



Cryptic diversity in the Western Balkan endemic copepod: Four species in one?



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ABSTRACT

We use mitochondrial (mtCOI) and nuclear (nH3) sequence data to investigate differentiation of *Eudiaptomus hadzici*, a freshwater copepod endemic to the Western Balkans. *E. hadzici* has a disjunct distribution and morphological differences were observed at regional scale. In the current study 6 out of 7 known populations are included. We applied several species delimiting approaches, distance based methods (K2P *p*-distance and Automatic Barcode Gap Discovery, ABGD) using the mtCOI, Bayesian phylogeny and the Bayesian method implemented in bPTP and BPP programs using the concatenated sequences of both genes. Phylogenetic and species delimitation analyses all suggest that the nominal species *E. hadzici* consists of four isolated, cryptic evolutionary lineages in the Western Balkans. Each of the four lineages inhabits a single lake or a group of lakes in close proximity. They exhibit major differences in secondary sexual characters, e.g. right antennule in males. Denticulation of spine on 13th segment is substantially distinct among the four lineages, having different number and shape of tooth-like protrusions. Gene flow and dispersal are restricted to very small spatial scale, but with local differences, implying that diverse historical and contemporary processes are operating at small spatial scales in *E. hadzici*. In order to further examine spatial and temporal diversification patterns, we constructed a dated species tree analysis using BEAST. Due to lack of reliable calibration points and taxa specific evolutionary rates, two evolutionary rates were applied and the faster one (2.6% myr) seems more plausible considering the geological history of the region. The divergence of *E. hadzici* lineages is dated from Early Miocene onwards with geographically close lineages diverging more recently, Late Miocene to Pleistocene and Pleistocene, respectively. Overall, our findings shed light on cryptic genetic complexity of endemics in one of European biodiversity hotspots. Moreover, this study represents one further example of integrative taxonomy, linking DNA methodology and classical taxonomy based on morphology. Therefore, it lays groundwork for future taxonomy and biogeography of freshwater microcrustaceans in the region.

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1. Introduction

Discovery of cryptic species has increased in recent years in different taxa, habitats, and regions, largely facilitated by the use of DNA barcoding methods (Bickford et al., 2007; Pfenninger and Schwenk, 2007). High cryptic diversity is expectedly revealed in poorly surveyed and geographically isolated regions. The Western Balkans is recognized as a European biodiversity hotspot, with high endemism particularly in subterranean (Gottstein Matočec et al., 2002) and freshwater fauna (Bănărescu, 2004), with coldwater

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springs and ancient lakes identified as regional hotspots of freshwater diversity (e.g. Previšić et al., 2014a; Sell and Spirkovski, 2004; Wilke et al., 2010). In microcrustaceans, awareness of the diversity of groundwater fauna has increased in recent decades (Gibert et al., 2009; Pipan and Culver, 2007). However, the region is still understudied, and consequently, many cryptic species have been recently discovered in different freshwater taxa, e.g. in fishes (Buj et al., 2014; Tsoumani et al., 2013), crayfishes (Klobučar et al., 2013), amphipods (Weiss et al., 2014), and caddisflies (Previšić et al., 2014a). In addition to revealing cryptic diversity, recent studies of freshwater taxa in the Western Balkans also try to explain the complex and interesting biogeographic patterns and underlying processes (e.g. Klobučar et al., 2013; Palandačić et al., 2015; Perea et al., 2010; Previšić et al., 2009, 2014b).

Traditional view that freshwater zooplankton taxa have mainly wide, even cosmopolitan distribution has been revised within the last decade, and a high degree of cryptic diversity has been revealed (e.g. Barrera-Moreno et al., 2015; Bohonak and Jenkins, 2003; Karanovic et al., 2015; Marrone et al., 2013; Soh et al., 2012). Zooplankton taxa employ differing modes of passive dispersal, e.g. birds, insects (Green and Figuerola, 2005; van de Meutter et al., 2008), wind and rain mediated dispersal have been suggested (Cohen and Shurin, 2003; Havel and Shurin, 2004), as well as dispersal via hydrological connections (Frisch and Threlkeld, 2005; Michels et al., 2001) and human-mediated means (e.g. Bilton et al., 2001). However, their potential high dispersal ability has been clearly differentiated from their actual dispersal. Molecular studies focusing on dispersal of passively dispersing freshwater invertebrates have shown that rates of movement are often low even on small scales, and that gene flow is not strong enough to counteract other contemporary or historical evolutionary factors (review in Bohonak and Jenkins, 2003). For freshwater calanoids, a combination of different factors such as founder effect, rapid local adaptation, and resilience due to large egg banks have been proposed as responsible for actual gene flow restriction regardless of their dispersal potential (Marrone et al., 2013).

Copepods are abundant and diverse in fresh waters, occurring as a major component of most planktonic, benthic and groundwater metazoan communities (Dussart and Defaye, 2001). They have successfully colonized restricted microhabitats and extreme environments like hot springs, glacial meltwaters or high mountain lakes (Boxshall and Defaye, 2008). According to Huys and Boxshall (1991) and Boxshall and Jaume (2000) all orders of copepods originated in marine waters. The Diaptomidae is the largest family within the order Calanoida, comprising of 515 valid species from inland waters distributed in Europe, Asia, North America, Africa and northern South America. It is speculated that the original colonization of continental waters by this family occurred in the northern supercontinent of Laurasia sometime after the break-up of Pangaea around 160 Mya (Boxshall and Jaume, 2000).

Many genera of Diaptomidae are endemic to particular continental areas, like the genus *Eudiaptomus*, having the highest species richness in the Palaearctic region; although it is also distributed in the Nearctic, Oriental and African region (Dussart and Defaye, 2002). Chad (2015) listed 29 *Eudiaptomus* valid species. Their biogeography, biodiversity and endemism in the Holarctic, like other species of Diaptomidae, have been profoundly affected by the Pleistocene glaciations. In Europe, there are two endemics, *Eudiaptomus padanus* (Burckhardt, 1900) and *Eudiaptomus hadzici* (Brehm, 1939), with ranges restricted to potential glacial refugia, the Apennine and the Balkan Peninsula, respectively. *Eudiaptomus hadzici* has a disjunct distribution from Slovenia in the north-westernmost part of the Dinaric Western Balkan ecoregion (freshwater ecoregion ER5 *sensu* Illies 1978) to Macedonia in the northern part of the Hellenic Western Balkan ecoregion (ER6, Illies 1978). It was first described from small glacial lakes on the Jablanica Mt. in Macedonia and thus regarded as a high mountain species (Brehm, 1939). Next it was reported from two different karstic lakes in Slovenia, then from the Mediterranean barrage lake Visovac in Croatia (Bukvić et al., 1999), and finally from two additional lakes in the Krka basin in Croatia (Fig. 1a). So far, the species is recorded from 7 distant and very different lakes at altitudes ranging from ca 30 to 2100 m a.s.l. (Bukvić et al., 1999). Its ecology was investigated only in Visovac where it is very abundant and present the whole year through (Bukvić et al., 1999). Some differences were observed in morphology of both, females (shape of the last thoracomer) and males (5th leg and right antennule 13th segment spine in males) between populations in lakes in Slovenia and Visovac compared to the original description,

however, specimens from the type locality in Macedonia were not available so far (Bukvić et al., 1999).

Integrative studies using convergence of morphology, ecology and molecular genetic data have significantly improved knowledge not only on evolutionary history and biogeography of microcrustacean taxa, but also on their taxonomy and systematics (e.g. Karanovic et al., 2015, 2014; Marrone et al., 2013; Soh et al., 2012). On the Balkan Peninsula, however, diversity and ecology, but particularly biogeography and evolutionary history of freshwater zooplankton microcrustacea are rather understudied. Considering observed morphological differences between *E. hadzici* populations and an overall growing evidence of cryptic diversity in freshwater calanoids, *E. hadzici* represents an ideal model species to investigate biogeographic and evolutionary patterns of a regional endemic in a biodiversity hotspot. In this study, we use concatenated mitochondrial (mtCOI) and nuclear (nH3) sequence data of *E. hadzici* covering its entire range (6 out of total 7 lakes) to reconstruct phylogenetic relationships and monophyly of populations. We also aim to examine the existence of potential cryptic species within the single *Eudiaptomus* species and to delineate these. In addition to Bayesian phylogeny, we use distance based mtCOI barcoding approaches to identify putative species limits. By inferring genetic structure and estimating timing of divergence of *E. hadzici* populations we aim to investigate phylogeographic history and determine major processes responsible for differentiation of *E. hadzici*. Finally, we propose taxonomic and systematic implications of our results.

2. Materials and methods

2.1. Sample collection, DNA extraction and PCR amplification

Sampling of *E. hadzici* was conducted to cover its whole distribution range in the Western Balkans (including populations from 6 out of total 7 lakes, Fig. 1a), and specifically to encompass particular populations presumed to represent potential cryptic species (Bukvić et al., 1999). It was also designed to cover finer scale population genetic structure, so in Croatia all 3 lakes where *E. hadzici* is known to occur were sampled. Moreover, in the largest lake, Visovac, two different sites were sampled (Fig. 1a). Detailed information on sampling sites and specimen is presented in Table A1. Sampling of *E. hadzici* was carried out using a plankton net with the mesh size of 60 µm. The net was towed horizontally and/or vertically, depending on the lake size and depth, and *E. hadzici* abundance. Samples were fixed in absolute ethanol and stored at +4 °C until the extraction. Prior to DNA extraction, *E. hadzici* specimens were identified using Einsle (1993). DNA extraction was carried out using the whole specimens with the QIAamp DNA Micro Kit (Quiagen) according to the manufacturer's protocol.

Initial amplification of the mitochondrial cytochrome oxidase c subunit (mtCOI) was performed with standard primers for the barcode region (LCO-HCO; Folmer et al., 1994). However, this was only successful for populations from oligotrophic lakes in Macedonia. For the remaining populations various species were amplified, coinciding with the most abundant prey in the respective lakes (e.g. Rotatoria, *Daphnia* sp.). Thus, for these populations specific primers were designed amplifying shorter fragments (Table 1). From all populations, the histone 3 gene (nH3) was amplified using primers H3AF and H3AR (Colgan et al., 1998, Table 1). All PCR reactions were set up in 20 µl reactions using 1 µl of DNA as a template, 0.5 µl of each primer at 10 µM, and 10 µl of HotStarTaq Plus Master Mix (Quiagen). Temperature profiles are listed in Table 1. All PCR products were checked on a 0.8% agarose gel, purified and sequenced by a commercial sequencing company

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