Freshwater and brackish bryozoan species of Croatia (Bryozoa: Gymnolaemata, Phylactolaemata) and their genetic identification

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Abstract. Freshwater and brackish species of bryozoans belong to the Phylactolaemata and Gymnolaemata class. Twelve species of bryozoans were recorded and morphologically determined at eight locations in the Black Sea and the Adriatic basin in Croatia. Twelve species of Bryozoa have been listed in the taxonomic index for Croatia (Conopeum seurati, Lophopus crystallinus Paludicella articulata, Cristatella mucedo, Fredericella sultana, Hyalinella punctata, Plumatella casmiana, Plumatella emarginata, Plumatella fruticosa, Plumatella fungosa, Plumatella geimermassardi and Plumatella repens). For the purposes of gene identification of recorded species, molecular markers for nuclear 18S and 28S genes, ITS2 region and mitochondrial COI gene were amplified. Genetic identifications of morphologically determined bryozoan species were confirmed using highly similar sequences local alignment analysis. Proliferation of freshwater bryozoan species over long distances with the help of the vector animals was confirmed by defining haplotypes on the base of 18S, 28S and ITS2 sequences associated with the Black Sea-Mediterranean waterfowl flyway.

Key words: Adriatic basin, Black Sea basin, local alignment, haplotypes, long-distance transfer

Introduction

Bryozoans are invertebrates that inhabit all types of aquatic ecosystems. Freshwater and brackish species of bryozoans are a poorly known group of animals even though they are widespread in most freshwater ecosystems. While the individual unit within colony has a size of only a few millimeters, by asexual budding it can form a large cooperative that can reach the size to tens of centimeters. With sponges and shellfish they belong to an important group of filter feeding animals (Wood et al. 2006). Phylum Bryozoa is divided into three classes: Phylactolaemata, Stenolaemata and Gymnolaemata (Woollacott & Zimmer 1977). Phylactolaemata contains about 80 freshwater and brackish species (Massard and Geimer 2008a; Wood 2002), Stenolaemata contains about 700 marine species, while Gymnolaemata contains about 5000 mostly marine species (Gordon 2003), whereas few species live in brackish waters. Although freshwater species are passively dispersed over large areas, their morphology shows little geographic variation (Frey 1995).

Their unique method of reproduction and small size allow them great potential for dispersal. Freshwater bryozoans create chitinous statoblasts, while brackish species generally produce hibernaculae that are easily spread with water currents. Statoblasts and hibernaculae are also transferred by waterfowl, fish, amphibians and reptiles so for that matter they serve as vectors for dispersal over long distances and therefore freshwater and brackish bryozoans can have cosmopolitan distribution. Recent studies have quantified transport in the field, confirming that a variety of long-distance migrants can carry invertebrates both internally and externally (Mayr 1963; Green & Figuerola 2005). Waterfowl feeding in swamp areas can ingest statoblasts, which can remain viable and germinate after passing through the digestive tract (Brown 1933; Charalambidou et al. 2003; Figuerola et al. 2005; Green et al. 2008). Those dispersal buds can attach to the feathers of waterfowl (Okamura & Hatton-Ellis 1995; Freeland 2001; Wood 2002) and can be dispersed over long distances. Distribution and gene flow are important factors for organisms such as bryozoans of fresh and brackish waters that pass through frequent local extinction because of drought or recolonization due to floods and dispersal vectors (Slatkin 1977; Whitlock 1992). Aforementioned statement is also important for bryozoans which inhabit discrete places such as ponds and lakes (Barrett et al. 1993; Berg & Garton 1994). In Croatia, freshwater and brackish bryozoan species have been reported in rivers, lakes and wetland areas of the Black Sea and the Adriatic basin.
Genetic markers are specific parts of the DNA molecule which may be unique to the identifiable individual, species or population. Commonly used markers for gene identification are 18S rRNA and 28S rRNA genes and the non-coding regions, such as the ITS, which are situated in nuclear genome. Non-coding regions of the ITS are often used for species gene identification because of its large number of variations within close group. This attribute can be explained by a weak evolutionary pressure on the non-coding DNA regions. Because of this, the ITS region sequences are recommended as a universal barcode region at the species level. Universal barcode region is also the gene for mitochondrial cytochrome c oxidase subunit 1—COI (Schulman et al. 2004).

Research history

At the global level, 88 species of freshwater and brackish bryozoans have been described. In the Palaearctic region 44 species of bryozoans that inhabit freshwater and brackish habitats were found (Massard and Geimer 2008a) of which 23 species are identified in Europe (Taticchi & Pieroni, 2005; Taticchi et al. 2006, 2008; Taticchi 2010; Wood & Okamura 2005). Early taxonomy research and species description of freshwater bryozoans in Europe began in 1741 when Trembley discovered and described *Lophopus crystallinus* from a pond on the country estate in the Netherlands. That marked the beginning of the study of freshwater bryozoans (Trembley 1743). Blumenbach (1780) discovered one of the most widely distributed species, *Fredericella sultana*, Pallas (1768) described *Plumatella fungosa*, also frequent species, while Cuvier (1798) recorded *Cristatella mucedo*. Allman (1856) devoted a monograph to the known species of Western Europe, and introduced the name Phylactolaemata. Marcus (1934) should be mentioned for his bryozoan investigations and Cori (1941) for his paper in "Handbuch der Zoologie" which contains the entire knowledge to that date of the histology of freshwater bryozoans. The most recent monograph is that of Lacourt (1968), from the Netherlands, which deals with all species known at the time and provides complete citations of phylactolaemate literature through the mid-1960s.

Data on species composition and distribution of freshwater and brackish members of bryozoans in southeast Europe are still incomplete. Massard & Geimer (2008b) recorded 11 species for Bulgaria and 10 for Romania, while Simić & Ostojić (1997) recorded 7 species for Serbia. A source from 2015 (Zorić et al.) mentions the appearance of non-native species *Pectinatella magnifica* (Leidy, 1851) in the 900 km long Danube River stretch between Budapest (Hungary) and the Romanian-Bulgarian border. Studies of freshwater and brackish bryozoan species in Croatia were neglected in history, until the announcement of the first results of research on the Krka River from 2013 where the first 9 freshwater and brackish bryozoan species for Croatia were recorded. Wöss & Novosel (2013) recorded 7 species of phylactolaemates and 2 species of gymnolaemates. Previously, the data from 2009, appearing in two graduate works from the Department of Biology, Faculty of Science, University of Zagreb, filled the list with 3 more species of phylactolaemates (Garašić 2009; Janjiš 2009) for a total of 12 freshwater and brackish bryozoan species recorded in Croatia.

Materials and methods

Sampling of the bryozoan specimens was conducted in the areas of the Black Sea basin: the Korana River, lake system Plitvice lakes, Jarun Lake in Zagreb and wetlands Lonjsko polje, Kopački rit and Crna Mlaka. Samples of freshwater and brackish species were collected in the area of the Adriatic basin: rivers Krka and Neretva. Plitvice lakes and the rivers Korana and Krka are situated on karst substrate and are characterized by limestone barriers resulting from water spray in interaction with aquatic organisms. Travertine barriers are responsible for the formation of Plitvice lakes, which today consists of 16 lakes connected by a cascading linear system. Crna Mlaka, Kopački rit and Lonjsko polje are permanent wetlands created by water seeping from the beds of nearby rivers, and their water level varies depend on seasonal water circulation of the rivers. Crna Mlaka is connected with the Kupa River, Kopački rit is located between the rivers Drava and Danube, while Lonjsko polje is located along the Lonja River, tributary to the Sava River. Jarun Lake is an artificial lake near the town of Zagreb, which is connected to the Sava River, the largest tributary of the Danube River in Europe. Neretva, the largest river which flows into the eastern Adriatic Sea, is a typical lowland river with large sediment loading on its delta. The delta is often flooded by the Adriatic Sea so the water at the river mouth is brackish. Brackish water is also present in the estuary of the Krka River. The Crna Mlaka wetland complex and delta of the Neretva River are declared as Ornithological Wildlife Sanctuaries registered on the Ramsar list. Locations and collection points are shown in Figure 1.
FIGURE 1. Sampling sites in Adriatic and Black Sea Drainage basins.

1 – JARUN LAKE (Zagreb)
2 – CRNA MLAKA (wetland)
3 – KORANA RIVER
4 – PLITVICE LAKES
5 – LONJSKO POLJE (wetland)
6 – KOPAČKI RIT (wetland)
7 – KRKA RIVER (7FW & 7BW)
8 – NERETVA RIVER (8FW & 8BW)
FW – freshwater sampling site
BW – brackish sampling site
Colony sampling included investigating natural substrates (submerged logs, branches, aquatic plants and rocks) by wading along the shore (Wöss 2004) and the samples were preserved in 96% ethanol. Colonies and their dispersal buds were identified with magnifying lens and stereomicroscope. For species identification the following keys have been used: Lacourt (1968), Wood & Okamura (2005) and Janjiš (2009). Data on valid names of freshwater and brackish bryozoan species appearing in Croatia have been collected from papers published from the 18th century and ending with the most recent sources. Locations of species findings in Croatia were listed in the graduation thesis from 2009 (Bračun; Garišić; Janjiš), 2013 (Dundović) and 2014 (Koletić) and supplemented by research from 2015 (Koletić et al.). Voucher specimens were deposited at Department of Biology at Faculty of Science at University of Zagreb in Croatia.

Isolation of DNA from 25 mg tissue samples was done with DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. Multiplication of nuclear ITS2 region [ITS2rück (5’-CGGGGATTCCGGCCTGGGCTCTTCCC-3’) & ITS2hin (5’-GGATCACTCGGCTCGTGCTCGATGAAG-3’); Ohst 2008], 18S [5F (5’-GCGAAAGCATTTGCCAAGAA-3’) & 9R (5’-GATCCCTTCCGAGGTTACCTAC-3’); Giribet et al. 1996] and 28S gene [1F (5’-ACCCGCTGAATTTAAGCATT-3’) & 3R (5’-CACCTTGGAGACCTGCTTTCA-3’); Féral et al. 1994] was performed with PCR reaction so that the species collected in Croatia can be confirmed at the molecular level. PCR analysis was carried out in 25 μL reaction volumes containing TopTaq Master Mix kit (QIAGEN), 1 μL of DNA, 1 μL of 10 μmol/L of each primer, and 22 μL of distilled RNA-free water. PCR products were purified using MinElute Reaction Cleanup kit (QIAGEN) and sequenced commercially by Macrogen Company (Netherlands).

Obtained sequences of COI, ITS2, 18S and 28S markers were verified with the Megablast program for local alignment of highly similar sequences (Altschul et al. 1990). Experimentally obtained bryozoan sequences were compared with the same species from the GenBank database to define the accuracy of the morphologically determined species via nucleotide sequence homology. Resemblance for 18S and 28S experimentally obtained sequences were compared with those published in Wood & Lore (2005) and Waeschenbach et al. (2012), for COI from Fuchs et al. (2009) and for ITS2 from unpublished paper of Taticchi et al. Identical sequences, groups of haplotypes, were verified with HaploFinder program. Assigned accession numbers for obtained sequences were stored in GenBank base and are listed in the Index of taxa with each species and the location where the sample of species tissue was sampled.

Results

This paper defines freshwater and brackish species of Bryozoa that are discussed in the previously available literature and which have been confirmed by field research, morphological determination and genetic identification. Ultimately, at 8 localities in Croatia (8 freshwater and 2 brackish sites - Figure 1), 12 freshwater and brackish species were recorded (Index of taxa). Two species were found in brackish environment, Conopeum seurati in the Krka River and Lophopus crystallinus in the Neretva River.

Isolated sequences of COI, 18S, 28S and ITS2 molecular markers of species collected in brackish and freshwaters in Croatia correspond to the sequences from the GenBank database (96-99%). Sequence analysis showed that the COI gene specificity can identify the sample to the systematic level of species with a large percentage of accuracy. Analysis of COI gene haplogroups showed that there was no correspondence between any other analyzed sequences whose sequence universality provided the most accurate estimate of species determination. ITS2 region also served in the determination of samples to the species level and has proven to be the best marker for genetic identification of bryozoan samples collected in Croatia. Although markers of 18S and 28S genes are known for their insufficient specificity, haplotypes of certain species were confirmed.

Comparison of ITS2, 18S and 28S sequences indicated the presence of five sets of haplotypes of freshwater bryozoan species sampled in Croatia. Identical sequences of ITS2 regions were confirmed in Hyalinella punctata sampled in marshland Crna Mlaka (KJ024843) and in the Korana River (KJ024836) and in Plumatella emarginata sampled in marshland Crna Mlaka (KJ024841) and in the Neretva River (KJ024832). Identical sequences of 18S gene were confirmed in Plumatella repens sampled in the Jarun Lake in Zagreb (KJ024815) and in the Neretva River (KJ024821) and in Plumatella casmiana sampled in marshland Crna Mlaka (KJ024818) and in the Neretva River (KJ024822). Identical sequence of 28S gene was confirmed in Plumatella casmiana sampled in marshland Crna Mlaka (KJ024827) and in the Neretva River (KJ024828).
## Index of taxa

Captions to index:

**Species name** (References)

<table>
<thead>
<tr>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>JL-FW-1</td>
<td>Jarun Lake (Zagreb); shallow artificial lake, clear water with aquatic vegetation; freshwater</td>
</tr>
<tr>
<td>CM-FW-2</td>
<td>Crna Mlaka; swamp valley, eutrophic water with aquatic vegetation; freshwater</td>
</tr>
<tr>
<td>KO-FW-3</td>
<td>Korana River; slow river flow in lowland area of Kordun region; freshwater</td>
</tr>
<tr>
<td>PL-FW-4</td>
<td>Plitvice lakes; lake surrounded with old branches and leaves in the water; freshwater</td>
</tr>
<tr>
<td>LO-FW-5</td>
<td>Lonjsko polje; swamp valley, eutrophic water with aquatic vegetation; freshwater</td>
</tr>
<tr>
<td>KP-FW-6</td>
<td>Kopački Rit; swamp valley, eutrophic water with aquatic vegetation; freshwater</td>
</tr>
<tr>
<td>KR-FW-7</td>
<td>Krka River; low eutrophicated and cold water with fast river flow; freshwater</td>
</tr>
<tr>
<td>KR-BW-7</td>
<td>Krka River; downstream flow from Skradinski Buk cascade; brackish water</td>
</tr>
<tr>
<td>NE-FW-8</td>
<td>Neretva River; slow river flow in lowland agricultural area; freshwater</td>
</tr>
<tr>
<td>NE-BW-8</td>
<td>Neretva River; river mouth to the Adriatic Sea; brackish water</td>
</tr>
</tbody>
</table>

**GenBank accession number (molecular marker)**

Phylum BRYOZOA Ehrenberg, 1831
Class GYMNOLEMAEATA Allman, 1856
Order CHEILOSTOMATA Busk 1859
Family ELECTRIDAE d'Orbigny, 1851
Genus CONOPEUM Gray, 1848
*Conopeum seurati* (Canu, 1928)
**KR-BW-7:** [KJ024835 (ITS2)]

Order CTENOSTOMATA Busk, 1852
Family PALUDICELLIDAE Allman, 1885
Genus PALUDICELLA Gervais, 1836
*Paludicella articulata* (Ehrenberg, 1831)
**PL-FW-4, KR-FW-7**

Class Phylactolaemata Allman, 1856
Order Plumatellida Allman, 1856
Family CRISTATELLIDAE Allman, 1856
Genus CRISTATELLA Cuvier, 1798
*Cristatella mucedo* Cuvier, 1798
**KR-FW-7**

Family FREDERICELLIDAE Hyatt, 1868
Genus FREDERICELLA Gervais, 1838
*Fredericella sultana* Blumenbach, 1779
**KR-FW-7**

Family LOPHOPIDAE Rogick, 1935
Genus LOPHOPUS Dumortier, 1835
*Lophopus crystallinus* Pallas, 1768
**LO-FW-5:** [KJ024817 (18S), KJ024833 (ITS2)]; **NE-BW-8:** [KJ024839 (ITS2)]

Family PLUMATELLIDAE Allman, 1856
Genus HYALINELLA Jullien, 1885
*Hyalinella punctata* (Hancock, 1850)
**CM-FW-2:** [KJ024814 (COI), KJ024819 (18S), KJ024836 (ITS2), KJ024842 (ITS2), KJ024843 (ITS2)]; **KO-FW-3:** [KJ024824 (18S), KJ024825 (18S)]; **LO-FW-5**
Genus PLUMATELLA Lamarck, 1816

**Plumatella casmiana** Oka, 1907


**Plumatella emarginata** Allmann, 1844


**Plumatella fruticosa** Allmann, 1844

JL-FW-1; PL-FW-4; LO-FW-5; KR-FW-7

**Plumatella fungosa** (Pallas, 1768)

LO-FW-5; KP-FW-6; KR-FW-7

**Plumatella geimermassardi** Wood & Okamura, 2004

LO-FW-5; KR-FW-7

**Plumatella repens** (Linnaeus, 1758)

JL-FW-1: [KJ024811 (COI), KJ024815 (18S), KJ024831 (ITS2)]; CM-FW-2; PL-FW-4; LO-FW-5; KR-FW-7; NE-FW-8: [KJ024821 (18S), KJ024829 (28S), KJ024838 (ITS2)]

Discussion

Genetic identification of the morphologically determined species of bryozoans collected in Croatia was verified by determining the haplotype structure using local alignment tools of nucleotide sequences. This form of identification confirmed the possibility of resident phylactolaemate species in a brackish environment. Although *Lophopus crystallinus* is not characterized as brackish species, additional research is needed to determine the limit values of the salinity at which certain species of bryozoans appear.

By determining five haplogroups with identical nucleotide sequences, the transfer of freshwater bryozoans over long distances with the help of the vector animals can be confirmed. The same species (*Plumatella emarginata*, *Plumatella repens* and *Plumatella casmiana*) that were recorded at two geographically remote locations (Crna Mlaka marsh and Jarun Lake in the Danube basin and delta of the Neretva River in Adriatic basin), in the wetlands known as the residence of waterfowl with identical sets of haplotypes of various molecular markers support that endeavor. A theory is also supported by the fact that these two locations are associated with the Black Sea-Mediterranean waterfowl flyway which suggest a high probability that the birds served as vectors for transference. Fortified haplotypes also prove mixing of bryozoan populations separately widespread in the Black Sea and the Adriatic basin which are separated by massive Dinara mountain range.

Migratory routes of waterfowl are just one of the ways of the arrival of new species of bryozoans in a particular area. For example, the arrival of non-native *Pectinatella magnifica* in the Danube River indicates that this species is now also associated in the Croatian fauna of freshwater bryozoans, because part of the flow between Hungary and the Romanian-Bulgarian stretch is in Croatia.

Acknowledgment

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