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Species diversity and origin of non-biting midges (Chironomidae) from a geologically young lake and its old spring system

PhD Thesis

Performed in Department of Invertebrate Zoology and Hydrobiology in Institute of Ecology and Environmental Protection

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Różnorodność gatunkowa i pochodzenie fauny ochotkowatych (Chironomidae) z geologicznie młodego jeziora i starego systemu źródlisk

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Summary

In the present thesis, using midges (Diptera Chironomidae) as flagship taxa of freshwater ecology, I am focusing on the interesting research model represented by the Skadar Lake system. It is a well-known hot-spot of freshwater biodiversity consisting of the geologically young lake Skadar (originated ca. 1200 years BP) and by its ancient system of springs (originated in the Pliocene). The main aim of my thesis was to reveal and compare the morphological and molecular species diversity of non-biting midges (Diptera, Chironomidae) inhabiting Skadar Lake and its spring system. Using a taxonomy-based approach for adult males and pupal exuviae, I identified 164 Chironomidae taxa providing the first insight into species diversity of the Skadar Lake basin. Results presented in my thesis extending the existing checklist with 152 taxa newly found in the Skadar Lake basin. DNA barcoding of larvae and mature males revealed a total of 168 Operational Taxonomic Units which is a higher result than the number of morphotypes obtained during morphological identification. Pursuing this goal, I additionally compared the level of species diversity with other central and southern European lakes. A comparison of species checklists from 13 other well-studied European lakes resulted that Lake Constance (Switzerland/Germany/ Austria) is the richest in species number, followed by the Skadar Lake.

The second aim was to investigate the influence of physical-chemical conditions on composition and distribution of chironomid assemblages in Skadar Lake basin. The obtained results suggest that shallow, coastal parts of the lake covered with macrophytes are inhabited by a higher number of species.

As a third aim, I developed and evaluated the first reference barcode library for Chironomidae from Skadar Lake basin. Moreover, using an expanded reference library and records deposited in Barcode of Life Database (BOLD), I estimated DNA barcoding efficiency for the European Chironomidae. My study provides COI barcodes for 770 Chironomidae individuals assigned, based on morphology, to 75 species collected in the Skadar Lake basin (all records from this area are new for online repositories) and confirms the usefulness of DNA barcoding for the identification of non-biting midges.

My fourth aim was to explore chironomid species distribution patterns in Europe using universal Barcode Index Number (BIN) with a discussion of problematic species groups, both

for traditional taxonomy and DNA barcoding. The results of my PhD thesis provide the first insight into the factual chironomid species diversity of the Lake Skadar basin, in comparison with chironomid fauna at the European scale. The results fill a significant gap in knowledge of biodiversity in the Balkan region. Based on the results of Chironomidae fauna investigation, I can conclude that the Skadar Lake basin is now well sampled and such a high representation of species from various sampling sites provides reliable estimation of the local chironomid fauna. Based on obtained results it is hard to predict the origin of the chironomids inhabiting the Skadar Lake basin based on the sequences uploaded so far to BOLD and on their known geographic distribution. The still insufficient number of sequences is distributed between the well-studied European regions and Skadar Lake basin. Additionally, the Skadar Lake basin could be recognized as a hot-spot of freshwater biodiversity but without species-level endemism.

General introduction

For decades, a reliable assessment of species diversity was an essential scientific problem undertaken by many research teams. In the past years, huge efforts have been made aiming to estimate global species diversity in many projects funded by national and international agencies. The question of global species diversity could not be resolved without referring to different scales in time and space. Estimating biodiversity at the regional scales seems to be crucial for further calculations on a wider, global, scale (Bond *et al.,* 2002; Crist *et al.,* 2003). Researchers aiming to explain the variability of biodiversity should refer to processes of migration, speciation and extinction (Vellend, 2003; Hubbell, 2001). The comprehensive knowledge of species inhabiting a particular environment represents the essential prerequisite for conservation biology and global biodiversity assessments. Besides, farsighted strategies preserving the biological diversity require an understanding of both, theoretical and empirical processes underlying species distribution at different levels (Gaston, 2000; Brown, 1989).

Due to its complex geological history and unique combination of geological and climatic factors, the Mediterranean region is recognised as one of the 25 most important biodiversity and endemism hot-spots worldwide (Myers *et al.,* 2000, Woodward 2009, Blondel *et al.,* 2010). Skadar Lake represents one of them and is a well-known hot-spot of freshwater biodiversity and endemicity (Pešić *et al.,* 2018).

The main aim of my thesis was to reveal and compare the morphological and molecular species diversity of non-biting midges (Diptera, Chironomidae) inhabiting Skadar Lake and the system of its springs. Pursuing this goal, I additionally compared the level of species diversity with other central and southern European lakes. **The second aim** was to investigate the influence of physical-chemical conditions on composition and distribution of chironomid assemblages in Skadar Lake basin. Results of these findings, except species diversity based on molecular data, are presented in **Chapter** I. **As a third aim**, I developed and evaluated the first reference barcode library for Chironomidae from Skadar Lake basin. Using a reference library, I estimated DNA barcoding efficiency for the European Chironomidae based on BOLD records. **My fourth aim** was to explore chironomid species distribution patterns in Europe using universal Barcode Index Number (BIN) with the discussion of problematic species groups, both for traditional taxonomy and DNA barcoding. In **Chapter II**, I presented results of the two latter aims together

with results upon the molecular diversity of species. Both chapters and presented in the form of manuscripts ready for submission to scientific journals.

The results of my PhD thesis provide the first insight into the factual chironomid species diversity of the Lake Skadar basin in comparison with chironomid fauna at the European scale. Moreover, I demonstrate a future direction for the field of taxonomy using DNA barcode-based species diagnoses. In-depth understanding of processes shaping faunas in biodiversity hot-spots will allow for planning reasonable and effective strategies preventing or at least minimising the loss of species diversity.

Skadar Lake

Figure 1. Northern part of the Skadar Lake

Skadar Lake is a shallow lacustrine ecosystem located in the outer part of the Dinaric Alps, in the south-western part of the Balkan Peninsula (Pešić *et al.*, 2019). Approximately two-third (229 km^2) of its surface belongs to Montenegro and about one-third (142 km^2) to Albania.

Length of the coast is 168 kilometres, from which 110.5 km on the Montenegrin side and 57.5 km in Albania. The lake is approximately 44 km long and 14 km wide with a surface area that seasonally fluctuates between 370 km^2 to 530 km^2. The average level of the lake is 6.52 m above sea level with a mean depth of 5 m. It is the biggest lake on the Balkan Peninsula, situated in Zeta-Skadar valley. It is located only 7 km from the Adriatic Sea and outflows to the sea through the River Bojana. Bojana is also connected to the Drin River by the 11-km long River Drinasa, and together they form a connection with the Ohrid Lake in the Republic of Northern Macedonia. The characteristic feature of the Lake Skadar water balance is high inflow from many temporary and permanent karstic springs. Some of them (called 'oko') are sublacustrine and in cryptodepression. The best known of those springs are Karuč and Raduš, the latter being also the deepest one - at least 60 m deep. Coast of the lake encompasses numerous bays, islets and capes (Figure 1). On the eastern side, it is mostly a swamp area covered with a wide reed zone. Only the western part of the lake, the coast is rocky, with numerous small islands (Pešić *et al.*, 2018).

The Skadar Lake catchment area covers 5,631 km^2, and the lake itself has several tributaries that flow into the lake. River Morača is the most important one and provides 63% of the total water inflow. Morača River also provides most of the sediments and nutrients to the lake (Pešić *et al.*, 2018). The most important tributaries of Lake Skadar enter the lake from the north: the Morača, the Crnojevića, the Orahovštica, the Karatuna and the Plavnica in Montenegro. The Rjolska and the Vraka rivers from the Albanian side. Precipitation and evaporation regime over the lake and its catchment, as well as lake bathymetry, causes large water levels fluctuations from 4.5 to 10.4 m above the sea level. The highest precipitation occurs during the spring while the lowest during late summer. During the year, water in the Lake Skadar renews 3.5 times which corresponds to a water residence time of ca. 100 days (Skarbøvik *et al.*, 2014). The climate of the Lake Skadar basin is Mediterranean, the temperatures in summer are high, providing extensive evaporation, while in the winter temperature is low but usually above the freezing point (Lasca, 1981).

Various habitats present in Lake Skadar support rich species diversity. There are 726 vascular plants, with more than 30 rare species, about 60 fish species (15 of them endemic to the Skadar basin), 15 amphibians, 30 reptiles, 281 bird species (90% of them are migratory species of international, conservation importance), and 57 mammals (Dhora and Sokoli, 2000; Bejko,

2011). The Montenegrin part of Lake Skadar (40,000 ha) was declared a National Park in 1983 (IUCN category II) by the Law on Shkoder Lake (Nat. Her. SRCG 33/83). Since 2005, the Albanian side is protected as “Managed Natural Reserve” (IUCN category IV) (Official Gazette SRCG 1991). In 1995, it was also added to the designation by the Federal Republic of Yugoslavia for inclusion in the RAMSAR World List of International Important Wetlands in recognition of its enormous value as an aquatic habitat (Site 3YU003). This decision has drawn international attention to the management of the lake and its surroundings. Since 2005, Bojana/Buna River has also been protected on the Albanian side with the status of the "Protected Waterscape/Landscape" (IUCN category V) (Katnic, 2013).

The Skadar Lake system represents a well-known hot-spot of freshwater biodiversity, with a high degree of endemicity, predominantly associated with the local network of karstic springs (Glöer *et al.*, 2009; Pešić and Glöer, 2013). However, the time of origin and diversification of the local faunal/floral elements inhabiting the Skadar Lake and its system of spring is uncertain (Grabowski *et al.*, 2018). Only recent studies of Middle-Late Holocene palaeoenvironmental and palaeoclimatic changes based on micropalaeontological evidence, including subfossil Ostracoda and Characeae, compared with palynological data and stable isotope curves provided a rational hypothesis about the time of origin of Lake Skadar. Mazzini *et al.* (2015) concluded that, at least from 4,560 to 1,200 cal year BP, Skadar Lake basin was a freshwater marshland fed by numerous springs, particularly in the north-western part of the valley. According to the author, the Skadar Lake originated quite abruptly from a vast flat marshland which lied on the depression and evolved into a lake by filling with water from springs by the changed course of the Drim River. Summarising the numerous hypotheses on the origin of the Skadar Lake origin, Grabowski *et al.* (2018) suggested the most probable combination of factors, such as the progressive sea level rise along the eastern Adriatic coastline, and the possible change course of the Drim River that overflowed the Skadar depression. It is a plausible scenario taking into account very dynamic tectonics in the Balkans. Thus, the Skadar Lake itself is very young, originated between 1274 – 1197 calibrated years before present (Mazzini *et al.*, 2015). In contrast to the lake, the Skadar basin with its system of springs is ancient and originated in the Pliocene, ca. 3 Ma, if not earlier (Grabowski *et al.*, 2018, 2019).

Chironomidae

In the present thesis, I focused on the dipteran family Chironomidae, non-biting midges, which is a flagship taxon in freshwater ecology. The knowledge upon the chironomid species inhabiting the Skadar Lake and its system of springs is still fragmentary and far from being complete (Płóciennik *et al.*, 2014; Płóciennik and Pešić, 2012; Jacobi, 1977; Karaman and Nedić, 1975; Janković, 1974). Chironomidae, with more than 6000 valid species described worldwide, grouped into 550 genera, is one of the largest and most diverse dipteran families (Ashe *et al.*, 2009; Ashe *et al.*, 2012; Pape *et al.*, 2011). They belong to the suborder Nematocera. Like all flies, chironomids are holometabolous. The chironomid life cycle is divided into four life stages, i.e. egg, larva, pupa, and adult. The time of development from an egg to adulthood depends on the species and season. It ranges from less than a week in the tropics to even seven years in the Arctic. Most species emerge within one to two weeks (Armitage *et al.*, 1995).

Larvae newly hatched from eggs, called larvulae, are short-living and disperse with water drift. They remain planktonic until a suitable habitat is found. The occurrence of four larval instars is universal in Chironomidae with unconfirmed reports of the fifth instar in Tanypodinae. The second instar is little studied and possesses intermediate features between larvulae and later instars. Third and fourth instars are best studied and have most morphological features useful for identification to genus level. Unfortunately, most of the larvae are very difficult to identify using morphology. Confusion is caused due to the complexity of Chironomidae taxonomy. The majority of the characters useful for larval identification are found on the sclerotised head capsule, with most of the differentiating characters located on the ventral side of the head. Problems with identification are caused by the fact that most of the features are very similar among taxa (not only those that are closely related). Most of the studies on chironomid communities have been hampered by the notorious difficulties of species-level identification, and thus the identifications were usually restricted to higher taxonomic levels (Nyman *et al.*, 2005) or referred to “larval types” (Real and Prat, 2000). Except for some predaceous species, all chironomids build a larval case on or within the substrate in which they live.

At larval stages, chironomids inhabit various freshwater ecosystems such as streams, rivers, ponds, lakes, dam reservoirs and, to a much lesser extent, brackish waters and soil. Some breed in isolated damp habitats such as tree-holes, pitcher plants, patches of moist soil, dung pats and even thin films of water on high-altitude glaciers. They are found from 5600 m asl on glaciers in Nepal down to depths of over 1000 m in Lake Baikal. Larvae of the genus *Baeoctenus* parasitise on the gills of the swan mussel, *Anodonta,* while larvae of the genus *Symbiocladius* parasitise mayfly nymphs (Coffman and Ferrington, 1996; Armitage *et al.*, 1995; Cranston, 1983; Wiens, 1975). The "blood midges" or "bloodworms" have haemoglobin in their hemolymph, which allows them to survive in low-oxygen habitats. Exceptional physiological and behavioural adaptations, such as the evolution of heat shock proteins and haemoglobin genes, let chironomids colonise extreme environments such as glacial rivers or highly polluted waters. In addition, due to their large populations, even in heavily eutrophic waters, chironomids play a prominent role in energy flow in freshwater ecosystems. Considering all the previous features, in association with the fast response to environmental changes, benthic chironomid communities have been considered to be among the most promising biological indicators of water and sediment quality (Lindegaard, 1995), both in lakes (Free *et al.*, 2009) and in running waters (Soulikidis *et al.*, 2009).

Before the completion of the larval stage, chironomids attach themselves to substrates with silken secretions and the pupation process starts. The **pupal stage** is short-lived when compared with the larval stage, it lives from a few hours to several days. When the developing adult matures, pupa frees itself from a silken chamber and, with help of collected air, swims to the water surface. Here, an adult emerges from the pupal skin (or exuviae) and flies away. The pupal exuvium remains floating on the water surface and can be moved by wind or water currents to downstream areas, vegetation or submerged obstacles. Pupal exuviae accumulated in such places can be collected for an efficient survey of species composition or emergence patterns (Gadawski *et al.*, 2016). Such a technique, named Chironomidae Pupal Exuviae Technique (CPET), is often used in bioassessment of aquatic ecosystems (Kranzfelder ,2015; Wilson and Ruse; 2005; Ferrington *et al.*, 1991). When using appropriate keys and descriptions, pupal exuviae are an excellent material to identify specimens to genus, and often species level (Prat and Rieradevall, 2016).

Mature Chironomidae mate mostly in aerial swarms, however, some species mate on the ground. Such swarms consist mostly of males. The less abundant females usually also fly in the mating swarm. The eggs are laid in gelatinous batches, shortly after mating. Such egg packages sink to the bottom or are attached by a gelatinous anchor cord to submerged vegetation or submerged objects. Some species lay eggs which flow under the water surface. Depending on the species, egg masses contain from less than 100 to more than 2000 eggs, which usually hatch within 24 to 36 hours (Armitage *et al.,* 1995). In various publications during the past decades, it has been repeated that non-biting midges do not feed as adults. Nowadays there is evidence of feeding as imagines. Their diet includes fresh fly droppings, nectar, pollen, honeydew, and various sugar-rich materials (Armitage *et al.,* 1995). Even if chironomids are considered as weak flyers, with a flight distance restricted by the adult life-span (4 to 8 days), they are able to migrate actively and/or passively (by anemochory) and colonise new habitats, even in a short time (Ferrington, 2008, Armitage *et al.,* 1995). However, despite the weak flying abilities, faunistic and autecological data indicate that most of the species are widespread in each biogeographic area, such as the Palaearctic (Moller Pillot, 2009). Historically, most Chironomidae species have been described based on morphological characters of adult males. By the fact that females are often difficult or impossible to identify using morphological characters, they are suffering a general lack of taxonomic attention (Ekrem, 2010).

Species concept and integrative taxonomy

Defining and recognising species has been a controversial issue in taxonomy for a long time. Taxonomy is a basis of biology, used widely to explore and understand biodiversity. It is a science and art of identifying, describing, classifying and naming extant and extinct species (or higher taxa). Species name provides a link to the knowledge about an organism. Delimiting species using empirical data relies on a hypothesis of what a species is, or in other words on a species concept. Concepts itself do not only define what species is but also clarify what speciation is. In modern science, taxonomists proposed more than 20 different approaches to species concept (Schlick-Steiner, 2009). With such a large number of concepts, it is not an easy or simple decision to adopt one. According to the study of Aldhebiani, (2018), following major species concepts are known: *i)* biological, *ii)* morphological, *iii)* ecological, *iv)* evolutionary, *v)*

coherent, *vi)* phenetic, *vii)* phylogenetic and *viii)* pluralistic. Whichever of the species concepts is chosen, it will influence the delimitation criterion and further choice, analysis and interpretation of data. According to the indicated division, for the purpose of my thesis, I have chosen the phenetic species concept, as a classification process based on similarities between present properties of organisms. **As it was already specified, the main aim of my thesis is to explore the species-level diversity of chironomids in Lake Skadar basin integrating both, the morphology-based taxonomy and molecular methods (DNA barcoding).** Morphological species concept states that "a species is a community, or a number of related communities, whose distinctive morphological characters are, in the opinion of a competent taxonomist, sufficiently definite to entitle it, or them, to a specific name" (Regan, 1926). Sometimes identification based on morphological characteristics is subjective and depends on expert opinion for key traits. Species identification using DNA barcoding is consistent with any species concept that a taxonomist uses to establish a named species. Fundamentally, barcoding ignores difficult issues of species concepts and boundaries, dynamics of biological classifications and taxonomic hypotheses. In DNA-based taxonomy, what is being estimated for a specimen is not necessarily its membership to a 'species', however defined. We call the taxa yielded by a grouping of specimens through a set of markers Operational Taxonomic Units. If two specimens yield sequences that are identical within some defined thresholds, they are assigned to the same Molecular Operational Taxonomic Units (Blaxter, 2004).

Nowadays, sound and reliable taxonomy is greatly needed, particularly during the challenge posed by the biodiversity crisis (Díaz, 2019). Many taxonomists began to suspect that the majority of species would remain undescribed and that some of them will probably go extinct before we have a chance to describe them (Pante, 2015). Describing and naming new species is a fundamental step when estimating species diversity and is the only way to be sure that researchers are talking about the same entity. It enables a broad perspective to compare data linked with species but obtained by different scientists. Moreover, only named species can be listed on endangered checklists and properly protected. Thereby we can save species and enable their survival (Mace, 2004). Considering the time, cost and specialist expertise required for sampling in the field and species identification traditional taxonomy is nowadays facing more challenges. Only implementing and merging new theories, methods and data from various

studies that focus on the origin, limits and evolution of taxa will bring new perspectives during the current taxonomy crisis (Padial, 2010; Pires and Marinoni, 2010).

Integrative methods are being increasingly used in species delimitation and have emerged as a consequence of combining various fields of biology, such as population ecology, behavioural sciences, molecular phylogenetics, phylogeography and evolutionary ecology. When morphological information is not sufficient, or doubtful, for reliable species identification, alternative techniques can provide additional verification. The most revolutionary changes in taxonomy were caused by the fast development of molecular techniques. For species identification in difficult orders of insects, such as Diptera, implementing molecular techniques and combining them with traditional approach provides additional decisive confirmation.

DNA barcoding

Linking morphology-based taxonomy with molecular techniques, such as DNA barcoding, can be particularly useful in molecular identification of specimens when subtle variation in morphological features are present among species. Major advantages of DNA barcoding in comparison to the traditional morphological approach were obtained in the case of immature, damaged, neglected organisms and when phenotypic characters are difficult to define. Likewise, if a researcher lacks the experience to identify taxa on the basis of their morphology (Ball *et al.,* 2005; Geraci *et al.,* 2011; Hebert *et al.,* 2003; Janzen *et al.,* 2005; Savolainen *et al.,* 2005; Montagna *et al.,* 2016). The use of molecular data, and molecular barcoding in particular, was successfully proposed by Hebert *et al.* (2003) and welcomed as such by taxonomists. The method was presented as one of the answers to "taxonomic impediment", to fill the knowledge gaps in our taxonomic system and the lack of well-trained taxonomists and curators.

DNA barcoding is a method of identifying organisms based on a short, standardised fragment of genomic DNA and has been developed for use by taxonomists, ecologists, conservation biologists and regulatory agencies (Hoy, 2013). DNA barcoding involves sequencing a short (typically 400–800 base pairs) 5' region of the mitochondrial *cytochrome c oxidase subunit I* (COI) gene, called "barcode" from taxonomically unidentified specimens

(Savolainen *et al.*, 2005). In the next step, it enables comparison of an obtained fragment with a reference library of DNA barcodes deposited in online repositories (Ratnasingham and Hebert, 2007). The individual DNA sequence can be used to identify organisms to species level based on the similarity of nucleotides. The capability to identify organisms starting from a molecular marker has been enhanced by the presence of standardised DNA-barcode reference databases, e.g., BOLD and GenBank (Ratnasingham and Hebert, 2007; Clark *et al.*, 2016).

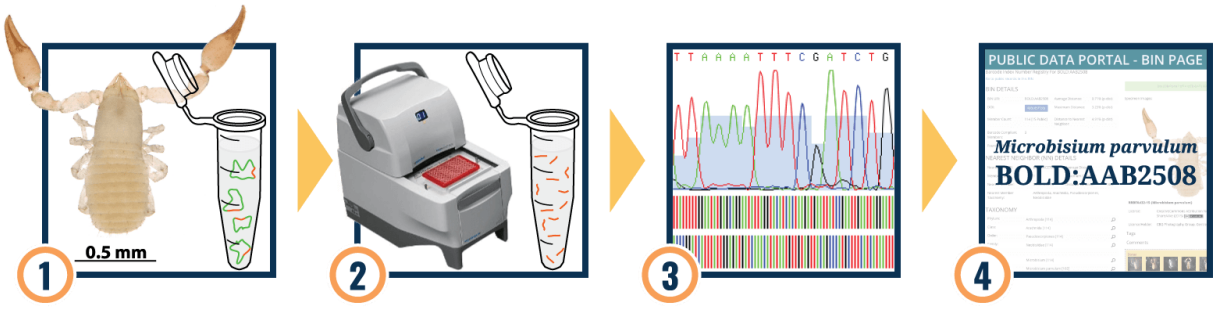

Figure 2. The process of DNA barcoding. Source: www.ibol.org

In the first step, the total DNA is isolated from a tissue sample. In the second step, the target DNA barcode region is amplified using Polymerase chain reaction (PCR). In the third step PCR products are sequenced and in the final step obtained sequences are compared against reference databases to find the matching species (Figure 2).

The DNA barcoding has been proposed, and successfully adopted (Yu *et al.*, 2012; Wang *et al.*, 2012; Carew *et al.*, 2013; Arribas *et al.*, 2016; Kimberley *et al.*, 2016), as an efficient method for species identification and habitat biodiversity assessment using standardised genetic markers. The mutation rate in COI can be fast enough to provide informative characters for delineation of closely related and sibling species and even to analyse phylogeographic patterns within a single species (Hebert *et al.*, 2003a, 2003b; Hunter *et al.*, 2008; Ilmonen, 2009). Despite the broad benefits that DNA barcoding can bring to a diverse range of biological disciplines, a number of shortcomings still exist. Previous studies have shown problems and limitations of the method such as mitochondrial heteroplasmy (the coexistence of multiple mitochondrial haplotypes in an individual) (Gandolfi *et al.*, 2017; Rubi *et al.*, 2018), the occurrence of symbiotic bacteria (Smith *et al.*, 2012, Kodandaramaiah *et al.*, 2013), the similarity of sequences in different species from distant geographic areas (Collins and Cruickshank, 2013; DeSalle *et al.*, 2005), introgression and hybrid speciation (Nesi *et al.*, 2011; Taylor and Harris, 2012; Kvist,

2013; Gay *et al.,* 2007; Martinsen *et al.,* 2001; Cong *et al.,* 2017; Zakharov *et al.,* 2009), incomplete lineage sorting (ancestral polymorphism) (Ballard and Whitlock, 2004; Heckman *et al.,* 2007; Willyard *et al.,* 2009, Mallo, 2016) or the presence of NUMTs - nuclear pseudogenes of mitochondrial origin providing multiple gene haplotypes, (Lopez *et al.,* 1994; Bensasson *et al.,* 2001; Richly and Leister, 2004; Brower, 2006; Song *et al.,* 2008; Zhang and Hewitt, 1997). NUMTs and symbiotic bacteria, like Wolbachia not yet been recorded in Chironomidae, but the other causes are possible explanations for the observed inconsistencies between morphological and molecular species clusters (Lin *et al.,* 2015). The above examples demonstrate that DNA barcoding is not trivial. It is important to consider the characteristics of the data in order to choose the most appropriate research methodology; or even better, to design the research project and the sequencing strategy taking into account the expected results and the most appropriate methods to analyse the data.

The use of DNA-based diagnostic characters for species identification within Chironomidae has proven effective for species recognition (Brondin *et al.,* 2003; Ekrem *et al.,* 2007; Ekrem *et al.,* 2010; Silva *et al.,* 2013; Lin *et al.,* 2015; Song *et al.,* 2016; Cranston and Krosh, 2012). Building up the knowledge upon chironomid fauna inhabiting the Skadar Lake basin, i.e. understanding patterns and processes that shaped the local fauna, the results of my thesis will have major implications in the development of farsighted strategies of freshwater biodiversity conservation. Since only the adult male stage is described for most non-European species, leaving out larvae and females, DNA barcoding has been shown also suitable to associate life stages (Carew *et al.,* 2005; Stur and Ekrem, 2011; Silva and Wiedenbrug, 2014). Midge larvae are very important for water quality assessments and they remain underutilised since the current sorting techniques cannot deliver species-level resolution at a reasonable cost. Nevertheless, DNA-based methods allow us to utilise non-biting midges in biomonitoring (Carew *et al.,* 2013; Sharley *et al.,* 2004; Brodin *et al.,* 2013; Cranston *et al.,* 2013). Beside the previously highlighted impact, the development of an accurate database of DNA barcodes for midges inhabiting the Skadar Lake system is of extreme interest for the existing databases (e.g., BOLD, GenBank). Developing a well-curated reference barcode library will enhance and serve for a taxonomic description of newly discovered cryptic species like it was done in previous studies (Anderson *et al.,* 2013; Lin *et al.,* 2018; Stur and Ekrem, 2015). Even now, such data are widely used in biogeography

studies and helps to resolve phylogenetic relationships (Krosh *et al.*, 2020; Stur *et al.*, 2019; Ekrem *et al.*, 2007; Cranston *et al.*, 2010; Damin *et al.*, 2011; Sari *et al.*, 2015).

The proposed expeditious and relatively cheap method of measuring the species diversity has to be considered essential in the water quality assessment on a large scale. Nowadays, serious discussion about the degradation of the freshwater habitats, which provide irreplaceable ecosystem services (e.g. drinking water, irrigation, hydropower, tourism etc.) and which are highly susceptible to environmental change, is under strong interests due to the heavy anthropogenic impact (Wilbur *et al.*, 1999, Hanson *et al.*, 2004). Without such knowledge, it is hard to imagine planning a reasonable and effective strategy preventing or at least minimising the loss of biodiversity. It must also be remembered that planning and implementing such a strategy is one of the top priorities of many EU and global agendas for the conservation of natural resources. The results achieved by this thesis are surely filling a large gap in knowledge of biodiversity in the Balkan region.

Chapter I. First insight into the diversity and ecology of non-biting midges (Diptera: Chironomidae) in the unique ancient Skadar Lake system (Montenegro/Albania).

1. Introduction

Chironomidae (non-biting midges), with nearly 7,500 species grouped into 550 genera, is one of the largest and most diverse dipteran families (Pape *et al.*, 2011). Chironomidae represents a flagship taxon in freshwater ecology and due to large populations, even in heavily eutrophic waters, chironomids play a prominent role in energy flow in various aquatic ecosystems (Armitage *et al.*, 1995). Chironomids are of relevant interest in basic/fundamental research since they can survive in extreme conditions of water temperature and level of pollution. Besides this, due to the fast response of larvae to environmental changes, chironomid communities has been considered to be among the most promising biological indicators of water and sediment quality (Lindegaard, 1995), both in lakes (Free *et al.*, 2009) and in running waters (Skoulikidis *et al.*, 2009). However, most of the studies upon chironomid communities have been hampered by the notorious difficulties in species identification and, thus, identifications were usually restricted to higher taxonomic level (Nyman *et al.*, 2005) or referred to larval morphotypes (Real *et al.*, 2000).

The Skadar Lake is a shallow lacustrine ecosystem located in the outer part of the Dinaric Alps, in the western Balkans (Pešić *et al.*, 2019), and represents a well-known hot-spot of freshwater biodiversity, with a high degree of endemicity (Pešić *et al.*, 2018). The time of origin and diversification of the local faunal/floral elements inhabiting the Skadar Lake and its system of spring is uncertain (Grabowski *et al.*, 2018). Recent studies have shown that the Skadar Lake itself is very young, originated between 1274 – 1197 BP (Mazzini *et al.*, 2015). However, the Skadar basin with its system of springs is ancient and originated in the Pliocene, ca. 3 Ma, if not earlier (Grabowski *et al.*, 2018). The faunistic knowledge on many groups, including Chironomidae, is still incomplete and fragmentary. Up to date, 38 species were reported from the Skadar Lake basin (Nedeljković, 1959; Janković, 1974; Karaman and Nedić, 1975; Jacobi, 1977; Jacobi, 1981; Płóciennik and Pešić, 2012; Płóciennik *et al.*, 2014; Pešić *et al.*, 2018; Pešić, 2018). The fact that Skadar Lake is a very recent lake fed by the geologically old system of

springs, makes it a very attractive model to test different assumptions about the biogeography and species composition of this aquatic insect group. Providing that Lake Skadar basin is a combination of diverse habitats, from the rocky environment through shallow parts of the lake covered by macrophytes to sublacustrine springs area, we hypothesise that they will be inhabited by different chironomid communities.

Despite the great value of the floral and faunal elements, this richness is greatly endangered. Due to the demographic movements and increasing human activity pressure the Skadar Lake region becomes internationally popular. Mass tourism is developing in an area which has almost no capability to protect the environment from huge volumes of visitors or monitor for illegal activities. The exploitation of natural resources leads to ecosystem degradation and water quality concerning (Stesevic *et al.,* 2007). The natural and cultural heritage of the Lake Skadar basin requires the implementation of an efficient management system at the level of the whole lake for the purpose of its continuous protection and the valorisation of this unique ecosystem. Especially in face of climatic changes and increasing demands for water increasingly impact the lake ecosystem.

The main aims of this study are: i) to investigate the composition and distribution of chironomid assemblages in Skadar Lake basin associated with physical-chemical conditions of the collecting sites, ii) to provide an updated list of Chironomidae species inhabiting the Skadar Lake basin, iii) to compare Chironomidae midges species diversity with other central and southern European lakes, and iv) to provide the actual state of the protection efforts undertaken for Skadar Lake fauna conservation. Moreover, by describing the valuable character of the basin and its uniqueness, we propose how non-biting midges fauna assessments may contribute to further protection efforts and raise the attractiveness of the whole region.

2. Material and methods

2.1. Study site

Skadar Lake is the largest lake in the Balkan Peninsula, with approximately two-third (229 km^2) of its surface belonging to Montenegro and about one-third (142 km^2) to Albania. The distance between the north-western and south-eastern part of the lake is 44 km long and greatest width is 13 km, with a surface area that seasonally fluctuates between 353 km^2 in dry

periods and 530 km^2 in wet periods (Pešić *et al.*, 2018). The lake makes an area of 2042 ha subjected to seasonal floods (Knežević and Todorovic, 2004). The most important tributaries of Lake Skadar enters from the north: Morača, Crnojevica, Orhovstica, Karatuna, Baragurska River in Montenegro and Rrjolli and Vraka Rivers in Albania. The Bojana River connects the lake with the Adriatic Sea, and the Drin River provides a link with the Ohrid Lake. The characteristic feature of Lake Skadar water balance is high inflow from a number of temporary and permanent karst springs, some of which are sublacustrine and in cryptodepression. Skadar Lake and its basin is a well-known hot-spot of freshwater biodiversity, however, it is not easy to classify Skadar Lake to any distinct lake-type because, in fact, it makes a combination of several types of lakes, e.g. i) the deeper, open part of the lake belongs to the oligotrophic type, ii) but the northern shallow part of the lake, covered by macrophytes, has all the characteristics of a eutrophic lake (Pešić *et al.*, 2009; Mrdak *et al.*, 2011; Pešić and Glöer; 2013; Zawal *et al.*, 2019) (and karst spring system). Such conditions connected with different types of bottom provides very specific habitats for freshwater macroinvertebrates.

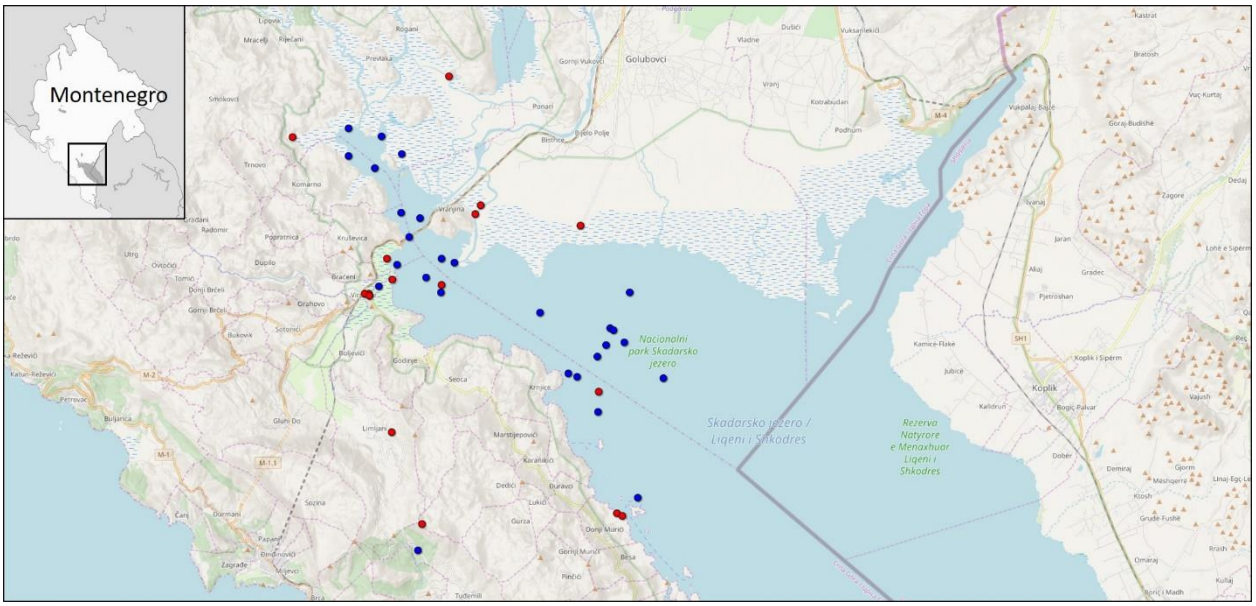

Figure 3. Map of sampling sites. Red points – sites where adult specimens were collected. Blue points – sites where pupal exuviae were collected. The arrow indicates three collecting sites outside of the area. Map source: OpenStreetMap contributors based on the Open Data Commons Open Database License.

2.2. Material collection

Pupal exuviae and imagines were collected during three expeditions to the Skadar Lake system, in spring (April/May) and autumn (September/October) 2014 and in summer (July/August) 2015, covering three seasons of chironomid emergence (Figure 3). Sites inaccessible by land were visited by boat. Imagines were collected by sweeping through coastal vegetation using entomological hand net from the lake and river shores, and attracted to light in the open areas, ensuring that the light was visible over a long distance above lake surface (Figure 4). For the latter purpose, 500W white-light bulb connected to a power generator was placed in front of a white screen (2m × 3m). The exposition started ca. 45 minutes before sunset and continued for three hours. The light sampling was performed during each expedition to maintain the repetition of sampling sites across the different collecting seasons and thus to get information on seasonal turnover of the communities. Individuals were picked up and then transferred to plastic bottles filled with 96% ethanol with appropriate labels. Pupal exuviae were gathered from the water surface of the open lake using a four-person boat, from the alluvial banks of the lake and from various habitats among the spring system surrounding the lake (Chironomidae Pupal Exuviae Technique - CPET). The material was collected using water-resistant hand-net and transferred to vials with 70% ethanol. For the purpose of further analysis, the studied area of the Lake Skadar basin was partitioned into the following zones: lake littoral, open lake, krenal, potamal and rhithral following Marziali and Rossaro, (2013). The studied habitats were classified as silt, silt_macrophytes, sand, stones and macrophytes. The above-adopted criteria to select the sampling sites were applied to maximise the representativeness of the whole range of habitats present in the area (i.e., open lake, shallow lake, springs, sublacustrine springs, rivers inflow) and to maximise the diversity of collected chironomids.

Figure 4. A) Light trap used for attracting adult Chironomids in Karuć, photo: Piotr Gadawski. B) Collecting individuals using entomological net, photo: Michał Grabowski.

2.3. Specimen identification

For the identification, chironomid imagines were picked up from the bulk samples and then grouped into morphotypes. In the next step, the individuals have been morphologically identified to the species level. Pupal exuviae were mounted on microscope slides using DMHF (dimethyl hydantoin formaldehyde resin) as a mounting medium. Exuviae were identified to the lowest taxonomic level based on the morphological characters. The material was sorted under Opta-TECH SK series stereomicroscope and species identification was performed under a Opta-TECH MK series microscope with 100×-1000× magnification, according to the diagnostic morphological characters, e.g. genital apparatus, wing, antenna and legs for adult males; diagnostic morphological characters of exoskeleton for exuviae). Taxonomic identification was performed based on recent literature (Reiss, 1968; Wiederholm, 1986; Wiederholm, 1989; Langton, 1991; Langton and Visser, 2003; Langton and Pinder, 2007; Giłka, 2011). The identified specimens have been deposited at the University of Lodz, Poland and at the University of Milan, Italy (under the trusteeship by Bruno Rossaro).

2.4. Environmental parameters

Beside the geographic coordinates, in each collecting site, the following parameters were measured: pH, electrical conductivity, NH_4, NO_3, TP, water temperature, $CaCO_3$, O_2 (Table S1). The depth was measured by Depth Sounder, ECHOTEST II provided by Plastimo, France. Substrate types (silt, silt_macrophytes, sand, stones) were divided by the Braun-Blanquet (1964)

methods modified by Stępień *et al.* (2019). The substrates were categorised empirically during sample collection depending on the grain size i.e., silt (< 2 mm grain), sand (< 2 mm grain size), stones (21-100 mm grain size), silt_macrophytes (silt covered with macrophytes). The physico-chemical parameters of water were measured at each site during macroinvertebrate sampling. Water temperature (used as an indirect factor influencing the emergence of imagines by its direct influence on larvae), pH, electrical conductivity and dissolved oxygen content were recorded with Elmetron CX-401 multiparametric sampling probe; $P\text{-}PO_4$, $N\text{-}NO_3$, $N\text{-}NH_4$ with Slandi LF205 photometer.

2.5. Statistical analysis

The obtained data were processed using the R environment (R Core Team, 2019). Separate analyses were carried out for the adult males and the pupal exuviae. Species diversity measurements were performed using the *vegan* package for R software (Borcard *et al.*, 2011; Oksanen *et al.,* 2018; htpp://www.r-project.org/). Shannon (H'), Simpson (S) and inverse Simpson (I) diversity indexes were calculated using all samples, according to the formulas presented by Shannon, (1948) and Simpson, (1949), respectively. A redundancy analysis (RDA) was performed with the support of the *Hmisc* package to reveal the relationship between species distribution and environmental variables (Harrell, 2019). RDA was carried out including only taxa present in at least 5 samples. Lakes for cluster analysis were selected based on the availability of Chironomidae checklists from well-studied central and southern European lakes (Reiss, 1968; Bazzanti, 1980; Lods-Crozet *et al.*, 1994; Petridis and Sinis, 1995; Specziár, 1998; Smiljkov *et al.*, 2001; Smiljkov *et al.*, 2006; Smiljkov *et al.*, 2008; Rossaro *et al.*, 2012; Tarrats *et al.*, 2017; Bitušík and Trnková, 2019). Cluster dendrogram with species similarity between the selected lakes was calculated using Ward's method (Ward, 1963) as a clustering criterion in R, package *Hclust* (Müllner, 2013). Briefly, hierarchical cluster analysis was performed using a set of dissimilarities (species) for the objects (lakes) being clustered. Initially, each lake was assigned to its own cluster and then the algorithm proceeded iteratively, at each stage joining the two most similar clusters, continuing until there is just a single cluster. In Ward's algorithm, using option 'ward.D2', dissimilarities were squared before clustering.

4. Results

4.1. Chironomidae species diversity and abundance

In total, 8845 individuals (adult males and pupal exuviae) were collected from 45 sites of the Skadar Lake and surrounding spring systems taking into account the seasonality of emergence (Figure 3 and 4). Out of that number, 3316 males and 631 pupal exuviae were collected during spring 2014, 2564 males and 463 pupal exuviae were collected during autumn 2014, as well as 1394 males and 476 pupal exuviae during summer 2015. The collected individuals were assigned to 164 Chironomidae taxa, but only 125 of them could be assigned to a complete valid name. It is a result of taxonomic impediment during identification of species-rich genera. In taxonomic literature under different abbreviations (e.g., Pe1 - Pupal exuviae 1, sp. A - species A, etc.) are described distinct morphotypes with visible differences but those forms are not yet described as a separate species. As an example on our species list 8 *Chironomus,* 11 *Cricotopus* and one *Polypedilum* morphotypes were identified using such abbreviations (Table S2) (Langton and Visser, 2003; Langton and Pinder, 2007). The collected material consisted of 7274 male adults, that were assigned to 86 distinct morphotypes and 82 correct species-rank names (Table S2). Fifty two species were recorded in spring 2014, and the most abundant were *Polypedilum nubeculosum* (Meigen, 1804), *Tanytarsus usmaënsis* Pagast, 1931 and *Chironomus riparius* Meigen, 1804. During autumn 2014 we collected 59 species, among which *Polypedilum nubeculosum*, *Paratanytarsus natvigi* (Goetghebuer, 1933) and *Cricotopus sylvestris* (Fabricius, 1794) dominated. Forty six species were found in summer 2015, with the highest abundance of *Procladius choreus* (Meigen, 1804), *Polypedilum nubeculosum* and *Cryptochironomus albofasciatus* (Stäger, 1839) (Table S2). In addition, we identified 133 morphospecies (95 valid species) based on 1571 pupal exuviae, among which 66 species were found in spring 2014 (most dominant: *Endochironomus albipennis* (Meigen, 1830), *Tanytarsus usmaënsis* and *Tanypus punctipennis* Meigen, 1818), 48 species were recorded in autumn 2014 (most abundant: *Chironomus plumosus* (Linnaeus, 1758), *Chironomus Pe.2,* and *Chironomus Pe.3*), and 60 species were found in summer 2015 (most abundant: *Kiefferulus tendipediformis* (Goetghebuer, 1921), *Paratanytarsus bituberculatus* (Edwards, 1929) and *Cladopelma virescens* (Meigen, 1818)) (Table S2). Additionally, we identified four genera not reported before from the Skadar Lake basin: *Sergentia*, *Stenochironomus*, *Stictochironomus* and *Thienemanniella*.

4.2. Species diversity and distribution

Diversity indices were calculated separately for adult males and pupal exuviae (Table S1). For adult males, the highest number of species (41) was observed in Virpazar.shop sampling site, which showed, consequently, the highest Shannon's diversity index (*H'*=2.56) and was followed by Vranjina with 36 species (*H'*=2.50). Calculated biodiversity indices revealed that highest species diversity was present in sites classified as *potamal* with median Shannon species diversity index *H'*=2.23; median Simpson's index D=0.85 and inverse Simpson A=6.86. The highest diversity was recorded on sites with *silt* as a substrate type *H'*=2.23; D=0.85 and A=6.86 (Table S1).

The highest number of species (41) identified based on the pupal exuviae was observed in the open lake SSL49 station (*H'*=2.80), but the highest Shannon's diversity index (*H'*=3.05) was observed in the open lake station 6. For pupal exuviae the highest species diversity indices were recorded for sites characterised as *potamal* with highest median Shannon species diversity index *H'*=2.49; median Simpson's index D=0.9 and inverse Simpson A=9.89. In the case of substrate type, the highest diversity was recorded on sites where macrophytes were present *H'*=1.79; D=0.81 and A=5.52 (Table S1).

4.3. Factors affecting Skadar Lake chironomid communities

Separate analyses were carried out with adult and pupal samples. 23 adult male species and 25 pupal species were included in the analysis with 12 environmental variables. The most frequent species selected in RDA are presented in Table S4. A large number of species present with respect to the low number of samples made difficult an exhaustive interpretation of the responses of each species.

Adult males:

The adults' distribution is influenced by the geographical position of the sites, the first two RDA axes separate high altitude, low water temperature from low altitude, high water temperature sites; while water conductivity separates sites according to a second axis (Figure 5). Eigenvalues emphasised that including the environmental variables and adult males' data (constrained ordination) the first 2 axes explained about 63% of the total variance. The first axis separates *Chironomus plumosus* and *Cricotopus bicinctus* (Meigen, 1818) from *Chironomus riparius* and *Polypedilum nubeculosum*, the second axis separates *Procladius choreus* and *Paratanytarsus*

spp.; the few stone substrates have negative values on the first axis (Figure 5). It could be noticed that conductivity, NH_4 and $CaCO_3$ contribute the most to the RDA 2. 51.49% of the variance is explained by the 1st axis and 11.82% by 2nd axis (Table S5).

Pupal exuviae:

The analysis on pupal exuviae show a similar response, separating high altitude, low temperature sites from the opposites, but in this case, an oxygen-nutrients axis is responsible for a second gradient. The first axis separates different *Chironomus* species, while the second axis separates Chironomini from Tanytarsini (Figure 5). It could be noticed that RDA 1 indicate the amount of Total Phosphorous and is collinear with NO_3^-. From the other hand RDA 2 seems to be more contributed by longitude, $CaCO_3$ and water temperature. Eigenvalues emphasised that, including the environmental variables and pupal exuviae data, the first 2 axes explained about 42% of the total variance. 1st axis=29.98% % of the variance is explained by the 1st axis and 12.27% by 2nd axis (Table S5).

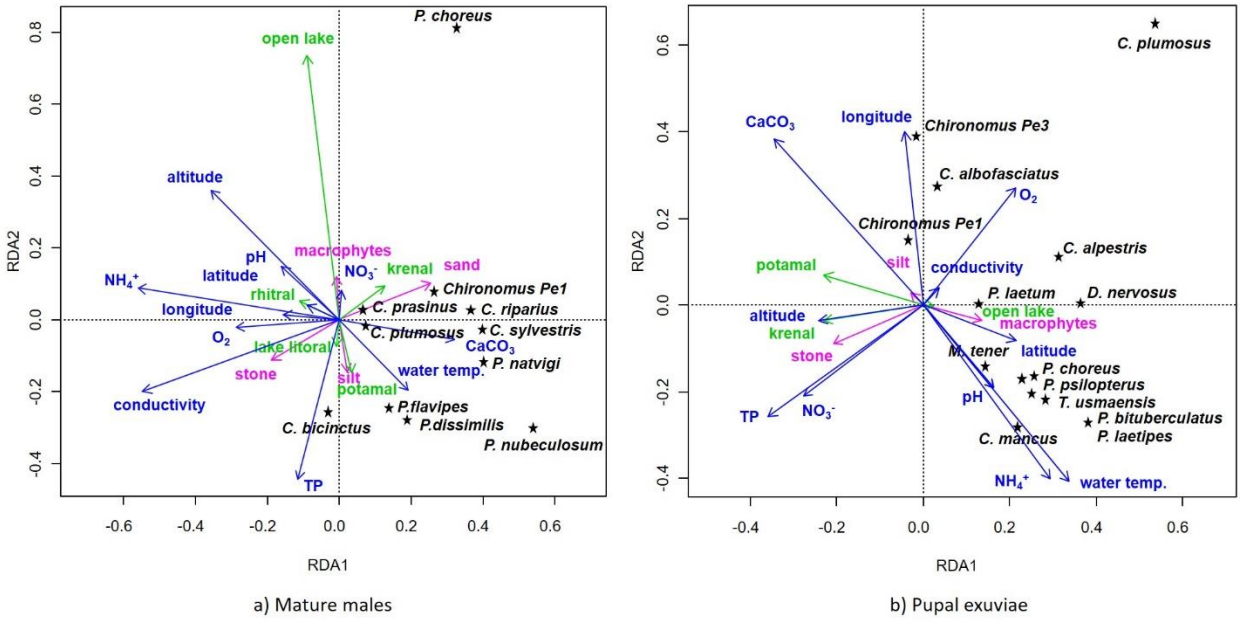

Figure 5. RDA plots of environmental variables, species and mean values of sites scores calculated for the same substrate (magenta) and habitat (green), abscissa 1st axis, ordinate, 2nd axis.

4.4. Comparative analysis

Comparing the list of species found in the 13 analysed European lakes resulted that Lake Constance (Switzerland/Germany/Austria) is the most species-rich waterbody with 174 taxa, followed by Lake Skadar with 164 taxa. The lowest number of species was reported from Prespa Lake (Macedonia/Albania/Greece) with 9 taxa and Tavropos (Greece) with 14 species. Cluster analysis revealed that Skadar Lake fauna was most similar to that in Lake Mantovo (Macedonia) with 52 species present in both lakes (Figure 6). The small lakes in Greece were separated from the large lakes, but Ohrid Lake had a peculiar position on the same branch with the Enol Lake in Spain. Similarities were also observed in species inhabiting lakes Bracciano (Italy) and Geneva (Switzerland/France) and together were grouped with Traunsee Lake (Austria). Ten taxa were present only in the Ohrid Lake, with the genus *Rheotanytarsus* recovered only in this lake, three are pupal exuviae of unidentified species and the other six taxa are widespread in Europe. A high number of 220 out of 382 species was reported from single lakes only.

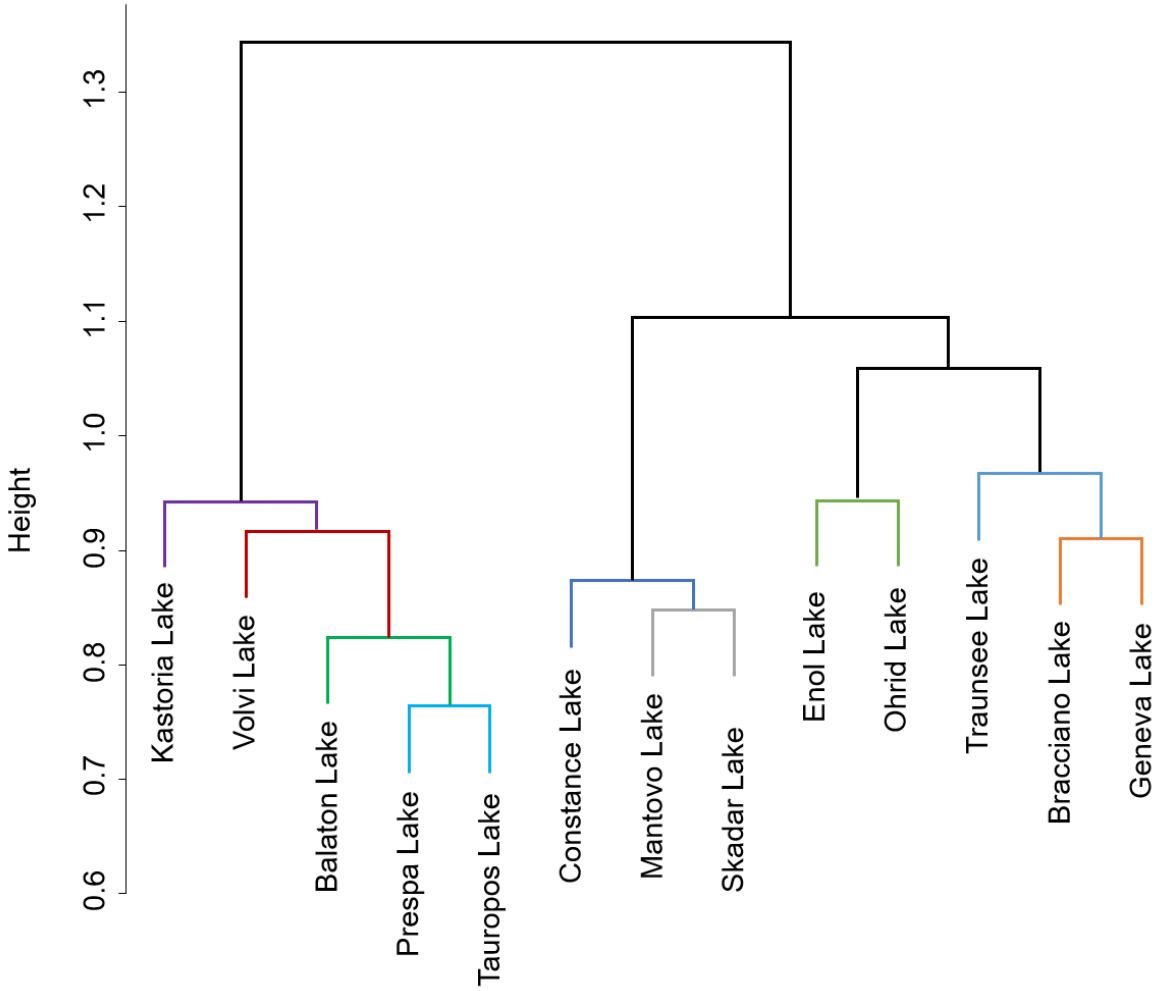

Figure 6. Cluster analysis based on Chironomidae from the Skadar Lake and central and southern European lakes studied previously. Ward.D2's method, starting from Jaccard similarities of species lists.

5. Discussion

5.1. Species diversity, abundance and distribution in the Skadar Lake basin

The updated list of species extends the existing checklist with 152 taxa newly found in the Skadar Lake basin (Table S2). All these species are widespread in Europe, including the Mediterranean Region. The number of new records based on the morphological characters of adult males (N=32) and exuviae (N=79) separately, confirms that the set of collected species depends on the sampling technique. The number of 54 taxa shared between imagines and

exuviae describes only a narrow fragment of total chironomid diversity inhabiting the Skadar Lake system. According to published data, 37 morphotypes (31 species assigned to a valid name, 6 to genus level) were reported so far from the Skadar Lake basin (Table S6) (Nedeljković, 1959; Janković, 1974; Jacobi, 1977, 1981; Karaman, 1981; Pulevic *et al.*, 2001; Płóciennik and Pešić, 2012). Among them, only 12 species are joint for both the present investigation and for the literature data. The highest number of species was collected in the Virpazar.shop sampling site situated on the shoreline of the Skadar Lake characterised as a slow-flowing channel which warm water and organic matter with silt and macrophytes. The second site with highest species diversity was Vranjina, which is situated in the mouth section of the Morača river just before it empties into the lake. In the case of pupal exuviae, the most species-rich sites were SSL49 and Station 6, situated in the centre of the small lake. In both stations, the presence of macrophytes favoured the species richness. Moreover, station 6 was situated close to the mouth of the Morača river, where the riverine water mixed with the lacustrine waters.

The high temperature is a limiting factor for many benthic invertebrates in the Mediterranean region. Numerous species occur only along the coast of Lake Skadar, in the vicinity of the sublacustrine springs or at the mouths of rivers where water parameters vary from those observed within the lake. The shallow coastal parts of the lake are much richer in species than the open, deeper parts (Pešić *et al.*, 2018).

Some of the reported species were characteristic for running waters e.g. *Brillia bifida* (Kieffer, 1909), *Conchapelopia pallidula* (Meigen, 1818), *Eukiefferiella brevicalcar* (Kieffer, 1911), *Eukiefferiella clypeata* (Thienemann, 1919), *Tanytarsus ejuncidus* (Walker, 1856), *Thienemannimyia laeta* (Meigen, 1818). The accumulation of knowledge upon chironomids suggests that the distribution area for many species is large and the absence of endemic species cannot be excluded (Rossaro *et al.*, 2019). Another factor possibly affecting rare species occurrence could be water pollution. An example is site SSL19 situated in the mouth of secondary inflow of Morača – main tributary with ammonia concentration up to 350 µg/L. During the spring and winter, when the water temperature in the lake is similar to that of the springs and rivers, some of the species were found in both habitats (e.g., *Orthocladius rivicola* Kieffer, 1911*; Cryptochironomus albofasciatus; Corynoneura edwardsi* Brundin, 1949*; Chironomus alpestris* Goetghebuer, 1934*; Paratanytarsus laetipes* (Zetterstedt, 1850)*; Tanytarsus chinyensis* Goetghebuer, 1934*; Stictochironomus; Chaetocladius perennis* (Meigen,

1830)*; Synendotendipes lepidus* (Meigen, 1830)). Chironomids of various feeding behaviours can be found in Lake Skadar. Collector-filterers feed on suspended material filtered from the water column. Larvae constructing tubes with catchnets inhabit diverse habitats (Armitage *et al.*, 1995) i.e., i) sediments (e.g., *Chironomus plumosus*; *Chironomus annularius* Meigen, 1818; *Glyptotendipes pallens* (Meigen, 1804)); ii) submerged substrata including wood (e.g., *Cladotanytarsus mancus* aggr. Giłka, 2001, *Polypedilum sordens* (van der Wulp, 1874)); iv) vascular plant tissues (e.g. *Polypedilum nubeculosum*, *Endochironomus tendens* (Fabricius, 1775)) (Pešić *et al.,* 2018). Larvae of the genus *Polypedilum* occur mainly in mud but several species are associated with aquatic plants (Sæther, 2010). The most abundant species, *Polypedilum nubeculosum* and *Chironomus plumosus*, inhabit sediments of an almost entire part of the Lake Skadar. Southern and southwestern shorelines of Skadar Lake are very poor in vegetation, which is limited to river outflow, gulfs and bays (Talevska *et al.,* 2009). Swamp soils are present along the coast in the north-western part of the Lake. Organic soils saturated with minerals are permanently under water, covered with typical swamp vegetation. In such places, where an accumulation of dead plants remains underwater in poor oxygen conditions, the 'deposit-feeders' (or collector-gatherers) can be found. They feed in habitats where organic matter accumulates such as areas with reduced water velocity in rivers and streams. Collecting-gathering is the most common feeding mode exhibited by chironomids and is represented within all subfamilies. Most chironomids feed this way at some period during their larval development (e.g., many Tanypodinae) (Oliver, 971; Baker and McLachlan, 1979). However, many taxa are deposit-feeders for most of the larval lifespan (Coffman and Ferrington, 1984; Armitage *et al.*, 1995). In Lake Skadar, some collector-gatherers, such as *Chironomus plumosus* and *Polypedilum nubeculosum*, are reported from the open lake sediments.

Adult males of *Paratanytarsus natvigi* were attracted by light and were collected using hand entomological net from all stations situated on the lakeshore of the Skadar Lake. This species prefers littoral zones where organic matter occurs and water temperature is higher. *C. plumosus* and *P. nubeculosum* are the most widely distributed and abundant species that can be used as a model organism in eco-toxicological sediment biotests (Meregalli *et al.,* 2002; Nowak *et al.,* 2006). Larvae of the genus *Chironomus* and *Polypedilum*, often form a substantial part of the benthic communities on muddy bottoms and previously were reported most frequently from ponds, ditches and water butts (Matena, 1990; Langton, 1991).

Southern/south-western shoreline and bottom of Lake Skadar is rocky, with boulders, stones and large gravel. Such types of habitats are well colonised by chironomids feeding as scrapers. Most of these species have well-developed mandibles that are used to shear the edible material from the surface of rocks, sediments, wood and other sunken objects. In Lake Skadar, the scrapers are represented mostly by the genera: *Acricotopus, Corynoneura* (e.g., *Corynoneura gratias* Schlee, 1968 and *C. edwardsi*), *Cricotopus* (e.g., *Cricotopus sylvestris* and *Cricotopus bicinctus*), *Dicrotendipes* (e.g. *Dicrotendipes lobiger (Kieffer, 1921); Dicrotendipes nervosus* (Stäger, 1839)*; Dicrotendipes notatus* (Meigen, 1818)*; Dicrotendipes pulsus* (Walker, 1856)); *Limnophyes* (e.g., *Limnophyes minimus* (Meigen, 1818) and *Limnophyes natalensis* (Kieffer, 1914)), *Orthocladius* (e.g., *Orthocladius abiskoensis* Thienemann & Krüger, 1937, *Orthocladius excavatus* Brundin, 1947, *Orthocladius luteipes* Goetghebuer, 1938, *Orthocladius oblidens* (Walker, 1856), *Orthocladius pedestris* Kieffer, 1909, *Orthocladius rivicola* Kieffer, 1911, *Orthocladius rubicundus* (Meigen, 1818)), *Parachaetocladius* (e.g. *Parachaetocladius abnobaeus* (Wülker, 1959)), *Paratrichocladius* (e.g., *Paratrichocladius rufiventris* (Meigen, 1830)) and *Pseudosmittia* (e.g., *Pseudosmittia trilobata* (Edwards, 1929)).

The eastern part of the lakeshore is characterised by a great diversity of submerged, floating and emerged macrophytes. It has a great influence on organic matter production and represents a significant element in Lake Skadar trophic network as well as it provides suitable habitats for many organisms. Many chironomid species feeding as shredders are associated with coarse particulate organic matter such as: i) alive vascular plants (e.g. *C. sylvestris* and *C. bicinctus, Endochironomus albipennis, Endochironomus tendens*, genus *Polypedilum* (e.g. *Polypedilum apfelbecki* (Strobl, 1900), *Polypedilum bicrenatum* Kieffer, 1921, *Polypedilum convictum* (Walker, 1856), *Polypedilum laetum* (Meigen, 1818) and *Stenochironomus* species); ii) submerged wood (e.g., *Stenochironomus*); iii) macro- or colonial algae (e.g. *Cricotopus* species); iv) leaf litter (e.g. *Brillia bifida and Brillia flavifrons* (Johannsen, 1905), some species of *Chironomus*) (Armitage *et al.,* 1995; Mrdak *et al.,* 2011; Pešić *et al.,* 2018). Aquatic macrophytes and algae are a typical habitat for *Cricotopus sylvestris*. *Cricotopus* species are known to inhabit a wide range of water bodies, from pristine streams and brooks to eutrophic ponds and brackish estuaries (Gresens *et al.,* 2012). Larvae of *C. sylvestris* group are very frequent in shallow lakes and ponds and are mostly associated with emergent and submerged

vegetation. They also feed on detritus, protozoans and rotatorians (Moller Pillot, 2013; Tarkowska-Kukuryk and Mieczan, 2013).

The characteristic feature of Lake Skadar water balance is high inflow from a number of temporary and permanent karst springs (Pešić and Glöer, 2013). Lower parts of the Skadar Lake basin are temporary flooded but in summer they dry and become meadows. Some parts of lake bottom are crypto-depressions and create sub-lacustrine springs where water entering the Lake is substantially harder than river water, probably owing to its direct limestone source (Lasca *et al.,* 1981; Grabowski *et al.,* 2018). There are about thirty of such springs. The deepest measured is Raduš about 60 m deep. The Chironomidae diversity on such depths is very low. There is one species reported from the depth of 55m: *Polypedilum convictum* (Jacobi, 1978). The largest inflow (ca. 62% of all the water that feeds the lake) comes from the Morača River (Montenegro). There were also high species diversities in the sampling sites situated on the end of the Morača river near its mouth. The most abundant species reported at these sites were e.g. *Polypedilum nubeculosum, Polypedilum scalaenum* (Schrank, 1803), *P. natvigi, Cladotanytarsus mancus and Paratanytarsus bituberculatus.* From other hand spring system surrounding lake has different taxa composition with most abundant species from genera: *Chironomus* (e.g., *Chironomus riparius*), *Cryptochironomus* (e.g., *C. albofasciatus*), *Eukieferiella* (e.g., *E. albipennis*), *Euorthocladius* (e.g. *E. rivicola*), *Polypedilum* (e.g. *Polypedilum nubeculosum*), *Tanytarsus* (e.g. *Tanytarsus usmaënsis), Procladius* (e.g. *Procladius choreus*).

5.2. Skadar Lake chironomid fauna comparison with European lakes

The different sampling strategies, frequency of sampling, different expertise of taxonomists, comparison based on species lists including various developmental stages or different lake trophy can heavily affect the conclusions. A comparison of the list of species from different lakes is not easy but based on performed cluster analysis we can conclude that only a few lakes are deeply studied, with most of the lakes that are poorly investigated. Only common species widespread in Europe are present in the analysed lakes. Taking into account the number of shared species, Mantovo Lake has been evidenced as the most similar to Skadar Lake in terms of taxa composition. The apparent taxonomic similarity between Skadar Lake and the large Mantovo and Constance lakes may be bound to the fact that these three water bodies were studied in detail and all are inhabited by a large number of species. The observed species

composition may be caused by different habitats observable in different lakes or by different sampling techniques. The high number of species observed in Skadar Lake is probably bound to the fact that both light traps and entomological hand net to collect adults and drift net to collect exuviae were used. Taxonomic identifications up to genus and species level are often intermingled. It is difficult to compare the checklist where genera are reported with a checklist in which more species of the same genus occur. It is not possible if a genus identified in one lake is the same species of the same genus in another lake. The perfect example is *Parachironomus vitiosus* Goetghebuer, 1921 which is cited only in Ohrid Lake, but the genus is cited in other 5 lakes, so the presence of *P. vitiosus* cannot be excluded in these 5 lakes. Moreover, *Chironomus* and *Cricotopus* species can be separated as pupal exuviae, but the correspondence with adults is unknown. Some of the species (e.g., *Benthalia carbonaria* (Meigen, 1804), *Chironomus dorsalis* Meigen, 1818, *Chironomus alpestris* Goetghebuer, 1934) were recently revised and their taxonomic status requires confirmation (Vallenduuk, 2013).

5.3. Future prospects

The present analysis is to emphasise the actual lack of comprehensive knowledge on the chironomids inhabiting European lakes. Skadar Lake, shows a high number of species, even if most of them are widespread in Europe. Analyses of adults and exuviae provide more reliable information than surveys based on sampling the sediments and identifying larvae as a part of zoobenthos. Efforts focused on implementing additional developmental stages will surely extend the accuracy of the obtained results. Additional sampling including seasonal aspect, all life stages and better spatial coverage of sampling sites may bring new, important data about species distribution and will help to monitor changes in their composition. Moreover, the obtained results suggest that, for comparative assessments, more effort should be put on sampling the littoral and inflow habitats. Identification with the use of innovative, rapid and relatively cost-efficient considering time, cost and specialist expertise required molecular tools can be considered. Such methods as the DNA barcoding and metabarcoding proved to be effective to study the spatial patterns of macroinvertebrate diversity starting from environmental or bulk samples, essential in the water quality assessment on a large scale. In the last years, DNA barcoding and metabarcoding approach have been applied to assess the animal biodiversity associated to complex environmental samples (e.g. Yu *et al.,* 2012; Arribas *et al.,* 2016) and in biomonitoring studies (Bohmann *et al.,* 2014; Carew *et al.,* 2018; Theissinger *et*

al., 2018). Even if that high precision is not required for biomonitoring studies, the obtained result could be simply filtrated to keep only the taxonomic level of interests. Considering the fact that morphological identification of larval specimens is limited mostly to the generic level (except for a few genera), the description of the community using larval instars loose information since this stage does not allow to reach a good taxonomic resolution. Further DNA-based studies based on Chironomidae from Skadar Lake basin may also reveal real (cryptic or/and endemic) diversity of chironomids. Adopting integrated approaches combining traditional taxonomy supported by molecular tools will bring conclusive results (Montagna *et al.,* 2016). Such integrative methods can be particularly useful in molecular identification of individuals if subtle variation in morphological features are present among closely related species if only one life stage is described (adult males) as well as in studying spatial diversity patterns based on the larval stages (Hebert *et al.,* 2003; Ball *et al.,* 2005; Savolainen *et al.,* 2005). Such studies may resolve taxonomical questions and help to assess the diversity of chironomids in every water system. It will allow interpreting results in a broad geographical context including poorly or not yet investigated lakes.

Nowadays, serious discussion about the degradation of freshwater habitats, which provide irreplaceable ecosystem services (e.g., drinking water, irrigation, hydropower, tourism etc.) and which are highly susceptible to environmental change, is under strong interests due to the heavy anthropogenic impact (Wilbur *et al.,* 1999, Hanson *et al.,* 2004). Without such knowledge, it is hard to imagine planning a reasonable and effective strategy preventing or at least minimising the loss of biodiversity. It must also be remembered that planning and implementing such a strategy is one of the top priorities of many EU and global agendas for the conservation of natural resources. The results achieved by scientists in the next few years will surely fill a large gap in knowledge of the biodiversity in the Balkan region.

6. Skadar Lake fauna conservation

The drainage area of Lake Skadar is about 5,500 km^2 and is inhabited by 540 000 people (310 000 inhabitants in Montenegro and 230 000 in Albania) concentrated in five municipalities (Bejko, 2011; Katnic, 2013; Ziu and Bejko, 2004). The local population, small businesses and industries are mainly interested in using Skadar Lake as a resource. The main occupations of the inhabitants include fishery, tourism and small-scale agriculture. However, population

density in the Skadar Lake National Park buffer zone and the Skadar Lake catchment is much higher, and therefore pressures on the resources are more substantial. The intensive agricultural activity in the Zeta Plain, with high usage of artificial fertilisers flowing through the Morača river, brings to Lake Skadar a large amount of waste, municipal and industrial water (over 69 million m3/year). Also, main activities of inhabitants in the Albanian part of the lake, such as intensive fishing, agriculture with food crops, vegetables and fruits, livestock around the major cities like Shkodra and Kopliku also negatively affects and intensifies eutrophication processes (Dhora *et al.*, 2012; Katnic, 2013).

Various habitats occurring in Lake Skadar support a rich assemblage of species (Dhora and Sokoli, 2000; Bejko, 2011). The Montenegrin part of Skadar Lake (40,000 ha) was declared a National Park in 1983 by the Law on Shkoder Lake, while the Albanian side since 2005 is a protected area as "Managed Natural Reserve" (Official Gazette SRCG 1991). In 1995 was also added for inclusion in the RAMSAR World List of International Important Wetlands in recognition of its enormous value as an aquatic habitat (Site 3YU003). This decision has drawn international attention to the management of the lake and its surroundings. Since 2005 Buna/Bojana River is also nationally protected on the Albanian side with status "Protected Waterscape/Landscape" (Katnic, 2013).

Despite the great value of the floral and faunal elements, this richness is greatly endangered. Mass tourism and exploitation of natural resources lead to ecosystem degradation and water quality concerning (Stesevic *et al.*, 2007). Among the main risks are the following ones: sewage and industrial pollution, degradation and loss of biodiversity as well as depletion of fish quantity (Bejko, 2011). The lake is affected by industrial and municipal waste from urban and industrial areas (e.g. Podgorica, Skadar, KAP - Kombinat Aluminijuma Podgorica - a giant aluminium processing plant), and by increasing use of artificial fertilisers and pesticides in agriculture (Rastall *et al.*, 2004; Mijovic *et al.*, 2006). In 2017 a dangerous idea showed up in public to build a luxury complex for 600 people and provide berths for 30 boats. It also proposes the construction of several big tourist infrastructures at Skadar Lake National Park and the removal of areas such as the precious Morača River delta – an Important Bird Area (IBA). For the last fifteen years, there is already an example of such destroying activities on Montenegrin coast which has been savagely destroyed by the large-scale constructions of flats, houses and hotels for the market (www.skadarlake.org).

Skadar Lake is known as a hot-spot of biodiversity which faunal and floral elements have developed in a unique physical environment where geology, geomorphology, hydrology and climate provide a wide variety of habitats. The Region is considered to be a reserve of European importance with the high relationship between species richness and area (SAR) = 0.875 (Mrdak *et al.,* 2011). The importance of Skadar Lake is explained by its situation where different components of flora and fauna meet: Mediterranean, oro-Mediterranean, Mediterranean-middle European, middle European, boreal and pontic (Mrdak *et al.,* 2011). All this gives a clear picture of Skadar Lake biodiversity importance and conservation needs. Conservation and protection of rare and endangered species should be a priority no matter if those species have some international or national conservation status. Skadar Lake has high importance as a diversity center for several groups such as algae, fish, birds, amphibians and lizards. Macroinvertebrates are still poorly studied (Mrdak *et al.,* 2011).

The current study cannot suggest any chironomid species requiring special protection measures but the level of revealed species diversity suggest that region should be more protected and additional initiatives should be implemented. Based on overall fauna from various groups inhabiting region we can predict how constant pollution will influence species diversity. Loss of Chironomidae diversity may have an implication on fish diversity and bird fauna. Different habitat protection projects may save or at least stop the loss of biodiversity. At this moment we do not know how this process changes through time. First scrupulous investigation of non-biting midges species (including imagines) diversity made from 1979 will help to monitor biodiversity in further time and investigate how protection programs influence biodiversity within the lake catchment. Well-planned strategy of environment protection will benefit not only for faunal and floral elements but also for local people from the region. Reasonable and well-controlled tourism will raise the incomes for Skadar Lake National Park and will help to improve or expand protection programs. Based on the present study, the stations with the highest chironomid diversity could be proposed for protection, but obviously, non-biting midges alone are not a good candidate for protection programmes. Although, presence of rare species can be a suggestion.

7. Summary and conclusions

- The collected individuals were assigned to 164 Chironomidae taxa providing insight into species diversity in the Skadar Lake basin. Identified mature males were assigned to 82 correct species-rank names and 95 species based on pupal exuviae identifications. The current study extends the existing checklist with 152 taxa newly found in the Skadar Lake basin. These results provide a significant improvement in the knowledge of the Skadar Lake chironomid fauna, essential for conservation purposes.
- The shallow, coastal parts of the lake covered with macrophytes where the water provide more suitable habitats for Chironomidae resulted in higher species diversity. An example is the Virpazar.shop sampling site where among imagines the highest number of species was collected. This site is situated on the lake shoreline where slow flowing channel supplies the Skadar Lake with warm water with organic matter. For pupal exuviae, the most species-rich site was SSL49, situated in the northern part of the lake where exuviae were transported by the wind and accumulated on the edge of macrophytes covering the lake surface.
- Based on the comparison made on the list of species from the 13 well-analysed European lakes it could be concluded that only a few lakes are deeply studied and Lake Constance (Switzerland/Germany/Austria) is the most species-rich waterbody, followed by the Skadar Lake. Only common species widespread in Europe are present in the analysed lakes.
- The observed species composition of European lakes may be caused by different habitats observable in different lakes or by different sampling techniques. The high number of species observed in Skadar Lake is probably bound to the fact that both light traps and entomological hand nets to collect adults and drift net to collect exuviae were used.
- These results, even if obtained on a model system, can be adopted as a starting point in farsighted policies of habitat conservation and management. Especially in a scenario where environmental changes occur rapidly, as in the case of human-driven changes.
- I hope that my results will help to monitor biodiversity in further time and investigate how protection programs influence biodiversity within the lake catchment. Even if the current study cannot suggest any chironomid species requiring special protection measures, the level

of revealed species diversity suggest that region should be more protected and additional initiatives should be implemented.

Chapter II. DNA barcoding reveals an unknown Chironomidae diversity from the freshwater biodiversity hot-spot of Skadar Lake: comparison between local and the European datasets.

1. Introduction

The study is focused on the dipteran family Chironomidae also known as non-biting midges, that is a flagship taxon in freshwater ecology. Chironomidae, with nearly 7,500 species grouped into 550 genera, is one of the largest and most diverse dipteran families (Pape *et al.*, 2011). At the larval stage, chironomids inhabit various freshwater ecosystems such as streams, rivers, ponds, lakes, dam reservoirs and, to a much lesser extent, brackish waters and soil. Exceptional physiological and behavioural adaptations, such as the presence of a highly effective system of heat shock proteins and hemoglobin as the hemolymph oxygen carrier, led chironomids to colonize extreme environments such as glacial rivers or highly polluted water (Panis *et al.*, 1995; Lee *et al.*, 2006; Ha and Choi, 2008; Bernabò *et al.*, 2011; Mazin *et al.*, 2018). In addition, due to the high habitat specificity and the fast response of larvae to environmental changes, chironomid communities has been considered to be among the most promising biological indicators of water and sediment quality in lentic and lentic and lotic waters (Lindegaard, 1995; Free *et al.*, 2009; Skoulikidis *et al.*, 2009). However, most of the studies on chironomid communities have been hampered by the notorious difficulties of species-level identification, especially for the larval stage and for females; thus specimens identification were usually restricted to higher taxonomic levels, i.e. genera/species groups (Nyman *et al.*, 2005) or to "larval types" (Real and Prat, 2000).

DNA barcoding is a system for fast and accurate species identification by using a short DNA sequence instead of the whole genome. The short DNA sequence is generated from the standard region of the genome known as a marker. The accurate species identification by DNA barcoding relies on a suitable DNA barcode, which refers to a standardized sequence, usually less than 1,000 base pairs of the genome (Hebert *et al.*, 2003). For animal identification, the most broadly used barcode marker is mitochondrial *cytochrome c oxidase subunit I* (COI). Molecular techniques, such as the DNA barcoding can be particularly useful in molecular identification and species description of Chironomidae specimens when subtle variation in

morphological features are present among closely related species when only one life stage is described, or when the investigator lacks the experience to identify taxa on the base of their morphology (Wiedenbrug *et al.*, 2009; Andersen *et al.*, 2013; Stur, 2015; Stur and Ekrem, 2015; Montagna *et al.*, 2016a; Giłka *et al.*, 2018; Lin *et al.*, 2018). The use of DNA-based diagnostic characters for species identification within Chironomidae has been also:

- effective for species recognition (Brodin *et al.*, 2013; Ekrem end Stur, 2007; Ekrem *et al.*, 2007; Sinclair and Gresens, 2008; Ekrem *et al.*, 2010; Krosch and Cranston, 2012; Silva *et al.*, 2013; Lin *et al.*, 2015; Failla *et al.*, 2016; Kondo *et al.*, 2016; Song *et al.*, 2016),
- suitable to associate life stages (Carew *et al.*, 2005; Stur and Ekrem, 2011; Silva and Wiedenbrug, 2014),
- used for resolving phylogenetic relationships (Ekrem *et al.*, 2007; Cranston *et al.*, 2010; Demin *et al.*, 2011; Sari *et al.*, 2015; Montagna *et al.*, 2016a, 2016b; Lin *et al.*, 2017; Stur *et al.*, 2019; Krosh *et al.*, 2020),
- used for biodiversity assessment and biomonitoring studies (Sharley *et al.*, 2004; Brodin *et al.*, 2013; Carew *et al.*, 2013; Carew *et al.*, 2015; Cranston *et al.*, 2013).

Among others, relevant advantages of using short DNA fragments to characterize the diversity of the organisms rely on the short time and low price required to pursue the proposed goals (Meier *et al.*, 2015; Baloğlu *et al.*, 2018). The cost of Chironomidae identification using morphology approach is high because it usually requires dissection and mounting of specimens onto microscopic slides which is time-consuming (~15-20 minutes *per* processed specimen) (Epler, 2001; Carew, 2007; Cranston, 2013; Wong, 2014). The capability to identify organisms based on DNA COI barcode has been enhanced by the presence of the open-access DNA-barcode reference databases (e.g., BOLD, http://www.boldsystems.org; GenBank, https://www.ncbi.nlm.nih.gov/genbank). By the 16th of May 2020, more than 314,000 species (over 8,261,000 records) were registered in the Barcode of Life Data System (BOLD).

In the present study, we propose to overcome this taxonomic impediment with the use of molecular tools such as the DNA barcoding and utility of the Barcode of Life Data System (BOLD) - open-access library of COI barcodes (Ratnasingham and Hebert, 2007). This method

has been proposed and successfully adopted as an efficient method for species identification and habitat biodiversity assessment using a standardized genetic marker (Hebert *et al.*, 2003; Yu *et al.*, 2012; Carew *et al.*, 2013; Arribas *et al.*, 2016). Moreover, the utility of Barcode Index Number (BIN) is an online framework greatly expedites the evaluation and annotation of described species and putative new ones while reducing the need to generate temporary names, a non-trivial issue in barcoding datasets. The BIN algorithm has been effectively tested on a broad set of taxonomic groups. The registry employs modern URI and web service functionality enabling integration with other databases (Ratnasingham and Hebert, 2013). In this study, we are taking advantage of DNA barcoding for specimen identification of European Chironomidae. The use of *a priori* defined thresholds vs optimal thresholds were here discussed. DNA barcoding as it stands is designed to identify organisms on the basis of a DNA sequence adopting a fixed threshold of nucleotide distance. Ratnasingham and Hebert, (2013) for assigning Barcode Index Number and OTU designation, estimated threshold of 2,2% based on six datasets of taxonomic groups such as birds, fishes, moths and butterflies, bees from two climatic regimes (temperate, tropics). Thresholds higher than optimal increased the number of cases where members of different species were assigned in a single OTU. Thresholds lower than the optimal value increased the cases where members of species were split into two or more OTUs. Optimal Thresholds (OT) based intra-interspecific nucleotide distance is estimated to increase the success of specimen identification from analysis based on a single dataset. OT corresponds to the values of nucleotide distances at which the sum of false positive (if an incorrect species name is assigned to the query) or false negative (heterospecifics with a value of nucleotide divergence lower than the threshold value) identifications reaches minimum values (Meyer *et al.*, 2005; Sonet *et al.*, 2013; Montagna *et al.*, 2016a). Using both methods for species delimitation requires a careful interpretation of the output (Collins and Cruickshank, 2013).

Skadar Lake is a shallow lacustrine ecosystem located in the outer part of the Dinaric Alps, in the south-western part of the Balkan Peninsula (Pešić *et al.*, 2019). Approximately two-third (229 km^2) of its surface belongs to Montenegro and about one-third (142 km^2) to Albania. The lake is approximately 44 km long and 14 km wide with a surface area that seasonally fluctuates between 370 km^2 to 530 km^2. The average level of the lake is 6.52 m. above sea level with a mean depth of 5 m. It is the biggest lake on the Balkan Peninsula, situated in Zeta-Skadar valley. The chironomid fauna of Lake Skadar is relatively well studied on the morphological

level (see the first chapter) under review). The fact that Skadar Lake basin contains a young lake fed by a geologically old system of springs, which has its origins in Pliocene (Grabowski *et al.*, 2018; Pešić *et al.*, 2019), makes it a very attractive model to test different assumptions about the biogeography of this aquatic insect group. DNA-based methods, which can be considered the gold standard for such type of studies, greatly help us to rapidly, and cost-efficiently, characterize the composition of chironomid fauna in the Lake Skadar basin and to create the first DNA barcode reference library of non-biting midges inhabiting the Skadar lake system, benefiting of the specimens collected during the collecting campaigns performed in 2014 and 2015.

The main aims of this study are: *i)* to develop and evaluate the first library of barcodes for Skadar Lake basin Chironomidae and reveal their species diversity and composition; *ii)* to estimate barcoding efficiency for the European Chironomidae fauna based on BOLD records; *iii)* to estimate the optimal intraspecific and interspecific thresholds for Chironomidae identification at family vs subfamily level; *iv)* to explore species distribution patterns in Europe using universal Barcode Index Number (BIN); *v)* to report and discuss species groups where molecular and morphological taxonomy disagree.

2. Materials and methods

2.1. Ethics statement

No species of Diptera Chironomidae are listed in national laws as protected or endangered. Specimens were collected partly from Skadar Lake National Park using the permission number 02-UPI/1070/3 provided by the Environmental Agency of Montenegro for V. Pesić.

2.2. Sample collection and identification

Sampling sites and methodology was described in the previous chapter. Briefly, larvae, pupae and imagines were collected from 72 sampling sites in Montenegro and Albania during four sampling campaigns: spring and autumn 2014, summer 2015 and summer 2018 (Figure 7). Both aquatic stages were collected from streams and rivers using a standard kick sampling procedure. From Lake bottom and macrophytes specimens were collected using a benthic dredge. Imagines were collected by sweeping through coastal vegetation using entomological

hand-net from the lake and river shores and attracted to light. For the latter purpose, a 500W white-light bulb connected to a power generator was placed at front of a white screen (2m × 3m) in the open areas, ensuring that the light was visible over a long distance above the lake surface. The above-adopted criteria to select the sampling sites were applied to maximize the representativeness of the whole range of habitats present in the area (i.e., open lake, shallow lake, springs, sublacustrine springs, rivers inflow) and to maximize the diversity of collected chironomids. Of the barcoded individuals, ca. 94% were sampled in Skadar Lake catchment. The main criteria adopted for the sampling site selection were (1) to maximize the representation of the local habitats (i.e., open lake, shallow lake, springs, sublacustrine springs, rivers inflow) and (2) to maximize the species diversity of chironomids. The collected individuals, regardless of their life stages, were picked from bulk samples, placed in absolute ethanol, and stored at -20°C for DNA extraction. Adult males have been morphologically identified to the species level. During the development of the reference barcode library, mature females were not analysed since their taxonomic identification is impossible to species level. Such specimens are sorted and kept for further analyses. With some exceptions, taxonomic identification of larvae and pupae is possible only to the genus level. Those individuals are kept as vouchers and will be used further to match obtained barcodes with developed on mature males reference library. Individuals were stored as vouchers after DNA extraction. When a new Barcode Index Number was discovered in the BOLD database, taxonomic identifications were double-checked if correspondence with a higher taxonomic level in the database occurred (Ratnasingham and Hebert, 2013). Identified voucher specimens are deposited in University of Lodz, Poland and the University of Milan, Italy under P. Gadawski's and B. Rossaro's curation, respectively.

Figure 7. Map of all sampling sites from Skadar Lake basin. Numbers in green marks indicate the number of sequences reported. Map source: OpenStreetMap contributors based on the Open Data Commons Open Database License.

2.3. DNA extraction, PCR and sequencing

DNA extraction was done in the Department of Invertebrate Zoology and Hydrobiology at the University of Lodz, Poland and in the Agricultural and Environmental Sciences - Production, Landscape, Agroenergy in the University of Milan, Italy according to the two following protocols. DNA from larvae was extracted by whole body lysis. In the case of adult males, the DNA was extracted from one/two legs (specimens > 2mm) or by whole body lysis (specimens < 2mm). In all cases the individuals/legs were incubated overnight in a lysis buffer with Proteinase K, both provided with DNA extraction kits. In the University of Lodz, the total DNA was extracted using Tissue Genomic Extraction Mini Kit provided by GenoPlast Biochemicals (Rokocin, Poland) and GeneMATRIX Tissue DNA Purification Kit provided by EURx (Gdansk, Poland) following the manufacturer protocol. At the University of Milan DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) as previously described (Montagna *et al.*, 2016a). DNA from 85 individuals was extracted and sequenced in the Centre for Biodiversity Genomics, Guelph, Canada. In general, the standard 658 bp barcode region of the mitochondrial *cytochrome oxidase subunit 1* (COI) was amplified by PCR for each individual, using barcode primer pairs LCO1490/HCO2198 (Folmer, 1994) and

C_LepFolF/C_LepFolR (Folmer *et al.*, 1994; Hebert *et al.*, 2004). PCR was performed in a final volume of 11 µL containing 5 µL of DreamTaq reaction Buffer (DreamTaq DNA Polymerase, 2x DreamTaq buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each, and 4 mM $MgCl_2$; Thermo Scientific Inc.), 0.8 µL of LCO1490 (5 µmol) primer, 0.8µL of HCO2198 (5 µmol) primer, 2.4µL of ultrapure water and 2µL of DNA template. PCR conditions included 94°C for 1 min followed by 5 cycles of 30s at 94°C, 1 min 30s at 45°C and 1 min at 72°C; 36 cycles of 94°C for 30s, 51°C for 1 min 30s and 72°C for 1 min; with the final extension of 5min at 72°C. Successful amplification was verified by the agarose gel electrophoresis. PCR products were purified using a mix of FastAP (1 U/µL, ThermoFisher Scientific) and Exonuclease I (20 U/µL ThermoFisher Scientific). Direct sequencing of the PCR product with the marker-specific primers was outsourced to Macrogen Europe (Amsterdam, the Netherlands). The obtained sequences were edited and primers removed using Geneious Pro 11 (Biomatters Ltd., Auckland, New Zealand; Kearse, 2012). The identity of the obtained COI sequences was verified using BOLD Identification Engine (Ratnasingham and Hebert, 2007). The presence of an open reading frame was verified for each obtained sequence during sequence edition and submission to the BOLD database. Consensus COI sequences were deposited in the BOLD (Dataset 'DS-CHBAL - Chironomidae from Skadar Lake basin', https://doi.org/10.5883/DS-CHBAL) and GenBank (BankIt2349074, Accession numbers MT534631 to MT535400).

2.4. Dataset composition and development

Overall 15,196 publicly available COI-5P sequences for European Chironomidae present in BOLD were mined to a newly created dataset and then downloaded as an alignment using built-in features with sequence name including process ID, taxon, subfamily and BIN number. The sequences were downloaded in the alignment form using MUSCLE with default parameters, resulting in a FASTA file excluding contaminants, records with stop codons and records flagged as misidentifications or errors (Edgar, 2004). In the next step, alignment was also edited and filtered to keep only the sequences identified to the species level with proper scientific names. This procedure was made using R 3.5.2 (R Core Team, 2020) package *spider* to exclude sequences belonging to species identified only to genus level or named with different morphotype abbreviations (Paradis and Schliep, 2018).

Chironomidae sequences obtained during this study were grouped in the SKADAR dataset. Sequences mined from BOLD were kept separately in a BOLD dataset and finally, we created a combination of both datasets named SKADAR + BOLD. The created datasets were kept separately in order to evaluate the efficiency of the data developed in this study. To estimate DNA barcoding efficiency for each Chironomidae subfamily, the obtained sequences from the dataset Skadar + BOLD were also split into sub-datasets to obtain datasets including only one subfamily each (Chironominae, Diamesinae, Orthocladiinae, Podonominae, Prodiamesinae, Tanypodinae, Telmatogetoninae).

2.5. Bioinformatics analysis

The intraspecific and interspecific nucleotide divergences were calculated for the datasets containing sequences identified to species level, starting from a pairwise distance matrix obtained using R library *spider* v1.4-2 (Brown, 2012) adopting Kimura 2-parameter (K2P) as nucleotide substitution model (Kimura, 1980). This model is standardly used in DNA barcoding (Nishimaki, 2019). With the same R package, the *Threshold optimisation analysis* was performed on the BOLD + SKADAR dataset and on each subfamily-level in order to calculate the value of nucleotide distance (optimal threshold; OT) that minimises the error related to molecular identification (as it has been performed by previous studies - Magoga *et al.,* 2018; Gibbs, 2018; Liu *et al.,* 2017; Amouroux *et al.,* 2017; Virgilio *et al.,* 2017; Virgilio, 2016; Fontaneto, 2015; Delić, 2017). The error is caused by the discordance between morphological and molecular identification and it is termed as a cumulative error (CE), calculated as the sum of the number of false positives (FP, conspecifics with a value of nucleotide divergence higher than the threshold value) plus the number of false negatives (FN, heterospecifics with a value of nucleotide divergence lower than the threshold value) (Meyer, 2005). The efficiency of molecular identification was estimated by performing the *Best Close Match* analyses, defined by Meier *et al.* (2006), on SKADAR Dataset, BOLD Dataset, and SKADAR + BOLD Dataset at family and subfamily level. The method compares each sequence of the dataset with the others included in it and checks if the best matches (i.e., pairs of sequences with the lowest values of nucleotide distance) are between sequences of organisms morphologically identified as the same species. Each best match results in one of the following four states: “correct”, when the two closest sequences under the defined threshold belong to the same species; “incorrect”, the opposite situation; “ambiguous”, when the closest match is represented by more than one

species; and, "no id" when no match is recorded under the chosen threshold. Predefined thresholds for each dataset were calculated using *Threshold optimisation analysis* in a *spider*, R package (Sonet, 2013). Minimum-spanning haplotype networks (Bandelt *et al.*, 1999) were reconstructed using PopART for five genera containing closely related and similar species, that are often misidentified (Leigh *et al.*, 2015). Distribution of Barcode clusters was performed by Barcode of Life Data System v4. The number of haplotypes was estimated using Fabox DNA Collapser and confirmed with BOLD data systems accumulation curve tool (Ratnasingham and Hebert, 2007; Villesen, 2007). Species included in the BIN analysis were selected based on the results of the haplotype network and *adhocTHR* (*red-flagged*) analyses performed on alignments (Table S8). Moreover, the BOLD barcode clustering method was implemented to provide possible explanations.

The Refined Single Linkage (RESL) algorithm was implemented directly in the Barcode of Life Data System and used to assign sequences to Operational Taxonomic Units. RESL was developed as a staged clustering process which employs single linkage clustering as a tool for the preliminary assignment of records to an OTU and a subsequent finishing step that employs Markov Clustering (MCL), a graph analytical approach (RESL, Ratnasingham and Hebert, 2013). Statistics for nucleotide divergences were calculated in BOLD using the Distance Summary tool on MUSCLE alignment adopting Kimura 2-parameter (K2P) as nucleotide substitution model (Ratnasingham and Hebert, 2007; Edgar, 2004). Venn diagram was calculated using an online tool http://www.interactivenn.net/ (Heberle *et al.*, 2015).

3. Results and discussion

3.1. *Chironomidae of Skadar Lake basin: species diversity, BIN analysis*

A total of 744 barcodes comes from the individuals collected in Montenegro and further 26 in Albania, from the Skadar Lake catchment. All the 770 sequences obtained during this study represent the first records of Chironomidae from Balkan Peninsula in BOLD. All the barcoded larvae and adult males were assigned to 165 different barcode clusters of which 65 (39.1%, 201 sequences) are unique and new for BOLD. Out of them, 94 BINs are concordant (56.6%), one is discordant (0.6%), formed of eight sequences of *Cricotopus sylvestris* (Fabricius, 1794) and five of *C. relucens* Hirvenoja, 1973 and finally, 71 BINs are represented by a single sequence (42.7%). 101 BINs (60.84%) were already present in the database. From a

total number of sequences, larvae were represented by 399 sequences assigned to 98 OTUs and 95 BINs, adult males (identified to species and genus level) were represented by 371 sequences assigned to 104 OTUs and 99 BINs. The library developed in BOLD contains 168 Operational Taxonomic Units (OTU) assigned to 88 distinct species. The dataset SKADAR consists of 341 sequences obtained from adult males identified to 75 distinct morphospecies. The average sequence length was 631 bp [range: 446-658], base composition of A=27.7%, C=17.9%, G=16.1% and T=38.3%. Similarities between Chironomidae species identified by morphology-based approach, BOLD BIN assignment and OTU delimitation are presented on the Venn diagram (Figure 8). Species assigned to OTUs and identified using morphology-based approach share 3 species. 15 species are present on lists revealed by BOLD BINs and OTUs approach. Altogether, BINs, OTUs and morphology-based approaches revealed 73 same species (Figure 8). List of taxa recorded on checklists identified using three mentioned approaches is presented in Table S10. Records of all European Chironomidae present in BOLD are assigned to 1289 Barcode clusters. The uploaded dataset represents 12.8% of the total number of barcode clusters from European non-biting midges. Before, the Balkan barcode library was represented only by records from Bulgaria (7 sequences) and Croatia (2 sequences).

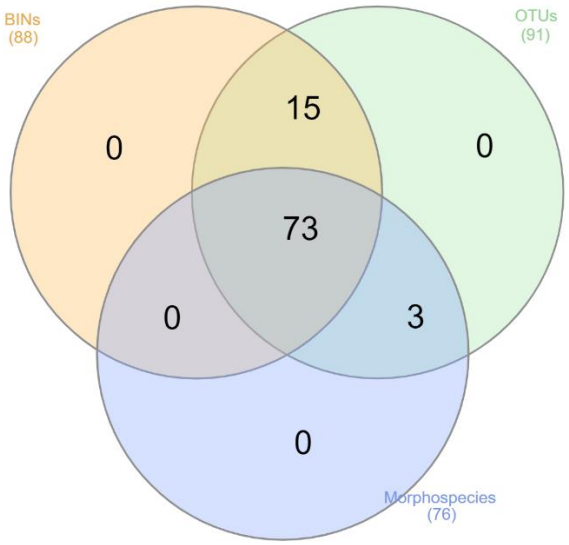

Figure 8. Similarities between Chironomidae species identified by morphology-based approach, BOLD BIN assignment and OTU delimitation

The previous study based on the taxonomic identification of mature males and pupal exuviae resulted in 125 correct species-rank names (86 for mature males and 133 for pupal exuviae) for the Skadar Lake basin (previous chapter). Comparing species checklists based on the morphology-based taxonomy and molecular approach, 53 species recorded on both lists. Species identification based on molecular data extended the existing checklist by about 22 new species (Table S2). All the species seem to be widespread in the Mediterranean region. Even if both checklists were based on the same collection with voucher preservation, there were differences in the number and composition of species. It is a result of additional improvement of mature male identification including results from a molecular approach, lack of sequences from pupal exuviae and vestigial failures during DNA isolation from tissue samples. As a result, 37% of Chironomidae fauna from Skadar Lake basin is now barcoded and publicly available in BOLD.

A basic comparison of molecular diversity among selected genera collected within Skadar Lake basin was performed to present a summary of the obtained dataset. Genera were analysed taking into account the number of sequences, haplotypes, BINs, identified morphospecies, values of K2P distance and mean intraspecific distance (Table 1).

Table 1. Diversity within most numerous in the number of sequences selected Chironomidae genera present in Lake Skadar.

Genus	Number of haplotypes	Number of BINs	Number of identified morpho species	Number of sequences	K2P distance				Mean within-species distance (%)
					min (%)	mean (%)	max (%)	SE (%)	
Cricotopus	32	11	8	34	3.26	14.47	22.84	0.02	2.46
Chironomus	52	8	8	55	3.53	15.55	22.4	0	0.79
Paratanytarsus	23	8	6	25	11.66	14.51	19.13	0.01	0.78
Polypedilum	44	8	7	44	11.57	17.03	21.59	0	1.13
Tanytarsus	30	8	8	32	13.62	17.62	22.53	0	0.63
Limnophyes	22	5	3	22	12.66	14.45	19.14	0.01	4.96
Procladius	22	4	2	22	8.95	9.99	11.09	0.02	0.91

Genus *Chironomus* and *Polypedilum* are represented by the highest number of sequences (52 and 44 respectively). On the opposite, genus *Procladius* and *Limnophyes* are represented by the lowest number of sequences (22). The highest number of 11 Barcode clusters within genera representing Skadar Lake basin fauna was recorded within genus *Cricotopus* (the lowest four within *Procladius*). *Cricotopus*, *Chironomus* and *Tanytarsus* were the most diverse among genera with 8 identified morphospecies. The lowest K2P distance was reported within the genus *Cricotopus*, 3.26% and 22.84% respectively. The highest mean value of K2P distance represented sequences of *Tanytarsus* (17.62%), the lowest *Procladius* (9.99%). The highest mean intraspecific distance was reported within genus *Limnophyes* (4.96%) and the lowest within *Tanytarsus* (0.63%).

The 65 newly formed BINs (201 sequences) are represented by the following species: *Cardiocladius capucinus* Zetterstedt 1850, *Chaetocladius perennis* (Meigen, 1830), *Cladotanytarsus mancus* (Walker, 1856), *Clinotanypus nervosus* (Meigen, 1818), *Cricotopus rufiventris* (Meigen, 1830), *Cricotopus sylvestris*, *Dicrotendipes pulsus* Walker, 1856, *Endochironomus albipennis* (Meigen, 1830), *Glyptotendipes pallens* (Meigen, 1804), *Glyptotendipes signatus* (Kieffer, 1909), *Guttipelopia guttipennis* (Wulp, 1874), *Limnophyes minimus* (Meigen, 1818), *Limnophyes natalensis* (Kieffer, 1914), *Microtendipes pedellus* (De Geer, 1776), *Orthocladius oblidens* (Walker, 1856), *Orthocladius pedestris* Kieffer, 1909*, *Parachironomus monochromus* (Wulp, 1874), *Paratanytarsus bituberculatus* (Edwards, 1929)*, *Paratanytarsus tenellulus* (Goetghebuer, 1921), *Polypedilum laetum* (Meigen, 1818)*, *Polypedilum nubeculosum* (Meigen, 1804), *Polypedilum pedestre* (Meigen, 1830), *Procladius culiciformis* (Linnaeus, 1767), *Tanytarsus brundini* Lindeberg, 1963. Asterisks (*) indicated species composed of single BIN.

3.2. Barcoding efficiency on Skadar vs European Chironomidae

The optimal threshold (OT) that minimizes the number of false-positive and false-negative identifications resulted in 3.27% of K2P nucleotide distance for the SKADAR dataset, with an associated cumulative error of 7 sequences out of 341 sequences (2%, FP=7, FN=0). In the case of the BOLD dataset, the OT resulted in a value of 0.65% of nucleotide distance, with a cumulative error of 948 sequences out of 10,025 (9,4%, FP=323, FN=625). As to be expected, the OT for SKADAR + BOLD datasets resulted in a value of 0.66% of nucleotide distance, with

a cumulative error of 1022 sequences out of 10,366 (9.8%, FP=382, FN=640) (Figure 9) (Table 2).

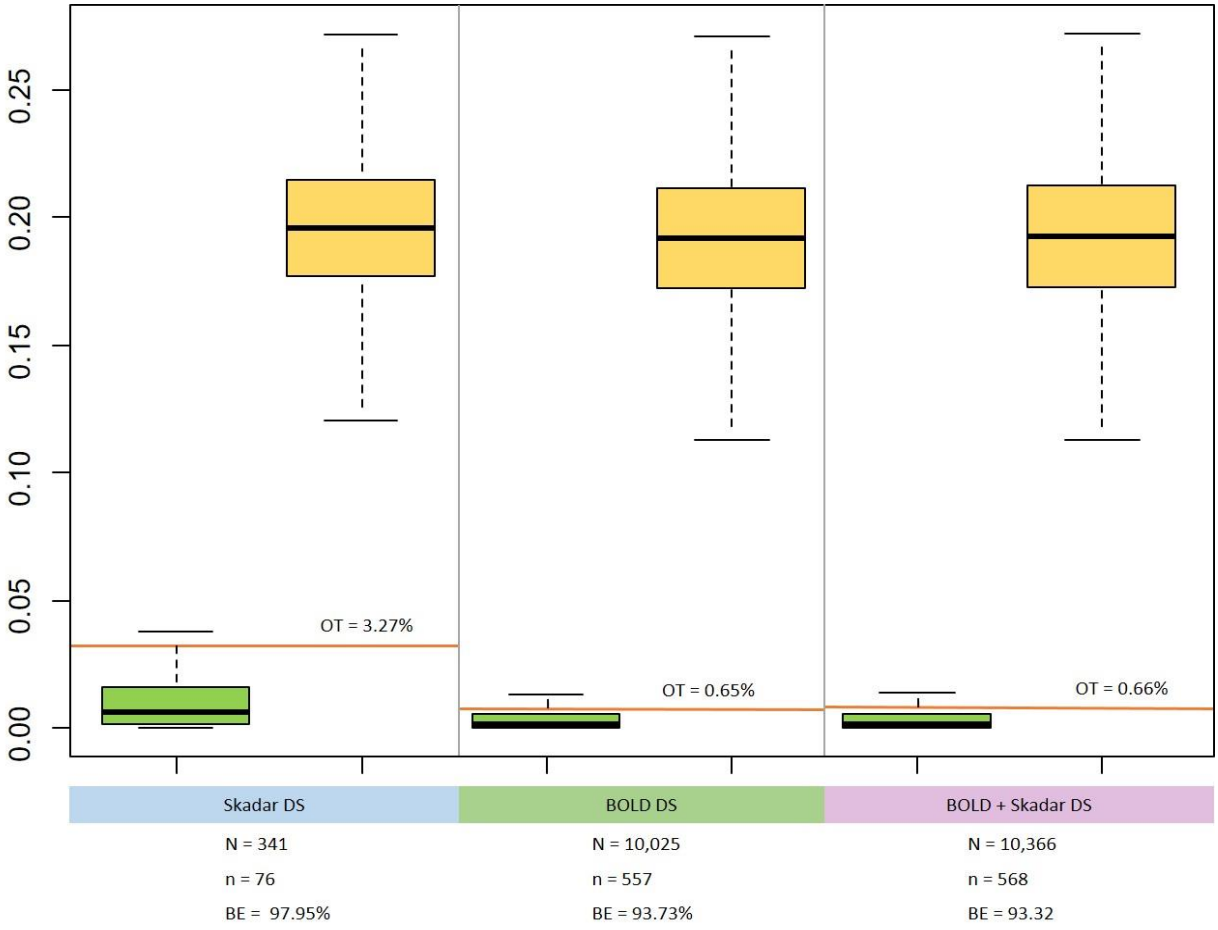

Figure 9. Inter and intraspecific K2P distances among three developed datasets. Green - intraspecific distance, orange - interspecific distance, OT - Optimal threshold; N - number of sequences; n - number of species; BE - Barcoding efficiency.

Table 2. Results of the barcoding efficiency analysis.

	ALL dataset (Family level)			BOLD + SKADAR DS (Subfamily level)						
	SKADAR DS	**BOLD DS**	**BOLD + SKADAR DS**	**Podonominae**	**Telmatogetoninae**	**Prodiamesinae**	**Diamesinae**	**Chironominae**	**Orthocladiinae**	**Tanypodinae**
Number of seq.	341	10025	10366	17	4	31	517	2317	7127	353
Correct	313	9295	9580	16	2	31	472	2155	6709	334
Incorrect		45	48				1	31	21	
Ambiguous		261	262				20	31	204	
No ID	30	424	476	1	2		24	100	193	19
Singletons (NO ID - NA)	21	101	94	1	2		3	35	44	10
Correct + singletons	334	9396	9674	17	4	31	475	2190	6753	344
Threshold	0.0327 (3.27%)	0.0065 (0.65%)	0.0066 (0.66%)	0.044 (4.40%)	0.01 (1.00%)	0.007 (0.70%)	0.0036 (0.36%)	0.019 (0.19%)	0.0065 (0.65%)	0.017 (1.70%)
Barcoding efficiency	97.94%	93.73%	93.32%	100.00%	100.00%	100.00%	91.88%	94.52%	94.75%	97.45%

The sum of cumulative errors obtained from the optimal threshold analyses performed on the subfamily level datasets, derived from the dataset SKADAR + BOLD, resulted in 933 sequences out of 10,366 (9%) (Table 2) (Figure 10). The highest error values calculated for subfamily datasets were observed for Orthocladiinae (605 sequences out of 7127 (8.4%, threshold = 0.65, Figure 10). The lowest error of nine sequences was reported for subfamily Tanypodinae from a total of 353 sequences (2.5%); the associated optimal threshold equalled 1.7%.

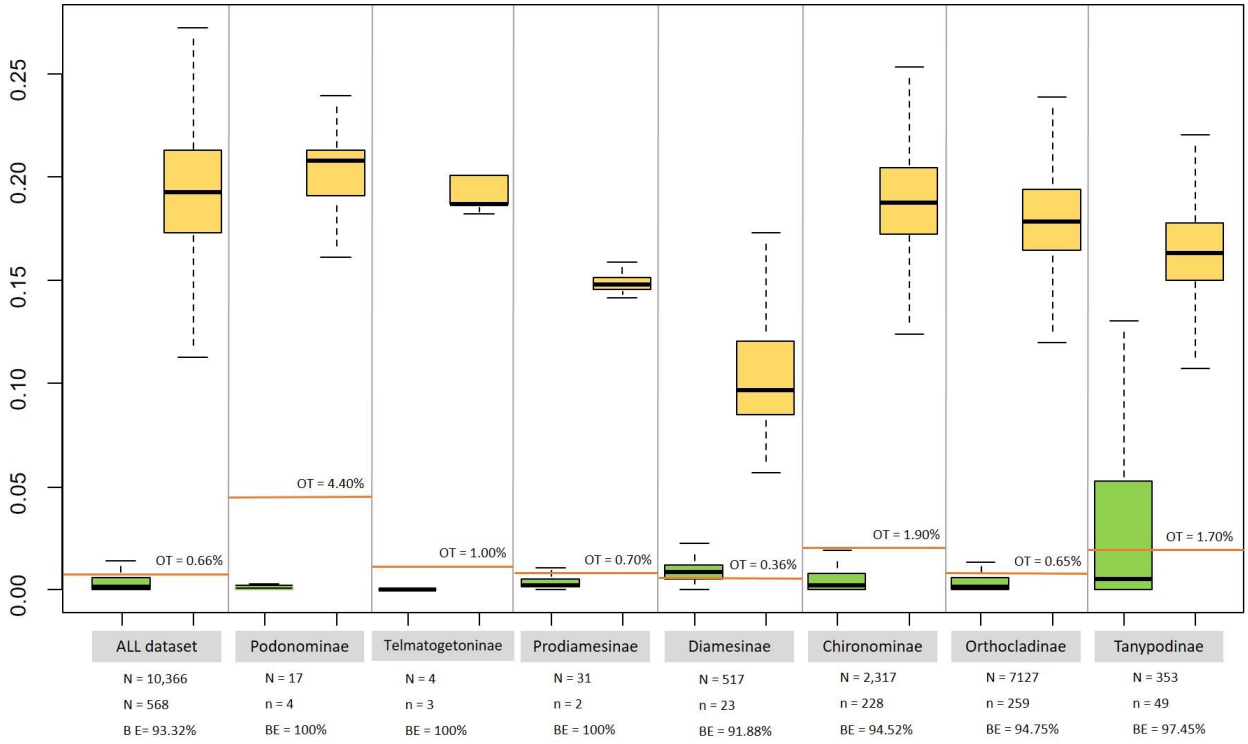

Figure 10. Inter and intraspecific K2P distances among Chironomidae subfamilies. Green - intraspecific distance, orange - interspecific distance, OT - Optimal threshold; N - number of sequences; n - number of species; BE - Barcoding efficiency

The barcode efficiency was evaluated separately for the three datasets defined above. *Best Close Match* analysis performed on the SKADAR dataset resulted in 97.9% of correct identifications (334 out of 341, with an optimal threshold of 3.27%) and nine missing identifications. The analysis for the sequences mined from BOLD, with the estimated OT of 0.65% resulted in 93.7% of correct identifications (9396 out of 10025), with 45 incorrect identifications,

261 ambiguous and 323 missing identifications. SKADAR + BOLD dataset evaluated through the *Best Close Match* analysis with an OT of 0.66% resulted in 93.3% of correct identification (9674 out of 10366), 48 incorrect identifications, 262 ambiguous and 382 missing identifications (Table S7). The 94 species consisting of single COI sequences were considered as correctly identified since no match with other heterospecific sequences occurred. Among all the analysed subfamilies, the highest number of incorrect and ambiguous identifications were listed among Orthocladiinae and Chironominae subfamily because of a high number of mined sequences. Problematic sequences were also present among Diamesinae.

A total of 262 sequences resulted in no match with conspecifics because of a pairwise nucleotide distance higher than the adopted OT (0.66%). Among these ambiguous cases, Orthocladinae were represented by following groups of misidentified/doubtful species/identifications: *Gymnometriocnemus kamimegavirgus* Sasa & Hirabayashi, 1993 - *G. volitans* (Goetghebuer, 1940); *Heterotrissocladius brundini* Sæther & Schnell, 1988 - *H. grimshawi* (Edwards, 1929) and *H. marcidus* (Walker, 1856); *Cricotopus rufiventris* (Meigen, 1830) - *Paratrichocladius rufiventris* (Meigen, 1830) and *Psectrocladius octomaculatus* Wülker, 1956 - *P. psilopterus* (Kieffer, 1906). Ambiguous sequences within the subfamily Chironominae were represented by: *Chironomus heteropilicornis* Wülker, 1996 - *C. pilicornis* Fabricius, 1787; *Microtendipes brevitarsis* Brundin, 1947 - *M. pedellus* (De Geer, 1776); *Polypedilum bicrenatum* Kieffer, 1921 - *P. tridens* Freeman, 1955; *Sergentia baueri* Wülker (1999) - *S. prima* Proviz & Proviz, 1997; *Dicrotendipes modestus* (Say, 1823) - *D. pulsus* (Walker, 1856); *Chironomus pseudothummi* Strenzke, 1959 - *C. riparius* Meigen (1803). In the subfamily Tanypodinae, we did not report ambiguous sequences.

3.3. European Chironomidae - species diversity, BIN analysis, haplotype networks

A total of 10,025 COI barcodes with the correct species-rank names were retrieved from BOLD (and, subsequently, from GenBank). A total of 770 Chironomidae sequences obtained from Skadar Lake catchment, together with the sequences retrieved from BOLD, were assembled into a dataset DS SKADAR + BOLD resulting in 10,366 sequences representing 567 species. The dataset includes sequences of 567 chironomid species with the mean number of haplotypes per species of 6.4 and average 18.2 sequences per species (Table 2). 101 species were represented by a single sequence. Average sequence length was 657 bp [range: 446-658]), base composition of

A=26.8%, C=17.5%, G=16.6, T=29.1%. Dividing sequences by sub-family led to obtaining datasets for the following seven subfamilies: *Chironominae* (2,317 sequences), *Diamesinae* (517 sequences), *Orthocladiinae* (7,127 sequences), *Podonominae* (17 sequences), *Prodiamesinae* (31 sequences), *Tanypodinae* (353 sequences), *Telmatogetoninae* (4 sequences). Within *Podonominae* (OT=4.4%), *Telmatogetoninae* (OT=1%) and *Prodiamesinae* (0.7%), three out of seven Chironomidae subfamilies we reported 100% of correct matches. Among other subfamilies, the efficiency was at the level of 97.4% for *Tanypodinae* (OT=1.7%, 344 out of 353 correct identifications, no incorrect), 94.7% for *Orthocladiinae* (OT=0.65%, 6753 out of 7127 correct identifications, 21 incorrect), 94.5% for *Chironominae* (OT=0.19%, 2190 out of 2317 correct identifications, 31 incorrect), and 91.8% for *Diamesinae* (OT=0.36%, 475 out 517 correct identifications, 1 incorrect) (Table 2). Values of OT estimated from European non-biting midges included in this study are comparable with results obtained from the delineation of species belonging to the genus *Tanytarsus* (OT=4–5%; Lin et al., 2015); Alpine Chironomidae (OT=0.7%-1.4%; Montagna *et al.,* 2016a); for Bavarian moths (OT=1.8%; Ratnasingham and Hebert, 2013); for *Gammarus* amphipods (OT=4%; Delić *et al.,* 2017); Euro-Mediterranean Chrysomelidae (OT=2.6%; Magoga *et al.,* 2018). COI sequences of closely related or most problematic for morphological identification at the species level within *Cricotopus* (two groups of species), *Chironomus*, *Diamesa*, *Micropsectra* and *Polypedilum* were here analysed and haplotype networks provided.

According to the BOLD, among selected species, the most sampled and widespread species in Europe were *Cladotanytarsus mancus* (Walker, 1856) and *Tanytarsus brundini* Lindeberg, 1963 represented by the highest number of different barcode clusters (12 and 9 respectively) (Table S8). Such high diversity may be explained by numerous aggregations of morphospecies within the C. *mancus* group. From the selected morphospecies, the most homogenous morphospecies, *Paratendipes albimanus* (Meigen, 1818) and *Orthocladius oblidens* (Walker, 1856) were represented by 1 and 2 barcode clusters, respectively. All of the species presented in Table S2 are widespread in the Mediterranean region (see the previous chapter). The lack of endemism in the list may be somewhat misleading because misidentified or undescribed taxa of generic rank are expected based on own data and some already described taxa do not closely match current generic concepts.

Genus *Cricotopus* (two groups of species)

Based on a test haplotype network calculated on the whole genus *Cricotopus*, sequences of the genus were divided into two groups, (1) consisting of 4 species (Figure 11); and (2) of 7 species (Figure 12) (Table S9). Only *C. sylvestris* and *C. glacialis* presented shared haplotypes (Figure 12).

Cricotopus (Isocladius) glacialis Edwards, 1922 and ***Cricotopus (Isocladius) sylvestris*** (Fabricius, 1794) are very similar in morphology and can be easily misidentified (Hirvenoja, 1973). Molecular data confirm the hypothesis that *C. glacialis* should be a junior synonym of *C. sylvestris* both species should be synonymised. Both belong to the same barcode cluster (BOLD:AAA5299) and all sequences of *Cricotopus glacialis* are assigned to this BIN. *C. glacialis* is restricted to Northern Europe (in BOLD is reported from Norway: 11 sequences from Svalbard archipelago, 7 from continental Norway, and 3 individuals from Iceland). Bruno Rossaro (personal communication) captured individuals probably belonging to *C. glacialis* in the Alps, but after a deep study of morphological characters, it was impossible to confirm such identification. In result, the species was not included in the Italian fauna (Rossaro *et al.*, 2019). Molecular data mined from the BOLD database indicate the presence of *C. sylvestris* in Sweden (17), Norway (13), Finland (4), Denmark (Zealand and Bornholm) (2) and Poland (1). Species was also reported from the Skadar Lake basin. We obtained sequences from 15 individuals collected in Montenegro.

The haplotype network provided interesting data and some mismatch of species identifications (Figure 12). For example, two specimens of *Cricotopus sylvestris* (Process ID: PGBAL250-19, PGBAL197-19) are grouped close to the *Cricotopus relucens* Hirvenoja, 1973 (PGBAL249-19). They were identified during this study and could be misidentified since they are assigned to the newly formed BIN (BOLD:AEA8330). It could be evidence of a *Cricotopus* species new for BOLD, which was not yet barcoded or even of species new for knowledge. Also, BOLD data are ambiguous because both specimens of *C. relucens* (PGBAL030-19 and PGBAL249-19) are clustered close to *C. sylvestris* from Sweden (BSCHI628-17) and all together assigned to the barcode cluster BOLD:AAA5299 which consists of mentioned species as well as *Cricotopus glacialis*, *Cricotopus sp. 3ES, Cricotopus laetus* Hirvenoja, 1973 and *Cricotopus sp*. It could be evidence of misidentification of *C. relucens* with *C. sylvestris,* since it is challenging to distinguish the two species based on morphological characters.

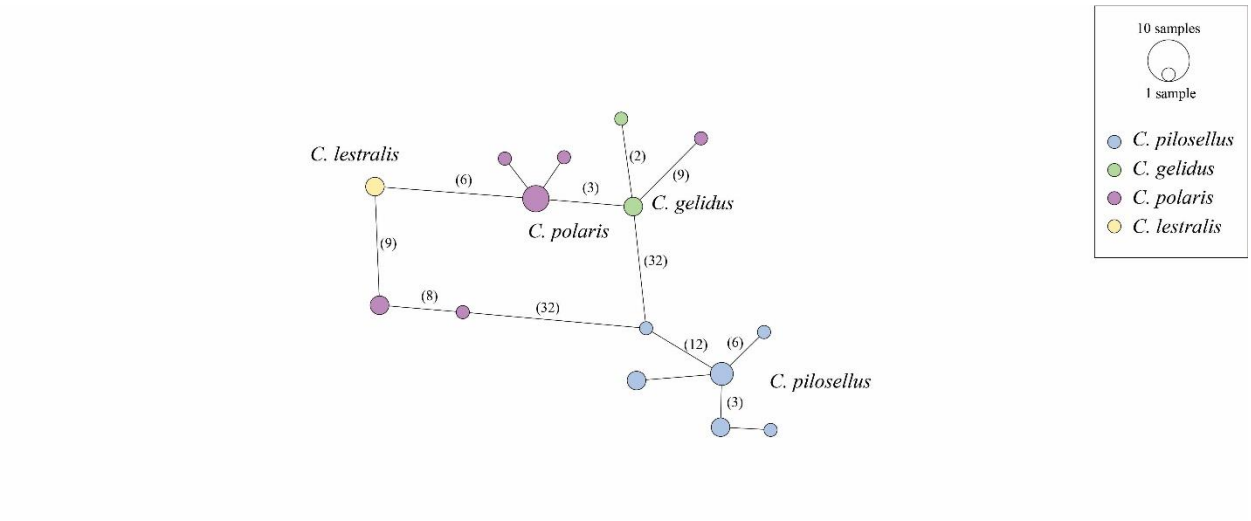

Figure 11. Haplotype minimum spanning network for the genus *Cricotopus* (1st group).

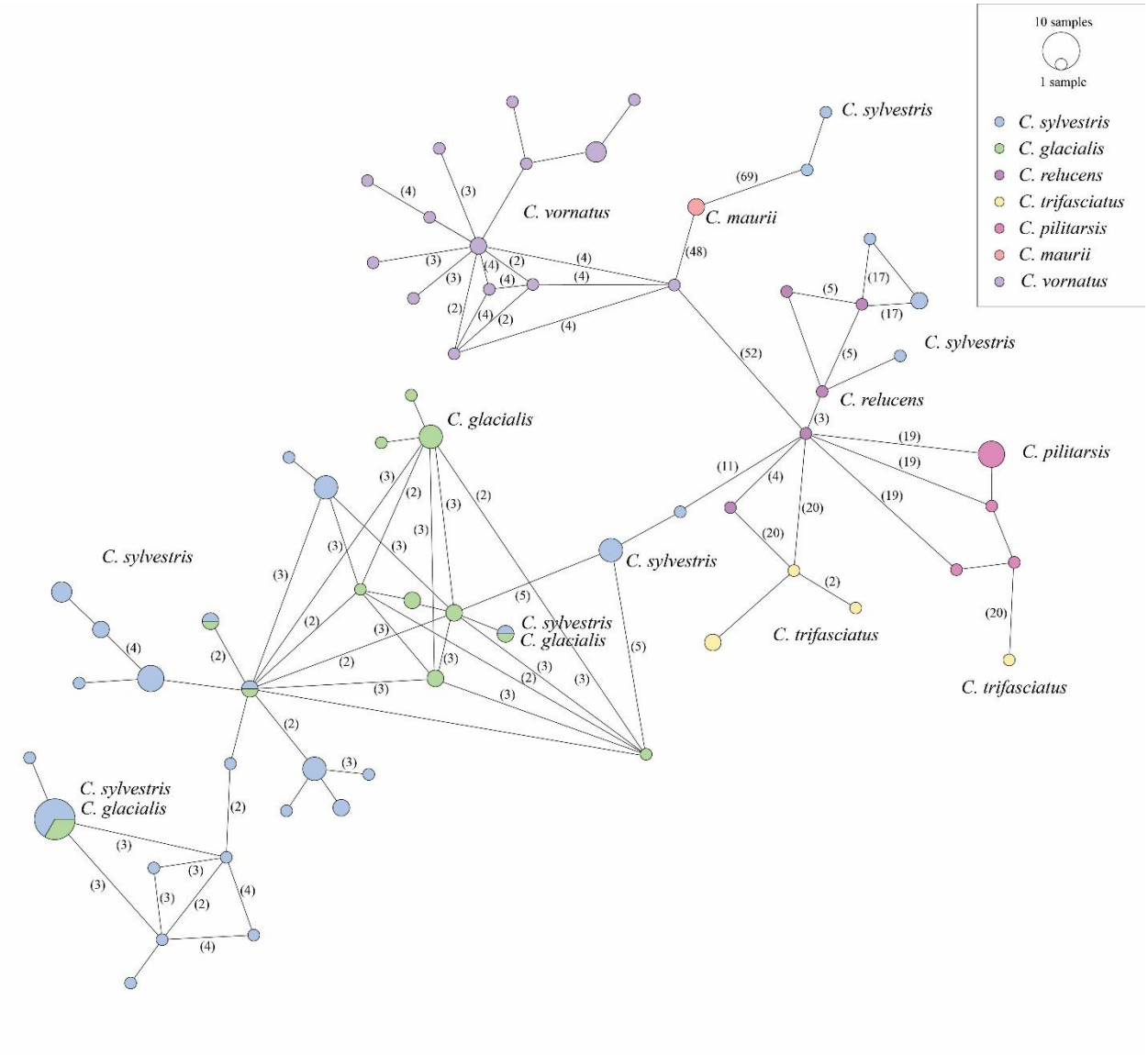

Figure 12. Haplotype minimum spanning network for the genus *Cricotopus* (2nd group).

The genus *Chironomus* was represented by 24 species (Table S9). Two groups of species: *C. pseudothummi* - *C. riparius* and *C. heteropilicornis* - *C. pilicornis* shared haplotypes (Figure 13).

C. pseudothummi, C. lugubris *and* ***Chironomus (Chironomus) riparius*** Meigen, 1808 can be difficult to separate by the morphology of adult males (Lindeberg and Wiederholm, 1979) and precise division can be done based on pupal exuviae (Langton and Visser, 2003). Rossaro, (2019) preliminary identified some exuviae as *C. lugubris*, but this species generally has a North-Central European distribution, even if it was found in Italy. Now the species has been reported also from

Switzerland and Hungary (Rossaro *et al.*, 2019). The specimens presented in BOLD were collected in Spitsbergen (Norway) and assigned to homogenous BIN (BOLD:AAB4581). ***Chironomus (Chironomus) sollicitus*** Hirvenoja, 1962 is restricted to Finland, but according to BOLD it was collected in Norway and is represented by a single sequence from the homogenous BIN (BOLD:AAI4306). ***C. salinarius*** is a well identifiable species from this group and is known to be restricted to highly saline waters. The individuals present in BOLD were collected from Denmark and Sweden in the strait between Malmo and Copenhagen. ***C. pseudothummi*** Strenzke, 1959 was not previously reported from the Mediterranean area, but was reared by B. Rossaro (personal communication) from the Adda river (Italy) (16.VI.2011, Rossaro *et al.*, 2019); the species has been reported from the Skadar Lake basin and its occurrence is supported by molecular data (sequences from 16 specimens already uploaded to BOLD). Based on 16 other records of ***C. pseudothummi*** present in BOLD, 14 specimens were collected in Sweden and two in Norway. Also nine records of ***C. lugubris*** were reported from Spitsbergen. ***Chironomus riparius*** seems to be well-sampled and based on 133 sequences is reported from France (39 specimens), Germany (27), Italy (54, including 44 from Sardegna), Bulgaria (5) and Sweden (3). The haplotype network built for *Chironomus* revealed 46 haplotypes of *Chironomus riparius*, but with high divergence (average 37 mutations). The sequences of this species are assigned to three BINs. It could be explained by some local differentiation due to the geographically wide specimen sampling.

C. heteropilicornis Wuelker, 1996 was identified based on karyotype and is restricted to Fennoscandia. ***C. pilicornis*** (Fabricius, 1787) has similar north-eastern distribution. The genitalia of *C. pilicornis* are very similar to those of *C. plumosus* (Wülker, 1996; Martin, 2017); so the two species can be easily misidentified. The molecular data available for *C. heteropilicornis* originate from Norway (5 barcodes), and those for *C. pilicornis*, from Sweden (2 barcodes). The sequences of *C. heteropilicornis* and *C. pilicornis* are assigned to one BIN (BOLD:ACX5781) which could indicate species misidentification or both species are in fact one species and should be synonymised. Another possible scenario suggests the effect of hybridisation or introgression process. Both the mentioned species can be easily misidentified with *C. plumosus*, but molecular data differentiate the clusters as separate species.

Chironomus (Chironomus) plumosus (Linnaeus, 1758) and ***Chironomus (Chironomus) curabilis*** Belyanina, Sigareva & Loginova, 1990 are very similar to each other. According to both, the COI sequences and karyotype (Polukonova, 2009), it can be prudentially suggested that *C.*

plumosus, is a synonym of *C. curabilis*. In Skadar Lake basin *C. plumosus* was reported based on morphological identification but BOLD identification tool using COI data, provided evidence for *C. curabilis.* All of its sequences are forming homogeneous BIN. *C. plumosus*, usually recognized as widely distributed in Europe, is not well represented in the BOLD database. *Chironomus plumosus* was reported from the Skadar Lake basin based on the morphological and molecular data. From the 24 available sequences, half was recently uploaded by me from specimens collected in Montenegro. Among others, 11 were collected from Sweden and one from Poland. All these sequences are grouped within 18 haplotypes and assigned to one BIN: BOLD:AAU2239, but it is not homogenous and includes also clusters with sequences of *C. usenicus*, *C. tentans* and *Cladotanytarsus atridorsum*.

The taxonomic status of ***Chironomus pallidivittatus*** and ***Chironomus tentans*** Fabricius, 1805 is in debate (Spies and Sæther, 2004). *Chironomus (Chironomus) pallidivittatus* sensu Edward, 1929 (not *Ch. pallidivittatus* sensu Malloch, 1915 which is distributed to Nearctic) reported from Europe does not have a clear status. All its 11 sequences were reported from Sweden. Four sequences of *C. tentans* were reported from Norway. Both species are grouped into one BIN (BOLD:AAE9024) which confirms their unclear taxonomic status.

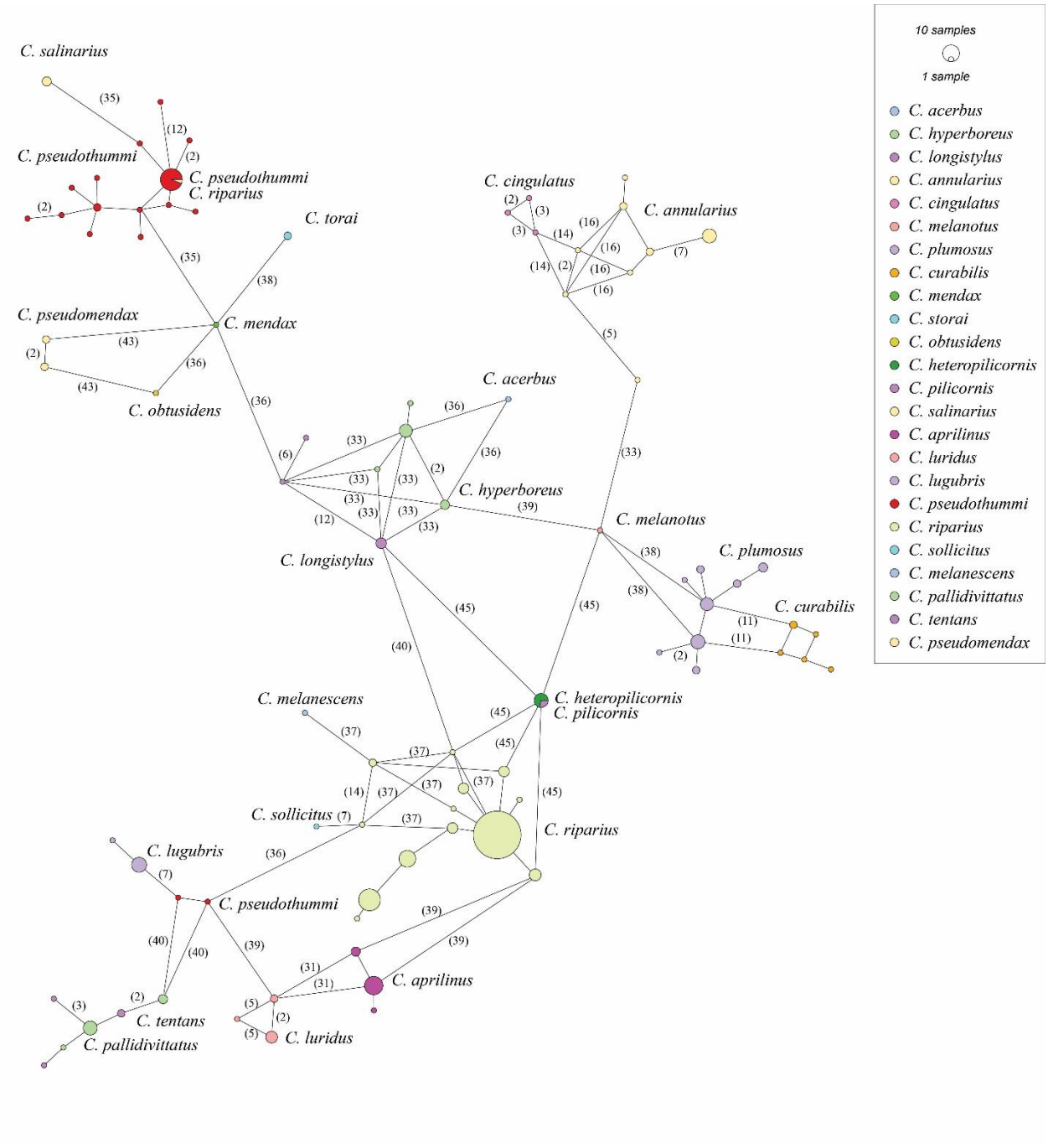

Figure 13. Haplotype minimum spanning network for the genus *Chironomus.*

The genus *Diamesa* was represented by 15 species (Table S9). Two groups of species: *D. bohemani* - *D. zernyi* and *D. tonsa* - *D. hyperborea* shared haplotypes (Figure 14).

Diamesa hyperborea Holmgren, 1869 distribution is restricted to North Europe, and it is very often misidentified with ***Diamesa tonsa*** (Haliday, 1856). Literature data indicate that ***Diamesa zernyi*** Edwards, 1933 is very similar to *Diamesa bohemani*, Goetghebuer, 1932, and it may be a case of intraspecific variation. The latter species was considered as restricted to North Europe (Serra-Tosio, 1973). Its presence in Italian Alps was supposed but not confirmed, even if ***D. bohemani*** is reported from France and Austria (Fauna Europaea, de Jong *et al.*, 2014). Based on molecular data mined from BOLD, five specimens of *D. tonsa* were collected in Norway and four specimens of *D. zernyi* were collected on Iceland. *D. bohemani* and *D. hyperborea* were much better sampled. Among 39 collected specimens of *D. bohemani*, 8 were collected in the continental part of Norway, 7 from the Bear Island and 24 from Spitsbergen. 33 specimens of the latter species were reported from the continental Norway (12) and 21 from the Bear Island. According to Montagna *et al.* (2016b), *D. tonsa* failed in specimen identification using molecular tools. The species was not well separated from *Diamesa cinerella* Meigen in Gisti, 1835 group, and thus the support of specialist entomologists is still required. Regarding the specimens belonging to the D. *zernyi* group, they were clearly separated from other groups based on molecular data, especially from the morphologically similar *D. cinerella*.

Among *Diamesa* species distributed in Europe, an interesting case is also *Diamesa insignipes* Kieffer, 1908. It includes 79 haplotypes (out of 238 sequences). It forms one barcode cluster, but with an average of two mutations, the haplotypes are not very divergent (Figure 4). Based on the BOLD data, this species was collected in Europe only in one sampling site in Germany (between Bonn and Koblenz) and all individuals belong to one BIN.

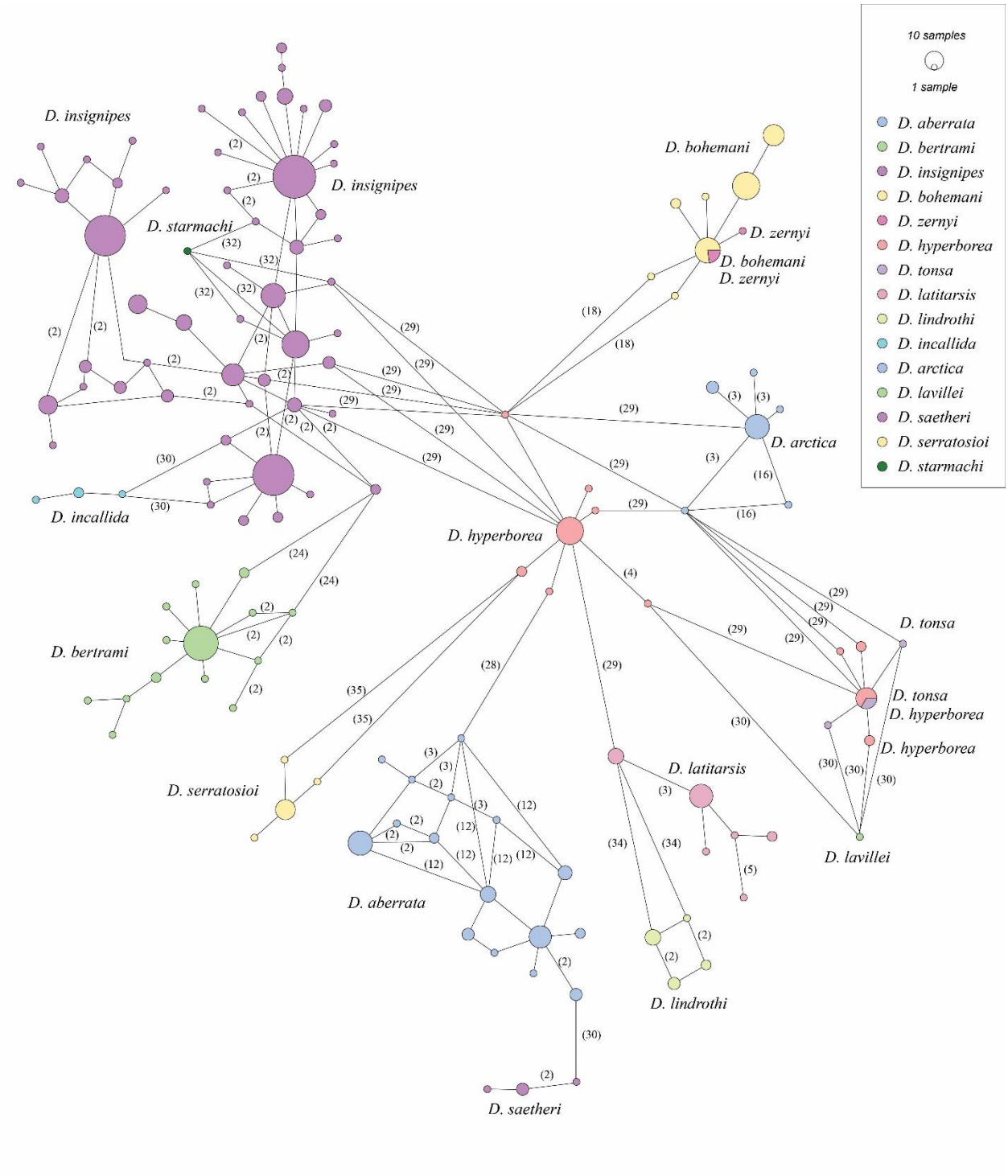

Figure 14. Haplotype minimum spanning network for the genus *Diamesa*.

The genus *Micropsectra* was represented by 25 species (Table S9) (Figure 15). No shared haplotypes were observed.

Micropsectra contracta, Reiss, 1965 is a junior synonym of ***Micropsectra apposita*** (Walker, 1856). ***Micropsectra junci*** is a distinct species, but the two species are very similar based on male genitalia (Sävedal, 1976). A distinction of both species based on ecological data is also not trivial. *M. apposita (=contracta)* is living only in cold water and it is characteristic of profundal zones of lakes, while *M. junci* lives in springs. These slight differences in temperature can be used for additional factor limiting their occurrence. In BOLD *M. apposita (=contracta)* is reported from south of Germany (4 specimens) and from Norway (9 specimens). Forty specimens of *M. junci* were reported from Norway (34), Finland (2), Luxembourg (2), Sweden (1) and Germany (1). Sequences of *M. junci* are assigned to five different barcode clusters. Both species (including specimens with junior synonym names) are present in one BIN: BOLD:AAC7823 which indicates species misidentification or both species are in fact one species based on molecular differences and should be synonymised. Another possible scenario suggests the effect of hybridisation or introgression process.

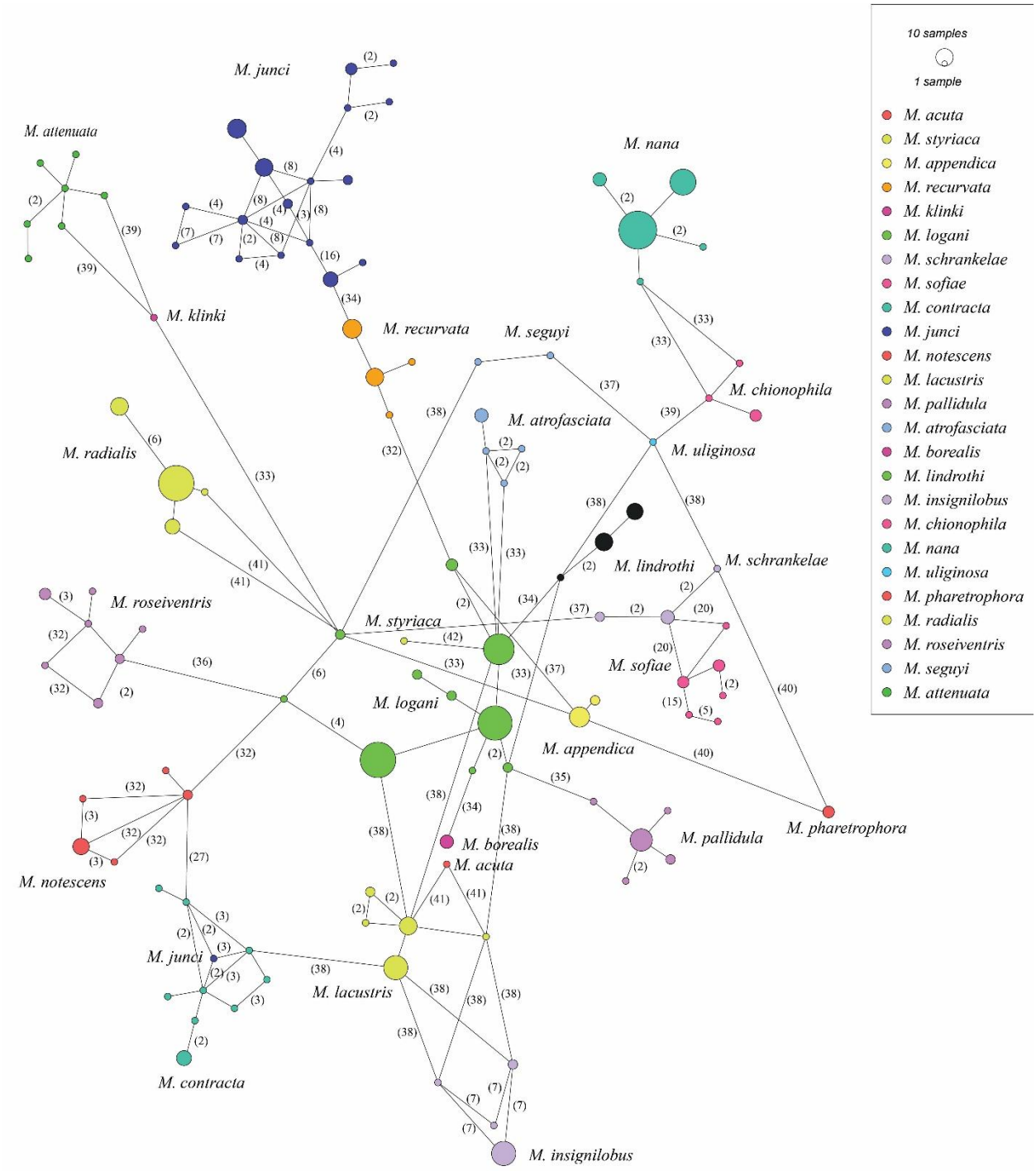

Figure 15. Haplotype minimum spanning network for the genus *Micropsectra*.

The genus *Polypedilum* was represented by 19 species (Table S9). Shared haplotypes were observed within one group *P. bicrenatum* - *P. tridens* (Figure 16).

Polypedilum* (Tripodura) *tridens Freeman, 1955 and ***Polypedilum* (Tripodura) *bicrenatum*** Kieffer, 1921, are considered distinct species, but are very similar based on morphology. In the literature, *P. tridens* is not reported from Europe, but only from Africa. In BOLD three sequences (3 haplotypes) are present. Two specimens were collected in Germany and one in Norway. Six specimens of *P. bicrenatum* (2 haplotypes) were collected in Sweden. All the published sequences are assigned to one BIN (BOLD:ACV3478) which could be hypothesised as a one species and the further taxonomic investigation is necessary. Sequences of ***Polypedilum albicorne*** (Meigen, 1838) which consists of 57 haplotypes and one BIN was obtained from specimens collected in Norway and Germany. ***Polypedilum nubeculosum*** (Meigen, 1804) included 26 sequences, 22 haplotypes and three BINs from Montenegro, Germany and Sweden.

Likewise problematic and red-flagged during the a*dhoc* analysis were *Psectrocladius octomaculatus* Wülker, 1956 (2 sequences) recorded in BOLD from Sweden and *Psectrocladius psilopterus* (Kieffer in Kieffer & Thienemann, 1906) (3 sequences) from Norway. Sequences of both species are assigned to the homogenous barcode cluster (BOLD:AAX8186). Morphologically they can be well separated both as adults and pupae (Wülker, 1956), but a high level of taxonomic expertise is required. This could indicate species misidentification or both species are in fact one species based on molecular data and should be synonymised. Another possible scenario suggest effect of hybridisation or introgression process.

Figure 16. Haplotype minimum spanning network for the genus *Polypedilum.*

5. Conclusions

- This study provides COI barcodes for 770 Chironomidae individuals assigned, based on morphology, to 75 species collected in the Skadar Lake basin (all records from this area are new for online repositories) and confirms the usefulness and efficiency of DNA barcoding for the identification of non-biting midges.
- Cases of barcoding failure in species identification were observed especially for the closely related species or for those where taxonomic identification demands a very high level of expertise in such genera as *Chironomus, Cricotopus, Polypedilum*. Barcoding failure could be also caused by the mitochondrial heteroplasmy, introgression, hybrid speciation, incomplete lineage sorting, the occurrence of symbiotic bacteria or the presence of NUMTs.
- Some species are reported for the first time in this study and are represented by single sequences. For those species, better sampling coverage is necessary to obtain more, good quality sequences for reliable comparisons.
- Species separation using *adho*c thresholds estimated on reference library could be a useful tool for more detailed study of diagnostic differences among groups of similar taxa. It could be done with reverse DNA-taxonomy approach – first using DNA barcoding for species separation and then back to the morphology looking for differentiating characters.
- The comparisons among optimal thresholds estimated at different taxonomic levels, *viz* family and subfamily, have underlined the importance of using taxon-specific thresholds to increase the efficacy of molecular identification. Moreover, the results of this study based on performed analysis on the developed dataset can be used for selecting ambiguous or incorrect identifications. Barcode misidentifications arising from an incomplete reference dataset of species could be flagged and then recognized as misidentifications. It will help to better curate reference barcode library and to share with the scientific community only confirmed data.
- With 65 (39.1%) unique barcode clusters from the Skadar Lake basin new for BOLD we can expect that further development of reference library of barcodes from mature females and larvae will surely extend hidden Chironomidae species diversity.

General discussion

For my PhD dissertation, I studied species diversity and the origin of non-biting midges (Diptera, Chironomidae) fauna from the geologically young lake Skadar and its old spring system (Montenegro/Albania), based on morphological and molecular characters. Pursuing this goal, I additionally compared the level of species diversity with other central and southern European lakes. The second aim was to investigate the influence of physical-chemical conditions on composition and distribution of chironomid assemblages in Skadar Lake basin. Results of these findings, except species diversity based on molecular data, are presented in Chapter I. As a third aim, I developed and evaluated the first reference barcode library for Chironomidae from Skadar Lake basin. Using a reference library, I estimated DNA barcoding efficiency for the European Chironomidae based on BOLD records. My fourth aim was to explore chironomid species distribution patterns in Europe using universal Barcode Index Number (BIN) with a discussion of problematic species groups, both for traditional taxonomy and DNA barcoding. By implementing the integrative taxonomy approach to my study, I provided evidence for hidden species diversity within a hot-spot of freshwater biodiversity which is undoubtedly Skadar Lake basin.

My PhD dissertation consists of two chapters. In the first chapter, I focused on providing the first insight into the diversity and ecology of non-biting midges inhabiting the unique ancient Skadar Lake system. Individuals for this analysis were collected during three sampling campaigns, based on seasonality of emergence and then identified to species level based on a taxonomy-based approach. Findings of Chapter I, focused on morphological identification of 8,845 specimens (adult males and pupal exuviae) and indicates the presence of 164 Chironomidae taxa which extends the existing checklist with about 152 taxa newly found in the Skadar Lake basin. In total, 7,274 male adults were assigned to 86 distinct morphotypes and 82 correct species-rank names. Based on 1,571 pupal exuviae, I identified 133 morphospecies assigned to 95 valid species (Table 1). It is a first scrupulous investigation of non-biting midges species diversity (including imagines) of Lake Skadar basin made from 1977 (Jacobi, 1977). In Chapter II, I presented results of molecular identification based on *cytochrome oxidase subunit I* gene (*CO1*) of 770 specimens, 399 larvae and 371 adult male specimens. According to the species separation methods based on molecular characters, 'species' in terms of taxonomy, here is represented by molecular *Operational Taxonomic Units* (OTUs) or by Barcode Index Number (BIN cluster) (Ratnasingham

and Hebert, 2013). Molecular identification of the collected chironomid individuals revealed the diversity of 168 OTUs and 165 BINs. From the total number of sequences, larvae were represented by 399 sequences assigned to 98 OTUs and 95 BINs since adult males were represented by 371 sequences representing 75 morphotypes assigned to 104 OTUs and 99 BINs. Taking into account that the larval stage is included exclusively in the molecular approach and pupal exuviae were analysed based on only morphological characters, it is hard to compare overall species diversity. Nevertheless, both approaches can be compared using identified adult males. The morphological taxonomy-based approach resulted in 86 distinct morphospecies which is eleven more than the number revealed by DNA barcoding. From the other hand, taking into account the number of analysed specimens: 7,274 by morphology and 371 by DNA barcoding, the disproportion is much bigger. It could be concluded that some of the species might be overlooked during taxonomical identification of such big collection. Analysing the number of OTUs and BIN clusters, both present a similar level of molecular diversity, (104 and 99, respectively). Both results are significantly higher than those obtained based on morphological taxonomy-based approach. Thus, these findings indicate that some records are wrongly identified or there is evidence of cryptic diversity. Up to date, 38 species were reported from the Skadar Lake basin (Nedeljković, 1959; Janković, 1974; Karaman and Nedić, 1975; Jacobi, 1977; Jacobi, 1981; Płóciennik and Pešić, 2012; Pešić *et al.,* 2018; Pešić, 2018). Besides the recent big contribution to the diversity of the Skadar Lake zoobenthos (Pešić *et al.,* 2018), previous studies made by local researchers were focused on Chironomidae larvae as a part of the whole benthic community. Since the most of larvae are very difficult to identify to species level using morphology, due to the complexity of their taxonomy and lack of expertise, the presented results might counterfeit the actual degree of chironomid diversity. Only 12 species are joint for both, the present investigation and for the literature data. It is a good point to state that for valuable overall species diversity assessments based on reliable results, it is necessary to include as many developmental stages as possible for which the identification allows deeper resolution. Integrating both, the taxonomy-based and molecular approach on the bigger collection may resolve concerns about factual species diversity within the Skadar Lake basin. In my opinion, by combining the results obtained from both approaches, this study contributes significantly to the knowledge of the diversity of aquatic biota of the lake. The results of my thesis provide an interesting and important insight of modern biodiversity loss and

contain original and valuable information that could be interesting not only for limnologists but also for nature conservation units.

In Chapter I, benefiting from faunistic information acquired from specimens collected during sampling seasons 2014 and 2015, in association with physical-chemical conditions of the collecting sites, I investigated the environmental factors influencing the composition and distribution of chironomid assemblages. Considering the mobility of collected pupal exuviae and adult male specimens (passive and active, respectively), the results were analysed separately.

The results of my thesis highlight the factors shaping non-biting midges species distribution among Skadar Lake basin. Based on the studied literature, I assumed that adult specimens collected on each sampling site will reflect the larval population inhabiting the closest habitat (Armitage *et al.*, 1995). The findings of this thesis indicate that for mature males better suitable habitats are situated in the shallow, coastal part of the lake where temperatures are higher. The performed RDA analysis does not provide any evidence of the influence of macrophytes but indicates the influence of water temperature as a factor shaping Chironomidae assemblages (Figure 6). Based on personal observations, both factors are highly connected and could be explained by the fact that high biomass of macrophytes growing from the bottom to the surface of the lake slows down the water flow. The heat accumulated in these areas is transferred at a slower rate to the colder parts of the basin. This observation is confirmed by the ecology of species identified in such sites, e.g. species from genus *Chironomus* or *Polypedilum* (Armitage *et al.*, 1995). Additionally, the increased amounts of recorded total dissolved phosphorus provide a good basis for growing macrophytes which are a source of detritus accumulating on the lake bottom. Dead organic matter turns into sediments which are the perfect habitat for larvae of deposit-feeders such as *Chironomus plumosus* and *Polypedilum nubeculosum* (Coffman and Ferrington, 1984; Armitage *et al.*, 1995). Excellent food availability together with high temperature allows to close life cycle in a short time and causes mass emergence. By collecting mature specimens using two main methods such as swiping through coastal vegetation and attracting by light exposed on a screen I was trying to maximize species diversity.

From another hand, exuviae were collected from the open lake and the presence of macrophytes is an important factor shaping the distribution of Chironomidae in Lake Skadar. Both factors can be explained by the limitation of the implemented Chironomidae Pupal Exuvial Technique (CPET)

technique on lakes. The open lake area is often exposed to the wind. Few hours after imago emergence, pupal exuviae are floating on the surface and could be easily transported by the wind. Usually, winds blow along the mountainous southern shoreline to the north of the lake. The northern, shallow part of the lake is in turn covered by floating on the surface macrophytes which accumulates all obstacles transported with the wind. Among them are numerous pupal exuviae, usually collected by hand net from the boat. Since exuviae can be passively transported (e.g. by anemochory) at short distances, I am fully aware that collecting pupal exuviae with simultaneous measuring water parameters in the place of the collection has drawbacks. By implementing CPET technique during sampling within Skadar Lake basin, and by studying literature data, I was conscious that during each season the differences in water parameters between sampling sites within the lake were negligible (Ruse, 2002; Ruse, 2010; Ferrington, 2008; Armitage *et al.*, 1995).

An additional result during the investigation of Chironomidae species composition achieved during my thesis was a comparison of the Skadar Lake species diversity with other central and southern European lakes. Literature review revealed that that only a few lakes were deeply studied and only common species, widespread in Europe, occur in all of them. It is not a surprise that the highest species diversity was recorded from the lakes studied in details. From the lakes chosen for this comparison, Lake Constance (Switzerland/Germany/Austria) was represented by the highest number of species (174 taxa), followed by the Skadar Lake (164 taxa). For Lake Constance, a possible explanation of such high number of recorded species among lakes included in the cluster analysis (Figure 6) could be the fact of collecting larvae together with imagines (Reiss, 1968). In case of Skadar Lake, it is a result of combining all developmental stages during sample collection, implementation of different sampling techniques (using hand net for exuviae collection, for imagines using entomological hand net and light attracting), replication of sampling campaigns taking into account seasonality, and sampling through a variety of habitats. Other lakes were sampled mostly by larvae (Bazzanti, 1980; Lods-Crozet *et al.*, 1994; Petridis and Sinis, 1995; Specziár, 1998; Smiljkov *et al.*, 2001; Smiljkov *et al.*, 2006; Smiljkov *et al.*, 2008; Rossaro *et al.*, 2012; Tarrats *et al.*, 2017), an exception were pupal exuviae and adult males collected by Bitušík and Trnková, 2019. For the European lacustrine chironomid fauna comparison, I have chosen only lakes where species diversity assessments were well developed. In the perfect scenario, lake selection should be based on lake trophy, presence of similar habitats, biogeography or investigation made based on selected developmental stages. In reality, such comparison could be

possible only based on larvae identification, mostly to genus level with a certain loss of diversity hidden within unseparated genera. I refused this strategy by comparing the lakes based on identifications made to species level for any chironomid developmental stage. Combining literature data obtained by the morphological taxonomy-based approach with the barcoded Chironomidae specimens recorded in BOLD Data System, it may be concluded that also barcoding data for most of the lakes are poor and lakes are not well investigated. A recent study of Theissinger *et al.* (2018), based on metabarcoding analysis of samples collected from seasonal wetlands in Germany, retrieved 30 chironomid species names out of 54 obtained chironomid OTUs based on BOLD database searches. The authors state also that, by applying molecular (metabarcoding) approach, they obtained 70% more chironomid species identifications than would be possible based on a traditional taxonomy-based determination of larval samples. The number of species assessments using Chironomidae molecular data is too low for reliable species diversity comparisons between lakes. For such analysis, the lacustrine fauna needs to be studied in detail. Therefore, the DNA-based determination approaches seem promising to support and complement the taxonomic assessment of chironomid community composition.

As a third aim, in Chapter II, I developed and evaluated the first reference barcode library for Chironomidae from the Skadar Lake basin. Moreover, by using a reference library I estimated the DNA barcoding efficiency for the European Chironomidae based on the barcode library developed in my thesis and on the publicly available BOLD records. Hereafter, I took advantage of DNA-based methods combined with open source databases, which together are considered as the gold standard for biogeographical and taxonomic studies. Implementing those methods greatly helped to rapidly, and cost-effectively, characterize the composition of chironomid fauna in the Lake Skadar basin and resulted in the first DNA barcode reference library of non-biting midges inhabiting the Skadar lake system. Four years after DNA-barcoding invention by Hebert *at al.* (2003), Ekrem *et al.* (2007) predicted that if a species is represented in a DNA sequence library, there is a very good chance for correct identification and pointed that a comprehensive DNA sequence library is essential for identification with DNA barcodes. It may sound trivial, but after 23 years of publication emergence, developing reference library still is and should be, the first and most important step. Moreover, taking into consideration the common and widespread species, there is a great chance for fast verification, if the identification is already correct for numerous records. The problem emerges when we are dealing with rare and not abundant species where

identification made with the morphological taxonomy-based approach and barcoding data are mutually exclusive. In such cases, re-investigation and taxonomic identification made by curator of the data and collection is necessary. The studies enclosed in Chapter II of my thesis confirm this statement. For species well represented by numerous sequences, it is easy to set the optimal thresholds for species delimitation by calculation of intra- and interspecific nucleotide distances. In theory, the optimal thresholds (OT) based on intra-interspecific nucleotide distance are estimated to increase the success of specimen identification from a single dataset (Meyer *et al.*, 2005; Sonet *et al.*, 2013). Thus, optimal thresholds calculated during this thesis for all chironomid subfamilies could be used in further species separation or description of species new for knowledge by using molecular data, like it was done by Giłka *et al.* (2018). Species separation using *adhoc* thresholds estimated on reference library could be a useful tool for more detailed study of diagnostic differences among groups of similar taxa with reverse taxonomy approach. The results of my PhD thesis as well as of literature data provide evidence that optimal thresholds calculated for the developed dataset are more reliable for species recognition than *a priori* fixed thresholds (Montagna *et al.*, 2016a; Meyer *et al.*, 2005, Virgilio *et al.*, 2012; Lin 2015).

Analysing the continuously growing BOLD Chironomidae reference library and publications emerging based on its records, I can conclude that there is a high need for further investigations which will provide more molecular data for non-biting midges as well as higher ranks of invertebrates. By putting more effort into reasonable sampling, every scientist can take benefit of such data. Well-developed and curated reference barcode library, which is also a key point of many worldwide initiatives, will help to resolve the still arising questions about diversity and distribution of many species inhabiting various regions, as well as will help to plan a reasonable and effective strategy preventing or at least minimizing the loss of biodiversity (Brooks *et al.*, 2004). A perfect example of such initiatives might be the International Barcode of Life consortium (iBOL) established in 2008, which provided up to now, more than 8,311,000 barcodes assigned to 222,000 animal species (www.boldsystems.org). iBOL is aiming to extend barcode coverage to 2.5 million species by 2026. Still, a lot needs to be done to complete the non-biting midges reference library. The 15,196 records of European Chironomidae is still a very low number. Despite a few leading institutions, such as NTNU University Museum (Norway), Bavarian State Collection of Zoology (Germany), Museum Alexander Koenig (Germany) and Swedish Museum of Natural History (Sweden) there are many gaps on European map of chironomid barcodes distribution. Based on

BOLD data, most of Chironomidae barcode distribution is centralized in a few areas in northern and central Europe. Certainly, the results of my PhD thesis will enrich our knowledge about the diversity and distribution of Chironomidae in Europe, especially in the southern edge where the data is scarce.

It is hard to predict the origin of the chironomids inhabiting the Skadar Lake basin based on the sequences uploaded so far to BOLD and on their known geographic distribution. The still insufficient number of sequences is distributed between the well-studied European regions and Skadar Lake basin. Interestingly, one question still remains unresolved, why among reported 75 morphospecies with valid names, there were no endemic species? According to Pešić *et al.* (2013), Skadar Lake spring system is characterized by a high degree of endemicity among gastropods, crustaceans, fishes, or other aquatic taxa. The progression of the chironomid knowledge suggests that the distribution area for many species is large and the absence of endemic species cannot be excluded (Rossaro *et al.*, 2019). Since numerous OTUs and BIN clusters remain unassigned to any species, I expect that endemic species may be hidden in molecular diversity. According to another possible scenario, chironomids are considered as weak flyers among insects, with a flight distance restricted to the adult life-span (4 to 8 days), they are able to migrate actively and/or passively (by anemochory) and colonize new habitats, even in a short time (Ferrington, 2008; Armitage *et al.*, 1995). However, despite the weak flying abilities, faunistic and autecological data indicate that most of the species are widespread in each biogeographic areas, such as the Palaearctic (Moller Pillot, 2009). Previous evidence support the hypothesis that 'everything is everywhere', suggesting the prominent role of ecology, in shaping the present species distribution, instead of the geographic barriers. From another side, the Lake Skadar basin together with its large karst spring system is definitely ancient, originated more than 2.5 million years from the present and isolated for most of this time. Some of the karst spring connections are likely to be hundreds of thousands years old and stayed in prolonged isolation, increasing the emergence of divergent and locally endemic lineages or even species (Jablonska *et al.*, 2020)At the same time others, even if isolated now, could be temporarily connected in the past (Grabowski *et al.*, 2018).

Further in-depth studies, based on the molecular data, should be undertaken upon the taxonomy of the local chironomid fauna (and not only) to reveal the actual level of endemism that is probably high, even if still not evidenced by the current but not closed species checklist (Pešić *et al.*, 2018). Further studies, during initial steps, should be focused on sampling developmental stages which

provides the best species-level resolution, such as mature males. It will help to develop reliable reference barcode library, which is fundamental during further assessments. Database coverage often will be incomplete, as there will be much un-barcoded biodiversity, especially in the early stages of barcoding projects. A complete reference barcode library is necessary for accurate identification of all queries, but this goal is very hard to reach considering time and sequencing costs.

Conclusions

The results of my PhD thesis provide the first insight into the factual chironomid species diversity of the Lake Skadar basin, in comparison with chironomid fauna at the European scale. The results fill a significant gap in knowledge of biodiversity in the Balkan region. Based on the results of Chironomidae fauna investigation, I can conclude that the Skadar Lake basin is now well sampled and such a high representation of species from various sampling sites provides reliable estimation of the local chironomid fauna. The collected individuals were assigned to 164 Chironomidae taxa based on a taxonomy-based approach providing insight into species diversity in the Skadar Lake basin. Identified mature males were assigned to 82 correct species-rank names and 95 species based on pupal exuviae identifications. DNA barcoding of larvae and mature males revealed a total of 168 Operational Taxonomic Units which is a higher result than the number of morphotypes obtained during identification based on morphological features. Results presented in the current thesis extending the existing checklist with 152 taxa newly found in the Skadar Lake basin. Based on obtained results it is hard to predict the origin of the chironomids inhabiting the Skadar Lake basin based on the sequences uploaded so far to BOLD and on their known geographic distribution. The still insufficient number of sequences is distributed between the well-studied European regions and Skadar Lake basin. Thus, findings of Chironomidae species diversity indicate that Skadar Lake basin could be a hot-spot of freshwater biodiversity.

Species separation using *adhoc* thresholds estimated on reference library could be a useful tool for more detailed study of diagnostic differences among groups of similar taxa with reverse taxonomy approach. The results of my PhD thesis, provide evidence that optimal thresholds calculated for a developed dataset of sequences are more reliable for species recognition than the *a priori* fixed thresholds. Moreover, the results provide an indicating tool for ambiguous or incorrect identifications. Barcode misidentifications arising from an incomplete reference dataset of species could be flagged and then recognized as misidentifications.

The results of my PhD thesis will enrich our knowledge about the diversity and distribution of Chironomidae in Europe, especially on the Balkan Peninsula. Further in-depth studies, based on the molecular data, should be undertaken upon the taxonomy of the local chironomid fauna (and not only) to reveal the actual level of endemism that is probably high (even if still not evidenced by the current species checklist). Additionally, a comprehensive sampling, in particular

the immature life stages, is required to provide more morphological characters and expand the DNA barcode database for chironomids. Using well-developed reference library will be possible to bind unidentified immature developmental stages with records identified based on integrative taxonomy. By continuous development and lowering the costs of molecular techniques, integrative taxonomy joining morphological and molecular data will be more available for Chironomidae taxonomists. This scenario will definitely provide the scientific community more reliable measures of the species diversity in one of the most abundant and species-rich groups of aquatic insects.

Finally, well-developed and curated reference barcode library which is also a key point of many worldwide initiatives will help to resolve still arising questions about diversity and distribution of many species inhabiting various regions, as well as will help to plan a reasonable and effective strategy preventing or at least minimizing the loss of biodiversity.

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Supplementary materials

Table S1. Environmental parameters and biodiversity indices calculated for pupal exuviae and adult males.

Sampling site	Stadium	Habitat	Sub-strate	Latitude [decimal degrees]	Longitude [decimal degrees]	Alti-tude [m]	pH	Cond-uctivity [µS]	$NH4^+$ [mgl/1]	NO_3^- [mgl/1]	Total phos-phorus [mgl/1]	Water temp. [°C]	$CaCO_3$ [mgl/1]	O_2 [mgl/1]	Year	Month	Day	Shannon (*H'*)	Simp son (D)	Inv-Simp (A)
ASL7	Male	potamal	sand	42.42839	19.29966	59	8.39	4.39	0.127	0.01	0.282	18.1	4.96	9.95	2014	9	30	0	0	1
ASL8	Male	rhithral	stone	42.42833	19.48444	162	8.39	4.39	0.127	0.01	0.282	18.1	4.96	9.95	2014	9	30	0	0	1
ASLP8	Male	Open-lake	silt	42.21	19.21	1	8.56	4.65	0.1	0.05		17.5	4.63		2014	10	1	0	0	1
Dolni.Murici	Male	Lake-litoral	stone	42.16410	19.21903	5	7.94	4.91	0.36	0.01	0.874	30.6	5.23	9.5	2014	10	5	1.7	0.77	4.26
LIM.River	Male	rhitral	stone	42.61888	19.93525	916	8.51	4.89	0.129	0.64	0.029	23		8.95	2015	7	19	2.59	0.89	8.91
Limljani	Male	Open-lake	macrop hytes	42.19476	19.10463	411	chironomidae attracted by light - mountains								2015	7	22	0.91	0.47	1.88
LSLZ2E	Male	rhithral	sand	42.3057	19.05402	11	7.65	2.89	0.147	0.01	0.089	17.9	6.74	8.75	2015	7	19	0.23	0.09	1.1
PL7V14	Male	krenal	sand	42.2725	19.20056	5	7.86		0.085	0.23	0.01	22.6	9.27	10.21	2014	5	7	1.44	0.6	2.53
SSL08_IM	Male	krenal	stone	42.16	19.12	576	8.3	2.76	0.276	0.01	0.01	11.4	6.42	9.42	2014	4	30	0.5	0.32	1.47
SSL09_IM	Male	potamal	silt	42.28	19.15	12	8.32	3.84	0.04	0.3	0.01	12	5.51	10.13	2014	4	30	2.33	0.88	8.22
SSL11	Male	Lake-litoral	stone	42.16306	19.22194	10	8.35	3.99	0.04	0.21	0.664	19	5.16	8.77	2014	4	30	0.68	0.45	1.81
SSL19	Male	potamal	silt	42.32848	19.13381	5	8.65	5.44	0.353	0.01	0.01	14.2		7.84	2014	5	3	1.33	0.69	3.18
SSL20	Male	potamal	silt	42.25	19.13	7	8.2	3.78	0.04	0.01	0.01	13	5.81	8.3	2014	5	5	2.23	0.85	6.86
SSL21	Male	lake-litoral	macrop hytes	42.25222	19.10472	1	8.16	4.54	0.072	0.01	0.017	30.9	8.71	6.86	2014	5	5	2.48	0.91	10.8
SSL22	Male	Lake-litoral	macrop hytes	42.26	19.102	2	7.89	2.78	0.4	0.01	0.031	17	6.81	9023	2014	5	5	1.67	0.79	4.84

Virpazar.boat	Male	Lake-litoral	macrophytes	42.24690	19.09294	9	8.35	4.51	0.04	0.01	0.01	29.8	7.82	7.15	2014	4	27	1.58	0.75	3.93
Virpazar-restaurant	Male	Lake-litoral	macrophytes	42.24614	19.09306	1	8.3	4.49	0.01	0.02	0.01	28	7.83	6.8	2015	7	25	2.16	0.82	5.6
Virpazar.shop	Male	Lake-litoral	macrophytes	42.24683	19.09091	5	8.35	4.51	0.04	0.01	0.01	29.8	7.82	7.15	2014	10	8	2.56	0.9	9.67
Vranjina	Male	potamal	silt	42.27681	19.14695	10	8.32	3.84	0.04	0.3	0.01	12	5.51	10.13	2014	10	9	2.5	0.89	9.06
ASLP1	Exuviae	open.lake	silt	42.21682	19.19444	1	8.09	3.75	0.166	0.01	0.103	20.4	6.2		2014	9	30	2.4	0.86	7.33
ASLP14	Exuviae	open.lake	silt	42.24736	19.22559	1	7.97	3.26	0.102	0.01	0.049	19.1	6.38		2014	10	3	1.31	0.68	3.16
ASLP16	Exuviae	open.lake	silt	42.25861	19.13653	1	8.09	3.27	0.149	0.27	0.496	16.5	6.78		2014	10	3	1.79	0.73	3.68
ASLP3	Exuviae	open.lake	silt	42.21555	19.19890	1	8.35	3.59	0.04	0.01	0.01	20.3	5.57		2014	10	2	2.61	0.88	8.06
ASLP5	Exuviae	open.lake	silt	42.22318	19.20920	1	8.33	3.71	0.04	0.01	0.01	19.9	5.83		2014	10	2	0.64	0.44	1.8
ASLP6	Exuviae	open.lake	silt	42.22758	19.21363	1	8.4	3.99	0.04	0.01	0.118	19	5.03		2014	10	2	0	0	1
ASLP7	Exuviae	open.lake	silt	42.23316	19.21740	1	7.47	3.67	0.061	0.01	0.028	19.3	5.37		2014	10	2	1.22	0.67	3.04
ASLP8	Exuviae	open.lake	silt	42.2024	19.2095	1	8.56	4.65	0.1	0.05		17.5	4.63		2014	10	1	0	0	1
Dolni.Murici	Exuviae	open.lake	stone	42.17	19.23	5	7.94	4.91	0.36	0.01	0.874	30.6	5.23	9.5	2014	10	5	1.99	0.85	6.76
lake.open	Exuviae	open.lake	silt	42.25285	19.12198	1	8.09	3.27	0.149	0.27	0.496	16.5	6.78		2014	10	3	1.14	0.55	2.23
LSLP7	Exuviae	open.lake	silt	42.26805	19.11348	1	8.02	3.61	0.206	0.14	0.01	26.7	1.8	6.28	2015	7	31	0	0	1
Skadar.Lake.1	Exuviae	open.lake	silt	42.21497	19.24290	1	8.24	3.96	0.04	0.01	0.01	19	7.61	9.58	2014	4	30	1.04	0.63	2.67
Skadar.Lake.10	Exuviae	open.lake	silt	42.24954	19.09796	1	8.2	4.51	0.04	0.01	0.01	29.8	7.82	7.15	2015	9	30	2.09	0.78	4.56
Skadar.Lake.11	Exuviae	open.lake	silt	42.27524	19.11911	1	8.37	3.65	0.304	0.1	0.01		1.7	10.51	2014	5	8	0	0	1
Skadar.Lake.3	Exuviae	open.lake	silt	42.23982	19.17993	1	7.64	3.7	0.112	0.01	0.01	28.6	1.6	4.35	2015	7	21	2.05	0.84	6.11
Skadar.Lake.4	Exuviae	open.lake	silt	42.24737	19.12972	1	8.45	3.9	0.04	0.01	0.612	19.7	4.63		2015	7	24	1.04	0.63	2.67
Skadar.Lake.5	Exuviae	open.lake	silt	42.25776	19.10727	1	8.12	2.77	0.03	0.01	0.03	19	5.2	8.63	2015	7	25	2.33	0.83	6.01
Skadar.Lake.6	Exuviae	open.lake	macrophytes	42.27740	19.10950	1	8.37	3.65	0.304	0.1	0.01		1.7	10.51	2015	7	23	3.05	0.93	15.13

Skadar.Lake.7	Exuviae	open.lake	macrophytes	42.29419	19.09589	1	8.14	3.62	0.078	0.01	0.01	28.8	1.3	7.33	2014	5	6	0	0	1
Skadar.Lake.8	Exuviae	open.lake	macrophytes	42.29867	19.08254	1	8.3	3.83	0.04	0.01	0.01	20	5.34	9.28	2014	10	2	0.46	0.22	1.29
Skadar.Lake.9	Exuviae	open.lake	macrophytes	42.30894	19.08253	1	7.96	2.6	0.167	0.01	0.024	24	6.24	7.73	2015	7	22	2.03	0.85	6.54
small.Lake	Exuviae	open.lake	macrophytes	42.30612	19.09944	1	8.03	3.86	0.04	0.23	0.01	17.8	4.88	10.21	2015	7	30	1.56	0.78	4.5
SSL08_PE	Exuviae	krenal	stone	42.15027	19.11777	576	8.3	2.76	0.276	0.01	0.01	11.4	6.42	9.42	2014	4	29	0.5	0.32	1.47
SSL09_PE	Exuviae	potamal	slit	42.26	19.13	10	8.32	3.84	50	0.3	10	12	5.51	10.13	2014	4	30	2.49	0.9	9.89
SSL36	Exuviae	open.lake	silt	42.22861	19.22305	1	8.4	3.99	0.04	0.01	0.118	19	5.03		2014	5	6	2.81	0.91	11.3
SSL49	Exuviae	open.lake	macro-phytes	42.29944	19.10972	1	8.22	3.66	0.216	0.01	0.01	29.5	1.5	7.19	2014	5	8	2.8	0.91	10.58

Table S2. Checklist of species from Skadar Lake basin. Legend: sg. – sub-genus; Pe. – Pupal exuviae; sp. – *species.*

Genus species	Authors	Adult male	Pupal exuviae	Taxon identified based on DNA barcoding	Taxon present in previous research
ABLABESMYIA	Johannsen, 1905				
sg. Ablabesmyia	Johannsen, 1905				
Ablabesmyia (Ablabesmyia) mallochi	(Walley, 1925)	**x**	**x**		
Ablabesmyia (Ablabesmyia) monilis	(Linnaeus, 1758)	**x**	**x**		**x**
Ablabesmyia longistyla	Fittkau, 1962			**x**	
ACRICOTOPUS	Kieffer, 1921				
Acricotopus lucens	(Zetterstedt, 1850)	**x**			
BENTHALIA	Lipina, 1939				
Benthalia carbonaria	(Meigen, 1804)	**x**			
BRILLIA	Kieffer, 1913				
Brillia bifida	(Kieffer, 1909)	**x**	**x**		
Brillia flavifrons	(Johannsen, 1905)		**x**		
BRYOPHAENOCLADIUS	Thienemann, 1934				
Bryophaenocladius illimbatus	(Edwards, 1929)	**x**			
Bryophaenocladius nigrus	Albu, 1974			**x**	
CARDIOCLADIUS	Kieffer, 1912				
Cardiocladius capucinus	(Zetterstedt, 1850)	**x**			
CHAETOCLADIUS	Kieffer, 1911				
sg. Chaetocladius	Kieffer, 1911				
Chaetocladius (Chaetocladius) algericus	Moubayed, 1989	**x**			
Chaetocladius (Chaetocladius) dentiforceps	(Edwards, 1929)		**x**		
Chaetocladius (Chaetocladius) perennis	(Meigen, 1830)	**x**			
CHIRONOMUS	Meigen, 1803				
sg. Chironomus	Meigen, 1803				
Chironomus (Chironomus) alpestris	Goetghebuer, 1934	**x**	**x**		

Chironomus (Chironomus) annularius	Meigen, 1818	**x**	**x**		
Chironomus (Chironomus) anthracinus	Zetterstedt, 1860	**x**	**x**		
Chironomus (Chironomus) cingulatus	Meigen, 1830			**x**	
Chironomus (Chironomus) curabilis	Belyanina, Sigareva & Loginova, 1990			**x**	
Chironomus (Chironomus) plumosus	(Linnaeus, 1758)	**x**	**x**		**x**
Chironomus (Chironomus) prasinus (=?*horni* Kieffer, 1918)	sensu Pinder 1978	**x**	**x**		
Chironomus (Chironomus) pseudomendax	Wulker, 1999			**x**	
Chironomus (Chironomus) pseudothummi	Strenzke, 1959			**x**	
Chironomus (Chironomus) riparius	Meigen, 1804	**x**	**x**		
Chironomus (Chironomus) salinarius	Kieffer in Thienemann, 1915		**x**		
Chironomus (Chironomus) tentans	Fabricius, 1805		**x**		
Chironomus (Chironomus) Pe 4 *(=?balatonicus* Devai, Wülker & Scholl, 1983)	sensu Langton 1991		**x**		
Chironomus (Chironomus) Pe 5	sensu Langton 1991		**x**		
Chironomus (Chironomus) Pe 6	sensu Langton 1991		**x**		
Chironomus (Chironomus) Pe 7	sensu Langton 1991		**x**		
Chironomus (Chironomus) Pe 8	sensu Langton 1991		**x**		
sg. Lobochironomus	Ryser, Wülker & Scholl, 1985				
Chironomus (Lobochironomus) Pe 1	sensu Langton 1991	**x**	**x**		
Chironomus (Lobochironomus) Pe 2	sensu Langton 1991		**x**		
Chironomus (Lobochironomus) Pe 3	sensu Langton 1991		**x**		
CLADOPELMA	Kieffer, 1921				
Cladopelma virescens	(Meigen, 1818)	**x**	**x**		
Cladopelma viridula	Linnaeus, 1767			**x**	
Cladopelma viridulum	(Linnaeus, 1767)	**x**			
CLADOTANYTARSUS	Kieffer, 1921				
sg. Cladotanytarsus	Kieffer, 1921				
Cladotanytarsus atridorsum	Kieffer, 1924	**x**			

Cladotanytarsus mancus aggr.	sensu Giłka 2001	**x**	**x**		
CLINOTANYPUS	Kieffer, 1913				
Clinotanypus nervosus	(Meigen, 1818)	**x**	**x**		**x**
CONCHAPELOPIA	Fittkau, 1957				
Conchapelopia pallidula	(Meigen, 1818)	**x**	**x**		
CORYNONEURA	Winnertz, 1846				
Corynoneura edwardsi	Brundin, 1949	**x**	**x**		
Corynoneura gratias	Schlee, 1968	**x**			
CRICOTOPUS	van der Wulp, 1874				
sg. Cricotopus	van der Wulp, 1874				
Cricotopus (Cricotopus) annulator	Goetghebuer, 1927		**x**		
Cricotopus (Cricotopus) bicinctus	(Meigen, 1818)	**x**	**x**		
Cricotopus (Cricotopus) Pe 1	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 3	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 5	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 6	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 7	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 9	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 10	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 11	sensu Langton 1991		**x**		
Cricotopus (Cricotopus) Pe 12	sensu Langton 1991		**x**		
Cricotopus fuscus	(Kieffer, 1909)			**x**	
Cricotopus relucens	Hirvenoja, 1973			**x**	
Cricotopus trifasciatus	(Meigen in Panzer, 1813)			**x**	
sg. Isocladius	Kieffer, 1909				
Cricotopus (Isocladius) sylvestris	(Fabricius, 1794)	**x**	**x**		
Cricotopus (Isocladius) Pe 4	sensu Langton 1991		**x**		
Cricotopus (Isocladius) Pe 8	sensu Langton 1991		**x**		
sg. Paratrichocladius	Santos Abreu, 1918				
Cricotopus (Paratrichocladius) rufiventris	(Meigen, 1830)	**x**	**x**		
Cricotopus (Paratrichocladius) skirwithensis	(Edwards, 1929)		**x**		

CRYPTOCHIRONOMUS	Kieffer, 1918				
Cryptochironomus albofasciatus	(Stäger, 1839)	**x**	**x**		
Cryptochironomus rostratus	Kieffer, 1921	**x**			
Cryptochironomus supplicans	(Meigen, 1830)		**x**		
CRYPTOTENDIPES	Beck & Beck, 1969				
Cryptotendipes pseudotener	(Goetghebuer, 1922)		**x**		
DICROTENDIPES	Kieffer, 1913				
Dicrotendipes lobiger	(Kieffer, 1921)	**x**	**x**		
Dicrotendipes nervosus	(Stäger, 1839)	**x**	**x**		
Dicrotendipes notatus	(Meigen, 1818)		**x**		
Dicrotendipes pulsus	(Walker, 1856)	**x**			
Dicrotendipes sp.	as for the present paper		**x**		
ENDOCHIRONOMUS	Kieffer, 1918				
Endochironomus albipennis	(Meigen, 1830)	**x**	**x**		**x**
Endochironomus tendens	(Fabricius, 1775)	**x**	**x**		**x**
EUKIEFFERIELLA	Thienemann, 1926				
Eukiefferiella brevicalcar	(Kieffer, 1911)		**x**		**x**
Eukiefferiella claripennis	(Lundbeck, 1898)		**x**		
Eukiefferiella clypeata	(Thienemann, 1919)	**x**	**x**		
FLEURIA	Kieffer, 1924				
Fleuria lacustris	Kieffer, 1924		**x**		
GLYPTOTENDIPES	Kieffer, 1913				
sg. Caulochironomus	Heyn, 1993				
Glyptotendipes (Caulochironomus) foliicola	sensu Contreras-Lichtenberg 1997		**x**		
sg. Glyptotendipes	Kieffer, 1913				
Glyptotendipes (Glyptotendipes) pallens	(Meigen, 1804)	**x**	**x**		
sg. Heynotendipes	Spies & Sæther, 2004				
Glyptotendipes (Heynotendipes) signatus	(Kieffer, 1909)		**x**		
GUTTIPELOPIA	Fittkau 1962				
Guttipelopia guttipennis	(Wulp, 1874)			**x**	

HARNISCHIA	Kieffer, 1921				
Harnischia curtilamellata	(Malloch, 1915)		**x**		
KIEFFERULUS	Goetghebuer, 1922				
Kiefferulus tendipediformis	(Goetghebuer, 1921)	**x**	**x**		
Kiefferulus sp.	as for the present paper		**x**		
KLOOSIA	Kruseman, 1933				
Kloosia pusilla	(Linnaeus, 1767)		**x**		
KRENOPELOPIA	Fittkau, 1962				
Krenopelopia binotata	(Wiedemann, 1817)	**x**	**x**		
LIMNOPHYES	Eaton, 1875				
Limnophyes minimus	(Meigen, 1818)	**x**			
Limnophyes natalensis	(Kieffer, 1914)	**x**			
Limnophyes pentaplastus	(Kieffer, 1921)			**x**	
MICROCHIRONOMUS	Kieffer, 1918				
Microchironomus deribae	(Freeman, 1957)		**x**		
Microchironomus tener	(Kieffer, 1818)	**x**	**x**		
MICROPSECTRA	Kieffer, 1908				
Micropsectra nana	(Meigen, 1818)		**x**		
Micropsectra lindrothi	Goetghebuer, 1931			**x**	
MICROTENDIPES	Kieffer, 1915				
Microtendipes chloris	(Meigen, 1818)			**x**	
Microtendipes pedellus	(De Geer, 1776)	**x**			**x**
NANOCLADIUS	Kieffer, 1913				
Nanocladius dichromus	(Kieffer, 1906)		**x**		
ORTHOCLADIUS	van der Wulp, 1874				
sg. Euorthocladius	Thienemann, 1935				
Orthocladius (Euorthocladius) luteipes	Goetghebuer, 1938	**x**	**x**		
Orthocladius (Euorthocladius) rivicola	Kieffer, 1911	**x**			
sg. Orthocladius	van der Wulp, 1874				
Orthocladius (Orthocladius) abiskoensis	Thienemann & Krüger, 1937	**x**	**x**		

Orthocladius (Orthocladius) excavatus	Brundin, 1947	**x**	**x**	
Orthocladius (Orthocladius) glabripennis	(Goetghebuer, 1921)		**x**	
Orthocladius (Orthocladius) oblidens	(Walker, 1856)	**x**	**x**	
Orthocladius (Orthocladius) pedestris	Kieffer, 1909	**x**		
Orthocladius (Orthocladius) rubicundus	(Meigen, 1818)	**x**	**x**	
PAGASTIELLA	Brundin, 1949			
Pagastiella orophila	(Edwards, 1929)		**x**	
PARACHAETOCLADIUS	Wülker, 1959			
Parachaetocladius abnobaeus	(Wülker, 1959)	**x**		
PARACHIRONOMUS	Lenz, 1921			
Parachironomus biannulatus	Staeger, 1839	**x**	**x**	
Parachironomus gracilior	(Kieffer, 1918)		**x**	
Parachironomus monochromus	(van der Wulp, 1874)		**x**	
Parachironomus varus	(Goetghebuer, 1921)		**x**	
Parachironomus sp.	as for the present paper		**x**	
PARATANYTARSUS	Thienemann & Bause, 1913			
Paratanytarsus abiskoensis	Reiss & Säwedal, 1981	**x**	**x**	
Paratanytarsus bituberculatus	(Edwards, 1929)	**x**	**x**	
Paratanytarsus dissimilis	(Johannsen, 1905)	**x**	**x**	
Paratanytarsus grimmii	(Schneider, 1885)		**x**	
Paratanytarsus inopertus	(Walker, 1856)	**x**	**x**	
Paratanytarsus laetipes	(Zetterstedt, 1850)	**x**	**x**	
Paratanytarsus lauterborni	(Kieffer, 1909)	**x**	**x**	**x**
Paratanytarsus natvigi	(Goetghebuer, 1933)	**x**		
Paratanytarsus penicillatus	(Goetghebuer, 1928)		**x**	
Paratanytarsus tenellulus	(Goetghebuer, 1921)	**x**		
Paratanytarsus sp.	as for the present paper		**x**	
PARATENDIPES	Kieffer, 1911			

Paratendipes albimanus	(Meigen, 1818)	**x**			**x**
Phaenopsectra punctipes	(Wiedemann, 1817)			**x**	
PHAENOPSECTRA	Kieffer, 1921				
Phaenopsectra flavipes	(Meigen, 1818)	**x**			
POLYPEDILUM	Kieffer, 1912				
sg. Pentapedilum	Kieffer, 1913				
Polypedilum (Pentapedilum) sordens	(van der Wulp, 1874)	**x**	**x**		
sg. Polypedilum	Kieffer, 1912				
Polypedilum (Polypedilum) laetum	(Meigen, 1818)	**x**	**x**		
Polypedilum (Polypedilum) nubeculosum	(Meigen, 1804)	**x**	**x**		**x**
sg. Tripodura	Townes, 1945				
Polypedilum (Tripodura) apfelbecki	(Strobl, 1900)		**x**		
Polypedilum (Tripodura) bicrenatum	Kieffer, 1921		**x**		
Polypedilum (Tripodura) scalaenum	(Schrank, 1803)	**x**	**x**		**x**
Polypedilum (Tripodura) tetracrenatum	Hirvenoja, 1962		**x**		
sg. Uresipedilum	Oyewo & Sæther,1998				
Polypedilum (Uresipedilum) convictum	(Walker, 1856)		**x**		**x**
Polypedilum (Uresipedilum) cultellatum	Goetghebuer, 1931			**x**	
Polypedilum Pe 5	sensu Langton 1991		**x**		
Polypedilum Pe 7	sensu Langton 1991		**x**		
Polypedilum Pe 9	sensu Langton 1991		**x**		
Polypedilum Pe 1	sensu Ferrarese in Langton 1991		**x**		
PROCLADIUS	Skuse, 1889				
sg. Holotanypus	Roback, 1982				
Procladius crassinervis	(Zetterstedt, 1838)			**x**	
Procladius culiciformis	(Linnaeus, 1767)			**x**	
Procladius (Holotanypus) choreus	(Meigen, 1804)	**x**	**x**		
Procladius (Holotanypus) denticulatus	Sublette, 1964		**x**		

Procladius (Holotanypus) sagittalis	(Kieffer, 1909)		**x**	
Procladius sp.	as for the present paper		**x**	
PSECTROCLADIUS	Kieffer, 1906			
sg. Psectrocladius	Kieffer, 1906			
Psectrocladius (Psectrocladius) limbatellus	(Holmgren, 1869)		**x**	
Psectrocladius (Psectrocladius) psilopterus	(Kieffer, 1906)		**x**	
Psectrocladius (Psectrocladius) sordidellus	(Zetterstedt, 1838)		**x**	
PSEUDOSMITTIA	Edwards, 1932			
Pseudosmittia trilobata	(Edwards, 1929)	**x**		
RHEOTANYTARSUS	Thienemann & Bause, 1913			
Rheotanytarsus curtistylus	(Goetghebuer, 1921)		**x**	
Rheotanytarsus sp.	as for the present paper		**x**	
RHEOCRICOTOPUS	Thienemann & Harnisch, 1932			
Rheocricotopus chalybeatus	(Edwards, 1929)			**x**
SERGENTIA	Kieffer, 1922			
Sergentia sp.	as for the present paper	**x**		
SMITTIA	Holmgren, 1869			
Smittia abiskoensis	Goetghebuer, 1940		**x**	
STEMPELLINA	Thienemann & Bause, 1913			
Stempellina bausei	(Kieffer, 1911)	**x**	**x**	
STENOCHIRONOMUS	Kieffer, 1919			
Stenochironomus sp.	as for the present paper	**x**	**x**	
Stenochironomus gibbus	(Fabricius, 1794)			**x**
STICTOCHIRONOMUS	Kieffer, 1919			
Stictochironomus pictulus	(Meigen, 1830)			**x**
Stictochironomus sticticus	(Fabricius, 1781)		**x**	
Stictochironomus sp.	as for the present paper	**x**		

SYNENDOTENDIPES	Grodhaus, 1987		
Synendotendipes impar	(Walker, 1856)	**x**	
Synendotendipes lepidus	(Meigen, 1830)	**x**	
TANYPUS	Meigen, 1803		
sg. Tanypus	Meigen, 1803		
Tanypus (Tanypus) punctipennis	Meigen, 1818	**x**	**x**
Tanypus sp.	as for the present paper		**x**
TANYTARSUS	van der Wulp, 1874		
Tanytarsus brundini	Lindeberg, 1963	**x**	**x**
Tanytarsus chinyensis	Goetghebuer, 1934	**x**	**x**
Tanytarsus cretensis	Reiss, 1987		**x**
Tanytarsus ejuncidus	(Walker, 1856)	**x**	**x**
Tanytarsus eminulus	(Walker, 1856)	**x**	**x**
Tanytarsus excavatus	Edwards, 1929	**x**	
Tanytarsus inaequalis	Goetghebuer, 1921	**x**	
Tanytarsus lactescens	Edwards, 1929	**x**	
Tanytarsus mendax	Kieffer, 1925	**x**	
Tanytarsus miriforceps	(Kieffer, 1921)		**x**
Tanytarsus striatulus	Lindeberg, 1976	**x**	
Tanytarsus usmaensis	Pagast, 1931	**x**	**x**
Tanytarsus volgensis	Miseiko, 1967		**x**
Tanytarsus Pe 1	sensu Langton 1991		**x**
Tanytarsus Pe 14	sensu Langton 1991		**x**
Tanytarsus Pe 15	sensu Langton 1991		**x**
Tanytarsus Pe 4	sensu Langton 1991		**x**
Tanytarsus sp.	as for the present paper		**x**
THALASSOMYA	Schiner, 1856		
Thalassomya frauenfeldi	Schiner, 1856	**x**	**x**
THIENEMANNIELLA	Kieffer, 1911		
Thienemanniella sp.	as for the present paper		**x**
THIENEMANNIMYIA	Fittkau, 1957		

Thienemannimyia laeta	(Meigen, 1818)		**x**
TVETENIA	Kieffer, 1922		
Tvetenia calvescens	(Edwards, 1929)	**x**	**x**
XENOCHIRONOMUS	Kieffer, 1921		
Xenochironomus xenolabis	(Kieffer, 1916)		**x**

Table S3. Median values of species diversity indices for adult males and pupal exuviae.

Habitat type	Adult males			Pupal exuviae		
	Shannon (H')	**Simpson (D)**	**Invsimp (A)**	**Shannon (H')**	**Simpson (D)**	**Invsimp (A)**
lake litoral	1.7	0.79	4.84	-	-	-
open lake	0.455	0.235	1.44	1.435	0.705	3.42
krenal	0.97	0.46	2	0.5	0.16	0.735
potamal	2.23	0.85	6.86	2.49	0.9	9.89
rhitral	0.23	0.09	1.1	-	-	-
stone	0.68	0.45	1.81	1.245	0.585	4.115
macrophytes	1.915	0.805	5.22	1.795	0.815	5.52
sand	0.23	0.09	1.1	-	-	-
silt	2.23	0.85	6.86	1.265	0.675	3.1

Table S4. RDA species scores for adult males and pupal exuviae.

Species	Life stage	RDA1	RDA2
P.P.nubeculosum	Adult male	1.1352	-0.3035
C.I.sylvestris	Adult male	0.8599	-0.0369
P.flavipes	Adult male	0.2942	-0.2432
P.H.choreus	Adult male	0.6935	0.8433
C.C.bicinctus	Adult male	-0.0681	-0.2843
C.C.plumosus	Adult male	0.0871	-0.001
C.C.prasinus	Adult male	0.1189	0.0311
C.C.riparius	Adult male	0.8954	0.0231
Chironomus.Pe.1	Adult male	0.4794	0.0861
P.dissimilis	Adult male	0.4013	-0.2835
P.natvigi	Adult male	0.8698	-0.1244
C.C.alpestris	Pupal exuviae	0.4371	0.0983
C.C.plumosus	Pupal exuviae	0.7529	0.5868
P.bituberculatus	Pupal exuviae	0.3981	-0.1998
C.mancus	Pupal exuviae	0.3108	-0.2491
Chironomus.Pe.1	Pupal exuviae	-0.0491	0.1256
D.nervosus	Pupal exuviae	0.5099	-0.0006
P.laetipes	Pupal exuviae	0.5191	-0.2397
C.albofasciatus	Pupal exuviae	0.0468	0.2473
Chironomus.Pe.3	Pupal exuviae	-0.0219	0.3457
M.tener	Pupal exuviae	0.2048	-0.1243
P.H.choreus	Pupal exuviae	0.3618	-0.1544
P.P.laetum	Pupal exuviae	0.1793	0.0017
P.P.psilopterus	Pupal exuviae	0.3274	-0.1591
T.usmaensis	Pupal exuviae	0.3508	-0.1933

Table S5. RDA and PCA eigenvalues with the percentage of variance explained by constrained (RDA) and unconstrained (PCA) axes.

	Adult males				Pupal exuviae			
	RDA1	**RDA2**	**PCA1**	**PCA2**	**RDA1**	**RDA2**	**PCA1**	**PCA2**
Eigenvalue	2.8987	0.6655	0.5348	0.1456	0.6244	0.2557	0.2912	0.1324
Proportion explained	0.5149	0.1182	0.095	0.0259	0.2998	0.1227	0.1398	0.0636
Cumulative proportion	0.5149	0.6331	0.9741	1	0.2998	0.4225	0.8142	0.8778

Table S6. Species list from previous research. Legend: gr. – from a group; sp. – *species;* agg. – species aggregate; cf. – *confer* – to compare, to be compared with.

Genus	Species	Reference
***ABLABESMYIA* Johannsen, 1905**		
	Ablabesmyia monilis (Linnaeus, 1758)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***CHIRONOMUS* Meigen, 1803**		
	Chironomus plumosus (Linnaeus, 1758)	Janković M. 1974. Dejstvo ekoloških faktora rasprostranjenje dominatnih vrsta Chironomidae u Skadarskom Jezeru
	Chironomus plumosus (Linnaeus, 1758)	Jacobi (1977; 1981), Zoobenthos from sublacustrine springs in Lake Skadar. The Biota And Limnology of Lake Skadar. Univerzitet "Veljko Vlahović" Institut Za Biološka I Medicinska Istraživanja u SRCG, Biološki Zavod Titograd, Yugoslavia. 251-263
	Chironomus semireductus (Lenz, 1924)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
	Chironomus semireductus (Lenz, 1924)	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
	Chironomus sp.	Jacobi (1977; 1981), Zoobenthos from sublacustrine springs in Lake Skadar. The Biota And Limnology of Lake Skadar. Univerzitet "Veljko Vlahović" Institut Za Biološka I Medicinska Istraživanja u SRCG, Biološki Zavod Titograd, Yugoslavia. 251-263
	Chironomus sp.	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***CLINOTANYPUS* Kieffer, 1913**		
	Clinotanypus nervosus (Meigen, 1818)	Janković M. 1974. Dejstvo ekoloških faktora rasprostranjenje dominatnih vrsta Chironomidae u Skadarskom Jezeru
	Clinotanypus nervosus (Meigen, 1818)	Jacobi (1977; 1981), Zoobenthos from sublacustrine springs in Lake Skadar. The Biota And Limnology of Lake Skadar. Univerzitet "Veljko Vlahović" Institut Za Biološka I Medicinska Istraživanja u SRCG, Biološki Zavod Titograd, Yugoslavia. 251-263
	Clinotanypus nervosus (Meigen, 1818)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
	Clinotanypus nervosus (Meigen, 1818)	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
***CRYPTOCHIRONOMUS* Kieffer, 1918**		

Cryptochironomus from gr. *conjugens* (Kieffer, 1921)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Cryptochironomus from gr. *defectus* (Kieffer, 1913)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Cryptochironomus from gr. *camptolabis* (Kieffer, 1913)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
***DIAMESA* Meigen, 1835**	
Diamesa sp.	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***ENDOCHIRONOMUS* Kieffer, 1918**	
Endochironomus albipennis (Meigen, 1830)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
Endochironomus from gr. *signaticornis* (Kieffer, 1913)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Endochironomus from gr. *tendens* (Fabricius, 1775)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
***EUKIEFFERIELLA* Thienemann, 1926**	
Eukiefferiella brevicalcar (Kieffer, 1911)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
Eukiefferiella ilkleyensis-type	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***NEOZAVRELIA* Goetghebuer & Thienemann, 1941**	
Neozavrelia sp.	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***LAUTERBORNIELLA* Thienemann & Bause, 1913**	
Lauterborniella brachylabis Edwards, 1929	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Lauterborniella brachylabis Edwards, 1929	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
***DICROTENDIPES* Kieffer, 1913 = Limnochironomus Kieffer, 1920**	
Limnochironomus from gr. *tritomus* Kieffer 1916	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
***MICROTENDIPES* Kieffer, 1915**	

Microtendipes pedellus agg. – sensu Moller Pillot 1984	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***MICROPSECTRA* Kieffer, 1909**	
Micropsectra type A – sensu Brooks *et al*. 2007	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***ORTHOCLADIUS* van der Wulp, 1874**	
sg. *Orthocladius* van der Wulp, 1874	
Orthocladius type S – sensu Brooks *et al*. 2007	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
sg. *Euorthocladius* Thienemann, 1935	
Orthocladius rivulorum Kieffer, 1909	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***PARATENDIPES* Kieffer, 1911**	
Paratendipes albimanus (Meigen, 1818)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***POLYPEDILUM* Kieffer, 1912**	
sg. *Pentapedilum* Kieffer, 1913	
Pentapedilum exsectum (Kieffer, 1916)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Pentapedilum exsectum (Kieffer, 1916)	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
Polypedilum breviantennatum Chernovskij, 1949	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
Polypedilum breviantennatum Chernovskij, 1949	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
sg. *Uresipedilum* Sasa & Kikuchi, 1995	
Polypedilum convictum (Walker, 1856)	Jacobi (1977; 1981), Zoobenthos from sublacustrine springs in Lake Skadar. The Biota And Limnology of Lake Skadar. Univerzitet "Veljko Vlahović" Institut Za Biološka I Medicinska Istrażivanja u SRCG, Biološki Zavod Titograd, Yugoslavia. 251-263
Polypedilum convictum (Walker, 1856)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.

Polypedilum from gr. *convictum* (Walker, 1856)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
sg. *Polypedilum* Kieffer, 1912	
Polypedilum nubeculosum - type sensu Brooks *et al.* 2007	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
Polypedilum from gr. *nubeculosum* (Meigen, 1804)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
sg. *Tripodura* Townes, 1945	
Polypedilum from gr. *scalenum* (Schrank, 1803)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
***POTHASTIA* Kieffer, 1922**	
Potthastia gaedii (Meigen, 1838)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***PROCLADIUS* Skuse, 1889**	
Procladius sp.	Jacobi (1977; 1981), Zoobenthos from sublacustrine springs in Lake Skadar. The Biota And Limnology of Lake Skadar. Univerzitet "Veljko Vlahović" Institut Za Biološka I Medicinska Istraživanja u SRCG, Biološki Zavod Titograd, Yugoslavia. 251-263
Procladius sp.	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Procladius sp.	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***PRODIAMESA* Kieffer, 1906**	
Prodiamesa olivacea (Meigen, 1818)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
Prodiamesa olivacea (Meigen, 1818)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Prodiamesa olivacea (Meigen, 1818)	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
***PARATANYTARSUS* Thienemann & Bause, 1913**	
Paratanytarsus lauterborni (Kieffer, 1909)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***PARATENDIPES* Kieffer, 1911**	

Paratendipes albimanus (Meigen, 1818)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***STICTOCHIRONOMUS* Kieffer, 1919**	
Stictochironomus from gr. *historio* Fabricius	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
***RHEOCRICOTOPUS* Brundin, 1956**	
Rheocricotopus cf. *effusus* (Walker, 1856)	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.
***TANYTARSUS* van der Wulp, 1874**	
Tanytarsus from gr. *gregarius* Kieffer, 1909	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Tanytarsus from gr. *mancus* (Walker, 1856)	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
***TRISSOCLADIUS* Kieffer, 1908**	
Trissocladius griseipennis Goetghebuer, 1913 = *Hydrobaenus lugubris* Fries, 1830	Nedeljković (1959), Karaman 1981; Skadarsko Jezero: studija organske produkcije u jednom krasnom jezeru. Posebno izd. Biol. Inst. 4: 1-156, Beograd.
Trissocladius griseipennis Goetghebuer, 1913 = *Hydrobaenus lugubris* Fries, 1830	Pulevic V., Hadžiablahovic S., Kasom G., RakocevicNedovic J., Nikcevic S., Pešic V., Ražnatovic A., Cirovic R., Saveljic D., Buškovic V., Dhimitër D., Lefter K., Fatbrdh S., Idriz H., Taulant B., Ferdinand B., Rrok S., Marash R. 2001. BIODIVERSITY DATABASE OF THE SHKODRA/SKADAR LAKE - checklist of species. Project: "Promotion of networks and exchanges in the countries of the South Eastern Europe." THE REGIONAL ENVIRONMENTAL CENTER for Central and Eastern Europe.
***TVETENIA* Kieffer, 1922**	
Tvetenia sp.	Plociennik and Pesić 2012, New records and list of non-biting midges (Chironomidae) from Montenegro. Biologia Serbica 34(1-2): 36-50.

Table S7. Table with a list of species and number of correct, incorrect and ambiguous identifications.

Species	*Best Close Match* Identification			
	correct	**incorrect**	**ambiguous**	**no ID**
Aagaardia protensa				1
Ablabesmyia aspera	3			1
Ablabesmyia longistyla	8			2
Ablabesmyia monilis	37			
Acamptocladius submontanus				1
Acricotopus lucens	10			
Allocladius bothnicus				1
Allocladius nanseni				1
Anatopynia plumipes	5			
Apsectrotanypus trifascipennis	6			
Arctopelopia barbitarsis	11			
Arctopelopia griseipennis	3			
Arctopelopia melanosoma	8			
Brillia bifida	9			4
Brillia longifurca	4			1
Bryophaenocladius aestivus	2			1
Bryophaenocladius dentatus	3			1
Bryophaenocladius flavoscutellatus	13			
Bryophaenocladius flexidens	20			
Bryophaenocladius ictericus	55			
Bryophaenocladius inconstans	3			
Bryophaenocladius nigrus				1
Bryophaenocladius nitidicollis	3			
Bryophaenocladius propinquus	2			
Bryophaenocladius scanicus				3
Bryophaenocladius subparallelus	5			
Bryophaenocladius subvernalis	2			
Camptocladius stercorarius	8			

Cardiocladius capucinus	2		2
Chaetocladius crassisaetosus	2		
Chaetocladius dissipatus	7		
Chaetocladius elisabethae			2
Chaetocladius gracilis			1
Chaetocladius grandilobus	5		
Chaetocladius holmgreni	42		
Chaetocladius incertus	12		2
Chaetocladius laminatus			1
Chaetocladius longivirgatus	9		
Chaetocladius melaleucus	7		
Chaetocladius minutissimus			1
Chaetocladius perennis	29		1
Chaetocladius piger	7		
Chaetocladius suecicus	10		
Chaetocladius tenuistylus			1
Chironomus acerbus			1
Chironomus annularius	13		3
Chironomus aprilinus	16		
Chironomus cingulatus			3
Chironomus curabilis	6		
Chironomus heteropilicornis	2	3	
Chironomus hyperboreus	11		
Chironomus longistylus	4		2
Chironomus lugubris	9		
Chironomus luridus	7		1
Chironomus melanescens			1
Chironomus melanotus			1
Chironomus mendax			1
Chironomus obtusidens			1
Chironomus pallidivittatus	11		
Chironomus pilicornis		2	

Chironomus plumosus	23			1
Chironomus pseudomendax	4			
Chironomus pseudothummi	16		14	2
Chironomus riparius	131	1		1
Chironomus salinarius	3			
Chironomus sollicitus				1
Chironomus storai	2			
Chironomus tentans	2	2		
Cladopelma virescens	14			
Cladopelma viridula				2
Cladopelma viridulum	7			2
Cladotanytarsus amandus				1
Cladotanytarsus atridorsum	18			2
Cladotanytarsus cyrylae				1
Cladotanytarsus difficilis	3			1
Cladotanytarsus gedanicus	10			2
Cladotanytarsus iucundus	6	1		
Cladotanytarsus mancus	23			7
Cladotanytarsus nigrovittatus	5			
Cladotanytarsus pallidus		1		1
Cladotanytarsus vanderwulpi				1
Cladotanytarsus wexionensis	2			
Clinotanypus nervosus	2			1
Conchapelopia hittmairorum	2			
Conchapelopia melanops	8			
Conchapelopia pallidula				2
Constempellina brevicosta	11			4
Corynoneura arctica	3			1
Corynoneura carriana	8			
Corynoneura edwardsi	6			
Corynoneura fittkaui	13			1
Corynoneura gratias	6			3

Corynoneura lacustris	8			
Corynoneura lobata	19			2
Corynoneura scutellata	2			
Corynoneurella paludosa	3			
Cricotopus albiforceps	2			
Cricotopus annulator	21			
Cricotopus beckeri	2			3
Cricotopus bicinctus	48			6
Cricotopus brevipalpis	2			
Cricotopus caducus	7			
Cricotopus coronatus	2			
Cricotopus festivellus	11			
Cricotopus fuscus	2			1
Cricotopus gelidus	2			1
Cricotopus glacialis	14	3	4	
Cricotopus intersectus	13			
Cricotopus laricomalis	5			3
Cricotopus lestralis	2			
Cricotopus magus	2			
Cricotopus maurii	2			
Cricotopus nivalis	20			1
Cricotopus obnixus	2			
Cricotopus ornatus	15			2
Cricotopus osellai	13			1
Cricotopus pallidipes				1
Cricotopus patens				1
Cricotopus perniger				1
Cricotopus pilitarsis	8			
Cricotopus pilosellus	8			2
Cricotopus polaris	8			2
Cricotopus pulchripes	3			1
Cricotopus relucens	2	2		1

Cricotopus reversus				3
Cricotopus rufiventris	257		11	
Cricotopus similis	241			
Cricotopus skirwithensis	406			
Cricotopus sylvestris	40	3	10	
Cricotopus tibialis	83			
Cricotopus tremulus	158			
Cricotopus triannulatus	8			
Cricotopus trifascia	11			1
Cricotopus trifasciatus	4			1
Cricotopus vierriensis				1
Cricotopus villosus				1
Cryptochironomus albofasciatus				2
Cryptochironomus redekei	4			
Cryptochironomus rostratus	2			
Cryptochironomus supplicans	15			2
Demeijerea rufipes	2			
Demicryptochironomus vulneratus				2
Diamesa aberrata	47			2
Diamesa arctica	18			1
Diamesa bertrami	39			
Diamesa bohemani	34	1	4	
Diamesa hyperborea	25		8	
Diamesa incallida	4			
Diamesa insignipes	238			
Diamesa latitarsis	20			1
Diamesa lavillei				1
Diamesa lindrothi	11			
Diamesa saetheri	5			
Diamesa serratosioi	11			
Diamesa starmachi				1
Diamesa tonsa			5	

Diamesa zernyi		1	3	
Dicrotendipes lobiger	11			
Dicrotendipes modestus		1	4	
Dicrotendipes nervosus	17			1
Dicrotendipes pulsus	7	1	3	
Diplocladius cultriger	4			2
Einfeldia pagana	4			
Einfeldia synchrona	3			1
Endochironomus albipennis				2
Endochironomus tendens	13			
Eukiefferiella brevicalcar	14			
Eukiefferiella claripennis	39			
Eukiefferiella coerulescens				1
Eukiefferiella devonica	7			1
Eukiefferiella dittmari	3			1
Eukiefferiella ilkleyensis	19			
Eukiefferiella minor	113			
Eukiefferiella pseudomontana				1
Eukiefferiella tirolensis				1
Glyptotendipes barbipes	7			
Glyptotendipes cauliginellus	9			2
Glyptotendipes imbecillis	2			1
Glyptotendipes lobiferus				2
Glyptotendipes pallens	19			2
Glyptotendipes signatus	2			1
Guttipelopia guttipennis	12			
Gymnometriocnemus autumnalis	3			
Gymnometriocnemus brevitarsis	5			
Gymnometriocnemus brumalis	37			3
Gymnometriocnemus kamimegavirgus	160	1	147	
Gymnometriocnemus marionensis				1
Gymnometriocnemus pallidus	195			

Gymnometriocnemus subnudus	213			1
Gymnometriocnemus volitans	2	3	5	
Halocladius fucicola	4			
Halocladius variabilis	38			
Halocladius varians	3			1
Heleniella ornaticollis	34			
Heterotanytarsus apicalis	30			
Heterotrissocladius brundini		1		
Heterotrissocladius grimshawi	4	1	6	
Heterotrissocladius marcidus	6		20	
Heterotrissocladius subpilosus	3			1
Heterotrissocladius zierli				1
Hydrobaenus conformis	17			
Hydrobaenus lapponicus	2			
Hydrosmittia oxoniana	9			1
Hydrosmittia ruttneri	7			
Kiefferulus tendipediformis	14			
Krenopsectra acuta		1		
Krenosmittia boreoalpina				1
Krenosmittia camptophleps	5			
Krenosmittia halvorseni	4			
Larsia atrocincta				1
Lasiodiamesa sphagnicola				1
Lauterborniella agrayloides				2
Limnophyes aagaardi	12			
Limnophyes asquamatus	27			1
Limnophyes bidumus	71			1
Limnophyes brachytomus	42			
Limnophyes difficilis	10			
Limnophyes edwardsi	25			
Limnophyes eltoni	12			
Limnophyes habilis	609			

Limnophyes madeirae			1
Limnophyes minimus	949		4
Limnophyes natalensis	36		6
Limnophyes pentaplastus	20		1
Limnophyes pumilio	57		2
Limnophyes schnelli	10		
Limnophyes vrangelensis			1
Macropelopia fittkau			1
Macropelopia nebulosa	11		1
Macropelopia notata	15		
Mesocricotopus thienemanni			1
Mesosmittia flexuella			1
Metriocnemus acutus			2
Metriocnemus albolineatus	57		1
Metriocnemus atriclava	2		
Metriocnemus beringensis			1
Metriocnemus brusti	25		1
Metriocnemus caudigus	3		
Metriocnemus eurynotus	188		3
Metriocnemus fuscipes	77		1
Metriocnemus intergerivus	5		
Metriocnemus picipes	190		
Metriocnemus tristellus			1
Metriocnemus ursinus	29		
Microchironomus tener	18		
Micropsectra acuta		1	
Micropsectra appendica	11		
Micropsectra atrofasciata	5		2
Micropsectra attenuata	7		
Micropsectra borealis	4		
Micropsectra chionophila	5		
Micropsectra contracta	13		

Micropsectra insignilobus	15			2
Micropsectra junci	36	1		3
Micropsectra klinki				1
Micropsectra lacustris	24			
Micropsectra lindrothi	14			
Micropsectra logani	87			1
Micropsectra nana	53			
Micropsectra notescens	9			2
Micropsectra pallidula	16			
Micropsectra pharetrophora	3			
Micropsectra radialis	41			
Micropsectra recurvata	17			
Micropsectra roseiventris	10			1
Micropsectra schrankelae	7			
Micropsectra seguyi	2			
Micropsectra sofiae	8			2
Micropsectra styriaca				1
Micropsectra uliginosa		1		
Microtendipes brevitarsis	15	1	1	1
Microtendipes chloris	2			1
Microtendipes confinis	5			
Microtendipes nigellus	2			
Microtendipes pedellus	5		2	1
Monodiamesa bathyphila	2			
Monopelopia tenuicalcar				2
Nanocladius dichromus	8	1		7
Nanocladius distinctus	2			1
Nanocladius minimus				1
Nanocladius rectinervis	9			
Natarsia punctata	17			1
Neostempellina thienemanni				1
Neozavrelia cuneipennis				1

Nilotanypus dubius	12				
Oliveridia tricornis					1
Orthocladius ashei					4
Orthocladius consobrinus	63				
Orthocladius decoratus	61				1
Orthocladius dentifer	3				
Orthocladius frigidus	49				
Orthocladius fuscimanus					1
Orthocladius gelidorum	20				
Orthocladius lamellatus	2				
Orthocladius lapponicus	2				
Orthocladius lignicola					1
Orthocladius nitidoscutellatus	42				3
Orthocladius oblidens	74				3
Orthocladius olivaceus	6				
Orthocladius pedestris	3				
Orthocladius rivicola	15				3
Orthocladius rivulorum	3				
Orthocladius rubicundus	133				
Orthocladius saxosus	39				1
Orthocladius schnelli	3				
Orthocladius subletteorum	4				
Orthocladius telochaetus	54				
Orthocladius thienemanni	5				5
Pagastia orophila		1			
Pagastiella orophila	3		6		
Paraboreochlus minutissimus	3				
Parachaetocladius abnobaeus					1
Parachironomus digitalis	8				
Parachironomus frequens	2				1
Parachironomus gracilior	7				
Parachironomus monochromus	5				1

Parachironomus parilis	4		
Parachironomus siljanensis	5		1
Parachironomus subalpinus	3		
Parachironomus tenuicaudatus			1
Paracladius alpicola	5		
Paracladius quadrinodosus			1
Paracladopelma camptolabis			2
Paracladopelma laminatum	4		
Paracladopelma undine	2		
Paracladopelma winnelli			2
Paracricotopus niger	5		
Paracricotopus uliginosus	13		
Parakiefferiella bathophila	10		
Parakiefferiella coronata			1
Parakiefferiella gracillima			1
Parakiefferiella scandica	12		
Parakiefferiella smolandica	2		1
Paralauterborniella nigrohalteralis	2		
Paralimnophyes longiseta			1
Paramerina cingulata	11		
Paramerina divisa	3		
Parametriocnemus boreoalpinus	6		
Parametriocnemus lundbeckii	4		
Parametriocnemus stylatus	26		3
Paraphaenocladius brevinervis	8		
Paraphaenocladius exagitans	12		1
Paraphaenocladius impensus	39		4
Paraphaenocladius intercedens	2		
Paraphaenocladius irritus	3		
Paraphaenocladius pseudirritus	16		1
Parapsectra uliginosa		1	
Parasmittia carinata	6		

Paratanytarsus abiskoensis				1
Paratanytarsus austriacus	42			
Paratanytarsus bituberculatus	5			2
Paratanytarsus dissimilis	9			1
Paratanytarsus grimmii				1
Paratanytarsus hyperboreus	7			1
Paratanytarsus inopertus	22			
Paratanytarsus laccophilus	16			2
Paratanytarsus laetipes	3			3
Paratanytarsus lauterborni	6			1
Paratanytarsus natvigi	13			5
Paratanytarsus penicillatus	10			3
Paratanytarsus setosimanus	3			
Paratanytarsus tenellulus				2
Paratanytarsus tenuis	4			1
Paratendipes albimanus	105			1
Paratendipes nudisquama				1
Paratendipes subaequalis	2			
Paratrichocladius rufiventris	2	3	2	
Parochlus kiefferi	7			1
Pentaneurella katterjokki	14			
Phaenopsectra flavipes	18			
Phaenopsectra punctipes	6			1
Polypedilum absensilobum				1
Polypedilum albicorne	76			
Polypedilum albinodus	3			
Polypedilum arundineti	8			
Polypedilum bicrenatum	6			
Polypedilum convictum	6			
Polypedilum cultellatum				4
Polypedilum laetum				1
Polypedilum nubeculosum	15			8

Polypedilum pedestre	3			2
Polypedilum pullum	3			
Polypedilum quadriguttatum	2			1
Polypedilum scalaenum	11			5
Polypedilum simulans				1
Polypedilum sordens	11			
Polypedilum tridens		3	1	
Polypedilum trigonus	3			
Polypedilum tritum				1
Polypedilum tuberculum				1
Polypedilum uncinatum	8			
Potthastia gaedii	3			2
Potthastia longimanus	3			2
Procladius appropinquatus				1
Procladius barbatus				1
Procladius choreus	2			
Procladius crassinervis	37			1
Procladius culiciformis	19			1
Procladius dentus	2			
Procladius flavifrons	4			
Procladius nigriventris	11			
Procladius rufovittatus				1
Procladius sagittalis	8			
Procladius signatus	8			
Prodiamesa olivacea	28			1
Propsilocerus saetheri	3			
Prosmittia jemtlandica	3			1
Protanypus caudatus	3			
Protanypus morio	5			
Psectrocladius barbimanus	8			
Psectrocladius calcaratus	4			1
Psectrocladius conjungens	2			

Psectrocladius fennicus	2			1
Psectrocladius limbatellus	45			2
Psectrocladius octomaculatus			2	
Psectrocladius oligosetus	4			
Psectrocladius oxyura	21			2
Psectrocladius platypus	4			
Psectrocladius psilopterus		1		1
Psectrocladius schlienzi	7			
Psectrotanypus varius	3			
Pseudochironomus prasinatus	4			2
Pseudodiamesa branickii	10			3
Pseudodiamesa nivosa	3			
Pseudokiefferiella parva	2			
Pseudorthocladius curtistylus	18	2	4	1
Pseudorthocladius filiformis	7			
Pseudorthocladius pilosipennis	8			
Pseudosmittia albipennis	21			
Pseudosmittia danconai				1
Pseudosmittia forcipata	5			
Pseudosmittia gracilis	2			
Pseudosmittia mathildae				1
Pseudosmittia trilobata	6			1
Psilometriocnemus europaeus	4			1
Rheocricotopus atripes	7			1
Rheocricotopus chalybeatus				1
Rheocricotopus chapmani	5			
Rheocricotopus effusus	26			1
Rheocricotopus fuscipes	26			
Rheocricotopus reduncus				1
Rheopelopia maculipennis	8			
Rheosmittia spinicornis	2			
Rheotanytarsus distinctissimus	3			

Rheotanytarsus illiesi				1
Rheotanytarsus pentapoda	20			
Rheotanytarsus ringei	189			1
Robackia demeijerei				1
Saetheria tylus				1
Schineriella schineri	2			
Sergentia baueri		1		1
Sergentia coracina	4			1
Sergentia prima	1	1	1	
Smittia aterrima	14			1
Smittia brevipennis	21			
Smittia edwardsi	8			4
Smittia extrema	10			
Smittia longicosta	71			
Smittia nudipennis	3			
Smittia paranudipennis	17			
Smittia pratorum				1
Smittia scutellosetosa	3			
Stackelbergina praeclara				1
Stempellina bausei	13			1
Stempellinella brevis	16			1
Stempellinella edwardsi	5			2
Stenochironomus gibbus	6			
Stictochironomus maculipennis	6			
Stictochironomus pictulus				3
Stictochironomus psilopterus	6			
Stictochironomus rosenschoeldi	2			
Stictochironomus sticticus	3			
Synendotendipes dispar				2
Synendotendipes impar	21			
Synendotendipes lepidus		1		3
Synendotendipes luski	2			1

Synorthocladius semivirens	22			
Tanypus kraatzi				1
Tanypus punctipennis	4			1
Tanytarsus aculeatus	6			
Tanytarsus adustus	5			
Tanytarsus albisutus				1
Tanytarsus anderseni	2			2
Tanytarsus bathophilus	6			2
Tanytarsus brundini	45			7
Tanytarsus buchonius				1
Tanytarsus chinyensis	5			
Tanytarsus curticornis				3
Tanytarsus debilis				3
Tanytarsus dispar	3			
Tanytarsus ejuncidus	11			
Tanytarsus eminulus	84			
Tanytarsus excavatus	7			1
Tanytarsus fennicus				1
Tanytarsus glabrescens				1
Tanytarsus gracilentus	13			1
Tanytarsus gregarius				1
Tanytarsus heliomesonyctios	15			1
Tanytarsus heusdensis	5	1		3
Tanytarsus inaequalis	11			6
Tanytarsus innarensis				1
Tanytarsus lactescens	2			
Tanytarsus latens	2			
Tanytarsus lestagei	4			1
Tanytarsus longitarsis		1		2
Tanytarsus lugens	2			1
Tanytarsus madeiraensis	2			
Tanytarsus medius	2			

Tanytarsus mendax			5
Tanytarsus miriforceps	3		1
Tanytarsus nemorosus	4		1
Tanytarsus niger			2
Tanytarsus nigricollis			1
Tanytarsus occultus	4		2
Tanytarsus palettaris	2		
Tanytarsus pallidicornis	2		
Tanytarsus palmeni			1
Tanytarsus paraniger			1
Tanytarsus pseudoheusdensis	2		
Tanytarsus recurvatus	7		2
Tanytarsus reei		1	
Tanytarsus signatus	6		
Tanytarsus sinuatus	7		
Tanytarsus striatulus			2
Tanytarsus sylvaticus			2
Tanytarsus telmaticus	3	2	4
Tanytarsus thomasi			2
Tanytarsus usmaensis	18		7
Tanytarsus verralli	2		5
Telmatogeton amphibius			1
Telmatogeton japonicus	2		
Telmatopelopia nemorum			1
Thalassomya frauenfeldi			1
Thienemannia fulvofasciata	2		
Thienemannia gracei	3		
Thienemannia libanica			1
Thienemanniella caspersi	20		
Thienemanniella majuscula	3		1
Thienemanniella obscura			1
Thienemanniella vittata	12		

Thienemanniella xena				1
Thienemannimyia carnea	15			1
Thienemannimyia fuscipes	2			1
Thienemannimyia lentiginosa	4			
Thienemannimyia northumbrica	3			
Thienemanniola ploenensis				1
Tokunagaia excellens	3			
Tokunagaia kibunensis	7			
Tokunagaia parexcellens	11			
Tokunagaia rectangularis	4			
Tokunagaia tonollii	5			
Tribelos intextum	2			
Trichotanypus posticalis	5			
Trissopelopia longimana				1
Tvetenia bavarica	32			1
Tvetenia calvescens	218			2
Tvetenia duodenaria	7			
Tvetenia tshernovskii	4			
Tvetenia verralli	5			
Virgatanytarsus arduennensis	9			
Xenochironomus xenolabis	4			
Xenopelopia falcigera	2			
Xenopelopia nigricans				1
Zalutschia tornetraeskensis	3			
Zavrelia pentatoma	3			1
Zavrelimyia barbatipes				1
Zavrelimyia melanura	6			
TOTAL with singletons as 'no id'	**9580**	**48**	**268**	**476**
Correct singletons				**94**
TOTAL with singletons as 'correct'	**9674**	**48**	**268**	**382**

Table S8. Selected Chironomidae species diversity and distribution in Europe.

Species	Number of sequences	Country of presence	BINs	Number of haplotypes	Number of OTUs	K2P distance			
						min (%)	mean (%)	max (%)	SE (%)
Cricotopus bicinctus	54	Montenegro Germany Sweden Norway Portugal	BOLD:AAI6018 BOLD:ACU9662 BOLD:AAT9677 BOLD:AAP5931	41	4	0	2.93	12.2	0
Cricotopus sylvestris	53	Montenegro Norway Finland Sweden Denmark Poland	BOLD:AAA5299 BOLD:AEA8330 BOLD:AEA8329	38	4	0	2.43	15.09	0
Limnophyes minimus	953	Montenegro Norway Germany Finland France Sweden	BOLD:AEB4645 BOLD:AAA8204 BOLD:AAM5218 BOLD:AAA8202 BOLD:ADE7302	303	6	0	0.5	21.12	0
Limnophyes natalensis	42	Montenegro Norway Germany Portugal Finland	BOLD:ABW5528 BOLD:AEB6944 BOLD:AAB7361 BOLD:AAT9715 BOLD:AAB7360 BOLD:ACT1270	41	5	0	8.79	21.55	0.01
Paratendipes albimanus	106	Montenegro Norway Germany Sweden	BOLD:AAO1037	57	1	0	0.28	1.58	0
	52	Montenegro	BOLD:AEB4993	45	11	0	3.99	18.22	0

Tanytarsus brundini		Norway	BOLD:AAP5616						
		Sweden	BOLD:ACQ8988						
		Finland	BOLD:AAB9119						
		Ukraine	BOLD:AAB9124						
			BOLD:ACQ8983						
			BOLD:ACQ8984						
			BOLD:AAB9122						
			BOLD:ACW3092						
Tanytarsus usmaensis	24	Montenegro	BOLD:AAP7166	20	4	0	2.41	4.38	0
		Norway	BOLD:ACY8157						
		Sweden	BOLD:ACT5665						
		Finland	BOLD:ACR6261						
		Poland	BOLD:ACR5765						
Orthocladius oblidens	77	Montenegro	BOLD:AEA9438	55	2	0	1	13.89	0
		Norway	BOLD:AAD8971						
		Iceland							
		Sweden							
		Germany							
Psectrocladius limbatellus	49	Montenegro	BOLD:ABZ5549	24	3	0	8.52	16.73	0.01
		Norway	BOLD:AAD4703						
		Sweden	BOLD:AAG5500						
		Finland	BOLD:AAU0274						
		Poland	BOLD:AAU0273						
Cladotanytarsus mancus	38	Montenegro	BOLD:AEB0319	33	4	0	2.11	5.08	0
		Norway	BOLD:AAJ1096						
		Sweden	BOLD:ACR8611						
		Poland	BOLD:ACY4677						
		Finland	BOLD:AAN5378						
			BOLD:ADK6135						
			BOLD:ADF2204						
			BOLD:ACR8609						
			BOLD:ADK1947						

			BOLD:ADK6134						
			BOLD:ACR8610						
			BOLD:ACR8612						
Chironomus riparius	138	Montenegro	BOLD:AAA7263	48	4	0	1.05	16.94	0
		France	BOLD:AAI4307						
		Italy	BOLD:AAU4044						
		Bulgaria							
		Germany							
		Sweden							

Table S9. Species included in the minimum-spanning haplotype networks analysis.

***Cricotopus* group 1**
C. lestralis
C. polaris
C. gelidus
C pilosellus
***Cricotopus* group 2**
C. sylvestris
C. glacialis
C. trifasciatus
C. pilitarsis
C. maurii
C. ornatus
C relucens
Chironomus
C. pseudomendax
C. tentans
C. pallidivittatus
C. melanescens
C. sollicitus
C. riparius
C. pseudothummi
C. lugubris
C. luridus
C. aprilinus
C. salinarius
C. pilicornis
C. heteropilicornis
C. obtusidens
C. storai
C. mendax
C. curabilis

C. plumosus
C. melanotus
C. cingulatus
C. annularius
C. longistylus
C. hyperboreus
C. acerbus
Diamesa
D. starmachi
D. serratosioi
D. saetheri
D. lavillei
D. arctica
D. incallida
D. lindrothi
D. latitarsis
D. tonsa
D. hyperborea
D. zernyi
D. bohemani
D. insignipes
D. bertrami
Micropsectra
M. attenuata
M. seguyi
M. roseiventris
M. radialis
M. pharetrophora
M. uliginosa
M. nana
M. chionophila
M. insignilobus

M. lindrothi
M. borealis
M. atrofasciata
M. pallidula
M. lacustris
M. notescens
M. junci
M. contracta
M. sofiae
M. schrankelae
M. logani
M. klinki
M. recurvata
M. appendica
M. styriaca
M. acuta
Polypedilum
P. laetum
P. nubeculosum
P. arundineti
P. trigonus
P. uncinatum
P. sordens
P. convictum
P. cultellatum
P. pullum
P. albinodus
P. tritum
P. pedestre
P. albicorne
P. tridens
P. bicrenatum

P. scalaenum
P. simulans
P. quadriguttatum
P. tuberculum

Table S10. List of shared taxa recorded on checklists identified using morphology-based approach, BINs and OTUs.

[OTUs] and [Morphospecies]	[BINs] and [OTUs]	[BINs] and [OTUs] and [Morphospecies]
Cricotopus trifasciatus	*Apsectrotanypus trifascipennis*	*Ablabesmyia longistyla*
Paratanytarsus inopertus	*Benthalia carbonaria*	*Ablabesmyia monilis*
Phaenopsectra punctipes	*Chironomus riparius*	*Bryophaenocladius nigrus*
	Chironomus transvaalensis	*Cardiocladius capucinus*
	Cricotopus maurii	*Chaetocladius perennis*
	Cricotopus similis	*Chironomus annularius*
	Cricotopus trifascia	*Chironomus cingulatus*
	Cryptochironomus albofasciatus	*Chironomus curabilis*
	Macropelopia nebulosa	*Chironomus plumosus*
	Macropelopia notata	*Chironomus pseudomendax*
	Micropsectra pallidula	*Chironomus pseudothummi*
	Paratanytarsus grimmii	*Cladopelma virescens*
	Prodiamesa olivacea	*Cladopelma viridula*
	Psectrotanypus varius	*Cladotanytarsus mancus*
	Tvetenia calvescens	*Clinotanypus nervosus*
		Conchapelopia pallidula
		Corynoneura gratias
		Cricotopus bicinctus
		Cricotopus fuscus
		Cricotopus relucens
		Cricotopus rufiventris
		Cricotopus sylvestris
		Cryptochironomus supplicans
		Dicrotendipes lobiger
		Dicrotendipes nervosus
		Dicrotendipes pulsus
		Endochironomus albipennis
		Endochironomus tendens
		Glyptotendipes pallens

Glyptotendipes signatus
Guttipelopia guttipennis
Kiefferulus tendipediformis
Limnophyes minimus
Limnophyes natalensis
Limnophyes pentaplastus
Microchironomus tener
Micropsectra lindrothi
Microtendipes chloris
Microtendipes pedellus
Orthocladius oblidens
Orthocladius pedestris
Parachironomus monochromus
Paratanytarsus bituberculatus
Paratanytarsus dissimilis
Paratanytarsus laetipes
Paratanytarsus lauterborni
Paratanytarsus tenellulus
Paratendipes albimanus
Phaenopsectra flavipes
Polypedilum absensilobum
Polypedilum cultellatum
Polypedilum laetum
Polypedilum nubeculosum
Polypedilum pedestre
Polypedilum scalaenum
Polypedilum sordens
Procladius crassinervis
Procladius culiciformis
Psectrocladius limbatellus
Pseudosmittia trilobata
Rheocricotopus chalybeatus

Stempellina bausei
Stenochironomus gibbus
Stictochironomus pictulus
Tanypus punctipennis
Tanytarsus brundini
Tanytarsus chinyensis
Tanytarsus ejuncidus
Tanytarsus excavatus
Tanytarsus inaequalis
Tanytarsus lactescens
Tanytarsus striatulus
Tanytarsus usmaensis

Streszczenie

Celem rozprawy jest odpowiedź na pytania dotyczące różnorodności i pochodzenia fauny muchówek z rodziny ochotkowatych (Diptera Chironomidae) Jeziora Szkoderskiego - unikatowego słodkowodnego układu modelowego na Półwyspie Bałkańskim. Jezioro Szkoderskie jest określane, jako „hot-spot" bioróżnorodności. Na kompleks ten składa się powstałe około 1200 lat temu jezioro oraz system geologicznie starych, plioceńskich źródlisk. Głównym celem mojej rozprawy było odkrycie i porównanie różnorodności gatunkowej ochotkowatych zamieszkujących Jezioro Szkoderskie oraz system jego źródeł na poziomie zarówno morfologicznym jak i molekularnym. W oparciu o identyfikację taksonomiczną dorosłych samców oraz wylinek poczwarkowych, zidentyfikowałem 164 taksony Chironomidae. Wyniki przedstawione w mojej rozprawie doktorskiej rozszerzają istniejącą listę gatunków o 152 taksony nowo odkryte w basenie Jeziora Szkoderskiego. Skutkuje to pierwszym, tak dokładnym zbadaniem składu gatunkowego unikalnych siedlisk niemal zupełnie niezbadanego akwenu, jakim jest Jezioro Szkoderskie. Dzięki zastosowaniu metod molekularnych, takich jak barkoding DNA wykazałem 168 operacyjnych jednostek taksonomicznych (OTU), co jest wynikiem wyższym niż liczba morfotypów uzyskanych podczas identyfikacji taksonomicznej dojrzałych samców i wylinek poczwarkowych.

Drugim celem mojej rozprawy było zbadanie wpływu czynników fizyko-chemicznych na skład i rozmieszczenie zbiorowisk Chironomidae w basenie Jeziora Szkoderskiego. Otrzymane wyniki wskazują, że płytkie, przybrzeżne części jeziora porośnięte makrofitami charakteryzują się najwyższą różnorodność gatunkową. Następnie porównałem poziom różnorodności gatunkowej ochotkowatych badanego jeziora z innymi jeziorami Europy Środkowej i Południowej. Porównanie przeprowadzone w oparciu o listy gatunków występujących w 13 dobrze zbadanych europejskich jeziorach wykazało, że Jezioro Bodeńskie (Szwajcaria/Niemcy/Austria) jest najbardziej różnorodnym pod względem liczby gatunków ochotkowatych zbiornikiem wodnym, a zaraz po nim badane przeze mnie Jezioro Szkoderskie.

Podczas realizacji trzeciego celu opracowałem pierwszą bibliotekę referencyjną barkodów DNA dla Chironomidae z basenu Jeziora Szkoderskiego. Ponadto, korzystając z opracowanej biblioteki referencyjnej oraz danych zdeponowanych w bazie Barcode of Life (BOLD), oszacowałem wydajność barkodingu DNA dla europejskich Chironomidae na poziomie rodziny,

jak i podrodziny. Wynikiem jest baza 770 sekwencji COI reprezentowanych przez 75 gatunków zidentyfikowanych w oparciu o cechy morfologiczne. Wszystkie sekwencje pochodzące z tego obszaru są nowe dla repozytoriów online i potwierdzają użyteczność tej metody do identyfikacji ochotkowatych.

Moim czwartym celem było zbadanie wzorców rozmieszczenia Chironomidae w Europie w oparciu o uniwersalny numer BIN (Barcode Index Number) oraz dyskusja problematycznych grup gatunków, zarówno w przypadku tradycyjnej taksonomii, jak i danych molekularnych. Wyniki mojej pracy doktorskiej dają pierwszy wgląd w faktyczną różnorodność gatunkową Chironomidae basenu Jeziora Szkoderskiego i jej porównanie z fauną Chironomidae w skali europejskiej. Uzyskane w ten sposób wyniki z pewnością odkrywają braki w wiedzy o różnorodności biologicznej Półwyspu Bałkańskiego. Na podstawie wyników badania fauny Chironomidae można stwierdzić, że basen Jeziora Szkoderskiego jest obecnie dobrze zbadany, i tak wysoka reprezentacja gatunków z różnych miejsc poboru prób zapewnia wiarygodne oszacowanie lokalnej fauny ochotkowatych. Bazując na uzyskanych wynikach trudno jest jednak przewidzieć pochodzenie fauny Chironomidae basenu Jeziora Szkoderskiego w oparciu o sekwencje zdeponowane w bazie BOLD oraz na podstawie ich rozmieszczenia geograficznego. Liczba sekwencji rozpowszechniona między dobrze zbadanymi regionami europejskimi i basenem Jeziora Szkoderskiego jest wciąż niewystarczająca. Ponadto, można stwierdzić, że Jezioro Szkoderskie wraz z jego systemem źródeł jest hot-spotem słodkowodnej różnorodności biologicznej, ale bez endemizmu na poziomie gatunków.