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Diversity of European freshwater cyclopoid species: phylogeny, morphology and ecology

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Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, červenec 2014

Martin Krajíček

Declaration:

I thereby declare that this thesis has not been submitted in order to obtain the same or any other academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed in the authorship order of the included publications and manuscript. All publications and other sources I used when writing this thesis have been properly cited.

In Prague, July 2014

Martin Krajíček

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Preface and acknowledgements

My name is Martin Krajíček. I was born 11. 3. 1983 in Prague, the Czech Republic. After graduating secondary school in 2001, I started to study Biology at Charles University in Prague. During my studies I found an interest in Hydrobiology and chose the Department of Ecology. I obtained a Master's Degree with the following thesis: 'Genetic diversity of Central European *Cyclops* (Crustacea, Copepoda) species' in 2007. I decided to continue my doctoral studies at the same department under the supervision of Dr. Martin Černý. My mentor and closest collaborator was Dr. Jan Fott, the greatest contemporary Czech specialist on the morphology and taxonomy of copepods of the genus *Cyclops*.

Here I would like to thank everyone who supported me during my study. At first I should mention **my parents**, Alena and Pavel, who stood by me all the time and gave me unreserved help on many levels. My special thanks belong to **Jan Fott** for his endless friendly source of inspiration and enthusiasm in copepod research. I should thank also my adviser, **Martin Černý**, for the nice support and help whenever I asked.

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Thank you all so much!

Martin :o)

Abstract

Cyclopoids are together with Calanoids and Harpacticoids a part of the largest, very diverse group of crustaceans and the most numerous aquatic metazoans of the world. The history of their research goes as far back as to the beginning of 19th century when the first cyclopoid copepods were described. The taxonomy of cyclopoids started to develop gradually since that time, adding new and more detailed methods and morphological characters, as well as a certain degree of taxonomical confusion. In last decades, the molecular-genetic techniques of DNA sequencing have become available offering a new independent tool for taxonomists. This work contains different studies concerning the morphology, taxonomy, ecology, distribution and colonisation of cyclopoid copepods, with the use of molecular tools as a uniting element.

Chapter 1 of this thesis summarizes basic knowledge about the taxonomy, morphology and biology of cyclopoid copepods and introduces the following chapters containing four studies presented as single publications. The taxonomy of copepods of the genus *Cyclops* is based mainly on the morphology which is sometimes ambivalent and some of the most problematic species groups are presented here. **Chapter 2** presents our unique results, the first reconstruction of phylogenetic relationships among 15 *Cyclops* species based on a comprehensive dataset of DNA sequences of six mitochondrial and nuclear markers. Additionally, a summarization of morphological microcharacters useful for species delineation is provided. In **Chapter 3** the mitochondrial sequence variation of two crustacean species, the cyclopoid *Eucyclops serrulatus* and the cladoceran *Daphnia longispina* from East European mountain lakes is compared, and their dispersal ability and patterns of colonisation are discussed. **Chapter 4** questions the cosmopolitan distribution and possible anthropogenic translocation of a freshwater copepod *Macrocyclops albidus* using molecular and morphological traits. The last paper, presented in **Chapter 5**, is focussed on the West Australian *Diacyclops* species of the *alticola*-group, and discuss the size differentiation and monophyly of these species using a molecular sequence data. Finally, in **Chapter 6** different methods useful in the research of copepods are summarized with a special emphasis on the DNA sequencing.

Abstrakt

Buchanky jsou spolu s vznášivkami a plazivkami součástí největší a velmi druhově bohaté skupiny korýšů a patří mezi nejpočetnější živočichy ve světových vodách vůbec. Historie jejich výzkumu sahá až do počátku 19. století, kdy byly popsány první druhy. Od té doby se začala jejich taxonomie pomalu vyvýjet, přibývaly nové metody výzkumu, jakož i taxonomické znaky, které ale mohly být do jisté míty i nejednoznačné či matoucí. Během posledních desetiletí se staly dostupnými molekulárně genetické techniky sekvenování DNA, které nabídly taxonomům novou nezávislou metodu. Tato práce obsahuje studie týkající se motfologie, taxonomie, ekologie, možností kolonizace a rozšíření buchanek, přičemž jednotícím elementem těchto prací je právě použití molekulárních metod.

Kapitola 1 shrnuje základní znalosti o taxonomii, morfologii a biologii buchanek a tvoří tím úvod pro následující části obsahující čtyři studie představené formou samostatných publikací. Taxonomie buchanek rodu *Cyclops* je založena převážně na morfologických znacích, které však někdy můžou být nejednoznačné. Zároveň se zde vyskytují některé problematické druhové skupiny. **Kapitola 2** tak představuje unikátní výsledky první rekonstrukce fylogenetických vztahů patnácti druhů buchanek rodu *Cyclops*, které jsou založené na rozsáhlém souboru sekvenčních dat šesti jaderných a mitochondriálních genů. Navíc jsou zde shrnuty morfologické znaky klíčové k rozlišení všech těchto druhů. **Kapitola 3** se zabývá srovnáním mitochondriální sekvenční variability u dvou druhů korýšů: buchanky *Eucyclops serrulatus* a perloočky *Daphnia longispina* z horských jezer ve východní Evropě. Diskutovány jsou také schopnosti šíření a kolonizace obou druhů. **Kapitola 4** nastoluje otázku kosmopolitního rozšíření a možnosti antropogenního zavlečení sladkovodního druhu buchanky *Macrocyclops albidus*. Tento problém je opět řešen s využitím molekulárních a morfologických metod. Poslední článek představený v **Kapitole 5** je zaměřen na západoaustralské druhy buchanky *Diacyclops* ze skupiny *alticola*. Možnost velikostní diferenciace a monofylie této skupiny je zkoumána na základě molekulárních dat sekvencí DNA. Závěrečná **Kapitola 6** shrnuje metody využitelné při studiu klanonožců, přičemž speciální pozornost je věnována technikám sekvenace DNA.

Chapter 1: Introduction

This chapter will describe some general aspects of Cyclopoid copepods. The basic knowledge about the taxonomy, morphology and biology of these invertebrates will be summarised. This part will be an introduction for the following Chapters 2 to 5, containing four projects presented as separate publications. The main aims of the whole thesis are specified at the end of this Chapter 1. Finally, Chapter 6 will summarise and discuss the main findings of previous chapters. Suggestions for further studies will be given as well.

General introduction

Copepods are the largest, very diverse group of crustaceans and the most numerous metazoans in the world water community. They are very ancient arthropods, but because of poor fossilization their remains are rarely found in the sediments (Frey, 1964). Only some subrecent remains have been identified, mainly spermatophores (Frey, 1964), and also diaptomid egg-sacs isolated from Late Quaternary lake sediments (12 000 to 10 000 ^{14}C years BP) in Denmark (Bennike, 1998). According to the present knowledge copepods include over 21000 species, 2600 genera and 240 families. They inhabit both sea and continental waters, including localities with extreme conditions like hot hydrothermal vents, cold polar ice-waters, phytotelmat or interstitial waters and subterranean caves. Copepods can be free-living, symbionts or parasites on other water animals. In water ecosystems they often form the dominant part of the zooplankton and as such they play an important role as secondary producers.

The subclass Copepoda consists of nine orders (Boxshall & Halsey, 2004) Cyclopoida being one of them. Traditionally, at the generic level the systematics of the cyclopoid copepods is based on the morphological structures and armature of the fifth leg (Kiefer, 1927; Monchenko, 1974; Rylov, 1948; Yeatman, 1959). Nevertheless, some authors (Kiefer, 1978; Lindberg, 1957; Morton, 1985; Reid, 1993) have suggested that this appendage should be considered a slightly conservative morphological character among the cyclopoid copepods, and could have poor taxonomic value as a generic discriminant. Apparently in some cases using this character alone considerably confuses the identification of some genera, which remain still vague and controversial (Reid, 1994). The present confusion in the delineation and identification of cyclopoid species is also caused by the fact that certain microcharacters, like the ornamentation of the antenna and antennule, and the armature of basis, coxa and couplers of the swimming legs, once considered unimportant, have been often neglected in descriptions and illustrations.

Taxonomy: a historical overview

The first cyclopoid copepods were described by Jurine (1820) from Lake Geneva as *Monoculus quadricornis viridis* (called *Megacyclops viridis* now) and *Monoculus quadricornis fuscus* (now *Macrocyclops albidus*), see Figure 1. Later on, the first species of the genus *Cyclops* (*C. strenuus*) was discovered by Fischer (1851), followed by descriptions of *C. furcifer* and *C. insignis* by Claus (1857), *C. abyssorum*, *C. lacustris* and *C. scutifer* by Sars (1863) and *C. vicinus* by Uljanin (1875). In 1892 Schmeil reduced all these species to a single, morphologically highly variable *C. strenuus* and to a morphologically stable *C. insignis*.

A **B**

Figure 1. Original drawings of first described cyclopoid copepods (after Jurine, 1820): (A) *Monoculus quadricornis viridis* (now *Megacyclops viridis*) and (B) *Monoculus quadricornis fuscus* (now *Macrocyclops albidus*).

A further step in taxonomy was taken by Koźmiński (1936) who introduced the method of morphometrical analysis. This method was based on the comparison of 18 quantitative characters between two species. However, indiscriminate evaluations of morphological characters led Lindberg (1957) to recognize as much as 52 species and subspecies, which contradicts the recent concept of taxonomical units in *Cyclops*.

The main researcher dealing with freshwater copepods in the 20th century was zoologist and taxonomist Fridrich Kiefer. He performed long term studies on the zooplankton in Lake Constance and its surroundings in South Germany. He described new species from samples collected during numerous scientific expeditions to Turkey, India and Mongolia (Kiefer, 1939), he

made a revision of the genus *Acanthocyclops* (Kiefer, 1976) and wrote a monograph on freshwater plankton-dwelling cyclopoids and calanoids (Kiefer, 1978).

Elrich K. Einsle, Kiefer's student, also become a very important copepodologist. Einsle carried out many ecological studies focussed on diapause, seasonal dynamics and the invasion of new zooplankton species. He studied interspecific and intraspecific variation of copepods using morphological and morphometric approaches. He established the use of chromatin diminution and enzyme electrophoresis in copepod taxonomy (Einsle, 1996b). Using these methods he distinguished and described two new species, *Cyclops heberti* and *Cyclops singularis*, from South Germany (Einsle, 1996a). Einsle also made a revision of the *Cyclops abyssorum*-group (Einsle, 1975, 1980) and described the patterns of spinule ornamentation on the caudal side of coxa of fourth swimming leg as an important character for *Cyclops* species differentiation (Einsle, 1985, 1996b). More information on Ulrich Einsle's contribution to the taxonomy of copepods can be found in Wyngaard (2000).

Looking in the history of Czech hydrobiology, Rudolf Šrámek-Hušek was an important scientist and populariser of science. Besides other areas he did research on the zooplankton of Černé jezero (Black Lake) in the Bohemian Forest (Šrámek-Hušek, 1937) where he described the new species *Cyclops bohemicus* (Šrámek-Hušek, 1944) based on Koźmiński's method of morphometric analysis, however this species is not currently considered valid. He also published the first identification key on copepods in the Czech language (Šrámek-Hušek, 1938, 1953) and studied copepods in the winter plankton of reservoirs and ponds (Šrámek-Hušek, 1954).

Research focussed on the morphology and taxonomy of Cyclopoid copepods continues up to the present. For example Maria Hołyńska and Hans-Uwe Dahms (2004) recently published a paper revealing the taxonomical potential of cephalothoracic appendages in the genus *Cyclops*. Hołyńska (2008) revised the delimitation and geographical distribution of *Cyclops ankyrae*, *C. divergens* (syn.: *C. singularis*) and C. *abyssorum*.

Most importantly, the molecular-genetic techniques of DNA sequencing have become available in last decades offering a new independent tool for taxonomists, allowing species re-descriptions (Alekseev, Dumont, Pensaert, Baribwegure, & Vanfleteren, 2006) as well as detailed studies of problematic species complexes (Bláha, Hulák, Slouková, & Těšitel, 2010). These DNA techniques have also revealed large sequence divergences in many common copepod species like *Eucyclops serrulatus* (Hamrová, Krajicek, Karanovic, Černý, & Petrusek, 2012) or *Macrocyclops albidus* (Karanovic & Krajicek, 2012), opening potential areas for future studies.

Morphology

According to their body plan there are three types of free-living copepods: Calaniformes, Cyclopiformes and Harpactiformes, corresponding with the orders Calanoida, Cyclopoida and Harpacticoida (Figure 2). As all parts of this thesis are focussed on cyclopoid copepods, only detailed description of Cyclopiformes will follow. Most freshwater copepods are small with mean adult length, without furcal setae, of about one millimetre (Dussart & Defaye, 2001). By comparison, marine free-living copepods may be more than 10 mm long and some parasitic forms may reach 30 mm.

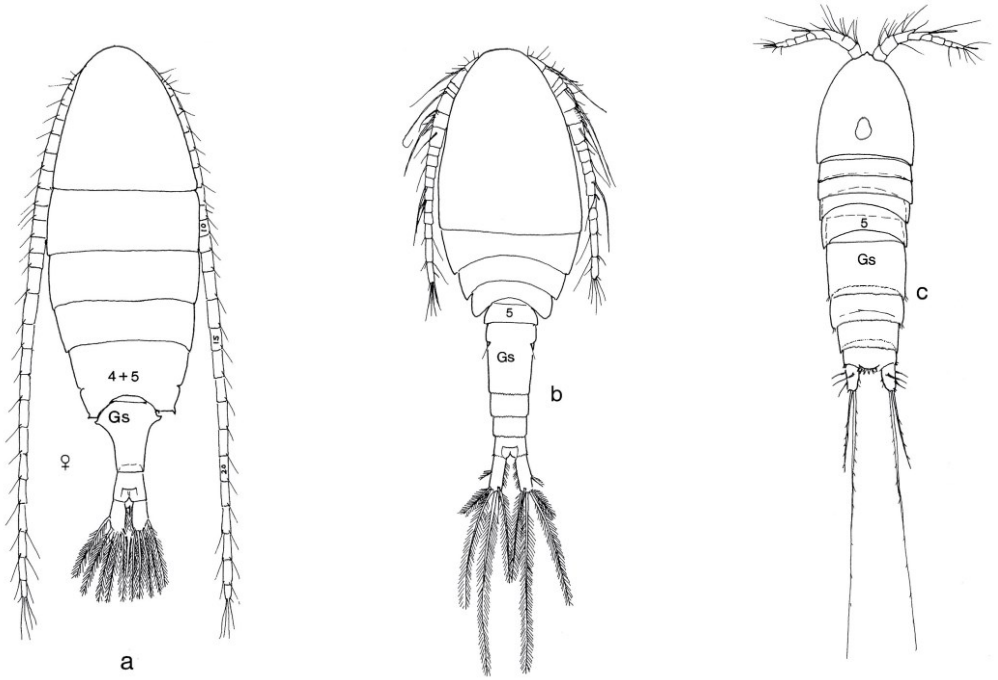

Figure 2. The three types of free-living copepods (♀): (a) Calaniformes, (b) Cyclopiformes, (c) Harpactiformes. Gs indicates the genital segment. (after Dussart and Defaye, 2001)

The body of cyclopoid copepods basically consists of 16 somites (Dussart & Defaye, 2001). The first six are fused into a cephalosome, including segments with the head, mouth appendages and first pair of swimming legs. The remaining 10 somites form the thoracic somites and urosomites. The cephalosome and the thoracic somites constitute the cephalothorax (sometimes called the prosome or metasome) (Dussart & Defaye, 2001). The urosome (abdomen) is limbless and comprises of the genital complex (genital segment in females) and the urosomites, ending in a furca. The furca is composed of two rami, each more or less ornamented, and bears six setae. Within a given species, these setae may be slightly variable in length according to ecological

conditions. The fifth thoracomer is fused with the first urosomite in males or with the genital segment in females.

The exoskeleton of the copepods is not just a simple defence shield. It has a great variety of ornamentation as integumentary structures or organs of sensory or secretory nature. Some of these ornamentations are species-specific, and some of them are related to ecological characteristics, giving information on the quality of the environment (Dussart & Defaye, 2001). Recent studies indicate the large taxonomic potential of these structures (Alekseev et al., 2006; Karanovic & Cho, 2012; Karanovic, Yoo, & Lee, 2012).

The cephalothorax (Dussart & Defaye, 2001) bears these extremities (Figure 3): antennula (A1), antenna (A2), mandibula (Md), maxillula (Mxl), maxilla (Mx), maxilliped (Mxp) and four pairs of swimming legs (P1 to P4). Antennules are uniramous (the exopodite is reduced to a seta and only the endopodite is developed); in cyclopoids they have 6 to 17 segments and rarely reach beyond the cephalothorax. The type of antennule is related to the mode of life (pelagic, benthic, littoral), and help in catching food. Some segments have a specific chaetotaxy as setae, spinules or aesthetascs of different shapes. The ornamentation of basipodite is used in species identification.

The mandibles are situated on either side of the mouth: they are strongly chitinized and transformed to a '*pars molaris*' with masticatory function. In herbivores, the teeth are small, numerous and arranged in two rows. They are generally impregnated by silica (opal) which increases the ability to crush hard algae, such as diatoms. In carnivores, the teeth are stronger and less numerous (Dussart & Defaye, 2001).

The maxillules are adapted to grasping and breaking up food and are typically biramous. The setal ornamentation of the maxillular palp can be used as a species-specific criterion. The maxillae are uniramous and the coxopodite can bear a special spine ornamentation which is used in taxonomy (Hołyński & Fiers, 1994).

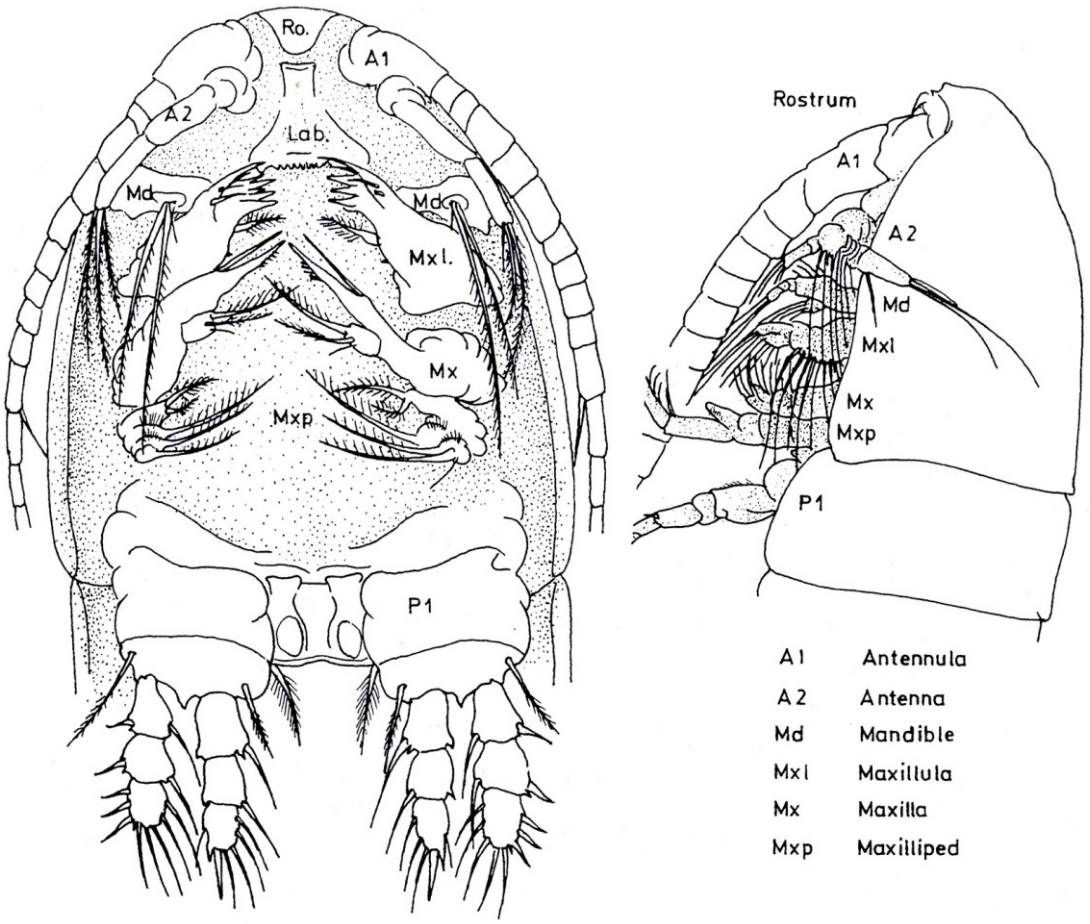

Figure 3. The cephalothorax of cyclopoid species. (after Einsle, 1996b)

Even though the antennules and antennae are active in locomotion, the four pairs of swimming legs (thoracic appendages) are essential for swimming and other movements in copepods (Dussart & Defaye, 2001). Each leg comprises a coxopodite and a basipodite, bearing three-segmented endopodites and exopodites (Figure 4). Each pair of coxopodites is connected by an intercoxal plate (coupler) ensuring simultaneous movement of the legs. The ornamentation of the P4 coxopodite and setation of the P4 coupler, as well as the aspect of humps of the coupler (extending beyond its margin or not) are used to identify species (Einsle, 1985, 1996b).

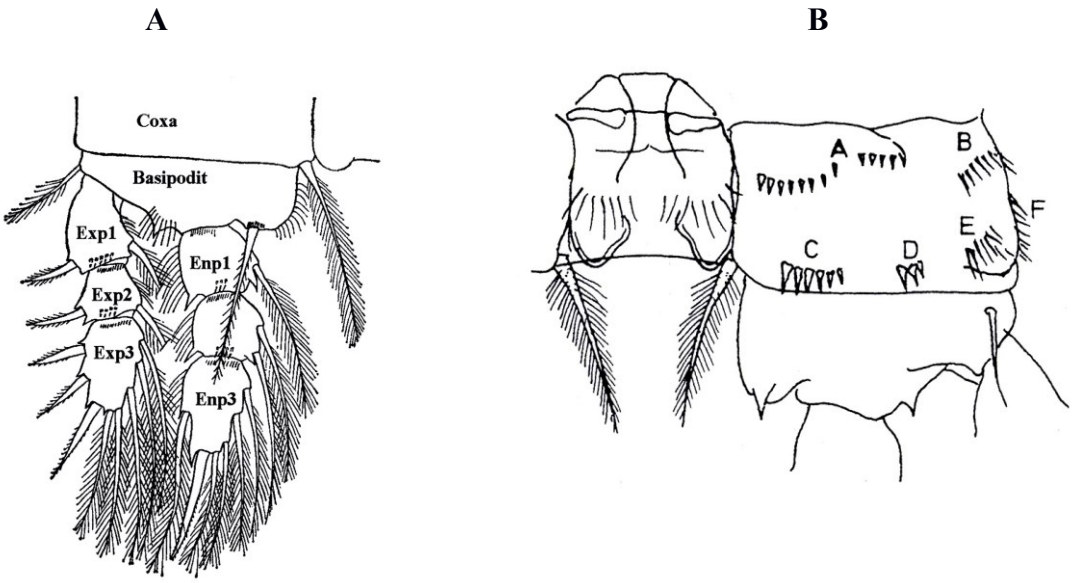

Figure 4. (A) Swimming leg of cyclopoid copepods. Exp1 to Exp3, articles of exopodite; Enp1 to Enp3, articles of endopodite. (after Ivo Přikryl, unpublished).
(B) Ornamentation of the P4 coxopodite with groups of spines (after Einsle, 1996b).

Biology

The development of cyclopoids is indirect, going on through two types of larval stages (naupliar and copepodite). Fertilized eggs hatche into the first naupliar stage (N1). It has a compact, non-segmented pear-shaped body with three pairs of appendages (antennules, antennae and mandibles) and two posterior setae (precursors of the furca). After moulting, the new larva (N2) starts to feed. After each moulting a new pair of appendages is added, the body prolongs and the last naupliar stage (N6) is followed by the first copepodite (C1). The characteristics of naupliar development have systematic value and Kiefer (1973) based a key of 30 Cyclopidae genera on the morphology of nauplii. So, identification of these instars to the generic level is possible, but requires long experience (Einsle, 1996b).

The change from N6 to C1 is the most radical one, because the segmentation of the body appears, as well as structures of the antennae, mandibles, oral appendages and the first pair of swimming legs. These copepodite stages resemble adults with respect to the body shape; single stages (C1 to C5) can be distinguished according to the number of body segments and pairs of legs (Figure 5). The definitive structure of all legs (number of segments and setation) is reached during the last moult leading to the adult. The body structure of adult female and male copepods differs due to sexual dimorphism.

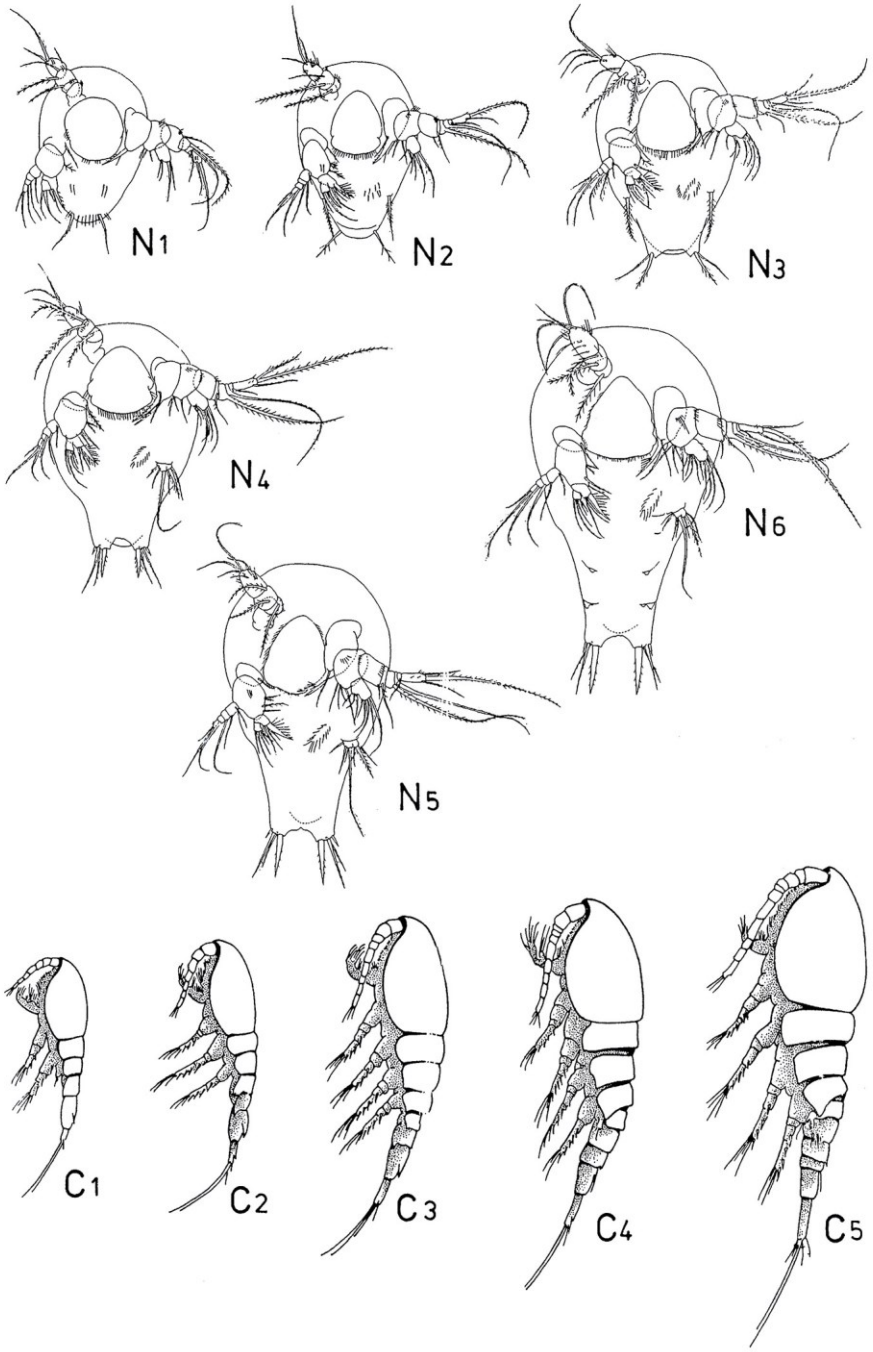

Figure 5. Development of cyclopoid copepods (after Dussart and Defaye, 2001), from N1 to N6: *Eucyclops serrulatus*, from C1 to C5: *Cyclops strenuus.*

Regarding the feeding behaviour, cyclopoids are usually considered to be omnivorous. They graze detritus and algae, and they can capture phytoplankton as well as other planktonic or benthic prey. In the course of postembryonic development, the diet changes with the size of the copepod and with metabolic needs. Sometimes they attack other copepods or even their own nauplii or copepodites. This happens more often in females than in males. Even cannibalism of males by

females after mating has been observed (Wyngaard & Chinnappa, 1982). Large adult females (*Cyclops* and *Megacyclops* species) can even attack fish larvae (Einsle, 1996b). Predaceous copepods detect their prey using the mechanoreceptors on their antennules, and they are able to sense changes in water disturbances (Strickler & Bal, 1973; Strickler, 1975). They also use chemoreception (Kerfoot & Peterson, 1979). A substance (probably hormonal), already present in the egg-sacs allow a female to recognize her own nauplii, preventing her from attacking them (Kahan, Berman, & Bar-El, 1988).

The copepods use various strategies like diapause and dormancy to survive unfavourable conditions. Diapause is an obligatory step in the development, induced by the necessity of changes in physiological processes. It affects only one stage, occurs regularly and proximal external factors play only a secondary role in this event. Dormancy, on the contrary, is a pause in development caused by environmental factors, and is reversible (Dussart & Defaye, 2001). Eggs, copepodites or adults move to the bottom of permanent lakes or ponds to enter diapause or dormancy. In temporary pools, the copepods survive long periods of desiccation, buried in the sediment. The main inducing factors are temperature and high degree of photoperiod.

For example *Cyclops scutifer* from an oligotrophic lake with high fish-predation pressure in South Norway stayed in diapause from the autumn till early spring (Elgmork, 1967). A later study of eight lakes in North America showed substantial differences in the length of the life cycle of this species, ranging from 0.5 year to 2 years and no diapause was observed (Elgmork, 2004). In large lakes, but also in ponds, *Cyclops* populations enter a diapause when a critical day-length is reached in March or April. The diapausing instars (fourth or fifth copepodite stages) show the behaviour of dormancy in or on the sediment, or remain active in benthic regions (Einsle, 1996b). Populations living in ephemeral habitats must be able to establish a new reproductive generation of adults, and they are often synchronous in the production of resting stages (fourth copepodites).

Aims of the thesis

This thesis is focussed on different aspects of cyclopoids concerning the morphology, taxonomy, ecology, distribution and colonisation, while the use of molecular tools is the uniting element. Even though the molecular-genetic techniques of DNA sequencing have been available for more than two decades, just a few genetic studies on the freshwater cyclopoid copepods have been done. The studies presented in this thesis should contribute to filling this gap. There are four major questions this thesis deals with:

- Morphological and molecular characterization of species of the genus *Cyclops* in Europe. Does the species delineation based on the currently used morphological characters match lineages resulting from the analysis of mitochondrial and nuclear DNA sequences? Is there a possibility to discover new, yet undescribed lineages? Do these lineages differ in any morphological character?

- The comparison of the mitochondrial sequence variation of the cyclopoid *Eucyclops serrulatus* and the cladoceran *Daphnia longispina* collected from East European mountain lakes. Is the difference in life cycle and dispersal ability of these species reflected in diverse patterns of haplotype diversity? Do remote mountain lakes harbour any cryptic lineages?

- Molecular and morphological variability of *Macrocyclops albidus* populations from Europe, USA, Australia and New Zealand. Is this species' distribution cosmopolitan or is there any evidence of cryptic speciation? Are the molecular tools useful for testing the hypothesis of anthropogenic translocation of freshwater copepods associated with early shipping activities?

- Molecular sequence characterization of three West Australian *Diacyclops* species of the *alticola*-group. Do mitochondrial sequence lineages correspond with recently described morpho-species? Is the *alticola*-group a monophyletic taxon?

Chapter 2:

The genus *Cyclops* in Europe: an integrative taxonomy approach reveals two new species and confirms thirteen others

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Copepods of the genus *Cyclops* become my main interest since I started the work on the master thesis in 2004. Doctor Jan Fott was a great mentor and experienced guide on my journey into the world of copepod morphology and taxonomy. Initial problems with the use of universal PCR primers for DNA barcoding were solved and a comprehensive dataset of DNA sequences of six mitochondrial and nuclear markers for 15 *Cyclops* species was obtained. These results are unique, allowing the first reconstruction of phylogenetic relationships among *Cyclops* species as well as a summarization of morphological microcharacters useful for species delineation.

Some scientists (Maria Hołyńska, personal communication) consider *Cyclops* to be relatively evolutionary young genus of cyclopoids. This hypothesis could be supported by the presence of taxonomically problematic species groups like *Cyclops abyssorum* (Einsle, 1980, 1996b). Our results revealed two molecularly distinct lineages and subsequent analysis of morphological microcharacters found reliable traits useful for their separation as a new species. The detailed description of these lineages will be the subject of a future study. The 13 remaining species identified by morphology using the recent taxonomic key of Einsle (1996b) were confirmed and well supported by the molecular characters. This reflects the relatively good state of *Cyclops* taxonomy

The genus *Cyclops* in Europe: an integrative taxonomy approach reveals two new species and confirms thirteen others

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Abstract

Despite the fact that copepods of the genus *Cyclops* are among the most common and dominant plankton taxa of lakes in the northern temperate zone, their taxonomy is still unclear. We have analysed an extensive array of *Cyclops* populations from Europe by means of molecular methods and an evaluation of morphological characters. Altogether 64 populations of *Cyclops* species were sampled, assigned to morphospecies, and the 12S rRNA mitochondrial gene sequenced. Selected populations of each morphospecies were additionally sequenced for three mitochondrial (16S rRNA, cytochrome b, COI) and two nuclear genes (18S rRNA, ITS1), and detailed micromorphological analysis was performed. Phylogenetic relationships were reconstructed using maximum likelihood and Bayesian methods. Our analysis has revealed fifteen lineages that can be regarded as different species. Thirteen of these match species defined by Einsle (1996b). The remaining two lineages did not match with any of the described species. A new combination was established - *Cyclops divergens* (Lindberg, 1936). Besides taxonomy our study also brings new insights into species ecology and distribution. Last but not least, a set of morphological traits was selected to facilitate species identification, and a working procedure for efficient dissection and preparation of permanent mounts was developed.

Keywords: *Cyclops*, genetic diversity, morphology, phylogeny, ecology

Introduction

The first seven *Cyclops* species (according to the recent conception: *C. strenuus, C. furcifer, C. insignis, C. abyssorum, C. lacustris, C. scutifer, C. vicinus*) were described in the second half of the 19th century. Since then the number of recognized species has been characterised by considerable fluctuation – from "lumping" together (Schmeil, 1892) [considering *C. furcifer*, *C. scutifer*, *C. abyssorum* and *C. vicinus* to be synonyms of *C. strenuus*] to "oversplitting" (Lindberg, 1957) [52 species and subspecies]. The last major revision of the genus (Einsle, 1996b) recognized 22 species worldwide. In addition to a critical evaluation of morphological traits, Einsle introduced two criteria that are independent of external morphology: patterns of chromatin diminution and enzyme electrophoresis. Later, Hołyńska and Dahms (2004) broadened the spectrum of morphological traits by a careful description of cephalothoracic appendages in 12 *Cyclops* species. However, the taxonomy of this genus by morphology alone remained problematic. Although DNA techniques have been available for two decades, a broad genetic study on the genus *Cyclops* has not yet been done. The main objective of the present study is filling that gap by combining DNA barcoding and morphological delimitation in a large group of 15 *Cyclops* species.

Material and methods

Study sites, sampling and species assignment

This study focuses on European species of the genus *Cyclops*, which were sampled from 64 localities between 2004 and 2013. The samples came from Albania, Austria, Bulgaria, the Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Montenegro, Norway, Poland, Romania, Russia, Slovakia, Spain, Sweden and Switzerland (Table 1), and included various habitats such as glacial lakes, rift lakes, a karst lake, reservoirs, fishponds, mining lakes, and riverine and ephemeral pools. Zooplankton were collected by plankton net tows from the shore or from an inflatable boat. Shallow pools were sampled by a plankton net attached to a pole, or water was scooped up by plastic jug and filtered through the net. All samples for subsequent molecular analysis were preserved in 96 % ethanol and stored in a refrigerator.

Table 1. List of analysed species and populations including numbers of individuals examined morphologically and genetically.

Species	Locality	Type	Country	Altitude (m)	Latitude (N)	Longitude (E)	Area (ha)	Sample date	Sampled by	Identified by	Nr. of morphol. examined individuals	Nr. of genetically analysed individuals					
												12S	16S	18S	COI	cytB	ITS-1
C. abyssorum	**Plešné**	glacial lake	CZ	1089	48,776	13,865	7.5	31.10.2011	JF	JF	9	3	2	2	1	3	2
	Prášilské	glacial lake	CZ	1080	49.075	13.400	3.7	23.9.2004	JF	JF	4	3					
	Vyšné Temnosmrečinské	glacial lake	SK	1725	49,189	20,038	5.6	24.8.2004	SM	JF		3	2	2	2	2	2
	Nižné Terianské	glacial lake	SK	1940	49.170	20.013	5.5	24.9.2004	MK	JF	3	2					
	Wielki Staw	glacial lake	PL	1225	50.758	15.693	8.5	14.10.2005	MK	MK		3					
	Östra Ringsjön	glacial lake	SE	53	55.868	13.551	2250	21.11.2005	JPN	JPN		1					
	Grand Saint-Bernard	glacial lake	SW	2447	45.868	7.167	3.6	6.9.2005	MC	MK		1					
	Lugano	glacial lake	SW	271	45,959	8,895	4870	7.10.2004	SM	JF	5	4	2	1		3	2
	Seebergsee	glacial lake	SW	1830	46,578	7,443	6.0	26.9.2010	MK	MK		3	2				
	Canelles	reservoir	ES	506	41.983	0.616	1569	5.8.2013	MRM	MRM	2	4					
	Redon	glacial lake	ES	2240	42.640	0,778	24	6.9.2010	MK	MK		3	2				
	d'Oô	glacial lake	FR	1507	42.741	0.492	42	8.9.2010	MK	JF	2	pg	3	2		1	1
	Jablan	glacial lake	ME	1791	43.167	19.061	0.8	20.8.2004	MK	MK		3	2				1
	Bulandet	humic pond	NO	5	61.296	4.629	0.035	28.7.2004	JPN	JPN		2					
	Ulvenvann	glacial lake	NO	184	59,813	10,354	260	11.10.2004	JPN	JPN	1	2	2	2		2	
	(Hvaler)	rock pool	NO	3	59.064	10.891	0.02	30.9.2010	MK	JF		2	2				1
	(Pyramiden)	arctic man-made reservoir	NO	80	78.656	16.181	3.6	12.7.2011	LN	MK	8	2	3	3			1
	(Tvärmine)	rock pool	FI	3	59,849	23,255	0.002	10.10.2010	MK	JF		2	2				
	Úlfljótsvatn	man-made lake	IS	97	64.101	-21.042	315	2.6.2010	MK	MK		2	2				1
C. bohater	**Schöhsee**	glacial lake	DE	22	54.165	10.441	78	4.2012	JPN	JPN	1	2	2				
C. divergens	**(Pole)**	abandoned fouled fishpond	CZ	477	49.416	13.787	0.04	9.4.2010	JF	JF	8	3	2	2		1	2
	Žďárské	abandoned humic fishpond	CZ	955	48.936	13.653	1.4	10.7.2007	VS	JF	5	4	1	2	2		
	Slapy	deep reservoir	CZ	271	49.766	14.415	1163	16.6.2007	JF	JF	2	3					
	(Praha, Komořany)	riverine pools	CZ	190	49.989	14.402	0.06	6.4.2004	JF	JF	9	2	2	3	1	2	2
	(Babiny)	ephemeral pool	CZ	570	50.601	14.136	0.001	14.8.2006	AP	JF	15	2					
	Banyoles	karst lake	ES	172	42.125	2.755	112	28.9.2012	MRM	MRM	2	6					
	Ebro	reservoir	ES	834	42,972	-4,048	625	15.7.2013	MRM	MRM	2	3					
	González Lacasa	reservoir	ES	993	42.177	-2.676	152	16.7.2013	MRM	MRM	2	1					
	Siurana	reservoir	ES	470	41.25	0.915	85	29.8.2012	MRM	MRM	2	5					
C. furcifer	**(Tchořovice)**	ephemeral pool	CZ	448	49.431	13.840	0.18	11.4.2004	JF	JF	2	3		3		1	2
	(Litoměřice)	ephemeral pool	CZ	142	50.524	14.112	0.07	16.4.2009	VS	JF		2					
C. heberti	**(Mušovský luh)**	ephemeral pool	CZ	170	48.910	16.591	0.001	28.3.2007	MK	JF		1	1				
	(Filena)	ephemeral pool	CZ	187	49.267	17.484	0.001	31.3.2007	MK	JF	2	2	2	1		1	1
	(Střeň)	ephemeral pool	CZ	225	49.705	17.156	0.4	2.4.2007	MK	JF		1					
	(Oldenburg, Wechloy)	permanent ditch	DE	3	53.150	8.166	0.001	8.8.2011	PM	MRM	2	5					
C. insignis	**(Praha, Komořany)**	riverine pools	CZ	190	49.989	14.402	0.06	26.12.2004	JF	JF	2	2		2	2	2	3
	(Průhonice)	ephemeral pool	CZ	300	49.991	14.556	0.002	8.4.2006	MK	MK		1					
C. kikuchii	**Štikárna**	fishpond	CZ	417	50.201	12.634	0.5	22.2.2008	IP	JF	2	4		1		1	2
	(Postřekov)	fishpond	CZ	430	49.447	12.827	0.6	15.4.2006	JB	MK		1					
C. kolensis	**Bajkal**	rift lake	RU	456	51.778	104.809	3172200	7.2010	TM	JF	2	pg		2		1	2
	Östra Ringsjön	glacial lake	SE	53	55.868	13.551	2250	21.11.2005	JPN	JPN		pg					2
C. lacustris	**Mjøsa**	glacial lake	NO	123	60.696	11.003	36500	28.10.2004	JPN	JF	1	4	4	2		1	2
C. ochridanus	**Ohrid**	rift lake	AL	693	40.950	20.710	35800	17.7.2004	SM, SS	JF	3	3	5	1	2		4
C. scutifer	**Maridalsvann**	glacial lake	NO	149	59.984	10.778	380	4.6.2008	JPN	JF		3		1			1
	Semsvann	glacial lake	NO	145	59.858	10.422	75	11.10.2004	JPN	JF		3		2		2	2
	Øvre Neådalsvatn	glacial lake	NO	728	62.777	8.991	499	19.8.1996	LL	JF	2						
C. strenuus	**(Praha, Komořany)**	riverine pools	CZ	190	49.989	14.402	0.06	5.4.2004	JF	JF		2		2		2	2
	(Smyslov pool)	ephemeral pool	CZ	460	49.419	13.803	0.003	7.4.2010	JF	JF	4	2					
	(Dolejší pool)	ephemeral pool	CZ	445	49.433	13.836	0.002	11.4.2004	JF	JF		3		1		1	2
	Postřekov	fishpond	CZ	430	49.447	12.827	0.6	15.4.2006	JJ	MK		2					
	Horní Polka	humic fishpond	CZ	948	48.943	13.671	1.0	10.7.2007	VS	JF		3					
	Lugano	glacial lake	SW	271	45,959	8,895	4870	7.10.2004	SM	JF		1					
Cyclops sp. X	**Velká Amerika**	mining (limestone) lake	CZ	335	49.959	14.196	1.2	21.4.2004	JF	JF		2			1		
	Barbora	mining (lignite) lake	CZ	242	50.643	13.750	65	11.5.2011	JF	JF		4	2	2			2
	Milada	mining (lignite) lake	CZ	146	50.654	13.950	252	8.11.2006	LH	JF	5	2					
	Plußsee	glacial lake	DE	29	54.182	10.445	13	8.2011	DCM	PK		1					
	Esrum	glacial lake	DK	7	56.032	12.410	1730	28.9.2010	MK	JF	2	4	2	2			2
	Östra Ringsjön	glacial lake	SE	53	55.868	13.551	2250	21.11.2005	JPN	JF	1	4					
	Murtensee	lowland lake	SW	429	46.932	7.085	4500	8.2011	MM	PK		2					
Cyclops sp. Y	**Zănoaga**	glacial lake	RO	1997	45.346	22.822	6.0	23.10.2005	MK	JF		2					
	Bucura	glacial lake	RO	2030	45.360	22.875	10	24.10.2005	MK	JF	4	3	2	2		2	2
C. vicinus	**(Praha, Nové Butovice)**	shallow reservoir	CZ	278	50.044	14.350	1.6	17.2.2007	JF	JF	2	2		1			2
	(Řečice)	ephemeral pool	CZ	442	49.432	13.847	0.0003	9.4.2006	JF	JF		2		2			1
	Piburgersee	mountain lake	AT	913	47.195	10.889	13.4	8.2011	PK	PK		2					
	Wörthersee	lowland lake	AT	439	46.625	14.153	1930	8.2011	RF	PK		2					
	Golyam Beglik	reservoir	BG	1526	41.807	24.119	410	13.8.2004	SM	MK		1					
	Eugui	reservoir	ES	627	42.973	-1.513	123	24.7.2013	MRM	MRM	2	1					
	González Lacasa	reservoir	ES	993	42.177	-2.676	152	16.7.2013	MRM	MRM	2	1					
	Zazari	reservoir	GR	598	40.626	21.549	190	16.7.2004	SM	MK		1					
	Šiško	glacial lake	ME	1660	42.898	19.672	2.9	6.10.2008	MK	MK		2					
	Bielersee	lowland lake	SW	429	47.086	7.174	3930	8.2011	MM	PK		2					
A. americanus	**(Hamburg, ZOO)**	pond	DE	12	53.602	9.938	0.08	9.5.2010	TK	TK		2		2		2	2
M. albidus	**(Hamburg, ZOO)**	pond	DE	12	53.602	9.938	0.08	9.5.2010	TK	TK		2	1	1		1	1
M. viridis	**(Smyslov pool)**	ephemeral pool	CZ	460	49.419	13.803	0.003	7.4.2010	JF	JF		2		2		2	3

Table 1. Continued

Nearby geographical name was used for localities with no official name; indicated by parentheses. pg, indicates PCR amplification of a pseudogene. Countries are abbreviated by their two-letter codes according to the International Organization for Standardization, standard ISO 3166: AL, Albania; AT, Austria; BG, Bulgaria; CZ, Czech Republic; DE, Germany; DK, Denmark; ES, Spain; FI, Finland; FR, France; GB, Great Britain; GR, Greece; IS, Iceland; ME, Montenegro; NO, Norway; PL, Poland; RO, Romania; RU, Russia; SE, Sweden; SK, Slovakia; SW, Switzerland; UA, Ukraine.

Identification was carried out according to Einsle (1996b). Specimens were assigned to established morphospecies by comparison of detailed descriptions and figures, not by the usage of the dichotomic key. The identification was subsequently checked using the microcharacters on cephalothoracic appendages described by Hołyńska and Dahms (2004); for species not included in their study (*C. bohater, C. ochridanus, C. vicinus, C. kikuchii*), these traits were newly established.

Molecular analysis

DNA from individual adult female copepods or from egg sacs was extracted in 50 µL of proteinase K solution, following the protocol of Schwenk (1998) and in some cases by the HotSHOT method (Montero-Pau, Gómez, & Muñoz, 2008). The number of analysed individuals per population differed, ranging from one to six (Table 1). For some populations, only a single specimen was analysed because of the scarcity of animals in the samples or because of failure during DNA isolation. For all 71 populations (sampled from 64 localities), a 430 bp long fragment of the mitochondrial gene for the small ribosomal subunit (12S) rRNA was amplified. Additionally, for further phylogenetic analyses, 15 populations (one for each species) were selected and parts of the following three mitochondrial and two nuclear genes were amplified: the small ribosomal subunit (16S) rRNA (350 bp), the cytochrome b subunit (Cytb, 360 bp), cytochrome c oxidase I (COI, 660 bp), the small ribosomal subunit (18S) rRNA (630 bp) and internal transcribed spacer 1 (ITS-1, 420–540 bp).

PCR reactions were run in 20 µL volume containing 1 x PCR Dream Taq buffer (Fermentas), 0.2 mM dNTPs, 4 mM $MgCl_2$, 0.4 mM of each primer, 0.6 U Dream Taq polymerase (Fermentas), and 4 µL of the DNA template. For the amplifications of specific

sequences, published primer pairs were used: 12S (Machida, Miya, Nishida, & Nishida, 2004), 16S (Braga, Zardoya, Meyer, & Yen, 1999; Palumbi & Benzie, 1991), 18S (Spears, Abele, & Kim, 1992), COI (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), Cytb (Merritt et al., 1998), ITS-1 (Chu, Li, & Ho, 2001); all are listed in Table 2. The PCR cycle consisted of the following steps: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 94 °C for 45 s, annealing for 45 s at 60 °C (for 12S, 16S, 18S, ITS-1) or at 48 °C (COI and Cytb), and elongation at 72 °C for 1.5 min, with final elongation at 72 °C for 6 min. PCR products were purified by ethanol precipitation or with a QIAquick Gel Extraction Kit (QIAGEN) and sequenced by dideoxynucleotide termination (using the primers marked in Table 2) at Macrogen, Inc., the Faculty of Science, Charles University in Prague, or the University of Valencia using Applied Biosystems PRISM 3730XL and 3130XL DNA Analyzer capillary sequencers. Specimens of *Acanthocyclops americanus*, *Macrocyclops albidus*, *Megacyclops viridis* from Germany and the Czech Republic, respectively, were used as an outgroup. All newly obtained sequences were deposited in GenBank and accession numbers will be provided in a published version of this manuscript.

Table 2. List of PCR primers

Gene	Primer	Sequence	Reference
12S	L13337-12S *	5'-YCTACTWTGYTACGACTTATCTC-3'	Machida et al. (2004)
	H13842-12S	5'-TGTGCCAGCASCTGCGGTTAKAC-3'	
	H13845-12S	5'-GTGCCAGCAGCTGCGGTTA-3'	
16S	16S-arL	5'-CGCCTGTTTATCAAAAACAT-3'	Palumbi & Benzie (1991)
	16S-CB *	5'-ATTCAACATCGAGGTCACAA-3'	Braga et al. (1999)
18S	18s329	5'-TAATGATCCTTCCGCAGGTT-3'	Spears et al. (1992)
	18sI- *	5'-AACTCAAAGGAATTGACGG-3'	
COI	LCO-1490 *	5'-GGTCAACAAATCATAAAGATATTGG-3'	Folmer et al. (1994)
	HCO-2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	
Cytb	UCYTB151-F *	5'-TGTGGRGCNACYGTWATYACTAA-3'	Merritt et al. (1998)
	UCYTB270-R	5'-AANAGGAARTAYCAYTCNGGYTG-3'	
ITS-1	SP-1-5′138	5′-CACACCGCCCGTCGCTACTA-3′	Chu et al. (2001)
	SP-1-3′ *	5′-ATTTAGCTGCGGTCTTCATC-3′	

* primer used for sequencing

Data analysis

All sequences were checked twice manually in MEGA v5 (Tamura et al., 2011). Initially, the dataset of 12S sequences was analysed. The sequences were aligned by the PRANK algorithm (Löytynoja & Goldman, 2005), a phylogeny-aware method specifically

developed to deal with alignments containing gaps, using the Prankster interface (http://www.ebi.ac.uk/goldman-srv/prank/prankster/). Afterwards the dataset was tested for poorly alignable regions using Gblocks Server 0.91b (Castresana, 2000; Talavera & Castresana, 2007), with default settings but allowing gaps, and the ambiguously aligned positions detected were subsequently excluded. This process resulted in a 350 bp-long alignment. The TrN+I+G model of evolution was chosen by corrected Akaike Information Criterion (AICc) in jModelTest 2 (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003). A maximum-likelihood (ML) tree was constructed in GARLI with 20 replicates, and bootstrapping was done with 1000 replicates. Bootstrap results were summarized using the SumTrees script of the DendroPy package v3.12.0 (Sukumaran & Holder, 2010). The ML tree obtained was visualized using FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and used as the basis for further species identification.

Next, the sequence data of other genes were analysed for selected populations (Table 1). The sequences for the protein coding genes (COI and Cytb) were aligned separately using ClustalW (Thompson, Higgins, & Gibson, 1994) and checked with translating into protein sequences in MEGA. The alignments of non-coding sequences (12S, 16S, 18S and ITS-1) were done using Prankster and Gblocks Server, as described above. Genetic distances for each gene were calculated under Kimura's two-parameter (K2P) model with pairwise deletion of missing data in MEGA. Substitution saturation was tested for each gene in DAMBE v5.3 (Xia, 2013) to inspect any loss in phylogenetic signal. SequenceMatrix (Vaidya, Lohman, & Meier, 2011) was used to concatenate the sequences from the six loci, resulting in a 2531 bp-long alignment.

Optimal partitioning schemes and substitution models of molecular evolution were selected with PartitionFinder v1.1.1 (Lanfear, Calcott, Ho, & Guindon, 2012; Lanfear, Calcott, Kainer, Mayer, & Stamatakis, 2014). The dataset was divided into 10 partitions (12S, 16S, 18S, ITS-1, the three COI and three Cytb codon positions) and all combinations were tested. For subsequent Maximum Likelihood (ML) inference in GARLI v2.0 (Zwickl, 2006), the following five-partition scheme was selected under the AICc: 12S and 16S with the GTR+I+G model; first codon positions of COI and Cytb with TrN+I+G; second positions of COI and Cytb with K81uf+I+G; 18S and third positions of COI and Cytb with HKY+G; ITS1 with GTR+G. For Bayesian inference (BI) in MrBayes v3.2.2 (Ronquist et al., 2012), the

three-partition scheme was selected under the Bayesian Information Criterion (BIC): 12S, 16S, first and second positions of COI and Cytb with the HKY+I+G model; 18S and ITS-1 with K80+I+G; third positions of COI and Cytb with HKY+G. Model parameters and branch lengths were unlinked between partitions.

A maximum-likelihood tree was constructed in GARLI with 20 replicates, and bootstrapping was done with 1000 replicates, as described above. Bayesian inference was performed in MrBayes with two replicates of four chains each for 15 million generations, sampling every 100 generations. Parameters for each partition were unlinked and rates were allowed to vary independently. The first 25 % of both runs were discarded as burn-in. Convergence of parameters and topologies were checked using the standard MrBayes diagnostics and assessed in Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). To prevent the potential loss of phylogenetic information caused by excluding selected alignment gaps with Gblocks Server, the software package SATé v2.2.7 (Liu et al., 2012) was used as another alternative. Its iterative algorithm involves repeated alignment and tree searching operations. The original data are divided into smaller subproblems by a tree-based decomposition and these subproblems are aligned and further merged for the inference of a phylogenetic tree. The alignments were done by the PRANK algorithm (Löytynoja & Goldman, 2005), subproblems merged by MUSCLE (Edgar, 2004) and trees estimated with an approximately-maximum-likelihood method by FastTree (Price, Dehal, & Arkin, 2010), all implemented in SATé.

Morphological analysis

For the detailed microscopic analysis of microcharacters, the following specific procedure for copepod dissection and preparation of permanent slides was developed. About 5 ethanol-preserved specimens were transferred in a small glass beaker and 1 ml of 10 % KOH was added. The beaker was covered with a large cover slip and heated at 80 ºC for 20 minutes (formalin-preserved specimens need to be heated at 90 ºC for 30 minutes; the optimal variant for different samples must be tested) to dissolve all the soft tissues of the specimens; only translucent chitinous envelopes remained. Washing off the hydroxide was done using a chamber made of a small plastic test tube, the bottom of which was replaced by a nylon netting of 40 μm mesh size. The washed copepods were then stained overnight in water with

a few drops of chlorazol black in ethanol. The stained copepods were transferred into a 1:1 mixture of 70 % ethanol and glycerol and heated at 80 ºC until the water and ethanol evaporated, which made them ready for dissection.

The subsequent dissection was performed in a drop of glycerol under a stereo microscope. Dissection needles consisted of short, well-sharpened tungsten wires of 0.3 mm diameter, attached to inoculating loop holders. For the morphological determination, the following parts were isolated and mounted individually in a series of permanent mounts: antennules (A1), maxillules (Mxl), maxillipeds (Mxp), the first pair of swimming legs (P1) and fourth pair of swimming legs (P4). The dissected parts were transferred, one at a time, on the tip of a needle from glycerol into a small drop of the synthetic mounting medium Hydro-Matrix (Micro-Tech-Lab) onto a slide. Due to the high viscosity of glycerol, the transfer of even the smallest objects (Mxl, Mxp) was relatively easy. After proper orientation of the transferred part (e.g. the caudal side of P4 upwards) the object was covered by a cover slip. If the drop of Hydro-Matrix was small enough, parts such as the antenna, maxilliped and swimming legs were pressed by the cover slip into a single plane, which allowed all important features to remain in focus at one time. After mounting, the slides were placed horizontally until the Hydro-Matrix medium solidified.

Microcharacters were observed and documented with the use of a compound microscope (objective lenses 40x and HI 100x, bright field or phase contrast) equipped with a digital camera. To remove artefacts and irrelevant objects, the photographs were retouched in Adobe Photoshop CS3 using the basic tools. All objects mounted on permanent slides are deposited at the Department of Ecology, Charles University in Prague. In addition to this, scanning electron microscope images were taken to examine the surface ornamentation of antennules and thoracic segments.

Results

Molecular analysis

In total, 540 *Cyclops* individuals were analysed with the molecular methods and 372 DNA sequences were obtained (Table 1). The amplification success rates varied for different genes. Primary, we tried to amplify the sequences of COI, a marker broadly used in phylogenetic studies (Hebert, Cywinska, Ball, & deWaard, 2003). However, the commonly

used COI primer pair (Folmer et al., 1994) worked just for a few populations (Table 1) and amplification with other COI primers (Machida et al. 2004; Dirt Steinke, unpublished data) was also usually unsuccessful. On the other hand, amplification of the 12S gene using a primer pair described by Machida (Machida et al., 2004) was the most successful, even though pseudogene sequences were amplified for a few individuals of *C. abyssorum*, *C. scutifer*, *C. kolensis* and *C.* sp. X. The genes for 16S and CytB were amplified in 73 % and 53 % of the species under study, respectively. Amplification of the nuclear genes for 18S and ITS-1 was successful in most cases (Table 1), except for two populations of *C. strenuus*, where the DNA of epibiont organisms (*Vorticella*) was amplified instead.

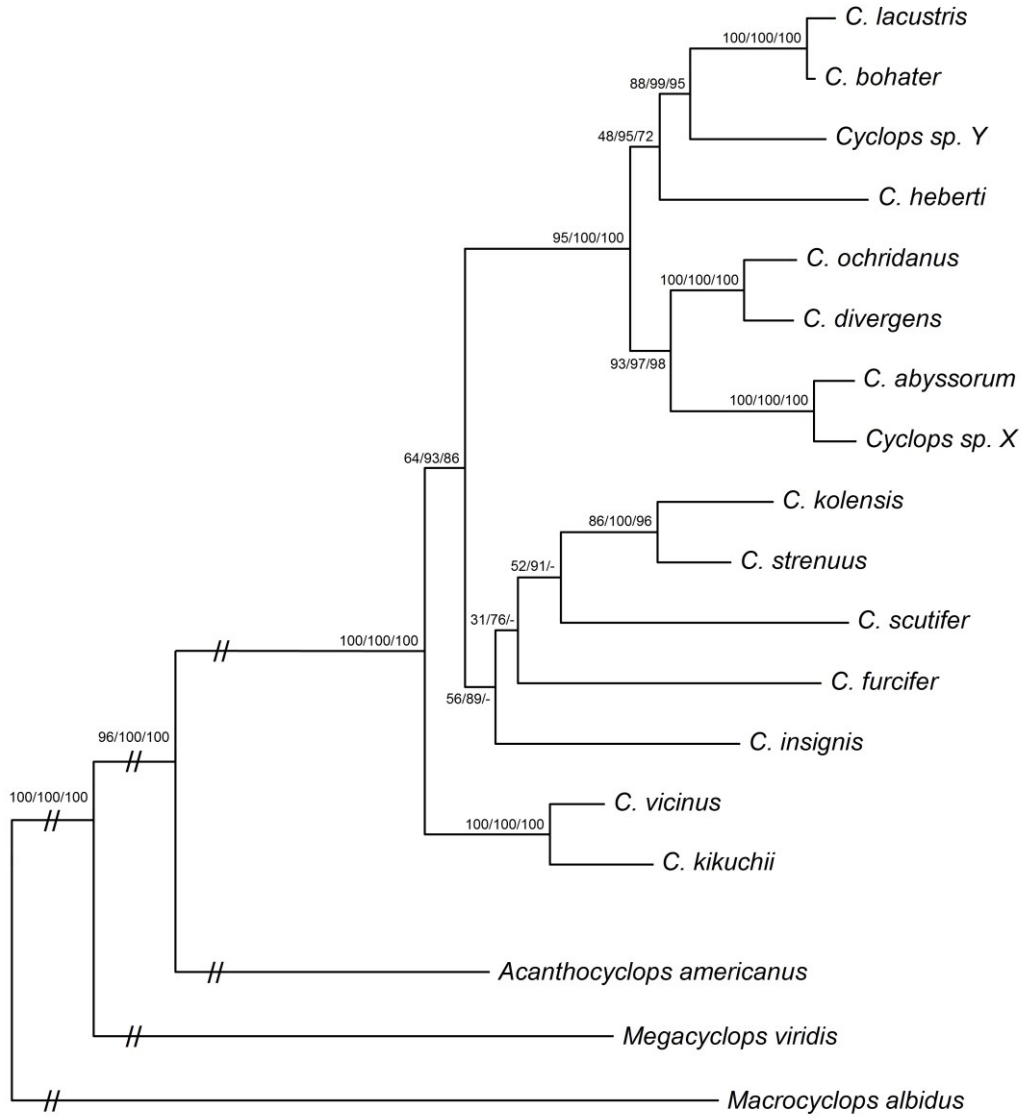


Figure 1. Phylogenetic relationship of *Cyclops* species.

The maximum likelihood tree was based on the 2531 bp-long alignment consisting of fragments of

mitochondrial genes for 12S rRNA, 16S rRNA, cytochrome b and cytochrome c oxidase I and the nuclear genes for 18S rRNA and internal transcribed spacer 1. Numbers at nodes indicate branch supports (as percents) assessed by maximum likelihood (GARLI), Bayesian inference (MrBayes) and approximately-maximum-likelihood (SATé).

The substitution saturation detected by DAMBE was low, thus all six studied gene partitions had a sufficiently high phylogenetic signal. The inter-specific K2P distances among *Cyclops* species were 0 – 0.7 % in 18S, 0.2 – 24.3 % in ITS-1, 4.0 – 19.0 % in 16S, 17.3 – 32.9 % in COI, 21.8 – 39.9 % in cyt b, and 1.3 – 42.2 % in 12S, while those within species were always lower.

The final phylogenetic tree of the concatenated dataset (Figure 1) was based on the ML topology obtained from GARLI, with the bootstrap support values of BI and the analysis of SATé shown. The ML and BI topologies were identical, while the SATé topology differed just in the position of one branch (*C. scutifer*). All 15 *Cyclops* species (including the two new lineages of *C.* sp. X and *C.* sp. Y) were well separated and their independent species status confirmed. Three main branches were distinguished on the phylograms: an upper branch (consisting of *C. lacustris*, *C. bohater*, *C.* sp. Y, *C. heberti*, *C. ochridanus*, *C. divergens*, *C. abyssorum* and *C.* sp. X), a middle branch (consisted of *C. kolensis*, *C. strenuus*, *C. scutifer*, *C. furcifer*, *C. insignis*) and a lower branch (with related species pair of *C. vicinus* and *C. kikuchii*). Some of the morphological traits were found to be specific for species in particular branches (Table 3). The outgroup species were isolated as well, with *A. americanus* the most closely related and *M. albidus* the most distant. The phylogenetic relationships revealed from 12S sequences (Figure 2) and from the concatenated dataset (Figure 1) were very similar.

The two lineages exhibiting differences in DNA sequences on the species level but not matching any described species were tentatively designated as *Cyclops* sp. X and *Cyclops* sp. Y. *Cyclops* sp. X was first found in the plankton of three man-made lakes in the Czech Republic, flooded basins resulted from mining lignite (Barbora, Milada) and limestone (Velká Amerika). Later findings came from Scandinavia (Esrum, Östra Ringsjön), Germany (Pluβsee) and Switzerland (Murtensee). *Cyclops* sp. Y came from two high mountain lakes in the Retezat Mountains, Romania (Bucura, Zănoaga).

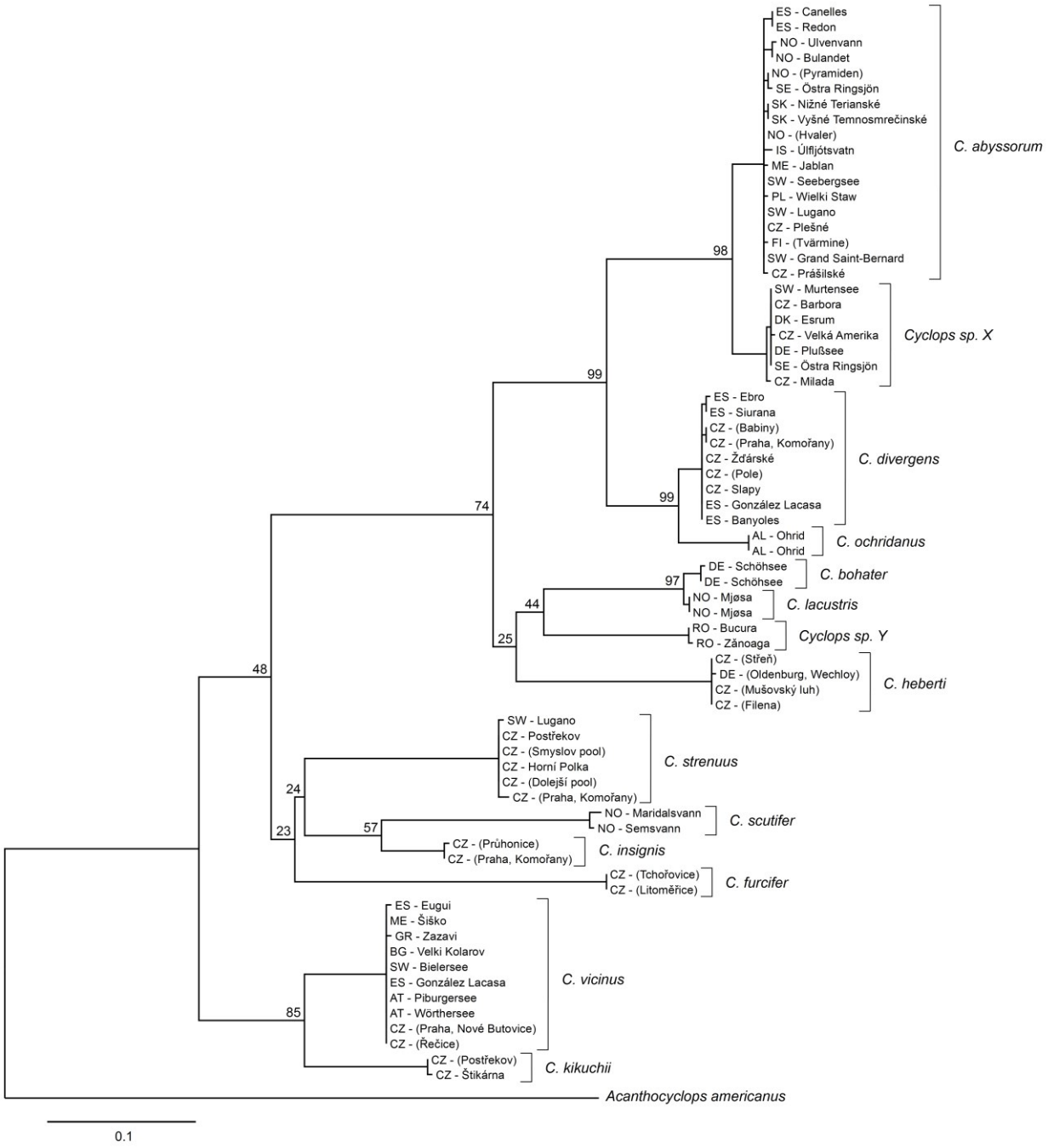
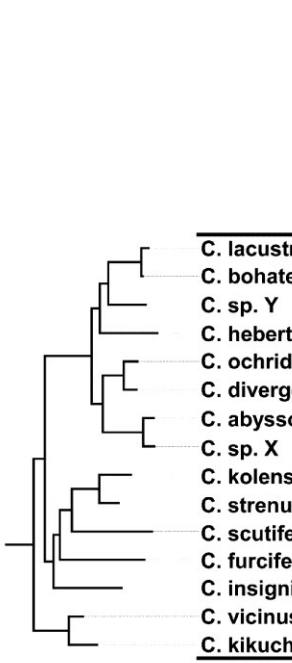


Figure 2. Sequence variation and relationship of *Cyclops* populations based on the mitochondrial 12S rRNA gene shown on a maximum likelihood tree. The scale bar represents genetic distance; numbers at nodes indicate branch supports (as percents). Countries are indicated by two-letter codes (see Table 1). Nearby geographical name was used for localities with no official name; indicated by parentheses.

Table 3. Phylogenetic relationship of *Cyclops* species and table of morphological characters.



	1) **A1 proximal segments:** small pits	2) **Maxillular palp:** proximal seta with long hairs	3) **Maxilliped syncoxopodite:** spinules long and oblique to the longitudinal axis	4) **P1 basipodite:** row of long spinules on the frontal surface	5) **P4 coxopodite:** groups of spines on the caudal surface	6) **P4 coxopodite - group C:** stout first spine	7) **P4 coupler humps:** distinctly extend the margin	8) **P4 coupler lateral setae:** thickened at the base	9) **P4 basipodite:** long hairs at the postero-interior angle	10) **Th4:** distinctly widest at the posterior tips	11) **Spine formula:** Bini (B), Terni (T) or Variable (var)	source
C. lacustris	n	**Y**	n	**Y**	A(B)CDE	n	n	n	n	n	**T**	E,F
C. bohater	n	**Y**	n	**Y**	A(B)CDE	n	**Y**	n	n	n	**T**	E,F
C. sp. Y	n	n	n	**Y**	AC(D)E	n	n	n	**Y**	n	**T**	F
C. heberti	**Y**	**Y**	n	**Y**	AC(E)	**Y**	**Y**	n	n	n	**T**	E,H&D,F
C. ochridanus	n	**Y**	n	n	ABC(D)	n	n	n	n	n	**T**	E,F
C. divergens	**Y**	**Y**	n	**Y**	ACDE	n	**Y**	n	n	**Y**	**T**	E,H&D,H, F
C. abyssorum	var	**Y**	n	**Y**	AC(D)E	n	**Y**	n	n	var	**T**	E,H&D,H, F
C. sp. X	n	**Y**	n	**Y**	ABC(D)E	n	var	n	n	n	**T**	F
C. kolensis	n	n	**Y**	**Y**	AC(D)	n	n	n	n	**Y**	B	E,H&D,F
C. strenuus	**Y**	n	n	**Y**	AC(D)	n	n	n	n	n	**T**	E,H&D,F
C. scutifer	n	n	**Y**	**Y**	ABCD	n	n	**Y**	n	**Y**	var	E,H&D,F
C. furcifer	**Y**	**Y**	n	n	ABC	n	n	n	n	n	var	E,H&D,F
C. insignis	**Y**	n	n	n	ABC	n	n	**Y**	n	n	B	E,H&D,F
C. vicinus	n	n	**Y**	n	ABCDE	n	n	n	n	**Y**	B	E,F
C. kikuchii	n	n	?	n	ABCDE	n	n	n	n	**Y**	B	E,F

A1, antennula; P1, first pair of swimming legs; P4, fourth pair of swimming legs; Th4, fourth thoracic segment; var, variable character; ?, character not examined.

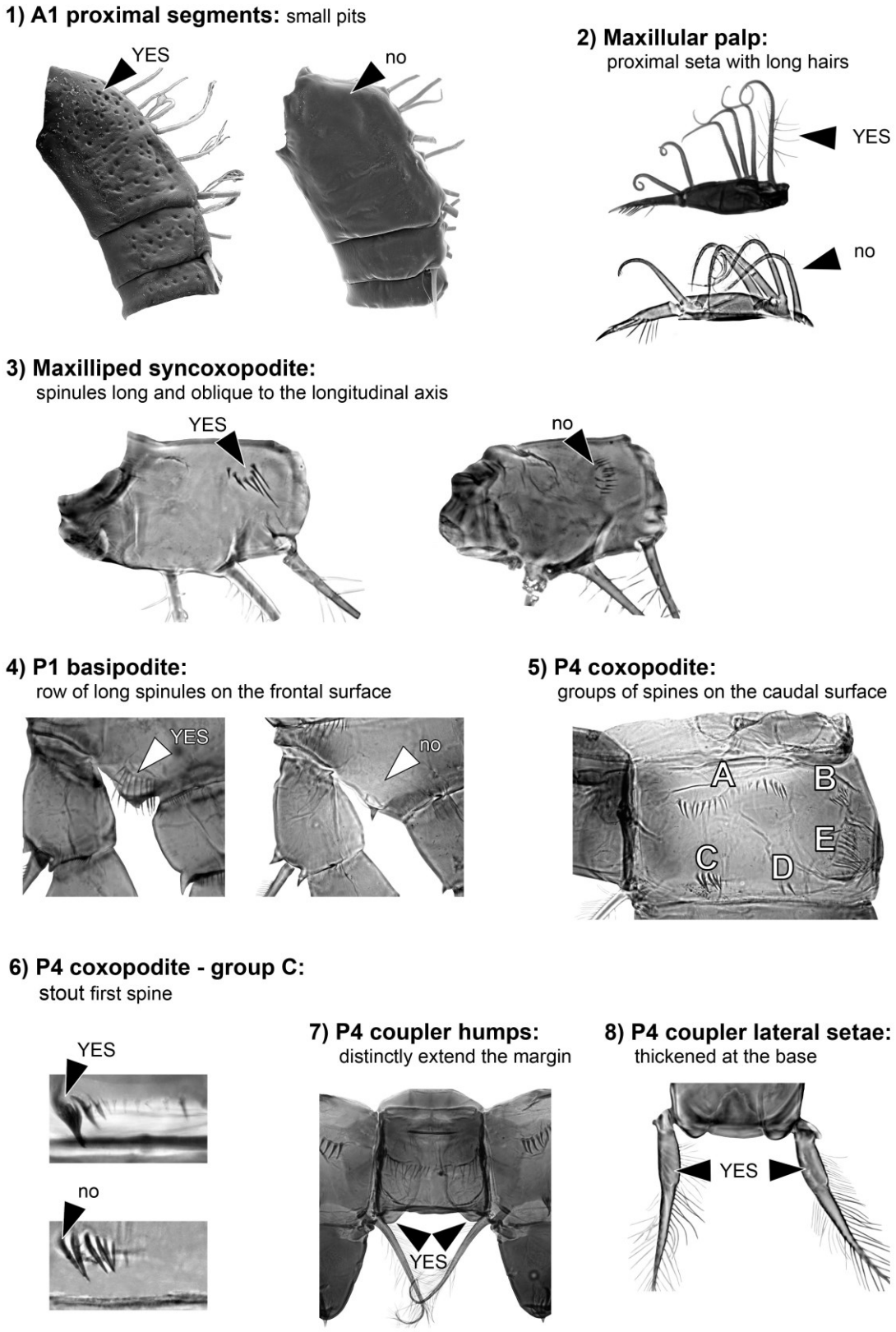


Figure 3. Ten morphological characters of Cyclops species

illustrated by scanning electron microscope images (character No. 1) or by photographs from optical microscope (characters No. 2 – 10). Mostly, both character states are displayed and marked. A1, antennula; P1, first pair of

swimming legs; P4, fourth pair of swimming legs; Th4, fourth thoracic segment.

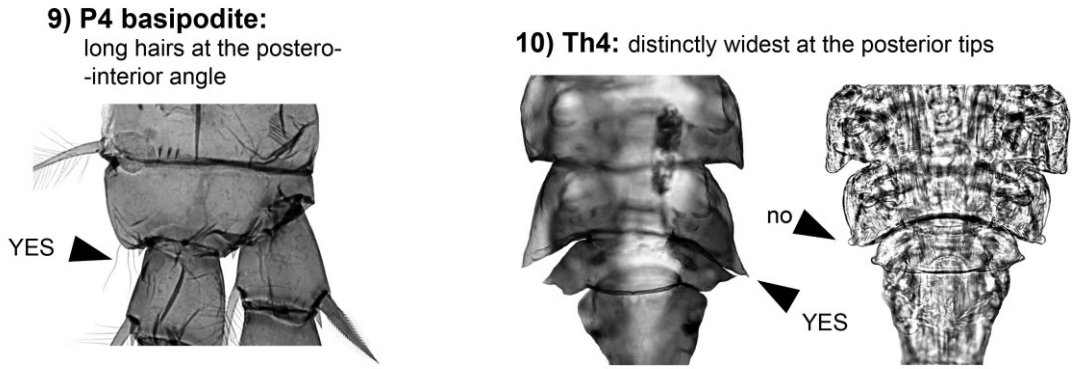


Figure 3. Continued

Morphological analysis

Permanent slides were prepared for all 15 morphospecies under study. In total, 122 individual copepods were dissected and observed for morphological characters (Table 1). The following microcharacters were considered to be relatively easy for examination and species identification (Figure 3 and Table 3): 1) The proximal segments of antennules (A1) may have small pits on the surface. These pits were mentioned by Einsle (Einsle, 1996a, 1996b) in *Cyclops heberti* and *C. divergens* (syn.: *C. singularis*) as "small deepened circles (tiny craters)" on the dorsal front side of the first four articles. The scanning electron microscope images also confirm this surface ornamentation on the dorsal parts of the thoracic segments and on the genital double-segment. When using an optical microscope, the ornamentation is readily observable on the surface of A1 articles, which can be therefore used as a standard for routine examination. 2) The proximal-most seta of the maxillular (Mxl) palp is equipped with long hairs in some species (Hołyńska & Dahms, 2004; Hołyńska, 2008). 3) The maxilliped syncoxopodite may be ornamented at the frontal surface with long spinules oriented obliquely to the longitudinal axis of the segment. Alternatively, the spinules are parallel to the axis or they are very short with no apparent orientation (Figures 9, 10 in Hołyńska and Dahms 2004). 4) The basipodite of first swimming legs (P1) may bear a row of long spinules on the frontal surface, above the large spine between the insertion of the endo- and exopodite. In other species the long spinules are missing (Hołyńska & Dahms, 2004). 5) The ornamentation of the coxopodite of the fourth swimming legs (P4) is an important character for species identification, firstly described by Einsle (Einsle, 1985, 1996b). Groups of spines at the

caudal surface are labelled A, B, C, D, E, F. Groups A and C are always present, the presence of groups B, D and E may be species-specific. Variation in the presence or absence among or within populations of the same species is marked by parentheses (Table 3). Group F was not considered here because of its high variation. 6) *Cyclops heberti* is easily recognizable by a single microcharacter: the first spine of group C is remarkably stout, the following spines in this line are much smaller. This character was well illustrated by Einsle (Einsle, 1996a, 1996b) but not mentioned by him in the text. 7) The coupler of P4 has two humps that can distinctly surpass the coupler margin or not. This character was used extensively by Einsle (Einsle, 1985, 1996b). 8) The P4 coupler lateral setae are thickened at the base in *C. scutifer* and *C. insignis*. 9) A single lineage (*Cyclops* sp. Y) is distinguishable by the presence of long hairs at the posterio-interior angle of the P4 basipodite. This character has not been recorded in any other *Cyclops* species so far. 10) The fourth thoracic segment (Th4) has different shape among *Cyclops* species when observed from the dorsal view, being widest in the posterior tips or in the middle of the segment. Evaluating this character requires the observation of both posterior tips in the same focal plane. 11) The spine formula expresses the number of spines of last exopodite articles of P1 – P4. Some species have spine formula 2 3 3 3 (Bini) and some 3 4 3 3 (Terni). In two species (*C. scutifer* and *C. furcifer*) the spine formula may be variable (Einsle, 1996b).

Comparing diagnostic traits on cephalothoracic appendages described by Hołyńska and Dahms (2004) with our findings on the same species (*C. abyssorum, C. divergens, C. heberti, C. kolensis, C. strenuus, C. scutifer, C. insignis, C. furcifer*), there is generally mutual agreement and little variation. One exception is the spinule ornamentation on the caudal surface of the antennal coxobasis, which, considering its complexity, was not included in this study. Regarding the spinule ornamentation of the caudal surface of P4 (trait No. 5, Table 3), it was confirmed that groups A and C are present in all *Cyclops* species. Group D, when present in a low number of spines, may vary down to zero within a single population (*C. abyssorum*, *C. strenuus*, *C.* sp X, *C.* sp Y). An interesting case is the distribution of group B, which was absent in all populations of *C. abyssorum* confirmed by molecular analysis. However, it was reported in some Scandinavian *abyssorum* populations by Einsle (1996b) and Hołyńska (2008), although with some variation. Surprisingly, we found the occasional occurrence of the B group in a population of *C. divergens* from a shallow astatic aestival pool and co-occurring with the tadpole shrimp *Triops cancriformis* (location Babiny).

Arranging the sequence of species in Table 3 according to their distribution in the phylograms makes it possible to estimate the distribution of morphological traits within the main branches. Unexpectedly, trait No. 2: long hairs on the proximalmost seta of the maxillular palp, occurs in the species of the upper branch (*C. lacustris*, *C. bohater*, *C. heberti*, *C. ochridanus*, *C. divergens*, *C. abyssorum*, *C.* sp. X), with the only exception of *C.* sp. Y. The remaining part of the phylogram is occupied by species without this trait (*C. kolensis*, *C. strenuus*, *C. scutifer*, *C. insignis*, *C. vicinus*, *C. kikuchii*), with the exception of *C. furcifer*. The spine formula in species of the upper branch is exclusively 3 4 3 3, while in the rest of phylogram the formula is 2 3 3 3 (*C. kolensis*, *C. insignis*, *C. vicinus*, C. *kikuchii*) or variable (*C. furcifer*, *C. scutifer*), with one exception: *C. strenuus*.

Discussion

Accepting that morphospecies are hypotheses that should be tested by different approaches (Dayrat, 2005), the main objective of this study was to test the present "morphological" state-of-the-art in *Cyclops* taxonomy by analysing the DNA sequences of particular genes. In this process the delineation of cohesive lineages was the primary task, which was followed by assignment of the lineages to the presently recognised morphospecies. This process led to 13 successful matches, solving some problems in the taxonomy of *C. abyssorum* and the discovery of two new species, which altogether considerably clarifies the current taxonomy in *Cyclops*.

One of the most problematic species of the genus is *Cyclops abyssorum*, with several described morphotypes or subspecies (Kiefer, 1978; Einsle, 1980, 1996b; Hołyńska, 2008). Our molecular data on populations assigned to *C. abyssorum* (based on the specimens from the type locality area, Ulvenvann) covered several Scandinavian populations, a population from a large and deep subalpine lake (Lugano, "*praealpinus*"-type) and several high mountain lakes (Pyrenees, Alps, Tatras, Dinarids, "*tatricus*"-type), but no substantial variation in 12S or 16S sequences was observed. The values for K2P distances did not exceed 1.6 % in 12S and 1.3 % in 16S, which is within the normal range of intraspecific variation. Regarding this species, it should be noted that all populations of *C. abyssorum* confirmed by molecular analysis come from glacial lakes and none from small shallow waters. The only exceptions are those from rock pools in coastal areas of Norway (Hvaler) and Finland (Tvärmine). These

habitats, although small, are cold and stable, which perhaps make them suitable for typical lake dwellers like *C. abyssorum*. Any findings of *C. abyssorum* from small and shallow waters in central and southern Europe are thus suspect and need revision.

In light of the fact that *C. abyssorum "divergens"*, *C. abyssorum "divulsus"* and similar morphotypes have been included in the range of variation of *C. abyssorum* (Kiefer, 1978; Dussart & Defaye, 1985; Einsle, 1996b), some findings identified as *C. abyssorum* were actually *Cyclops divergens* (Lindberg, 1936) comb. nova. That is the case of *Cyclops* from Spanish and Czech reservoirs (Armengol, 1978; Jaume, 1993; Brandl & Lavická, 2002).

We named *Cyclops divergens* the morphotype that corresponds to *C. abyssorum divergens* described by Lindberg in 1936. Later on, the same author described other closely related subspecies (*mauritaniae, divulsus, corsicanus* etc.) which Dussart and Defaye (1985) placed within the same group; however, because the date of publication is more recent, these names would most probably constitute younger synonyms. Hołyńska (2008) synonymized the species that Einsle (1996b) described as *C. singularis* with *C. abyssorum divergens* (Lindberg). The type locality of *C. singularis* was Litzelsee, an ephemeral pool in a depression in a cultivated field. This species was described with the use of chromatin diminution and enzyme electrophoresis methods, and until that time it had been considered together with *C. heberti* as a variation of *C. furcifer*. Our results clearly separate all these three species. Whereas the ecological valence of *C. furcifer* and *C. heberti* is as yet limited to small water bodies, the habitat of *C. divergens* is very broad.

Since 2003 we collected this species repeatedly in the plankton of the Slapy reservoir, and molecular analysis undoubtedly identified it as *C. divergens*. It was also later identified from the plankton of other deep canyon-shaped reservoirs in the Czech Republic. Our molecular analyses of *Cyclops* from reservoirs or karst lake in Spain (Banyoles, Ebro, González Lacasa, Siurana) also confirm *C. divergens*. This is in accordance with the suggestion of Hołyńska (2008) that the distribution of *C. divergens* (*C. abyssorum divergens* in her interpretation) is not restricted to shallow, often astatic waters, but covers deep water bodies as well. Its occurrence in the plankton of riverine reservoirs is not surprising if we take into account the downstream dispersal from floodplain pools by floods.

Cyclops strenuus, the first described species of the genus, is an ecologically variable species that can be found in different types of pools, fishponds, reservoirs and lakes.

According to our observations it often dominates brown-water pools in woodlands or on the edges of forests, while pools in meadows and fields are inhabited by other species (*C. divergens*, *C. heberti*, *C. furcifer*, *C. vicinus*).

The most frequent species in the Czech Republic, from where we have the most data, is *Cyclops vicinus*. This species is common in fishponds and reservoirs as well as in small astatic waters. We found it in reservoirs and lakes in Spain, Switzerland, Austria, Bulgaria, and Greece, as well as in the glacial mountain lake Šiško, Montenegro (1660 m). Interestingly, in spite of this large area most of the analysed individuals shared a single 12S haplotype, which suggests this species has excellent dispersal ability and has expanded recently.

Cyclops kikuchii and *C. vicinus* are the only species pair which cannot be distinguished by any of the traits mentioned in Table 3, but they are well separated on both phylograms. The main morphological character used to differentiate them is the relative length of the terminal furcal setae (Einsle, 1996b).

Cyclops sp. X seems to be widely distributed in Europe but until now it has not been recognized as a distinct species. It has been reported either as *C. strenuus* (Bosselmann, 1974) or *C. abyssorum*. A full description and naming as a new species is beyond the scope of this study.

Cyclops sp. Y bears some resemblance (size, furca, genital double segment) to *Cyclops ricae* (Einsle, 1996b; Monchenko, 1977) known from lake Ritza, Great Caucasus. The taxonomic status of *Cyclops* sp. Y can only be evaluated after DNA analysis of the Ritza population.

It may seem that because of their universality, molecular methods are more feasible than morphological ones in copepods, which need cumbersome dissection and special expertise. But the two approaches are complementary and one cannot stand without the other. The negative fame of copepod dissection comes from insufficient know-how as the time-tested technique has rarely been described in detail. By applying the method described here, one finds out that dissection of even small structures is relatively easy and can be mastered in several hours. Because not all obtained photographs of analysed microcharacters could be included in this paper, a new website www.copepoda.cz was established for this purpose. Different sections contain photo galleries that can help with correct species identification.

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Chapter 3:

Congruent patterns of lineage diversity in two species complexes of planktonic crustaceans, *Daphnia longispina* (Cladocera) and *Eucyclops serrulatus* (Copepoda), in East European mountain lakes

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My long-term interest in the Balkan Mountains resulted in a representative collection of zooplankton samples from different mountain ranges covering Romania, Bulgaria, Macedonia, Albania, Montenegro and Bosnia and Herzegovina. The first analysis confirmed the presence of the cyclopoid *Eucyclops serrulatus* and the cladoceran *Daphnia longispina* in many of the lakes. We decided to add several samples from the Tatra Mountains in Slovakia as well as a few lowland reference localities from Europe and compare the mitochondrial sequence variation in both species.

We were expecting significant differences in lineage diversity because of the different reproductive modes and dispersal abilities of both studied taxa. Then, we were interested if mountain lakes will harbour some cryptic lineages of *Daphnia* or *Eucyclops*. The preliminary results showed huge sequence divergences in the lowland *Eucyclops* populations indicating possible cryptic speciation. In total eight highly divergent *Eucyclops* clades were found, but mountain areas were inhabited just by two of them. This result didn't confirm our hypothesis that mountain lakes could be a substantial source of cryptic speciation.

Also *E. serrulatus* doesn't seem to be more dispersal limited than *D. longispina*, even though cladocerans produce long-lived dormant eggs ideal for dispersal. This indicates that copepods likely have some dispersal advantage that is not yet known or they are somehow more successful in dominating new localities. This hypothesis might be supported by other research on the colonisation of new temporary ponds (Frisch & Green, 2007). The high amount of discovered cryptic lineages in *Eucyclops* also represents possibilities for further research in the future.

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Congruent patterns of lineage diversity in two species complexes of planktonic crustaceans, *Daphnia longispina* (Cladocera) and *Eucyclops serrulatus* (Copepoda), in East European mountain lakes

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Cladocerans and copepods are globally important freshwater zooplankton groups, differing in reproductive modes and dispersal abilities. We compared genetic variation of two common taxa of these crustaceans, the *Daphnia longispina* species complex (known to harbour multiple cryptic lineages) and *Eucyclops serrulatus* (morphologically and ecologically variable morphospecies), in lakes of ten Eastern European mountain ranges. We expected to discover cryptic lineages in both groups, and to observe different geographical patterns of diversity because of differences in life cycles. Within *E. serrulatus*, limited sampling through lowland habitats indeed showed the presence of eight highly divergent clades, probably cryptic species, but most of these were not found in the studied mountain lakes. Such a pattern was congruent with the diversity of the *D. longispina* complex. Regional coexistence of multiple clades within respective species complexes (two in *Eucyclops* and three in *Daphnia*) was observed only in the Tatra Mountains (on the Polish–Slovak border). In all other studied mountain ranges (in the Balkans), only single lineages of *Daphnia* and *Eucyclops*, respectively, were present, showing similar intraspecific patterns and no evidence for stronger dispersal limitation in *Eucyclops* than in *Daphnia*. Our results indicate that substantial cryptic variation may be expected in seemingly widespread copepod taxa. However, detection of cryptic lineages is not a general pattern in mountain lakes, although these habitats harbour substantial genetic diversity in crustacean zooplankton.

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INTRODUCTION

Detailed molecular studies of freshwater zooplankton taxa have shown that their global diversity is much higher than previously assumed. Species considered as cosmopolitan or widespread have often been discovered to be cryptic complexes, a phenomenon observed in most major zooplankton groups, including cladocerans (e.g. Rowe, Adamowicz & Hebert, 2007; Petrusek *et al*., 2009; Xu *et al*., 2009, 2011), copepods (e.g. Lee, 2000; Grishanin *et al*., 2005; Karanovic & Krajicek, 2012), and rotifers (e.g. Gómez *et al*., 2002; Xiang *et al*., 2011).

The diversity of cladocerans has been relatively well studied, and some cladoceran taxa, especially *Daphnia* O.F. Müller, 1785, have also been subjects of numerous molecular analyses. Still, only about

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45–50% of cladoceran species may be considered well described and valid, and many taxa are likely to represent species complexes (Korovchinsky, 1996; Forró *et al*., 2008). This is also true for *Daphnia*, which has become a model taxon in many fields of biology. Based on patterns of diversity emerging from molecular studies, its diversity had been estimated to about 200 species (Hebert & Taylor, 1997). This estimate, probably still a conservative one, contrasts with 75 species listed in the recent global monograph on this genus (Benzie, 2005) or almost 90 species considered valid in the checklist compiled by A. A. Kotov within the Freshwater Animal Diversity Assessment project (http://fada.biodiversity.be/group/show/17). The widespread existence of undescribed species within the genus is supported by phylogenetic and biogeographical analyses (Adamowicz *et al*., 2009), and genetic tools facilitated recent taxonomic revisions (Petrusek *et al*., 2008a) as well as characterization and subsequent formal description of new *Daphnia* species (e.g. Kotov, Ishida & Taylor, 2006; Juračka, Kořínek & Petrusek, 2010).

In contrast to daphnids, the evolutionary history and genetic diversity of another important group of freshwater planktonic crustaceans, copepods, is understudied (Boxshall & Defaye, 2008). In our study, we highlight this by comparison of a widespread group of European *Daphnia*, the *Daphnia longispina* species complex, with that of the cyclopoid genus *Eucyclops* Claus, 1893. The *D. longispina* complex is an ecologically important cladoceran taxon, particularly widespread in lake habitats of northern temperate to boreal zones. Some of the widespread species, particularly *D. longispina* O.F. Müller, 1785 and *Daphnia galeata* G.O. Sars, 1864, are often the key grazers in pelagic environments, and have become important study species for ecological and evolutionary studies, e.g. on interspecific hybridization (Schwenk & Spaak, 1995; Petrusek, Seda & Macháček, 2008b), diel vertical migrations (e.g. De Meester, Weider & Tollrian, 1995), and host–parasite interactions (e.g. Wolinska & Spaak, 2009; Yin *et al*., 2012). However, owing to substantial phenotypic variation and relatively few clearly defined species-specific characteristics, the taxonomy of the complex has been confusing. Application of genetic tools in studies of its diversity has resulted in lumping of various forms together (Petrusek *et al*., 2008a) as well as in resurrecting earlier described species (Nilssen *et al*., 2007) and uncovering cryptic variation (e.g. Petrusek *et al*., 2008a; Ishida *et al*., 2011). Interestingly, an analysis of pelagic *Daphnia* diversity from a relatively limited number of lowland lakes in north-eastern Europe resulted in additional discovery of multiple highly divergent lineages within the complex, suggesting that this region is an important centre of diversity (Petrusek, Thielsch & Schwenk, in press).

Species of the genus *Eucyclops* occur as planktonic or littoral epibenthic forms in ponds and lakes worldwide, and represent an ecologically important group of freshwater copepods. More than 135 nominal species and subspecies are known to date (Dussart & Defaye, 2006). However, the taxonomic knowledge of *Eucyclops* is incomplete. Several studies have reported cryptic species, which have been revealed by detailed morphometric analyses (Frost, 1989; Boileau, 1991; McKinnon, Kimmerer & Benzie, 1992; Reid, 1998). Genetic study within the genus (Alekseev *et al*., 2006) has been limited to the analysis of a highly conservative 18S rRNA gene, which, however, is not very suitable for analyses of diversity of relatively closely related lineages that often form species complexes.

For a long time, the type species of the genus, *Eucyclops serrulatus* Fischer, 1851, has been reported from water bodies around the world and was considered to be cosmopolitan, until some taxa were separated from it (Dussart & Fernando, 1985; Alekseev, 2000, 2008; Suárez-Morales & Walsh, 2009). The presumed geographical range of *E. serrulatus* still includes several biogeographical regions. Most records are from the Palaearctic where it extends from North Africa, the Mediterranean basin, and continental Europe across Siberia and perhaps Central Asia (Alekseev *et al*., 2006) to Japan (Ito, 1957; Alekseev & Defaye, 2011). Furthermore, there are numerous older records from various other regions, such as islands in the Pacific (Lindberg, 1955) and Malaysia (Lim & Fernando, 1985), and also recent ones from Australia and North America (Alekseev & Defaye, 2011).

Despite the recent redescription of *E. serrulatus* from its Russian type locality (Alekseev *et al*., 2006), little is still known about its diversity. Three morphological forms, differing in setae morphology of caudal rami and swimming legs, were distinguished within a single water body at the type locality but all easily mated and had fertile offspring (Alekseev *et al*., 2006), suggesting that such morphological differences might not be taxonomically relevant. These morphotypes were also confirmed in a few other European localities, but vast areas of the distributional range of *E. serrulatus* remain unexplored.

Daphnia and *Eucyclops* differ in their reproduction mode, and hence have different diapause and dispersal abilities. *Daphnia* species reproduce by cyclical parthenogenesis, the alternation of asexual and sexual reproduction with production of dormant eggs, whereas *Eucyclops* species reproduce strictly sexually (Gyllström & Hansson, 2004). Long-lived dormant stages ensure easy dispersal of cladocerans, whereas cyclopoid copepods diapause as short-lived immature

instars (Hairston, 1996). Both of these zooplankton taxa disperse passively via animal or environmental vectors (Proctor, Malone & DeVlaming, 1967; Cáceres & Soluk, 2002), and thus the formation of long-lived resistant propagules in cladocerans should increase their dispersal success on a large scale. In addition to that, in parthenogenetic cladocerans, establishment of new populations is possible from a single propagule, whereas copepods require both sexes to colonize a new habitat. This suggests that *Daphnia* should have an advantage over *Eucyclops* when colonizing new and remote water bodies, such as alpine lakes. Despite this, initial studies on *E. serrulatus* showed it to be one of the most widespread taxa in mountain lake habitats (Jersabek *et al*., 2001), and it is assumed to be relatively well dispersed by waterfowl (Frisch & Green, 2007).

Mountain lakes often harbour populations of zooplankton species that differ morphologically and/or genetically from their lowland counterparts. There are many copepod species adapted to the harsh conditions of alpine lakes (e.g. Liss *et al*., 1998; Jersabek *et al*., 2001; Tolotti *et al*., 2006), and endemic cladocerans are found in these habitats, particularly in the tropics (e.g. Kotov, Sinev & Berrios, 2010; Van Damme & Eggermont, 2011).

Studies on mountain *Daphnia* have also revealed unique forms and genotypes (e.g. Manca, Cammarano & Spagnulo, 1994; Mergeay *et al*., 2008). High diversity may be observed even within relatively small regions. For example, substantial variation in phenotypes of the *D. longispina* complex was observed a century ago in a small mountain range, the Tatra Mountains, the northern range of the Carpathians (Lityński, 1913). Genetic analyses confirmed the presence of three different species of this complex, including two populations of *Daphnia lacustris* G.O. Sars, 1862, a species that is, apart from this mountain range, known at present from Fennoscandia only (Petrusek *et al*., 2007). Similarly high morphological variation of the *D. longispina* group in the Balkan mountain regions was observed by Pljakic (1961). Although several researchers have studied different groups of freshwater zooplankton in the Balkans (for a review see Ostojić, 2010), no studies have focused on genetic diversity of local planktonic crustaceans. As important glacial refugia had been located in this region, both a traditional Mediterranean one in the Balkans and extra-Mediterranean refugia in the Carpathians and elsewhere (Habel & Assmann, 2010), we presume that taxa that survived the harsh glaciation periods in lowland water bodies may have established in the mountain lakes that became available for colonization after glacier melting, and that such a process could have been reflected in the preservation of substantial taxon and genetic diversity in these habitats.

Based on the previously observed patterns of *Daphnia* lineage diversity in the Tatra Mountains, discovery of several cryptic lineages of the *D. longispina* complex in north-east European lowland lakes, and high phenotypic variation documented from the lakes in the Balkans, we thus hypothesized that substantial variation, including the presence of cryptic species, might be found in East European mountain water bodies. To test this prediction, we analysed genetic variation of populations of the *D. longispina* complex and *E. serrulatus* from 38 lakes in ten mountain ranges, and compared them with reference data from other European habitats. Considering the globally understudied genetic diversity in *Eucyclops*, and substantial morphological variation of *E. serrulatus*, we expected that a detailed analysis would reveal it to be a species complex. Our additional aim was to compare the patterns of intraspecific diversity of mountain *Daphnia* and *Eucyclops*, which might be related to their different dispersal potential. We thus hypothesized that patterns of *Eucyclops* diversity will show more regional clustering than *Daphnia* because of limited long-range dispersal.

MATERIAL AND METHODS

Study sites and sampling

Cladocerans of the *D. longispina* complex and cyclopoids identified as *E. serrulatus* were analysed from samples collected from 38 East European mountain lakes sampled between 2007 and 2009 (Table 1). The studied lakes belong to three major European mountain areas (Fig. 1): (1) the Carpathians (20 lakes) represented by the Tatra Mountains (Slovakia) and Retezat (Romania); (2) the Macedonian–Thracian massif (six lakes) represented by Rila (Bulgaria), Pirin (Bulgaria), and Šar Planina (Macedonia); (3) the Dinaric Alps (12 lakes) represented by the mountain ranges Prokletije (Albania, Montenegro), Bjelasica (Montenegro), Durmitor (Montenegro), Zelengora (Bosnia and Herzegovina), and Treskavica (Bosnia and Herzegovina). Nine lakes were inhabited by both studied taxa; in the others either the *D. longispina* complex or *E. serrulatus* were found (Table 1).

From the Tatra Mountain region, only populations of *D. longispina sensu stricto* were included in Table 1 and most analyses, as other species present in this mountain range (*D. lacustris* and *D. galeata*) have already been dealt with in other publications (Petrusek *et al*., 2007; Hamrová, Goliáš & Petrusek, 2010; Hamrová, Mergeay & Petrusek, 2011), and did not have any counterparts in other studied mountain ranges (see Results). Nevertheless, we used the sequence data from populations of these species (two of *D. lacustris*: Toporowy Staw Niżni and

Table 1. List of the sampled lakes, number of analysed individuals, and detected haplotypes in *Daphnia* and *Eucyclops*

Lake	Mountain range	Country	Altitude (m)	Latitude (N)	Longitude (E)	Area (ha)	*Daphnia*		*Eucyclops*		
							N	Hapl.	*N*	Hapl.	Clade
Carpathians											
Capie	Tatra Mts	SK	2075	49.168	20.036	3.0			3	2	II
Dolné Roháčske	Tatra Mts	SK	1562	49.206	19.744	2.2	13	4	3	1	II
Horné Roháčske	Tatra Mts	SK	1719	49.206	19.736	1.4	4	2			
Jamské	Tatra Mts	SK	1448	49.132	20.013	0.7	1	1			
Malé Čierne	Tatra Mts	SK	1566	49.208	20.225	0.07	1	1			
Malé Hincovo	Tatra Mts	SK	1921	49.174	20.057	2.2			3	1	II
Nižné Jamnícke	Tatra Mts	SK	1732	49.203	19.770	1.1	14	5			
Nižné Rakytovské	Tatra Mts	SK	1307	49.126	20.025	0.1	1	1			
Satanie	Tatra Mts	SK	1894	49.171	20.064	0.2	8	2			
Tretie Roháčske	Tatra Mts	SK	1648	49.209	19.737	0.2	7	3			
Velke Žabie	Tatra Mts	SK	1921	49.172	20.077	2.7			3	1	II
Vyšné Furkotské	Tatra Mts	SK	1698	49.144	20.031	0.4	9	1			
Vyšné Jamnícke	Tatra Mts	SK	1839	49.202	19.763	0.4	1	1	3	1	II
Vyšné Račkové	Tatra Mts	SK	1697	49.200	19.805	0.7	9	3			
Vyšné Rakytovské	Tatra Mts	SK	1307	49.125	20.027	0.2	1	1			
Vyšné Wahlenbergovo	Tatra Mts	SK	2157	49.164	20.026	5.2			3	1	I
Zadni Staw Polski	Tatra Mts	PL	1890	49.213	20.013	6.5			2	1	II
Bucura	Retezat	RO	2031	45.360	22.875	8.9	5	2	2	1	I
Lia	Retezat	RO	1910	45.352	22.877	0.6			1	1	I
Păpuşa	Retezat	RO	1855	45.329	22.899	0.1			1	1	I
Macedonian–Thracian massif											
Bliznaka	Rila	BG	2243	42.199	23.317	9.1			2	2	I
Sulzata	Rila	BG	2535	42.197	23.311	0.7	3	3			
Trilistnika	Rila	BG	2216	42.206	23.318	2.6			2	2	I
Ribno Banderishko	Pirin	BG	2194	41.738	23.415	6.5	4	4			
Zhabeshko	Pirin	BG	2230	41.738	23.423	0.6			1	1	I
Bogovinjsko	Šar Planina	MK	1941	41.951	20.792	2.1			4	1	I
Dinaric Alps											
Dashit	Prokletije	AL	2171	42.530	20.077	1.2	4	4			
Jezerce '7'	Prokletije	AL	1763	42.468	19.816	0.5	4	2			
Jezerce '8'	Prokletije	AL	1760	42.467	19.812	0.4			3	1	I
Kocajve	Prokletije	AL	2107	42.533	20.077	0.1			4	1	I
Hridsko	Prokletije	ME	1968	42.571	20.035	1.4	4	1	4	1	I
Blatina	Bjelasica	ME	1771	42.876	19.688	0.8			2	1	I
Malo Šiško	Bjelasica	ME	1792	42.889	19.673	0.4	3	3	3	1	I
Modro	Durmitor	ME	1609	43.086	19.072	0.3	4	3	1	1	I
Valovito	Durmitor	ME	1695	43.096	19.066	0.2	1	1	1	1	I
Velke Škrčko	Durmitor	ME	1686	43.136	19.015	3.0			1	1	I
Jugovo	Zelengora	BA	1537	43.375	18.532	1.1	5	2	3	1	I
Veliko	Treskavica	BA	1555	43.606	18.376	1.9	4	2	4	2	I
Reference *Eucyclops* localities											
Postřekov		CZ	429	49.447	12.826	0.6			2	1	I
Tupadly		CZ	202	50.447	14.472	1 m^2			1	1	III
Hamburg		DE	12	53.602	9.938	0.5			2	1	IV
Suontee		FI	91	61.736	26.111	N/A*			1	1	VIII
Bois de Boulogne		FR	44	48.867	2.264	8.3			2	1	III
Fedderate reservoir		GB	89	57.543	–2.182	0.5			3	1	VII
Inglesham-Lechlade		GB	74	51.676	–1.705	0.2			2	1	V
Lechlade		GB	77	51.707	–1.683	0.1			2	2	V, VI
Whitway		GB	134	51.335	–1.334	0.1			2	1	I
Wigry		PL	127	54.078	23.084	2187			1	1	VIII
Blejsko		SI	289	46.365	14.097	137			2	1	II
Seebergsee		SW	1832	46.578	7.443	6.1			2	1	II
Khotov		UA	129	50.331	30.466	2.5			2	1	IV

*Area was not estimated for Suontee, an interconnected system of many large lowland lakes.
Clade, assignment of *Eucyclops* cf. *serrulatus* to clades (as in Fig. 3); Hapl., number of detected haplotypes; Mts, Mountains; *N*, number of analysed individuals.
Countries are indicated by their two-letter codes according to the International Organization for Standardization, standard ISO 3166: AL, Albania; BA, Bosnia and Herzegovina; BG, Bulgaria; CZ, Czech Republic; DE, Germany; FI, Finland; FR, France; GB, Great Britain; ME, Montenegro; MK, Macedonia; PL, Poland; RO, Romania; SI, Slovenia; SK, Slovakia; SW, Switzerland; UA, Ukraine.

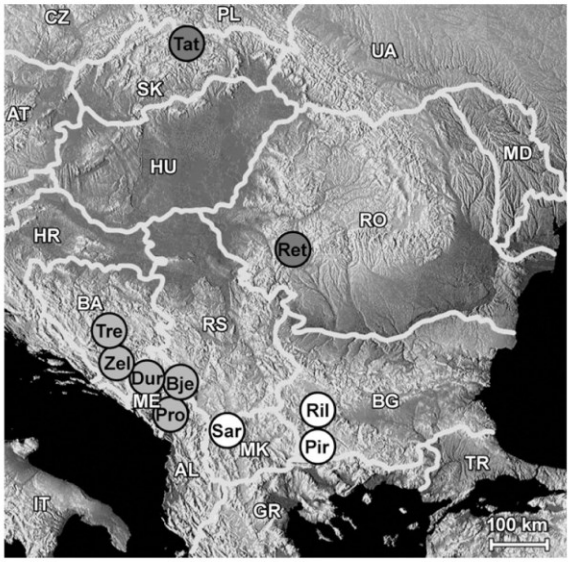

Figure 1. Map of the sampled Eastern European mountain ranges (Bje, Bjelasica; Dur, Durmitor; Pir, Pirin; Pro, Prokletije; Ret, Retezat; Ril, Rila; Sar, Šar Planina; Tat, Tatra Mountains; Tre, Treskavica; Zel, Zelengora). The main mountain regions are differentiated by shading: the Carpathians in dark grey, Macedonian–Thracian massif in white, and Dinaric Alps in light grey. Countries are indicated by two-letter codes (see Table 1).

Toporowy Staw Wyżni; four of *D. galeata*: lakes Štrbské, Nižné Temnosmrečianské, Nižné Žabie Bielovodské, and Morskie Oko; Petrusek *et al*., 2007; Hamrová, 2011) in the tree demonstrating the overall patterns of variation of the *D. longispina* complex in mountain lakes of the region (Fig. 2A).

Altogether, we analysed representatives of 23 *D. longispina* and 24 *E. serrulatus* populations from East European mountain lakes, together with additional 12 reference *E. serrulatus* populations from various European lowland localities (the Czech Republic, Finland, France, Germany, Great Britain, Poland, Slovenia, and Ukraine) and one population from a mountain lake in the Swiss Alps (Table 1).

Zooplankton was collected by plankton net tows from the lake shore or from a rubber boat, and preserved in 96% ethanol. Samples were initially visually screened for the presence of the target taxa, and species were determined using the taxonomic keys of Flössner (2000) and Einsle (1993) for *Daphnia* and *Eucyclops*, respectively. *Eucyclops* individuals were identified as *E. serrulatus* if they exhibited the following traits considered characteristic for this taxon: 12-segmented antennule with smooth membrane without denticles along three distal-most segments; caudal rami four to seven times longer than wide, with long row of spinules along the outer margin and spinular outer terminal caudal seta (Einsle, 1993; Alekseev *et al*., 2006).

Molecular analysis

DNA from individual crustaceans was extracted in 50 µL (*Eucyclops*) or 100 µL (*Daphnia*) of proteinase K solution, following the protocol of Schwenk *et al*. (1998). The number of analysed individuals per population differed, ranging from one to 14 (on average three; Table 1). Only a single specimen could be analysed from some populations because of the scarcity of animals in the samples, or failure in DNA isolation. Part of the mitochondrial gene for the small ribosomal subunit (12S) rRNA was amplified for both species. Owing to primer availability, the sequenced fragment differed in length for the two taxa, being 528 bp for *Daphnia* and *c.* 383 bp for *Eucyclops*. For further phylogenetic analyses, part of the mitochondrial gene for cytochrome *b* (327 bp) and part of the nuclear gene for the small ribosomal subunit rRNA (18S; 596 bp) were amplified for selected *Eucyclops* specimens representing divergent clades.

For *Daphnia*, PCR reactions in 20 µL volume contained 1 × PCR buffer with $(NH_4)_2SO_4$ (Fermentas, Burlington, Canada), 0.15 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 0.4 µM of each primer, 1% dimethyl sulphoxide, 0.5 U Taq polymerase (Fermentas), primers (12S-F: 5′-ATG CAC TTT CCA GTA CATCTA C-3′; 12S-R: 5′-AAA TCG TGC CAG CCG TCG C-3′; Taylor, Hebert & Colbourne, 1996), and 2 µL of the DNA template. The PCR cycle consisted of the following steps: initial denaturation at 92 °C for 3 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and elongation at 72 °C for 1.5 min, with final elongation at 72 °C for 5 min.

For *Eucyclops*, reactions in 35 µL volume contained 1 × PCR Dream Taq buffer (Fermentas), 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 0.4 µM of each primer, 0.6 U Dream Taq polymerase (Fermentas), and 7 µL of the DNA template. Previously published primer pairs were used for 12S (L13337-12S: 5′-YCT ACT WTG YTA CGA CTT ATC TC-3′; H13845-12S: 5′-GTG CCA GCA GCT GCG GTT A-3′; Machida *et al*., 2004), cytochrome *b* (UCYTB151-F: 5′-TGT GGR GCN ACY GTW ATY ACT AA-3′; UCYTB270-R: 5′-AAN AGG AAR TAY CAY TCN GGY TG-3′; Merritt *et al*., 1998), and 18S (18 s329: 5′-TAA TGA TCC TTC CGC AGG TT-3′; 18sI: 5′-AAC TCA AAG GAA TTG ACG G-3′; Spears, Abele & Kim, 1992). The PCR cycle consisted of the following steps: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 94 °C for 45 s, annealing for 45 s at 60 °C (for 12S) or at 48 °C (for cytochrome *b* and 18S), and elongation at 72 °C for 1.5 min, with final

elongation at 72 °C for 6 min. All PCR products were purified and sequenced using the forward primers on an ABI PRISM 3730XL DNA capillary sequencer by a third party (Macrogen, Seoul, Korea).

Newly obtained sequences have been submitted to GenBank under accession numbers JX134322–359 for *Daphnia*, and JX134280–321, JX134360–383 for *Eucyclops*. *Daphnia longispina* sequences from several Tatra Mountain lakes had been already deposited in GenBank during our previous studies (Petrusek *et al*., 2007; Thielsch *et al*., 2009) under accession numbers DQ337929–937, DQ337939, FJ178325–329, and FJ178332–333.

Data analysis

The sequences were aligned for each gene separately by ClustalW (Thompson, Higgins & Gibson, 1994) in MEGA v. 5.0 (Tamura *et al*., 2011). Mean divergences between distinct clades were calculated using the Kimura two-parameter (K2P) model, with gaps treated with partial deletion, in MEGA v. 5.0. For *Daphnia* as well as *Eucyclops*, we constructed 12S haplotype networks for the most common clade found in the mountain lakes, using the program TCS v. 1.21 (Clement, Posada & Crandall, 2000). The networks were based on 528- and 383-bp-long alignments for *Daphnia* and *Eucyclops*, respectively (sequences of the respective *Eucyclops* clade were re-aligned for this purpose). Furthermore, we demonstrated the variation in *Daphnia* populations (including *D. lacustris* and *D. galeata* from the Tatra mountains) in a tree constructed by the maximum likelihood method using the Tamura–Nei model of evolution with a proportion of invariant sites (TN93 + I). Model selection (based on the Bayesian information criterion) and tree reconstruction were carried out in MEGA v. 5.0.

When constructing the parsimony network of the *D. longispina* haplotypes, we further included already available representative sequences of this species from other regions of Europe (GenBank accession nos. DQ337938, DQ536400, EF375827, EF375832–839, EF375841–846, FJ178314, FJ178318–323, FJ178330–331, FJ178334–339, FJ178341, FJ178343–345; Nilssen *et al*., 2007; Petrusek *et al*., 2007, 2008a; Thielsch *et al*., 2009) and additional ones from Spain (Hamrová, 2011; M. Ventura, E. Hamrová, A. Miró, A. Petrusek, D. Buñay, L. De Meester & J. Mergeay, unpubl. data), the Czech Republic, Denmark, Germany, and Norway (A. Petrusek, unpubl. data). The sequences of other *Daphnia* species from the Tatra Mountains included in the tree are available under accession numbers DQ337926–928, DQ337940, and JX134359.

The phylogenetic relationship amongst divergent *Eucyclops* lineages was assessed from the concatenated data set of all three genes (12S, cytochrome *b*, and 18S, altogether 1299 bp long) by Bayesian inference in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003). Most variable loop regions in 12S sequences could not be reliably aligned, and we excluded these from further analyses by processing the 12S alignment in GBLOCKS SERVER v. 0.91b (Castresana, 2000), using default settings but allowing gaps within blocks. We thus obtained a 376-bp-long alignment (93% of the original 404 bp). Cytochrome *b* and 18S could be aligned unambiguously, resulting in 327- and 596-bp-long alignments, respectively. The model of evolution was set for each gene separately [generalized time-reversible with proportion of invariant sites and gamma distributed rate heterogeneity (GTR + I + G) for cytochrome *b* and 12S, and GTR + G for 18S]; these parameters were set to match most closely the models chosen by Akaike information criterion in jModelTest (Guindon & Gascuel, 2003; Posada, 2008). The Bayesian analysis was performed in two parallel runs of four Monte Carlo Markov chains for six million generations; trees were sampled every 100 generations, with 25% discarded as burn-in. Two independent MrBayes analyses resulted in identical topologies.

RESULTS

In total, we analysed 110 *Daphnia* and 84 *Eucyclops* individuals (Table 1). All analysed *Daphnia* individuals from newly sampled lakes of East European mountains (i.e. other than those from the Tatra Mountains) belonged to a single species, *D. longispina s.s.* (Fig. 2A). No other species or divergent lineages were detected throughout the sampled lakes in Romania and the Balkans. *Daphnia longispina* haplotypes detected in East European mountains represent a substantial proportion of the species' variation and were scattered throughout most of the haplotype structure representing other European lowland and mountain localities (Fig. 2B). Only one haplotype found in lowland habitats (a pond in Göteborg, Sweden, and Goksjø Lake in southern Norway) was also recorded in an East European mountain lake (Lake Jugovo in the Zelengora mountains, Bosnia and Herzegovina; a split oval in Fig. 2B).

The simultaneous presence of divergent haplotypes was detected within several mountain ranges, suggesting independent colonization events by *Daphnia*. These included Prokletije and Treskavica (light grey 'Pro' and 'Tre' in the network in Fig. 2B). In the latter, a single analysed lake harboured very divergent haplotypes. Particularly high, up to 1.9% divergence, was the haplotype variation observed in the Tatra Mountains (dark grey 'Tat' in Fig. 2B). On the contrary, some patterns of regional haplotype diversity of *D. longispina* were consistent with local radiations. These include the West Tatra Mountains (top dark

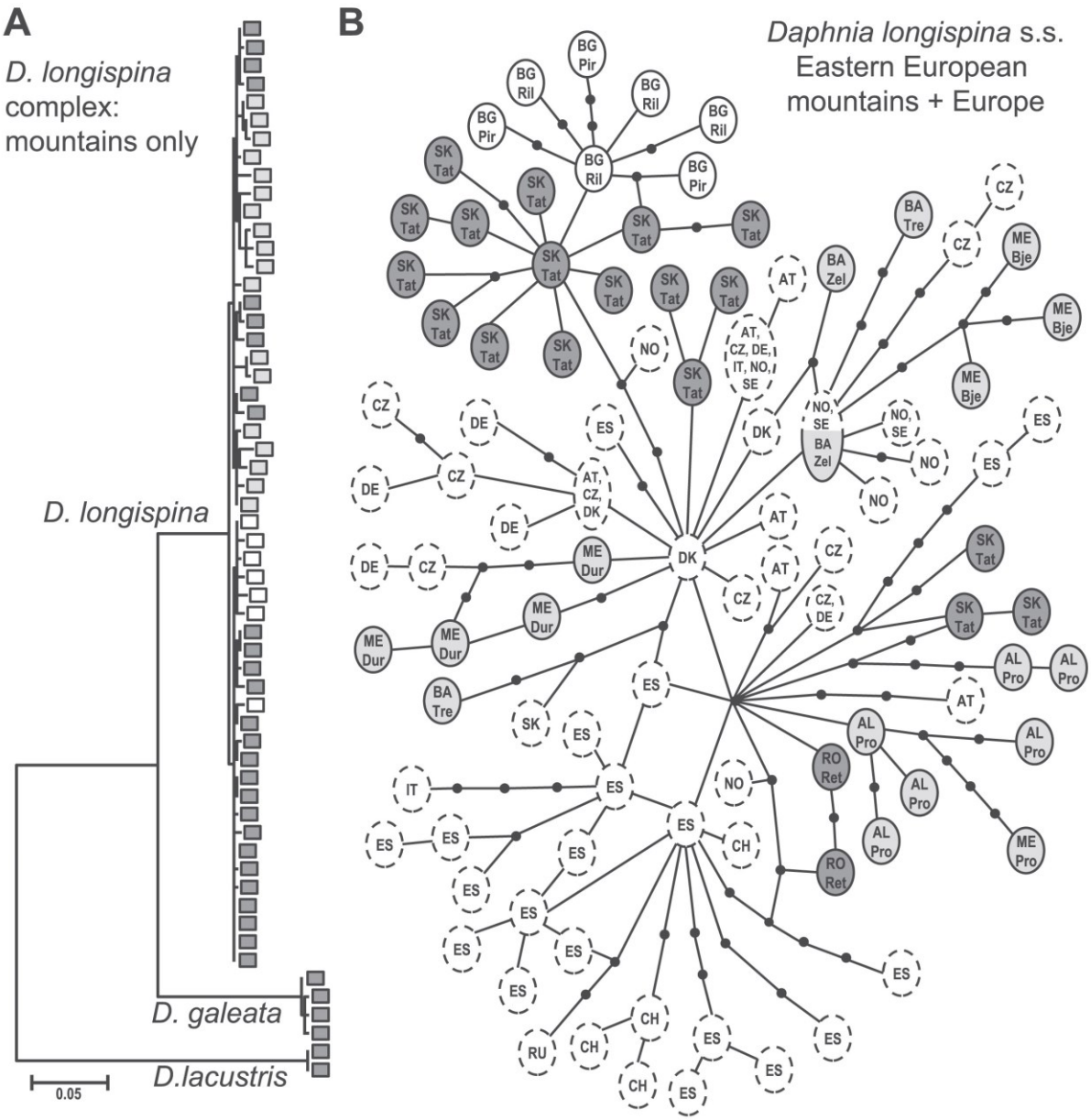

Figure 2. Sequence variation of the 528-bp-long fragment of the 12S rRNA gene within the *Daphnia longispina* complex from lakes of the studied East European mountain ranges. This is shown in a maximum likelihood tree (A) consisting only of sequences from the studied region (each haplotype represented once per lake), and in a parsimony network (B) of haplotypes of *D. longispina s.s.*, amongst which 63 reference sequences from other European localities were also included. Three main mountain regions from this study are differentiated by shading: the Carpathians in dark grey, Macedonian–Thracian massif in white, and Dinaric Alps in light grey. Haplotypes from other localities, only included in the network, are enclosed by dashed lines. Mountain range abbreviations: Bje, Bjelasica; Dur, Durmitor; Pir, Pirin; Pro, Prokletije; Ret, Retezat; Ril, Rila; Tat, Tatra Mountains; Tre, Treskavica; Zel, Zelengora. Countries are indicated by two-letter codes (see Table 1).

grey cluster in Fig. 2B), Retezat, Macedonian–Thracian massif (i.e. Rila and Pirin together), Prokletije, Bjelasica, and Durmitor. No haplotype sharing was observed amongst studied mountain ranges. The greatest divergences within regions were 1.9, 1.5, and 1.3% in the Carpathians, Macedonian–Thracian massif, and Dinaric Alps, respectively.

Limited sampling of European *E. serrulatus*, which included apart from the mountain lakes only 12 lowland sites, yielded eight genetically divergent lineages (Fig. 3), differing by 18.9 to 45.4% at 12S and 23.7 to 53.9% at cytochrome *b* (all values given as K2P distances; see Table 2). The within-clade variation did not exceed 4.4% for 12S and 7.8% for the more variable cytochrome *b*, with the exception of clade IV containing populations from Germany and Ukraine, which differed by 8.3 and 15.1%, respectively. The variation in the conservative nuclear gene for 18S rRNA was much lower, being invariant amongst four clades (I to III, which form a well-supported monophyletic lineage, and VI), and not exceeding 0.84% (between clade IV on the one hand, and clades VII and VIII on the other).

All analysed mountain *Eucyclops* populations were grouped in clades I and II (see Fig. 3). Clade I contained all mountain populations from south-eastern Europe, one population from the Tatra Mountains (Vyšné Wahlenbergovo Lake), and two lowland populations from the Czech Republic and Great Britain. Lakes in the Tatra Mountains were otherwise dominated by clade II (six lakes with K2P distances not exceeding 0.3%), which also included an alpine population from Switzerland and a lowland one from Slovenia. The remaining populations from European lowland habitats belonged to other divergent lineages (clades III to VIII). Some morphological differences were observed amongst these lineages (in length–width ratio and angle of caudal rami branches; M. Krajíček, unpubl. data) but all these fell within the ranges generally considered as intraspecific variation of *E. serrulatus* (Alekseev *et al*., 2006). Despite limited sampling of lowland reference sites, we even recorded two genetically divergent lineages coexisting at a single locality (clades V and VI in Lechlade, Great Britain).

The 12S haplotype variation within clade I, widespread in East European mountains, is presented in the network in Figure 3. There are two clusters in the network, their central haplotypes diverging by 2.7%. Haplotypes from different mountain regions are scattered throughout the network without any apparent geographical pattern, with haplotypes of individuals from the Carpathians, Macedonian–Thracian massif, and Dinaric Alps found in both of these clusters. The central haplotype of the top cluster was shared by individuals from seven lakes from six different mountain ranges. The second cluster consisted of representatives from all main regions (four mountain ranges) and included two lowland populations (from the Czech Republic and Great Britain).

DISCUSSION

Our study, the first analysing genetic variation of planktonic crustaceans in 'uncharted' waters of the Balkans, brought results interesting for both taxonomy and biogeography of these groups. The hypothesis about cryptic variation of the studied taxa in East European mountain lakes was only partly supported. Based on the results of a previous study on the *D. longispina* complex in the Tatra Mountains (Petrusek *et al*., 2007), we expected that other mountain ranges included in our study would also harbour multiple *Daphnia* lineages of this complex. However, such a pattern was not observed, as only *D. longispina s.s.* was revealed in the analysed populations from the Balkan Peninsula and Romania, and no cryptic lineage was found.

In contrast, our results support the view that *E. serrulatus*, considered a widespread, variable species with wide ecological tolerance, is actually a cryptic species complex. Eight deeply divergent lineages have been revealed in analysed populations morphologically assigned to *E. serrulatus* (Fig. 3). However, the variation observed in East European mountain lakes was substantially lower, as only two lineages were found there. The lineage distribution of both studied species complexes, cladoceran and copepod, was remarkably similar in the studied mountains. The Tatra Mountains on the Slovak–Polish border, the northern-most studied mountain range, were exceptional in our data set because of the presence of multiple lineages of both complexes (two of *E. serrulatus*, Fig. 3, and three of *D. longispina*, Fig. 2A; Petrusek *et al*., 2007). In all other mountainous regions studied by us, only one widespread lineage of each species complex was detected. The reasons for this contrast in lineage diversity are unclear. Environmental characteristics between lakes sampled in the Tatra Mountains and more southerly located mountain ranges do not seem to differ substantially. The studied populations are evolutionarily relatively young, as most of the studied mountain lakes were unavailable for colonization before the end of the last glaciation in the mountains (*c*. 8000 years ago). Possibly, taxon composition differed in source populations from which the different mountain ranges were colonized, e.g. as a result of survival of some lineages in other than southern glacial refugia, or because of the distance from boreal lakes where *D. lacustris*, present in the Tatra Mountains, is common (Nilssen *et al*., 2007).

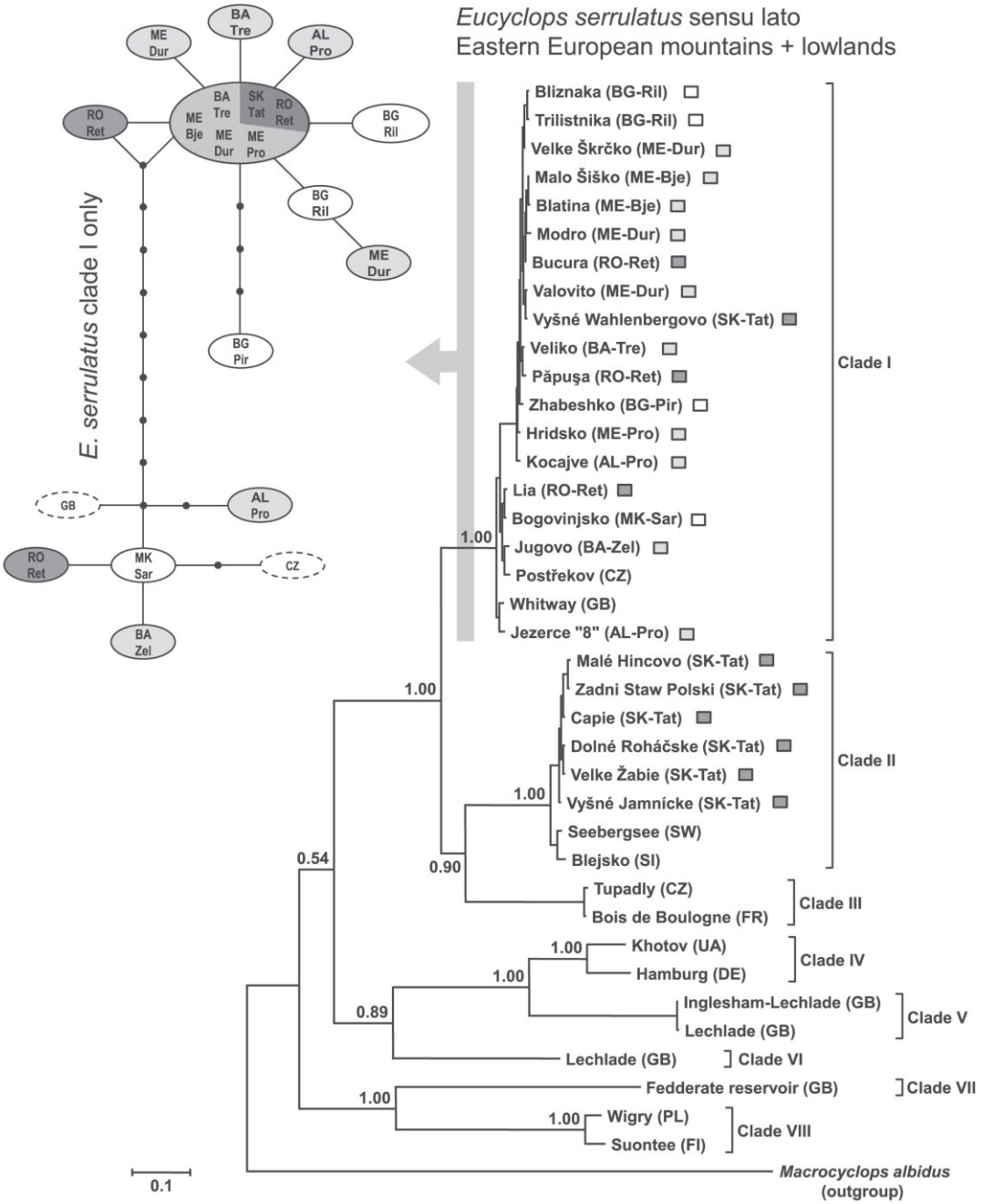

Figure 3. Relationship of eight clades of the *Eucyclops serrulatus* complex, assessed by Bayesian inference of phylogeny, and haplotype variation of the 12S rRNA gene within clade I. The phylogenetic tree was based on the 1299-bp-long alignment consisting of fragments of mitochondrial genes for 12S rRNA and cytochrome *b*, and the nuclear gene for 18S rRNA. The scale bar represents genetic distance; numbers at nodes indicate branch support (as posterior probabilities). Haplotype network representing the variation within clade I is based on 43 sequences of the 383-bp-long 12S rDNA fragment. Individuals from the three main mountain regions are indicated by different shading (as in Figs 1, 2) in both tree and network: the Carpathians in dark grey ($N = 24$), Macedonian–Thracian massif in white ($N = 9$), and Dinaric Alps in light grey ($N = 26$). Mountain range abbreviations: Bje, Bjelasica; Dur, Durmitor; Pir, Pirin; Pro, Prokletije; Ret, Retezat; Ril, Rila; Sar, Šar Planina; Tat, Tatra Mountains; Tre, Treskavica; Zel, Zelengora. Countries are indicated by two-letter codes (see Table 1).

Table 2. Mean between-group genetic divergences of the clades of the *Eucyclops serrulatus* complex (as indicated in Fig. 3). Values are given for analysed fragments of the mitochondrial genes for cytochrome *b* (above diagonal) and 12S rRNA (below diagonal). Divergences were calculated as Kimura two-parameter distances (with pairwise deletion of gaps in the 12S alignment)

Clade	I	II	III	IV	V	VI	VII	VIII
I	–	0.24	0.28	0.39	0.42	0.45	0.45	0.46
II	0.19	–	0.33	0.46	0.42	0.44	0.49	0.44
III	0.22	0.24	–	0.47	0.43	0.42	0.44	0.47
IV	0.36	0.39	0.41	–	0.28	0.39	0.54	0.41
V	0.39	0.41	0.41	0.23	–	0.40	0.53	0.43
VI	0.34	0.35	0.39	0.35	0.42	–	0.49	0.43
VII	0.36	0.38	0.43	0.38	0.45	0.42	–	0.52
VIII	0.38	0.42	0.44	0.41	0.44	0.45	0.34	–

High morphological variation of *Daphnia* observed in the lakes of the former Yugoslavia (Pljakic, 1961) might indeed represent only a variation within *D. longispina* rather than the presence of multiple taxa. Many phenotypically different populations within the Tatra Mountains have been shown to belong to this species (Petrusek *et al*., 2007; Hamrová *et al*., 2010). However, we cannot exclude that other mountain localities in south-eastern Europe not studied by us harbour additional lineages of both species complexes. None of the *E. serrulatus* lineages found in the mountain lakes were restricted to high elevations; it is therefore likely that some of the other clades found by us only in lowland habitats could also colonize mountains. Owing to the relatively low number of analysed specimens from individual populations, we also cannot rule out that additional lineages may have coexisted with those found by us in some of the studied lakes.

Nevertheless, our study suggests that although mountain lakes represent important reservoirs of genetic or morphologic diversity of zooplankton species, the detection of cryptic lineages in these habitats is apparently not a general pattern, at least in Europe. Similarly to the lakes studied by us, *D. longispina* was the only member of the complex recorded in a number of genetically analysed populations from the Pyrenees (Hamrová, 2011; M. Ventura, E. Hamrová, A. Miró, A. Petrusek, D. Buñay, L. De Meester & J. Mergeay, unpubl. data). The regional coexistence of two lineages of the *E. serrulatus* complex and three lineages of the *D. longispina* complex in mountain lakes, as observed in the Tatra Mountains, thus does not seem to be the rule. This contrasts with the patterns observed in mountain lakes in the tropics, where endemic cladoceran species as well as distinct *Daphnia* lineages are often found (Kořínek & Villalobos, 2003; Mergeay *et al*., 2008; Kotov *et al*., 2010; Van Damme & Eggermont, 2011).

Intraspecific haplotype variation of *D. longispina* and *E. serrulatus* clade I revealed some general patterns similar for both the cladoceran and the copepod, but also substantial differences. The topology of the haplotype networks for both analysed species cannot be compared directly, as the length of the analysed 12S fragment differed between them. However, the mtDNA variation clearly revealed that each taxon colonized at least certain mountain ranges more than once, and multiple distinct lineages of both taxa were found in each of the three studied main regions, although the colonization patterns themselves most likely differed between the species and regions.

We could not find any clear indications that *E. serrulatus* is more dispersal-limited than *D. longispina*. There were neither regionally restricted mountain clades (clade II, widespread in the Tatra Mountains, was also found in Slovenia and Switzerland) nor haplotype clusters suggesting prevailing short-range dispersal and local diversification of the copepod. On the contrary, one haplotype of *E. serrulatus* clade I was particularly widespread, being present in at least six mountain ranges. It is not possible to assess whether this represents a long-range dispersal amongst mountains, or gradual expansion of a lineage carrying this haplotype across the continent. Short distance dispersal is relatively easy for zooplankton (Allen, 2007), but dispersal on large scales may be limited (Havel & Shurin, 2004). Given their reproductive modes, this should affect copepods more than cladocerans. However, both *Daphnia* and *Eucyclops* were apparently able to cross the barriers between the continents a number of times in the past (see Adamowicz *et al*., 2009 for *Daphnia*, and the reports on distribution of *E. serrulatus sensu lato* given above), which suggests ability for long-distance dispersal. Although *Eucyclops* reproduce exclusively sexually with no production of resistant dormant propagules, relatively good dispersal via water-birds has been observed (e.g. Frisch, Green & Figuerola, 2007). Waterfowl nesting or occasionally stopping on isolated mountain lakes are the most likely natural

vectors of crustacean zooplankton in these habitats. Possibly, the dispersal of the most widespread haplotype of *E. serrulatus* clade I across the south-east European mountain ranges may have been facilitated by the fact that these lie on important bird migratory routes (e.g. Zehtindjiev & Liechti, 2003).

Our results nevertheless confirm that cryptic diversity in widespread freshwater copepod taxa is likely to be high. *Eucyclops serrulatus* was previously considered to be a cosmopolitan species (Dussart & Defaye, 1985), but subsequent studies challenged this view (Dussart & Fernando, 1990; Ueda, Ishida & Imai, 1996). Detailed morphological examination of micromorphological patterns and integumental pore mapping, as well as molecular analysis of sequences of nuclear small subunit (18S) ribosomal DNA was carried out by Alekseev *et al*. (2006). They found three morphotypes in the material from the species' type locality, all of which easily mated and had fertile offspring, and molecular analysis of three distant populations (type locality at St. Petersburg and populations from Siberia and Belgium) showed no variation in the 18S sequences. However, as our results show, even clades relatively divergent at mitochondrial markers (I, II, III, and VI) show no variation in the studied 18S fragment. Similar patterns of genetic diversity as in *E. serrulatus* were observed also for another common cyclopoid copepod taxon *Macrocyclops albidus* (Jurine, 1820), with no variation in the 18S sequences in populations from all around the world, but substantial variation of 12S sequences, up to 33.3% (Karanovic & Krajicek, 2012). Furthermore, the same pattern (high 12S but no 18S divergence, at least at studied fragments of the respective genes) may be also observed in morphologically well-defined species pairs within the genus *Cyclops*, such as *Cyclops abyssorum* G.O. Sars, 1863 and *Cyclops lacustris* G.O. Sars, 1863, and *Cyclops strenuus* Fischer, 1851 and *Cyclops vicinus* Ulyanin, 1875 (M. Krajicek, unpubl. data).

The mitochondrial 12S rRNA gene evolves faster and offers better resolution for closely related taxa than 18S rRNA (Machida *et al*., 2004; Audzijonyte *et al*., 2005). A recent study on the copepod genus *Acanthocyclops* Kiefer, 1927 (Bláha *et al*., 2010) reported K2P genetic distances of 12S rRNA gene between well-described species of between 12.4 and 19.4%. Variation of this gene amongst 12 different *Cyclops* species ranged between 3.2 and 40% (M. Krajicek, unpubl. data). These values are comparable, and often substantially lower, than the 12S variation amongst *E.* cf. *serrulatus* clades observed by us (see Table 2). Thus, although high divergences are occasionally found between interbreeding lineages in invertebrates (e.g. King, Tibble & Symondson, 2008), and we cannot rule out that some of the clades detected by us actually belong to the same biological species or to hybridizing species pairs, we suggest that *E. serrulatus* most likely represents a very speciose cryptic complex.

A more detailed study including highly variable nuclear markers may reveal whether hybridization and introgression occur amongst at least some of these divergent *Eucyclops* lineages, as has been well documented within the *D. longispina* complex (e.g. Ishida *et al*., 2011). Unlike in *Daphnia*, these phenomena have been little studied in freshwater copepods. However, a recent study on *Acanthodiaptomus pacificus* from Japan reported sharing of a single nuclear ribosomal internal transcribed spacer variant in populations from the contact zone of two distinct mitochondrial lineages, apparently because of introgressive hybridization (Makino & Tanabe, 2009). As hybridization is widespread throughout the animal kingdom (Schwenk, Brede & Streit, 2008), it seems likely that this record for copepods is not exceptional.

The application of genetic tools will certainly change the view on the diversity of *Eucyclops*, and copepods in general. Our limited sampling of a single species complex across lowland water bodies throughout Europe has already resulted in the discovery of eight distinct lineages, so it is likely that the *E. serrulatus* complex is much more diverse. This trend of discovering cryptic variation is apparent also in other copepod groups (Thum & Harrison, 2008). The view on patterns of freshwater copepod diversity has substantially changed in recent decades; the original idea that many species are cosmopolitan has been refuted, and most of the species (90%) are assumed to be endemic to their zoogeographical region (Boxshall & Defaye, 2008). This is similar to the shift in the view on the diversity of cladocerans, which changed in the second half of the 20th century (Forró *et al*., 2008). In both groups, such a 'paradigm shift' is well supported by both morphological and genetic analyses. The taxonomic revision of the *E. serrulatus* complex is warranted, but it is likely to be only one of many copepod taxa in which genetic methods will reveal numerous cryptic lineages.

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Chapter 4:

When anthropogenic translocation meets cryptic speciation globalized bouillon originates; molecular variability of the cosmopolitan freshwater cyclopoid *Macrocyclops albidus* (Crustacea: Copepoda)

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The work on this paper was the beginning of a nice collaboration with Tom Karanovic. He spent several years in Australia and New Zealand studying copepod fauna and noticed many morphological similarities with the European species. We were discussing the possibility of the cosmopolitan distribution of copepod crustaceans and decided to test his hypothesis (Karanovic, 2005) about possible anthropogenic translocation of freshwater copepods associated with early shipping activities by European settlers. In those days, sailing ships carried plenty of wooden barrels with water from a local European river, used mostly for cooking and washing and refilled wherever possible (Hood, 2003). This way of transport would have been a great way for freshwater species to get to new localities, for example from harbours in England or Germany to Australia and New Zealand (Karanovic, 2005).

We compared the patterns of DNA sequence variation of four genes and 11 populations of *Macrocyclops albidus* (from England, Scotland, France, Germany, USA, New Zealand and Australia) to test the interpopulation variability. The resulting phylograms were then tested against two competing hypothesis (anthropogenic translocation versus natural dispersal) and complemented by comparative morphology of microcharacters.

One of the most surprising results was the presence of the same haplotype in the highly disjunct populations in Western Australia, Germany and USA. This cannot be explained by any mode of dispersal other than anthropogenic translocation. However, our data were not able to confirm our hypothesis that the cosmopolitan distribution of some cyclopoid copepods is the result of early shipping activities. On the other hand, our phylogenetic analysis revealed four well-defined clades and subsequent morphological analysis of microcharacters found a high number of differences. This indicates the importance of these microstructures on the species level and show how they can be used to effectively discriminate closely related congeners. We can conclude that the taxonomy of *Macrocyclops albidus* s. l. needs clarification and probably a re-description. Morphological microcharacters resulted from this work could be used as a basis for further research.

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When anthropogenic translocation meets cryptic speciation globalized bouillon originates; molecular variability of the cosmopolitan freshwater cyclopoid *Macrocyclops albidus* (Crustacea: Copepoda)

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Abstract – Invasive species are a global problem, which costs the world economy billions of dollars and world ecosystems millions of tons of herbicides, pesticides and other cides. Anthropogenic translocation of freshwater copepods associated with early shipping activities was postulated for some time, but was never tested with molecular tools. Here, we examine global molecular diversity of one cyclopoid species, test if the current cosmopolitan distribution is a result of anthropogenic translocation or natural dispersal, and investigate a possibility of cryptic speciation. We use patterns of haplotype frequency of DNA and RNA sequences of four genes (12S, 16S, 18S and cytB) and 11 populations (from England, Scotland, France, Germany, USA, New Zealand and Australia) to test inter- and intrapopulation variability, and three different methods (neighbour joining (NJ), maximum likelihood (ML) and maximum parsimony (MP)) for reconstructing their phylogenetic relationships. They were then tested against two competing hypotheses, and complemented by comparative morphology of microcharacters. Reconstructed phylogenies present strong evidence for anthropogenic translocation, with the same haplotype found in highly disjunct populations in Western Australia, Germany and the USA. Four different clades were revealed with the 12S, 16S and cytB genes, probably representing four cryptic species. Morphological examination of females of two clades contributed a set of microcharacters that can be used in the future taxonomic revision of this species complex. We prove for the first time that cuticular pores and sensilla are homologous structures. This research provides evidence for both homogenization of world freshwater fauna and our inadequate methods of identifying some of its most common species.

Key words: Invasive species / ecosystem degradation / freshwater / globalized fauna / haplotype frequency

Introduction

Invasive species pose among the greatest threats to biodiversity, ecosystem integrity, agriculture, fisheries and public health (Lee *et al.*, 2003), but mechanisms of invasions are not yet fully understood (Dunstan and Johnston, 2007). Economic costs associated with the more publicized invaders, such as weeds, agricultural pests, mussels and plant pathogens, were estimated in the United States by Pimentel *et al.* (2000) to be around 137 billion dollars. Almost one-half of all plant species in the British and Irish flora represent introduced elements (Preston *et al.*, 2002). Aquatic ecosystems have also been heavily impacted. Introduced seaweeds can account for a significant proportion of the total aquatic flora (Johnson, 2007), and about 260 species have now been identified as alien to their native range (Hewitt *et al.*, 2007). In some cases, these invaders, freed of the natural controls of their native range, can proliferate in new waterways, displace native species and significantly degrade ecosystems. Well-known examples include the zebra mussel in North America (May *et al.*, 2006), and the northern Pacific sea star in Tasmania (Ross *et al.*, 2006). The latter was first collected in Tasmania in 1986, but its true identity was not realized until 1992, which shows the importance of taxonomic expertise in the study of invasive species. In many cases of invasive

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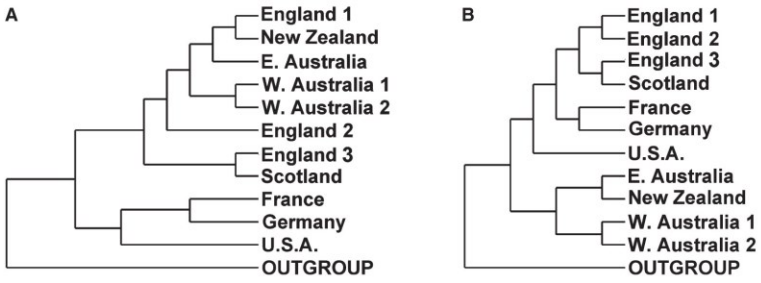

Fig. 1. Two competing hypothetical phylogenies of *M. albidus* (Jurine, 1820) that may explain its cosmopolitan distribution, and especially the presence of disjunct populations in Australia and New Zealand: (A) anthropogenic translocation associated with early shipping activities by European settlers; (B) natural passive dispersal by different vectors, such as wind and on bird feet.

species, there is direct evidence of their competition with local commercially fished fauna (Strain and Johnson, 2009). Freshwater habitats are not less impacted. Invasion by common carp and red swamp crayfish in shallow lakes in Japan have been followed by changes from a macrophyte-dominated clear water state to a phytoplankton-dominated turbid water state (Matsuzaki *et al.*, 2009). Over 170 non-indigenous plant and animal species are now established in the Laurentian Great Lakes (Grigorovich *et al.*, 2003). In some cases, species have been introduced intentionally to replace closely related congeners that had gone extinct, like the North American amphipod crustacean, which was introduced to the Werra River in Germany (Bulnheim, 1985). The extensive construction of reservoirs over the past century has radically altered the environmental landscape on a global scale, further facilitating invasions (Havel *et al.*, 2005).

Copepod crustaceans are also well-known invaders. Anthropogenic translocation associated with shipping activities sometimes results in introduction of marine and estuarine species to ecosystems outside their historic ranges through ballast water discharge (Reid and Pinto-Coelho, 1994; Lee, 1999). A review of passive copepod dispersals by Reid and Pinto-Coelho (1994) showed that from 21 species, where intercontinental introduction was known or presumed, at least 10 were introduced in ships' ballast waters; other significant vectors being aquaculture and tropical aquatic plants. Calanoid copepods were the most dominant, followed by cyclopoids and harpacticoids. Subsequent records include that of one Afro-Asian cyclopoid species in the Cayman Islands by Suarez-Morales *et al.* (1999), two Asian calanoids and one cyclopoid in the San Francisco Estuary by Orsi and Ohtsuka (1999), two Ponto-Caspian harpacticoids in the nearshore sands of Lake Michigan by Horvath *et al.* (2001); one tropical calanoid in Japan by Ohtsuka *et al.* (2005); and possibly one brackish cyclopoid in Western Australia (Karanovic, 2008). A particularly well-studied example is that of the calanoid copepod *Eurytemora affinis* (Poppe, 1880), which exhibited rapid and repeated invasions of freshwater from brackish and marine habitats (Lee, 1999; Lee *et al.*, 2003, 2007; Winkler *et al.*, 2008). Ballast water is today recognized as a major vector of non-indigenous species invasion globally (Grey *et al.*, 2007; Zvyaginstev and Selifonova, 2008), and many management options have been developed so far (for a review, see Gregg *et al.*, 2009).

Recent studies on freshwater copepods in Australia and New Zealand (Karanovic, 2004, 2005, 2006) also suggested the presence of some "cosmopolitan" cyclopoids in lotic and subterranean habitats, which was hypothesized by Karanovic (2005) to be a result of early shipping activities by European settlers. In those days, sailing ships would carry over 100 wooden butts, containing the water that came straight out of a local European river, and was used mostly for cooking and washing and refilled wherever possible (see Hood, 2003: 8). So, when Captain Cook in 1769 first landed in New Zealand (Horwitz, 2000) and refilled his butts with local freshwater, the first few cyclopoids could have been introduced (Karanovic, 2005). Support for this hypothesis was found by the absence of morphological variability (suggesting a bottleneck) between some highly disjunct Australian and New Zealand populations in heavily populated areas, the ability of many cyclopoids to survive adverse conditions in different life stages, the absence of cosmopolitan harpacticoids and calanoids in Australia and New Zealand (with resting stages only as eggs or cysts in benthos), and the presence of indigenous stygobitic bathynellids, isopods and amphipods for example. We test this hypothesis here using patterns of haplotype frequency of DNA and RNA sequences for several disjunct populations of the cosmopolitan *Macrocyclops albidus* (Jurine, 1820). If the hypothesis is correct, then our reconstructed phylogenies should be similar to that in Figure 1(a), with the New Zealand and Australian populations being most closely related to those from England. If, however, the current cosmopolitan distribution of this species is a result of natural dispersal and not anthropogenic translocation, then our phylogenetic trees may be similar to that in Figure 1(b).

Material and methods

Specimens of *M. albidus* were collected in 11 locations in Europe, North America, Australia and New Zealand (Tab. 1). *Cyclops abyssorum* G.O. Sars, 1863 from three locations in Slovakia and *Eucyclops serrulatus* (Fischer,

Table 1. List of material examined with specimen numbers for different sequences; see text for generic names and authors of the specific names.

Species	Country	Locality	Coordinates	Date	Collector	12S	16S	18S	cytB
C. abyssorum	Slovakia	Tatra Mt., Litvorove	49.177°N, 20.129°E	22 Sep 2005	V. Sacherova	–	M05	–	–
C. abyssorum	Slovakia	Tatra Mt., Nizne Temnosmrecinske	49.189°N, 20.029°E	26 Sep 2004	M. Krajicek	C13	–	–	–
C. abyssorum	Slovakia	Tatra Mt., Vysne Temnosmrecinske	49.189°N, 20.038°E	24 Aug 2004	S. Markova	–	–	L10	–
E. serrulatus	Czech Republic	Tupadly, pond	50.447°N, 14.472°E	30 Apr 2010	D. Vondrak	–	–	–	O10
M. albidus	Australia (LR)	Lake Richmond, Perth, WA	32.283°S, 115.712°E	11 Dec 2009	T. Karanovic	Q17, R38	S13	Q35	P01
M. albidus	Australia (LZ)	Lake Zot, Armidale, NSW	30.492°S, 151.636°E	15 Dec 2009	P. Hancock	Q18	–	–	–
M. albidus	Australia (MR)	Margaret River, spring, WA	33.787°S, 115.006°E	13 Feb 2010	T. Karanovic	Q20, Q21	–	S06	–
M. albidus	England (L)	Lechlade, trout fishery, GL7	51.707°N, 1.683°W	19 May 2010	T. Karanovic	P08, R33	S08	S15	S19
M. albidus	England (SL)	Staindale Lake, near Lockton, YO62	54.301°N, 0.648°W	8 Jul 2008	T. Karanovic	–	–	Q41	–
M. albidus	England (W)	Whitway, pond, RG20	51.335°N, 1.334°W	19 May 2010	T. Karanovic	P07, R34	S09	–	S21
M. albidus	France	Paris, Bois de Boulogne, pond	48.867°N, 2.264°E	20 Apr 2010	V. Alekseev	P11	S10	S16	–
M. albidus	Germany	Hamburg, creek near Zoo	53.602°N, 9.938°E	9 May 2010	T. Karanovic	P06, R32	S07	S14	S18
M. albidus	New Zealand	Porirua, pond	41.121°S, 174.857°E	28 Dec 2009	J. Bradford	Q19, S03	–	Q37	S02
M. albidus	Scotland	Fedderate, reservoir	57.543°N, 2.182°W	16 Jul 2008	S. Markova	Q22, R36, R37	S11, S12	Q39	S22
M. albidus	USA	New Orleans, pond	29.979°N, 89.951°W	18 Apr 2010	G. Wyngaard	P12	–	–	–

1861) from one location in the Czech Republic were used as outgroups in different molecular analyses. All sequences are deposited at GenBank (accession numbers from JN656662 to JN656704).

Samples were collected with plankton nets and preserved in 96 + % ethanol. DNA was extracted from individual whole specimens in 50 µl proteinase K solution, using the protocol of Schwenk *et al.* (1998). Fragments of four different genes (mitochondrial 12S rRNA (430 bp), 16S rRNA (380 bp), cytochrome *b* (360 or 430 bp) and nuclear 18S rRNA (650 bp)) were amplified using a combination of primers given in Table 2. The 35 µl PCR was done in a Bio-Rad iCycler Thermal Cycler and contained 7 µl of DNA template, 1 × PCR buffer, 0.2 mM deoxynucleotides, 2.5 mM $MgCl_2$, 0.4 µM primers and 0.6 U Taq polymerase. The PCR protocol consisted of 4 min initial denaturation at 95 °C, followed by 40 cycles consisting of denaturation at 94 °C for 45 s, annealing at 48 °C (for 18S and cytB) or 60 °C (for 12S and 16S) for 45 s and extension at 72 °C for 1.5 min. A final extension at 72 °C lasted for 6 min. PCR products were purified and sequenced on ABI automatic capillary sequencer (Macrogene, Seoul, Korea) using primers marked in Table 2.

Obtained sequences were checked manually and aligned by ClustalW algorithm (Thompson *et al.*, 1994) in MEGA version 5 (Tamura *et al.*, 2011). The alignment was checked again and trimmed to a fragment length available for all individuals (399 bp for 12S, 313 bp for 16S, 595 bp for 18S and 328 bp for cytB); all sites were unambiguously aligned. The best evolutionary model for each dataset was established by Akaike Information Criterion, performed with jModelTest (Guindon and Gascuel, 2003; Posada, 2008). The following models of nucleotide substitution were selected for maximum likelihood (ML) analysis: for the 12S dataset the Hasegawa–Kishino–Yano model (Hasegawa *et al.*, 1985) with gamma distributed rate heterogeneity (HKY + G), for the 16S dataset the General Time Reversible model (Tavaré, 1986) with gamma distributed rate heterogeneity (GTR + G), for the 18S dataset the Tamura–Nei (TN) model (Tamura and Nei, 1993) with uniform rates (TN), for the cytB dataset the HKY model (Hasegawa *et al.*, 1985) with significant proportion of invariable sites (HKY + I). Neighbour joining (NJ) analysis of all datasets used the TN model (Tamura and Nei, 1993) with uniform rates (TN). Maximum parsimony (MP) analysis of all datasets was computed using the Close-Neighbour-Interchange (CNI) method on random trees, with 10 initial trees (random addition) and 500 bootstrap replicas. All phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5 (Tamura *et al.*, 2011). Five hundred bootstrap replicates were performed to obtain a relative measure of node support for the resulting trees. Average pairwise NJ distances for each dataset were also computed in MEGA version 5 using the TN model.

Specimens for morphological observations of microcharacters were dissected and mounted on microscope slides in Faure's medium (Stock and Von Vaupel Klein,

Table 2. List of primers. Asterisk marks those used for sequencing reaction.

Gene	Primer	Sequence	Reference
12S	L13337-12S*	5'-YCTACTWTGYTACGACTTATCTC-3'	Machida *et al.* (2004)
12S	H13845-12S	5'-GTGCCAGCAGCTGCGGTTA-3'	Machida *et al.* (2004)
16S	16S CB*	5'-ATTCAACATCGAGGTCACAA-3'	Braga *et al.* (1999)
16S	16Sar-L	5'-CGCCTGTTTATCAAAAACAT-3'	Palumbi *et al.* (1991)
18S	18s329	5'-TAATGATCCTTCCGCAGGTT-3'	Spears *et al.* (1992)
18S	18sI*	5'-AACTCAAAGGAATTGACGG-3'	Spears *et al.* (1992)
cytB	UCYTB144F*	5'-TGAGSNCARATGTCNTWYTG-3'	Merrit *et al.* (1998)
cytB	UCYTB272R	5'-GCRAANAGRAARTACCAYTC-3'	Merrit *et al.* (1998)
cytB	UCYTB151-F*	5'-TGTGGRGCNACYGTWATYACTAA-3'	Merrit *et al.* (1998)
cytB	UCYTB270-R	5'-AANAGGAARTAYCAYTCNGGYTG-3'	Merrit *et al.* (1998)

1996). All line drawings were prepared using a drawing tube attached to a Leica MB2500 phase-interference compound microscope, with N-PLAN (5 ×, 10 ×, 20 ×, 40 × and 63 × dry) or PL FLUOTAR (100 × oil) objectives. Specimens for molecular analysis were examined without dissecting under a compound microscope (objective 63 × dry) in propylene glycol ($CH_3CH(OH)CH_2OH$) and, after examination, were preserved in 100% ethanol. Morphological terminology follows Karanovic (2008). All specimens examined morphologically for microcharacters are deposited in the National Institute of Biological Resources (NIBR), Seoul.

Results

Molecular analyses

Amplification success rates were quite different for different genes, the best ones being those for the 12S (close to 95%). For the other three genes the PCR-amplification efficiency was much lower, the lowest one being for the cytB (< 35%), which is expected given that the cytB is the fastest evolving gene here. Low amplification rates suggest that we are yet to find an optimal procedure and combination of primers for this group and each gene. We did, however, test most primers available for copepods, and spent a lot of time on the optimization of the PCR protocol (finding the optimal annealing temperature on the temperature gradient). The ingroup was well defined in all analyses, and the topology of the resulting cladograms did not differ depending on the phylogenetic method used, which shows that our data were robust and informative for the analysis, despite relatively short fragments of each gene. This is further confirmed by very high retention and consistency indexes in all MP analyses, and especially for the 12S.

Phylogenetic analysis of the 12S sequence data (Fig. 2) revealed at least four well-defined clades, each supported with high bootstrap values, especially in the MP and NJ analyses: England/France/Scotland-clade, New Zealand-clade, Australia (LR)/(LZ)-clade and Australia (MR)/USA/Germany-clade (for locality data see Tab. 1). Average pairwise NJ distances between the New Zealand and Australia (LR)/(LZ) clades are about 7.3% (see Tab. 3), while those among other clades are all in excess of 12.3%, and those between the New Zealand and Australia (MR)/USA/Germany clades in excess of 27%. Such large divergence values are generally indicative of distinct species by comparison with other crustaceans, even for much faster evolving genes like the COI (Lefébure *et al.*, 2006), and are well within accepted values for distinct species in better studied non-related animal groups (Seddon *et al.*, 1998). They are also very similar to those between well-defined (and mostly described) species of the cyclopoid genus *Cyclops* Müller, 1785 (M. Krajicek, in preparation). This result prompted our analysis of additional genes and morphological characters. Another interesting result of the 12S sequence data analysis is the presence of the same haplotype on two opposite sides of Australia (Lake Richmond (Q17 and R38) in Western Australia and Lake Zot (Q18) in New South Wales), which confirms some previous observations on the lack of morphological variability between highly disjunct cyclopoid populations on this continent (Karanovic, 2004, 2005), and provides evidence for the homogenization of freshwater fauna here. An even more surprising result was the presence of the same haplotype in highly disjunct populations in Australia (Q20 and Q21), Germany (P06 and R32) and the USA (P12) (see Fig. 2). This result we interpret as evidence for anthropogenic translocation, but it is probably not associated with early shipping activities (further discussed below). The resulting 12S cladogram does not fully support any of the two competing hypotheses (compare Figs. 1 and 2), suggesting rather a much more complex history of this species complex. This is best illustrated by the presence of two completely different haplotypes in south-western Western Australia, one in Lake Richmond (Q17 and R38) and another in Margaret River (Q20 and Q21).

The 16S sequence dataset was much more limited, due to the limited PCR-amplification efficiency, but all phylogenetic analyses (Fig. 3) confirmed that two populations from England (W and L) are closely related to those from France and Scotland, which was a well-defined clade in our 12S analysis (Fig. 2). The divergence values in this clade are even smaller for 16S sequences (Tab. 4), with no difference whatsoever between the French and English populations, and only 0.7% divergence between them and the Scottish population. The population from Lake

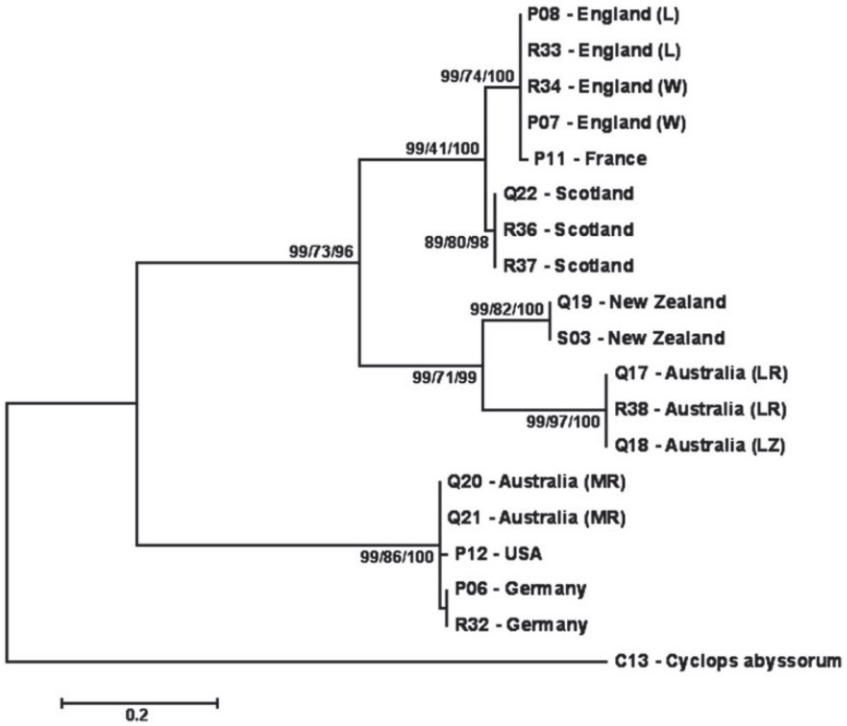

Fig. 2. One of six equally parsimonious trees based on 12S sequence data (CI = 0.921; RI = 0.964) for 18 specimens from 10 different locations of *M. albidus* (Jurine, 1820). The numbers on the branches representing bootstrap values for three different methods (MP/ML/NJ). The outgroup in this analysis was *C. abyssorum* G.O. Sars, 1863, collected in Nizne Temnosmrecinske, Slovakia (Tab. 1). The cladogram is drawn to scale and specimen numbers correspond to those in Table 1.

Richmond in Western Australia (S13) is quite distant from this clade, with average pairwise distances being in excess of 8% (Tab. 4), while the German population shows divergence values in excess of 10% when compared with the England/France/Scotland-clade and 13% when compared with the Australian haplotype. From our experience (Karanovic and Cooper, 2011), these divergence values in copepods would be indicative of distinct species even for COI sequences, and generally the 16S evolves more slowly than COI (Pesole *et al.*, 1999). In comparison with other crustaceans, for example, the maximum uncorrected divergence values between species of opossum shrimps from the genus *Mysis* Latreille, 1802 were 6% for the 16S (Audzijonyte *et al.*, 2005).

Our 18S sequence dataset was also somewhat limited (Fig. 4), but no differences were observed between any sequences of *M. albidus s.l.* This was expected, as nuclear genes generally evolve much slower than mitochondrial ones (Pesole *et al.*, 1999; Audzijonyte *et al.*, 2005), and it is consistent with our research on other cyclopoids with evidence of cryptic speciation (T. Karanovic and M. Krajicek, in preparation). This result probably indicates that cryptic species in the *M. albidus* complex are all relatively young, which would explain why they were never found together in the same locality. As their distributions are not allopatric, and do not conform to any zoological patterns associated with natural dispersal, we interpret this as further evidence of anthropogenic translocation.

Finally, our cytB sequence dataset was most limited, as we managed to PCR-amplify multiple specimens only of the first clade recognized in the 12S analysis. Nevertheless, the reconstructed phylogeny (Fig. 5) shows a similar topology to those reconstructed with the 12S and 16S sequences. Two English specimens from two different locations (S21 and S19) cluster together with the Scottish specimen (S22), with divergence values between them of 5.1 and 7.8% (Tab. 5). These values are all within intraspecific variability levels, in comparison with other crustaceans (Audzijonyte *et al.*, 2005), as the cytB evolves at a similar rate or slightly faster than COI (De Filippis and Moore, 2000; Feldman and Omland, 2004). The divergence values among the other three sequences (Australia (LR), New Zealand and Germany), and between them and the English/Scottish clade, are all in excess of 23%, suggesting four distinct species, as already indicated by the 12S and 16S sequences.

Comparative morphology

We only had adult females left for morphological observation from the representatives of the first and fourth

Table 3. Pairwise NJ distances (Tajima-Nei model) among 12S sequences between 19 specimens from 10 different locations of *M. albidus* (Jurine, 1820), and the outgroup *C. abyssorum* G.O. Sars, 1863 (for locality data and specimen numbers see Tab. 1).

Specimen	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. C13 – *C. abyssorum*																		
2. P06 – Germany	0.522																	
3. P07 – England (W)	0.517	0.252																
4. P08 – England (L)	0.517	0.252	0.000															
5. P11 – France	0.517	0.256	0.003	0.003														
6. P12 – USA	0.523	0.005	0.252	0.252	0.256													
7. Q17 – Australia (LR)	0.543	0.283	0.156	0.156	0.159	0.292												
8. Q18 – Australia (LZ)	0.543	0.283	0.156	0.156	0.159	0.292	0.000											
9. Q19 – New Zealand	0.504	0.269	0.132	0.132	0.135	0.277	0.073	0.073										
10. Q20 – Australia (MR)	0.529	0.003	0.248	0.248	0.252	0.003	0.288	0.288	0.273									
11. Q21 – Australia (MR)	0.529	0.003	0.248	0.248	0.252	0.003	0.288	0.288	0.273	0.000								
12. Q22 – Scotland	0.518	0.240	0.016	0.016	0.019	0.240	0.150	0.150	0.123	0.236	0.236							
13. R32 – Germany	0.522	0.000	0.252	0.252	0.256	0.005	0.283	0.283	0.269	0.003	0.003	0.240						
14. R33 – England (L)	0.517	0.252	0.000	0.000	0.003	0.252	0.156	0.156	0.132	0.248	0.248	0.016	0.252					
15. R34 – England (W)	0.517	0.252	0.000	0.000	0.003	0.252	0.156	0.156	0.132	0.248	0.248	0.016	0.252	0.000				
16. R36 – Scotland	0.518	0.240	0.016	0.016	0.019	0.240	0.150	0.150	0.123	0.236	0.236	0.000	0.240	0.016	0.016			
17. R37 – Scotland	0.518	0.240	0.016	0.016	0.019	0.240	0.150	0.150	0.123	0.236	0.236	0.000	0.240	0.016	0.016	0.000		
18. R38 – Australia (LR)	0.541	0.282	0.156	0.156	0.159	0.291	0.000	0.000	0.072	0.287	0.287	0.149	0.282	0.156	0.156	0.149	0.149	
19. S03 – New Zealand	0.500	0.270	0.133	0.133	0.136	0.278	0.073	0.073	0.000	0.274	0.274	0.123	0.270	0.133	0.133	0.123	0.123	0.073

clades recognized in our 12S analysis (see Fig. 2). We studied all morphological macro- and micro-characters in two specimens from the USA, ten from Scotland, three from France, and two from England (W) (for locality data, see Tab. 1). For comparison of homologous microstructures in different specimens, rows or groups of spinules were provisionally assigned letters of the Greek alphabet, and pores and sensilla Arabic numerals. A majority of morphological characters showed remarkable similarity, down to the size and number of spinules in homologous rows and number and position of pores on most appendages and prosomal somites, which is to be expected from a species complex and is not presented here. Shape and ornamentation of the mandibular cutting edge (Figs. 6(d) and 9(d, e)) suffice as an illustration.

We found a surprisingly high number of differences as well that are not variable in the studied representatives of these clades. Presented here first are significant morphological features of the USA population (Figs. 6–8), followed by the same structures in the Scottish population, with distinguishing characters marked with black arrows (Figs. 9–11), and then a few cases of minor variability within the first clade, as observed in the specimens from England (W) (Fig. 12).

The habitus shape (Figs. 6(a) and 9(a)) is more robust in the Scottish population, with a more rounded cephalothorax, but this character is not so reliable, as it depends somewhat on the relative position to free thoracic somites in preserved specimens. Relative length of the outer principal caudal seta is more reliable, and it can also be observed without dissecting the specimens (Figs. 6(a) and 9(a)). Scottish specimens also have a somewhat wider anal operculum (Figs. 7(b) and 10(b)) and a longer inner spine on the fifth leg (Figs. 8(b) and 11(d)). Most differences, however, were discovered in the pore/sensillum pattern on the genital double-somite, spinule pattern on the antenna, and shape and ornamentation of the fourth swimming leg.

All homologous pores and sensilla on the ventral side of the genital double-somite are present in both clades (Figs. 7(a) and 19(a)), but the position of the pore No. 19 is much more posterior in the first clade (arrow in Fig. 10(a)). Dorsally, however, this clade is missing pores and sensilla nos. 1, 4, 8, 12 and 13 (arrows in Fig. 10(b)), and the element No. 7 is a sensillum, not a pore. The latter character is the first evidence that pores and sensilla are in fact homologous structures. Usefulness of pore and sensillum pattern in the delineation of closely related species was previously nicely demonstrated by Alekseev *et al.* (2005) in the cyclopoid genus *Eucyclops* Claus, 1893, although for prosomal somites, not for urosomal ones. Note that all pores in No. 10 were asymmetrical in all examined specimens.

Most groups of spinules on the basis of antenna are present in both clades (Figs. 6(b, c), 9(b, c) and 12(b)), but the first clade has an additional σ-row of spinules on the posterior surface (arrow in Fig. 9(c)), and the ε-row on the anterior surface is considerably shorter and with more slender spinules (arrow in Fig. 9(b)).

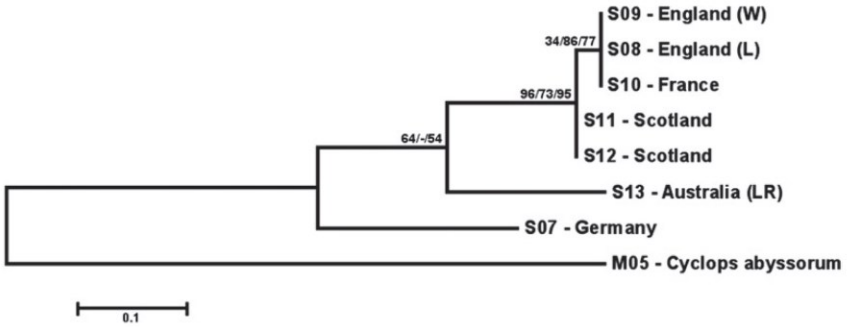

Fig. 3. One of 18 equally parsimonious trees based on 16S sequence data (CI = 0.937; RI = 0.666) for seven specimens from six different locations of *M. albidus* (Jurine, 1820). The numbers on the branches representing bootstrap values for three different methods (MP/ML/NJ). The outgroup in this analysis was *C. abyssorum* G.O. Sars, 1863, collected in Litvorove, Slovakia. The cladogram is drawn to scale and specimen numbers correspond to those in Table 1.

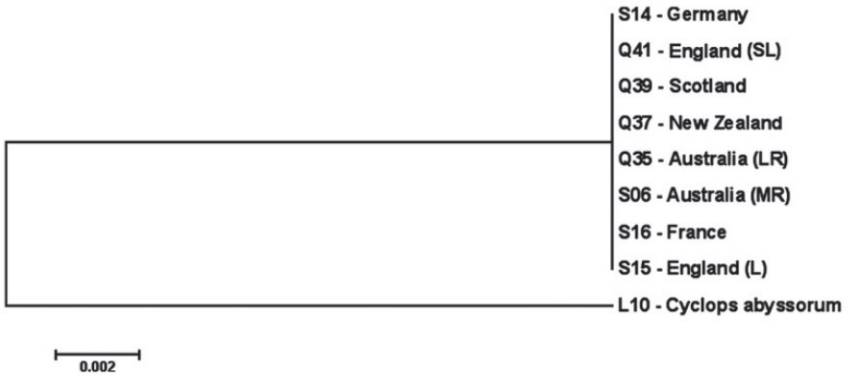

Fig. 4. NJ tree based on the 18S sequence data for eight specimens from as many different locations of *M. albidus* (Jurine, 1820). The outgroup in this analysis was *C. abyssorum* G.O. Sars, 1863, collected in Vysne Temnosmrecinske, Slovakia. The cladogram is drawn to scale and specimen numbers correspond to those in Table 1.

Table 4. Pairwise NJ distances (Kimura 2-parameter model) among 16S sequences between seven specimens from six different locations of *M. albidus* (Jurine, 1820) and the outgroup *C. abyssorum* G.O. Sars, 1863 (for locality data and specimen numbers, see Tab. 1).

Specimen	1	2	3	4	5	6	7
1. M05 – *C. abyssorum*							
2. S07 – Germany	0.472						
3. S08 – England (L)	0.450	0.100					
4. S09 – England (W)	0.450	0.100	0.000				
5. S10 – France	0.450	0.100	0.000	0.000			
6. S11 – Scotland	0.466	0.104	0.007	0.007	0.007		
7. S12 – Scotland	0.466	0.104	0.007	0.007	0.007	0.000	
8. S13 – Australia (LR)	0.474	0.130	0.091	0.091	0.091	0.083	0.083

Fourth swimming legs (Figs. 6(e), 8(a), 11(a, b, c) and 12(c, d)) of the two clades differ in the length/with ratio of the coxa (arrows in Fig. 11(a, b)), number of spinules in the η-row on the posterior surface of coxa and the shape of that row (arched in the first clade; arrow in Fig. 11(a)), presence/absence of the pore No. 3 on the anterior side of coxa (arrows in Fig. 11(b)), angle between the two apical spines on the third endopodal segment and their relative length (the outer one always considerably longer in the first clade; arrows in Fig. 11(c)), and the relative length of the distal inner seta on the same segment (also arrows in Fig. 11(c)). Other small differences include number of spinules in different rows. For example, the β-row on the posterior side of the intercoxal sclerite bears 20–21 spinules in the American specimens (Fig. 6(e)) and 28–30 in the Scottish ones (Fig. 11(a)). This morphological

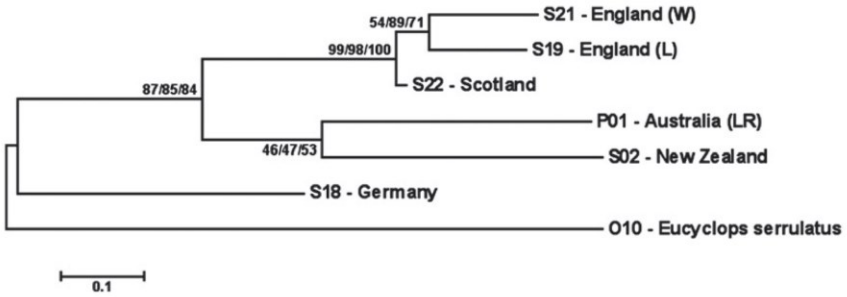

Fig. 5. Single most parsimonious tree based on cytB sequence data (CI = 0.859; RI = 0.642) for six specimens from as many different locations of *M. albidus* (Jurine, 1820). The numbers on the branches representing bootstrap values for three different methods (MP/ML/NJ). The outgroup in this analysis was *E. serrulatus* (Fischer, 1851), collected in Tupadly, the Czech Republic. The cladogram is drawn to scale and specimen numbers correspond to those in Table 1.

Table 5. Pairwise NJ distances (TN model) among cytB sequences between six specimens from as many locations of *M. albidus* (Jurine, 1820) and the outgroup *E. serrulatus* (Fischer, 1851) (for locality data and specimen numbers, see Tab. 1).

Specimen	1	2	3	4	5	6
1. O10 – *E. serrulatus*						
2. P01 – Australia (LR)	0.477					
3. S02 – New Zealand	0.545	0.241				
4. S18 – Germany	0.432	0.265	0.304			
5. S19 – England (L)	0.463	0.295	0.255	0.328		
6. S21 – England (W)	0.492	0.282	0.239	0.307	0.078	
7. S22 – Scotland	0.423	0.241	0.233	0.259	0.051	0.051

feature is probably connected with increased number of spinules in other rows on this appendage in the first clade, and should not be considered as an independent character.

Discussion

It is beyond the scope of this paper to revise the taxonomy of the *M. albidus* complex, as this would involve re-examination of all available type material for various species and subspecies described in the past that are now considered to be junior subjective synonyms of this species (for a comprehensive list of synonyms, see Dussart and Defaye, 2006). The problem is more compounded by the fact that the type material of *M. albidus* is lost (Peter Schwendinger and Muséum Genève, personal communication), and designating neotypes from the type locality would be futile in the age of a homogenized world's freshwater fauna (Rahel, 2007; Schram, 2008). Specimens of this species that lived in that area in 1820 may have been replaced a number of times with other closely related species, which could have been described from elsewhere and with the type material still extant. We could have designated one of the specimens examined here morphologically as a neotype, but consequent revision of the complex may prove it to be conspecific with one of the current synonyms of *M. albidus*, which would only add unnecessary confusion.

Our analysis of the 12S sequences (Fig. 2) showed the presence of the same haplotype in the highly disjunct populations in Western Australia, Germany and the USA, which cannot be explained by any modes of dispersal known for freshwater fauna other than anthropogenic translocation. Another identical haplotype on two opposite sides of the Australian continent (Fig. 5), more than 3,300 km apart, can also probably be explained in the same way, as much smaller distances in Europe show greater divergences within clades. Both examples are strong evidence for the homogenization of the world freshwater cyclopoid fauna, which emphasizes difficulties in interpreting biogeography in a globalized world and further add to the current biodiversity crisis. Evidence from the fossil record suggests that speciation appears to be more strongly related to invasion than extinction; when invasion intensity is greatest speciation rates decline and vicariant speciation in particular is retarded (Stigall, 2007). That, in combination with generalist survival, suggests that one consequence of the modern biodiversity crisis will be the establishment of a species-poor but biogeographically widespread fauna – not unlike the observed biotas of the latest Ordovician and Devonian. Unfortunately, current museum practices are not designed to cope with this challenge of a globalized fauna. They are hardly adequate to study species-level changes in a certain area, let alone population-level changes; while most homogenization probably happens on the population level (humans themselves are a good example here). If we are to be able to

Fig. 6. *M. albidus s.l.* (Jurine, 1820), USA, adult female (NIBRIV0000232633): (A) habitus dorsal view; (B) basis of antenna, anterior view; (C) basis of antenna, posterior view; (D) cutting edge of mandibula, anterior view; (E) intercoxal plate, coxa, and basis of fourth swimming leg, posterior view. Greek letters identify different ornamentation elements. All scales 100 μm.

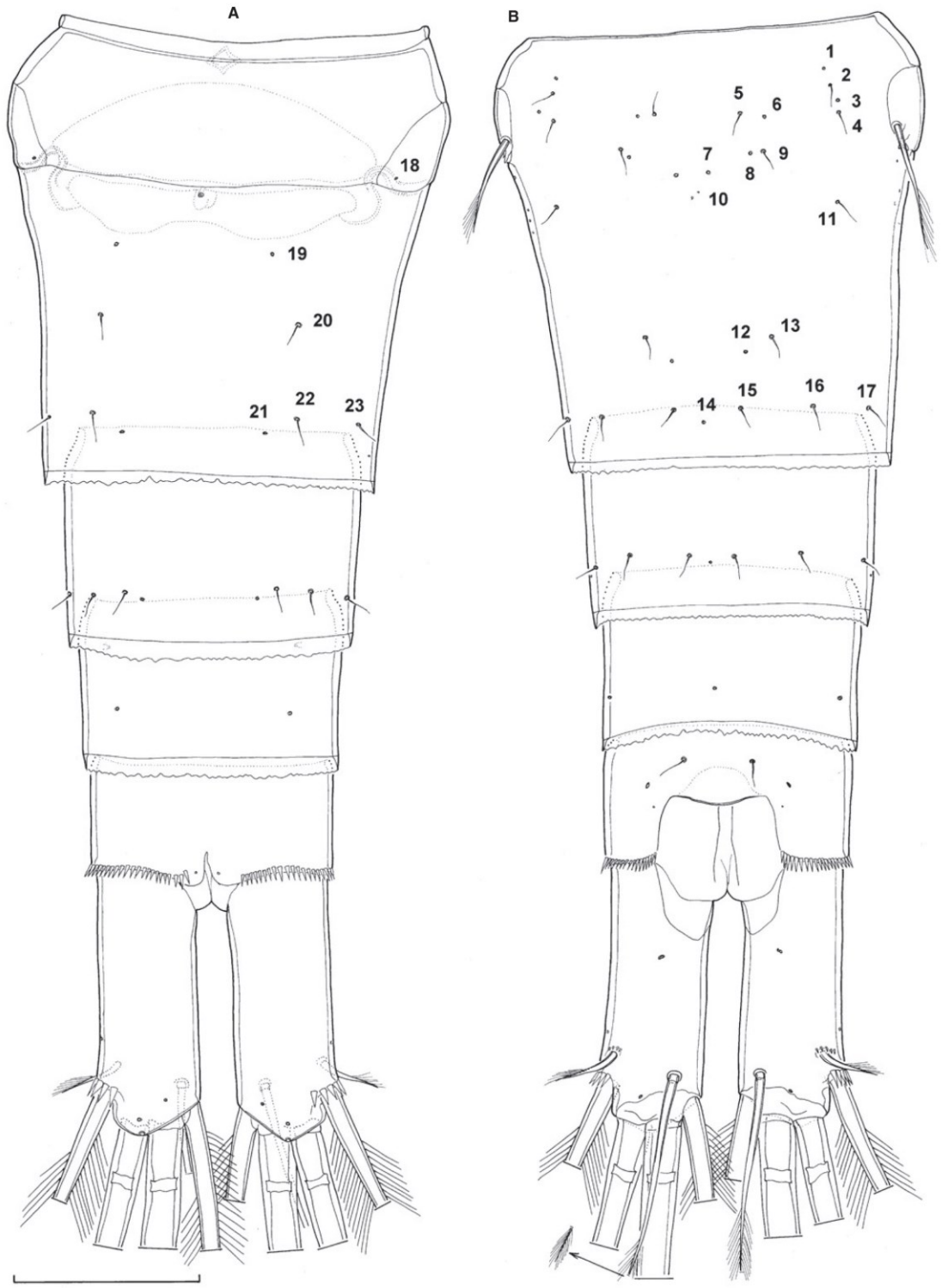

Fig. 7. *M. albidus s.l.* (Jurine, 1820), USA, adult female (NIBRIV0000232633): (A) urosome, ventral view; (B) urosome, dorsal view. Numbers identify different pores and sensilla on genital double somite. Scale: 100 μm.

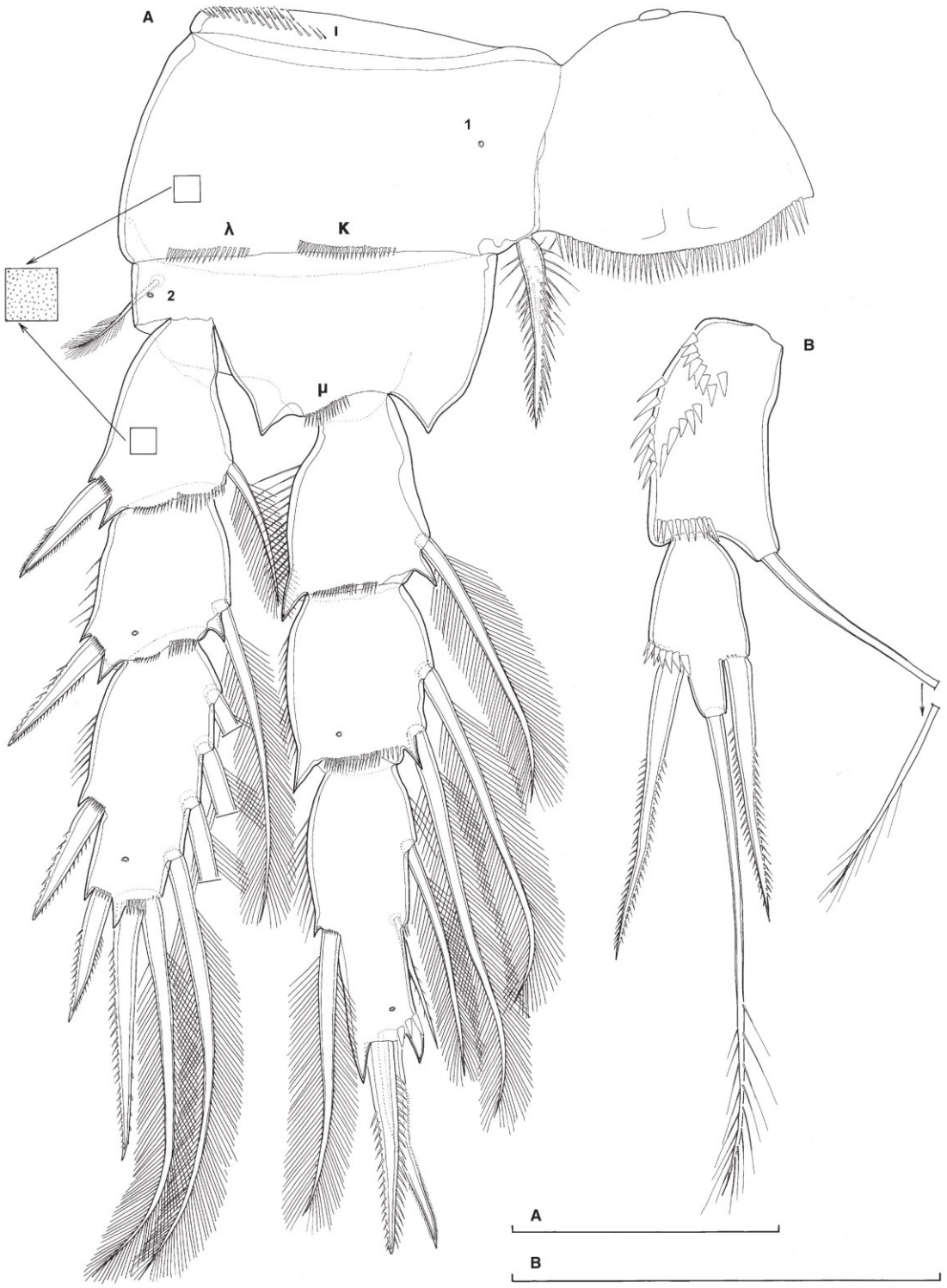

Fig. 8. *M. albidus s.l.* (Jurine, 1820), USA, adult female (NIBRIV0000232633): (A) fourth swimming leg, anterior view; (B) fifth leg, anterior view. Greek letters identify different rows of spinules and numbers identify pores on coxa and basis. Both scales 100 μm.

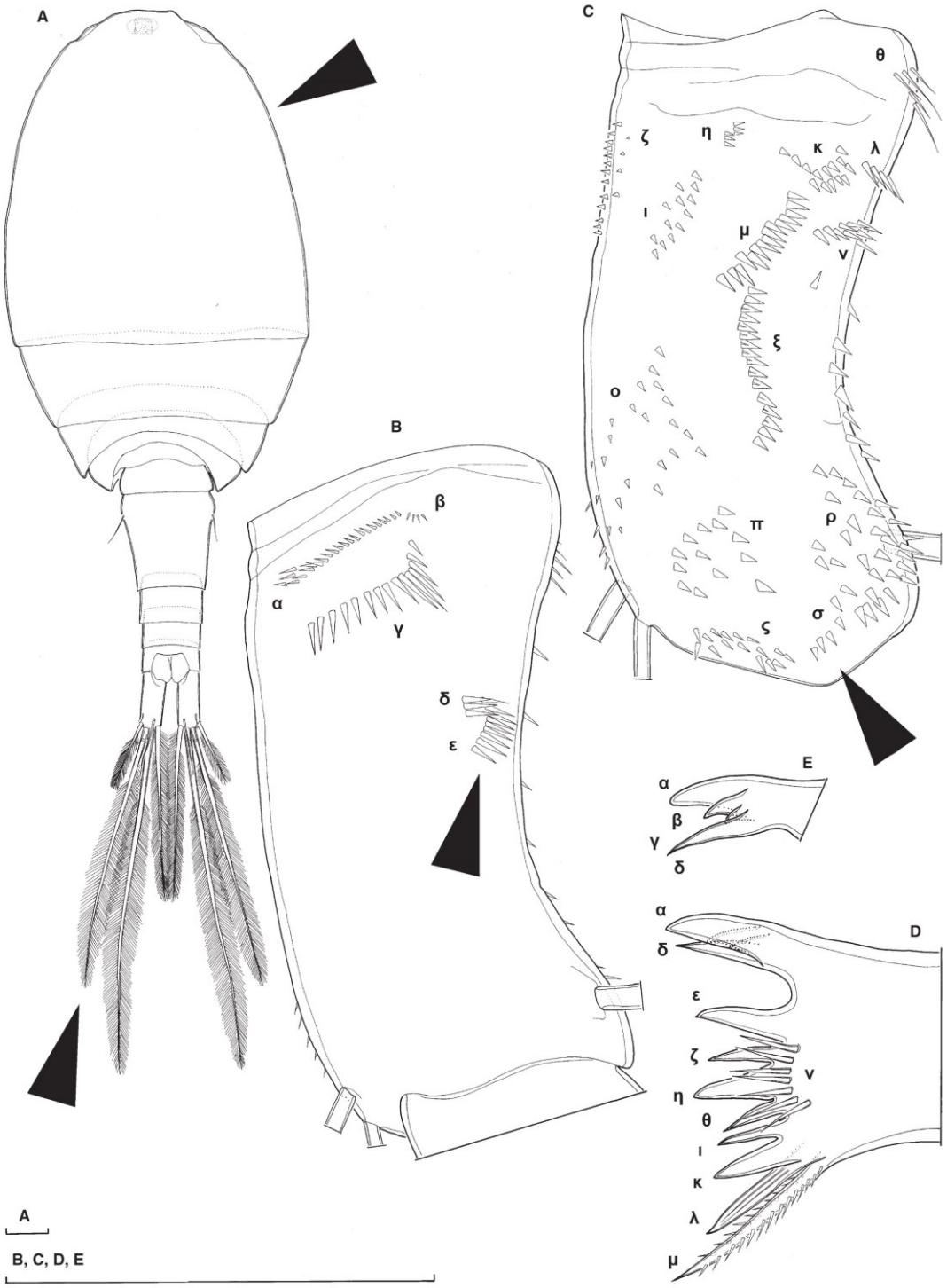

Fig. 9. *M. albidus s.l.* (Jurine, 1820), Scotland, adult female (NIBRIV0000232634): (A) habitus dorsal view; (B) basis of antenna, anterior view; (C) basis of antenna, posterior view; (D) cutting edge of mandibula, anterior view; (E) quadricuspidate ventral spine on cutting edge of mandibula, posterior view. Greek letters identify different ornamentation elements and arrows point out most distinguishing features. Both scales 100 μm.

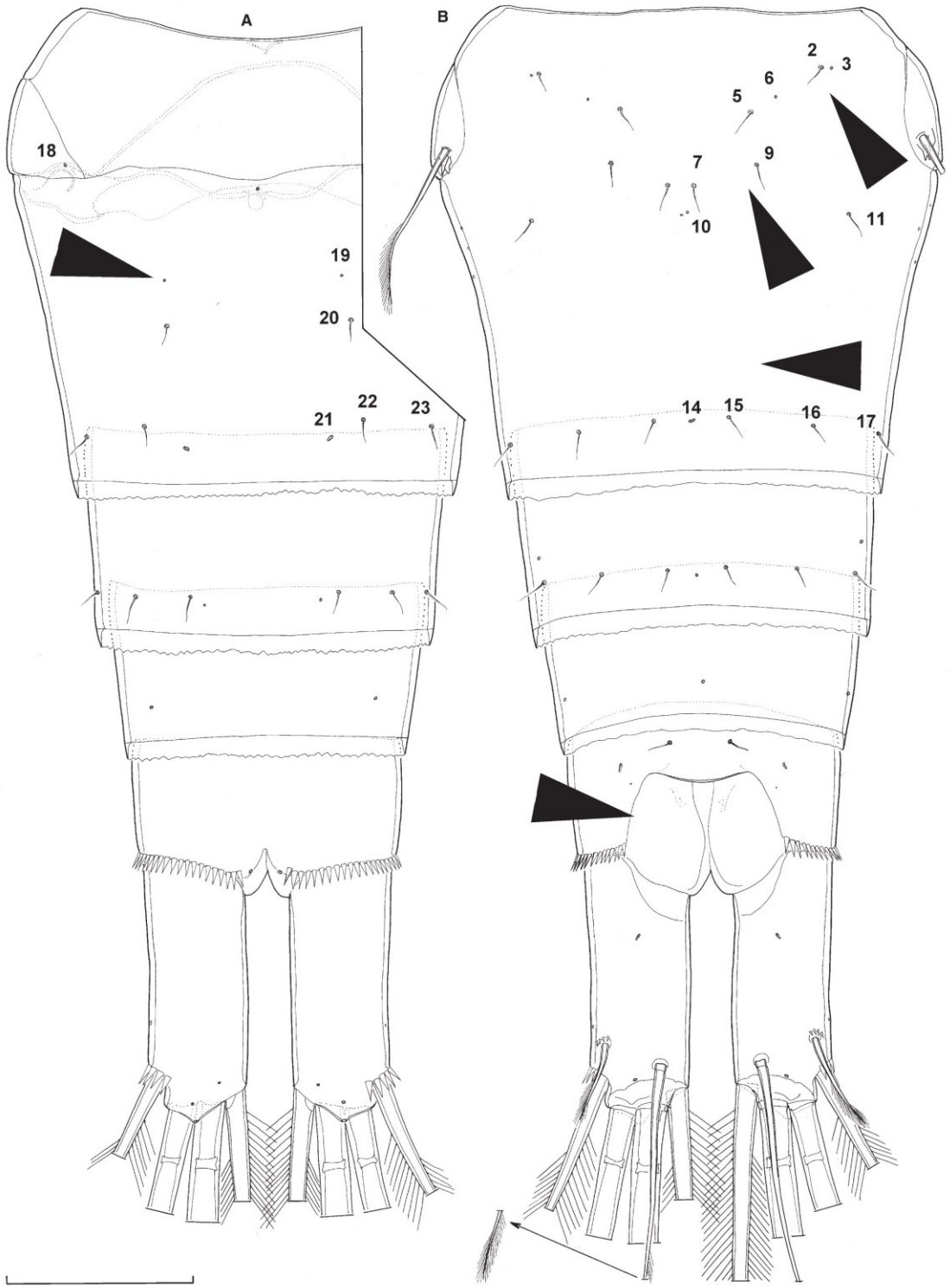

Fig. 10. *M. albidus s.l.* (Jurine, 1820), Scotland, adult female (NIBRIV0000232634): (A) urosome, ventral view; (B) urosome, dorsal view. Numbers identify different pores and sensilla on genital double somite and arrows point out most distinguishing features. Scale 100 μm.

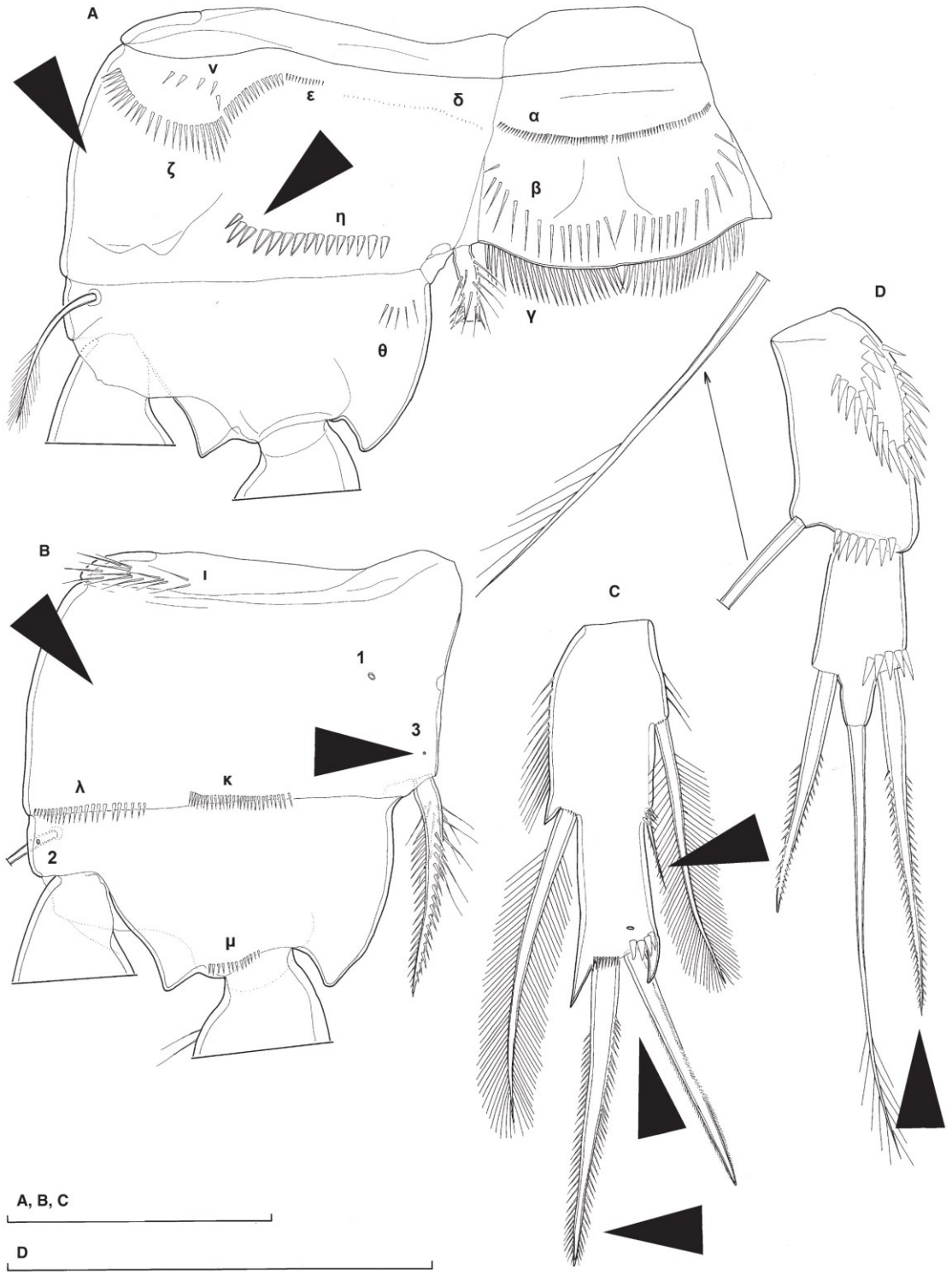

Fig. 11. *M. albidus s.l.* (Jurine, 1820), Scotland, adult female (NIBRIV0000232634): (A) intercoxal plate, coxa and basis of fourth swimming leg, posterior view; (B) intercoxal plate, coxa and basis of fourth swimming leg, anterior view; (C) third endopodal segment of fourth swimming leg, anterior view; (D) fifth leg, anterior view. Greek letters identify different rows of spinules, numbers identify pores on coxa and basis, and arrows point out most distinguishing features. Both scales 100 μm.

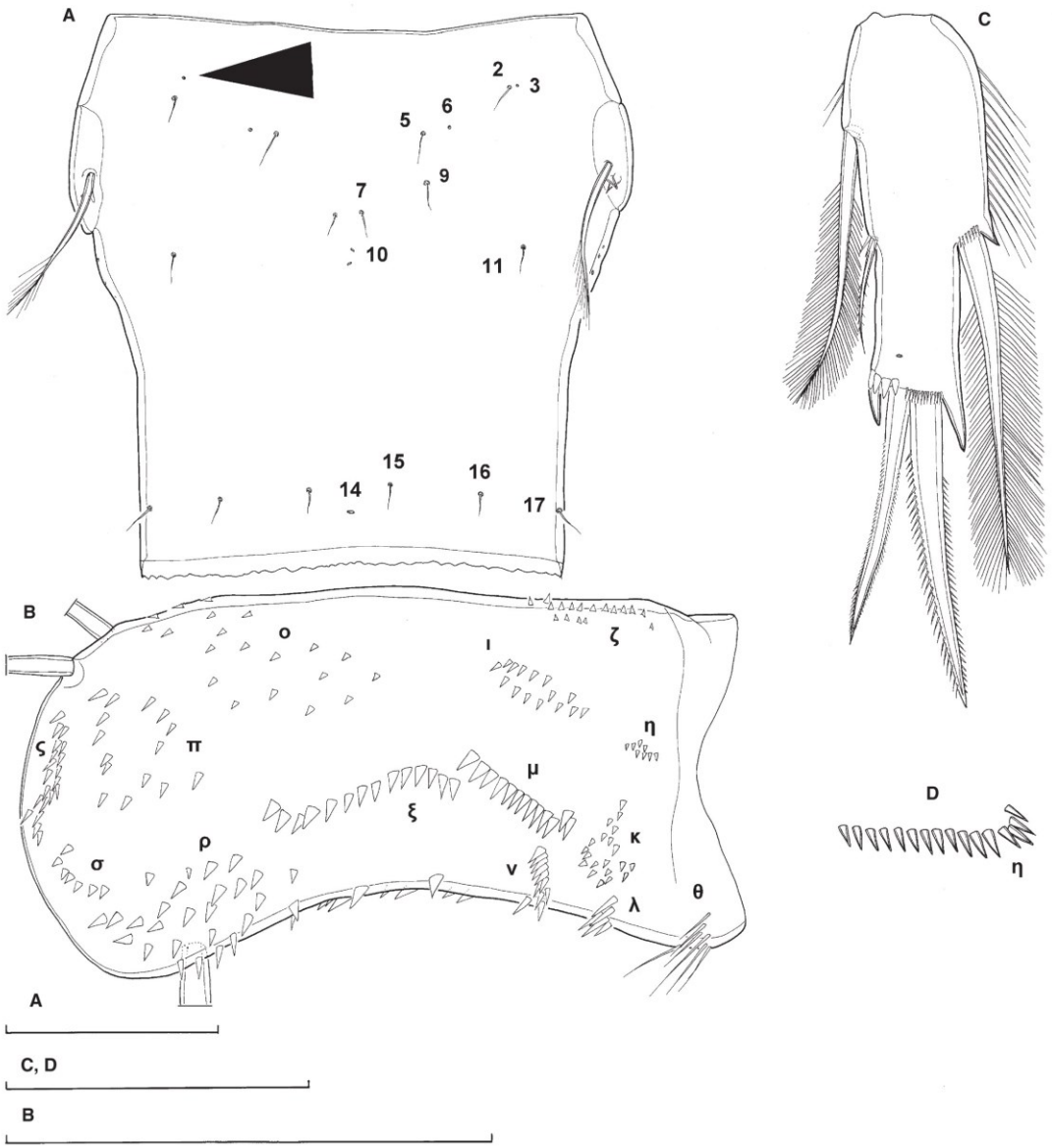

Fig. 12. *M. albidus s.l.* (Jurine, 1820), England (W), adult female (NIBRIV0000232635): (A) genital double-somite, dorsal view; (B) basis of antenna, posterior view; (C) third endopodal segment of fourth swimming leg, anterior view; (D) distal row of spinules on posterior surface of fourth swimming leg coxa; greek letters identify different ornamentation elements, numbers identify pores and sensilla on genital double somite and arrow points out asymmetry in ornamentation. All scales 100 μm.

follow these changes adequately, a massive, world-wide sampling of all taxa is needed, and curation of such a collection has to transcend national boundaries. Such a collection would enable studies like this one to be performed on a routine basis. Most museums are funded today by states rather than federal national governments, and national collections (such as the NIBR in Korea) are extremely rare. None of them are funded sufficiently to study global diversity by themselves, or to curate collections that would enable researchers to pursue such goals. Individual samplings for isolated projects and groups are, of course, less effective and more costly.

One of the aims of this study was to examine global molecular diversity of *M. albidus*, and this is only partly fulfilled with 11 populations from three continents (Tab. 1). We also had a few specimens from Asia, but they were not properly preserved for DNA analysis, and all our attempts at PCR-amplifying any of the four genes

studied were unsuccessful. We were not able to obtain any specimens from Africa or South America, although the species has been reported a number of times from both continents (Dussart and Defaye, 2006). One has to expect that more haplotypes will be discovered in future studies, when more material becomes available, and that some of the existing ones will be reported from other places. We hope these studies will follow shortly, as we think that *M. albidus s.l.* is a very good model organism for studies of the homogenization of the world freshwater fauna for a number of reasons: cosmopolitan distribution, relatively large size for a cyclopoid copepod, easy to disperse passively, easy to identify morphologically (with only six valid species in the genus), and with a probably very long history of anthropogenic translocation. Our analyses also showed several cases of incongruence among the support values for MP, ML and NJ, which may reflect relatively poor fit for the particular model of evolution used, or a weak support at those nodes. Further studies are needed to answer these questions too.

Unfortunately, in this study, we were not able to confirm the hypothesis of Karanovic (2005) that the cosmopolitan distribution of some cyclopoid copepods, and especially their presence in Australia and New Zealand, is a result of early shipping activities by European settlers (Fig. 1(a)). None of the reconstructed phylogenies (Figs. 2–5) showed a close relationship of the English and Australian/New Zealand populations. This, however, still does not mean that the hypothesis is false, as the original English populations may have been replaced by invaders from other places, or we have just not been able to find the ancestral population in England. This country went through enormous changes in the last 300 years, as did many other parts of the world. It is also possible that the original invaders in Australia have been replaced by new invaders. Hopefully, further studies on more populations and other species (T. Karanovic and M. Krajicek, in preparation) will shed more light on these interesting questions. In general, however, we were able to disprove that the current cosmopolitan distribution of *M. albidus* is entirely the result of natural dispersal (Fig. 1(b)), both by finding the same haplotype in highly disjunct and zoogeographically unrelated places (Fig. 2) and by discovering cryptic speciation in this complex.

The latter came as a surprise, considering the long history of studies on this species, very few members in the genus, and all of them being extremely similar morphologically and traditionally mostly discriminated by details of the ornamentation on the caudal rami. Detailed examination, however, of the females of two of these cryptic species (Figs. 6–12) showed a number of distinguishing features, especially in the fine ornamentation of the genital double-somite, antenna and fourth swimming leg. These characters have been underestimated, considered as intraspecific variability, or, most commonly, overlooked in the past, due to an inadequate quality of microscopes used or procedures followed. Current studies on copepods that combine molecular and morphological tools (Alekseev *et al.*, 2005; Sakaguchi and Ueda, 2010; Karanovic and Cooper, 2011) show the importance of these microstructures on the species level, and how they can be used to effectively discriminate closely related congeners. Unfortunately, it means that we have been greatly underestimating the diversity of these small crustaceans in the past.

As mentioned above, our 18S analysis probably indicates that the cryptic species in the *M. albidus* complex are relatively young. Further support for this is that no two clades were found sympatrically (in the same water body), although some of them do live in relatively close places (Margaret River and Lake Richmond in Western Australia, and France and Germany for example); but our sample size in most cases was relatively small. If proven in further studies, this would indicate that they probably exclude each other by competing for the same resources, and that their original (pre-anthropogenic) distribution was allopatric. These are indeed handy properties for experimental studies on invasive species, and copepod crustaceans are ideal experimental organisms due to their small size, short generation time and easy maintenance (Winkler *et al.*, 2008). Whereas phylogenetic analyses can show the direction of niche shifts (best known on oceanic islands), we do not really understand how a population may enter a new habitat or exploit new resources, persist for many generations in the face of abiotic and biotic pressures to which it is not well adapted, and undergo genetic and phenotypic changes that enhance fitness and correlatively render it a new species (Abbott *et al.*, 2003). Human-introduced invasive species may serve as good surrogates for understanding processes of colonization and niche shifting (Levin, 2003), and we can probably gain a new perspective on these phenomena by viewing new species as successful invaders, and by using invasive species as a model system for understanding the early stages of speciation (Lee *et al.*, 2003, 2007). Both new species and introduced populations, for example, go through similar bottlenecks (May *et al.*, 2006).

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Chapter 5:

First molecular data on the Western Australian *Diacyclops* (Copepoda, Cyclopoida) confirm morpho-species but question size differentiation and monophyly of the *alticola*-group

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This small study was my first publication, also in cooperation with Tom Karanovic. It is focussed on a specific subterranean water habitat in Western Australia consisting of numerous isolated calcrete aquifers that lie along paleodrainage channels. These channels, ranging in diameter from tens kilometres to hundreds of meters, are known as a significant biodiversity hot-spot (Humphreys, 2008). The diversity of stygofauna is most dependent on the size of a calcrete, and the larger ones may harbor a much more diverse copepod fauna including endemic species (Karanovic & Cooper, 2011, 2012). Size differentiation among sympatric congeners of copepods of the genus *Diacyclops* was observed (Karanovic, 2006) and considered an important evolutionary phenomenon.

We decided to use molecular tools to test three *Diacyclops* species belonging to the *alticola*-group collected from bores in the Pilbra region of Western Australia. The difference in the size of these species was significant, allowing their separation even during the sorting under the dissecting microscope. Nevertheless, most other morphological characters were highly conservative. We examined the molecular diversity based on mitochondrial (12S rRNA) and nuclear (18S rRNA) genes of three subterranean interstitial species belonging to *Diacyclops alticola*-group (*D. humphreysi*, *D. scaloni* and *D. sobeprolatus*) supplemented with two planktonic congeners *D. bicuspidatus* and *D. bisetosus*. Two outgroup species (*Macrocyclops albidus* and *Eucyclops serrulatus*) were tested as well.

Our results represent the first 12S sequence data for the genus *Diacyclops* and confirm all three species of the *Diacyclops alticola*-group despite their conservative morphology. However, our analysis based on both tested genes show that the relationship between *D. scaloni* on the one side and the *D. humphreysi* / *D. sobeprolatus* clade on the other is much more remote than the morphological data would suggest. This also indicates a possible paraphyly of the *alticola*-group and emphasizes the importance of the use of molecular tools in systematics. A possibility of cryptic speciation in the "cosmopolitan" *D. bisetosus* is also suggested due to K2P average pairwise distance of 12S sequences exceeding 7 % in specimens collected from a single locality.

BRILL

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CRUSTACEANA

FIRST MOLECULAR DATA ON THE WESTERN AUSTRALIAN *DIACYCLOPS* (COPEPODA, CYCLOPOIDA) CONFIRM MORPHO-SPECIES BUT QUESTION SIZE DIFFERENTIATION AND MONOPHYLY OF THE *ALTICOLA*-GROUP

BY

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ABSTRACT

Size differentiation has been considered an important phenomenon in evolution, and in situ speciation was hypothesized in the past for the parapatric subterranean Western Australian *Diacyclops* Kiefer, 1927 species from the *alticola*-group, based on morphological evidence. Aims of this study are to: derive their preliminary molecular phylogenies based on mitochondrial (12S) and nuclear (18S) genes; test if morpho-species are supported by molecular data; examine monophyly of the *alticola*-group; and test whether the size differences evolved in situ after colonization by a single ancestral species or resulted from different phylogeny. Analyses of the 12S sequences reveal at least six well defined clades, each corresponding to one morpho-species. The divergences are very high between all species, suggesting only a remote relationship, with those between sympatric species with significant size difference being in excess of 27%. Surprisingly, all analyses show very high bootstrap values for the clade formed by two cosmopolitan surface-water species, *Diacyclops bisetosus* (Rehberg, 1880) and *D. bicuspidatus* (Claus, 1857), despite numerous morphological differences. The 18S dataset also supports only a remote relationship between *Diacyclops scanloni* Karanovic, 2006 and two other Western Australian members of the *alticola*-group: *D. humphreysi* s. str. Pesce & De Laurentiis, 1996 and *D. sobeprolatus* Karanovic, 2006. Preliminary analyses suggest absence of in situ speciation and parallel evolution in the Western Australian *Diacyclops*, interspecific size differentiation being a result of different phylogeny. The *alticola*-group may be polyphyletic, and we recognize morphological characters that define two main lineages. A possibility of cryptic speciation in the cosmopolitan *D. bisetosus* is also suggested, and several sequences of *Diacyclops* available from GenBank are recognized either as contamination or misidentification.

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RÉSUMÉ

La différenciation par la taille a été considérée comme un phénomène important dans l'évolution, et l'hypothèse de la spéciation in situ a été proposée dans le passé pour les espèces souterraines parapatriques du genre *Diacyclops* Kiefer, 1927 appartenant au groupe *alticola*, à partir des données morphologiques. Les objectifs de cette étude sont : élaborer leur phylogénie moléculaire préliminaire à partir de gènes mitochondriaux (12S) et nucléaires (18S) ; tester si les espèces morphologiques sont soutenues par les données moléculaires ; examiner la monophylie du groupe alticola ; et enfin tester si les différences de taille ont évolué in situ après colonisation par une seule espèce ancestrale ou si elles résultent d'une phylogénie différente. Les analyses des séquences de 12S révèlent au moins six clades bien définis, correspondant chacun à une espèce morphologique. Les divergences sont très élevées entre toutes les espèces, suggérant seulement une relation très éloignée, avec celles parmi les espèces sympatriques ayant une différence de taille significative de plus de 27%. De façon surprenante, toutes les analyses montrent des valeurs de bootstrap très élevées pour le clade formé par deux espèces cosmopolites d'eaux de surface, *Diacyclops bisetosus* (Rehberg, 1880) et *D. bicuspidatus* (Claus, 1857), malgré de nombreuses différences morphologiques. De même, les données du 18S soutiennent seulement une relation éloignée entre *Diacyclops scanloni* Karanovic, 2006 et deux autres membres du groupe *alticola* d'Australie Occidentale : *D. humphreysi* s. str. Pesce & De Laurentiis, 1996 et *D. sobeprolatus* Karanovic, 2006. Des analyses préliminaires suggèrent l'absence de spéciation in situ et une évolution parallèle chez les *Diacyclops* d'Australie Occidentale, la différence de taille interspécifique étant le résultat d'une phylogénie différente. Le groupe *alticola* pourrait être polyphylétique, et nous reconnaissons les caractères morphologiques qui définissent deux lignées principales. Une possibilité de spéciation cryptique chez l'espèce cosmopolite *D. bisetosus* est aussi suggérée, et plusieurs séquences de *Diacyclops* disponibles sur GenBank sont reconnues soit comme contamination, soit comme fausse identification.

INTRODUCTION

Subterranean waters of Western Australia are becoming known as a significant hot-spot for faunal diversity on a global scale (Humphreys, 2008; Guzik et al., 2011), with numerous isolated calcrete aquifers that lie along palaeodrainage channels, and range in diameter from tens of kilometres to hundreds of meters (Humphreys, 2001, 2006). Highly porous and carbonate rich calcrete sediments represent an ideal habitat for various groups of stygofauna (aquatic subterranean fauna), including dytiscid beetles (Watts & Humphreys, 2006), amphipods (Finston et al., 2007), isopods (Wilson, 2008), bathynellids (Cho et al., 2006a, b), ostracods (Karanovic, 2007) and copepods (Karanovic, 2004, 2006). The majority of stygobitic species evolved within individual calcretes following independent colonization by epigean ancestors (Cooper et al., 2002, 2007, 2008; Leys et al., 2003; Guzik et al., 2008; Leys & Watts, 2008). The diversity of stygofauna is mostly dependent on the size of the calcrete, and typically includes one to three species from each major group, most of them endemic to that site (Karanovic, 2004, 2006, 2007; Finston et al., 2007; Leys & Watts, 2008). An example of a typical Western Australian calcrete is that at Sturt Meadows, where multiple studies from a very dense grid of bores revealed only two copepod species (Allford et al., 2008; Bradford et al., 2010). Some other recent studies (Karanovic & Cooper, 2011a, b, 2012) have

TABLE I
Body length of six *Diacyclops* Kiefer, 1927 taxa from the Pilbara region

Species	Female length (μm)				Male length (μm)			
	Minimum	Maximum	Average	*n*	Minimum	Maximum	Average	*n*
D. cockingi	409	802	597	30	375	662	514	17
D. einslei	477	604	527	8	446	452	448	3
D. h. humphreysi	360	488	432	18	321	404	372	6
D. h. unispinosus	326	492	418	18	324	377	352	5
D. scanloni	474	712	610	8	448	546	496	7
D. sobeprolatus	423	715	514	12	388	429	403	5

All data from Karanovic (2006); see text for authors of the specific names.

shown that larger calcretes may harbor a much more diverse copepod fauna, with up to four sympatric harpacticoid congeners and up to ten copepod species in a single bore. In these cases, a significant size differentiation among sympatric congeners was observed, which suggested this process to be potentially an important evolutionary force in subterranean habitats.

The only other well documented case of closely related sympatric congeners of copepods with a significant size differentiation was that of the genus *Diacyclops* Kiefer, 1927 in the Pilbara region of Western Australia (Karanovic, 2006), although this was never tested using molecular tools. Body length information was not a very good indicator of their size on its own (table I), because the copepod body has telescopic somites that can be extended or contracted depending on many factors during and after their collection and fixation (Huys & Boxshall, 1991). The difference in size, however, was so significant and devoid of intermediate stages, that one was led to hypothesize their separate specific statuses even during the preliminary identification and sorting under the dissecting microscope, often before even their generic status could be established with any certainty (fig. 1). Apart from their size, most other morphological characters are highly conservative. Six species were recorded so far from the Pilbara region, and one subspecies is endemic to Barrow Island, all of them belonging to the *alticola*-group: *Diacyclops cockingi* Karanovic, 2006; *Diacyclops einslei* De Laurentiis, Pesce & Humphreys, 1999; *Diacyclops humphreysi* s. str. Pesce & De Laurentiis, 1996; *Diacyclops humphreysi unispinosus* Karanovic, 2006; *Diacyclops scanloni* Karanovic, 2006; *Diacyclops sobeprolatus* Karanovic, 2006; and *Diacyclops reidae* De Laurentiis, Pesce & Humphreys, 1999 (see Pesce & De Laurentiis, 1996; De Laurentiis et al., 1999; Karanovic, 2006). Karanovic (2006), however, considered the validity of *D. reidae* problematic, possibly being described after an aberrant specimen of *D. einslei*. Only two other *Diacyclops* species are known from Australia, both very remotely related to the members of the *alticola*-group and to each other, and both known

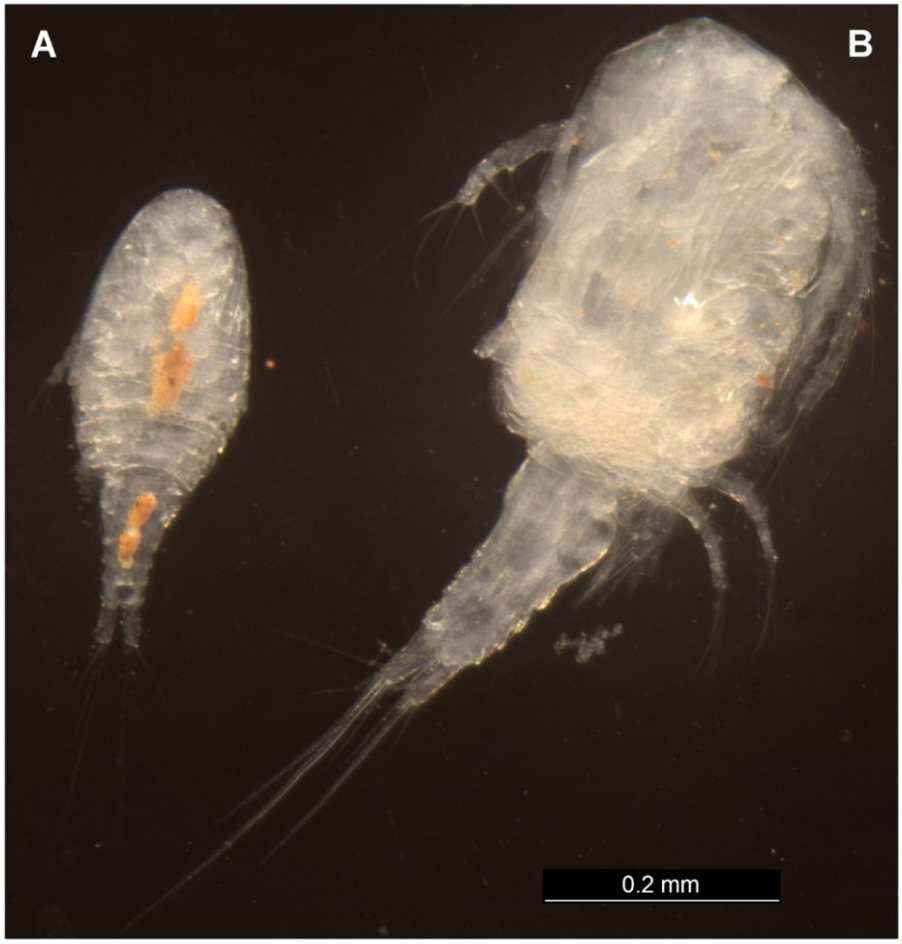

Fig. 1. Two morphologically very similar and sympatric *Diacyclops* Kiefer, 1927 species from the Pilbara region with a significant size difference (both collected at the FMG tenement Solomons, from bore SM2872, 21 January 2010): A, *D. humphreysi humphreysi* Pesce & De Laurentiis, 1996; B, *D. scanloni* Karanovic, 2006, with somewhat squashed prosome. Scale bar 0.2 mm. This figure is published in colour in the online edition of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/15685403.

from surface waters in eastern Australia: the cosmopolitan *D. bisetosus* (Rehberg, 1880), and the Tasmanian-Victorian endemic *Diacyclops cryonastes* Morton, 1985 (see Morton, 1985; Dussart & Defaye, 2006). Additionally, the cosmopolitan *Diacyclops bicuspidatus* (Claus, 1857) has been recorded recently in New South Wales (Karanovic, unpublished data), but its presence in Australia (along with that of *D. bisetosus*) could be a result of anthropogenic translocation associated with early shipping activities (Karanovic, 2005; Karanovic & Krajicek, 2012).

It is beyond the scope of this paper to revise the taxonomy of the genus *Diacyclops*, which is the largest Cyclopidae Rafinesque, 1815 genus (Dussart & Defaye, 2006), and recognized to be polyphyletic or at least paraphyletic by many researchers (Monchenko & Von Vaupel Klein, 1999; Monchenko, 2000; Karanovic, 2005). The general agreement among taxonomists seems to be that the

genus would have to be split into several monophyletic lineages, many of which are recognized as species groups today (Reid & Strayer, 1994; Pesce, 1996), but revised together with the closely related genus *Acanthocyclops* Kiefer, 1927. The *alticola*-group was proposed by Karanovic (2006) for six subterranean *Diacyclops* species and one subspecies from Western Australia, in addition to the Indian *D. alticola* Kiefer, 1935 and the Madagascan *D. longifurcus* Shen & Sung, 1963. They all have a 12-segmented female antennula, three-segmented rami of all swimming legs, and the outer apical spine on the fourth leg endopod longer than the inner one.

Recent intensive sampling of two areas in the Pilbara, done as a part of impact assessment and monitoring projects for the mining industry, produced several specimens of four closely related species from the *alticola*-group, which gave us an opportunity to study them using molecular tools. They were found to live in sympatry (Karanovic, 2006), always exhibiting a significant difference in size (fig. 1). Body size determines many aspects of life history, such as energy balance, resource utilization, competition, dispersal or reproduction rates (Kubota & Sota, 1998; Sota et al., 2000; Leyequién, 2006). Differences among similar species whose distributions overlap geographically are normally accentuated in areas where the species live sympatrically, but are minimized or lost in those where their distributions do not overlap (Brown & Wilson, 1956), and character displacement has been considered an important phenomenon in speciation (Mayr, 1963; Nagel & Schluter, 1998; Berner et al., 2009). The process is driven by competition for limited resources (Bolnick & Fitzpatrick, 2007), and in subterranean interstitial environments size differentiation would enable different closely related species to explore and utilize voids of different size, thus avoiding competition (Gibert et al., 1994; Culver & Pipan, 2009). The process often results in parallel speciation (Rundle et al., 2000).

This is a phenomenon well known in Australian calcrete habitats for diving beetles, where the fauna of a single calcrete typically consists of three species of very different sizes, with 13 cases of sympatric sister species pairs being reported in different calcretes (Leys et al., 2003; Leys & Watts, 2008). Even sympatric speciation was considered at one stage as a possible explanation (Cooper et al., 2002, 2008; Leys et al., 2003; Bradford et al., 2010), however, evidence for considerable population structuring within calcretes makes it difficult to rule out parapatric or allopatric modes of speciation (Guzik et al., 2008; Juan et al., 2010). Although theoretical work suggests that speciation can occur despite initially high gene flow, empirical evidence for sympatric (Savolainen et al., 2006; Ryan et al., 2007) or parapatric (Foster et al., 2007; Quesada et al., 2007) speciation remains thin (Berner et al., 2009). In copepods, some recent studies (Karanovic & Cooper, 2012) on the genus *Schizopera* Sars, 1905 in a small subterranean area in the Yilgarn region documented closely related sympatric species with a significant body size difference. At least three different size classes, and with at

least two species in each size class, suggested a possibility of interspecific size differentiation as a main evolutionary mechanism, as well as parallel evolution of similar traits (size in this case). However, molecular phylogenies based on a 623-bp fragment from the mitochondrial COI gene revealed that both explosive radiation and multiple colonisations were responsible for this richness, but no evidence for parallel evolution was found, interspecific size differentiation probably being a result of different phylogeny.

Aims of this study were to: derive molecular phylogenies of Australian *Diacyclops* species based on mitochondrial and nuclear genes; test if morpho-species are supported by molecular data; examine monophyly of the *alticola*-group; and test if the size differentiation is a result of parallel evolution or different phylogeny. To test if the *Diacyclops* morpho-species are a result of in situ speciation (and parallel evolution) or different phylogeny (and thus colonisation history), we examined them for mitochondrial 12S rRNA and nuclear 18S rRNA haplotypes. For phylogeny to have a significant influence, populations of the same ecomorph must be more closely related to each other than to populations of different ecomorphs (Rundle et al., 2000). Investigating these phenomena in different copepod orders (Harpacticoida and Cyclopoida) and in different regions (Yilgarn and Pilbara), and comparing them with studies on diving beetles, may allow us to exclude any phylogenetic or historical environmental influence. This can hopefully lead to more comprehensive conclusions about size differentiation in subterranean habitats, as well as about the origin and evolution of stygofauna in different regions. The genus *Diacyclops*, for example, is completely absent from the Yilgarn region (Karanovic, 2004), while it is a dominant element in the fauna of the neighbouring Pilbara region (Karanovic, 2006).

MATERIAL AND METHODS

Most samples studied here were collected in the Fortescue Metals Group Ltd (FMG) Solomon tenement, Pilbara region of Western Australia, by a private environmental consulting company (Subterranean Ecology), and entrusted to the senior author for morphological identification (table II). Several samples were collected from the BHP Billiton (BHP) OB23 tenement, also in the Pilbara region, and also by Subterranean Ecology. They resulted from various impact assessment and monitoring projects. Specimens were collected from or near proposed or existing mine sites, but due to the sensitivity of such data no further information about mining operations or plans will be given here. Locality data and number of specimens analysed for this study are listed for every species, including precise coordinates (table II). These samples were collected with haul-nets (mesh size 50 or 150 μm) from groundwater bores. Bores are holes mainly made by mining companies or agricultural enterprises for the purpose of water monitoring and

TABLE II

List of material examined with specimen numbers for different sequences; see text for generic names and authors of the specific names

Species	Country	Locality	Coordinates	Date	Collector	12S	18S
D. bicuspidatus	Ukraine	Kiev, Khotov, pond	50.331°N 30.466°E	21 Apr 2010	V. Monchenko	Q15, Q16	–
D. bisetosus	Japan	Shiga, Maibara, rice paddy	35.369°N 136.346°E	07 Oct 2009	T. Karanovic	Q11, Q12, Q14	Q33
D. humphreysi	Australia	WA, FMG, Solomon, bore SM2308	22.123°S 117.747°E	25 Jan 2010	E. Volschek	Q05, Q06	Q29
		WA, FMG, Solomon, bore NILE	22.124°S 117.868°E	24 Jan 2010	E. Volschek	Q01, Q02	Q28, S04
D. scanloni	Australia	WA, FMG, Solomon, bore SM3633	22.122°S 117.872°E	20 Jan 2010	E. Volschek	Q09	Q31
		WA, FMG, Solomon, bore SM2872	22.124°S 117.871°E	21 Jan 2010	E. Volschek	Q07	S05
D. sobeprolatus	Australia	WA, BHP, OB23, bore W262	23.306°S 119.862°E	22 Nov 2009	P. Bell	Q10	Q32
		WA, BHP, OB23, bore W152	23.266°S 119.885°E	22 Nov 2009	P. Bell	Q03, Q04	–
M. albidus	Australia	WA, Perth, Lake Richmond	32.283°S 115.712°E	11 Dec 2009	T. Karanovic	Q17, R38	Q35
E. serrulatus	Germany	Hamburg, pond	53.602°N 9.938°E	09 Apr 2010	T. Karanovic	–	W06
	Poland	Wigry, pond	54.078°N 23.084°E	11 Oct 2010	D. Vondrák	–	X37
	Czech	Tupadly, pond	50.447°N 14.472°E	30 Apr 2010	D. Vondrák	–	W10

abstraction or mineral exploration, usually from 5 to 20 cm in diameter, and lined entirely, or in part, by PVC tubing (the casing). Haul-nets are simple plankton nets of a different size suitable for the bore; collars can range from 20 to 150 mm in diameter and are made of stainless steel. Weighed nets (using simple fishing leads) were lowered down into the bore with a bottle screwed on its distal part and then hauled through the water column, usually six times. Samples were preserved in the field in cold 100% ethanol, kept on ice or in a refrigerator, and sorted in a laboratory. Four species from the *alticola*-group were collected in these two locations: *Diacyclops cockingi*, *Diacyclops humphreysi* s. str., *Diacyclops scanloni* and *Diacyclops sobeprolatus*; the first one only represented with several decomposed specimens that were not suitable for PCR-amplification.

Two other *Diacyclops* species were included in our molecular analysis, both cosmopolitan and surface-water dwellers, and both previously reported from Australia: *Diacyclops bicuspidatus* and *D. bisetosus* (table II). *Macrocyclops albidus* (Jurine, 1820) was intended as an outgroup for our molecular analyses. Specimens of *Eucyclops serrulatus* (Fischer, 1851) were used as an additional outgroup in our 18S analyses. These samples were collected with plankton nets and preserved in 96% or 99.9% ethanol.

All specimens were examined morphologically in propylene glycol ($CH_3CH(OH)CH_2OH$) prior to DNA extraction using a dissecting microscope Leica M205C, and a compound microscope Leica MB2500, equipped with phase-interference kit and N-PLAN objectives (especially using the 63× dry objective). After examination they were returned in 100% ethanol. Morphological terminology follows Karanovic (2008), while biospeleological terminology follows Humphreys (2000).

DNA was extracted from individual whole specimens in 30 μl proteinase K solution, using the protocol of Schwenk et al. (1998). Fragments of two different genes (mitochondrial 12S rRNA (430 bp), and nuclear 18S rRNA (650 bp)) were amplified using a combination of primers given in table III. The 35 μl PCR reaction was done in a Bio-Rad iCycler Thermal Cycler and contained 7 μl of the DNA template, 1× PCR buffer, 0.2 mM deoxynucleotides, 2.5 mM $MgCl_2$, 0.4 μM primers and 0.6 U *Taq* polymerase. The PCR protocol consisted of 4 min initial denaturation at 95°C, followed by 40 cycles consisting of denaturation at 94°C for 45 s, annealing at 48°C (for 18S) or 60°C (for 12S) for 45 s and extension at 72°C for 1.5 min. A final extension at 72°C lasted for 6 min. PCR products were purified and sequenced on ABI automatic capillary sequencer (Macrogene, Seoul, South Korea) using primers marked in table III.

Obtained sequences were checked manually and aligned for each gene separately by the ClustalW algorithm (Thompson et al., 1994) in MEGA version 5 (Tamura et al., 2011). Most variable loop regions in 12S sequences could not be

TABLE III
List of primers

Gene	Primer	Sequence (5′ → 3′)	Reference
12S	L13337-12S*	YCTACTWTGYTACGACTTATCTC	Machida et al. (2004)
12S	H13845-12S	GTGCCAGCAGCTGCGGTTA	Machida et al. (2004)
18S	18s329	TAATGATCCTTCCGCAGGTT′	Spears (1992)
18S	18sI*	AACTCAAAGGAATTGACGG′	Spears (1992)

* Primer used for sequencing reaction.

reliably aligned, and were excluded from further analyses by processing the 12S alignment in Gblocks Server v. 0.91b (Castresana, 2000), using default settings but allowing gaps within blocks. We thus obtained a 403 bp long alignment (96% of the original 419 bp). The 18S dataset could be aligned unambiguously, resulting in a 596-bp-long alignment. Each dataset was analysed in MEGA version 5 (Tamura et al., 2011) with (1) maximum likelihood (ML) analysis using the General Time Reversable model with uniform rates (GTR) and the Close-Neighbour-Interchange (CNI) method, (2) maximum parsimony (MP) analyses using the CNI method on Random Trees and (3) neighbour joining (NJ) analysis using the Kimura 2-parameter (K2P) model, with gaps treated with partial deletion. One thousand bootstrap replicates were performed to obtain a relative measure of node support for the resulting trees. Average pairwise NJ distances for each dataset were also computed in MEGA version 5 using the K2P model. GenBank numbers for specimens listed in table II (in brackets) as follows: JN656684 (Q17), JN656666 (Q35), JX134402 (X37), JX134394 (W06), JX124393 (W10), JX236042 (Q16), JX236043 (Q15), JX236044 (Q14), JX236045 (Q12), JX236046 (Q11), JX236047 (Q10), JX236048 (Q09), JX236049 (Q07), JX236050 (Q06), JX236051 (Q05), JX236052 (Q04), JX236053 (Q03), JX236054 (Q02), JX236055 (Q01), JX236056 (Q33), JX236057 (Q32), JX236057 (Q28), JX236059 (Q29), JX236060 (S04), JX236061 (Q31), JX236062 (S05). BLAST analyses of GenBank were also done using MEGA.

RESULTS

DNA was extracted and 12S and 18S fragments were successfully PCR-amplified from 16 and 11 whole copepod specimens respectively (table II). BLAST analyses of GenBank, and also comparisons with our unpublished sequences of other cyclopoid genera, revealed that the sequences obtained are copepod in origin and not contaminants, and three of the GenBank 18S sequences (HQ008752.1, AY643529.1 and HQ008745.1), from *Diacyclops crassicaudis* (G. O. Sars, 1863), *Acanthocyclops vernalis* (Fischer, 1853) and *A. brevispinosus* (Herrick, 1884), re-

spectively, were included in our analyses (deposited by Grishanin et al., 2005; Wyngaard et al., 2011). A number of other 18S sequences of both identified and unidentified species ascribed to the genus *Diacyclops* are available from GenBank, as unpublished results from the Lake Baikal sequencing project (GU066263, 066268-066272, 066274, 066275, 066277-066281 and 066285-066289), but unsuccessful alignments with our 18S sequences exposed these as probably not copepod in origin. Impossible alignment also suggested that the 18S sequence published for *Diacyclops uruguayensis* (Kiefer, 1935) by Wyngaard et al. (2011) is either a contamination or a misidentification (GenBank accession number HQ008753.1). Our results represent the first 12S sequence data for the genus *Diacyclops*.

The ingroup taxa formed a monophyletic group in all analyses, and the topology of the resulting cladograms did not differ significantly depending on the phylogenetic method used. Relatively high retention and consistency indexes in the MP analyses (for 12S: No. of trees = 2; Ci = 0.777, Ri = 0.863; for 18S: No. of trees = 49; Ci = 0.852; Ri = 0.913) suggested that our data were relatively robust and informative for the analysis, despite short fragments of each gene.

Basic frame of phylogeny based on the 12S sequence dataset (fig. 2) revealed at least six well defined clades, most supported with high bootstrap values, and each corresponding to one previously recognized morpho-species. The average pairwise distances between *Macrocyclops albidus* and any of the *Diacyclops* species were in excess of 35% (table IV), and this result is not surprising, as *Macrocyclops* Claus, 1893 and *Diacyclops* belong to two different subfamilies of the family Cyclopidae Rafinesque, 1915 (see Boxshall & Halsey, 2004; Dussart & Defaye, 2006), and the former shows most morphological character states in their plesiomorphic form in the whole family (Karanovic & Tang, 2009). All morpho-species are also well defined, with the lowest 12S divergences (ranging from 22.6 to 23.5%) being those between the two cosmopolitan species (*Diacyclops bicuspidatus* and *Diacyclops bisetosus*). This is a surprising result considering their numerous morphological differences (they differ much more morphologically than any of the members of the *alticola*-group; see Dussart, 1969; Monchenko, 1974), but the clade was well supported in all our analyses (98% in ML, see fig. 2; 99% in MP and NJ). Surprisingly high divergences between the three Western Australian members of the *alticola*-group indicate that they are not as closely related as previously thought, and as suggested by their conservative morphology (Karanovic, 2006), and the monophyly of the *alticola*-group group was not supported in any of our analyses. Two sympatric species from the Solomon tenement, *Diacyclops humphreysi* and *Diacyclops scanloni* (fig. 1), are only remotely related, with the pairwise distances all being in excess of 27%, with an average value of 28%. Our analyses suggest a sister relationship between *Diacyclops sobeprolatus* and *D. humphreysi*, which are also morphologically most similar species (Karanovic, 2006), but the support for this clade is not very high in our ML analysis (49%,

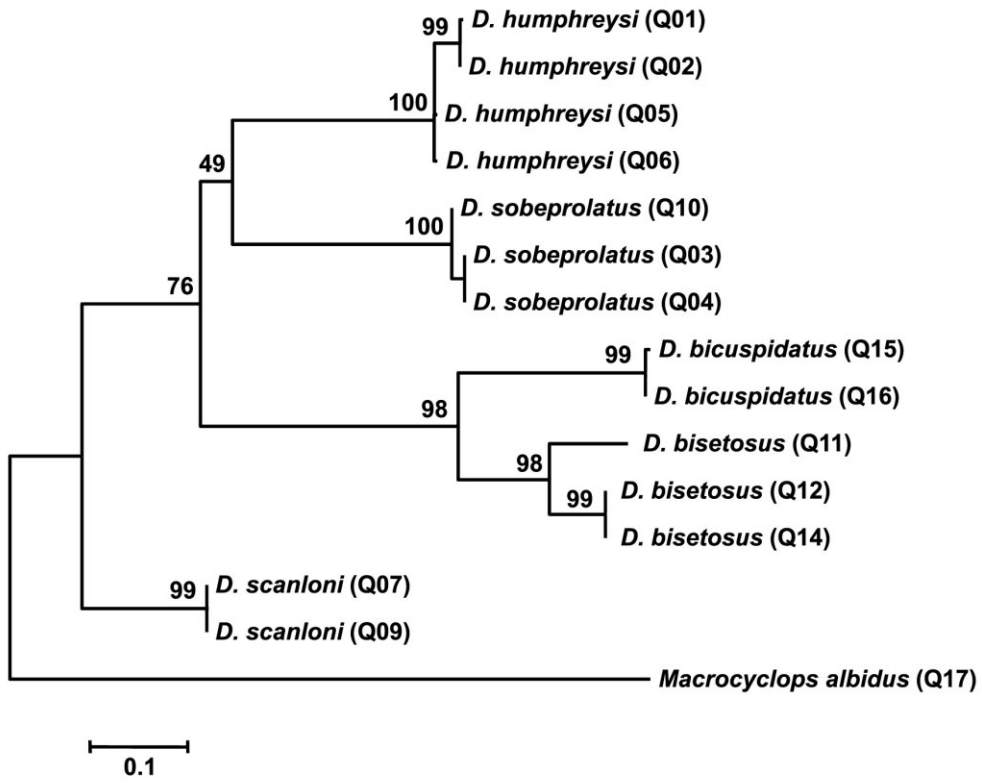

Fig. 2. Maximum likelihood (ML) tree based on 12S data from five *Diacyclops* Kiefer, 1927 species from eight different locations, constructed using MEGA v 5.0.3 and General Time Reversable model with uniform rates (GTR) and Close-Neighbour-Interchange (CNI) method. The outgorup is *Macrocyclops albidus* (Jurine, 1820), from Lake Richmond in Western Australia. The cladogram is drawn to scale, specimen codes in brackets correspond to those in table II, and the numbers above branches represent bootstrap values from 1000 pseudoreplicates.

see fig. 1) and it is only slightly better in our NJ analysis (78%). Despite great morphological similarity (they can be distinguished confidently only by the relative length of the dorsal caudal seta and body size when found together), the pairwise distances between these two species are surprisingly high, being between 27 and 30.4% (table IV), which suggests a long evolutionary history in this group of

TABLE IV

Average pairwise NJ distances (Kimura 2-parameter model) among 12S sequences between six morpho-species of cyclopoid copepods (lower diagonal) and within morpho-species (diagonal)

Species	1	2	3	4	5	6
1 *Diacyclops humphreysi*	0.029					
2 *Diacyclops sobeprolatus*	0.291	0.008				
3 *Diacyclops scanloni*	0.280	0.306	0.000			
4 *Diacyclops bisetosus*	0.343	0.337	0.332	0.071		
5 *Diacyclops bicuspidatus*	0.362	0.365	0.342	0.229	0.005	
6 *Macrocyclops albidus*	0.442	0.430	0.357	0.412	0.443	–

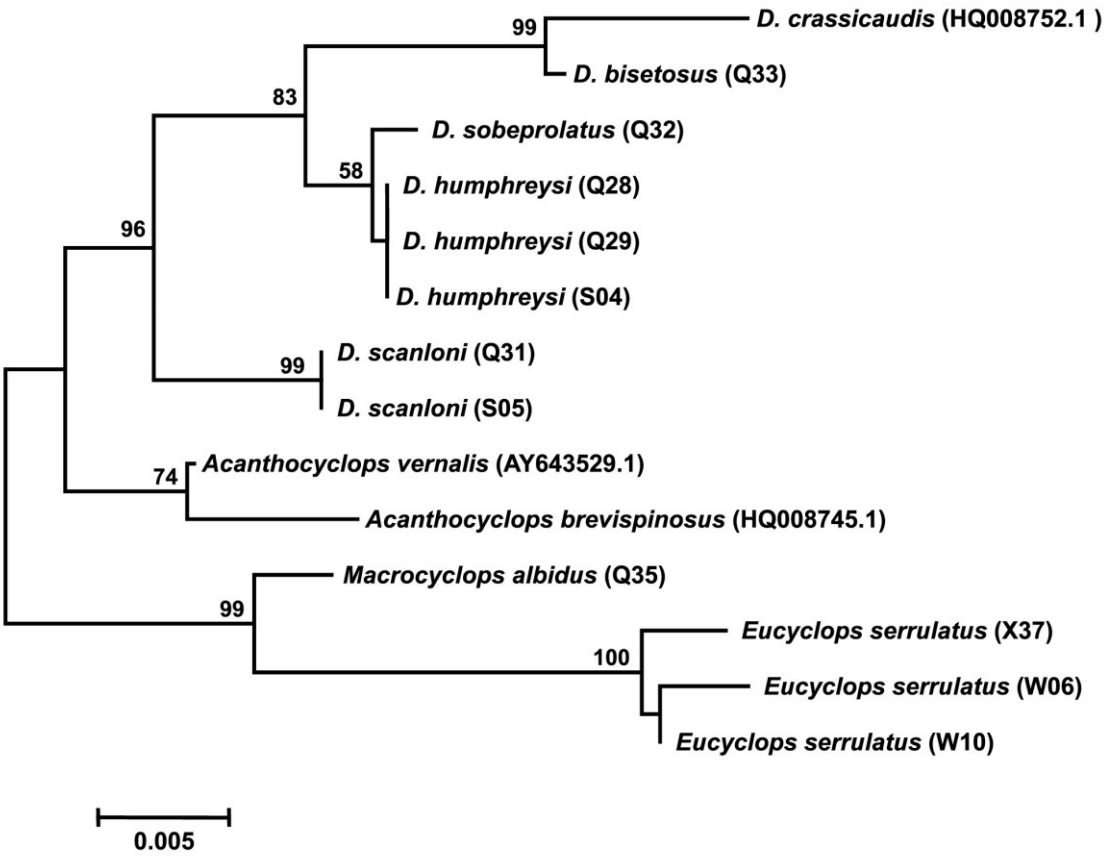

Fig. 3. Maximum likelihood (ML) tree based on 18S data from five *Diacyclops* Kiefer, 1927 and two *Acanthocyclops* Kiefer, 1927 species (from eight and two locations respectively), constructed using MEGA v 5.0.3 and General Time Reversable model with uniform rates (GTR) and Close-Neighbor-Interchange (CNI) method. The outgorups are *Macrocyclops albidus* (Jurine, 1820) from Lake Richmond in Western Australia, and *Eucyclops serrulatus* (Fischer, 1851) from Poland, Germany, and the Czech Republic. Sequences for *Acanthocyclops vernalis* (Fischer, 1853), *A. brevispinosus* (Herrick, 1884), and *Diacyclops crassicaudis* (G. O. Sars, 1863) are from GenBank (accession numbers in brackets). The cladogram is drawn to scale, specimen codes in brackets correspond to those in table II, and the numbers above branches represent bootstrap values from 1000 pseudoreplicates.

subterranean *Diacyclops* species in Western Australia. The clade that suggests a sister relationship of the *bicuspidatus/bisetosus* and *humphreysi/sobeprolatus* clades is moderately supported in the ML analysis (76%, fig. 2).

The highest divergences within morpho-taxa were those between three specimens of *D. bisetosus* (from 0 to 10.6%), which all came from the same rice paddy (table II), and this result indicates a possibility of cryptic speciation in this cosmopolitan species. Four specimens of *D. humphreysi* from two different bores show divergences between 0.2 and 4.4%, and three specimens of *D. sobeprolatus* from two bores differ from 0 to 1.2%. These are all indicative of intraspecific variability (Lefébure et al., 2006; Karanovic & Krajicek, 2012). Most specimens

TABLE V

Average pairwise NJ distances (Kimura 2-parameter model) among 18S sequences between nine morpho-species of cyclopoid copepods (lower diagonal) and within morpho-species (diagonal)

Species	1	2	3	4	5	6	7	8	9
1 *Diacyclops humphreysi*	0.000								
2 *Diacyclops sobeprolatus*	0.002	–							
3 *Diacyclops scanloni*	0.014	0.012	0.000						
4 *Diacyclops bisetosus*	0.012	0.014	0.026	–					
5 *Diacyclops crassicaudis*	0.021	0.022	0.035	0.008	–				
6 *Acanthocyclops vernalis*	0.017	0.019	0.014	0.022	0.031	–			
7 *Acanthocyclops brevispinosus*	0.024	0.026	0.020	0.029	0.038	0.007	–		
8 *Macrocyclops albidus*	0.029	0.031	0.026	0.035	0.044	0.022	0.022	–	
9 *Eucyclops serrulatus*	0.043	0.040	0.042	0.048	0.050	0.038	0.038	0.022	0.016

(excluding those of *D. bisetosus*) that came from the same locality showed zero divergence between their sequences.

Our 18S sequence dataset was somewhat limited (table II) but all morpho-species are well supported clades in this analysis as well, and the ingroup (*Diacyclops* + *Acanthocyclops*) is well defined (fig. 3). Members of the subfamily Eucyclopinae Kiefer, 1927 (*M. albidus* and *E. serrulatus*) form a well supported clade (99% support in ML), which is in contrast to some recent studies involving larger datasets (Wyngaard et al., 2011). Also, the genus *Diacyclops* is well supported, while the monophyly of the *alticola*-group is not. The average divergence rates between taxa (table V) are much smaller than those recoded for 12S, but this was expected, as 18S is a highly conservative gene (Pesole et al., 1999; Audzijonyte et al., 2005; Karanovic & Krajicek, 2012). Also not surprisingly, the 18S sequences show no intraspecific variability even between different sites (specimens of *D. humphreysi* and *D. scanloni* were collected at two different sites each), except in *Eucyclops serrulatus* which is probably a species-complex. The most interesting result of our 18S phylogenetic analyses is a very remote relationship of *D. scanloni* and two other Western Australian members of the *alticola*-group (fig. 3), with the average pairwise distances all in excess of 1.2%. Two widely distributed and surface water species, *D. bisetosus* and *D. crassicaudis*, form a well supported clade (99% in ML). All analyses also suggested a sister relationship between the surface-water *Diacyclops* clade (*bisetosus/crassicaudis*) and the *humphreysi/sobeprolatus* clade, which may indictate that the *alticola*-group is in fact polyphyletic. The 18S cladogram (fig. 3) also supports a sister relationship between *D. sobeprolatus* and *D. humphreysi*, just as the 12S sequence data (fig. 2) and morphological characters (Karanovic, 2006) do, but the support for this clade is again not high in any of our analyses (58% in ML).

DISCUSSION

The key findings of this study of the Australian *Diacyclops* are that morphospecies are well supported with molecular data despite their conservative morphology, the *alticola*-group is most probably polyphyletic, and our preliminary analyses suggest absence of in situ speciation and parallel evolution, with the interspecific size differentiation being a result of different phylogeny instead. A possibility of cryptic speciation in the cosmopolitan *Diacyclops bisetosus* is also suggested, and several 18S sequences of *Diacyclops* available from GenBank are recognized either as contamination or misidentification.

Among seven Australian taxa of the *alticola*-group two lineages were recognized on the basis of the presence/absence of inner seta on the first exopodal segments of all swimming legs (see key to species in Karanovic, 2006: 99), although this character was not considered as phylogenetically informative, and the monophyly of the Australian taxa was advocated. The first group, with the inner seta present, included *Diacyclops einslei*, *Diacyclops reidae* and *Diacyclops scanloni*; the second group included *Diacyclops cockingi*, *Diacyclops humphreysi* s. str., *Diacyclops humphreysi unispinosus* and *Diacyclops sobeprolatus*. Our phylogenetic analyses based on both 12S and 18S sequences suggest that the relationship between *D. scanloni* on one side and the *humphreysi/sobeprolatus* clade on the other is much more remote than what morphological data would suggest. The 18S cladogram (fig. 3) even suggests a sister relationship between the surface-water *bisetosus/crassicaudis* clade and the *humphreysi/sobeprolatus* clade, which would render the *alticola*-group polyphyletic. This sheds a new light on the phylogenetic importance of the inner setae on the first exopodal segments, and forced us to reexamine other morphological characters in the two groups (all published in Karanovic, 2006). The fifth leg looks very different in these two groups, with a much more slender distal segment and longer apical seta in *D. cockingi*, *D. humphreysi* s. str., *D. humphreysi unispinosus* and *D. sobeprolatus*, while the apical seta is much shorter and apical spine more robust in *D. einslei*, *D. reidae* and *D. scanloni* (see figs. 24E, 28F, 31A, 36D, G, 38C, 46E in Karanovic, 2006). The molecular and morphological analyses suggest that these two groups may represent two monophyletic lineages, which originated from different surface-water ancestors. They probably reduced the number of antennular segments through convergent evolution in subterranean habitats, where long antennulae may be a disadvantage for exploring smaller crevices in interstitial spaces. All seven Western Australian endemics have the outer apical spine on the fourth leg endopod longer than the inner one, which is a character they share with the surface-water cosmopolitan *D. bicuspidatus*, but not with *D. bisetosus*. This was the main reason we included both in our molecular analysis (besides both being recorded in Australia previously), as

we expected this character also to be reflected in our cladograms (i.e., we expected *D. bicuspidatus* to be a sister clade of the *alticola*-group, and *D. bisetosus* to be a sister clade of the former). That, however, was not the case, and the 12S cladogram suggests that the two surface-water species are more closely related to each other than to any of the Western Australian congeners (fig. 2). A possible polyphyly of the *alticola*-group in a well defined zoogeographical region shows that molecular characters will have to be considered in any future revision of the genus.

Relatively high divergence rates between three specimens of *D. bisetosus* (in excess of 10%; fig. 2; table IV) are generally indicative of distinct species by comparison with other crustaceans, even for much faster evolving genes like the COI (Lefébure et al., 2006), and are well within accepted values for distinct species in better studied non-related animal groups (Seddon et al., 1998). As all three specimens came from the same rice field (table II), this may suggest a possibility of two cryptic species in this complex. This is not surprising, as Monchenko (2000) found evidence for cryptic speciation in the *D. bicuspidatus* complex using cross-breeding studies, and Karanovic & Krajicek (2012) discovered cryptic speciation in the *Macrocyclops albidus* complex using a combined morphological/molecular approach. These are all cosmopolitan freshwater taxa, with a long and troubled taxonomic history (Dussart & Defaye, 2006), which may owe their very wide distribution to anthropogenic translocation associated with early shipping activities (Karanovic, 2005; Karanovic & Krajicek, 2012), or any subsequent human-mediated passive dispersal mechanism (fisheries, aquaculture, aquaristics, etc.).

Our analyses of both 12S and 18S sequences present preliminary evidence for absence of in situ speciation and parallel evolution in the Western Australian *Diacyclops*, interspecific size differentiation being probably a result of different phylogeny. This is most apparent in the case of two sympatric species in the FMG Solomon tenement, *D. humphreysi* s. str. and *D. scanloni*, which show a remarkable size differentiation (fig. 1). Both 12S and 18S data (figs. 2, 3) show that these two species are only remotely related. This inidicates that their size difference did not originate in response to a recent parapatry, driven by competition for limited resources. However, it should be said that the fact that two taxa come from different ancestors in the phylogeny does not rule out that selection in the aquifer could drive or maintain their size difference. Very high divergence values among the Western Australian *Diacyclops* species (especially for the 12S sequences) suggest that they may be an old component of the stygofauna in this arid Australian state, possibly originating from different surface-water species that lived here during a more humid climate in the Pliocene (Byrne et al., 2008). This is, however, just a speculation, as no molecular clock calibrations were used in our analyses.

The main conclusions of this study are similar to those of Karanovic & Cooper (2012), who examined a possibility of size differentiation in a different group

of copepods, with different genes, and in a different region. They studied an explosive radiation of the harpacticoid genus *Schizopera* in one of the larger calcretes in the Yilgarn region, combining haplotype frequency of the mtCOI gene and comparative morphology of microcharacters. There, up to four, and commonly three, species live sympatrically in the same bore, almost always with a significant difference in size. They described eight new species and subspecies from that small area, and suggested a possibility of another three cryptic species. However, they found no evidence for in situ speciation and parallel evolution with character displacement, the interspecific size difference being a result of different phylogeny in all cases. Reconstructed phylogenies revealed that both explosive radiation and multiple colonisations were responsible for this extraordinary richness, that sister species have parapatric distributions and niche partitioning in the area of overlap but no difference in size, and that *Schizopera* is a recent invasion in these habitats. In situ speciation from the same ancestral source is still to be found in cyclopoid or harpacticoid copepods, as opposed to more than 13 documented cases in dytiscid beetles (Cooper et al., 2002, 2008; Leys et al., 2003; Leys & Watts, 2008; Bradford et al., 2010), which may imply that different evolutionary forces are at work in different stygofauna groups. Karanovic & Cooper (2011b) provided evidence that even two different harpacticoid genera have a different colonisation history in the same palaeochannel in Western Australia, with the members of the genus *Kinnecaris* Jakobi, 1972 colonising the channel downstream and being represented just with allopatric species, and the genus *Schizopera* colonising upstream and with numerous sympatric and parapatric species. These two genera, however, belong to two different families, one of which has most of its diversity in marine environments (Karanovic & Cooper, 2012), while the other is freshwater in origin and probably started colonising subterranean waters in Australia just after the Permo-Carboniferous glaciations (Karanovic, 2004, 2006; Karanovic & Cooper, 2011a, b), which spread throughout much of what subsequently had become the Gondwana supercontinent and covered the entire Australian plate (Frakes, 1999; Playford, 2003). Both molecular and morphology based phylogenetic studies continue to provide new and amazing insights into the evolution of Darwin's wrecks of ancient life (Juan et al., 2010), and we hope they will stimulate other areas of research in subterranean environments, as well as their conservation and responsible management. This paper presents only preliminary results, based on a limited dataset, but they come from some of the remotest corners of our planet, where sampling is further complicated by restricted access due to numerous mining tenements.

Amplification success rates were different for the two chosen genes, those for the 12S being much higher (close to 90%) than those for the 18S (slightly below 50%). This is surprising, given that the 12S is a faster evolving gene.

Low amplification rates may be partly due to a relatively small size of copepod specimens and correspondingly low amount of DNA isolate, but more probably because we are yet to find an optimal procedure and combination of primers for this group and each gene (Karanovic & Cooper, 2011b). We did, however, test most primers available for copepods, and spent a lot of time on the optimization of the PCR protocol (finding the optimal annealing temperature on the temperature gradient). Recently, Karanovic & Krajicek (2012) were able to detect cryptic speciation in a global study of the *Macrocyclops albidus* complex, using 12S in combination with three other genes (16S, 18S, and cytB) and morphological microcharacters. Bláha et al. (2010) detected a possible cryptic species in the *Acanthocyclops vernalis* complex, also using 12S. This gives us confidence in the divergence values interpretation in the genus *Diacyclops*, as all three genera live in similar habitats and belong to the same family and their genes should evolve at similar rates. In the *M. albidus* complex, just as in most other animal groups, of the four genes cytB evolves fastest, followed by 12S, 16S and 18S. Possibilities of cryptic speciation in the cosmopolitan *D. bisetosus* (suggested by our 12S analyses; fig. 2, table IV) and *Eucyclops serrulatus* (suggested by our 18S analyses; fig. 3, table V) are thus worth investigating further with more markers and in combination with a study of morphological microcharacters.

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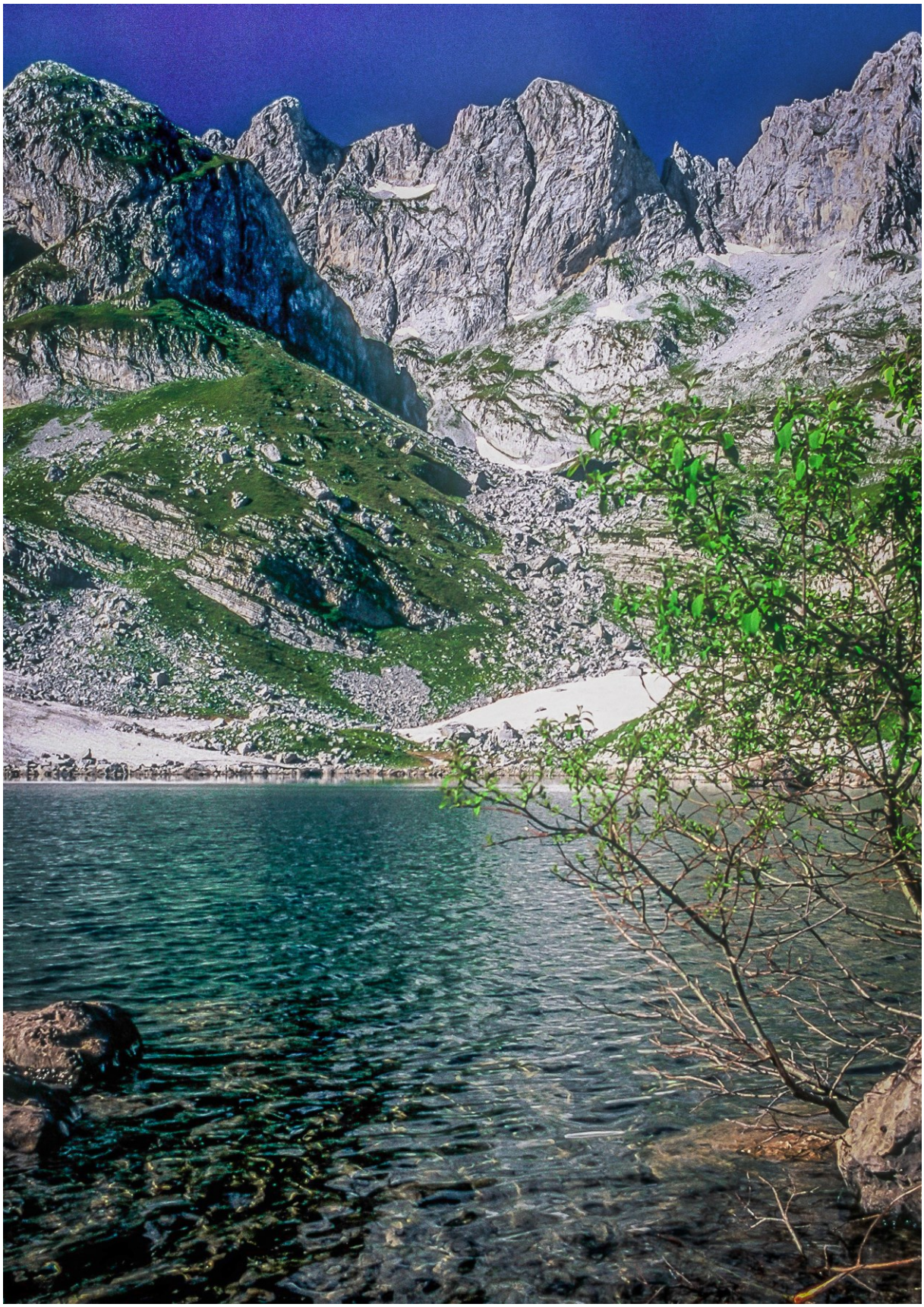

Chapter 6: General discussion and conclusions

This chapter will discuss and conclude all my work presented previously in Chapters 2 to 5. I was involved in different studies concerning the morphology, taxonomy, ecology, distribution and colonisation of cyclopoid copepods. Nevertheless, the use of molecular tools was the uniting element, so I'd like to focus mainly on this part of the work. Firstly the different methods useful in the research of copepods will be summarized, then the DNA sequencing will be discussed in more detail. Advantages and drawbacks of this method will be concluded and suggestions for further research mentioned.

Methods overview

Sampling methods should be adapted to the biotope and studied animals. Plankton nets are used to collect in lakes, pools and ponds. For qualitative sampling in open water, a simple conical net with 70 to 100 μm mesh size can be used. The net can be thrown from the bank and pulled at a constant speed of not more than 0.5 m s^{-1}. The use of a boat may be necessary for sampling deeper areas in the centre of a lake. The plankton trap of Schindler-Patalas (Schindler, 1969) is useful for quantitative sampling in lakes. In littoral areas and in the marginal vegetation of lakes and ponds, a hand-held, cone-shaped net is most efficient. When sampling swamps, bogs and shallow ephemeral pools, water can be scooped up by a plastic jug and filtered through a net.

Before fixation, it is sometimes useful to narcotise the specimens, because the addition of formalin or 96% ethanol may provoke violent reactions like ejection of the gut contents, dropping off egg-sacs or contraction of muscles, making the subsequent observations more difficult. Different narcotization solutions may be used (Dussart & Defaye, 2001), but slow addition of the preservation medium is the easiest method. Samples can be preserved adding 40% formalin to a final concentration of 5% in the sample, or in 96% pure ethanol in case further molecular analysis will be performed.

For preparations, it is recommended to dissect the preserved copepods in a medium of high viscosity, i.e. glycerine or propylene glycol (which preserves DNA). It is sometimes useful to clear or stain animals before the observations are made. To study integumental structures, it is possible to dissolve internal tissues by heating the specimens in a 10% KOH solution at 80 °C for 20 to 120 minutes (the optimal time should be tested). The integument is

then washed during a few minutes in distilled water and stained with Chlorazol Black or Rose Bengal. Stained copepods are then transferred back into the glycerol. The preparation method used for observing *Cyclops* species in our study was described in detail in Chapter 2 of this thesis.

After general observations, it is necessary to dissect the animals to study each appendage separately. The dissection is carried out under a stereomicroscope using two sharp tungsten needles mounted on a holder. All drawings or photographs should be made on a compound microscope equipped with a camera lucida or digital camera, using a set of objectives, including 100x oil immersion for details. Phase contrast illumination is useful especially for the examination of microcharacters such as pores, spinules and sensillae (Dussart & Defaye, 2001). Scanning electron microscopy (SEM) is also a technique of great help for the study of microcharacters, particularly when the appendages are tiny or have a complex 3-dimensional structure.

Biochemical methods have become progressively more available during the last decades, so taxonomists could also include these techniques. Studies of enzyme polymorphism by electrophoresis (Boileau & Hebert, 1988; Einsle, 1996b), cytogenetic methods as karyotyping, observing chromatin diminution patterns (Dorward & Wyngaard, 1997; Einsle, 1962, 1996a; Leech & Wyngaard, 1996), measuring DNA content and genome size (Fara, Berdalet, & Arin, 1996; Wyngaard, Rasch, Manning, Gasser, & Domangue, 2005; Wyngaard & Rasch, 2000) have become popular.

Using the DNA sequences as barcodes (Hebert, Cywinska, Ball, & DeWaard, 2003) has become widely accessible with the development and price reduction of polymerase chain reactions (PCR) and DNA sequencing techniques. Despite the fact that these techniques have been available for more than two decades, much research have been done only on marine calanoid copepods (Bucklin, Frost, Bradford-Grieve, Allen, & Copley, 2003; Bucklin & Kochert, 1996; Bucklin & LaJeunesse, 1994; P K Lindeque, Harris, Jones, & Smerdon, 1999; P. K. Lindeque, Harris, Jones, & Smerdon, 2004), with just a few projects focussed on freshwater cyclopoids (Alekseev et al., 2006; Bláha et al., 2010). The studies presented in this thesis should equilibrate this imbalance at least a little.

DNA sequencing and related methods

Although there are many molecular biology techniques useful in studies of copepods, this part will be focussed just on methods associated with the DNA sequencing. This approach was applied in all projects included in this thesis; therefore process of the DNA extraction, polymerase chain reaction (PCR), nucleic-acid purification and sequence data analysis will be discussed as well.

After the discovery of a double helix structure of DNA by Watson and Crick (1953) it took several decades before first fragments of DNA were analysed for their sequence in the laboratory. The Maxam-Gilbert sequencing method (Maxam & Gilbert, 1977) required radioactive labelling of DNA, while the Sanger sequencing method (Sanger & Coulson, 1975; Sanger, Nicklen, & Coulson, 1977), using fewer toxic chemicals and lower dose of radioactivity, was progressively modified and developed (with fluorescent labelling, capillary electrophoresis and general automation) and thus allowed more efficient sequencing at lower costs. Nowadays, sequencing is performed as a service by universities or commercial companies (e.g. Macrogene) and the costs were reduced to mere 5 U.S. dollars per sample.

DNA extraction is the initial part of all sequencing analyses. It used to be complicated, time-consuming and labour-intensive process in the past, but currently, there are many specialized methods, such as solution-based and column-based protocols, that can be used (Tan & Yiap, 2009). They usually consist of these steps (Aljanabi & Martinez, 1997; Green & Sambrook, 2012): 1) Chemical or physical breaking of cells to expose the DNA. 2) Removing membrane lipids, proteins and RNA. 3) DNA purification.

Pure ethanol is the best solution for preserving samples for further DNA analyses, nevertheless even older formalin-preserved zooplankton samples could be used when processed using special protocols (Bucklin & Allen, 2004; Coombs, Gough, & Primrose, 1999; Lin et al., 2009; Schander & Kenneth, 2003).

The protocol of DNA isolation according to Schwenk et al. (1998) was used for all presented studies. The pre-mixed solution contains tween, TrisHCL, KCl, Nonidet P40 (non-ionic, non-denaturing detergent produced by Sigma-Aldrich, USA), proteinase K and water. Individual specimens were washed in distilled water for one hour, placed in 50 µl of pre-mixed solution and heated at 57 °C for 4 hours. According to my experience, the mechanical crushing of copepods is not necessary. Moreover, some researchers (Sofia V. Moudrova, personal communication) use the remaining cuticular exoskeleton for subsequent analysis,

which allows getting both molecular and morphological data from a single specimen. The same aim could be reached by separating and isolating DNA just from egg-sacs. When using this protocol, purification of the extracted DNA is not necessary and about 5 µl of the solution could be added directly into the PCR reaction. The DNA isolate could be stored in the refrigerator for several months or in the freezer for several years without degradation.

There are also several commercial kits for spin column-based nucleic acid extraction and purification based on the ability of nucleic acids to bind to the solid phase of silica under certain conditions. This characteristics could be useful also in various applications for biosensors, "lab on a chip" devices, and other new technologies that require rapid, high quality DNA extraction at minimal cost (Cady, Stelick, & Batt, 2003; Tian, Hühmer, & Landers, 2000; Wolfe et al., 2002). The application of these technologies is huge, ranging from primary research to environment monitoring and global health care. Some researchers believe that "lab on a chip" technology may be the key to powerful new diagnostic instruments (Yager et al., 2006). The goal of these researchers is to create microfluidic chips that will allow healthcare providers in poorly equipped clinics to perform various diagnostic tests with no laboratory support.

The polymerase chain reaction (PCR) is a biochemical technology used to amplify a single or a few copies of a piece of DNA by a factor of more than 10 million with very high specificity (Saiki, Gelfand, Stoffel, & Scharf, 1988). This method was developed in 1983 by Kary Mullis (Bartlett & Stirling, 2003) and is based on cycles of repeated heating and cooling of the reaction mixture allowing the DNA melting and enzymatic replication. A thermostable DNA polymerase is used for assembling new DNA strands from the nucleotides. PCR primers complementary to the target region specify the area of amplification (Saiki et al., 1988). There are various applications and modifications of this method, e.g. the nested PCR using two primer pairs for higher specificity (Puig, Jofre, & Lucena, 1994), or the real-time PCR for precise quantitative measurement of starting amount of DNA (Heid, Stevens, Livak, & Williams, 1996). However, the basic principle remains always the same.

The use of proper PCR primers is the most important factor of this method. Our experience with analysing the *Cyclops* species (Chapter 2 of this thesis) suggests that finding suitable primers could be a long process, especially when no sequences of related species are known. Trying already designed universal primers as well as primers for related species groups would be the first choice. The online databases of primer sequences like PrimerBank

(http://pga.mgh.harvard.edu/primerbank) are focussed mainly on human and mouse genes, so a detailed search among published papers is necessary. When some related sequences are known, it is possible to use Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast), an online tool for primer design. This program combines the features of both Primer3 (http://primer3.sourceforge.net), an open-source PCR primer design software and BLAST (http://blast.ncbi.nlm.nih.gov), a web-based algorithm for comparing primary biological sequences stored in nucleotide and protein databases. Beside this, other primer design tools could be used, e.g. the downloadable PerlPrimer (http://perlprimer.sourceforge.net) or the web-based OligoAnalyzer (http://scitools.idtdna.com/analyzer/Applications/OligoAnalyzer).

After obtaining suitable primers, there are other possibilities of further optimization to improve the performance of PCR and minimize failure. As the PCR is an extremely sensitive method, there are many risks of contamination from any DNA present in the lab environment. Apart from wearing fresh laboratory gloves or using DNA-removers to clean the working place, it is convenient to divide the lab into two separate areas: one used for preparation and handling of pre-PCR reagents and the setup of the PCR reaction, the other for post-PCR processing, such as gel electrophoresis or PCR product purification (James, 2010). The purity and yield of the PCR reaction products also depends on the annealing temperature (Rychlik, Spencer, & Rhoads, 1990) which can be optimised using the PCR reaction with thermal gradient.

Amplified DNA fragments are than concentrated and purified using commercial spin column-based protocols (Tan & Yiap, 2009) or the ethanol precipitation (Bucklin, 2000; Chu, Li, & Ho, 2001; Green & Sambrook, 2012; Imai, Cheng, Hamasaki, & Numachi, 2004) as a cheaper alternative. There are various methods for the DNA quantification (Ahn, Costa, & Emanuel, 1996; Dell'Anno & Fabiano, 1998), but the microvolume UV-Vis spectrophotometric measurement (e.g. using the NanoDrop spectrophotometer) or the gel electrophoresis with molecular-weight size marker are the most common options (James, 2010). Then the DNA concentration in samples is adjusted according the sequencing laboratory requirements.

Obtained DNA sequences are checked manually evaluating the quality of chromatograms and then could be processed with variety of methods for the sequence alignment (Notredame, 2002), substitution model selection (Darriba, Taboada, Doallo, & Posada, 2012; Johnson & Omland, 2004) and phylogeny reconstruction (Guindon & Gascuel,

2003; Liu et al., 2012; Price, Dehal, & Arkin, 2010; Ronquist et al., 2012; Stamatakis, 2006; Tamura et al., 2011; Zwickl, 2006). Many of these methods are used and discussed in Chapters 2 to 5, while the most accurate and sophisticated approach is applied in Chapter 2.

Suggestions for further studies

DNA sequence analyses performed in all four studies presented in Capters 2 to 5 revealed new lineages suggesting (cryptic) speciation. In most cases adequate morphological differences in microcharacters were also found. These results indicate the importance of microcharacters, support the integrative approach to the taxonomy (Dayrat, 2005) and represent possibilities for further research in the future.

The fast development of new molecular-genetic technologies like the next generation sequencing (Metzker, 2010) also opens many new research options for future phylogenetic, biogeographic and ecological studies.

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