Conservation genetics, molecular phylogeny and phylogeography of freshwater snails (Mollusca: Gastropoda) of high conservation concern in Hungary. OTKA-NNF 78185 Project.

Final Report

Zoltán Fehér, Hungarian Natural History Museum

Introduction

In past decades, biodiversity is being lost on Earth at an unprecedented rate. As biodiversity is the foundation of life, its long-term conservation, including not only conservation of species, but that of intra-specific diversity as well as the variability of functional ecosystems, is an essential component of the sustainability. In order to preserve biodiversity in Hungary, there are site- and species based protection as well, but species for protection have mostly been selected in ad hoc manner. As resources for conservation are always limited, it would be necessary to use objective methods in setting priorities, in order to maximise the benefits of any conservation actions. Therefore, systematic conservation planning is of increasing significance in conservation biology and environmental management. Unfortunately there are often insufficient distribution data and data on population trends. Moreover, these data are sometimes biased by the poor taxonomic knowledge and there are hardly any data on the intraspecific diversity (number, distribution and diversity of the deeply diverged intraspecific lineages, which practically are considered as "evolutionary significant units". As classical morphological methods are insufficient in themselves for the better understanding, conservation and management of diversity, it seems necessary to apply molecular methods.

The aim of our project was to clarify taxonomical, filogenetic and filogeographic questions which are associated to endemic mollusc species or other mollusc species of conservation concern. We planned to apply classic morphological as well as molecular methods. We hoped that revealing intraspecific diversity, philogenetic relations and distribution history of these species and clarifying taxonomic problems will help to make effective conservation decisions.

Our main objectives were the following:

- To confirm the distinct species status of *Theodoxus prevostianus* by nuclear markers and to find a reasonable explanation for the paraphiletic *Theodoxus prevostianus* - *Theodoxus danubialis* mitochondrial phylogenetic tree.

- Based on the mitochondrial COI gene fragment, to reveal intraspecific lineages of *Bythinella pannonica*, as well as to determine intraspecific variability within and genetic distance between these lineages. We also aimed to study the spatial dependence of intraspecific variability, to estimate the times of main phylogenetic / demographic events. Based on the above, we were to infer the biogeographical history of the species. These findings were expected to be generalized to other *Bythinella* species, and to promote the better understanding of the phylogeny and distribution history of the whole genus.

- To confirm or reject the distinct species status of *Bythinella hungarica* and *Bythinella molcsanyi* within *Bythinella austriaca* species complex.

During the fieldwork of this project, we have collected further species, a part of which was also studied – other *Theodoxus* and *Bythinella* species, *Valvata macrostoma*, *Granaria frumentum*, *Corbicula spp., Aspasita spp.*

Results

Basic research

While *Theodoxus prevostianus* and *Theodoxus danubialis* species are clearly distinguishable by shell morphology, similarities of their opercula and radulae indicate close relationship. Mitochondrial COI tree, based on material collected throughout the ranges of both species, was found to be polyphyletic. This is in accordance with the results of (Bunje 2007), based on less representative material. New COI data made it clear that the centre of diversity (and the location of the presumed Pleistocene refugium) of *T. danubialis* is in the Upper-Sava system rather than in the Lower-Danube. At the beginning of the project, we hypothesized that polyphyletic mithocondrial gene tree is the consequence of multiple introgression events and reciprocally monophyletic nuclear gene trees were expected to find. To the contrary, phylogenies based on all the three nuclear sequences (ATP synthase gene beta subunit second intron (ATPSb-iII); ATP synthase gene α subunit; actin gene) were found to be polyphyletic, and therefore rejected our hypothesys. This seems to support the idea of Bunje (2007), i.e. today's *T. danubialis* is a young species, which has evolved from a lineage of the fluvial *T. prevostianus* in the Pleistocene, while remnant *T. prevostianus* populations are relict lineages of the Pleistocene *T. prevostianus*. (Fehér et al. 2009 (a,d)).

We have calculated intraspecific diversity and divergence of mitochondrial COI gene sequences of *T. transversalis* specimens of eight remnant populations, representing the whole range of the species. Low diversity and divergence – only three closely related haplotypes – were found, which seems to be the consequence of a historical bottleneck. In our view, the whole Holocene range has been colonized by descendants of a bottlenecked population. Low genetic diversity, however, was not an obstacle for this species to obtain a relatively wide range and abundance until the recent past. Comparison with other *Theodoxus* species of similar sized historical ranges raises the idea that possibly reduced genetic diversity accounts for the species' high sensitivity to water quality and habitat alteration. (Fehér et al. 2010 (a), Fehér et al. submitted (a)).

We have studied the intraspecific variability of *Bythinella pannonica* and its spatial distribution, based on mitochondrial COI gene, and tried to infer the species' distribution history. We have found two deeply diverged intraspecific clades, which have split sometimes in the Pleistocene (probably earlier than 1 My) due to allopatric fragmentation. As the original picture was obscured by more further events (more recent fragmentations, long and short range dispersals as well as restricted gene flow), we can just suppose that the rarer clade has been evolved originally in the Bükk Mts and the other one in the Karst. The rarer clade has three well distinguishable sub-clades, which are restricted to three narrow areas (Kács and Sály in the southern Bükk Mts; Szinva drainage in the eastern Bükk Mts; Drienovec in the eastern part of the Slovak Karst). Due to the complex distribution history, populations of the two genetically different lineages can inhabit habitats of a few hundred meters far from each other. In recent years, *Bythinella* was frequently used in molecular phylogenetic studies. Up to now, however, there were not any reference study on the intraspecific variability of a morphologically distinguishable *Bythinella* species of a relatively wide range (like *B. pannonica*). In this species, we have found that ca. 5 % difference in mt COI gene – which is a widely used barcoding sequence – is far within the frames of intraspecific variability. This questions the conclusions of Bichain et al (2007), who have defined 1,5% COI difference as a species treshold in the French *Bythinella* species. (Fehér et al. submitted (b)).

We refound successfully *Bythinella molcsanyi* in its type location (Stațiunea Izvoare, Igniş Mts., Maramureş county, Romania). This worth of note because this species has not been collected since its description in 1941, and its type series was believed to be disappeared, and its distinct species status was a matter of debate (Bába 1997). *B. molcsanyi* was collected in some springs and streams within a few km^2

range in the Igniş Plateau. Both genital morphology and mitochondrial phylogeny (COI) proves its monophyly and separation from *Bythinella austriaca* and other known *Bythinella* species. (https://sis.iucnsis.org/SIS/155780).

During the search for *Bythinella molcsanyi*, we have collected *Bythinella* populations in several locations of Maramureş county (Igniş Mts., Lăpuş Mts., Ţibleş Mts., Rodnei Mts., Gutâi Mts.). Beside the recently described *B. grossui* and *B. viseuiana* (Falniowski et al 2009), we have found further *Bythinella* species, which are new to science. These species are not yet described as shell and genital morphology were not congruent with molecular (mt COI) phylogeny, and therefore further studies seem necessary. However, it is obvious that this part of the Eastern Carpathians is a diversity hot spot, which was overlooked up to now. (unpublished result).

We have tested the concept of Fauna Europaea (Bank 2011), which says that *Bythinella austriaca* sensu stricto is not identical to *Bythinella* populations occurring in northern Hungary, eastern Slovakia and Southeastern Poland; and names this latter taxon as *B. hungarica*. Based on mt COI sequences, Hungarian *Bythinella cf. austriaca* populations are almost identical with those of the Northern Carpathians and the Alps (Benke et al. 2010). Genital- and shell morphology has led us to the same conclusion: they belong to the same species. Our results has shown that *Bythinella hungarica* should be treated as a synonym. According to our recent field experience, *B. austriaca* is relatively frequent in northern Hungary (Északi-középhegység) and therefore its conservation concern shouldn not be overvalued. (https://sis.iucnsis.org/SIS/155335 and https://sis.iucnsis.org/SIS/155498).

Further results of our fieldwork on the Balkans and the Carpathian Basin:

We have found *Bythinella drimica* in some locations of South and North Albania (confirmed by morphology and molecular markers). Up to now, this species was believed to be a narrow range species endemic to Macedonia. Our findings have modified the known size of the extent of occurrence and the number of known populations, resulting that this species could be downgraded to Least Concern (LC) from Endangered (EN) category on the IUCN Red List. (Fehér and Erőss 2009, Murányi et al. submitted, https://sis.iucnsis.org/SIS/156099).

Studying *Granaria frumentum* material in the Balkans, we have found *G. f. hungarica* subspecies as far as to northern Albania – extending the known geographic range of this subspecies – and we have found and described a subspecies, which is new to science (*G. f. subaii*). (Fehér et al. 2010 (b)).

Molecular markers (mt COI) confirmed the distinct species status of *Aspasita triarius*, *A. triadis* and *A. trinodis*. In the Bükk Mts. (Hungary), we have found an *Aspasita* species, which is new to science. (in preparation).

We have discovered a *Valvata macrostoma* population in the Bodrogköz region (Hungary). This viable population was not known previously. This led us to revise the *Valvata macrostoma* (syn.: *V. pulchella*) material of the three largest state-owned Mollusca collections in Hungary. The majority of the studied material were either fossil/subfossil, incorrectly determined or debris collected material. Our results have revalued the conservation concern of *V. macrostoma* in Hungary, as it has proved to be significatly rarer than it was believed previously. (Varga and Fehér 2010).

Molecular markers of Danubian populations of the invasive *Corbicula*, could not confirm that the two morphotypes occurring in the Danube drainage belongs to two different species. Meta analysis of our sequences and those of the GenBank database confirmed that the more frequent morphotype belongs to the widespread and frequent *C. fluminea* lineage. The other one, however, is not identical to *C. fluminalis*, occurring in the Near East, but belongs to a lineage, which was only found in invasive European populations, and its native origin is still uncertain. (Bódis et al. submitted).

Methodology

About molecular studies, our first methodological objective was to develop a routine protocol for sequencing mitochondrial cytochrome oxidase gene subunit I (mt COI) (ca. 650–700 bp) for our study objects. Methodological development was initiated with *Lozekia*, *Kovacsia* and *Theodoxus* species (Fehér et al. 2009 (b)), and proceeded with all *Bythinella* spp. and those species, which were involved in the meantime of the project. We have used LCO1490 and COI-H primers (Machordom et al. 2003). This latter one is shorter by six bases than the HCO2198 member of the earlier described primer pair (Folmer 1994). By the optimalization of the PCR conditions they were proper primers for all the study species. In same cases not only the PCR conditions but the DNA isolation method required specific optimalization steps (*Aspasita spp.,* which were isolated with DNAzol) and few populations of *B. pannonica* demanded the application of QIAamp microkit for DNA isolation.

We aimed to extract DNA and sequence mitochondrial COI gene from dry museum samples (mummies) of *Theodoxus prevostianus*, originated from extinct populations. Despite our efforts, we did not reach this objective. The crucial problem was the degradation of tissue and DNA of the old specimens and the presence of preservatives. As protocols applied to fresh speciemens did not work, we tried to modify them in several points, applying techniques developed to extract DNA from small and forsenic samples (Qiagen tissue Kit, QIAamp microkit suitable for small and forensic samples, CTAB and DNAzol based methods, GenomePlex WGA Kit which amplifies the whole genome and increases the amount of the template). The resulted DNA was detectable on agarose gel but according to its size it was degradated and not suitable for PCR amplification of ~600 bp fragments. Although different Taq polimerases and protocols (Fermentas Dream Taq, Finnzymes Phusion polimerase, AmpliTaq Gold, additonal BSA, DMSO) were tested it did not succeed to get usable sequences of 550–700 bp. A possible solution is to design inner primers and to amplify COI fragments separately, this however, is beyond the scope of this project.

The second methodologial objective was to find such nuclear sequences – and to develop routine protocols for them –, which are polymorph enough to reveal intraspecific relations, similar to mitochondrial COI. The second intron of ATP synthase beta subunit gene (ATPSb-iII) was the first investigated nuclear region. (Wörheide et al. 2008). The PCR amplifications were successful for all the studied *Theodoxus* species (*T. prevostianus, T. danubialis, T. transversalis* és *T. subterrelictus*) producing ~650 bp fragments. As for *Bythinella*, only *Bythinella austriaca* gave a PCR product (~1100 bp), but not any other *Bythinella* species. This marker did not show significant intraspecific polymorphism, but can be a useful tool in species distinction, as ATPSb-iII and COI phylogenies were mostly concordant.

The next target was the single intron of ATP synthase α subunit. (Jarman et al. 2002, Frey et al. 2008). Since the primers amplifying this intron and partially the flanking exons were developed not for mollusc species, optimalization of annealing temperature, primer, Taq and DMSO/BSA concentrations was a long procedure. Finally we succeded to come by sequences for all the *Theodoxus* species. These showed size and sequences variations between species. The sequence size was ~400 bp (PCR fragment size 500 bp) for *Theodoxus transversalis* and 550 bp (PCR fragment size 650 bp) for *Theodoxus prevostianus* and *Theodoxus danubialis*. We found intraspecific genetic variations within *Theodoxus prevostianus* and *Theodoxus danubialis* species (approx. 21 polymorphic positions within 550 bp). Among the polymorphic positions there were heterozygotic as well. The amplification of the intron of ATP synthase α subunit was rather unsuccessful for *Bythinella* species so far (only few specimens gave PCR products with these primers). Results with *Theodoxus* species show that the intron of ATP synthase α subunit is a promising marker for the analysis of intraspecific variability within molluscs, but it can be applied routinely if specific primers will be designed in order to increase the effectivity of the PCR.

The third studied sequence was the actin encoding gene. (Donald et al. 2005, Adema 2002). In this case we could successfully optimalize the PCR protocol for *Theodoxus prevostianus* and *T. danubialis*. The amplifications resulted 750 bp (PCR fragment size 800 bp) long sequences that proved to be beta-actin according to BLAST seach. It has shown much lower intraspecific polimorphism than mitochondrial COI, therefore it seems less usable for studying intraspecific variability.

Applied research

European freshwater mollusc species has been assessed according to IUCN categories. Study species were also involved and assessments based partly on the results of this project. It is available in the public database of the IUCN (www.iucnsis.org/). Four of the study species have been assessed to any of the threatened categories; *T. prevostianus* (EN), *T. subterrelictus* (EN), *T. transversalis* (VU), *B. molcsanyi* (VU).

Our study has shown that being the last representative of a distinct ancient lineage, the *Theodoxus prevostianus* population of Kács is of high conservation concern and it should be treated as a distinct conservation unit. This served as a theoretical basis to that programme, which was launched in 2010 and aimed to re-introduce this species to the Lator-kút spring in Sály, in order to make a „security copy" of the Kács population. (Fehér et al. 2009 (c), Fehér et al. 2011).

We have found hardly any intraspecific variability within the remnant *Theodoxus transversalis* populations. In the lack of intraspecific variability there is no sense to treat any of those populations as distinct conservation unit, and therefore each stable population – most of which are located in Hungary (Rába River, Upper-Tisza River, Bódva River) – are of the same conservation concern. (Fehér et al. submitted (a)).

Though *Bythinella pannonica* was assessed as Least Concern by IUCN criteria, this species is protected in Hungary. Our study has shown the relatively large intraspecific variability is distributed unevenly, i.e. some rare lineages are restricted to narrow ranges. If one accept the premise that intraspecific genetic diversity plays a very important role in the long-term survival and adaptability of a species and hence it need to be preserved, conservation activity should focus on these populations, namely Kács and Sály in the southern Bükk Mts., Szinva Stream and Közép spring in the eastern Bükk Mts. as well as Drienovecké kúpele in the Slovak Karst. This might be particularly important because Szinva and Sály populations are actually threatened by water extraction. (Fehér et al. submitted (b)).

Other scientific outputs

In the framework of this project, six papers were published in scientific journals and two abstracts were published in congress abstract books. Further four papers are submitted for publication.

Gene sequences were uploaded to public databases: GenBank (www.ncbi.nlm.nih.gov/Entrez/) and Barcode Of Life (http://www.boldsystems.org/).

Involving an MSc student, this project has broadened the scope of those researchers in Hungary who are dealing with molecular phylogeny.

Based on the results of this project, we managed to receive an official permission from the National Inspectorate for Environment, Nature and Water to re-introduce *T. prevostianus* specimens from the Kács population to the Lator-kút spring in Sály. This re-introduction programme has been launched in 2010.

Directions for future research

During the fieldwork of this project, further taxa have been collected and a part of them were also involved in molecular/morphological studies. We have found some taxa, which are new to science (*Bythinella spp.* in Maramures county, Romania, an *Aspasita sp.* in the Bükk Mts., Hungary) and which are yet to be described. In these cases, mitochondrial phylogenetic trees were not congruent with morphological data, therefore we plan to use nuclear genes in the future.

In order to apply routinely ATP syntase gene alpha subunit, we plan to design specific primers, which will hopefully be applicable for a wide range of mollusc species.

Publications in the framework of this project

Fehér Z; Bozsó M; Krízsik V; Major Á; Szabó K; Pénzes Zs: A fekete bödöncsiga [Theodoxus prevostianus (C. Pfeiffer, 1828)] intraspecifikus diverzitása és annak természetvédelmi vonatkozásai, p. 66. In: Körmöczi L (szerk.) 8. Magyar Ökológus Kongresszus, Szeged: Előadások és poszterek összefoglalói. Szeged, Aug 26-28., 2009 (a)

Fehér Z; Bozsó M; Szabó K; Pénzes Zs: Phylogeny and phylogeography of the Lozekia–Kovacsia species group (Gastropoda: Hygromiidae)., J Zool Syst Evol Res 47(4): 306-314., 2009 (b)

Fehér Z; Erőss Z: Contribution to the Mollusca fauna of Albania. Results of the field trips of the Hungarian Natural History Museum between 1992 and 2007., Schriften zur Malakozoologie 25: 3-21, 2009

Fehér Z; Sólymos P; Majoros G: Javaslat és akcióterv a fekete bödöncsiga [Theodoxus prevostianus (C. Pfeiffer, 1828)] populációinak egykori élőhelyeire történő visszatelepítésére. Manuscript submitted to the National Inspectorate for Environment, Nature and Water, 9 pp., 2009 (c)

Fehér Z; Zettler M; Bozsó M; Szabó K: An attempt to reveal the systematic relationship between Theodoxus prevostianus (C. Pfeiffer, 1828) and Theodoxus danubialis (C. Pfeiffer, 1828) (Mollusca, Neritidae)., Mollusca 27(2): 95-107, 2009 (d)

Fehér Z, Albrecht C, Major Á, Krízsik V: Extremely low intraspecific COI gene diversity suggests a historical bottleneck in the vulnerable striped nerite, Theodoxus transversalis (Mollusca, Gastropoda, Neritidae) – In : Pesic V (ed.) ISEM4 – IV International Symposium of of Ecologists of Montenegro, 2010 (a)

Fehér Z; Deli T; Sólymos P: Revision of Granaria frumentum (Draparnaud, 1801) subspecies occurring in the eastern part of the species' range, Journal of Conchology 40: 201-217, 2010 (b)

Varga A; Fehér Z: A ritka Valvata macrostoma Mörch, 1864 (Mollusca, Valvatidae) vízicsiga faj hazai előfordulási adatainak revíziója, Folia Historico Naturalia Musei Matraensis 34: 11-16, 2010

Fehér Z, Majoros G, Ötvös S, Sólymos P: Proposed re-introduction of the endangered black nerite, Theodoxus prevostianus (Mollusca, Neritidae) in Hungary, Tentacle 19:36-39, 2011

Bódis E, Fehér Z, Nosek J, Oertel N, Tóth B: A comparative study of two Corbicula morphs (Bivalvia, Corbiculidae) inhabiting River Danube, International Review of Hydrobiology (submitted)

Fehér Z, Albrecht C, Major Á, Sereda S, Krízsik V: Extremely low mitochondrial diversity in the endangered striped nerite, Theodoxus transversalis (Mollusca, Gastropoda, Neritidae) – a result of ancestral or recent effects?, Fundamental and Applied Limnology (submitted (a))

Fehér Z; Major Á, Krízsik V: Phylogeography and intraspecific diversity of the Northern Carpathian endemic spring snail, Bythinella pannonica (Frauenfeld, 1865) (Gastopoda: Hydrobiidae), Biodiversity Journal (submitted (b))

Murányi D; Kontschán J; Fehér Z: Zoological collecting sites of the Hungarian Natural History Museum and the Hungarian Academy of Sciences in Albania, between 2004 and 2010, Opuscula Zoologica Budapest (submitted)

Congress and workshop participations

8. Magyar Ökológus Kongresszus, Szeged, 2009.08.26–28 (Z. Fehér, V. Krízsik)

XXXIII. Magyar Malakológus Találkozó, Dunasziget, 2009.08.20–22 (Z. Fehér)

IUCN Mollusc Red List Assessment Workshop, Környezetvédelmi Minisztérium–Természettudományi Múzeum, Budapest, 2009.11.23–27 (Z. Fehér)

IUCN Mollusc Red List Assessment Workshop, Zoological Society of London, 2010.02.1–5 (Z. Fehér)

XXXIV. Magyar Malakológus Találkozó, Selmecbánya, 2010.09.17–19 (Z. Fehér)

IUCN Mollusc Red List Assessment Workshop, Naturhistorisches Museum Bern, 2010.09.27–10.02 (Z. Fehér)

4th International Symposium of Ecologists of Montenegro, ISEM4, Budva, Montenegro, 2010.10.06–10 (Z. Fehér, D. Murányi)

Congress presentations

Fehér Z; Bozsó M; Krízsik V; Major Á; Szabó K; Pénzes Zs: A fekete bödöncsiga [Theodoxus prevostianus (C. Pfeiffer, 1828)] intraspecifikus diverzitása és annak természetvédelmi vonatkozásai. 8. Magyar Ökológus Kongresszus, 2009.08.26–28, Szeged. (poster presentation)

Fehér Z: A fekete bödöncsiga (Theodoxus prevostianus) természetvédelmi helyzete a legújabb molekuláris filogenetikai kutatások tükrében. XXXIII. Magyar Malakológus Találkozó, Dunasziget, 2009.08.20–22. (oral presentation)

Fehér Z: A sávos bödöncsiga és a tornai patakcsiga természetvédelmi vonatkozásai a legújabb molekuláris kutatások tükrében. XXIV. Magyar Malakológus Találkozó, Selmecbánya, Slovakia, 2010.09.17–19. (oral presentation)

Fehér Z; Albrecht C; Major Á; Krízsik V: Extremely low intraspecific gene diversity suggests a historical bottleneck in the vulnerable striped nerite, Theodoxus transversalis (Mollusca, Gastropoda, Neritidae). 4th International Symposium of Ecologists of Montenegro, ISEM4, Budva, Montenegro, 2010.10.6–10. (poster presentation)

References

Adema C. M. Comparative study of cytoplasmic actin DNA sequences from six species of *Planorbidae* (*Gastropoda: Basommatophora*). J. Moll. Stud., 68, 17-23, 2002.

Bank R. Fauna Europaea: Mollusca, Gastropoda. Fauna Europaea version 2.4 http://www.faunaeur.org, 2011, April.

Bába K. Ein Beiträg zur Molluskenfauna des Rozsály-Berges (Gutin-Gebirge). Malakológiai Tájékoztató, 16: 51–55, 1997.

Benke M., Brändle M., Albrecht C., Wilke T. Pleistocene phylogeography and phylogenetic concordance in cold-adapted spring snails (Bythinella spp.). Molecular Ecology, 18, 890-903, 2009.

Bichain J.-M., Gaubert P., Samadi S., Boisselier-Dubayle M.-C. A gleam in the dark: phylogenetic species delimitation in the confusing spring-snail genus Bythinella Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Amnicolidae). Mol. Phylogenet. Evol., 45(3): 927–941, 2007.

Bunje P. M. E. Fluvial range expansion, allopatry, and parallel evolution in a Danubian snail lineage. Biological Journal of the Linnean Society, 90, 603-617, 2007.

Donald K. M., Kennedy M., Spencer H. G. Cladogenesis as the results of long-distance rafting events in south pacific topshells (*Gastropoda, Trochidae*). Evolution, 59(8), 1701-1711, 2005.

Falniowski A., Szarowska M., Sirbu I. Bythinella Moquin-Tandon, 1856 (Gastropoda: Rissooidea: Bythinellidae) in Romania: its morphology, with description of four new species. Fol Malacol., 17: 33–48. 17(1), 33-48, 2009.

Folmer O., Black M., Hoeh W., Lutz R., Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology, 3(5), 294-299, 1994.

Frey M. A., Vermeij G. J. Molecular phylogenies and historical biogeography af a circumtropical group of gastopods (Genus: *Nerita*): implications for regional diversity patterns in the marine tropics. Molecular Phylogenetics and Evolution, 48, 1067-1086, 2008.

Jarman S. N., Ward R. D., Elliott N. G. Oligonucleotide primers for PCR amplification of Coelomate Introns. Mar. Biotechnol., 4, 347-355, 2002.

Machordom A., Araujo R., Erpenback D., Ramos M.-A. Phylogeography and conservation genetics af endangered European *Margaritiferidae* (*Bivalvia: Unionoidea*). Biological Journal of the Linnean Society, 78, 235-252, 2003.

Wörheide G., Epp L. S., Macis L. Deep genetic divergences among Indo-Pacific populations of the coral reef sponge Leucetta chagosensis (*Leucettidae*): founder effects, vicariance, or both? BMC Evolutionary Biology, 8, 24, 2008.