phylogeny of this group. Recent work on phylogeny of the sponges indicates that morphological features are often dissonant with molecular data.

Here we present the first attempt to clarify over 20 years old taxonomic position of E. subterraneus using molecular markers. We combined three genes i.e. two nuclear and one mitochondrial: 18S rDNA, ITS2 (internal transcribed spacer 2) and COI (cytochrome oxidase I). Genes with different evolutionary rates were selected in order to obtain better resolution and confidence of phylogenetic trees. These genes have been routinely used in similar phylogenetic analyses of various sponge taxa on similar taxonomic levels (Blanquer and Uriz, 2007; Itskovich et al., 2008; Meixner et al., 2007; Redmond et al., 2007; Sole-Cava and Worheide, 2007; Worheide et al., 2004).

2. Materials and methods

2.1. Taxon sampling and identification

Samples of *E. subterraneus* were collected in the type locality cave Tounjčica špilja and in the spring cave Izvor špilja Gojak by cave diving (Fig. S1A, Supplementary data). A part of the material used for molecular analyses was stored at -80°C, while the other part was preserved in 96% ethanol and used for morphological analyses. Species identification was performed based on a microscopic study of the skeleton and gemmules (Fig. S1B and S1C, Supplementary data).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen). DNA fragments were amplified using previously described primers and PCR conditions: 1662 bp long 18S rDNA (Peterson and Addis, 2000) and 668 bp long COI (Folmer et al., 1994). We constructed custom PCR primers for amplification of 361 bp ITS2 fragment; ITS2F 50CGGCTCGTGCGTCGATGAAGAAC30 and ITS2R 50CGCC GTTACTGGGGGAATCCCTGTTG30. PCR products were checked by electrophoresis in 1.5% agarose gel, excised and purified using the QIAquick Gel extraction kit (Qiagen) and directly sequenced using the ABIPRISM 3100automatic sequencer. Sequences were deposited in NCBI GenBank database under the following accession numbers: 18S rDNA – FJ715438, CO1 – FJ715439 and ITS2 – FJ715436. 28S rDNA was also sequenced and deposited under the accession number FJ715437, but it was not used in the analyses. Other sequences used in this study were obtained from NCBI's GenBank.

For a complete list of GenBank sequence accession numbers used in the analyses see Table 1.

Table 1. List of the species included in the study and corresponding GenBank accession numbers of all analysed sequences.

Baikalospongia bacillifera	Lubomirskiidae	DQ176780.1	DQ176775.1	DQ778319.1
Baikalospongia dzhegatajensis	Lubomirskiidae	EF025856.1		
Baikalospongia fungiformis	Lubomirskiidae		EF095190.1	DQ778328.1
Baikalospongia intermedia	Lubomirskiidae	EU000567.1	AY769090.1	AY662500.1
Baikalospongia martinsoni	Lubomirskiidae			DQ778320.
Baikalospongia recta	Lubomirskiidae	EU000569.1		DQ778323.1
Baikalospongia robusta	Lubomirskiidae			AY662503.1
Corvomeyenia sp.	Metaniidae	DQ176781.1	DQ176774.1	DQ178649.1
Echinospongilla brichardi	Potamolepidae	EU000573.1		
Ephydatia cooperensis	Spongillidae	DQ087505.1	AF140354.1	DQ178652.1
Ephydatia fluviatilis	Spongillidae	DQ176777.1	AY578146.1	DQ178657.1
Ephydatia muelleri	Spongillidae	DQ176778.1	AF121110.1	DQ178654.1
Eunapius carteri	Spongillidae	DQ167175.1	DQ167160.1	AY662508.1
Eunapius coniferus	Spongillidae			EF151939
Eunapius fragilis	Spongillidae	DQ176779.1	AF121111.1	DQ178651.1
Eunapius ryuensis	Spongillidae			EF151948
Eunapius sinensis	Spongillidae			EF151949
Eunapius sp. MM-2004	Spongillidae			AY662508
Eunapius sp. BW0101	Spongillidae			EF151946
Eunapius sp. BW0118	Spongillidae			EF151934
Heterorotula multidentata	Spongillidae			AY662498.1
Lubomirskia abietina	Lubomirskiidae	DQ167170.1	DQ167156.1	DQ778321.1
Lubomirskia baikalensis	Lubomirskiidae	EU000568.1	DQ176776.1	DQ778322.1

Lubomirskia fusifera	Lubomirskiidae			DQ778327.1
Lubomirskia incrustans	Lubomirskiidae			DQ778324.1
Nudospongilla sp.	Spongillidae		DQ927323.1	
Ohridospongilla sp.	Incertae sedis	EF025855.1		
Pachydictyum globosum	Malawispongiidae	DQ167177.1		
Pachydictyum incrustans	Malawispongiidae	DQ167178.1		
Rezinkovia echinata	Lubomirskiidae			DQ778326.1
Rezinkovia abietina	Lubomirskiidae			AY662502.1
Spongilla lacustris	Spongillidae	AJ843883.1	AF121112.1	DQ178653.1
Spongilla vastus	Spongillidae	DQ167180.1	DQ167166.1	
Swartschewskia papyracea	Lubomirskiidae	EU000571.1	DQ167157.1	DQ778325.1
Trochospongilla horrida	Spongillidae	EF025854.1	AY609320.1	
Trochospongilla pennsylvanica	Spongillidae	DQ087503.1	DQ087507.1	

2.3. DNA sequence alignment

Sequences were aligned using the default gap opening–gap extension parameters (15.0–6.66) in ClustalW 1.7 (Thompson et al., 1994). BioEdit 5.09 (Hall, 1999) was used for editing of alignments. Gblocks 0.91b (Castresana, 2000) was used to determine and exclude ambiguously aligned regions in ITS2, under following parameters: minimum number of sequences for a conserved position: 14; minimum number of sequences for a flanking position: 22; maximum number of contiguous non-conserved positions: 8; minimum length of a block: 10; allowed gap positions: with half; use similarity matrices: yes. Final alignments were 1616, 617 and 182 bp long, for 18S rDNA, COI and ITS2 regions, respectively. Analyses of COI gene were performed on nucleotide sequence. No gaps needed to be postulated in COI and 18S rDNA alignments. Alignments are available upon request.

2.4. Phylogenetic analyses

Phylogenetic analyses were performed by parsimony (MP) and maximum likelihood (ML) in PAUP v4.0b10 (Swofford, 2000), and by Bayesian analysis with Markov Chain Monte

Carlo sampling in MrBayes v3.1 (Huelsenbeck and Ronquist, 2001) on Apple Macintosh PowerPC G4. A partition-homogeneity test (ILD-test) was used to assess congruence between partitions (18S, ITS2 and COI dataset). All analyses were applied for each separate dataset as well as for the combined dataset (all three partitions merged in one dataset).

MP analyses were performed with a heuristic search option (1000 random taxon-addition replicates) with tree-bisectionreconnection (TBR) branch swapping, all characters equally weighted. Gaps in ITS2 were treated as fifth-state characters. Number of parsimony-informative characters was 30, 13 and 90, for 18S, COI and ITS2 genes, respectively. Bootstrap values were calculated from 1000 replicates.

The hierarchical likelihood ratio test criterion (hLRT) implemented in Modeltest v3.06 (Posada and Crandall, 1998) was used to select the best-fit maximum likelihood model and parameters for each gene. The estimated parameters (TVM+G for COI, TIM+I+G for 18S and HKY+I+G for ITS2) were used in ML and Bayesian searches, while "alldata" character set was partitioned, and for each partition the preferred model was applied in Bayesian analysis. Searches were performed with four parallel Markov chains; 1,500,000 generations; sampling frequency one in every hundred trees; consensus tree constructed based on the trees sampled after burn-in. The convergence of Markov chains was checked through standard deviations of split frequencies and log-likelihood scores for each run.

As an outgroup in all phylogenetic analyses we used *Corvomeyenia* sp., a freshwater sponge that appeared basal among freshwater taxa in former phylogenetic analyses (Addis and Peterson, 2005; Itskovich et al., 2007; Meixner et al., 2007; Redmond et al., 2007).

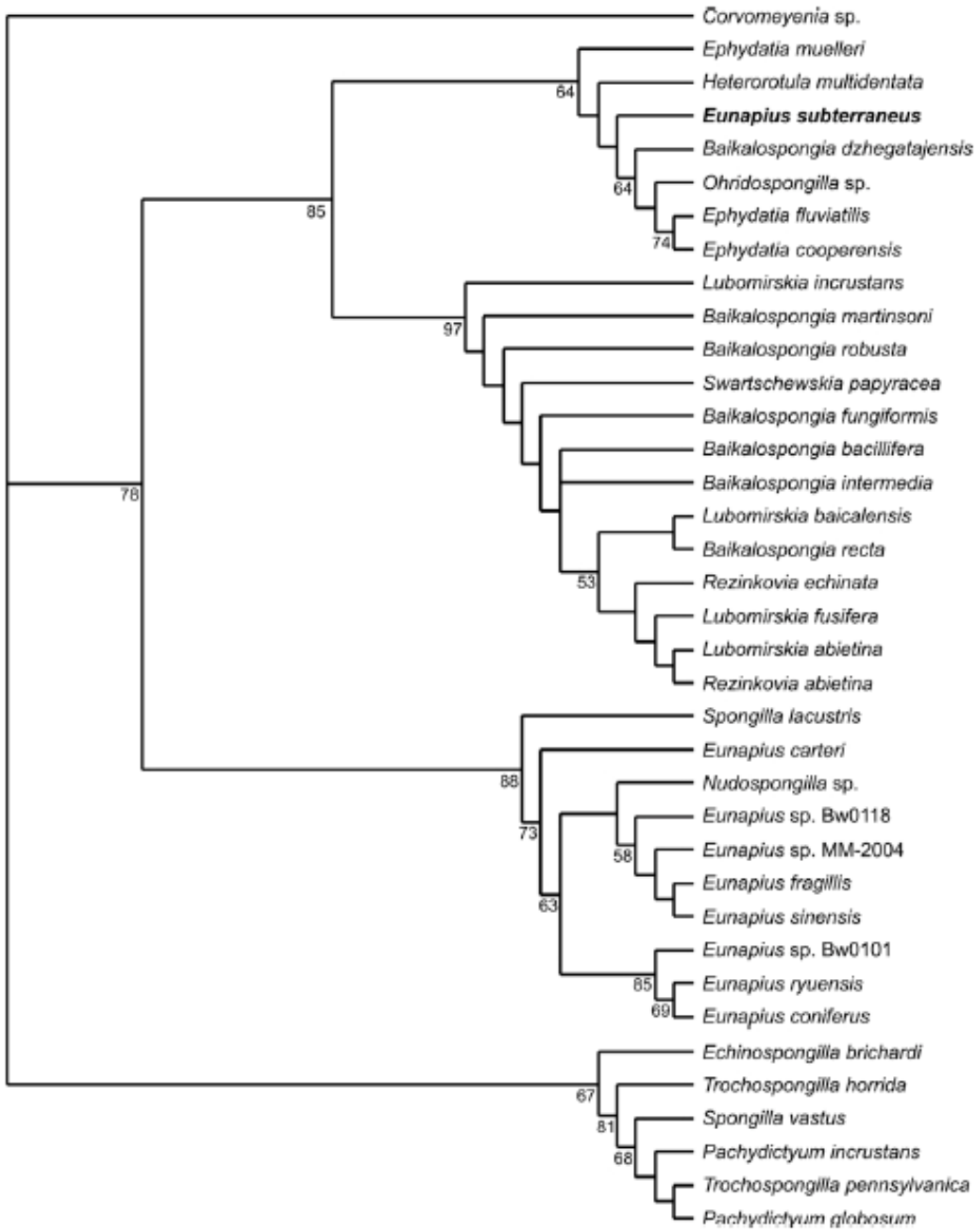
3. Results

3.1. Separate data partitions 18S rDNA and COI

The analyses of separate 18S rDNA and COI data partitions included three species from the genus *Eunapius*: *E. fragilis, E. carteri* and *E. subterraneus*. All the methods employed resulted in similar and fairly well resolved trees with *E. subterraneus* positioned within the well supported group that contained all species of *Ephydatia* genus and all members of the Lubomirskiidae family. Two other species of *Eunapius* (*E.*

carteri and E. fragilis) are grouped in a separate clade together with *Spongilla lacustris* and *Nudospongilla*. Analyses of both markers result in a third clade consisting of *Trochospongilla horrida, T. pensylvanica, Pachydictyum globosum* and *Spongilla vastus* (with 18S) or *T. horrida, T. pensylvanica, P. globosum, P. incrustans, S. vastus* and *Echinospongilla brichardi* (with COI). However, the relations among these three main clades are not well resolved (Figs. S3 and S4, Supplementary data).

A.



B.

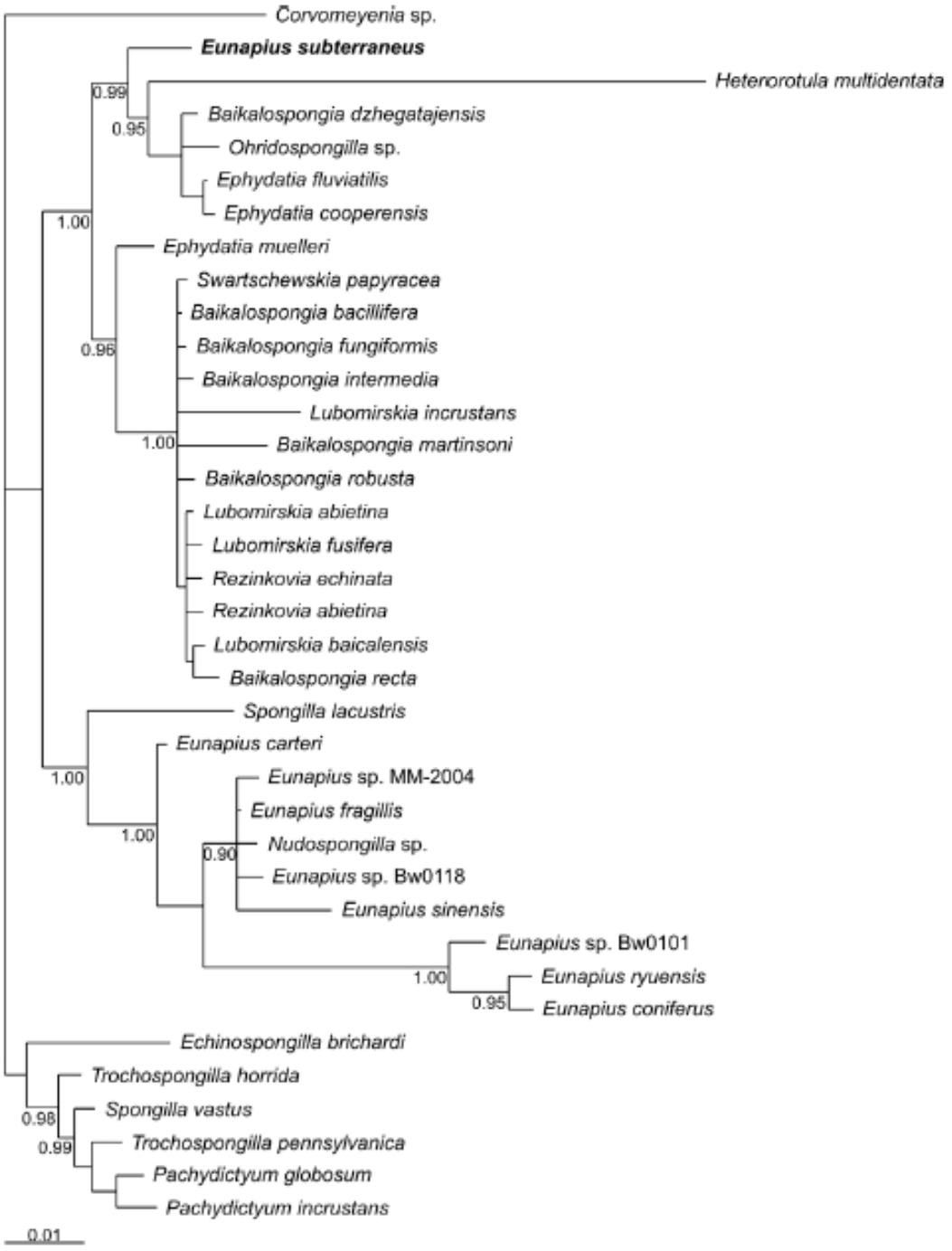


Fig. 1. Phylogenetic relationships among freshwater sponge taxa based on multiple alignment of concatenated nucleotide sequences of COI, 18S rDNA and ITS2 rDNA regions. The species names are indicated in the tree. (a) MP strict consensus tree with bootstrap values (resulting

from 1000 bootstrap replicates) indicated on the nodes (for BPs above 50%). (b) Bayesian strict consensus tree (calculated from trees obtained after burn-in) with PP values above 0.9.

3.2. ITS2 rDNA

In analyses of the ITS2 region we included eight species of the genus *Eunapius* (*Eunapius carteri, Eunapius coniferus, Eunapius fragilis, Eunapius ryuensis, Eunapius sinensis, Eunapius* sp. MM-2004, *Eunapius* sp. BW0101 and *Eunapius* sp. BW0118). In MP analysis *E. subterraneus* is positioned in a highly unresolved clade with all *Ephydatia* species and *Heterorotula multidentata*, being a weakly supported sister clade to a group of all other *Eunapius* species (supported with 100% BP) and in addition *Spongilla lacustris*. All Lubomirskiidae species are grouped in a separate clade with high support (100% BP) (Fig. S2, Supplementary data). Bayesian search resulted in a generally highly unresolved tree with only two well supported groups (Lubomirskiidae with 0.91 PP, and all species of *Eunapius* with the exception of *E. subterraneus*, with 1.00 PP), while *E. subterraneus*, *Heterorotula multidentata, Spongilla lacustris* and all *Ephydatia* species group closer to "*Eunapius*" clade, but their interrelationships could not be resolved.

3.3. Alldata

The ILD-test indicated no incongruence between the data partitions (P = 0.01), so we combined all data in one dataset. Total length of the combined dataset was 2415 characters.

MP search resulted in very large number of equally parsimonious trees of 396 steps. The overall resolution within the strict consensus tree (calculated from 1000 trees, Fig. 1) is higher than in the analyses of separate genes, while the topology is mostly in congruence with that of separate 18S, ITS2 and COI data partitions. Bayesian search of the combined dataset resulted in topology mostly compatible with the MP tree, with higher posterior probability values than in the analyses of separate data partitions for most of the nodes (Fig. 1). ML analysis gave the tree of identical topology (not shown).

In all of the analyses of the combined dataset *E. subterraneus* is positioned within the clade of *Ephydatia* and all Lubomirskiidae species, and in addition *Ohridospongilla* sp. and *Heterorotula multidentata* with high support (85% BP in MP and 1.00 PP in Bayesian). The relations within this group are again mostly unresolved in both MP and Bayesian analyses. All other *Eunapius* species are positioned in a well supported group

with *Nudospongilla* sp. and *Spongilla lacustris* in both MP and Bayesian analysis (88% BP, 1.00 PP values).

4. Discussion

The current taxonomic position of *Eunapius subterraneus* is based on morphological characters, but there are some important differences from the typical *Eunapius* morphology. *E. subterraneus* has a poorly developed pneumatic layer vs. large columnar or polygonal air spaces of the gemmular pneumatic coat which is emphasized as one of the main characteristics of the genus *Eunapius*. Furthermore, foramina of *E. subterraneus* are tubeless while foramen in the genus *Eunapius* is invariably tubular (Penney and Racek, 1968) (Fig. S1, Supplementary data). These differences could merely be a consequence of adaptations to a changed habitat or they might have more serious taxonomical implications. Therefore, characterization at the molecular level was employed in order to resolve the taxonomic position of this unique and endangered sponge, to broaden our general understanding of freshwater sponge radiation and speciation, and to reevaluate the usefulness of morphological characters in freshwater sponge phylogeny studies.

We have chosen three molecular markers that were previously used in phylogenetic studies of various sponge taxa: COI, ITS2 and 18S rDNA regions (Blanquer and Uriz, 2007; Meixner et al., 2007; Sole-Cava and Worheide, 2007; Worheide et al., 2004). Sole-Cava and Worheide (2007) and Meixner et al. (2007) noticed that the COI gene is very conserved in sponges, having an almost identical degree of sequence divergence as the 18S gene. These two markers are not efficient in discriminating between closely related species, and should therefore be used in mutual combination, and/or with other genetic markers. ITS rDNA is more variable and has been used to resolve closely related sponge genera and species as well as populations (Itskovich et al., 2008; Meixner et al., 2007), but it exhibits a high level of intragenomic variation and should also be used with caution (Worheide et al., 2004). Therefore, according to previous findings on the utility of these gene regions, we used them with caution regarding their efficiency when used as single marker, and we employed an "alldata" approach, which resulted in enhanced resolution and confidence of our trees.

Based on our results (Fig. 1, see also Figs. S2, S3 and S4 in Supplementary data) there is no clade supporting *Eunapius* as a monophyletic group, either in analyses of separate data partitions or in "alldata" analyses. Combined molecular markers placed *E.*

subterraneus within the more or less unresolved clade comprising several Spongillidae genera (with the exclusion of other *Eunapius* species) and all Lubomirskiidae. Another clade consisting of all other *Eunapius* species, *Spongilla lacustris* and *Nudospongilla* sp. is well defined in both MP and Bayesian analysis of concatenated sequences of all three markers. Neither any single marker nor the concatenated marker dataset placed *E. subterraneus* in a clade containing all other *Eunapius* species. This is a clear indication that *E. subterraneus* most probably shares a more recent common ancestor with other freshwater sponge genera.

Relationships within other freshwater sponge taxa are also very problematic and remain unclear. There is no congruence between morphological and molecular data, and based on molecular markers many families are unsupported. In our study all molecular markers reflect paraphyly of Spongillidae with respect to Lubomirskiidae. ITS2 is the only marker supporting the monophyly of Lubomirskiidae (100% bootstrap), however, there is no support for Lubomirskiidae being a separate family. Interestingly, *Baikalospongia dzhegatajensis* is not nested within Lubomirskiidae. Although only COI data for this species are available, its placement outside Lubomirskiidae is additionally supported by biogeographical data as noted by Itskovich et al. (2008). This species inhabits lake Chagytai (Dzegetai Kul) in Mongolia 750 km from lake Baikal. Para- phyly of Spongillidae with respect to the Lubomirskiidae was previously reported and furthermore the inclusion of Lubomirskiidae into Spongillidae was proposed (Addis and Peterson, 2005; Itskovich et al., 2007). In our phylogenetic tree of concatenated sequences (18S, ITS2 and COI) *Pachydictyum* spp. grouped with *Trochospongilla* spp. and *Spongilla vastus* making Spongillidae paraphyletic with respect to Malawispongiidae as in Meixner et al. (2007). *Ohridospongilla* sp. clusters with *Ephydatia* spp., *Heterorotula multidentata* and Lubomirskiidae indicating that its phylogenetic position is close to Spongillidae. However, it is not clear whether Meixner et al. study (2007) used *Ochridaspongia* sp. (Malawispongiidae) or *Ohridospongilla* sp. (Inc. sed.).

The presence of gemmules and their morphological traits are diagnostic characters in the current classification at the family, genus and species level (Manconi and Pronzato, 2002). However, molecular genetic studies indicate that gemmular traits are not as universally informative as previously thought and that they may not accurately reflect the phylogenetic relationships among freshwater sponge taxa. Our results place *E. subterraneus* (with strongylate to oxeate gemmuloscleres) in a clade with *Heterorotula multidentata* and *Ephydatia* species (birotulate gemmuloscleres), Lubomirskiidae, *Ohridospongilla* sp. and *Ephydatia cooperensis* (no gemmules). Reduction or loss of gemmules occurred in several freshwater sponge families (Lubomirskiidae,

Malawispongiidae and Metschnikowiidae) but also at lower taxonomical levels, e.g. *E. cooperensis* in contrast to all other *Ephydatia* species (Addis and Peterson, 2005; Manconi and Pronzato, 2002). The fact that non-gemmulating sponges are usually restricted to ancient lakes indicates that stable habitats favour absence of cryptobiosis which probably evolved more than once as indicated by both molecular and biogeographical data (extremely disjunct areals). Meixner et al. (2007) proposed a model in which only a few cosmopolitan sponge species gave rise to various endemic species, and according to our results *E. subterraneus* seems to fit this model.

Subterranean habitats are generally characterized as inhospitable. The absence of light, lack of food and the relative stability of other abiotic parameters are the conditions to which subterranean animals have to adapt. Since *Eunapius subterraneus* is the only underground freshwater sponge in the world, the question is why did it inhabit the subsurface and how did it survive there. The lack of light could be beneficial in the case of this species due to the absence of photosynthetic competitors for space. Another advantage is the relative constancy of abiotic parameters, especially temperature. Since the caves are situated in the shallow karst area the scarcity of food is not so pronounced.

Any attempt to trace the origin of *E. subterraneus* is inevitably difficult and somewhat speculative. *E. subterraneus* is thought to be a remnant of Neogene Dinaride lake system fauna (Sket, 1986). After the final uplift of the Dinarides during the oligocene–miocene, the process of karstification began on tectonically fractured carbonates, exposed to atmospheric influence (Pavelić, 2002; Surić, 2005). Water eroded porous rock and created underground systems. That way the ancestral sponges had the opportunity to invade this habitat although they were not stygobitic yet. Spongillidae are known to inhabit caves where conditions are appropriate (Vacelet, 1994; Sket and Velikonja, 1984). Sponge population inhabiting the caves was protected from the Pleistocene climatic fluctuations. In contrast, surface-level populations were subjected to repeated glacial cycles which finally led to their extinction. The fact that even today there are no representatives of Porifera in surface waters of rivers Dobra and Mrežnica (Bilandžija et al., 2007; Matoničkin, 1988; Sket and Velikonja, 1986) supports this idea. *E. subterraneus* adapted to life in the underground and seemingly lost the ability to inhabit surface waters even in present day favourable conditions. Due to geographical position and turbulent geological past of the Dinarides, Dinaric caves served as a refuge for many other species including other unique stygobites such as *Marifugia cavatica* – the only stygobitic serpulid and *Congeria kusceri* – the only stygobitic bivalve (Sket, 1999).

Presently *E. subterraneus* populations are found at six localities in Dobra and Mrežnica river catchments. However, the porous karst provides underground connections between them at least during the high water level periods (Bahun, 1968). Current distribution of this species can therefore be explained by below-surface dispersal. In attempting to pinpoint the location where ancestral sponge first inhabited the underground, under the assumption that hydrogeology of the area did not significantly change since, Mala Kapela Mountain stands out as a likely candidate. Its *E. subterraneus* population inhabits underground waters which are (1) at least 150 m higher above sea level and (2) upstream of the Ogulin-Plaški Valley where all other known *E. subterraneus* populations are found. Dispersal from Mala Kapela Mountain to other localities is thus much more likely than dispersal from any other locality to Mala Kapela Mountain.

Our results strongly support the placement of *E. subterraneus* outside the *Eunapius* genus and they clearly show a high level of disagreement between current freshwater sponge classification based on morphology and molecular data. Understanding of the evolutionary events in the process of speciation of *E. subterraneus* could be increased by extending the molecular studies to other populations of this species as well as by studying general principles and other examples of freshwater sponge radiation and speciation. Further analyses with larger sequence data sets (i.e. additional molecular markers) and species coverage should be performed in order to gain more conclusive results about freshwater sponge phylogeny.

In conclusion, we emphasize the need for detailed reexamination of morphological characters employed in freshwater sponge taxonomy and for a revision of the taxonomic position of *Eunapius subterraneus* in the light of novel molecular data.

Acknowledgments

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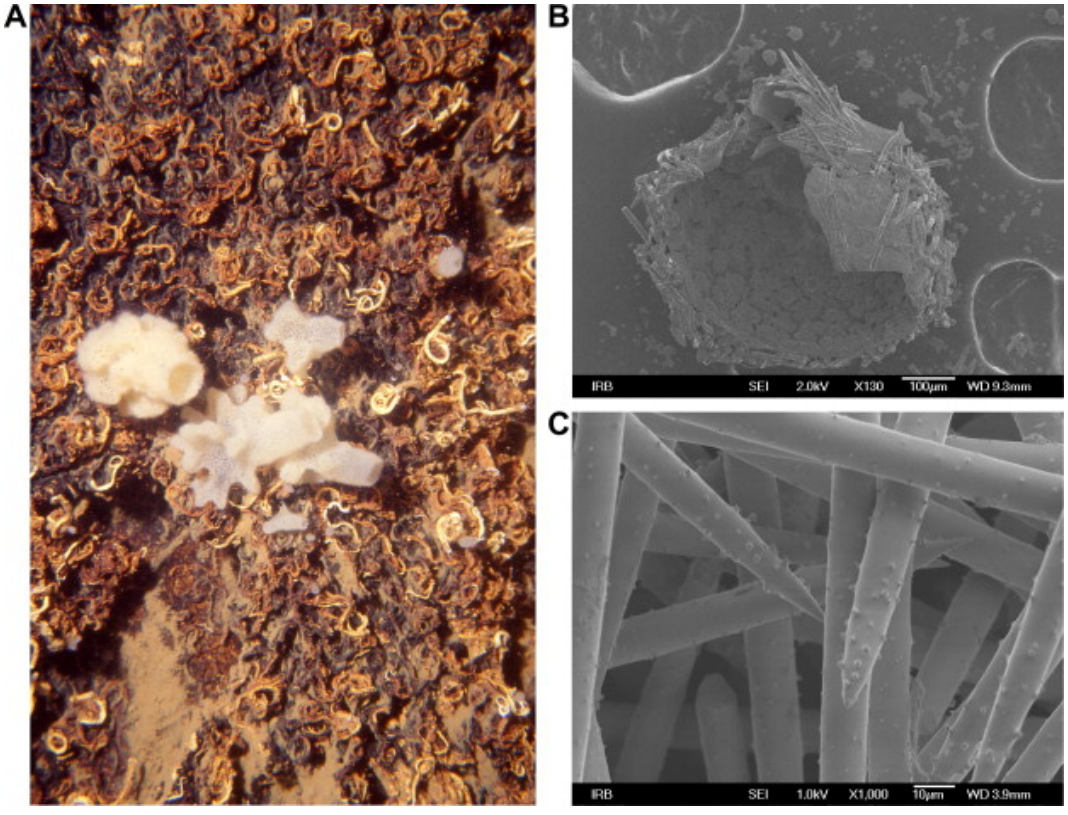
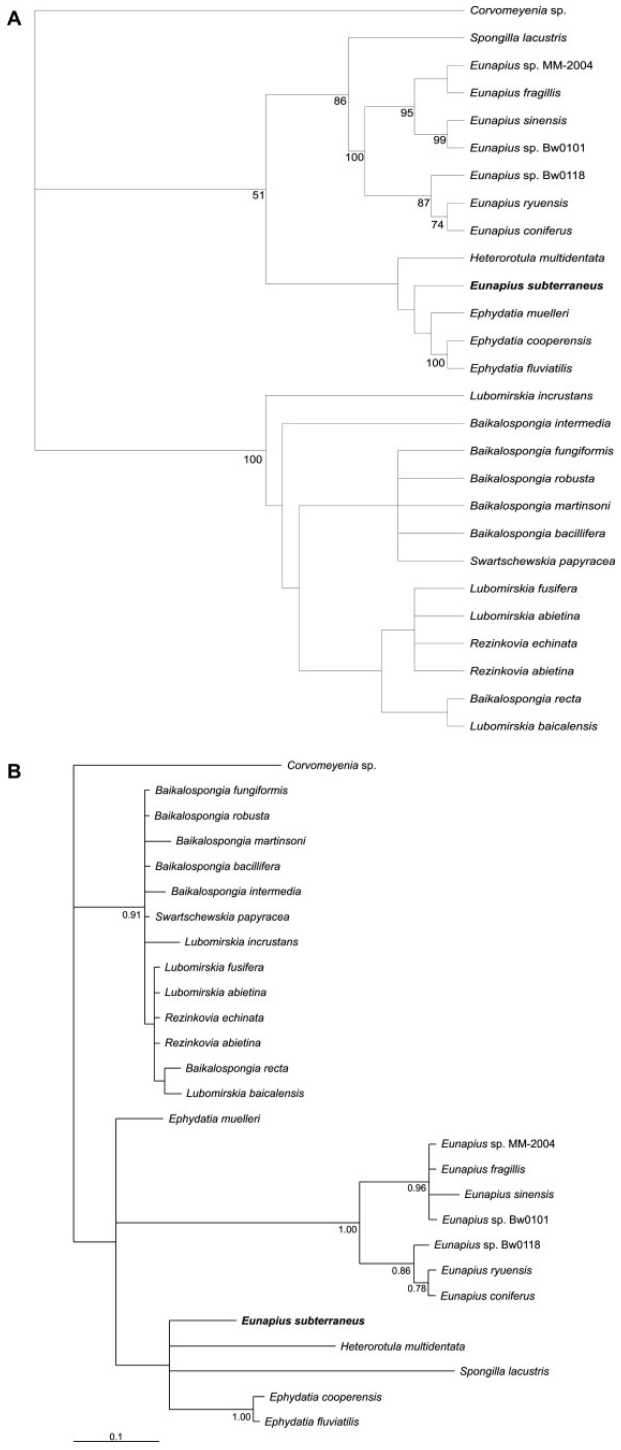
Appendix A. Supplementary data

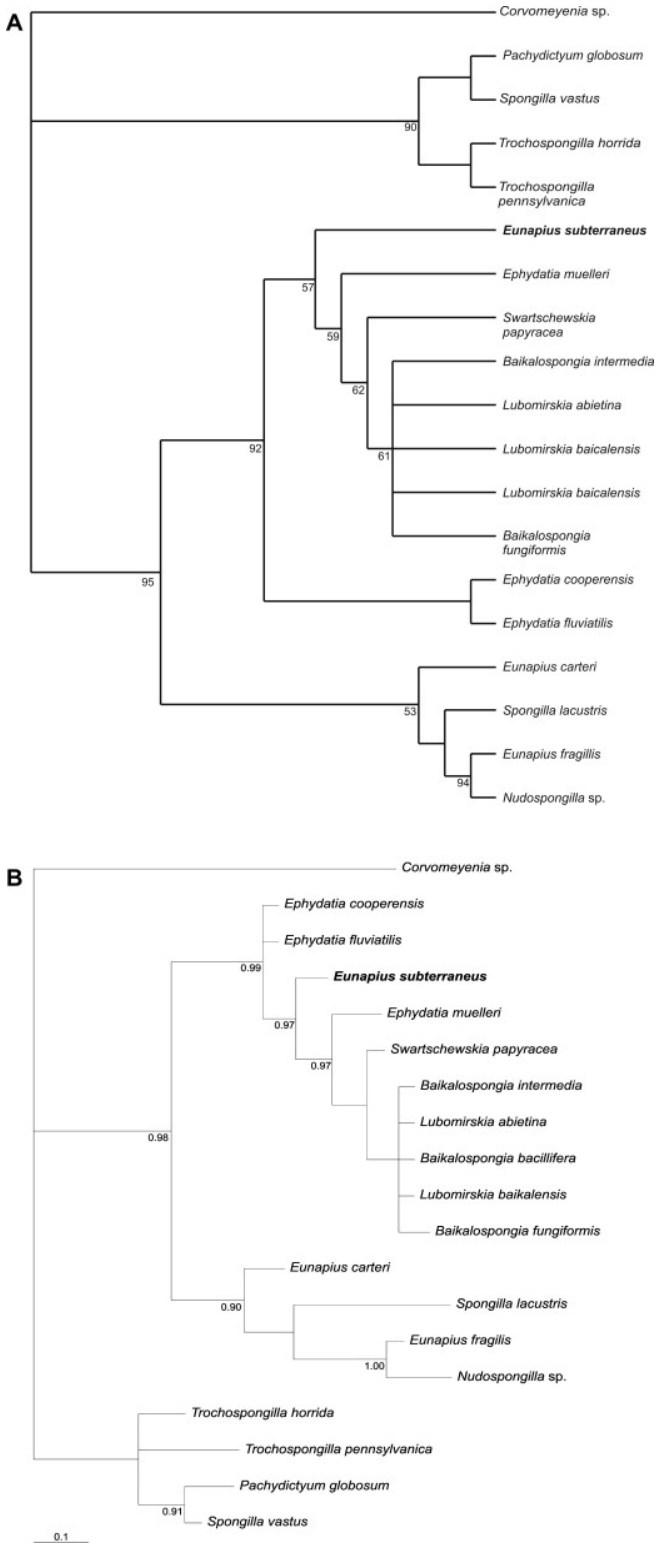
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.12.019.



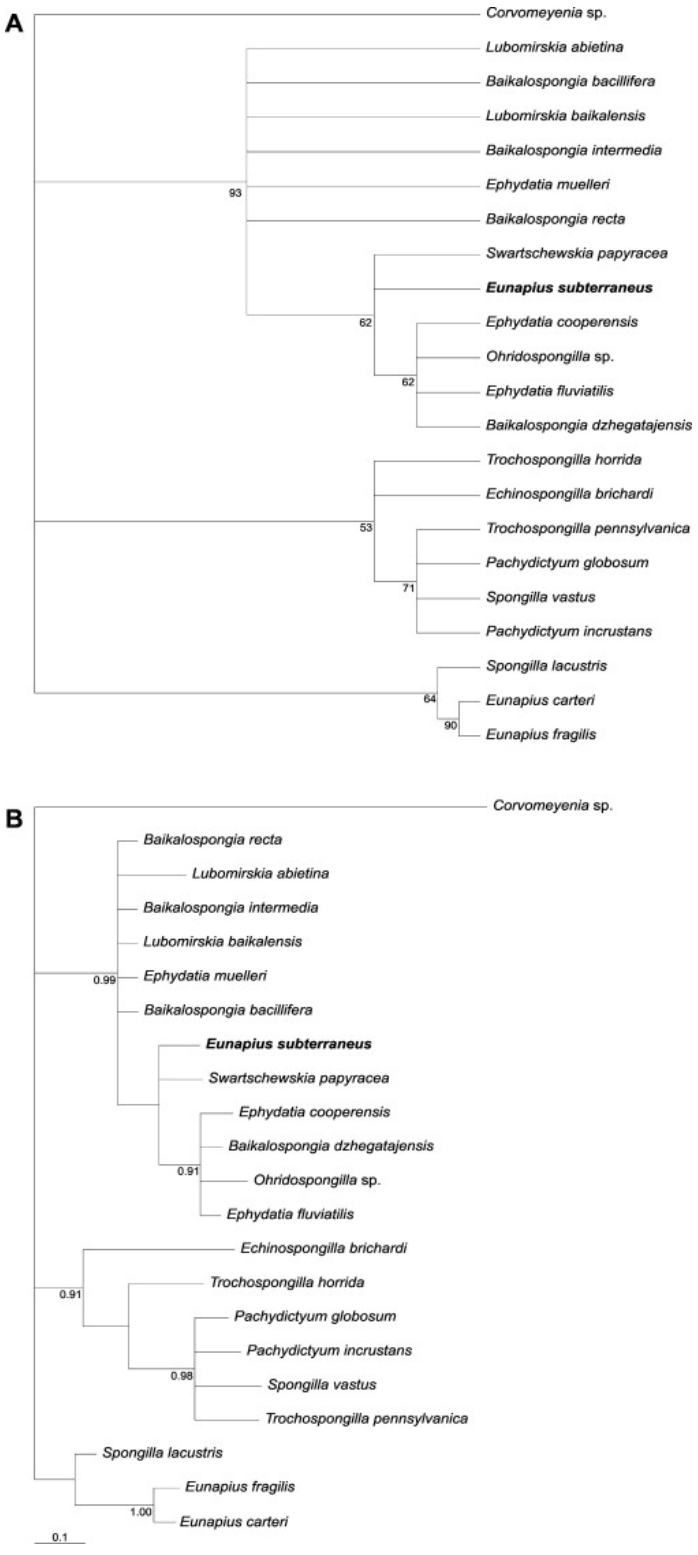
Supplementary Fig. 1. *Eunapius subterraneus* morphology (a) photography of habitus *in situ* from cave Tounjčica špilja (photo by: B. Jalžić). (b) Scanning electron microscope image of spicules. (c) Scanning electron microscope image of gemmule cross section.



Supplementary Fig. 2. Phylogenetic trees resulting from analyses of ITS2 rDNA marker. (a) MP strict consensus tree with bootstrap values (resulting from 1000 bootstrap replicates) indicated on the nodes (for BPs above 50%). (b) Bayesian strict consensus tree (calculated from trees obtained after burn-in) with PP values above 0.9.



Supplementary Fig. 3. Phylogenetic trees resulting from analyses of 18S rDNA marker. (a) MP strict consensus tree. (b) Bayesian strict consensus tree.



Supplementary Fig. 4. Phylogenetic trees resulting from analyses of COI marker. (a) MP strict consensus tree. (b) Bayesian strict consensus tree.

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Evolutionary history of relict *Congeria* (Bivalvia: Dreissenidae): unearthing the subterranean biodiversity of the Dinaric Karst

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Abstract

Background

Patterns of biodiversity in the subterranean realm are typically different from those encountered on the Earth's surface. The Dinaric karst of Croatia, Slovenia and Bosnia and Herzegovina is a global hotspot of subterranean biodiversity. How this was achieved and why this is so remain largely unresolved despite a long tradition of research. To obtain insights into the colonisation of the Dinaric Karst and the effects of the subterranean realm on its inhabitants, we studied the tertiary relict *Congeria*, a unique cave-dwelling bivalve (Dreissenidae), using a combination of biogeographical, molecular, morphological, and paleontological information.

Results

Phylogenetic and molecular clock analyses using both nuclear and mitochondrial markers have shown that the surviving *Congeria* lineage has actually split into three distinct species, i.e., *C. kusceri, C. jalzici* sp. nov. and *C. mulaomerovici* sp. nov., by vicariant processes in the late Miocene and Pliocene. Despite millions of years of independent evolution, analyses have demonstrated a great deal of shell similarity between modern *Congeria* species, although slight differences in hinge plate structure have enabled the description of the two new species. Ancestral plesiomorphic shell forms seem to have been conserved during the processes of cave colonisation and subsequent lineage isolation. In contrast, shell morphology is divergent within one of the lineages, probably due to microhabitat differences.

Conclusions

Following the turbulent evolution of the Dreissenidae during the Tertiary and major radiations in Lake Pannon, species of *Congeria* went extinct. One lineage survived, however, by adopting a unique life history strategy that suited it to the underground

environment. In light of our new data, an alternative scenario for its colonisation of the karst is proposed. The extant *Congeria* comprises three sister species that, to date, have only been found to live in 15 caves in the Dinaric karst. Inter-specific morphological stasis and intra-specific ecophenotypic plasticity of the congerid shell demonstrate the contrasting ways in which evolution in the underground environments shapes its inhabitants.

Keywords:

Dinaric Karst; Subterranean habitats; Cave bivalve; *Congeria*; Dreissenidae; New species; Ecophenotypic plasticity

Background

Subterranean habitats are often colonized, either actively or passively, by unusual and highly distinctive animals, which in many cases are remnants of the surface fauna that once lived above them. These animals are often referred to as living fossils, or relict species. *Congeria kusceri* Bole, 1962, the only known troglobiotic bivalve [1], is a good example of this.

During the Tertiary, most of Europe was covered by a vast aquatic ecosystem of swamps and lakes spreading from the Swiss molasse Basin to Lake Aral in Central Asia. Within this system, known as the Paratethys, a spectacular radiation of many molluscs and other animal taxa occurred [2]. Here, the Dreissenidae Gray, 1840, a family of freshwater bivalves, flourished and diversified [3]. All of the five dreissenid genera [4] evolved in the Neogene lake systems of the Paratethys but only three have survived until the present day: *Mytilopsis* Conrad, 1857, *Dreissena* van Beneden, 1835 and *Congeria* Partsch, 1835. Many different *Congeria* species inhabited the Paratethys. Harzhauser & Mandic [3] identified 16 species and 11 subspecies, while Kochansky-Devide & Sliskovic [5] identified ~30 species from Miocene deposits in Croatia and Bosnia and Herzegovina alone. By the end of the Miocene, however, all but one had become extinct. *Congeria kusceri*, the only species known to have survived this dramatic period, is restricted today to but a few caves in the Dinaric Karst.

The Dinaric Karst extends for about 56,000 km^2 along an 800 km arc from Trieste, Italy in the north, throughout most of Slovenia (SI), Croatia (HR) and Bosnia and Herzegovina (BA) to Albania in the south and is intersected by a network of caves, pits, underground lakes, rivers and streams containing one of the most complex and diverse subterranean faunas in the world [6,7]. It has been argued that the causes of such high subterranean biodiversity in the Dinarides lie in its complex geological history and intensive karstification that enabled multiple entries into the subterranean realm [8].

Congeria species, unlike other cave animals, have a rich fossil record, and can provide new insights into the timeframe, sources and causes leading to the biodiversity hotspot within the Dinaric Karst.

Congeria kusceri was first discovered in the 1930's in deposits of empty shells, but a living population was not found until 20 years later in Žira cave in Popovo polje, southern Herzegovina, allowing J. Bole to describe the species in 1962. Later, additional living populations were found in distant areas of the Dinarides [9,10]. Recent extensive field researches have resulted in the discovery of a total of 15 known *Congeria* populations (Jalžić & Bilandžija, unpublished data). Because of such a small number of sites, habitat destruction, and declines in population numbers, the species is listed as vulnerable (VU) in the Red List of European freshwater molluscs [11] and, in Croatia, *C. kusceri* is assessed as critically endangered (CR) [12].

It is currently assumed that there is only one species of stygobiotic bivalve - *Congeria kusceri* - that has a wide, holodinaric, distribution. Subterranean habitats are, however, subjected to fragmentation, leading potentially to lineage isolation and speciation. Conversely, the extreme character of the subterranean karst environment drives convergent adaptations in its inhabitants, resulting in cryptic morphologies and possibly masking real diversities [13,14]. Accordingly, widely-distributed cave animals have split into a number of lineages with small fragmented ranges [15], as demonstrated by molecular studies of several groundwater Dinaric taxa – the olm, *Proteus anguinus* Lorenti, 1768 [16], the cave shrimp, *Troglocaris*[17,18] and the water louse *Asellus aquaticus* (Linnaeus, 1758) [19].

In this study we have gathered biogeographical and paleontological data and used both molecular and morphological analyses to address several questions. We deal with contentious issues regarding the phylogenetic position and affinities of *Congeria* within the Dreissenidae. We have examined the question of *Congeria* lineage diversifications in separate parts of the Dinaric Karst, and explored the evolutionary history that ultimately

caused a shift, uniquely amongst bivalves, towards a subterranean way of life. Finally, we have reported the effects of the underground environment on *Congeria* shell morphology. For the first time, therefore, this study combines several approaches to provide a new understanding of the evolutionary biology of *Congeria* and uncovers speciation events leading to the description of new *Congeria* species.

Results

Biogeography

Congeria is restricted to only 15 caves (Figure 1) which belong to four geographic regions, each hydrologically isolated from the others: one is in the Kupa River basin, Bela Krajina region (SI), three in the Lika River basin, Lika region (HR) and three in the Sana River basin in north-western Bosnia (BA). The remaining eight populations occur in the Neretva River basin in southern Dalmatia (HR) and Herzegovina (BA).

Phylogenetic analyses

The final dataset consisting of all four concatenated gene markers contained 3847 nucleotides. Of these, 1033 sites were variable and 636 were parsimony informative. MP analysis resulted in 294 equally parsimonious trees (length 1598). Five runs of ML analysis computed in Garli resulted in the same topology, and the log-likelihood scores were similar in each run. Independent Bayesian runs converged to the stationary distribution. Inspection in Tracer showed acceptable ESS (effective sample size) values and good mixing of chains.

MP, ML and Bayesian concatenated trees were well resolved with most main branches showing high statistical support (>95% MP and ML bootstrap values and >98% BPP). Lower bootstrap and posterior probabilities were associated with the north-western

Bosnian populations, probably due to lower resolution in the sequences at this shallow phylogenetic level (Figure 2).

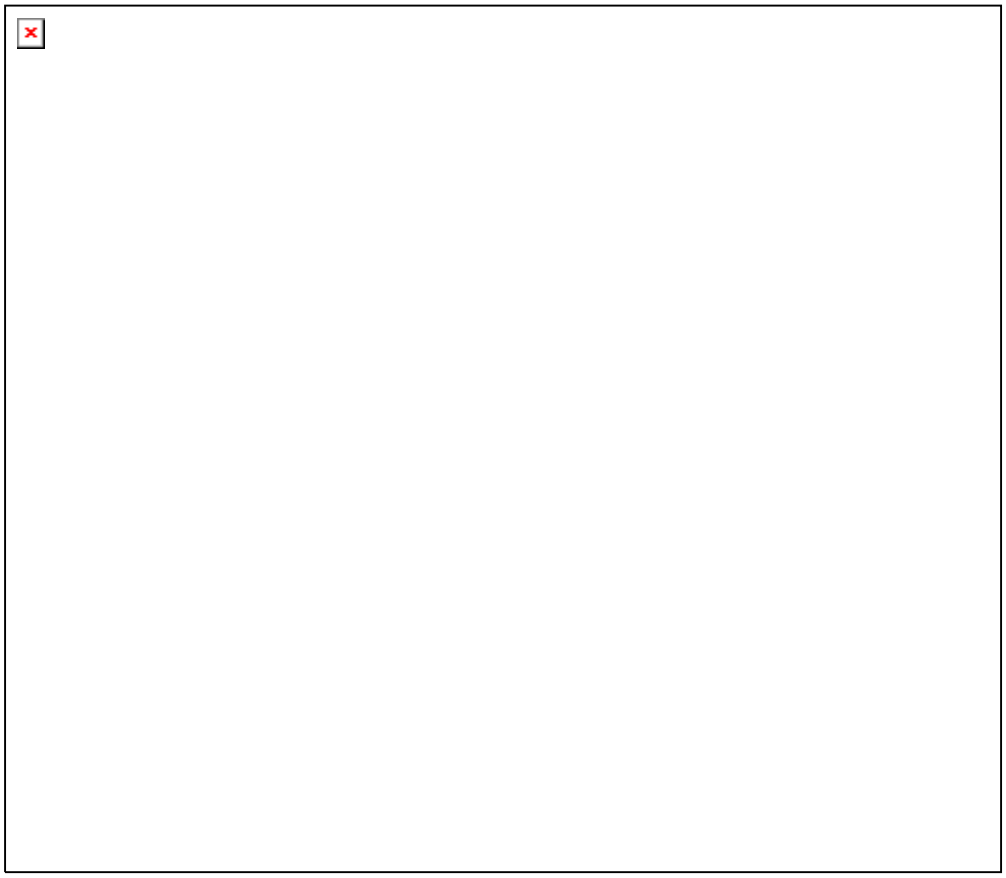

Figure 1. A map of the Dinaric Karst showing all known localities where living populations of *Congeria* occur. (**i**). Bela Krajina Region, (SI): I, Izvir Jamske Školjke. (**ii**). Lika Region, (HR): M, Markov Ponor; L, Lukina jama–Trojama Cave System; Dp, Dankov Ponor. (**iii**), north-western Bosnia, (BA): O, Oko; S, Suvaja; Db, Dabarska Pećina. (**iv**), Neretva Basin, southern Dalmatia (HR) and Herzegovina (BA): T, Tihaljina; Jas, Jasena Ponor; P, Pukotina u Tunelu Polje Jezero–Peračko Blato; J, Jama u Predolcu; G, Gradnica; Z, Žira; D, Doljašnica; Pl, Plitica.

All genetic markers and phylogenetic reconstruction methods employed supported the Dreissenidae as well as the three extant genera as monophyletic clades. Within the

family, the first split isolated *Dreissena,* leaving *Mytilopsis* and *Congeria* as sister groups. The only exceptions to this overall topology were due to conflicting phylogenetic signals in the *16S* and *18S rRNA* trees, but without good statistical support.

Congeria was, thus, always monophyletic and divided into three subclades, although in some cases not with high support. Each of the subclades was restricted to a distinct geographic region: one subclade comprised populations from the Lika and Kupa River basins, another comprised individuals from the Sana River basin and the third comprised all southern populations from the Neretva River basin.

Divergence dating

Separate molecular clock analyses using either the lognormal or exponential clock models, different prior distributions on the mean of the branch rates and on calibration nodes gave concordant divergence times in all but one instance (see below), demonstrating that the results are robust and not dominated by the choice of models and priors. The crown node of the family was estimated at 37.4 million years ago (MYA) (mean node age), which corresponds to the Priabonian Age and the occurrence of the first identifiable dreissenid fossils. The timing of the first split within *Dreissena* was set with lognormal prior placing a minimum hard bound at 11.6 MYA, when the genus first appeared in the fossil record. Accordingly, the first split, between *D. rostriformis bugensis* and the remaining three *Dreissena* spp., was estimated at 12.7 MYA. *Dreissena polymorpha* branched off next at 6.9 MYA. Finally, *D. blanci* and *D. presbensis* separated at 1.9 MYA (Figure 3).

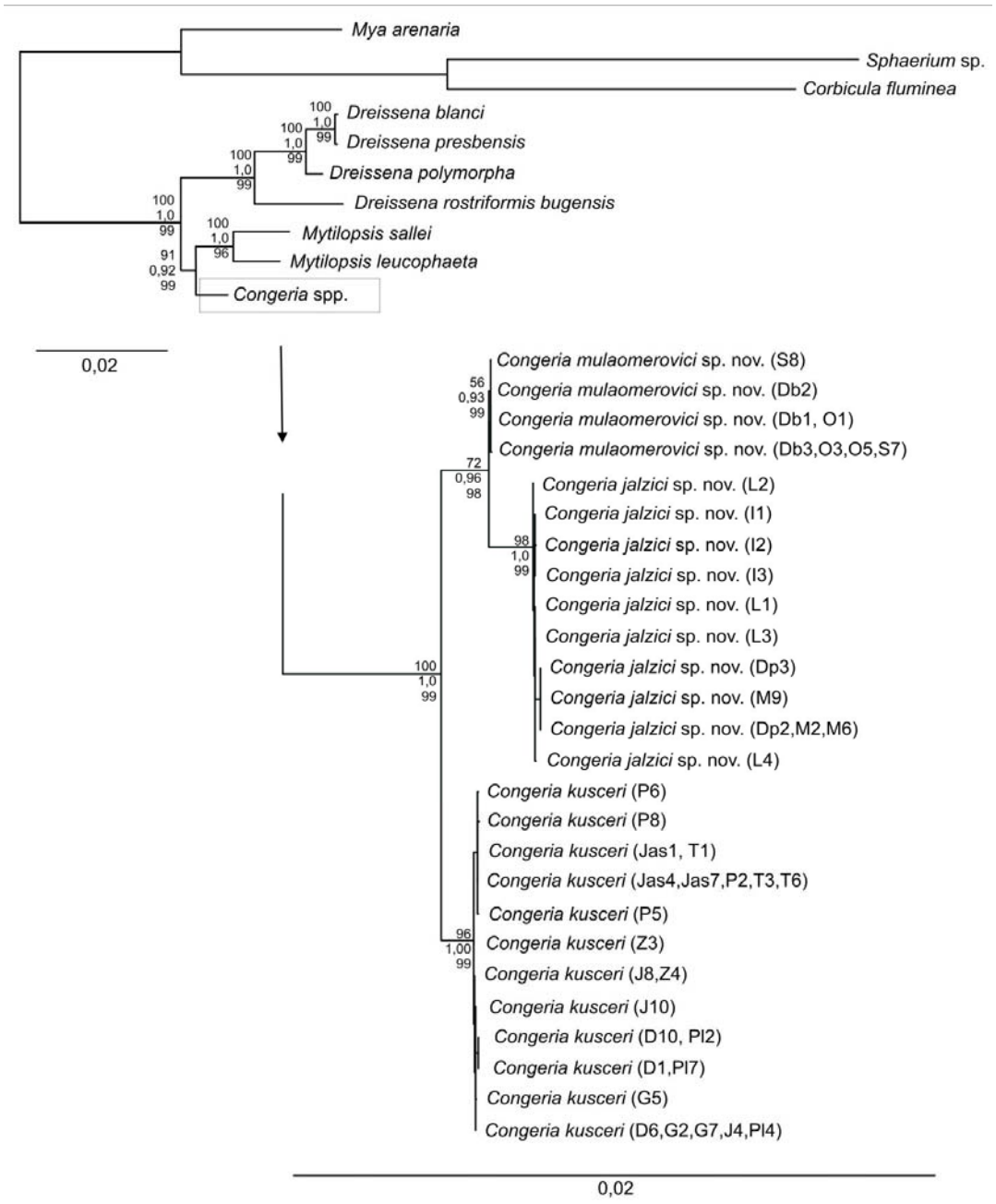

Figure 2. Maximum likelihood phylogram based on combined nuclear (*18S* and *28S rRNA*) and mitochondrial (*COI* and *16S rRNA*) gene fragments. Numbers on the nodes indicate ML bootstrap values (uppermost value), Bayesian posterior probabilities (in the middle) and MP bootstrap values (lowest value). Abbreviations next to *Congeria* branches stand for localities. See Figure1 legend for locality abbreviations.

The split between *Congeria* and *Mytilopsis* lineages was estimated to have occurred between 22.6 MYA, and the two *Mytilopsis* species split between 12.7 MYA. The estimates of the splits within *Congeria* differed according to the molecular clock model used. The exponential clock model placed these divergence events deeper in the past than the lognormal clock model did.

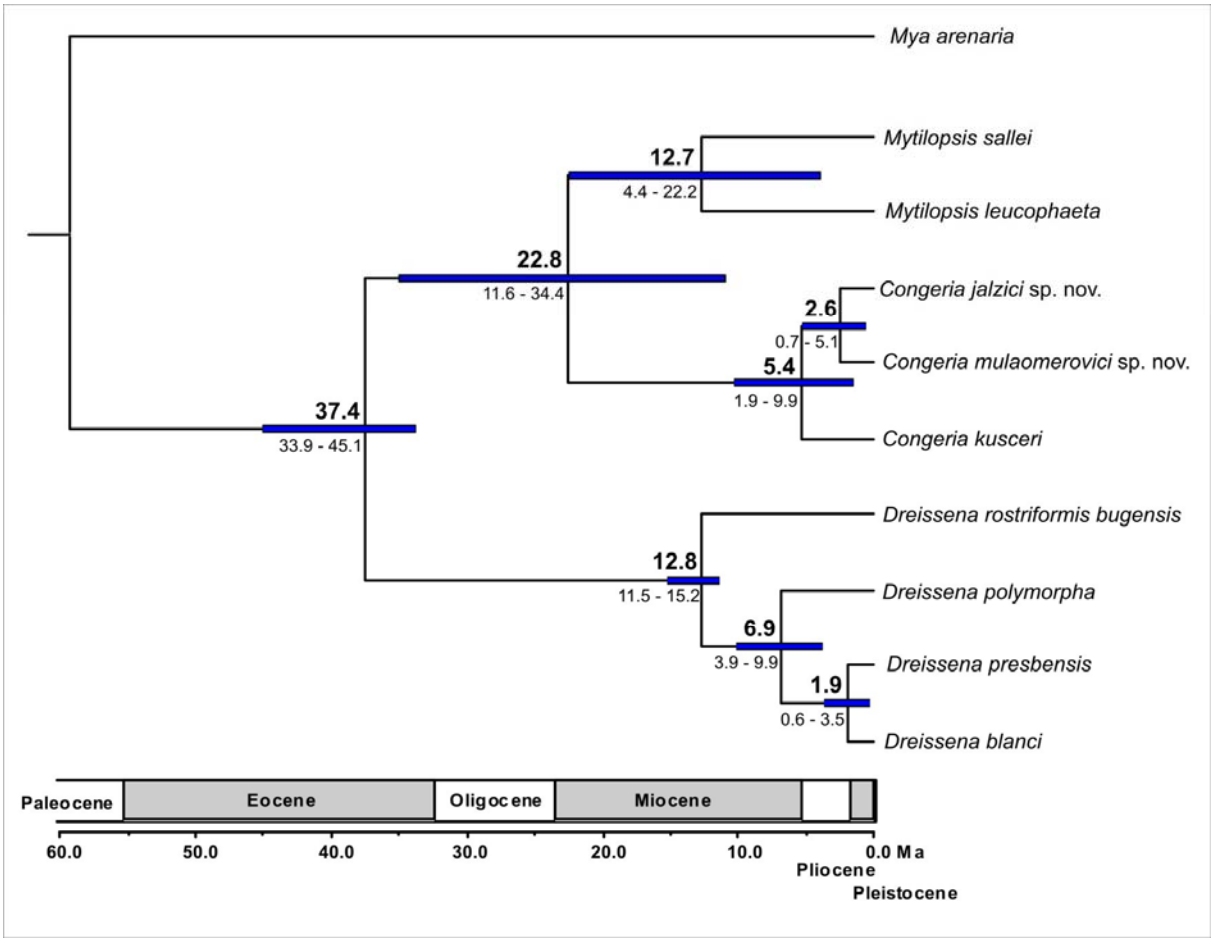

Figure 3. Age estimates of evolutionary divergence events within Dreissenidae. Maximum clade credibility chronogram based on BEAST analysis (lognormal clock model) of concatenated sequences of four genes (*18S*, *28S* and *16S rRNA* and *COI*). Mean divergence ages are shown above the nodes and 95% highest posterior density intervals (95% HPD) are given in parentheses below nodes and denoted by blue horizontal bars. Major geological periods are indicated in million years on the time scale bellow the tree. Bayesian posterior probabilities

were 1.0 for all nodes except for *Mytilopsis*□+□*Congeria* node (0.99) and *C. jalzici*□+□*C. mulaomerovici* node (0.99).

Descriptions of the new species

DREISSENOIDEA Gray in Turton, 1840

Dreissenidae Gray in Turton, 1840

Congeria Partsch, 1835

Type species, *Congeria subglobosa* Partsch, 1835, subsequent designation by Pilsbry, 1911. [=*Enocephalus* Münster, 1831 (*nomen nudum*)].

***Congeria jalzici* sp. nov. Morton & Bilandžija, 2013**. (Figures 4, 6A and 6B, 7A and D)

Material examined

HOLOTYPE. General Collection of Recent Molluscs, Croatian Natural History Museum, Zagreb (CNHM, Reg. No.: 10346). Locality: Markov Ponor, Lipovo Polje, Lika, Croatia (Co-ordinates: WGS84 x=44°45′57″: y=15°10′53″). Leg: B. Jalžić and H. Bilandžija, 2008–2009. Shell length: 11.7mm; height: 7.0mm; width: 8.0mm (Figure 4).

PARATYPES: Specimens 1–3, General Collection of Recent Molluscs, Croatian Natural History Museum, Zagreb (CNHM, Reg. No.: 10347); Specimens 4–6, The Natural History Museum, London (Reg. No's.: NHMUK 20110180–20110182). Locality: Markov Ponor, Lipovo Polje, Lika, Croatia (Co-ordinates: WGS84 x=44°45′57″: y=15°10′53″). Leg: B. Jalžić and H. Bilandžija, 2008–2009 (Table 1).

Figure 4. *Congeria jalzici* sp. nov. The holotype from Markov Ponor, Lipovo polje, Lika, Croatia. Croatian Natural History Museum, Zagreb, Croatia (Reg. No.: 10346).

Table 1. Shell dimensions of *Congeria jalzici* sp. nov.

Shell length	Shell height	Shell width (mm)
11.4	6.7	6.8
10.2	5.9	6.8
11.1	6.6	6.9
11.5	6.4	7.3
11.0	6.7	7.1
10.7	6.3	6.9

VOUCHER MATERIAL: Specimens 1 & 2, ecophenotypes of *Congeria jalzici* sp.nov.: General Collection of Recent Molluscs, Croatian Natural History Museum, Zagreb (CNHM, Reg. No.: 10348); Specimens 3 & 4, The Natural History Museum, London (Reg. No's.: NHMUK 20110183 & 20110184). Locality: Lukina Jama – Trojama Cave

System, Northern Velebit, Lika, Croatia. (Co-ordinates: WGS84 x=44°46′04″: y=15°01′52″). Leg: B. Jalžić, 2010 (Table 2).

Table 2. Shell dimensions of *Congeria jalzici* sp. nov. ecophenotypes

Shell length	Shell height	Shell width (mm)
11.6	7.6	6.0
12.4	7.4	6.7
11.5	7.3	6.2
10.9	6.7	6.4

Description

Shell small, up to 13 mm in length, approximately equivalve, and distinctly inequilateral. Shell generally wider than it is tall, but often only slightly so. Periostracum brown. Distinctly heteromyarian with the swollen posterior face generally round; anterior narrowly rounded with the beaks pointed downwards. Postero- and antero-ventrally convex, although typically concave mid to antero-ventrally around a distinct byssal notch. Valve margins uniform, except ventrally around byssal notch where they are sinusoidal to varying degrees. An external, opisthodetic, ligament. Anterior adductor muscle scar situated on a small septum whose internal face is characteristically and smoothly rounded. Apophysis tiny, situated dorsal to the septum and located (partially hidden) under the resilifer and bears the tiny scar of the anterior byssal retractor muscle.

Remarks

As with its sister species, *Congeria kusceri*, the shell of *Congeria jalzici* sp. nov. is variable in form, but the septum is small and distinctively concave. Hence, the anterior

adductor muscle scar of the former is much larger and has a near straight internal margin aligned with the straight septum margin.

The ecophenotype of *Congeria jalzici* sp. nov. from the Lukina Jama – Trojama Cave System is different, in terms of shell form, from the specimens obtained from the type locality. It has a near transparent shell, with the periostracum only obvious as a yellow – light brown marginal fringe. Its internal shell septum is even smaller than that of conspecifics from Markov Ponor and the shell has a less triangular form in cross-section.

Etymology

Congeria jalzici sp. nov. is named after Branko Jalžić, Croatian Natural History Museum, in honour of his achievements in the field of cave biology in the Dinarides and in appreciation of his invaluable help during this research.

***Congeria mulaomerovici* sp. nov. Morton & Bilandžija, 2013**. (Figures 5, 6C, 7C)

Material examined

HOLOTYPE. Collection of Molluscs, The National Museum of Bosnia and Herzegovina, Sarajevo (Reg. No.: 470). Locality: Oko, Lušci Palanka, north-western Bosnia, Bosnia and Herzegovina (Co-ordinates: WGS84 x=44°42′08″: y=16°28′04″). Leg: B. Jalžić, 2011. Shell length: 11.8mm; height: 6.8mm; width: 8.0mm (Figure 5).

PARATYPES: Specimens 1–3, The National Museum of Bosnia and Herzegovina, Sarajevo (Reg. No.: 471); Specimens 4–6, The Natural History Museum, London (Reg. No's.: NHMUK: 20110469/1,2,3); Specimens 7–9, General Collection of Recent Molluscs, Zoology Department, Croatian Natural History Museum, Zagreb (CNHM, Reg. No.: 10348). Locality: Oko, Lušci Palanka, Bosnia and Herzegovina. Leg: B. Jalžić, 2011 (Table 3).

Table 3. Shell dimensions of *Congeria mulaomerovici* sp. nov.

Shell length	Shell height	Shell width (mm)
12.0	7.3	7.6
10.9	7.2	6.6
10.8	6.7	6.3
10.6	6.7	6.4
11.2	7.1	7.1
10.4	6.1	6.7
10.2	6.2	6.1
10.4	6.4	6.5
10.0	6.2	6.0

Figure 5. *Congeria mulaomerovici* sp. nov. The holotype from Oko, Lušci Palanka, north-western Bosnia, Bosnia and Herzegovina. The National Museum of Bosnia and Herzegovina, Sarajevo, Bosnia and Herzegovina (Reg. No.: 470).

Description

Shell small, up to 12 mm in length, approximately equivalve but distinctly and acutely inequilateral. Shell usually wider than it is tall, but often only slightly so. Periostracum uniformly brown. Distinctly heteromyarian with the postero-dorsal slope straight and, hence, sharply pointed; anteriorly also pointedly rounded. Ventrally flattened, although somewhat concave antero-ventrally around a slight byssal notch. Valve margins uniform, except ventrally around the byssal notch where they are slightly sinusoidal to varying degrees. The beaks point downwards. An external, opisthodetic, ligament. Anterior adductor muscle scar situated on a small septum whose internal face is smoothly sinusoidal. Apophysis small, situated dorsal to the septum and located (partially hidden) under the resilifer and bears the tiny scar of the anterior byssal retractor muscle.

Remarks

As with its sister species, *Congeria kusceri* and *Congeria jalzici* sp. nov., the shell of *Congeria mulaomerovici* sp. nov. is variable in overall form but is more distinctively pyramidal dorsally. Further, the septum is sinusoidal, such that the anterior adductor muscle scar is bean-shaped.

Etymology

Congeria mulaomerovici sp. nov. is named after Dr. Jasminko Mulaomerović, Centre for Karst and Speleology, Sarajevo, an eminent researcher of the karst in Bosnia and Herzegovina, and in appreciation of his support during our research.

A comparison of shell form

Congeria jalzici sp. nov. Seen from the right (Figure $6A_1$), the shell is antero-dorsally keeled and deeply convex at the midpoint around the keel. Seen from the dorsal aspect (Figure $6A_2$), the shell is posteriorly pointed and laterally inflated. The ventral valve

margins (Figure $6A_3$) are straight posteriorly, anteriorly they are sinusoidal around a large byssal gape (BG). The separated umbones (U) are clearly obvious when seen from the anterior aspect (Figure $6A_4$). The shell is flattened ventrally and the greatest shell width (x---y) is situated close to the ventral side making the shell of *C. jalzici* sp. nov. distinctly mytiliform. From the posterior aspect (Figure $6A_5$), the shell is more rounded laterally and concave centrally.

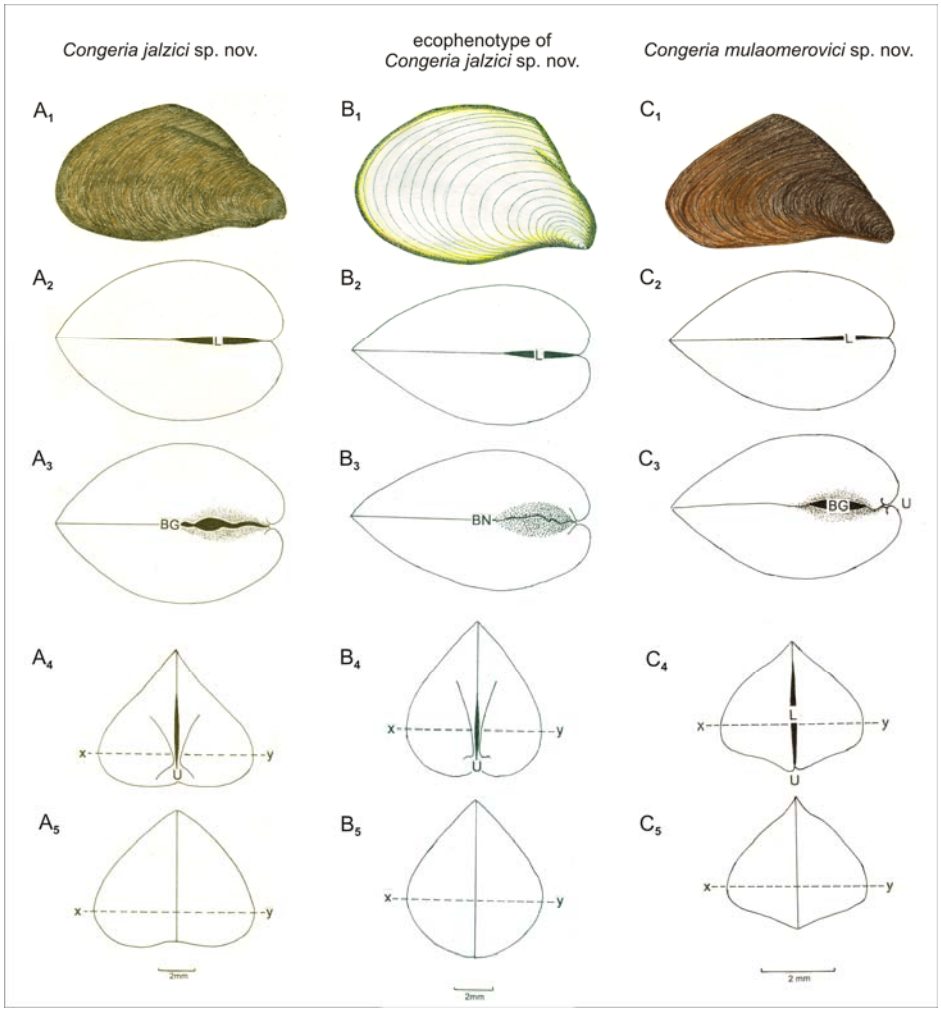

Figure 6. Shells of *Congeria*. A shell of (A) *Congeria jalzici* sp. nov. from Markov Ponor, (B) the ecophenotype of *Congeria jalzici* sp. nov. from the Lukina Jama-Trojama Cave System, and (C) *Congeria mulaomerovici* sp. nov. from Oko. 1, right lateral view; 2, dorsal view; 3, ventral

view; 4, anterior view; 5, posterior view. (x---y indicates the greatest shell width). Abbreviations: BG - Byssal gape; BN - Byssal notch; L - Ligament; U - Umbo. x-----y equals the greatest width of the shell.

Ecophenotype of *Congeria jalzici* sp. nov.. The shell of *C. jalzici* sp. nov. from the Lukina Jama – Trojama Cave System is distinctly less antero-dorsally keeled than conspecifics from the type locality (Figure $6B_1$) and therefore less concave at the mid antero-dorsal point. It is also posteriorly more rounded, less anteriorly convex and concave postero-ventrally around the byssal notch. Seen from the dorsal and ventral aspect (Figure $6B_2$, $6B_3$), the shell is like its type locality conspecifics except there is not a byssal gape although there is a shallow byssal notch (BN). In cross-section (Figure $6B_4$), the left and right valves are not indented as in type conspecifics but are more smoothly rounded to create a more drop-shaped form. The shell is less flattened ventrally, except at the valve margins, which are concave anteriorly. The greatest shell width (x---y) is situated at a point more dorsally than in type locality conspecifics and thus is not mytiliform. From the posterior aspect (Figure $6B_5$), the shell is distinctly rounded laterally and is not flattened ventrally as in type locality conspecifics. The shell of this population of *C. jalzici* sp. nov. is clearly not adapted to flowing waters as are conspecifics from the type locality.

Congeria kusceri. The shell of *Congeria kusceri* has been described by Morton *et al.* (1998, Figures seven-sixteen).

Congeria mulaomerovici sp. nov.. The shell of *C. mulaomerovici* sp. nov. is somewhat antero-dorsally keeled but at a point more anteriorly than in *C. jalzici* sp. nov. (Figure $6C_1$). It is dorsally peaked, almost pyramidal. Seen from the dorsal aspect (Figure $6C_2$), the shell is posteriorly pointed and laterally inflated. The ventral valve margins (Figure $6C_3$) are slightly curved posteriorly, anteriorly they are somewhat sinusoidal around a large byssal gape (BG). The right valve overlaps the left somewhat posterior and, especially, anteriorly such that the umbones (U) are distinctly unequally situated, the

left more anterior than the right. The umbones are also less separated than in *C. jalzici* sp. nov.. The valves are slightly laterally indented in cross-section (Figure $6C_4$, $6C_5$) and the shell is ventrally keeled. As a consequence, the greatest shell width (x---y) is situated more dorsally than in *C. jalzici* sp. nov. so that the whole form of the shell is less mytiliform.

A comparison of hinge plates

Congeria jalzici sp. nov. (Figure 7A). Internally, the shell possesses a large posterior adductor muscle scar (PA), internal to which is the scar of the posterior byssal retractor muscle (PBR). There is a thick pallial line (PL), especially posteriorly and a small, bean-shaped anterior adductor muscle scar (AA) located on a shell shelf or septum, internal to the downwardly directed umbones. The long thin ligament is situated on a resilifer and extends approximately half way up the anterior slope of the shell. Underneath the resilifer, just above the shell shelf is a tiny apophysis on which is located the scar of the anterior byssal retractor muscle (ABR). There is a deep byssal notch (BN). The shell shelf (Figure $7A_1$) has a distinctively curved inner margin (arrowed).

The ecophenotype of *Congeria jalzici* sp. nov. (Figure 7D). The shell is altogether more delicate than in *C. jalzici* sp. nov. from its type locality. Similarly, the internal muscle scars are smaller and more delicate – indeed they are difficult to discern in such a thin, near-translucent, shell but their arrangement is approximately the same. In the ecophenotype of *C. jalzici* sp. nov., the shell septum is extremely delicate, but has the same form, a distinctively curved inner margin (Figure $7D_1$). The scar of the tiny anterior adductor muscle (AA) is located just internal to the umbo (U). The apophysis with its scar of the anterior byssal retractor muscle (ABR) is similarly proportionally smaller than in *C. jalzici* sp. nov. from its type locality.

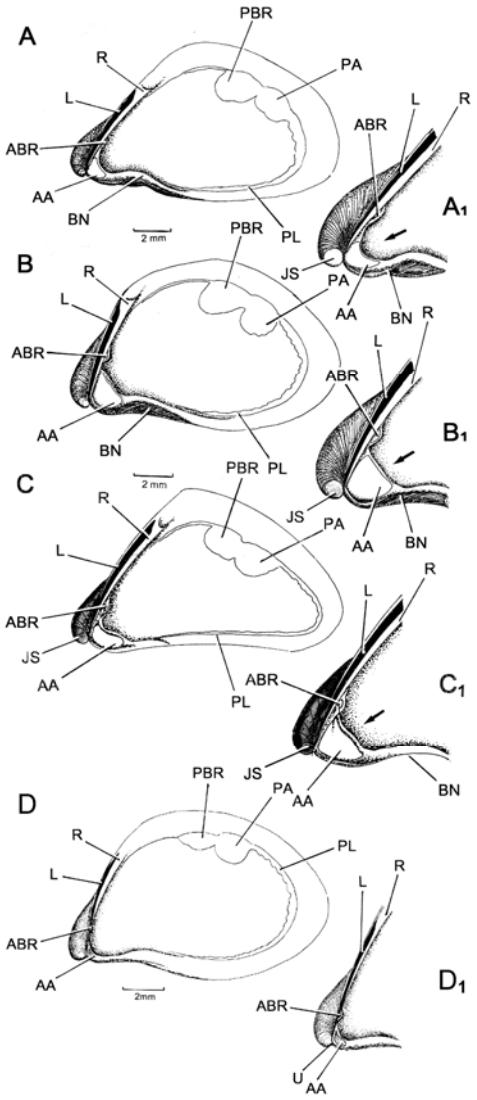

Figure 7. Internal shell structures. Internal views of the shells of **A**, *Congeria jalzici* sp. nov., **B**, *Congeria kusceri*, **C**, *Congeria mulaomerovici* sp. nov., and **D**, the ecophenotype of *Congeria jalzici* sp. nov. from the Lukina Jama-Trojama Cave System. **A**$_{I}$, **B**$_{I}$, **C**$_{I}$, and **D**$_{I}$ are details of the hinge plates of the four specimens with the arrows pointing to the septa on which is inserted the anterior adductor muscles. Abbreviations: AA - Anterior adductor muscle scar; ABR - Anterior retractor muscle scar; BN - Byssal notch; JS - Juvenile shell; L - Ligament; PA - Posterior adductor muscle scar; PBR - Posterior byssal retractor muscle scar; PL - Pallial line; R - Resilifer; U - Umbo.

Congeria kusceri (Figure 7B). The arrangement of the internal muscle scars are approximately the same as in *C. jalzici* sp. nov.. In *C. kusceri*, however, the shell septum is proportionally larger than in *C. jalzici* sp. nov., as are the scars of the anterior adductor muscle (AA) and the anterior byssal retractor muscle (ABR) situated on its also proportionally larger apophysis. The shell septum of *C. kusceri* (Figure $7B_1$) has a straight inner margin (arrowed) and the apophysis is located much closer to the shell septum.

Congeria mulaomerovici sp. nov. (Figure 7C). The shell is more steeply pointed dorsally and is distinctively more pointed posteriorly than in both *C. kusceri* and *C. jalzici* sp. nov., but the byssal notch is small. The arrangement of the internal muscle scars is approximately the same as in the previous two species. In *C. mulaomerovici* sp. nov., however, the shell septum is approximately mid way in size between the other two *Congeria* species as is the scar of the anterior adductor muscle (AA). The apophysis with its scar of the anterior byssal retractor muscle (ABR) is approximately the same size as in *C. kusceri* but, of all the three species it is located the closest to the shell septum and, in fact, partially beneath it (Figure $7C_1$). The shell shelf has a sinusoidal inner margin (arrowed).

Discussion

The Dreissenidae is an excellent candidate group to study evolutionary processes that shape close relatives into biologically and ecologically diverse sets of species. Here we have focused on the most rare and exceptional taxon in the family - *Congeria* - a Tertiary relict that underwent significant changes in morphology, biology and ecology to be the only survivor of a once widespread and diverse genus.

Phylogenetic relationships

Our results have shown that each of the three extant dreissenid genera form a monophyletic group. Although our *Mytilopsis* species representation is far from exhaustive, this confirms previous taxonomic understandings [20]. The sister relationship of *Congeria* and *Mytilopsis* is evident from both molecular and morphological characters. The presence of an apophysis is a common feature that separates them both from *Dreissena* but it has also been used as an argument to merge these two genera into one, that is, *Congeria* [21]. An apophysis is, however, the ancestral feature of the Dreissenidae and its sole use to infer dreissenid relationships has led to conclusions such as the polyphyletic origin of *Dreissena*. This view was most recently supported by Sket [22], who formally proposed the placement of *Congeria kusceri* into *Mytilopsis*. In a detailed study of the morphology of *C. kusceri*, however, Morton et al. [1] provided additional evidence to distinguish the species from others comprising *Mytilopsis*. Further, many aspects of the biology and ecology of *C. kusceri* are unique. In addition to its distinctive reproductive strategy, *C. kusceri* exhibits a wholly characteristic life history that involves extreme longevity (decades) [1] unlike the short lived (2–3years), opportunistic, non-brooding, representatives of *Dreissena* and *Mytilopsis* [23,24]. Furthermore, our molecular clock analysis placed the timing of divergence between these two extant lineages at 22.6 MYA, arguing in favour of a special and distinctive placement for *Congeria.*

The results have demonstrated that instead of only one holodinaric species, as was previously thought, *Congeria* comprises at least three distinct species: *C. kusceri*, *C. jalzici* sp. nov. and *C. mulaomerovici* sp. nov.. Separate lineages have formed in the geographically and hydrologically isolated regions of the Dinaric Karst. Along with fragmentation of karstic underground habitats, both the sessile lifestyle and the reproductive strategy of *C. kusceri*[25] would not facilitate dispersal, so it is argued that speciation occurred after vicariant isolation of lineages in separate hydrological basins. Within the *C. jalzici* sp. nov. lineage, however, the isolated Slovenian population lacks

any obvious genetic distinction, but shows slight and consistent differences in shell. Even if undiscovered populations exist between the Bela Krajina and Lika regions, it is unlikely that there is any gene flow present, because several hydrological basins and the divide between the Black Sea and the Adriatic Sea catchments separate these two populations. Without the possibility of communication, the most likely explanation for such a common genetic similarity would be a relatively recent split.

Effects on morphology

Over half of the known *Congeria* sites contain only empty shell deposits that were flushed to the surface by underground water currents. In order to be able to assign this material to any of the three *Congeria* species, we had to focus on finding distinctive shell characters. Shell morphometric measurements (Additional file 1) have, however, demonstrated that no single dimension differed significantly between all three species and the two species that are genetically and geographically most distant are most similar morphometrically. Shell morphology is, moreover, intra-specifically variable in all three species of *Congeria*, and *C. jalzici* sp. nov. is particularly remarkable in terms of a demonstrable shell plasticity. That is, two populations of this species, living in the same hydrological system (Figure 8), have significantly different shell morphologies. In the flowing waters of Markov Ponor, *C. jalzici* sp. nov. is characterised by a heteromyarian, ventrally flattened, shell whereas conspecifics from the Lukina Jama–Trojama Cave System at −1421 m below ground, are extremely delicate with ventrally concave shells and a reduced apophysis. In contrast to all other known *Congeria* localities where strong currents form during high water levels, the waters of the deep karst aquifer in the Lukina Jama–Trojama Cave System seem to be static and to rise and fall only slowly. The shape of *Congeria* shell and also the fact that tubes of *Marifugia cavatica* Absolon & Hrabe, 1930, grow perpendicular to the walls of the cave (B. Jalžić, personal information) point to this conclusion. The example of *C. jalzici* sp. nov. shows how the dreissenid shell is, to a great extent, shaped by environmental conditions and can be

misleading, when viewed alone, in interpreting phylogenetic relationships within the family.

Finally, subtle differences between the three *Congeria* species have been identified in terms of hinge plate morphology. These include the sizes of shell septum and the anterior adductor muscle scar, the form of the inner margin and the position of the apophysis. These characters, although also showing variability, were consistent in all cave populations comprising one phylogenetic lineage (including the ecophenotype of *C. jalzici* sp. nov.). Interestingly, Schütt [21] distinguished a number of dreissenid species also on the detailed structure of the hinge plate/resilifer/apophysis. Despite the difference in opinion with regard to the generic placement of these taxa, it is evident from both Schütt's and our studies that the only useful shell characters for distinguishing between these species of Dreissenidae relate to the hinge plate.

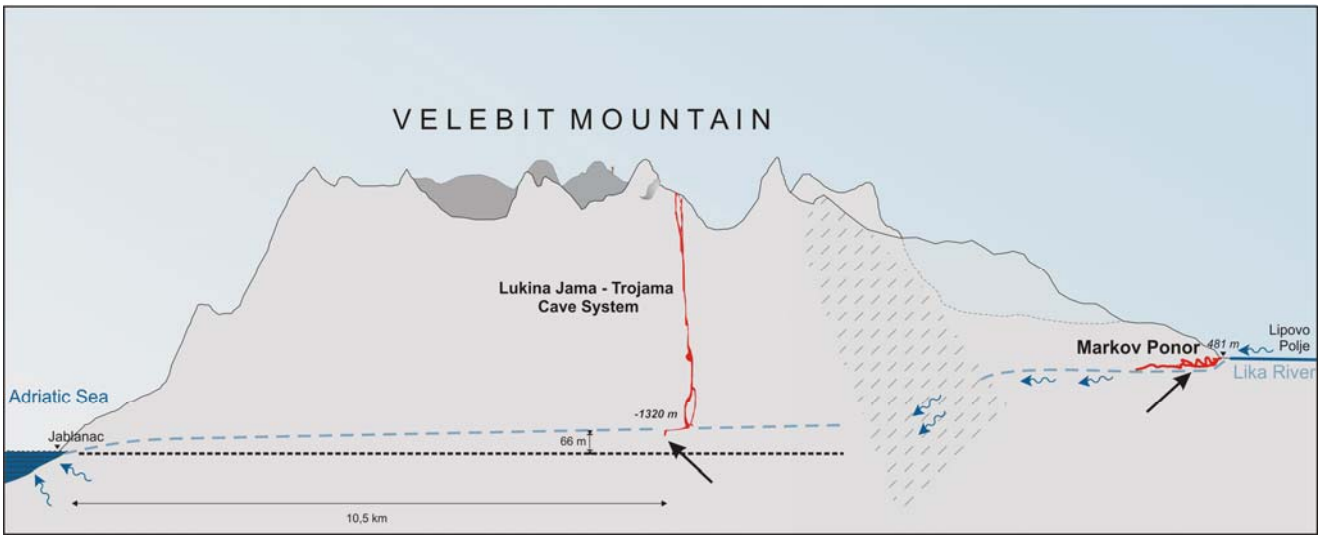

Figure 8. A cross section through Velebit Mountain, Lika, Croatia. The Markov Ponor and Lukina Jama – Trojama Cave System are hydrologicaly connected via underground conduits.

Divergence dating and evolutionary history

Our molecular clock estimates of divergence events within the Dreissenidae differ from those published previously [20,26,27]. All previous studies have utilised a strict clock for the timing of speciation events. In recent years, however, new methods have been developed that account for rate variation and assume uncorrelated rates of evolution [28,29], and these have been applied in the present study.

Although a wide uniform prior of 33.9-55.8 MYA was used to calibrate the origin of the Dreissenidae, the posterior estimates narrowed the divergence time to 37.4 MYA. This age estimate corresponds to the time frame of the formation of the Paratethys Sea [30] as well as the occurrence of the first certain dreissenid fossils [31]. Our study was not designed for divergence dating within *Dreissena* and the species coverage of this genus is incomplete in our dataset (for associated problems see Wilke [32]), but there is some correlation between our *Dreissena* divergence estimates and those reported in studies of Stepien et al. [20,26,27]. Their divergence ranges vary slightly from study to study, but they collectively place the split between *D. rostriformis bugensis* and *D. polymorpha* at around 10–15 MYA, which is consistent with the fossil record and supports our calibration choices. Our estimates of the divergence of *D. polymorpha* and the Balkan *D. blanci/D. presbensis* clade at 6.9 MYA is more ancient then reported by Stepien et al. [27]. The occurrence of *D. polymorpha* in lower Pliocene deposits from the southernmost remnants of Lake Pannon [3], suggest that this species followed the immigration route into the eastern Paratethys, presumably through the Dacian Basin [2], rather later. This implies that the common ancestor of the Balkan clade started migration towards the central Balkans during the late Miocene. Sometime during the Pliocene it settled in Lake Ohrid, which became the source of today's *Dreissena* biodiversity in the region [33]. This is in accordance with the late Miocene/early Pliocene timeframe of changes in distributions of *Dreissena* lineages [27]. The divergence between the two Balkan clades of *Dreissena* happened after ancestors of *D.*

blanci invaded the southern Ionian region from Lake Ohrid [33] around 1.9 MYA, according to our estimates.

Our results show a divergence time of 22.6 MYA for the split between the ancestors of the extant *Mytilopsis* species from the Americas and the ancestors of stygobiotic *Congeria* spp. This estimate roughly corresponds to the Oligocene/Miocene boundary and follows after the isolation and the establishment of the Paratethys as an independent biogeographic unit in Late Oligocene [2]. This is in accordance with Nutall [4], who proposed that founder populations of extant *Mytilopsis* lineages invaded the New World in the Late Oligocene. Another possible scenario is a gene flow blockade between *Mytilopsis* populations from both sides of the Atlantic as a result of the isolation of the Paratethys. Following this event, the New World *Mytilopsis* evolved independently of their relatives that remained in Europe. After the split with *Congeria,* that occurred in the long-lived Lake Pannon about 11.6 MYA [3], the European lineages of *Mytilopsis* became extinct.

According to the exponential clock model, the first split within the extant lineages of *Congeria* happened around 8 MYA, what approximately corresponds to the ages of the holodinaric groups of *Troglocaris* and *Proteus*, although 7.5-8.5 MYA was the lowest estimate of the inferred ranges for these taxa [34]. The lognormal clock gave different estimates and the discrepancy is probably due to the fact that the exponential model assumes most branch rates are small. Due to their generation times, reproductive strategies and pronounced adaptability that enabled high invasiveness, most dreissenids would not be expected to have slow mutation rates. The lognormal clock therefore probably gives better estimates. The lognormal clock estimate of 5.4 MYA corresponds approximately with the disappearance of *Congeria* from the fossil record, which coincided with large paleogeographical changes in the region involving the final disappearance of both Lake Pannon and the bordering Dinaride Lake System [3]. Although the Dinaride Lake System is more frequently suggested as the source of

stygobiotic *Congeria* ancestors [1,22] the adjacent Lake Pannon offers another possible alternative.

Lake Pannon was a long-lasting lake where *Congeria* originated and went through an exceptional radiation [2,3]. Its disappearance gradually progressed from north to south, and by the late Miocene/early Pliocene it occupied only the extreme south of its previous range, an area bordering the Dinaric Karst. Part of the Lake Pannon fauna has survived to the present day by immigration into other regions before the lake finally vanished. For example, Lake Pannon is considered to be the source of the Ponto-Caspian fauna and it also had an impact on the fauna of Balkan lakes such as Lake Ohrid [2]. According to one of the biogeographical scenarios presented by Albrecht et al. [35], the ancestor of endemic Balkan *Dreissena* migrated from the Pannon basin into central Balkan lakes that are situated even further from Lake Pannon than the neighbouring Dinarides. The origin of *Asselus aquaticus* (Linnaeus, 1758) lineages is also considered to be the western Pannonian region, from where it colonised the rest of Europe including the Dinaric Karst around 4–5 MYA [36]. It is, therefore, possible that the ancestral stock of the subterranean *Congeria* lineages invaded the Dinaric Karst from Lake Pannon. If so, this must have happened prior to the first divergence event that separated today's north-western and south-eastern lineages. Perhaps it coincided with the split of the "Dinaro-Caucasian" lineage of *Troglocaris* into Dinaric and Caucasian clades, which was estimated at 6–11 MYA [17], implying that there must have been a faunal interchange between the Dinaric water systems and the Paratethyan basins of Central Europe up to that time period.

The second divergence event occurred in the northern portion of the present distribution and separated the northern Bosnian populations of *Congeria mulaomerovici* sp. nov. from *Congeria jalzici* sp. nov. at about 2.5 MYA according to the lognormal clock. This occurred much later than the isolation of the northern Bosnian populations from the remaining *Proteus* (4.4-5.4 MYA) and *Troglocaris anophthalmus* (Kollar, 1848) lineages (3.7-5.3 MYA) [17,34]. Along with divergence time estimates of various Dinaric

groundwater taxa, the phylogeographical patterns are incongruent as well [15,16,18,34]. The disparities may be the result of dissimilarities in biology of these animals and/or a complex geological history of the Dinaric Karst. Progressive karstification alters hydrological relationships over time and consequently the distributions of phylogenetic lineages are often not concordant with present day hydrological regimes, for example in *Asellus aquaticus*[19], *Troglocaris anophthalmus*[18] and *Congeria jalzici* sp. nov..

On the other hand, the disparities in the divergence estimates may be a result of availability and employment of different molecular clock methodologies. In comparison to the other groundwater Dinaric taxa, hard-shelled dreissenids have a rich fossil record that can possibly enable more reliable time divergence dating. Unfortunately, plasticity of the dreissenid shell makes linking of cave *Congeria* lineages with any of the fossil species highly speculative so the questions regarding the last surface ancestor and its colonisation of the underground remain to be answered. Further studies are needed to resolve these issues and to create an integrated picture of the processes that shaped the subterranean biodiversity of the Dinaric Karst.

Conclusions

In conclusion, *Congeria* is a distinct member of the Dreissenidae that is separated from its closest extant relatives - *Mytilopsis* - by ~22-23 million years of independent evolution. The exact origin of the subterranean *Congeria* lineage is problematic because the innate plasticity of the dreissenid shell, as demonstrated in this study, does not allow the cave *Congeria* lineage to be related to the fossil dressenids of either the Lake Pannon or the Dinaride Lake Systems. Isolation in the Dinaric Karst underground has driven the speciation of the three allopatric lineages of *Congeria*: *C. kusceri*, *C. jalzici* sp. nov., and *C. mulaomerovici* sp. nov., herein identified morphologically and

genetically. Inter-specific morphological stasis of shell forms has not been interrupted during the colonisation of caves or subsequent speciations. Instead, a plesiomorphic shell form has been retained and remained relatively unaffected by the millions of years that the tree species have spent in isolation from each other. The divergent shell form within a single lineage illustrated by the ecophenotype of *C. jalzici* sp. nov. has possibly arisen as an adaptation to a specific subterranean microhabitat.

The tasks of understanding the evolutionary history of *Congeria* spp., especially their origin and colonisation of the subterranean habitats as well as how genotypic and ecophenotypic components interact, provide new interdisciplinary challenges.

Methods

Taxon sampling and identification

Samples of *Congeria* from all 15 caves (Figure 1) known to harbour living populations have been examined. Individuals were partly collected between 2008 and 2011. Others were obtained from the Croatian Natural History Museum, and the University of Ljubljana. *Dreissena polymorpha* Pallas, 1771 was collected from Lake Jarun, Zagreb, Croatia. *Dreissena rostriformis bugensis* (Andrusov, 1897) was obtained from Ijsselmeer, Lelystadt, The Netherlands, and *Mytilopsis sallei* (Récluz, 1849) was obtained from Hong Kong, China.

DNA extraction, amplification cloning and sequencing

The fragments of two mitochondrial (*COI* and *16S rRNA*) and two nuclear (*28S* and *18S rRNA*) genes were sequenced from one individual of *Dreissena polymorpha*, *Dreissena rostriformis bugensis, Mytilopsis sallei* and 44 different *Congeria* specimens from 15 locations including, for the first time, the type locality of *C. kusceri*, Žira ponor. DNA was extracted using DNeasy Blood & Tissue kit (Qiagen) or i-genomic DNA extraction kit

(Intron). The primers, PCR reaction components and cycling conditions are indicated in Additional file 2. PCR products were separated by electrophoresis in 0.5 to 1.5% agarose gels, excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen) or MEGAquick-spin PCR & Agarose Gel DNA Extraction System (Intron). DNA fragments were cloned into either pGEM-T or pGEM-T Easy Vector Systems (Promega) or were sequenced directly using an ABI PRISM 3100 automatic sequencer (Applied Biosystem).

PCR products were sequenced on both strands and inspected manually for ambiguities. The resulting sequences have been deposited in GenBank. Sequences from *Dreissena blanci* (Westerlund, 1890), *Dreissena presbensis* Kobelt, 1915, *Mytilopsis leucophaeta* (Conrad, 1831) and three selected outgroups were retrieved from GenBank. Accession numbers are listed in Table 4. Outgroups were chosen according to Park & O'Foighil [37] and Taylor et al. [38]. Since there is no consensus regarding the group most closely related to the Dreissenidae, *Mya arenaria* Linnaeus, 1758, *Corbicula fluminea* (O.F. Müller, 1774) and *Sphaerium striatinum* (Lamarck, 1818) were selected as outgroups.

Table 4. List of species and GenBank Accession numbers of sequences used in this study

Species	Source	Specimen	18S rRNA	28S rRNA	16S rRNA	COI
Congeria kusceri	Plitica, Popovo polje, BA	Pl2	JX099472	JX099493	JX099451	JX099431
		Pl4		JX524681	JX524654	JX524708
		Pl7		JX524682	JX524655	JX524709
Congeria kusceri	Žira, Popovo polje, BA	Z3	JX099475	JX099496	JX099454	JX099434
		Z4		JX524686	JX524659	JX524713

Congeria kusceri	Doljašnica, Popovo polje, BA	D1		JX524664	JX524637	JX524692
		D6		JX524665	JX524638	JX524691
		D10	JX099460	JX099481	JX099439	JX099419
Congeria kusceri	Gradnica, Neum, BA	G2		JX524666	JX524639	JX524693
		G5	JX099461	JX099482	JX099440	JX099420
		G7		JX524667	JX524640	JX524694
Congeria kusceri	Jama u Predolcu, Metković, HR	J4		JX524669	JX524642	JX524696
		J8		JX524670	JX524643	JX524697
		J10	JX099464	JX099485	JX099443	JX099423
Congeria kusceri	Pukotina u Tunelu Polje Jezero-Peračko Blato, Ploče, HR	P2		JX524679	JX524652	JX524706
		P5		JX524680	JX524653	JX524707
		P6	JX099470	JX099491	JX099449	JX099429
		P8	JX099471	JX099493	JX099450	JX099430
Congeria kusceri	Jasena, Vrgorac, HR	Jas1	JX099465	JX099486	JX099444	JX099424
		Jas4		JX524671	JX524644	JX524698
		Jas7		JX524672	JX524645	JX524699
Congeria kusceri	Tihaljina, Ljubuški, BA	T1	JX099474	JX099495	JX099453	JX099433
		T3		JX524684	JX524657	JX524711
		T6		JX524685	JX524658	JX524712
Congeria mulaomerovici sp.nov.	Oko, Lušci Palanka, Northern Bosnia, BA	O1	JX099469	JX099490	JX099448	JX099428
		O3		JX524677	JX524650	JX524704
		O5		JX524678	JX524651	JX524705
Congeria mulaomerovici sp.nov.	Suvaja, Lušci Palanka, Northern Bosnia, BA	S7		JX524683	JX524656	JX524710
		S8	JX099473	JX099494	JX099452	JX099432
Congeria	Dabarska	DB1	JX099459	JX099480	JX099438	JX099418

mulaomerovici sp.nov.	Pećina, Sanski Most, Northern Bosnia, BA	DB2		JX524660	JX524633	JX524687
		DB3		JX524661	JX524634	JX524688
Congeria jalzici sp.nov.	Markov Ponor, Lipovo Polje, Lika, HR	M2		JX524675	JX524648	JX524702
		M6		JX524676	JX524649	JX524703
		M9	JX099468	JX099489	JX099447	JX099427
Congeria jalzici sp.nov.	Dankov Ponor, Lipovo Polje, Lika, HR	DP2		JX524662	JX524635	JX524689
		DP3	JX473583	JX524663	JX524636	JX524690
Congeria jalzici sp.nov.	Lukina Jama – Trojama Cave System, Northern Velebit, Lika, HR	L1	JX099466	JX099487	JX099445	JX099425
		L2		JX524673	JX524646	JX524700
		L3		JX524674	JX524647	JX524701
		L4	JX099467	JX099488	JX099446	JX099426
Congeria jalzici sp.nov.	Izvir Jamske Školjke, Metlika, Bela Krajina, SI	I1	JX099462	JX099483	JX099441	JX099421
		I2	JX099463	JX099484	JX099442	JX099422
		I3		JX524668	JX524641	JX524695
Mytilopsis sallei	Lam Tsuen River, Shatin, Hong Kong, China		JX099476	JX099497	JX099455	JX099435
			JX099477		JX099456	
Mytilopsis leucophaeata	GenBank		AF305704	EF414468	EF414448	HM100258
Dreissena polymorpha	Jarun Lake, Zagreb, HR		JX099478	JX099499	JX099458	JX099437
Dreissena bugensis	Ijsselmeer, Lelystadt, The Netherlands		JX099479	JX099498	JX099457	JX099436
Dreissena presbensis	GenBank		-	EF414469	EF414449	EF414491
Dreissena blanci	GenBank		-	EF414471	EF414459	EF414483
Sphaerium spp.	GenBank		*S.corneum*	*S.corneum*	*S.striatinum*	*S.striatinum*

			AM774537	AM779711	AF152041	AF120667
Mya arenaria	GenBank		AF120560	FM999792	AY377618	AF68
Corbicula fluminea	GenBank		AF120557	DQ343848	AF038999	U47647

DNA sequence alignment

Sequences were aligned using the ClustalW option in BioEdit [39], and the resulting alignments were inspected manually and tested using Gblocks Server [40,41]. The regions identified as problematic and aligned poorly were excluded from subsequent analyses. The *COI* fragment showed significant variation, which was confirmed with the test for substitution saturation [42] implemented in Dambe [43]. Since there were no topological differences between the analyses ran with or without the 3rd codon position, the COI 3rd codon position was included in all phylogenetic analyses.

Phylogenetic analyses

The final dataset consisted of 1736 bp of the *18S rRNA* gene, 1046 bp of the *28S rRNA*, 470 bp of the *16S rRNA* and 595 bp of the *COI* gene fragment. The best-fit partitioning schemes as well as nucleotide substitution models for each partition were calculated using the PartitionFinder [44]. "Branchlengths" were set to unlinked allowing branch length to be estimated independently for each subset, "search" to all in order to analyse all possible partitioning schemes, and "models" were set to mrbayes or to all, depending on whether the results were used for setting up Bayesian or ML analyses, respectively.

The rate heterogeneity test performed in PAUP [45] showed significant incongruence between different partitions. The phylogenetic analyses were, therefore, performed for each gene fragment separately. Inspection of the results revealed inconsistencies in the

positions of *Dreissena* spp. in the *18S rRNA* tree and the position of *Mytilopsis* spp. in the *16S* gene tree. Since none of the conflicting branches had good posterior probabilities or bootstrap support, concatenated trees were constructed using Bayesian, MP and ML methods.

Maximum parsimony (MP) analyses were conducted in MEGA 5 [46]. The trees were obtained using the default settings. All sites were equally weighted and gaps were partially deleted (sites were deleted if gaps were present in more than 5% of the sequences). The resulting phylogeny was tested by 5,000 bootstrap replications.

Several independent Bayesian searches were run in MrBayes version 3.1.2 [47] for altogether 10 million generations with a sampling density of 1/100. The starting tree was random and partitioning scheme and substitution model type fixed according to results in PartitionFinder while model parameter values were estimated. First 17% of the generations that had average standard deviation of the split frequencies above 0,01 were discarded as burn in. Additionally, mixing of chains and ESS values were checked using Tracer [48]. Bayesian posterior probabilities (BPP) were estimated from the 50% majority-rule consensus tree.

Five replicates of maximum likelihood (ML) searches were performed in Garli [49]. Partitions and model types were set as determined in PartitionFinder while model parameter values were estimated by the program. All program settings were default except "streefname" which was set to random enabling multiple searches with random starting trees, "modweight" which was set to 0.003 according to developer's instructions in order to ensure that partitioned models are properly optimised and during 200 bootstrap replicates "genthreshfortopoterm", the first part of termination condition, was set to 10,000. Bootstrap consensus trees were obtained in Geneious Basic.

Divergence times were calculated on a reduced dataset using the relaxed clock in Beast 1.6.2. The dataset consisted of one sequence from the putative type locality of each *Congeria* lineage. *Mya arenaria* was included as an outgroup. The dataset was partitioned by different gene fragments, and substitution models were unlinked. To ensure that the dataset was robust enough for the divergence dating and that posterior ranges were not dominated by prior choices, we used both lognormal and exponential clocks, explored different prior distributions on various parameters (calibration nodes and means of the branch rates) and ran the analysis using sampling from the prior only. The Birth-death model was used as a tree prior.

Based on fossil data, we set up two points to calibrate the tree. The first undisputed dreissenid fossil is from the Priabonian Age (33.9–37.2 MYA), but there is also a questionable record from the Ypressian Age (48.6–55.8 MYA) [31]. We, therefore, used uniform prior spanning entire Eocene (55.8–33.9 MYA) to calibrate the family node. The origin of *Dreissena* was used as a second calibration point. *Dreissena* has a clear morphological feature, the lack of a shell apophysis for the attachment of the anterior byssal retractor muscle, which distinguishes it from other, both extant and fossil, genera. Although this feature has previously been considered a polyphyletic trait (references in Müller et al., 1999, e.g., Papp, 1950; 1985; Lueger, 1980; Taylor in Gray, 1988), our and other genetic studies [27,35] clearly demonstrate that *Dreissena* and, therefore, the loss of the apophysis, is of monophyletic origin. *Dreissena* first appeared in Lake Pannon 11.6 MYA [3]. Accordingly, we used lognormal prior with the onset of 11.6 MYA and a standard deviation of 0.75 to include the beginning of the Sarmatian Period (12.7 MYA) within the 95% of the prior probability density, because part of the Lake Pannon fauna, including dreissenids, originated in the Samartian Paratethyan Lakes [2,50].

With the final settings, we ran three independent runs and a total of 60 million generations, which after 15% burn in, yielded 51 million trees. We compared independent chains in Tracer to ensure that the chains had reached stationarity and

converged to the same posterior distribution. There was no significant difference between the runs, and LogCombiner was thus used to pool all estimates into one file.

Competing interests

The authors declare no competing interests.

Authors' contributions

HB conceived and designed the study, performed field work, carried out the molecular genetic studies, participated in the phylogenetic and morphological analysis, performed molecular clock analysis and drafted the manuscript. BM performed the morphological studies and statistical analysis and drafted the manuscript. MP participated in the phylogenetic analysis, HĆ conceived, designed and coordinated the study. All authors read and approved the manuscript.

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Additional file 1. Shell morphometrics. The file contains details of the methods and results of morphometric shell measurements.

Results

Morphometric shell measures differed between the three putative species of *Congeria* (Table S1). Differences in the median values of shell length among the three species groups were significant (H = 0.238; df = 2; *P* = 0.010). Both pairwise median shell length and median shell width of *C. mulaomerovici* sp. nov. differed significantly from that of the two sister species, whereas *C. jalzici* sp. nov. and *C. kusceri* median shell lengths and widths were not significantly different from each other ($p > 0.05$). Overall shell height medians were significantly different. Median shell height of *C. mulaomerovici* sp. nov. differed significantly from *C. kusceri* but not *C. jalzici* sp. nov., and nor did *C. jalzici* sp. nov. differ from *C. kusceri*. Shell width median differences were highly significant.

The results of linear regression undertaken separately for each of the putative species of *Congeria* indicated that length to height, and height to width length have a very good linear predictability to each other ($r^2 > 0.8$).

Comparison of species specific shell morphometrics by general linear modelling of the linear regression lines of shell length (SL) as a function of shell height (SH), shell length (SL) of shell width (SW) and shell height (SH) of shell width (SW) were not significantly different with regard to their slopes. Comparison of linear regression line intercepts, however, showed that *C. jalzici* sp. nov. deviated significantly from *C. kusceri* and *C. mulaomerovici* sp. nov., whereas the intercept difference between *C. kusceri* and *C. mulaomerovici* sp. nov. was not significant ($p > 0.05$) (Fig.S1).

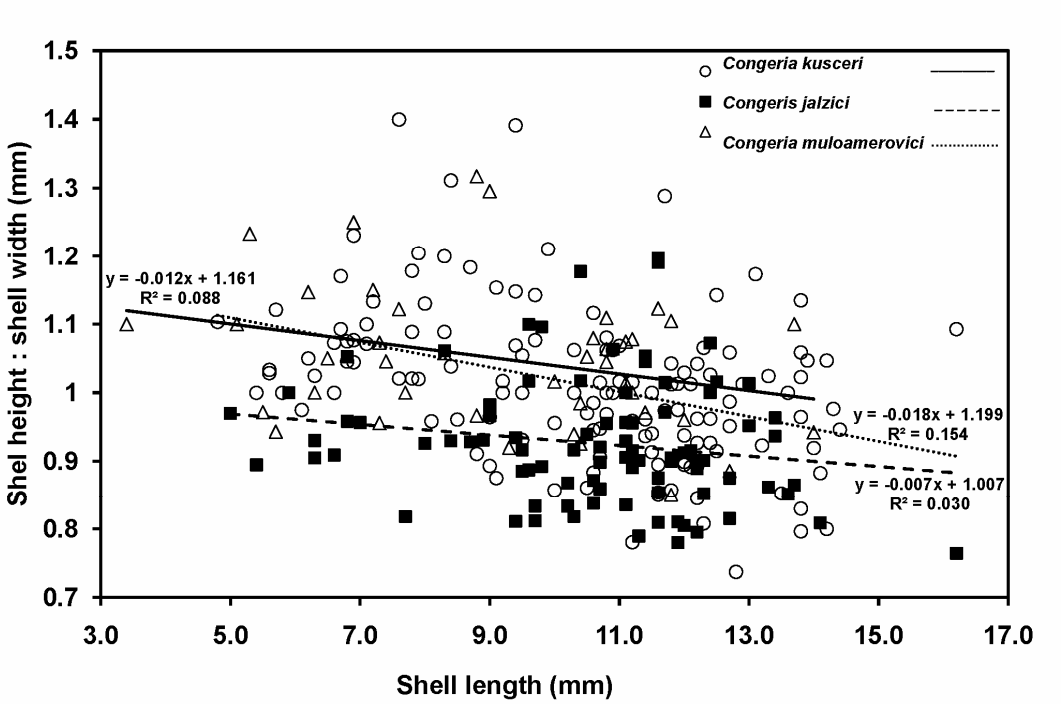

Figure S1. Shell morphometrics of the three putative species of *Congeria*.

Congeria kusceri (O), *Congeria jalzici* sp. nov. (●) and *Congeria mulaomerovici* sp. nov. (Δ). Linear correlations between shell length and shell height/shell width are low ($r^2 < 0.2$).

Table S1. Statistical tests of differences in morphometric shell measures between the three putative species of *Congeria*. An overall comparison of medians was tested using Kruskal-Wallis One Way ANOVA on Ranks, and all Pairwise Multiple Comparison Procedures using Dunn's Method. Significance levels are indicated by: *** = $p \leq 0.001$; ** = $p \leq 0.01$; * = $p \leq 0.05$; and ns = not significant.

	SL, mm		**SH, mm**		**SW, mm**	
Overall comparisons of median	H	*P*	H	*P*	H	*P*
	0.238	**	8.245	*	14.306	***
Pairwise comparisons of median	Q	*p*	Q	*p*	Q	*p*
C. kusceri vs. *C. jalzici*	-	ns	-	ns	-	ns
C. kusceri vs. *C. mulaomerovici*	2.772	*	2.790	*	3.182	*
C. jalzici vs. *C. mulaomerovici*	2.862	*	-	ns	3.710	*

Methods

Intact shells of all *Congeria* specimens were measured using Vernier calipers along their greatest length and maximum heights and widths to the nearest 0.5 mm. These datasets were pooled for an analysis of shell dimension differences between populations and putative species and examined for statistically significant differences. Because the normality test for differences in One Way Annova did not support significant differences for all three shell dimensions, an overall comparison between groups was made using a Kruskal-Wallis One Way ANOVA on Ranks, and all Pairwise Multiple Comparison Procedures using Dunn's Method. Comparisons of species-specific shell dimensions were made using a general linear modeling of regression lines (linear), slope and intercept. The general linear modelling was carried out using RStudio (v. 0.95.262) to test for differences in slope and intercept of regression lines of the three putative species [34]. Tests were carried out using Sigmastat.

Additional file 2. The PCR primers, reactions and conditions used in this study. The file contains details on the PCR primers, reaction components and cycling conditions used in the study.

Region	Primers	Primer sequence	Primer reference	PCR conditions	PCR reaction
COI	HCO2198	taaacttcagggtgaccaaaaaatca	[57]	94ºC 5min 94ºC 30s 50ºC 30s 35x 72ºC 40s 72ºC 5min	25 µL PCR mix* 15 pmol each primer 80-100 ng DNA 35-40 pmol Mg water to 50 µL
	COL1	ttgtgrgctggcttggtg	This study		
*18S***	18SKBPF	ctggtgccagcagccgcgg	[58]	94ºC 5min 94ºC 30s 50ºC 45s 35x 72ºC 70s 72ºC 5min	25 µL PCR mix 20 pmol each primer 80-100 ng DNA water to 50 µL
	18SKBPR	tggtgcccttccgtcaattcc			
	18SMR	ttgatccttctgcaggttcac	[59]		
	18SMF	aacctggttgatcctgccag			
28S^{+}	D1F	gggactacccctgaatttaagcat	[41]	94ºC 3min 94ºC 30s TA^{++} 30s 72ºC 60s 72ºC 5min	25 µL PCR mix 15 pmol each primer 80-100 ng DNA 35-40 pmol Mg 50 ng BSA water to 50 µL
	D2F	tcagtaagcggaggaa			
	D23F	gagagttcaagagtacgtg			
	D4RB	tgttagactccttggtccgtgt			
	D6R	ccagctatcctgagggaaacttcg			
	D6Rb	ggttccctccgaagtttcc	This study		
16S	16SLRN	cgcctgtttatcaaaaacat	[60]	94ºC 2min 94ºC 30s 50ºC 30s 30x 72ºC 60s 72ºC 5min	25 µL PCR mix 15 pmol each primer 80-100 ng DNA 35-40 pmol Mg water to 50 µL
	16SLRJ	ctccggtttgaactcagatca			

* ReadyMix Taq PCR Reaction Mix with $MgCl_2$ (Sigma)

** The *18S rRNA* marker was amplified in two overlapping fragments, one with 18sKBPF/18sMR and the other with 18sMF/18sKBPR primer pairs.

$^{+}$ The *28S* gene fragment was amplified with either the most external primer pair (D1F/D6R) or with different combinations of primers.

$^{++}$ The Touch down PCR program was used to amplify the *28S rRNA* fragment. The annealing temperature was set at 52ºC during the first five cycles, 55ºC during the next five cycles and 58ºC for the last 25 cycles.

3. DISCUSSION

There are several features that make caves an ideal ecosystem for understanding the processes of evolution. The cave ecosystems are rather simple and very similar throughout the world. A small number of abiotic components characterize the underground habitats and these are relatively constant and known. The biotic component is fairly simple as well. A limited number of animal groups have successfully colonized the underground. For example there are no cave mollusks other then gastropods and just one bivalve, there is not one representative of phylum Echinodermata and of the phylum Chordates only fish and amphibians have obligatory cave representatives. Further, the number of species within one cave is very limited. The richest cave with respect to biodiversity is Vjetrenica in south Herzegovina (BIH) with a total of 219 taxa, of which 101 are obligatory cave species [35]. However, 101 taxa include both terrestrial and aquatic animals from different types of habitats so the number of inhabitants per single habitat type is even smaller. Apart from being relatively simple, the subterranean habitats have one other very important advantage: the direction of the evolution is known. Namely, the ancestors of all cave dwellers came from the surface habitats. During their colonization of the subsurface, they went through the process of adaptation to, as already pointed out, a very uniform habitat with similar selection forces operating regardless of time and place. These similar selection pressures repeatedly resulted in the evolution of the same or similar morphological, physiological and ethological adaptations. Knowing the molecular basis of adaptive traits can help in understanding the environmental and developmental interactions in the evolution of novel phenotypes.

The loss of melanin pigment

The hallmarks of cave adaptations are the regression of eyes and melanin pigment. These traits evolved convergently in a vast majority of cave inhabitants. So far, the molecular basis of the loss of melanin is known only in the cavefish *Astyanax mexicanus*. The vast majority of cave inhabitants, however, are invertebrates so

identifying the causes that have led to loss of melanin in invertebrates is necessary to understand the evolutionary forces behind the occurrence of albinism in the caves. Unfortunately, the melanin synthesis pathway is not studied in the majority of invertebrates. The only exceptions are insects, however, vast majority of cave insects (especially those living in Croatian caves) are not albino. Luckily, there is an exception, insects from the family Cixiidae. There is not one known cave-dwelling cixiid from the Dinaric Karst, although several cave populations from different parts of Croatia are recorded (unpublished data) but still not investigated properly. Despite their uncertain taxonomical and ecological status, cixiids offer several advantages and were therefore used in this study. Cixiids have colonized caves in different parts of the world and of different geological setting [32,36]. For example the ones from the islands of Hawaii, USA, live in lava tubes as opposed to limestone caves in Croatia. Another very important feature of these insects is their feeding preference. Cixiidae are confined to the underground habitats where roots penetrate through the cave ceilings providing a sufficient amount of food. Therefore, they are not food-limited like the majority of other cave inhabitants and can be used to test one of the most common hypothesis of cave biology - energy economy as the cause for the regression of melanin pigmentation and other traits.

The melanogenic substrate assay has identified that the first step in the melanin synthesis pathway is affected and causes the albino phenotype in cixiids from both Hawaiian lava tubes and Croatian limestone caves. Similarly, different populations of *Astyanax* have acquired albinism by independent mutations in different regions of the same gene - *oca2* [20], which also functions during the first step. Moreover, the results of melanogenic substrate assay performed on a variety of cave invertebrates including the sponge *Eunapius subterraneus* and the bivalve *Congeria kusceri* point to the first step of melanin synthesis as being affected in diverse cave taxa (unpublished data). From these results it seems that the evolution of albinism is marked by both parallel and convergent changes at the first step of melanin synthesis pathway. Why the first step is repeatedly affected in various cave animals?

While there has been little doubt that the evolution of constructive traits is adaptive for life in caves, the evolution of regressive traits has caused a lot of debate and many different theories have been developed to explain this phenomenon [37,38]. Many of these theories are outside the framework of modern evolutionary theory and therefore nowadays completely abandoned. At present, natural selection and neutral mutation are the two main and opposite evolutionary forces used to explain the regression of traits in cave dwellers.

The explanations for repeatedly affecting the first step of melanin synthesis within the neutral mutation theory could be that the gene or genes operating at this step are more prone to mutations because of their size or position. The size of *oca2* gene (345 kb in humans) [39] and its position in the region associated with frequent crossovers and deletions provides support for this explanation. However, the fact that Bradić et al [40] discovered signs of selection in the OCA2 of several independently evolved *Astyanax* cave populations discards all neutral explanations, at least in the case of *Astyanax*. Another possibility is the existence of a developmental constraint. The first step may be a frequent target because all genes operating at other steps in the pathway are pleiotrophic and therefore can't be eliminated without affecting other functions and processes. However, there are a few types of albinism in humans and these are caused by mutations in several different genes showing that other genes can be mutated without detrimental effect on their carriers [41]. The other explanations would fall within the frame of natural selection. Natural selection can directly choose individuals bearing mutations in the first step of melanin synthesis because it saves energy which is quite limited in the caves. However, several facts are not in line with the expectations of this theory. For one, melanin synthesis is not costly as it starts with an amino acid [42]. Second, Cixiidae, as already mentioned, are not food limited and also there are nutrient rich habitats within the caves and in different ecosystems where the occurrence of albinos is frequent. Although energy saving must be beneficial, it seems that it may not be the driver of the evolution of albinism. The last hypothesis assumes the existence of another, pleiotrophic, function that is affected by mutation in the first

step of melanin synthesis. This other function might have a beneficial advantage for the animal and hence be selected for.

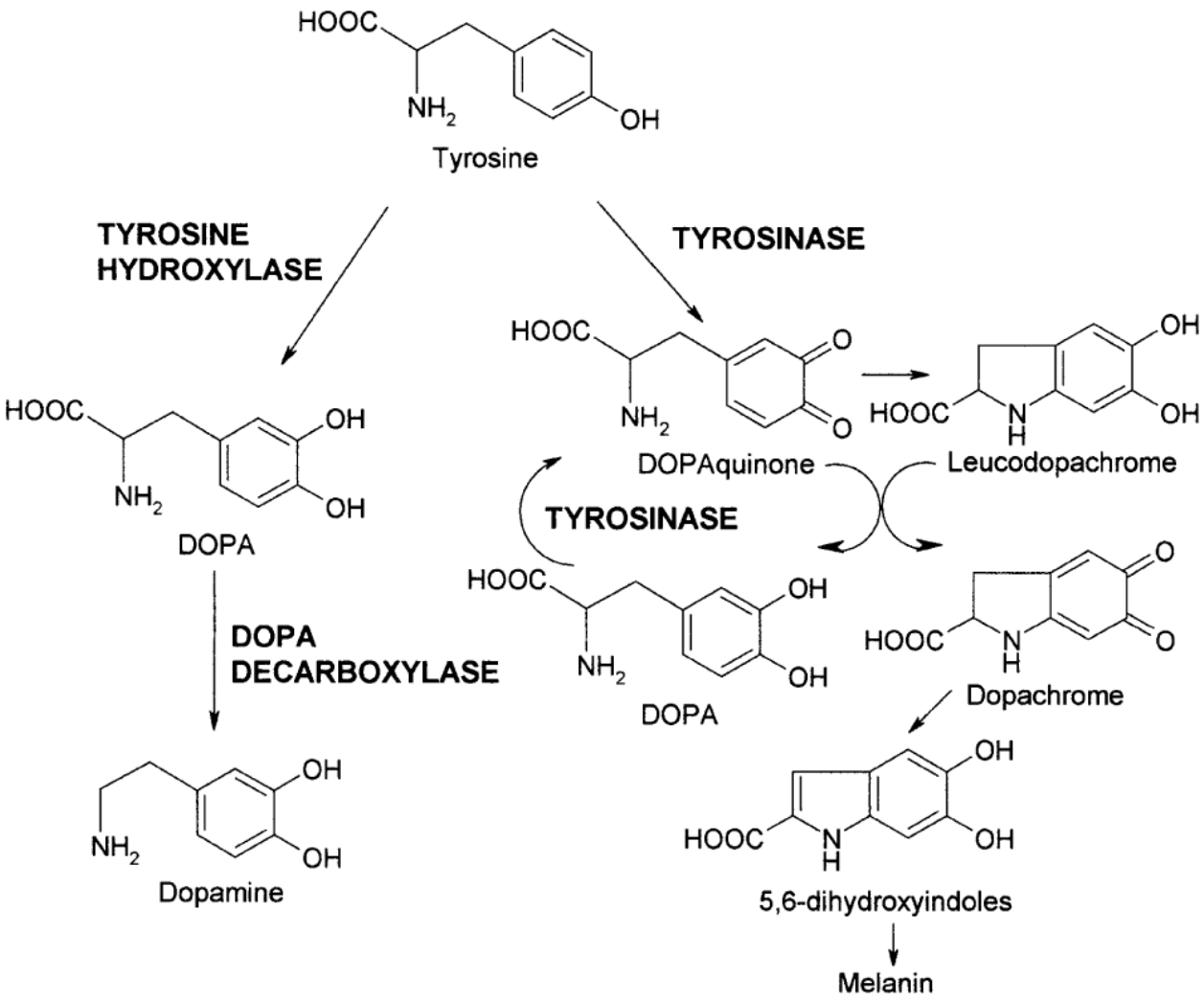

Figure 7. Both melanin and catecholamine synthesis pathways start with the same precursor, L-tyrosine. In the catecholamine pathway, L-tyrosine is converted to L-DOPA by TH which is converted to dopamine by DDC **[43]**. Dopamine gets further converted to norepinephrine by the enzyme called Dopamine β hydroxylase.

L-tyrosine, the precursor of melanin synthesis is also a precursor of catecholamine synthesis (Figure 7). The utilization of L-tyrosine in the melanin synthesis is stopped by

blocking the first step and L-tyrosine can be used in the synthesis of catecholamines. In support of this theory is the fact that increased levels of L-tyrosine, dopamine and norepinephrine were found in larvae and adult brains of *Astyanax mexicanus* fish from cave Pachon as well as increased levels of norepinephrine in the kidneys of adults when compared to surface populations (SF). Furthermore, after knocking down OCA2 in SF larvae by translation blocking morpholino, the levels of L-tyrosine and dopamine increased [44]. This suggests that a block in the melanin synthesis can increase the levels of L-tyrosine, the main precursor of catecholamine synthesis and lead to higher levels of catecholamines, potentially beneficial for life in the caves. Catecholamines are neurotransmitters and hormones involved in numerous physiological functions and in general have an influence on the overall homeostasis and state of the organism [45,46]. This is the first demonstration of the potential adaptive value for a regression of melanin pigmentation in cave animals.

Molecular phylogeny of the cave sponges and bivalves

The colonization of the caves by surface animals is accompanied by strong selection forces that produce a series of distinct morphological, physiological and other adaptations in many diverse groups of animals. Consequently, morphology of cave inhabitants is often misleading and results in erroneous identification of these taxa, their inaccurate placement into higher systematical categories, the lack of perception of divergence due to cripticity etc. Thus, the molecular phylogenetic analyses have become a vital tool in the biospeleology research.

The molecular analysis of cave sponge *Eunapius subterraneus* revealed its incorrect taxonomical position. The cave sponge is more closely related to freshwater sponges from the cosmopolitan genus *Ephydatia* Lamouroux 1816 then to its congenerics. The same study identified a series of problems in the phylogeny of the whole group of freshwater sponges, suborder Spongillina. Not only are several genera paraphyletic or

polyphyletic but the systematics at the level of families is not concordant with the molecular data. For example, the whole family Lubomirskiidae, endemic to Lake Baikal, is nested within Spongillidae, as a sister clade to *Ephydatia* and a few other sponge species including the cave sponge. Close affinity to genus *Ephydatia* is true for a number of other endemic taxa. For example, the endemic sponge from Chagatai and Tore Khol lakes (south Siberia) *Baikalospongia dzhegatajensis* (Rezvoj, 1936) is most closely related to *Ephydatia fluviatilis* (Linnaeus, 1758) [47], as are several endemic species from the order Malawispongiidae, for example sponge from Lake Ohrid assigned to endemic genus *Ohridaspongia* sp. Arndt 1937. Also, endemic species *Cortispongilla barroisi* (Topsent 1892), from the order Malawispongiidae, and *Ephydatia syriaca* Topsent 1910 from Lake Kinneret in Middle East have been synonymised with *Ephydatia fluviatilis* [48].

It is easy to understand the lack of corroboration between molecular phylogeny and classical systematics of the sponges. Their body plan is very primitive and a limited number of morphological characters that can be employed together with pronounced phenotypic plasticity (see later) make sponges the most difficult of all metazoans for classification. Clear taxonomic definitions are lacking even at the level of orders [49].

Due to a series of molecular analysis [47,50–52] done in the last decade, a pattern in the evolution of freshwater sponges emerges. The colonization of freshwater realm was successfully accomplished by a single common ancestor in the Jurassic. It subsequently populated freshwater bodies in all continents except Antarctica diverging into a series of species and genera [53]. Some of the widespread species, seemingly most often from the genus *Ephydatia*, colonized long-lived lakes or other stable habitats such as caves. Occasionally they went through a radiation like in Lake Baikal.

The ability to form gemmules probably had a major impact on a dispersal success of freshwater sponges [54]. Gemmules are dormant bodies that consist of a group of totipotent cells enclosed in the protective capsule. They are a form of asexual reproduction but their main role is in surviving the hostile environments. However,

following colonizations of constant environments, a reduction in the number of gemmules or their complete loss often occurred. The current practice to separate those species into their own systematic category led to formation of new genera (such as *Clypaetula* Addis and Peterson 2005) or orders (for example Lubomirskiidae and Malawispongiidae) which were in subsequent molecular analyses shown to be invalid or at least problematic.

The molecular analysis of cave bivalves, assigned to *Congeria kusceri* species, revealed a situation of overlooked biodiversity. Cave bivalves actually comprise 3 divergent species. This is not surprising since bivalve populations have a holodinaric but disjunctive distribution. Eight populations, including the type one from the cave Žira Ponor, live in Neretva basin, south Dalmatia and Herzegovina, and belong to *C. kusceri* species. Three populations in north-western Bosnia live in River Sana basin and were assigned to *C. mulaomerovici* Morton & Bilandžija 2013, while 3 populations from Lika River basin in Croatia and 1 population in Kupa River basin in Slovenia were described as *C. jalzici* Morton & Bilandžija 2013. Interestingly, *C. jalzici* is distributed not just in two separate river basins but these basins belong to separate sea drainages. Lika River flows into Adriatic Sea while Kupa River belongs to Black Sea catchment. The distribution of *C. jalzici* is therefore not concordant with the contemporary hydrogeological regime, which is true for many subterranean taxa (see later).

Morphological stasis and phenotypic plasticity

This study has identified conflicts between phenotypic and genetic differentiation in both cave sponges and bivalves. Cave sponge exhibits several distinct body morphologies (Figure 8), two already described [29] and two subsequently discovered: small round and big flattened habitus. Only one habitus is present within a single locality and there is no geographical or hydrological relationship that underlies the occurrence of different morphologies in different localities. Therefore, the body of the

cave sponge is shaped by environmental factors. This conclusion is further corroborated by molecular phylogenetic analyses showing no significant genetic distinction between populations harboring diverse morphologies (unpublished data). The same analyses showed no genetic distinction between the two cave sponge subspecies. The subspecies *E. s. mollisparspanis* was separated from the nominal *E. s. subterraneus* on the basis of skeletal morphology (the size and spinulation of megascleres) [30]. Thus, skeletal features can't be used as a robust taxonomical marker either.

Figure 8. Different habitus of several cave sponge populations: Rokina Bezdana Cave (top left), Zeleno jezero Lake (top right), Rudnica VI (bottom left) and Tounjčica Cave (bottom right).

In the suborder Spongillina one of the major characters used for species determination, but also higher level systematic, is the presence, shape and anatomy of gemmules. However, as already mentioned, many sponges that live in stable habitats have lost the ability to produce gemmules.

Cave sponge didn't entirely loose gemmules but their number is reduced. In the context of being closely related to *Ephydatia* that has birotules forming the gemmular layer, the fact that cave sponge gemmuloscleres are oxae (and in fact it is debatable whether these are real gemmulosceleres or megascleres tangentially encapsuling gemmules [53]) can be considered as a reduction due to life in more stable but not entirely constant environment. It happens rarely, but if the dry season lasts very long, water levels in the caves can drop considerably and, as encountered during a fieldtrip in 2008 (unpublished data); cave sponges can be left out in the dry. That is probably the reason why cave sponges didn't entirely lose their gemmules although reduction in the number and structure has happened.

In conclusion, apart from body shape and skeletal morphology, the presence and anatomy of gemmules too can be misleading in deciphering relationships of freshwater sponges. The plasticity of sponges is well known and can be even manipulated in the lab [55]. As already pointed out, it is one of the underlying causes for a notoriously difficult systematics and species identification of sponges. Molecular analyses are therefore invaluable tool for distinguishing cases of phenotypic plasticity from phylogenetic diversification of freshwater sponges.

Cave bivalves show an interesting pattern of morphological evolution. The shape of Lukina Jama – Trojama bivalves is different and much less robust compared to all other populations due to a life in part of a deep phreatic aquifer in the heart of Velebit Mountain where water dynamics is apparently not as violent as elsewhere. Bivalves in Lukina Jama are localized in just one small part of the sump where only vertical changes of water levels occur and there are no horizontal flushes that would rip bivalves off the substrate.

During last 5-6 My, which is the age of the last common ancestor, a diversification into three separate *Congeria* species happened. Basically the same shell plan of bivalves from all localities except Lukina Jama indicates either exceptional morphological stasis or outstanding convergence. Either way, it can be concluded that the bivalve shell is a highly plastic trait so its use in deciphering relationships among dreissenids is at this point limited. A thorough reexamination of shell characters is needed to identify the ones that will accurately reflect phylogenetic relationships.

Comparative phylogeography of subterranean aquatic taxa

A disagreement between hydrogeology and phylogenetic pattern of a certain lineage is a situation frequently encountered in cave biology that can be manifested in various ways. The same clade can be distributed across the divide between sea drainages as demonstrated in *Congeria jalzici*. Other examples include the isopod *Asellus* sp. [27] and a few lineages of cave shrimp: "Para-pretneri" clade in the "Dinaro-Caucasian" lineage [26] and "Western Slovenian" clade of *Troglocaris anophthalmus* lineage [56]. A recent find of cave sponge population at the bottom of Lukina Jama – Trojama System places the cave sponge in this category too. This is its only locality in the Lika River basin that drains towards Adriatic Sea. Preliminary results, though, do show some level of genetic differentiation (unpublished data) but to which extent it remains to be determined. Small genetic distances can be interpreted as either the consequence of slow molecular evolution, a known fact for sponges [49] or a relatively recent split. Future analyses will resolve this issue and determine whether sponges from Lukina jama should be placed in a separate taxonomical category.

The same clade can be distributed over a wide range of apparently separate river drainages. "Adriatic" lineage of *T. anophthalmus* is outstanding in lacking any significant genetic differentiation across a large area, contrary to model developed by Trontelj et al. [57]. It is distributed in a number of river basins, starting from Gacka River basin in Lika across catchments of rivers Zrmanja, Krka, Neretva, Trebišnjica and there is even a

population on the Island of Brač [56]. Another example is *Proteus*, whose "Adriatic" lineage is also distributed from Krka drainage in northern Dalmatia to Popovo polje in southern Herzegovina.

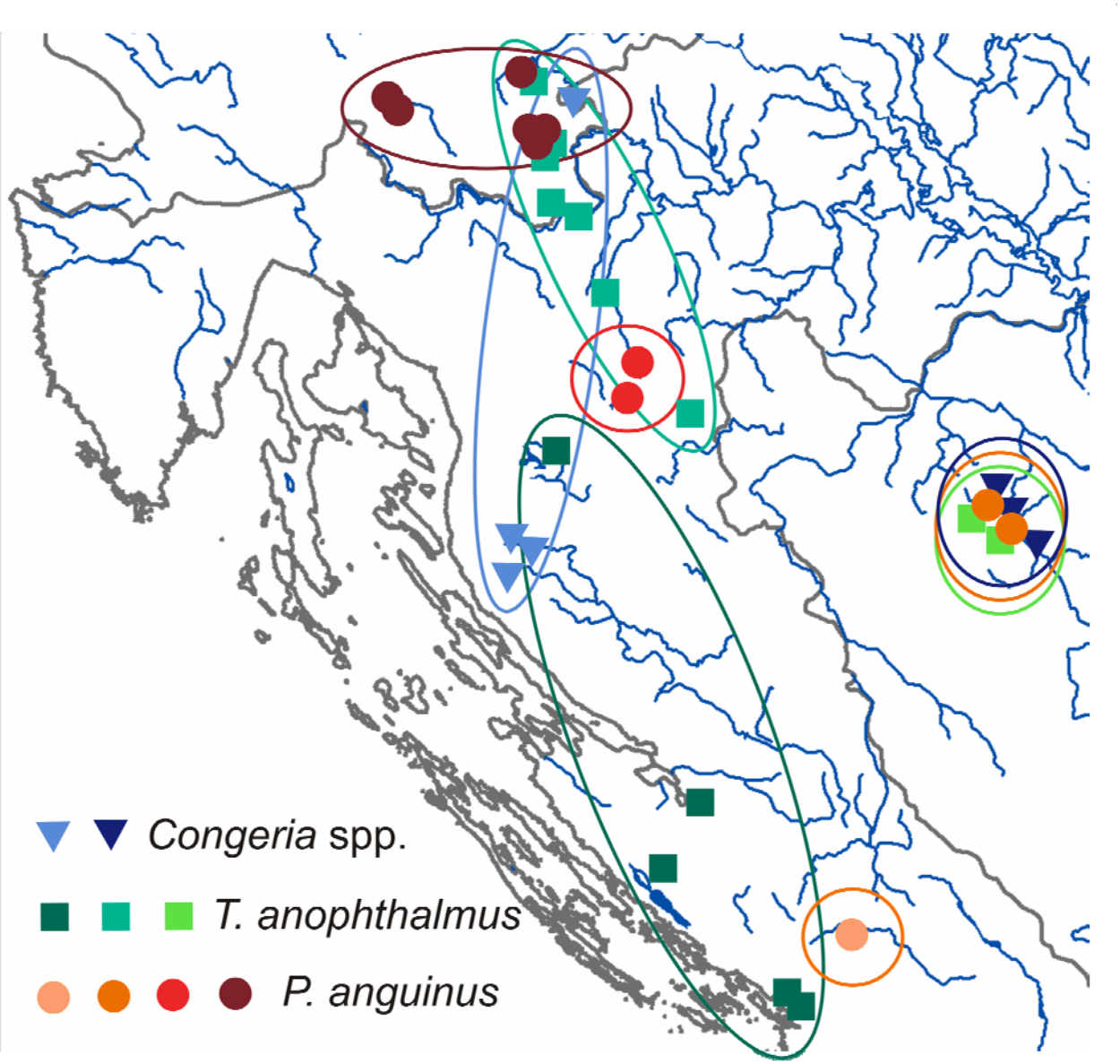

Figure 9. The distributions of phyletic lineages of the olm, cave shrimp and cave bivalve species in part of the Dinaric Karst.

It is expected that co-distributed lineages go through the same historical evolutionary processes resulting in the same phylogeographical patterns. However, there are cases of co-distributed cave taxa not showing identical phylogeographical patterns within the same region (Figure 9). For example, *C. jalzici* encompasses populations from Lika basin in Croatia and Bela Krajina population from eastern Slovenia. On the other hand the olm populations from Bela Krajina belong to "eastern Slovenian" lineage while "Lika" lineage of olm occupies localities in Ogulin region and in the Gacka River basin [25]. Unfortunately there are no records of *Proteus* in the Lika River basin so far [58,59] but both Ogulin region and Gacka River basin lie in between Bela Krajina and Lika basin so it can be concluded that the distribution of lineages of these animals is not concordant. The third pattern within the same region is seen in *T. anophthalmus*. "Adriatic" clade of shrimp encompasses populations from Popovo polje to Gacka River, but no sample from Lika basin was included in the study. Since Gacka River is further north then Lika River it can be assumed that the "Adriatic" lineage would also include *Troglocaris* from Lika basin [56]. This is contrary to both *Congeria* and *Proteus* phylogeography where one clade is distributed in Lika (*Congeria*) or Gacka (*Proteus*) basin and populations further south are included in a different clade. Another lineage of shrimp is distributed from eastern Slovenian localities to Kordun in Croatia (including Ogulin region) contrary to both *Congeria* and *Proteus*. As already mentioned *C. jalzici* is distributed further to the south and includes Lika populations. *Proteus* has "Lika" lineage in wider Ogulin region that is separated from eastern Slovenian populations.

Lastly, there are examples of genetically differentiated populations inhabiting the same hydrological system such as Soča population of *T. anophthalmus* complex which is hydrologically interconnected with Pivka River where "western Slovenian" lineage is identified [56].

Only a small number of Dinaric cave taxa have been investigated so far, and there is a problem with lack of coverage in distribution between different taxa and/or lack of sampling in the same localities but some general biogeographical patterns can be

portended. The region between eastern Slovenian Bela Krajina throughout Kordun and up to Lika is full of inconsistencies (Figure 9): many taxa and phyletic lineages are distributed across separate river basins as well as across sea catchment divides and phylogeographical patterns among *Troglocaris, Proteus* and *Congeria* lineages are discordant. Interestingly, located approximately in the centre of this region is Ogulin area, one of the biodiversity hotspots of aquatic subterranean fauna within the Dinaric Karst (together with Planina-Postojna and Popovo polje). Perhaps the same geodynamicity that caused conflicting phylogeographical patterns also shaped the rich biodiversity of Ogulin groundwater. If so, perhaps the same can be extrapolated to the whole region of Dinaric Karst.

Conversely, all taxa examined so far from Sana basin near Sanski Most and Lušci Palanka form their own separate lineages there: *C. mulaomerovici*, "Krajina" clade of *Proteus* and even both lineages of *Troglocaris* found in the region ("anophthalmus" and "Dinaro – Caucasian") have speciated each into its own species [26,60] (Figure 9). Clearly, the northwestern Bosnian region is a distinct biogeographical entity that has been completely isolated for some time. According to the molecular clock analyses both *Troglocaris* species from this region are older than 3,7 My. The isolation of the northwestern Bosnian group of *Proteus* from the rest of populations is older than 4,4 My [61] and *C. mulaomerovici* has been isolated from the rest of *Congeria* for 2,6 My.

Finally, whenever any of the phyletic lineages has an unusually large range it is distributed along the Dalmatian coast and in the Dalmatian hinterland and Herzegovina. As already mentioned, one lineage of cave shrimp and one lineage of the olm have such a wide range, encompassing several separated hydrographical basins [56,60] that flow mostly in parallel to the Adriatic Sea and have no surface nor in most cases known subterranean connections between each other. The lack of genetic structure in these lineages can be a consequence of either the presence of gene flow or the lack of time that has passed after the vicariant events. A wide range of Adriatic lineage of *Troglocaris was* explained as a consequence of dispersal. Recent range expansion was

indicated by mismatch analysis [56], however a more detailed sampling and the inclusion of more markers would be needed to prove this rather controversial hypothesis. An alternative hypothesis that is a recent split between these basins could have coincided with the sea level fluctuations during Pleistocene. The Adriatic Sea was gradually rising and reached its current level less than 20000 years ago [62]. Prior to that, river Po was running as far as present day middle Adriatic and most of today's rivers were presumably part of this basin, so faunal exchange could have existed here much longer than in other regions.

Genetic differentiation between populations in the same hydrological system, lack of genetic differentiation across apparently separated river beds and sea drainages, phylogeographical patterns not being concordant among different co-distributed taxa are different examples of conflicting biogeographical patterns compared to present day hydrological regimes. Moreover, different regions of the Dinaric karst produce specific genetic patterns in their subterranean fauna. From this it can be concluded that the subterranean habitats are not as stable from the geological perspective. The karstic realm is constantly reshaped and the karstic landscape we see today is geologically very young. Karstification frequently alters hydrological relationships among different basins and over time and this dynamicity has a great influence on the evolution of the biological component of the ecosystem. Distributions of phylogenetic lineages of different aquatic cave taxa are under a large influence of historical factors, perhaps more than present day hydrological regimes. An overview of the past events and processes is thus necessary to understand today's biodiversity patterns.

In conclusion, comparative biogeography can provide insights into the evolution of the subterranean fauna, but also the karst landscape itself, which is especially important since paleogeographical information on the evolution of Dinarides is largely missing. The picture will become more comprehensive and clearer as more subterranean aquatic taxa are investigated.

Molecular dating of subterranean lineages

According to the paleomagnetic and magnetostratigraphic results, the surface of the Dinaric karst started forming after Eocene. Before that the limestone was covered by impermeable rocks. The oldest sediments in caves in Slovenia, where the only research has been done, date to 5,5 Mya meaning that caves themselves but potentially also first inhabitants could be older [1]. The fossil remnants of *Marifugia cavatica* were dated to 3,6 Mya [63] meaning that already then the Dinaric underground harbored cave dwellers. According to molecular clock results the ages of the lineages and potential timing of first cave colonizations are as follows: 7-7,5 Mya for cave sponge *Eunapius subterraneus* [64], 6-8 Mya for *Troglocaris*, 8,8–16 Mya for *Proteus* [61] and the split between Northwestern Dinaric and The Central European clades of *Asellus* occurred 4-5 Mya [65]. However, all these datings were performed by using global molecular clock which assumes constant rates of evolution within and between all lineages. In the case of dreissenids, there are several limitations for the use of global molecular clock.

The paleogeographical evolution of the Paratethys, the site of origin and radiations of Dreissenidae, was dynamic and involved frequent changes in the aquatic environment. Lakes were being formed, expanded and contracted, repeatedly being connected to either the sea or to one another, and then isolated again [66–68]. The species living within these lakes that failed to adapt to these constant changes became extinct. Dreissenids went through several cycles of extinction and renewal during these periods [69]. The extant representatives of the family, after their ancestors survived all the challenges of the Cenozoic, adapted successfully to different environments. Some of the present day species are confined to stable habitats, such as ancient lakes, for example *Dreissena presbensis* and *D. caputlacus*, or the subterranean waters of the Dinaric karst (*Congeria* spp.), and these now have relatively limited ranges. Other modern species settled in either the turbulent waters of the Ponto-Caspian region (*Dreissena polymorpha, D. rostriformis bugensis*) or in the Gulf of Mexico (*Mytilopsis leucophaeta*, *M. sallei*) and have undergone recent, near global, range expansions and invasions. In

conclusion, the turbulent historical evolution of the Dreissenidae and the variety of life history traits and ecological niches that are currently exhibited by this species group do not conform to the expectations of a strict clock.

Further, species living in subterranean habitats exhibit a number of physiological adaptations, such as lowered metabolism, longer life spans and changes in life history traits from r to K selection [8] all of which are evident in stygobiotic *Congeria kusceri* [31,70]. These traits are either known or expected to have an impact on mutation rates [71,72] and would be expected to change the rate of evolution in the subterranean branch of the family tree. It is therefore not plausible that equal rates of evolution would occur either between or within the different branches in the phylogeny that involved both surface and cave representatives. New methods that account for rate variation, and assume uncorrelated rates of evolution [73,74] have been developed recently. However, their applicability is limited to groups with fossil record such as bivalves. For all the rest of taxa only tentative dating using global clock can be preformed. However, general patterns can possibly emerge by combining all the molecular dating information.

The calculated ages of the whole group of cave shrimp *Troglocaris*, the olm *Proteus* and the cave sponge are much older (late Miocene) then ages of *Asellus* and *Congeria* (very end of Miocene and Pliocene). Further splits within these groups, to northwestern and southeastern lineages occurred within Miocene (8,8-16 Mya) for *Proteus* and in the case of *Congeria* and *Troglocaris* the splits happened at the end of Miocene and in Pliocene respectively. Differences in the divergence estimates among the various Dinaric subterranean taxa can be the result of the different molecular clock methodologies available and employed. However, different diversification dynamics of various cave Dinaric taxa is also possible. As described previously, not only the divergence time estimates, but also the phylogeographical patterns among various Dinaric groundwater taxa are discordant. The complex geological history of the Dinaric Karst including intensive karstification, as previously discussed, but also different biological features of

the studied taxa can result in the observed disparities. Dissimilarities in biology and life history traits can cause different responses in diverse taxa to the same evolutionary pressures.

Detailed paleogeographical data for the Dinaric Karst are unfortunately lacking and, hence, it is unknown which events might have caused the diversification of any of the subterranean lineages during these time periods. In comparison to the other groundwater Dinaric taxa, hard-shelled dreissenids have a rich fossil record that possibly can enable more reliable time divergence dating and shed light on the processes of colonization of Dinaric underground habitats in general. Unfortunately, any attempt to link cave *Congeria* lineages with any of the fossil species is very problematic due to the plasticity of the dreissenid shell. A detailed morphological investigation that will identify the characters that reliably reflect phylogenetic relationships within this group is needed to recognize the ancestral lineage and distinguish which of the hypotheses about the cave *Congeria* spp. origin is correct, the Dinaride Lake system or Lake Pannon. Future studies of other widely distributed subterranean Dinaric taxa and other fossil-rich animal groups (such as gastropods) are needed to resolve these issues and to create an integrated picture of the processes that shaped the subterranean biodiversity of the Dinaric Karst.

One important question remains regarding *Congeria*. At what point during its evolutionary history did *Congeria* colonize the subterranean habitats and what are the adaptations that accompanied such an immigration period. Judging from low dispersal abilities, it seems most likely that the divergence events occurred on the surface and were followed by independent immigrations underground. If lineages of *Congeria* did colonize the underground independently, they had to acquire some of the same adaptations by convergence. Such a scenario was suggested for other groundwater Dinaric taxa for which more evidence exists, for example *Proteus anguinus* [61] *Asellus aquaticus* [27] and *Troglocaris* spp. [26].

Dinaric karst as a global hotspot of subterranean biodiversity

Ever since the emergence of the Adriatic carbonate platform from Mesozoic Tethys Ocean, it has been subjected to a complex evolution and frequent perturbations. After the onset of karstification which was and is very intense in the region, the vast underground system started forming and different subterranean habitats were accessible for colonization. Since ancestors of all cave animals came from surface habitats, a requirement for high biodiversity in the subterrain must be a high biodiversity on the surface from which colonization of the underground could proceed. Both in the present day and during geological history species richness was high in the Dinarides as well as in the adjacent areas.

Throughout the Tertiary most of Europe was covered by a vast aquatic realm called Paratetys (Figure 9). The evolution of Paratetys is characterized by an extraordinary interplay of sea-level fluctuations, changes in climate, immigrations, radiations and extinctions that resulted in very high species richness and outstanding endemicity, both of which peaked in faunal assemblages of Dinaride Lake system and Lake Pannon [66,67,69,75]. The Lake Pannon had almost 6 My of non-interrupted evolution where the radiation of many different animal groups occurred. Since drying out of Paratetys progressed from west to east, Lake Pannon presumably also served as a refuge for part of the western Paratethyan fauna. However, Lake Pannon too was gradually filled with sediments and by the end of the Miocene its remnants were confined to the southernmost part of the basin, parallel to the Sava river, along the northern foots of the Dinarides [76,77]. Dinaride Lake system was a long-lived bioprovince as well, comprised of numerous small lakes and situated between the rest of Paratethyan basins and the Mediterranean. Its faunal assemblages are characterized by high endemicity, especially of the mollusks.

Presently the Dinarides are situated in the western part of the Balkan Peninsula, within the European biodiversity hotspot. Due to its position beneath the ice borders Balkan provided a refuge throughout the Pleistocene climatic changes. From here,

recolonization of Europe by many aquatic taxa started [65,78,79] after conditions were favorable again. As far as Dinaric subterrain, it too provided shelter and presumably multiple colonizations [80] of both thermophiles during glaciations and cryophiles during interglaciations were taking place during most of the Pleistocene.

Animals colonized underground either actively to exploit new niches after process of karstification begun or, passively, to escape from severe climates or other destructive changes in the surface. The distinction between these two modes of cave colonizations can be made by building a phylogenetic tree and/or by the presence of the close relative on the surface. The example for the active colonization is water louse *Asellus aquaticus*. Both the presence of surface populations and the phylogenetic data showing that cave + surface populations within one basin are more closely related than all different cave populations are, point to the fact that *A. aquaticus* actively and in parallel colonized several different hydrological basins from the adjacent surface water bodies. Another example comes from cave cixiids from the island of Mljet. Its species description is on the way and this species has been assigned to an endemic genus known from Biokovo Mountain. There were not enough specimens collected to do a phylogenetic analysis but the presence of surface relative in the region points to the fact that cixiids actively colonized the caves exploring new niches [36]. On the contrary, there are no native dreissenids in the Dinarides and several searches of surface streams in Ogulin region confirmed that no freshwater sponges live in Mrežnica or Dobra River [30,81, personal data]. So both the cave sponge and the bivalve have no native surface relative which makes their relictual status very plausible.

Once in the underground, animals were subjected to numerous hydrological rearrangements and vicariant events that caused further subdivions of their lineages. As indicated by comparative phylogeography, present day hydrogeology is very young in a geological sense and biogeography of phyletic lineages is often not in accordance with it. Frequent changes of the limestone have a great influence on shaping the

phylogenies of its inhabitants and often result in numerous lineages confined to small ranges.

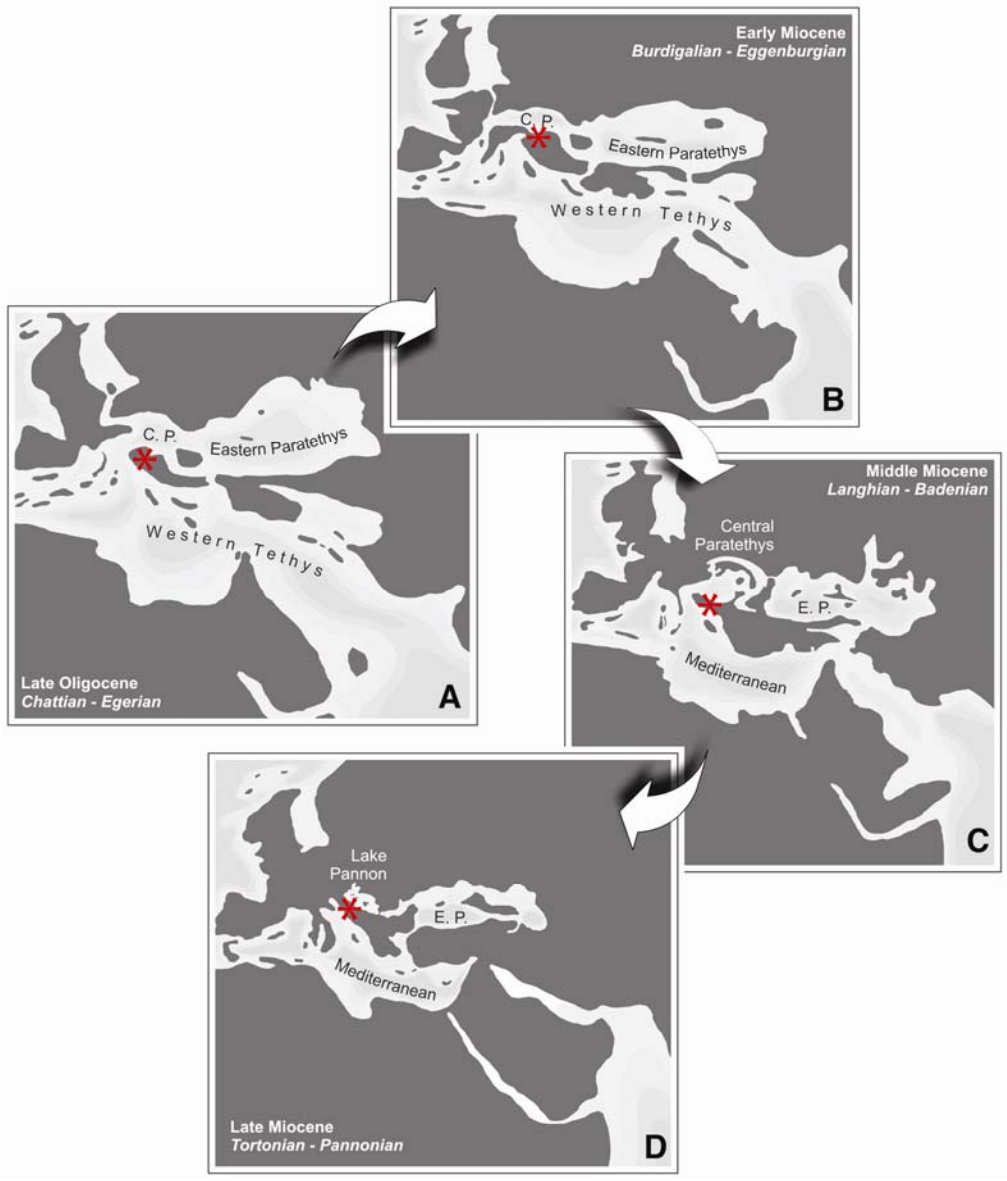

Figure 10. The evolution of Paratetys from late Oligocene to late Miocene [82]. Paratethys was formed in Early Oligocene by isolation from Tethys. Already in Late Oligocene Central and Eastern Paratetys became established. Central Paratetys became more isolated and gradually vanished giving place to brackish Lake Pannon. Throughout the tertiary Dinarides (denoted with red asterix) were positioned between the Paratetys and Mediterranean.

In conclusion, many different processes and circumstances have acted in concert to result in Dinaric karst harboring the richest subterranean fauna in the world. However, in recent times, the subterranean fauna of the Dinaric Karst is threatened by a range of human activities [6]. Such a status is particularly applicable to some of the organisms in this study. *Congeria* was assessed as vulnerable (IUCN Category VU) in the Red List of European freshwater mollusks [83] and, in Croatia, *C. kusceri* is critically endangered (IUCN Category CR) due to habitat destruction and declines in population numbers [84]. Subspecies of the cave sponge *E. s. subterraneus* was assessed as endangered (EN) [85] and the other subspecies *E. s. mollisparspanis* is vulnerable (VU) [86] due to groundwater pollution and large hydrotechnical changes in the region. The information described here will assist in their more effective protection and conservation.

4. CONCLUSIONS

Independently evolved cave cixiids from Croatia and Hawaii have lost the ability to produce melanin pigment due to an evolutionary change at the first step of melanin synthesis pathway.

The first step of melanin synthesis is also affected in several independently evolved cave populations of fish *Astyanax mexicanus* and a number of albino cave invertebrates. The fact that convergence in the loss of melanin is present even at the metabolic level, suggests that natural selection may play an important role in the evolution of albinism in cave animals.

Both melanin and catecholamine synthesis pathways begin with L-tyrosine, indicating a potential pleiotrophic effect of the loss of melanin. The elevated catecholamine levels in cave fish compared to surface fish provide support for this hypothesis.

Evolutionary forces that act during the colonization of the underground habitats and subsequent adaptations drive the evolution towards the same set of morphological and physiological changes in diverse cave animals. Consequences of the repeated acquirement of the same phenotypes (most strikingly exemplified by the convergent loss of eyes and pigments in numerous cave animals) are erroneous systematic positions or masked and cryptic biodiversity. Molecular tools are therefore vital in correctly assessing phylogenetic positions and relationships of various cave taxa.

As shown by the molecular analyses, the cave sponge *Eunapius subterraneus* is more closely related to sponges from the genus *Ephydatia*. Detailed phylogenetic and phylogeographical analyses of the cave sponge have revealed that the most important morphological characters used in freshwater sponge taxonomy are influenced by the environmental processes. Phenotypical plasticity and morphological simplicity have caused great discrepancies between the current systematics of freshwater sponges and the molecular phylogenetic data. The whole group of freshwater sponges (Spongillinae) needs a thorough systematical revision.

The molecular phylogenetic analyses have revealed that the cave bivalve encompasses 3 distinct species. Along with already known *C. kusceri*, there are two newly described species *C. jalzici* and *C. mulaomerovici*. Each of the three species is confined to its separate hydrogeological basin except *C. jalzici* which occupies localities from both the Black and Adriatic Sea catchments. A recent split between these basins is the most probable explanation for such biogeographical pattern. Both the habitat fragmentation and severe K-selected life history traits argue in favor of vicariance as a cause of divergence between the three *Congeria* species.

The shell of *C. jalzici* is an extraordinary example of phenotypic plasticity in the bivalves. Phenotypically monomorphic populations from different species exhibit deep genetic divergences, while morphologically distinct populations of *C. jalzici* lack any genetic differentiation. The shell morphology is widely used in bivalve systematic and in paleontological research it is completely irreplaceable. The example of *C. jalzici* shows that a thorough reexamination of various shell features is needed in order to find robust characters that can accurately reflect true phylogenies.

Comparison of phylogeographies of various aquatic cave taxa has revealed that the biogeographical patterns of numerous cave inhabitants are more often shaped by historical factors then by present day hydrological regimes indicating that the present day karstic landscape and its hydrology is very young. Comparative biogeography can provide insights into the evolution of both the subterranean fauna and the karst landscape itself. Together with molecular dating, it provides a powerful tool for elucidating the causes and events that shaped the present day biodiversity hotspot in the underground of the Dinarides.

Congeria is the only Dinaric subterranean animal investigated so far with abundant fossil record that can be used to calibrate the molecular clock and therefore possibly provide a more accurate understanding of the timeframe and circumstances of the Dinaric underground colonization. Family Dreissenidae first appeared at the end of Oligocene Epoch. Genus *Congeria* appeared in Lake Pannon where it went through an

exceptional radiation. All of the numerous *Congeria* species died out at the end of Miocene Epoch, exactly when the ancestor of cave *Congeria* lineage was dated. From this it seems that the ancestor of cave bivalves colonized Dinarides from the remains of drying-out Lake Pannon. The other possibility is that the ancestors of cave *Congeria* lineage lived in Dinaride lake system. Plasticity of dreissenid shell, as seen in *C. jalzici* lineage, prevents comparison of cave *Congeria* with fossil lineages at this point which is needed to discern the correct hypothesis.

Complex geodynamics and turbulent climate, the persistent high biodiversity in surface habitats both in the present day and during geological history, high biodiversity in the Dinarides as well as in adjacent areas (e.g. Lake Pannon), vast underground system with well developed contact zones through which colonization could have proceeded, different colonization advantages for the fauna (exploitation of the new niches as well as refugium from changing surface conditions), intensive karstification resulting in more fragmented habitat that further subdivided the lineages are all different causes that were involved in shaping the rich subterranean biodiversity of the Dinaric Karst.

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6. SUMMARY

One of the most notable adaptations of cave animals is albinism, the loss of melanin pigment, which has evolved in most animals that are adapted to life in caves. The results of a melanogenic substrate assay showed that all cave animals thus far tested have lost their pigment due to a change in the first step of melanin synthesis pathway, the conversion of L-tyrosine to L-DOPA. Therefore, the evolution of albinism is convergent at both the phenotypic and metabolic levels. L-tyrosine is a precursor of another biosynthetic pathway, the synthesis of catecholamines (dopamine, noradrenaline, and adrenaline), which suggests a possible pleiotrophic effect of the loss of melanin pigment: excess of L-tyrosine substrate could be used advantageously in the alternative pathway. The attempts to explain the evolution of regressive traits have caused many controversies since the beginning of cave biology research, and our data suggest that the natural selection may be a driver for the evolution of albinism.

Strong selection operating during the colonization of caves by various taxa results in the same set of adaptations and often similar morphologies which obstruct the recognition of true phylogenetic positions and relationships. For example, the closest surface relative of cave sponge *Eunapius subterraneus*, Sket & Velikonja, 1984, according to molecular data, belongs to the genus *Ephydatia*. In addition, phylogenetic analysis revealed many inconsistencies in taxonomy of freshwater sponges, even at the level of families. The lack of robust morphological characters coupled with the fact that many of the traits show plasticity (body shape, spicules, gemmules) makes the classification of sponges in general very challenging.

Another frequent outcome of using molecular analysis in cave fauna studies is overlooked biodiversity and this is the result of cave bivalve *Congeria kusceri* Bole 1962 research. The phylogenetic data revealed that cave bivalves have separated into three distinct species, the already recognized *C. kusceri* and two newly described species: *C. mulaomerovici* Morton & Bilandžija, 2013. and *C. jalzici*, Morton & Bilandžija, 2013. Each species is confined to its own hydrological region except *C. jalzici* which exhibits a specific pattern. It encompasses isolated populations living in basins of different sea

catchments. Further, functional adaptation to a distinct hydrological regime in Lukina Jama-Trojama Cave System resulted in very different shell morphology of bivalve population living there, making *C. jalzici* an extraordinary example of phenotypic plasticity.

The rich fossil record of dreissenids enabled the use of recently developed and more reliable molecular clock tools. The epicentre of evolution of the whole family Dreisssenidae was the Paratetys and the genus *Congeria* appeared and radiated in Lake Pannon. From there, it is hypothesized, the ancestor of extant cave *Congeria* colonized the Dinarides and caves. The last common ancestor of all three species evolved 5,4 Mya, which fits the timeframe when all other *Congeria* species vanished from the fossil record. Subsequent divergence occurred during the Pliocene Epoch, about 2,6 Mya. It is not known exactly when during their evolution these lineages have colonized the caves.

Comparative phylogeography together with molecular dating can provide valuable insights and contribute to the formation of an integrated picture of the Dinaric karst and its underground fauna evolution. Very rich subterranean biodiversity of the Dinaric karst was shaped by a combination of events and circumstances that took place mostly during geological history: the high biodiversity and therefore the productivity of surface habitats both in the past and today, the complex and turbulent geological evolution of both Dinaric and adjacent regions, and the excessive karstification of limestone bedrock that created a vast and diverse underground network of habitats but also frequently changed hydrological relationships causing further subdivision of lineages are some of the factors that influenced the biodiversity seen today. This research added further information to this story but, perhaps more importantly, provided valuable information that can help address conservation issues of these rare and endangered animals.

7. SAŽETAK

Albinizam, redukcija ili gubitak pigmenta melanina, jedna je od najuočljivijih adaptacija podzemnih životinja i evoluirao je kod svih skupina koje su uspješno kolonizirale podzemlje. Rezultati in vitro testa „melanogenic substrate assay" (test melaninskih prekursora) pokazali su da su sve do sad testirane životinje izgubile pigment promjenom na prvom koraku biosinteze melanina koji uključuje konverziju L-tirozina u L-DOPA. Dakle, konvergentna pojava albinizma očita je na razini fenotipa ali i na metaboličkom nivou. Aminokiselina L-tirozin prekursor je još jednog metaboličkog puta, onog sinteze kateholamina (dopamina, noradrenalina, adrenalina) što upućuje na mogući pleiotropni efekt gubitka melanina. Naime, L-tirozin koji se ne potroši za sintezu melanina može se usmjeriti i iskoristiti za sintezu kateholamina. Pokušaji razjašnjavanja regresivne evolucije kod špiljskih životinja izazvali su brojne polemike od početka biospeleoloških istraživanja. Naši rezultati upućuju da je prirodna selekcija odigrala važnu ulogu u evoluciji albinizma kod špiljskih životinja.

Snažan selekcijski pritisak koji djeluje tijekom kolonizacije podzemlja rezultirao je nizom istih prilagodbi i time sličnih morfologija kod različitih životinja. Posljedica je otežano određivanje točnog taksonomskog položaja i srodstvenih odnosa špiljskih životinja. Na primjer, molekularne su analize pokazale da je najbliži vanjski srodnik špiljske spužvice *Eunapius subterraneus*, Sket & Velikonja, 1984, spužva iz roda *Ephydatia*. Iste su analize pokazale veliko neslaganje s trenutnom klasifikacijom slatkovodnih spužvi, čak na razini porodica. Nedostatak robustnih morfoloških obilježja i izrazita fenotipska plastičnost mnogih karaktera (gemula, spikula, habitusa) čini sistematiku svih spužvi izuzetno problematičnom.

Filogenija špiljskog školjkaša, *Congeria kusceri* Bole 1962, primjer je drugačijeg ishoda upotrebe molekularnih markera u istraživanjima podzemne faune, zanemarene bioraznolikosti. Naime, utvrđeno je da je došlo do divergencije špiljskih školjkaša i specijacije u tri odvojene vrste. Uz otprije poznatu *C. kusceri*, opisane su još dvije vrste, *C. mulaomerovici* Morton & Bilandžija, 2013. i *C. jalzici*, Morton & Bilandžija, 2013. Svaka od njih obitava u zasebnom hidrološkom sustavu, osim *C. jalzici* koja pokazuje

neobičan obrazac. Naime, areal vrste obuhvaća lokalitete koji se nalaze u odvojenim morskim slivovima. Nadalje, izrazito drugačija morfologija ljuštura populacije iz jamskog sustava Lukina jama-Trojama rezultat je adaptacije na poseban hidrološki režim u tom lokalitetu. *C. jalzici* je stoga izvanredan primjer fenotipske plastičnosti.

Bogat fosilni zapis porodice trokutnjača (Dreissenidae) omogućio je upotrebu nedavno usavršenih i pouzdanijih metoda molekularnih datiranja. Evolucija trokutnjača odvijala se u Paratetisu, a rod *Congeria* se pojavio te prošao i kroz iznimnu radijaciju u Panonskom moru. Od tamo je, prema hipotezi, predak današnjih špiljskih školjkaša kolonizirao Dinaride i podzemlje. Posljednji zajednički predak svih špiljskih školjkaša datiran je na prije 5.4 milijuna godina, kada sve druge vrste roda *Congeria* izumiru. Daljnje divergencije događaju se tijekom pliocena, prije 2,6 milijuna godina. Nije poznato kada su se točno školjkaši nastanili u podzemlju.

Kombinacija komparativne filogeografije i molekularnog sata može ponuditi vrijedne uvide te pomoći u formiranju integrirane slike evolucije Dinarskoga krša i njegove podzemne faune. Vrlo bogata bioraznolikost podzemlja Dinarida rezultat je niza čimbenika i procesa: velika bioraznolikost nadzemnih staništa tijekom prošlosti, ali i danas, kompleksna i turbulentna geološka evolucija Dinarida, ali i susjednih regija, intenzivna karstifikacija koja je stvorila golemu mrežu raznovrsnih podzemnih staništa te potom uzrokovala česte promjene u hidrogeološkim odnosima što je za posljedicu imalo dalju diversifikaciju filetskih linija neki su od čimbenika koji su oblikovali bioraznolikost kakvu vidimo danas. Ova su istraživanja doprinijela rasvjetljavanju tog procesa, ali, još važnije, ponudila su i vrijedne informacije koje će pomoći u zaštiti i očuvanju tih rijetkih i ugroženih životinja.

8. CURRICULUM VITAE

I was born on the 5th of May 1981 in Zagreb. I finished primary and high school education in Ivanić Grad. I started Biology studies at the Faculty of Science, University of Zagreb, during which I got interested in cave biology which has been the main focus of my research ever since. During my student days I was an active member of Biospeleological Section in the Biology Students Association where I was involved in several projects. I graduated with a thesis entitled: Ecological and morphological characteristics and biogeography of freshwater underground sponge *Eunapius subterraneus* Sket & Velikonja (Spongillidae, Demospongia) in 2005. During my college education I received scholarships from the town of Ivanić Grad and Zagreb County.

Since 2002 I have been a member of Croatian Biospeleological Society, where I performed the function of Secretary between 2005 and 2010. I participated in over thirty national and international programs and projects of various scopes, 8 of which I coordinated or worked as an associate manager. Research programmes have been mostly involved in faunal inventories or ecological and biogeographical investigation of selected species. Many projects encompassed various educational activities so I am an author of 2 exhibitions (and was a professional associate on two more), 8 popular articles, 3 popular booklets and have organised 2 discussion forums on cave fauna endangerment and conservation issues.

At the end of 2007 I was appointed as a young researcher at the Rudjer Boskovic Institute under the supervision of Helena Ćetković, PhD. Within the Laboratory for Molecular Genetics I continued working on subterranean biology using a variety of molecular tools that enabled a more evolutionary aspect of cave life research.

I have published 6 original scientific papers, including 5 as a first author and 3 full papers in conference proceedings. I am a co-author of the Red Book of Croatian Cave Dwelling Funa and an editor of the first volume of The Cave Type Localities of Croatian Fauna Atlas. I am a first author on 13 of 33 conference reports, and have given oral presentations at 5 conferences, two times as an invited speaker.

I have supervised and trained two biology students from Zagreb University. Inga Patarčić recieved the University of Zagreb Provost Award. Dajana Hmura conducted her graduation thesis in our laboratory and was invited to give an oral presentation about her results at the International Symposium on "Evolution of Balkan Biodiversity". In addition, our poster received first prize at the International Conference on Subterranean Biology in 2012.

I have been a guest researcher at The Marine Biological Station of the University of Paris Vi in Roscoff, France in 2011 and 2012 and have spent 3 months in the Biology Department at the University of Maryland, USA during the first part of 2013. I have participated in the European Molluscs Expert Workshop organized by IUCN in 2009 and in Training on Habitats Directive monitoring & reporting organized by the State Institute for Nature protection in 2013. I am a member of European Society for Evolutionary Developmental biology. In 2013 I was awarded a scholarship, "For Women in Science", from the Loreal and Croatian National Commission for UNESCO at the Ministry of Culture.

9. PUBLICATIONS LIST

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5. Harcet M, Bilandžija H, Bruvo-Mađarić B, Ćetković H (2010) Taxonomic position of *Eunapius subterraneus* (Porifera, Spongillidae) inferred from molecular data – A revised classification needed? Molecular Phylogenetics and Evolution 54: 1021–1027.

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