FACULTY OF FISHERIES AND PROTECTION OF WATERS
UNIVERSITY OF SOUTH BOHEMIA IN ČESKE BUDĚJOVICE

Molecular and morphological aspects within *Acanthocyclops* Kiefer, 1927

Morfologické a molekulární aspekty v rámci rodu *Acanthocyclops* Kiefer, 1927

Martin Bláha

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I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

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Supervisor:

Prof. MSc. Zdeněk Brandl, CSc.
University of South Bohemia in České Budějovice (USB)
Faculty of Science (FS)
Branišovská 31
370 05 České Budějovice
Czech Republic

Head of Laboratory of Ethology and Nutrition of Fish and Crayfish, USB FFPW, RIFCH:

Assoc. Prof. Dipl.-Ing. Pavel Kozák, Ph.D.

Dean of Faculty of Fisheries and Protection of Waters:

Prof. Dipl.-Ing. Otomar Linhart, DSc.

Board of doctorate study defence with referees:

Prof. Dipl.-Ing. Petr Ráb, DSc. / Assoc. Prof. MSc. Josef Matěna, CSc. – head of the board
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Prof. Jacky Cosson – international referee

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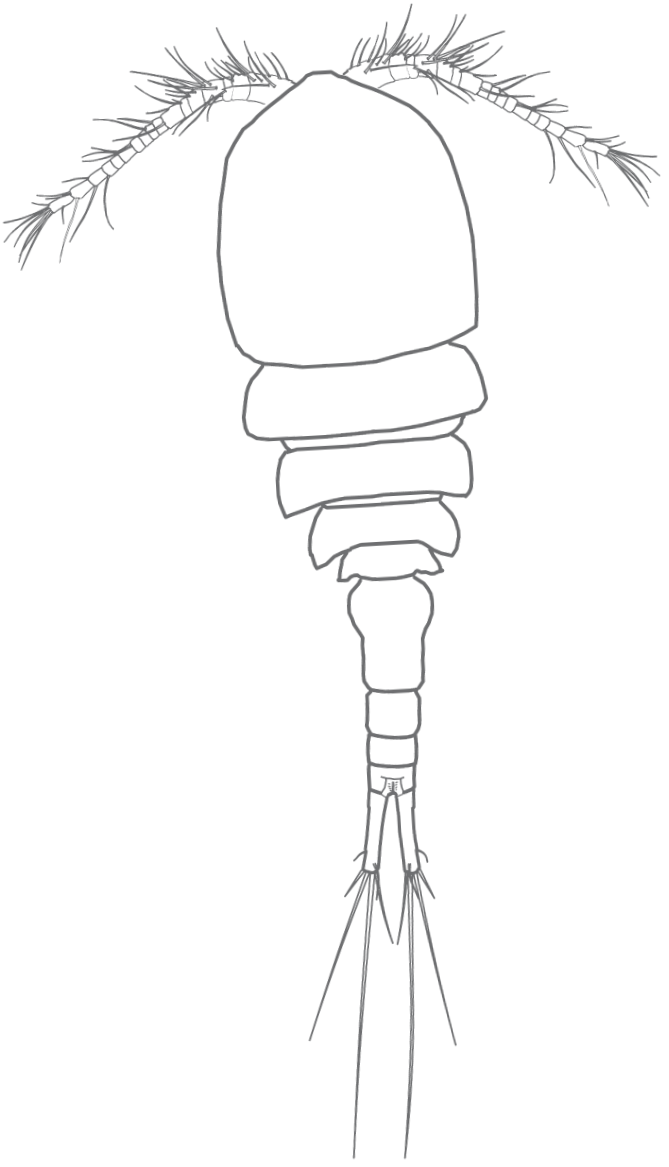
CONTENT

CHAPTER 1

GENERAL INTRODUCTION



1. INTRODUCTION

The freshwater copepods inhabit almost all types of biotopes from subterranean caves to water collected in bromeliad leaves or leaf litter on the ground as well as streams, rivers and lakes. The name „Copepod" arose from Greek *Kope* for „oar" and *Podos* for „foot". Hence copepod means oar-footed, referring to the pair of swimming legs on the same somite that are moved together, like the oars of a sculling shell.

Ecologically, copepods represent an important interlinks in the food chain connecting microscopic food particles to juvenile fish (e.g. Sommer, 2008; Takahashi et al., 2008). Copepods have also potential to act as a control mechanisms for malaria by consuming mosquito larvae (e.g. De Roa et al., 2002; Pernia et al., 2007), and contrariwise they represent intermediate hosts of many human and animal parasites as well (e.g. Lahnsteiner et al., 2009; Moravec, 2009).

The external morphology of copepods is quite conservative, with elongated, segmented body covered by chitinous exoskeleton. Moreover in terms of cyclopoid copepods, body is divided into several functional parts such as prosome and urosome. The prosome is composed of cephalosome and four thoracal somites each bearing particular appendages, and urosome consists of genital doublesomite, two urosomites and anal somite. The body is usually terminated by two furcal rami bearing either six furcal setae (Dussart and Defaye, 2001).

1.1. COPEPOD TAXONOMY

Planktonic crustaceans are in general susceptible to certain morphological phenotypic plasticity, especially in terms of water flea (Cladocera). The body shape and size change according to different biotic and abiotic factors (temperature, food availability, predator presence etc.) resulting in extension or shortening of particular body parts, and presence of spinular rows or patches (Lass and Spaak, 2003; Laforsch and Tolrian, 2004; Petrusek et al., 2009; Zuykova and Bochkarev, 2010). Although with no such a visible modifications as commonly reported in water fleas, copepods also undergo changes induced by environmental factors. These changes are in most cases in type of armature (seta/spine appearance) or in extension/shortening of furcal rami (Coker, 1932a).

Moreover, especially the type of armature, in the meaning of setae or spine presence and their number, were used as a species specific character in many Cyclopoid Copepod species for a long time. The genera such as *Acanthocyclops* or *Diacyclops* are notoriously known examples of complicated interspecies taxonomical relationships (Monchenko, 2000; Stoch, 2001; Dodson et al., 2003). Species within genera are characteristic by high phenotypic plasticity, which resulted in clustering of similar species into cryptic species complexes. In addition, a lot of such a species are living sympatricaly, superficially morphologically almost indistinguishable.

At the beginning, copepod taxonomy was based on differences in size, number of exopodal spines in swimming legs, and later more characteristics were added. Consequently, Kiefer (1927, 1929) established traditional systematic of the cyclopoid copepods, especially at generic level. Kiefers' systematic classification was based on the structure and armature of the fifth leg, followed by others (Rylov, 1948; Yeatman, 1959; Dussart, 1969; Monchenko, 1974). Kiefer also established the system where family Cyclopidae was subdivided into three subfamilies as follow: the Halicyclopinae, Eucyclopinae and Cyclopinae, and recognized most of subgenera including *Acanthocyclops* within the subfamily Cyclopinae.

With emergent demands for precise species discrimination especially within such problematic species complexes, other more promising methods were introduced. The most common was breeding

compatibility, originated from the principle of species, established by Dobzhansky (1937) and Mayr (1941) as a "biological species concept". A primary purpose of breeding experiments was to determine whether morphologically similar populations correspond with reproductively compatible groups. When morphological traits failed to distinguish interspecies differences, the cytogenetic and molecular approaches were widely used (e.g. Eisnlei, 1993; Grishanin and Akifiev, 2000; Wyngaard and Rasch, 2000).

Although quite labour, not so dependent on technique equipment, analyses of chromosome number were carried out much earlier (Krüger, 1911; Chambers, 1912). However, just molecular methods analysing differences in the DNA sequences open new chapter in biological science as well as in species discrimination and understanding variety of evolutionary trends. So far, the majority of published molecular studies deal with marine copepods (e.g. Bucklin et al., 1999, 2003; Lee, 2000; Caudill and Bucklin, 2004; Thum, 2004), and it seems that markers widely used for them are less efficient in freshwater copepods. However several studies on freshwater copepods have recently appeared (Alekseev et al., 2006; Grishanin et al., 2005, 2006; Ki et al., 2009; Bláha et al., 2010; Wyngaard et al., 2010).

1.2. EVOLUTION OF *Acanthocyclops vernalis-robustus* GROUP TAXONOMY

In the middle of 19th century, the two probably the most discussed *Acanthocyclops* species were described. The first one was described by Fisher (1853) as a *Cyclops vernalis* and second one by Sars (1863) as a *Cyclops robustus*.

More detailed description of both species was later provided by Sars (1918). However, the main discriminating criterion was still the spine formula and the nature of the setae, *C. vernalis* and *C. robustus* were attributed with spine formula 2 3 3 3 and 3 4 4 4, respectively, varying to 3 4 4 4 in *A. vernalis* quite often.

The American copepodologist, C.L. Herrick (1882, 1884) described representatives of *Cyclops* synonymized *C. vernalis* with *C. parcus* and *C. robustus* with *C. brevispinosus*. Soon after, stated that these species were same as species reported from Europe (Coker, 1934; Yeatman, 1944; Dodson, 1994). In addition, Marsh (1892) described another species *C. americanus*. This species was first identified in Europe by Lowndes (1926, 1928b) who gave remarks for its distribution and provided its detailed description (see also Monchenko, 1974; Alekseev, 1998; Alekseev et al., 2002). However, later the name *A. americanus* was used as a synonym for both *A. vernalis* (Gurney, 1933) and *A. robustus* (Petkovski, 1975 – f. *limnetica*; Kiefer, 1976; Dussart and Fernando, 1989), and after all rejected by Kiefer (1976).

Due to enormous morphological plasticity determined by Lowndes (1928a), Dodson (1994), Lescher-Motoue (1996), Caramujo and Boavida (1998) or Dodson et al. (2003), precise species determination was difficult. Moreover, it became clear, that using character such as number of spines/setae in exopodite of swimming legs undergoing high variation, and is therefore limited for proper species discrimination. Thus, similar *Acanthocyclops* species were grouped into clusters called *robustus* or *vernalis* species group according to major differentiate characteristic. These major discriminating characteristics, i.e. the shape of genital double somite and ratio of two apical spines in enp3 P4, resulted from revision of *vernalis-robustus* group provided by Kiefer (1976), who compared a lot of populations from both sides of Atlantic Ocean.

Also other attempts to find species specific characteristics failed usually due to high phenotypic plasticity apparent even among siblings (Dodson, 1994; Dodson et al., 2003). Nevertheless, Dodson (1994) and Dahms and Fernando (1997) redescribed *A. brevispinosus* differing from other *Acanthocyclops* members ecologically and morphologically.

Mirabdullayev and Defaye (2002, 2004) have greatly contributed to solving morphologically *robustus* species group. The new species delineation was based on characters of enp3 P4, shape of receptaculum seminis, and also some of microcharacters attributed to mouth appendages. Based on detailed morphological study of Sars' and Kiefer' samples as well as a number of specimens identified as *A. robustus* from Europe and North America, they separate new species, namely *A. trajani* (Mirabdullayev and Defaye, 2002) and *A. einslei* (Mirabdullayev and Defaye, 2004), and redescribed *A. robustus*.

1.3. DIFFERENTIAL DIAGNOSES OF SPECIES WITHIN *Acanthocyclops vernalis-robustus* SPECIES COMPLEX

Acanthocyclops vernalis-robustus species complex contains presently known, taxonomically valid species *A. robustus*, *A. trajani*, *A. einslei*, *A. brevispinosus* and *A. vernalis*. These species differ from each other by following characteristics.

Acanthocyclops einslei and *A. brevispinosus* markedly differ from other *robustus* species complex in the site of lateral spine insertion in enp3 P4, which is nearer to the apical end of the segment. Whereas the other three species has this seta/spine located more proximally, near the centre of the segment. These two species differ from each other by pattern of spinules in proctodeum. The *A. brevispinosus* have patchy pattern of spinules in proctodeum but in *A. einslei* is presented as a single row (Mirabdullayev and Defaye, 2004).

Acanthocyclops robustus differs from known species of *vernalis-robustus* complex by the ornamentation of the basipodite of antenna having spinules near the exopodal seta, and from *A. trajani* by missing spinules in claw-like seta in basipodite of maxilla, and by length ratio of innermost and outermost furcal seta (Mirabdullayev and Defaye, 2002).

Acanthocyclops vernalis differs from all species from *robustus* complex (*A. trajani*, *A. einslei* and *A. brevispinosus*) in the shape of genital double somite tapered in proximal part into blunt lobes on either side, and in ratio of two apical spines in enp3 P4 with inner always shorter than outer, but having opposite pattern in *A. trajani* as already mentioned by Kiefer (1976) or Dodson (1994). However, these authors synonymised *A. trajani* with *A. robustus*.

1.4. DISTRIBUTION AND ECOLOGY OF *Acanthocyclops vernalis-robustus* SPECIES COMPLEX

Acanthocyclops robustus s.s. inhabits waterbodies of Scandinavia, Canada and northern regions of USA. According to Mirabdullayev and Defaye (2002) records of this species from northern Russia are expected as well. These authors described *A. robustus* as a species that seems to be "probably planktonic", however Sars (1918), in original description attributed this species as a "true bottom-form, keeping constantly close to the ground". Additional data about distribution and ecology of this species are poor, since most of studies dealing with *A. robustus* dealt in fact with *A. trajani* (e.g. Einsle, 1977; Vijverberg and Richter, 1982; Roche, 1990; Caramujo and Boavida, 1999).

Acanthocyclops trajani has holarctic distribution, except Scandinavia (Mirabdullayev and Defaye, 2002). Occurrence in South America is also expected, since references about *A. robustus* from this area appeared in literature (e.g. Trochine et al., 2006; González et al., 2008) and as mentioned above, most of ecological studies of *A. robustus* dealt in fact with *A. trajani*. (e.g. Ponyi, 1967; Purasjoki and Viljamaa, 1984; Caramujo and Boavida, 1998; Hopp and Maier, 2005). However without any figure which clearly displaying the fourth swimming leg is quite difficult to make a final consideration. Moreover,

this species inhabits ponds, fishponds, lakes and reservoirs in all of which, it is the dominant copepod species. During summer, usually reach a high density of 40000 ind m^{-3} (Vijverbegr, 1977; Alekseev et al., 2002). Purasjoki and Viljamaa (1982) also reported occurrence in eutrophicated brackish water of Helsinki bay, reaching densities up to 1.10^6 ind.m^{-3}, indicating tolerance to certain salinity.

Acanthocyclops einslei inhabits waterbodies of Eurasia (except Scandinavia) and North America (Mirabdullayev and Defaye, 2004). Occurrence even in Australia can be presumed based on detailed description and figures provided by Morton (1985), and implying rather *A. einslei* than presented *A. robustus*. This species can be found in ponds, ditches and littoral zone of larger lakes cooccuring also with *A. trajani* (Mirabdullayev and Defaye, 2004).

Acanthocyclops brevispinosus occurs in Canada and North America, being found usually in larger lakes and ponds as a typical planktonic species (Mirabdullayev and Defaye, 2004), although Dodson (1994) mentioned discovery of this species from temporary ponds or stream littoral under water reservoirs. Since this species is already known for more than hundred years, probably due to long time confusion with other members of *Acanthocyclops* species complex, knowledge about ecology is still poor.

Acanthocyclops vernalis has probably worldwide distribution with well documented records from all continents. However reliability of these records is questionable (Einsle, 1996). Especially in North America is still being regarded as a complex of several reproductively separated species (Dodson et al., 2003; Grishanin et al., 2006) that can be found in different habitats and conditions from small ponds, ditches, temporary pools to littoral parts of larger reservoirs. The species strictly inhabit litoral parts of water bodies and never reach pelagic zone. The species is also frequently found in groundwater and interstitial space (Fryer, 1985; Jersabek et al., 2001; Alekseev et al., 2002). However in certain conditions such as acidified lakes, it inhabits open water (Nillsen and Wærvågen, 2003; Hořická et al., 2006). In conditions of Central Europe normally occurs during spring and autumn with diapause at fourth copepodid stage (Einsle, 1996).

1.5. MOLECULAR METHODS IN ANALYSES OF *Acanthocyclops vernalis-robustus* COMPLEX

Within such a complicated copepod genus, which *Acanthocyclops vernalis-robustus* definitely is, morphological approaches have limited using.

Molecular studies become powerful tool for species discrimination on DNA level, and for assessing species phylogenetic relationships. In terms of *Acanthocyclops vernalis-robustus*, phylogenetic relationships are hardly available due to phenotypic plasticity in certain characteristics usually using for species discrimination. In addition, molecular studies revealed hidden cryptic species within *Acanthocyclops* species (Bláha et al., 2010) and also other copepods with highly conserved morphology (e.g. Lee and Frost, 2002; Chen and Hare, 2008; Thum and Harrison, 2009).

The most useful molecular markers inferring phylogenetic relationships of lower taxonomic units (genera or species level) are mitochondrial genes whereas nuclear genes are used rather for assessing phylogenetic relationships between higher taxonomic groups (Avise, 1994, 2000). The widely used is mitochondrial gene for Cytochrome Oxidase subunit I (COI) (Bucklin et al., 1999; Hill et al., 2001; Machida et al., 2004; Chen and Hare, 2008), large subunit of ribosomal ribonucleic acid (16S rRNA), (Bucklin et al., 1992, 1995; Lindeque et al., 1999; Caudill et al., 2004), or their combination (Lee, 2000; Lefébure et al., 2006). In terms of nuclear genes most studies analysed partial segments of 18S ribosomal DNA (rDNA) (Thum, 2004; Bucklin et al., 2003) and ITS (Internal transcribed spacer) region of ribosomal DNA (Ki et al., 2009a; Marzsalek et al., 2009; Thum and Harrison, 2009).

However, some molecular markers were quite useful in calanoid copepods, but less efficient in

cyclopoid copepods, which is case of COI and 16S rRNA. These genes have never been used in studies of cyclopoid copepods. Contrariwise widely used are nuclear genes. From the small amount of molecular studies concerning freshwater cyclopoid copepods (Alekseev et al., 2006; Ki et al., 2009b; Bláha et al., 2010; Wyngaard et al., 2010), just three contributed to solving *Acanthocyclops* problem (Grishanin et al., 2005, 2006; Bláha et al., 2010). In most cases nuclear sequences show low variability in the species level e.g. in *Eucyclops* (Alekseev et al., 2006), Mesocyclops (Wyngaard et al., 2010) as well as in *Acanthocyclops* (Grishanin et al., 2005).

IN THIS THESIS

The overall aim of present thesis was to make comprehensive study combined morphological and molecular approaches to better understand complicated situation within *Acanthocyclops* species complex.

The specific objectives were to:

- describe developmental stages of newly described species (*A. trajani* and *A. einslei*), and to provide distinguishing characteristics;
- determine taxonomic position of newly described species within *Acanthocyclops* species complex using molecular methods;
- obtain new insight into the morphological and molecular patterns of the *Acanthocyclops* species complex.

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CHAPTER 2

MOLECULAR AND MORPHOLOGICAL PATTERNS ACROSS *Acanthocyclops vernalis-robustus* SPECIES COMPLEX (Copepoda, Cyclopoida)

Bláha, M., Hulák, M., Slouková, J., Těšitel, J., 2010. Molecular and morphological patterns across *Acanthocyclops vernalis-robustus* species complex (Copepoda, Cyclopoida). Zoologica Scripta 39 (3), 259–268.

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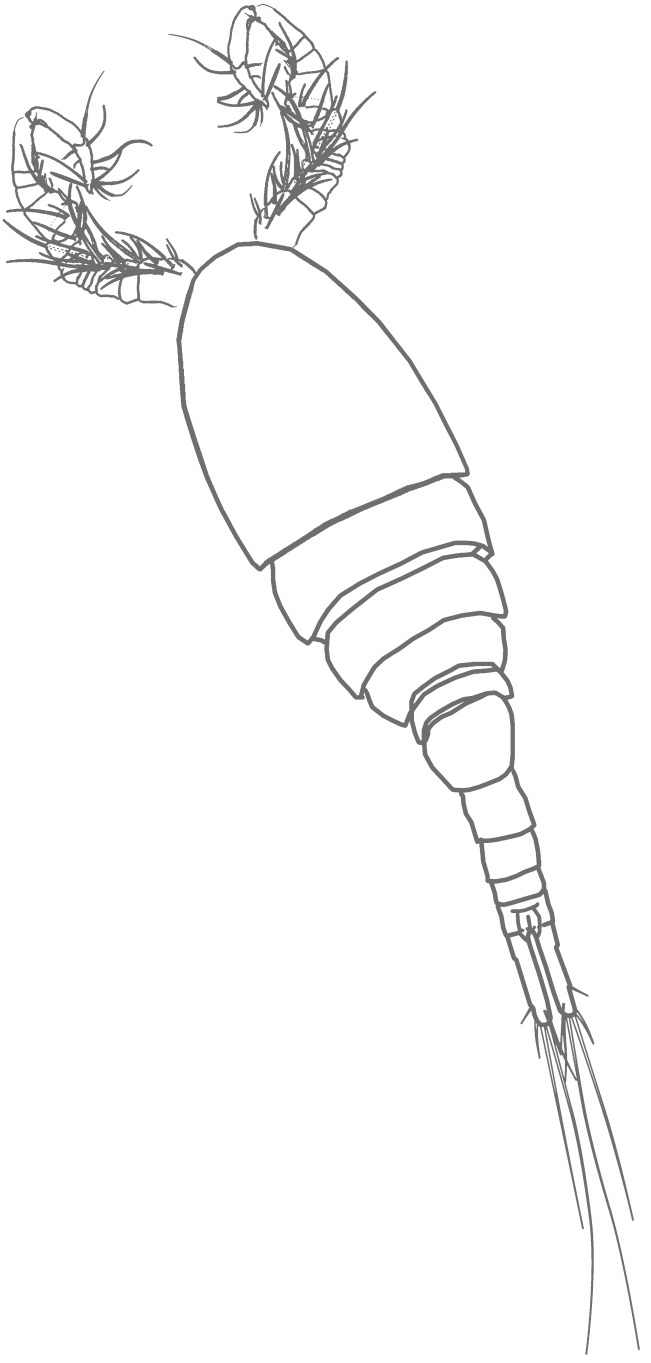

Zoologica Scripta

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Molecular and morphological patterns across *Acanthocyclops vernalis-robustus* species complex (Copepoda, Cyclopoida)

MARTIN BLÁHA, MARTIN HULÁK, JANA SLOUKOVÁ & JAKUB TĚŠITEL

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Morphological traits within *Acanthocyclops* (Kiefer, 1927) are highly variable, and morphology is too constrained to give complete information of phylogenetic relationships. This study combined morphological and molecular techniques to investigate the taxonomic and phylogenetic relationships of three species of *Acanthocyclops* (*Acanthocyclops trajani*, *Acanthocyclops einslei* and *Acanthocyclops vernalis*) inhabiting continental Europe. Morphological indices subjected to principal component analysis (PCA) separated sample populations into three distinct clusters corresponding with the taxonomic status of the species analysed. In addition, the taxonomy status of *A. trajani* and *A. einslei* was in agreement with molecular data; however, the intraspecific variation in sequences of 12S rRNA was lower in contrast to specimens morphologically determined as *A. vernalis*, which were divided into two deeply divergent clades, based on mtDNA sequence divergences. Moreover, high sequence divergence (26%) between these clades indicated the existence of another species that may not be a sister taxon of *A. vernalis* s.s. Results point to the need for further taxonomic work on *Acanthocyclops*.

Corresponding author: *Martin Bláha, University of South Bohemia, Faculty of Fishery and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Zátiší 728/II, 38925 Vodňany, Czech Republic. E-mail: blaha@vurh.jcu.cz*
Martin Hulák, University of South Bohemia, Faculty of Fishery and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, 38925 Vodňany, Czech Republic. E-mail: hulak@vurh.jcu.cz
Jana Slouková, Charles University in Prague, Faculty of Science, Department of Ecology, Viničná 7, 128 44 Prague 2, Czech Republic. E-mail: vodnarka@centrum.cz
Jakub Těšitel, University of South Bohemia, Faculty of Science, Department of Botany, 37005 České Budějovice, Czech Republic. E-mail: jakub.tesitel@centrum.cz

Introduction

Throughout the animal kingdom there are numerous species that show subtle morphological differences from sister taxa. Morphological stasis represents an evolutionary constant, and cryptic metazoan diversity predictably affects estimates of earth's animal diversity (Pfenninger & Schwenk 2007). Recent molecular phylogenetic and phylogeographical research has provided a powerful tool in the recognition of divergent clades that would have escaped notice because of their close morphological convergence (e.g. Lee 2000; Lee & Frost 2002; Mathews *et al.* 2008). This is the case within *Acanthocyclops* (Kiefer 1927), which is among the five most speciose genera of cyclopoid copepod subfamilies, Cyclopinae, with more than 60 valid species and subspecies. Many of them are cosmopolitan or Holarctic, living in surface or subsurface fresh waters. A few species are strictly subterranean (Boxshall & Halsey 2004; Dussart & Defaye 2006). However, understanding the taxonomy and phylogenetic relationships among them has remained a challenge. This, together with incomplete descriptions, has resulted in many species with uncertain status (Reid *et al.* 1991; Einsle 1996) and a progressively complex taxonomy that relies on only a few quite stable characters. Confounding effects include high phenotypic plasticity with extensive intraspecific morphological variation, as well as interspecific morphological similarity because of high morphological stasis, which can result in undetected cryptic speciation (Hebert 1998).

One relevant example of a cryptic species complex in the genus *Acanthocyclops* is the *Acanthocyclops vernalis* or

vernalis-robustus species complex. But in fact, each of the species *A. vernalis* and *Acanthocyclops robustus* is itself a species complex (e.g. Petkovski 1975; Kiefer 1976). These complexes are poorly defined, and different geographical regions have been subjected to different taxonomic treatments. In the past, the designation *A. robustus* has been frequently applied to individuals inhabiting most Holarctic habitats. More recently, Mirabdullayev & Defaye (2002) reported its existence only in Scandinavia and North America, whilst *A. vernalis*, is described by Kiefer & Fryer (1978), Purasjoki & Viljamaa (1984), and Einsle (1996) as inhabiting the entire Holarctic region. Currently, *A. robustus* is considered to be a separate species, as it differs morphologically from all species of the *A. robustus* species complex by the ornamentation of the basipodite of antenna, with spinules near the exopodal seta. Additionally, Mirabdullayev & Defaye (2002, 2004) described two new species (*Acanthocyclops trajani* and *Acanthocyclops einslei*) in the *A. robustus* species complex and have re-described *A. robustus* based on Sars's collection and newly collected material.

The newly described species have been previously referred to as *A. robustus* (*A. trajani*) and either *A. vernalis* or *A. robustus* (*A. einslei*) or morphological varieties of either species (Petkovski 1975; Caramujo & Boavida 1998). The morphological traits of *Acanthocyclops* are highly variable, and morphology is inadequate for understanding phylogenetic relationships within the genus. To overcome this constraint, our study, based on three independent data sets of nuclear and mtDNA, and morphological divergence, extends the data on genetic and phylogenetic relationships among species complexes of the cyclopoid genus *Acanthocyclops*.

The primary purpose of this study was to develop a phylogenetic framework of newly described species from Europe (*A. trajani* and *A. einslei*) belonging to the *A. robustus* species complex. The objectives were: (i) to clarify whether the phenotypic subdivision and morphological variability is related to genetic divergence; (ii) to test the predictions based on morphological investigations of cryptic diversity; and (iii) to obtain insights into the morphological and molecular evolution of the *Acanthocyclops* species complex.

Materials and methods

Collection, preservation and determination

Samples were collected from ponds, temporary pools, rivers, lakes, and reservoirs of central Europe using an 80-μm mesh size plankton net (Table 1). Samples were preserved in 96% ethanol. Adult females were independently identified, as to species, by two researchers (JS, MB) according to Mirabdullayev & Defaye (2002, 2004) and Einsle (1996). Because, we were unsuccessful in obtaining specimens of North American *Acanthocyclops* species and *A. robustus*, the morphological study addresses only three nominal species, *A. trajani*, *A. einslei*, and *A. vernalis*.

Morphology

In total, 179 individuals of *Acanthocyclops* species from 22 populations were measured. Specimens were immersed in a drop of lactic acid to clear non-exuvial material. Phase contrast photographs of whole body (dorsal view) and the dissected fourth pair of swimming legs were taken with a binocular microscope Olympus BX51 fitted with an Olympus E-510 digital camera. Subsequently, measurements were obtained using Quick PHOTO CAMERA 2.2 software (Olympus, Hamburg, Germany). Measurements of the fourth swimming leg distal endopodite (enp3P4) were made: length (L enp3P4), width (W enp3P4), distance from the beginning of enp3P4 to the site of inner lateral seta/spine insertion (Lo), and lengths of internal apical spine (IAS) and external apical spine (EAS). Length (Lfu) and width (Wfu) of furcal rami and length of furcal setae (Si, Smi, Sme, Se) were also recorded (Fig. S1). Statistical significance of morphological indices was assessed with statistical software Statistica 6.0, using the non-parametric Kruskal–Wallis test.

Molecular analyses

Total genomic DNA was extracted from whole individuals using E.Z.N.A.® Tissue DNA Mini Kits (Peqlab, Erlangen, Germany) following the manufacturer's protocol. Fragments including part of the mitochondrial gene 12S rRNA (430 bp) and nuclear 18S rDNA (620) were amplified using PCR primers L13337 and H13845 for 12S rRNA (Machida *et al.* 2004) and primers 18s329 and 18sI for 18S rDNA (Grishanin *et al.* 2005). The PCR reaction was done in an Eppendorf Master Gradient cycler. The amplification reaction consisted of 5 μL of PPP Master mix [50 mM Tris–HCl, pH 8.8, 40 mM $(NH_4)_2SO_4$, 0.02% Tween 20.5 mM $MgCl_2$, 400 μM dATP, 400 μM dCTP, 400 μM dGTP, 400 μM dTTP, and 100 U/mL Taq-Purple DNA polymerase], 0.3 μL of each primer (10 pmol/μL), 1 μL genomic DNA, and sterile water to a final volume of 15 μL. The PCR protocol consisted of 2 min initial denaturation at 95 °C, followed by 5 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and another 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min. A final extension at 72 °C lasted for 7 min. For sequencing, the PCR products were purified by the Nucleospin® (Macherey-Nagel, Düren, Germany). Purified products were subsequently sequenced on ABI automatic capillary sequencer

Table 1 List of analysed *Acanthocyclops* species populations.

Taxon	Country collection locality/acc. no. GenBank	Population code	Type of locality (altitude)	Latitude (N)	Longitude (E)	Analysed gene	Haplotype
Acanthocyclops einslei							
Czech republic							
Lužnice river, Majdalena*		Ecz1	Pool in river inundation area	48°58′21″	14°51′53″	12S	E1
Lužnice river, Halámky*		Ecz2	Pool in river inundation area	48°51′1″	14°54′29″	12S	E3
Strakonice, Hluboká Pálenina*		Ecz3	Fishpond	49°14′39″	13°52′6″	12S	E2
Volyňka river, Strakonice*		Ecz4	River littoral	49°14′8″	13°53′44″	12S	E2
Sokolov		Ecz5	Pool	50°12′16″	12°38′39″	12S	E2
Blanice river, Vodňany*		Ecz6	River littoral	49°9′38″	14°9′ 68″	12S	E2, E3
Bohuslavice*		Ecz7	Temporary pool	49°49′13″	16°55′56″	12S	E2
Strakonice, Sousedovice		Ecz8	Pool	49°13′47″	13°52′17″	12S	E4
Slovakia							
Velké Kapušany		Esk1	Temporary pool	48°30′	22°02′	12S	E2
Acanthocyclops trajani							
Czech republic							
Třeboň, Velký Tisý		Tcz1	Extensive fishpond	49°4′2″	14°42′30″	12S	T1
Jaroslavice, Zámecký*		Tcz2	Fishpond	48°45′40″	16°14′10″	12S	T2, T3
Strakonice, Hluboká Pálenina*		Tcz3	Fishpond	49°14′39″	13°52′6″	12S	T1
Strakonice, Močidlo*		Tcz4	Extensive fishpond	49°13′47″	13°52′26″	12S	T1, T4
Bohuslavice*		Tcz5	Temporary pool	49°4913″	16°55′56″	12S	T1
Doubravice, Mostek*		Tcz6	Fishpond	49°44′29″	16°57′42″	12S	T1
Klopina		Tcz7	Fishpond	49°48′25″	17°1′7″	12S	T1
Šumvald		Tcz8	Fishpond	49°49′2″	17°6′52″	12S	T1
Dolni Libina*		Tcz9	Fishpond	49°51′9″	17°6′12″	12S	T1
Blatná, Vitanov*		Tcz10	Fishpond	49°25′6″	13°49′29″	12S	T1
Paštiky*		Tcz11	Fishpond	49°26′32″	13°53′56″	12S	T1
Smyslov		Tcz12	Fishpond	49°25′11″	13°48′37″	12S	T1
Police*		Tcz13	Fishpond	49°48′20″	16°59′56″	12S, 18S	T1
Nové Hrady, Písař		Tcz14	Fishpond	48°48′1″	14°46′18″	12S, 18S	T1
Žadlovice		Tcz15	Fishpond	49°45′7″	16°54′8″	12S	T1
Spain							
Rio Guadiana, Badajoz		Tsp1	River littoral	38°51′34″	7°01′	12S, 18S	T1
Portugal							
Lagoa da Vela		Tpt1	Eutrophic lake	40°16′01″	8°46′60″	12S	T1
Greece							
Doiranis*		Tgr1	Lake	41°12′22″	22°45′12″	12S	T5
Petron		Tgr2	Lake	40°44′59″	21°46′47″	12S	T6
USA							
Short Pond 1, Chippewa County, WI/AY643524–26		S115, S130, S142	Shallow lake	45°23′41″	91°11′84″	18S	–
Acton Lake, Butler County, OH/AY643530–32		AC8–AC10	Eutrophic lake	39°55′77″	84°73′45″	18S	–
Trek Pond, WI/AY643522		Tre1	Urban lake	43°06′06″	89°52′37″	18S	–
Acanthocyclops vernalis							
Czech republic							
Lužnice river, Majdalena		Vcz1	River littoral	48°58′ 21″	14°51′ 54″	12S	V1
Velky Močál*		Vcz2	Moss lake (920 m)	50°23′32″	12°37′59″	12S	V6
Strakonice, Hluboká Pálenina*		Vcz3	Fishpond	49°14′39″	13°52′6″	12S	V1–V4
Volyňka river, Strakonice*		Vcz4	River littoral	49°13′ 34″	13°53′ 58″	12S, 18S	V1
Kralický Sněžník*		Vcz5	Spill (1300 m)	50°12′4″	16°50′54″	12S	V7
Labe river, Pardubice		Vcz6	River littoral	50°2′58″	15°46′46″	12S	V1
Strakonice, Sousedovice*		Vcz7	Temporary pool	49°13′ 49″	13°52′ 39″	12S	V1,V5
Strakonice, Sousedovice		Vcz8	Forest pool	49°13′ 33″	13°52′ 16″	12S	V7
Slovakia							
Rimavská Baňa		Vsk1	Temporary pool	48°30′ 38″	19°55′ 54″	12S	V8,V9
Bulgary							
Todorini Oči*		Vbu1	Ice lake (2100 m)	41°45′	23°25′	12S	V10

Table 1 (Continued).

Taxon	Country collection locality/acc. no. GenBank	Population code	Type of locality (altitude)	Latitude (N)	Longitude (E)	Analysed gene	Haplotype
Montenegro							
Velké Skrčko*		Vmn1	Ice lake (2000 m)	43°8′8"	19°0′55"	12S	V11
Switzerland							
Lac du col du Gd St Bernard*		Vsu1	Ice lake (2450 m)	45°52′06"	7°10′03"	12S, 18S	V12
USA							
Short Pond 1, Chippewa County, WI/AY643523		S102	Shallow lake	45°23′41"	91°11′84"	18S	–
Parejko Pond, Chippewa County, WI/AY643521		Pa26	Shallow lake	45°23′41"	91°11′84"	18S	–
State Highway 14, Dane County, WI/AY643527–29		CD60, CD61, CD69	Road ditch	43°09′31"	89°60′22"	18S	–
Megacyclops viridis							
Czech republic							
Moravičany		MV	Forest pool	49°45′12"	16°59′8"	12S	–
Mesocyclops thermocyclopoides/EF581894		MO				18S	–

*Populations used in morphological analysis.

(series 373) (Macrogene, Seoul, Korea). Fragments of 12S rRNA (430 bp) and 18S rDNA (620 bp) were sequenced using the same primers as those used for the amplification.

Sequence data analyses

All together, 150 individuals representing the three species collected were used in sequencing and phylogenetic analysis. *Megacyclops viridis* (Jurine, 1820) (details in Table 1) and *Mesocyclops thermocyclopoides* Harada, 1931 (acc. no. in GenBank EF581894) were used as outgroups for 12S rRNA and 18S rDNA, respectively. For comparative purposes, the set of 18S rRNA *Acanthocyclops* sequences from Nearctic populations from GenBank were used (acc. nos AY643521–AY643531). DNA sequences for each species were aligned using CLUSTAL W (Thompson *et al.* 1997) incorporated in MEGA version 4 (Tamura *et al.* 2007). Sequence divergences between and within main clades were calculated for the distinct clades (species) sequences (excluding outgroups) using DNASP version 4.90.1 (Rozas *et al.* 2003).

Phylogenetic analyses

The phylogenetic relationships of *Acanthocyclops* species were constructed using partial 12S rRNA gene and a part of the 18S rDNA gene sequences. Bayesian analysis was conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). A Markov Chain Monte Carlo (MCMC) analysis was run for 2 million generations, with two parallel runs of four chains run simultaneously, and sampled every 100th generation. The first 25% of sampled generations were discarded as a burn-in process. The remaining trees were used to construct the phylogram. The best-fit model of nucleotide substitution selected by ModelTest 3.7 (Posada & Crandall 1998) was the General Time Reversible plus Gamma (GTR + Γ) for 12S data set and the Hasegawa-Kishino-Yano (HKY) for the 18S dataset. These models were chosen based on the likelihood score and Akaike information criterion (AIC) from 28 models. In addition, phylogenetic analyses were conducted using the maximum parsimony and neighbor-joining method executed in MEGA version 4 (Tamura *et al.* 2007). The MP tree was obtained using the close-neighbor-interchange algorithm (Nei & Kumar 2000) with search level 3 (Felsenstein 1985). The neighbor-joining method for constructing a tree based upon maximum composite-likelihood and the Kimura 2-parameter algorithm was used.

Divergence time was estimated from the Kimura 2-parameter distance, calculated using MEGA version 4 on the mitochondrial 12S data set, assuming a clock-like mutation rate for mitochondrial DNA. Substitution rates of 0.9% (decapod 16S gene – Schubart *et al.* 1998) and of 1.4% per million years (decapod COI gene – Knowlton & Weigt 1998) have been used in previous studies. A substitution rate of 1.2% per million years was used in the present study.

Relationships among haplotypes were inferred using the statistical parsimony method (Templeton *et al.* 1992). A parsimony network was estimated with Network software version 4.109 (Bandelt *et al.* 1999) using the default 0.95 probability connection limit.

Results

Sequence variation and alignments

Of 56 ingroup specimens from 36 locations sequenced for 12S rRNA, 22 haplotypes were detected. The 12S rRNA

sequences were unambiguous, with no indels, and contained 105 variable and 87 parsimony informative sites. Pairwise Kimura 2-parameter (K2P) genetic distances among four main clades, designated A, B, C, and D, ranged from 0.124 to 0.194. Observed K2P distances between ingroups and outgroup, represented here by *M. viridis*, were 0.253–0.272.

Mitochondrial gene tree

All phylogenetic methods (Bayesian analysis, MP, NJ) resulted in trees that did not differ in main topology, i.e. all specimens were assigned to the same four main clades, and the relationships among these clades was stable (Fig. 1A). The few differences observed with the different methods were mostly related to terminal branch swapping. Clades were distinct from one another; sequence divergences among them ranged from 20% (between clades A and B) to 27.6% (between clades A and C).

Clades A and B, represented here by species *A. trajani* and *A. einslei*, formed monophyletic clades. Average sequence divergences within these two clades were 3.3% and 2.5%, respectively. Clades C and D contained well-supported lineages (V12 and V4), differing from the rest of the clade by 7% and 4%, respectively. Sequence divergences within the rest of clades C and D were almost the same and ranged from 0.7% to 8%.

18S rRNA gene tree and concordance among the mitochondrial and the nuclear phylogenies

Nuclear DNA was applied to test the similarity of European and American individuals by using existing sequences in GenBank. Phylogenetic analyses revealed two major genetically divergent and well-supported clades corresponding to *A. trajani* and *A. vernalis* morphotypes, i.e. Grishanin's specimens from Wisconsin and Ohio populations were clustered together with specimens from Europe, undoubtedly identified as either *A. trajani* or *A. vernalis*, and composed two main clades (Fig. 2S). The cluster pattern of these clades within the nuclear tree was almost identical to the pattern of the mitochondrial DNA tree.

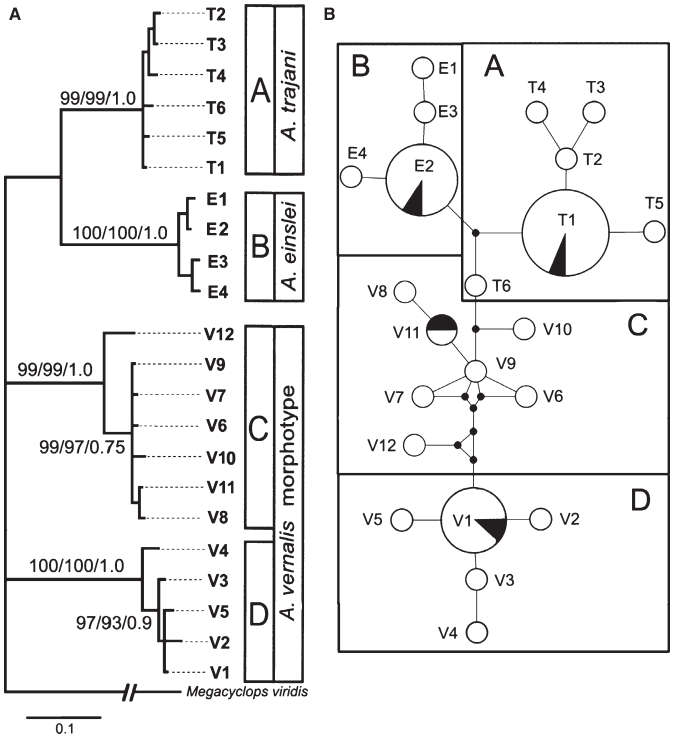

Fig. 1 A–B. Phylogenetic relationships within *Acanthocyclops* based on mitochondrial 12S rRNA. —A. Fifty per-cent majority-rule consensus tree of the Bayesian Inference (BI) showing relationship of the main haplotypes. The node support: bootstrap ML/MP/BI. A–D: main clades in the *Acanthocyclops* species complex (seeTable 1 for the location of main haplotypes). —B. Haplotypes association of *Acanthocyclops* species. Each dark node within parsimony network represents a hypothetical missing or unsampled ancestral haplotype. Circle size corresponds to the number of individuals sharing the particular haplotype. A–D corresponds to main clades in consensus tree.

Haplotype network

Specimens represented 36 populations from European locations (Table 1). DNA sequence analysis of mtDNA identified 22 haplotypes among analysed species (Fig. 1B). *Acanthocyclops trajani* (clade A) formed six haplotypes. Central haplotype T1 includes most of the population from the Central Europe; however, individual specimens from Spain and Portugal were also represented. Connected haplotypes (T2–T4) comprise populations from ponds in the Czech Republic and populations from Greece (T5 and T6). *Acanthocyclops einslei* (clade B) is characterized by four haplotypes. The most divergent are haplotypes within *A. vernalis* morphotype clade C, which originated in mountain lakes within the Czech Republic (V6, V7) and the Balkan countries (V10, V11) and in isolated periodic pools in South Bohemia (V11) and Slovakia (V8, V9). Lineage V12 is represented by a population (Vsu1) in the Swiss western Alps. Sequence divergences between this haplotype and the remaining haplotypes in clade C were 11.3–18.0%. *Acanthocyclops vernalis* morphotype clade D formed four haplotypes. Central haplotype V1, together with other haplotypes in this clade (V2–V4), originated in a single pond. Divergences between haplotype V4 and the others within the clade ranged from 2.8% (V1) to 18% (V2).

The age of the *Acanthocyclops* species complex and the time scale for the diversification can be only approximately estimated, as no fossil calibration exists for copepods. Using the range of genetic distances found in the literature for other crustaceans, the probable time of divergence of particular clades was assessed. Kimura 2-parameter distances between clade A (*A. trajani*) and clade B (*A. einslei*) were 0.124–0.147, which corresponds to a divergence time of ~10–12 MYA. Clades C and D (*A. vernalis* morphotypes), with distances of 0.143–0.184, probably diverged 12–15 MYA. *Acanthocyclops* species separated from a common ancestor with *M. viridis* approximately 21.0–22.6 MYA.

Morphological variation within the* Acanthocyclops *species complex

In total, 179 individuals of *Acanthocyclops* species from 22 populations were measured (Table 2). Rather than simple length characteristics, length ratios of furcal rami, furcal setae, and enp3P4 (Lf: Wf, Si: Lfu, Si: Smi, Si: Sme and L: W, L: Lo, IAS: EAS of enp3P4) were used as input for analyses. Significant differences were found (Kruskal–Wallis test; d.f. = 2; $P < 0.001$) among species for all indices, with the exception of Lo: L enp3P4, in which *A. einslei* showed significant differences from the two other species, and IAS: W enp3P4 and L: W enp3P4, in which *A. trajani* is significantly different from two other species.

The principal component analysis (PCA) of selected morphometric indices depicted three clearly defined groups (A, B, and C) corresponding to species recently described by Mirabdullayev & Defaye (2002, 2004) (Fig. 2). The groups form a gradient along the first axis, strongly correlated to several morphometric indices (Si: Se, Si: Lfu, Si: Sme, Si: Smi, IAS: EAS, IAS: L enp3P4, and IAS: W enp3P4). Three first axes explain 58.7%, 14.1%, and 10.3% of variability. Cluster A individuals differ from the two other clusters in several self-correlated characteristics (furcal setae indices and IAS: EAS). Individuals in cluster B markedly differ from other *Acanthocyclops* species in the site of lateral spine insertion in enp3P4, i.e. *A. einslei* has the site of insertion nearer the apical end of the segment, whereas, in the other two species, this seta/spine is more proximal, near the centre of the segment. Cluster C individuals have opposite pattern in apical spines ratio (IAS: EAS; inner apical spine is always shorter than outer) compared to cluster A, and also the other

Table 2 Measurements of *Acanthocyclops* species (adult females).

	Acanthocyclops trajani		*Acanthocyclops einslei*		*Acanthocyclops vernalis*	
	Mean ± SD	Min – max	Mean ± SD	Min – max	Mean ± SD	Min – max
Lfu: Wfu	4.85 ± 0.45[a]	3.68–5.61	5.18 ± 0.57[a]	4.07–6.20	4.91 ± 0.84[a]	3.41–6.75
Si: Lfu	0.93 ± 0.11[a]	0.61–1.14	0.75 ± 0.07[b]	0.56–0.98	0.62 ± 0.08[c]	0.44–0.81
Si: Smi	0.25 ± 0.02[a]	0.19–0.30	0.19 ± 0.02[b]	0.15–0.23	0.17 ± 0.02[c]	0.12–0.23
Si: Sme	0.36 ± 0.03[a]	0.26–0.42	0.28 ± 0.02[b]	0.22–0.32	0.24 ± 0.03[c]	0.18–0.30
Si: Se	1.81 ± 0.18[a]	1.40–2.17	1.67 ± 0.15[b]	1.34–2.25	1.41 ± 0.17[c]	0.95–1.89
L: W enp3 P4	2.72 ± 0.29[a]	2.28–3.60	2.50 ± 0.22[b]	2.19–3.08	2.41 ± 0.38[b]	1.87–3.42
Lo: L enp3 P4	0.61 ± 0.02[a]	0.58–0.66	0.76 ± 0.03[b]	0.62–0.81	0.61 ± 0.03[a]	0.53–0.70
IAS: EAS	1.18 ± 0.09[a]	1.00–1.50	1.03 ± 0.04[b]	0.95–1.14	0.86 ± 0.06[c]	0.71–0.99
IAS: L enp3 P4	0.87 ± 0.07[a]	0.67–1.09	0.7 ± 0.07[b]	0.56–0.90	0.61 ± 0.07[c]	0.48–0.81
IAS: W enp3 P4	2.37 ± 0.32[a]	1.79–3.19	1.75 ± 0.21[b]	1.41–2.12	1.48 ± 0.30[b]	0.98–2.00
N	89		39		51	

N, number of analysed individuals; values with identical superscripts within a lines did not differ significantly ($P < 0.001$, K–W test).

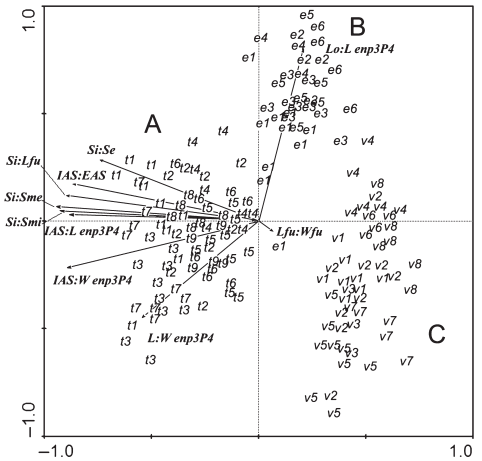

Fig. 2 Principal component analysis (PCA) populations' clustering of *Acanthocyclops* species based on the morphological characteristic (indices). Particular morphometric indices (details in text) are indicated by arrows. —A. *t1–t9*: populations of *Acanthocyclops trajani* (Tcz2, Tcz3, Tcz4, Tcz5, Tcz6, Tcz9, Tcz10, Tcz11 and Tgr1, respectively). —B. *e1–e6*: populations of *Acanthocyclops einslei* (Ecz1, Ecz2, Ecz3, Ecz4, Ecz6 and Ecz7, respectively). —C. *v1–v8*: populations of *Acanthocyclops vernalis* (Vcz2, Vcz3, Vcz4, Vcz5, Vcz7, Vbu1, Vmn1 and Vsu1, respectively).

traits show the lowest values in comparison with clusters A and B (except Lfu: Wfu), i.e. a clear tendency of decreasing ratios from *A. trajani* (cluster A) to *A. vernalis* (cluster C), with *A. einslei* (cluster B) showing mid-range values, was observed (Table 2).

Discussion

Genetic differentiation among Acanthocyclops trajani *and* Acanthocyclops einslei: *is there an agreement between morphological and genetic data?*

The current taxonomic classification in European populations of *A. trajani* and *A. einslei*, which is based on morphology according to Mirabdullayev & Defaye (2002, 2004) is confirmed here by the DNA sequence analysis of the mitochondrial 12S rRNA gene fragment. However, discrimination of *Acanthocyclops* species based on adult morphology is confounded by an apparent phenotypic plasticity of many traits. Therefore, to overcome these challenges to morphological analysis, this study focused on characteristics of the distal endopodite of the fourth swimming leg (enp3P4), proposed by Mirabdullayev & Defaye (2002, 2004) to be most useful as an identification marker in *Acanthocyclops* taxonomy. By applying relative size of body parts, we eliminated the effect of body size, determined mainly by environmental factors which can have an important influence on intraspecific morphological variability among populations (Dodson *et al.* 2003).

Based on PCA analysis, all specimens were clearly assigned to the clusters which correspond to species recently described by Mirabdullayev & Defaye (2002, 2004). Our results indicate that the site of lateral seta insertion (Lo: L enp3P4) unambiguously differentiates *A. einslei* from other *Acanthocyclops* species, and the ratio of the two apical spines in enp3P4 (IAS: EAS) differentiates *A. trajani* from *A. vernalis* (Table 2). Another useful trait (not used in our analyses) discriminating *A. vernalis* from *A. trajani* and *A. einslei* is shape of genital double-segment. In *A. trajani* and *A. einslei* is broadly rounded in its anterior part whereas in *A. vernalis* extended into 'blunt lobe' on either side as reported also by Kiefer & Fryer (1978) and Dodson (1994). In addition, PCA based on morphometric indices identified three distinct groups in the analysed samples (A, B, and C; Fig. 2), with the first three components accounting for 83.1% of the total variance. The first principal component explains 58.7% of the total variance and serves to distinguish the three species examined.

The 12S rRNA sequences in both species studied differed at least 20% between two mitochondrial lineages (A, B). The degree of intraspecific diversity observed for *A. trajani* (3.3%) and *A. einslei* (2.5%) is similar to that of *Lepidurus articus* [0.3–3.4% (King & Hanner 1998)] and *Daphnia* species [0.5–2.0% (Petrusek *et al.* 2007; Thielsch *et al.* 2009)]. However, on a broader scale, populations of *D. pulex* widely geographically separated were shown to be more divergent (7.2%; Mergeay *et al.* 2005). Molecular variance in *A. trajani* and *A. einslei* approached the minimum interspecific distances reported for other crustacean taxa (5.6–19.4%) (e.g. Petrusek *et al.* 2004, 2008; Parmakelis *et al.* 2008). Thus the observed sequence differences are clearly within the range of interspecific differences, while the sequence differences within lineages A (3.3%) and B (2.5%) were in the range of intraspecific variation. Moreover, the sequence divergence (20%) between *A. trajani* and *A. einslei* might arguably be substantial enough to indicate divergence into two biological species. Determining whether the lineages of the *A. vernalis* morphotype identified in this work represent full species or intraspecific units will require additional work that considers mating compatibility, gene flow at nuclear loci, and ecological and physiological divergence.

The majority of analysed *A. trajani* and *A. einslei* individuals represented a single haplotype which exhibited almost no spatial structure and high mitochondrial female gene flow. In contrast, individuals of the *A. vernalis* clade

C exhibited specific spatial structure due to isolation of habitat, represented here mostly by glacial lakes (Table 1), although results based solely on a mitochondrial marker in a small number of analysed individuals should be interpreted with caution. From the present distribution, and as the dispersal abilities and reproductive strategies of both species are poorly understood, further sampling and molecular markers of higher resolution are needed for more detailed phylogeographic information.

Cross-comparison with Grishanin* et al. *(2005) lineages

To assign our samples and those of Grishanin *et al.* (2005), we compared European and American specimens belonging to the *A. vernalis* complex. According to the morphological description of Dodson *et al.* (2003) who used the same populations as Grishanin *et al.* (2005), we expected the designation of either *A. trajani* or *A. vernalis* in the Grishanin's study although they called them simply *A. vernalis* complex. The data from nuclear 18S rDNA phylogeny were in accordance with mitochondrial phylogeny of the *A. vernalis* morphotype clade C and D; however, only a limited number of sequences from Palearctic specimens were used. Dissimilarity of the Swiss specimen sequence (Vsu1), apparent from mitochondrial phylogeny, was documented here by formation of a subclade including two other genetically distant Nearctic populations (PA26, S102) (Fig. 2S). Dissimilarity of these specimens was reinforced by reproductive isolation apparent from crossbreeding experiments carried out by Grishanin *et al.* (2006), indicating species status different from other populations in *A. vernalis* morphotype clades.

A paleobiogeographic scenario of* Acanthocyclops *evolution in Europe

Divergence time estimates indicated that divergences among clades A–D took place 10–15 MYA. This may concur with the theory that Pleistocene glaciations provided increased opportunities for divergence of species (e.g. Caudill & Bucklin 2004; Mathews *et al.* 2008). More likely Miocene glaciations, playing an important role in the divergence of several freshwater species such as crayfish (Trontelj *et al.* 2005), isopod (Verovnik *et al.* 2005), and copepods (Rocha-Olivares *et al.* 2001; Thum & Harrison 2009) played a substantial role in the initial divergence of *Acanthocyclops* species. The last ice age most likely formed the current distribution of many species (e.g. Hewitt 2004). More precise calibration of molecular clocks is needed, based on analyses either of closer relatives or congeneric species. Without fossil calibration of molecular clocks, however, it is difficult to estimate precise time of species origin.

Is there an additional sibling species of* Acanthocyclops vernalis *in continental Europe?

The specimens morphologically determined as *A. vernalis* showed higher genetic diversity than previously described species. Moreover, on the bases of several lines of evidence, the phylogenetic tree based on the 12S rRNA gene fragment revealed the possible existence of two cryptic species complexes among the individuals identified as *A. vernalis* (Fig. 1A; clades C and D). The two mtDNA lineages in the *A. vernalis* morphotype did not group together in the phylogenetic analysis. The genetic divergences among these lineages do not overlap with those within the clades. In addition, each of the detected lineages contained two clades with high bootstrap support for both mitochondrial lineages (Fig. 1A). The level of sequence divergence between lineages C and D was at least 20%. This implies that: (i) the two lineages represent different species and (ii) the two species might not be sister taxa.

Additionally, the origin of populations from clade D is inter-connected water bodies such as ponds, pools, or rivers. On the other hand, the individuals in lineage C were found in isolated sites such as glacial lakes in Switzerland and Bulgaria (Table 1). The persistence of morphological uniformity disguising genetic divergence in Palearctic populations is most likely similar to that in Nearctic, North American specimens, in which Dodson *et al.* (2003) and Grishanin *et al.* (2005) claimed the existence of several cryptic lineages, based on reproductive isolation and different chromosome numbers. Yang *et al.* (2009) reported the chromosome number in *A. vernalis*, *A. einslei*, and *A. trajani*; however, their samples of *A. vernalis* from the vicinity of Oldenburg (Germany) did not include a sufficient number of populations to reflect possible diversity in chromosome numbers. We could presume existence of populations with different chromosome numbers, similar to North American populations within the *A. vernalis* species complex as reported by Grishanin *et al.* (2005). Generally speaking, the arguments mentioned above are commonly used as evidence of independent evolutionary history and specific status of lineages (Avise & Ball 1990).

Conclusions

In addition to morphological data, the analysis of mitochondrial DNA is a useful tool in distinguishing species, but neither alone can be guaranteed to identify all species. Current evidence shows that species that diverged several million years ago can closely resemble one another in morphology. Clear genetic differences among cryptic species allow species identification and, hence, the separation of intraspecific from interspecific morphological variation. In the present study, mitochondrial phylogenies and

morphological analysis of European populations of *A. trajani* and *A. einslei* were concordant and corroborated the existence of two distinct species. On the other hand, mtDNA sequences revealed hidden diversity among European populations of *A. vernalis*, which together with high sequence divergence suggests new cryptic species complexes among individuals designated as *A. vernalis*. Understanding whether the new cryptic species complexes identified in this work represent full species or intraspecific units will require further detailed study of species morphology and determination of morphological indices appropriate for species identification.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1A–B Drawings of the main morphological characteristics used in this study. Acronyms are defined in the text. A. third endopodite of fourth swimming leg. B. furcal rami with furcal setae. The drawing is representative of *A. einslei*.

Fig. S2 Phylogenetic relationships within *Acanthocyclops* based on nuclear 18S rDNA. Fifty percent majority-rule consensus tree of the Bayesian Inference (BI) shows relationship of the particular populations. The node support: bootstrap ML/MP/BI. Labelling of the main clades corresponds with phylogenetic tree based on 12S rRNA, (see Table 1 for the location of particular populations).

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CHAPTER 3

DESCRIPTIONS OF COPEPODID AND ADULT *Acanthocyclops trajani* (MIRABDULLAYEV & DEFAYE 2002) AND *A. einslei* (MIRABDULLAYEV & DEFAYE 2004) (Copepoda: Cyclopoida) WITH NOTES ON THEIR DISCRIMINATION

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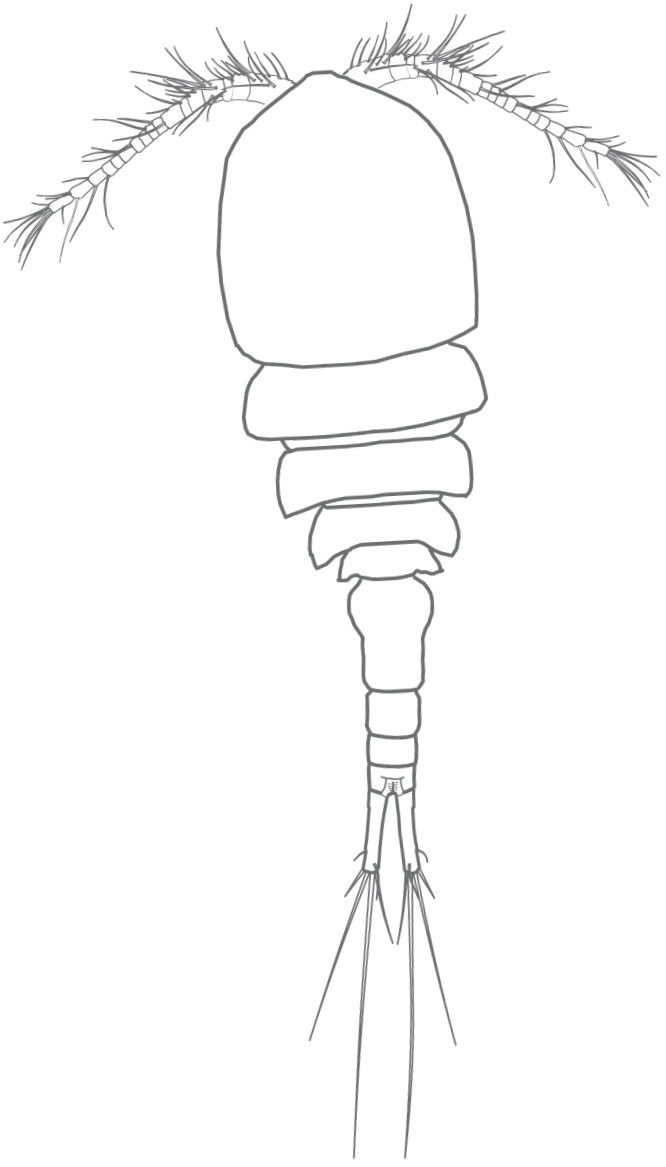

Fundam. Appl. Limnol., Arch. Hydrobiol. Vol. 177/3, 223–240 **Article**
Stuttgart, July 2010

Descriptions of copepodid and adult *Acanthocyclops trajani* (Mirabdullayev & Defaye 2002) and *A. einslei* (Mirabdullayev & Defaye 2004) (Copepoda: Cyclopoida) with notes on their discrimination

Martin Bláha[1]

With 15 figures and 2 tables

Abstract: The copepodid phases and adults of *Acanthocyclops trajani* and *Acanthocyclops einslei* were studied to record their distinguishing characteristics. Morphological examination showed that copepodids of both species were very similar, and showed an identical pattern of articulation and ornamentation of appendages and antennules. Differences in the distal endopodid of the fourth swimming leg and antennal ornamentation were found in later stage copepodids of both species. *A. trajani* had a higher ratio of apical spines on the distal endopodid of the fourth swimming leg compared to *A. einslei*, as well as a higher segment length/width ratio, and site of lateral spine insertion, which is more distal in *A. einslei* compare to *A. trajani*. Based on morphological descriptions of the copepodids of *A. trajani* and *A. einslei*, reported in this study, discrimination of later stage copepodids is feasible and may serve as a tool for basic ecological studies of zooplankton communities that is not dependent on the presence of adult specimens.

Key words: copepods, morphology, development, ornamentation.

Introduction

The life cycle of cyclopoid copepods includes two developmental phases: the naupliar phase with a phenotype distinct from the adult and the copepodid phase, which resembles adult forms (e.g. Ferrari & Dahms 2007). Developmental stages are generally present in the environment in larger numbers than are adults. Copepods are ubiquitous and often abundant in freshwater bodies, where they play an important role in freshwater food webs, supporting fish, larval insects, and other aquatic animals. Plankton community studies in the field are often hampered by inadequate knowledge of copepod developmental stages and the inability to assign copepodid or naupliar instars to species.

The recently described *Acanthocyclops trajani* and *A. einslei* that were removed from the *A. vernalis-robustus* species complex are common planktonic species occurring in fishponds, lakes, and reservoirs as well as pools and pond channels (Mirabdullayev & Defaye 2002, 2004). *A. trajani* was reported as typical for the pelagic zone of eutrophic aquatic ecosystems (Lescher-Motoué 1996, Caramujo & Boavida 1998), although in small waterbodies, such as fishponds, it is found together with *A. einslei*, which generally inhabits littoral zones (Mirabdullayev & Defaye 2004). Both species commonly coexist with other copepods, e.g. *Cyclops vicinus*, *Eucyclops serrulatus*, *Macrocyclops albidus*, *Megacyclops viridis*, and *Mesocyclops leuckartii* (Maier 1998, Frisch & Wohltmann 2005).

Author's address:

[1] University of South Bohemia in České Budějovice, Faculty of Fishery and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zátiší 728/II, 389 25 Vodňany, Czech Republic.

blaha@vurh.jcu.cz

www.schweizerbart.de
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The currently available keys to copepods are based on adult morphology, although naupliar and copepodid stages are more abundant than adults in aquatic ecosystems and comprise a substantial portion of the population. Distinguishing adults requires considerable practice and experience in working with copepods. There are dozens studies attempting to describe copepod developmental stages from the origins of copepodology (e.g. Ewers 1930, Dukina 1956, Dahms & Fernando 1993, 1995, Dahms et al. 2007, Ivanenko et al. 2008, Chullasorn et al. 2009), but there remains a lack of detailed descriptions of naupliar and copepodid phases. Previous studies of *A. robustus* dealt with *A. trajani* (e.g. Petkovski 1975, Purasjoki & Viljamaa 1984), and only two (Caramujo & Boavida 1998, Turki et al. 2002) studied naupliar and copepodid phases of this species.

The major aim of this study was to describe the morphology of copepodid developmental stages in *Acanthocyclops trajani* and *A. einslei* and to provide distinguishing characteristics together with notes for discriminating *A. trajani* and *A. einslei* from each other.

Material and methods

Adult females of *A. trajani* were collected from the Velká Outrata fishpond near the Research Institute of Fish Culture and Hydrobiology in Vodňany. Adult females of *A. einslei* were collected from the River Volyňka near the town of Strakonice. Ovigerous females were identified according to Mirabdullayev & Defaye (2002, 2004), and kept in 0.5 litre glass beakers at 18 °C in a light-dark cycle of 12:12 h until nauplii hatched, and then removed. Nauplii and copepodids were fed twice weekly with 2 ml of *Chlorella* sp. and *Paramecium* sp. culture, respectively.

Copepodids of various stages, and later adults, were taken from the culture, treated with lactic acid to clear non-exuvial material, and dissected in glycerol under a binocular microscope. Dissected appendages were immersed in a drop of glycerine on a cover slip for observation. Phase contrast photographs of each pair of swimming legs and other body parts were taken using an Olympus BX51 binocular microscope fitted with an Olympus E-510 digital camera. All drawings were made from digital photographs.

Body length was measured in dorsal view from the tip of the cephalothorax to the end of the furcal rami. Measurements were obtained using Quick PHOTO CAMERA 2.2 software (Olympus) from digital photography. At least ten individuals were measured for body length and five individuals for length ratios of swimming legs. All analyses were conducted using Statistica for Windows (StatSoft Inc.). The data are presented as mean ± SD.

Abbreviations used in text: A1 = antennule; C1–C5 = copepodid stages 1 to 5; P1–P4 = swimming legs 1 to 4; P5 = fifth leg; P6 = sixth leg; enp1–3 = endopodid of swimming leg; exp1–3 = exopodid of swimming leg; ae = aesthetasc.

Results

Description of *Acanthocyclops trajani* copepodids

Copepodid 1

The body comprises a four-segmented prosome and a single segment urosome. Mean body length was 398 ± 54 µm (n = 10) (Fig. 1A). Cephalothorax with

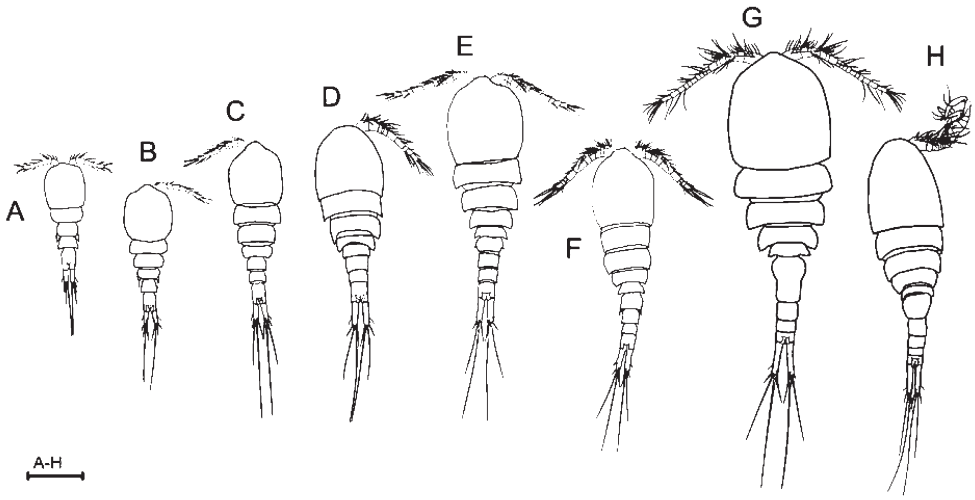

Fig. 1. *Acanthocyclops trajani*. General appearance of copepodid and adult instars. **A** – C1, **B** – C2, **C** – C3, **D** – C4; **E** – C5 ♀, **F** – C5 ♂, **G** – adult ♀, **H** – adult ♂ Scale bar: 200 µm.

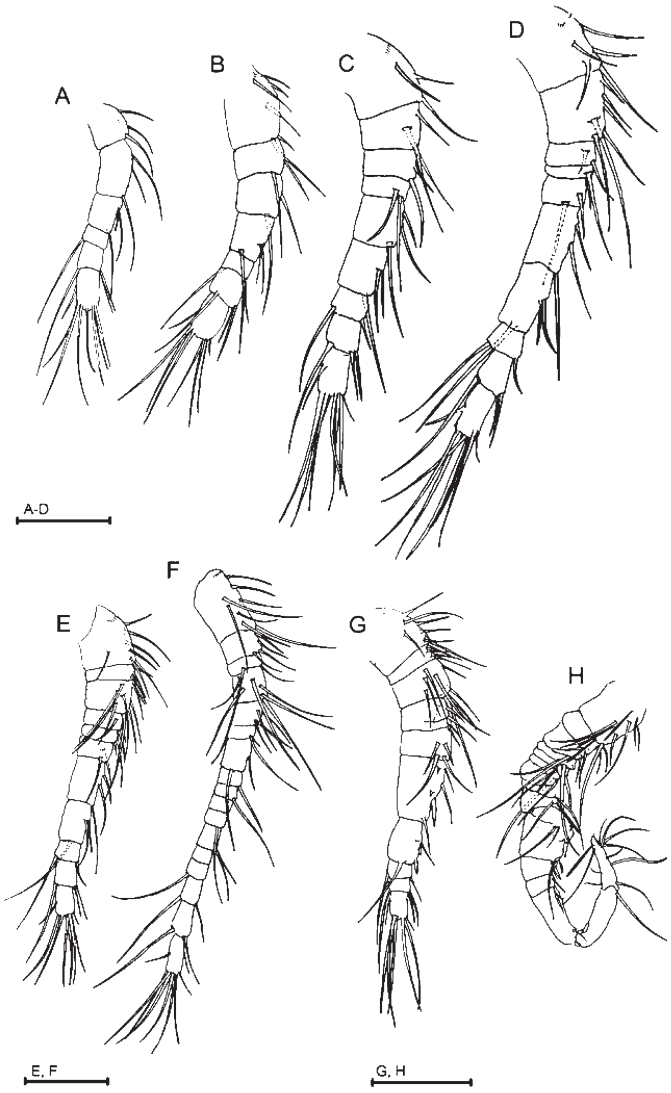

Fig. 2. *Acanthocyclops trajani*. Antennular development from C1 to adult. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀; **G** – C5 ♂; **H** – adult ♂. Scale bar: 50 µm. (A – D), 100 µm (E – H).

antennule, antenna, mandible, maxillule, maxilla, maxilliped and first pair of swimming legs. Second segment of the prosome bears the second pair of swimming legs. The third segment of the prosome bears outer long setae and an inner short spine at the posterolateral angle representing the third swimming leg. Ratio of length to width of the caudal rami is 2.5:1. Each ramus armed with six setae, the innermost and outermost plumose, and middle two are naked. Lateral setae located in centre of the caudal rami. Dorsal setae located distally in the posterior two thirds of the caudal rami.

Antennule six-segmented (Fig. 2A), with ornamentation as follows: 3, 3, 2 + ae, 1, 2 + ae, 7 + ae.

Antenna four-segmented (Fig. 3A). Basipodid bears two naked setae at the distal angle. Exopodid present as naked long seta. Endopodid comprises three segments, bearing one, four, and six distal setae. Terminal segment of the endopodid bears spinular row on inner margin in distal half of segment.

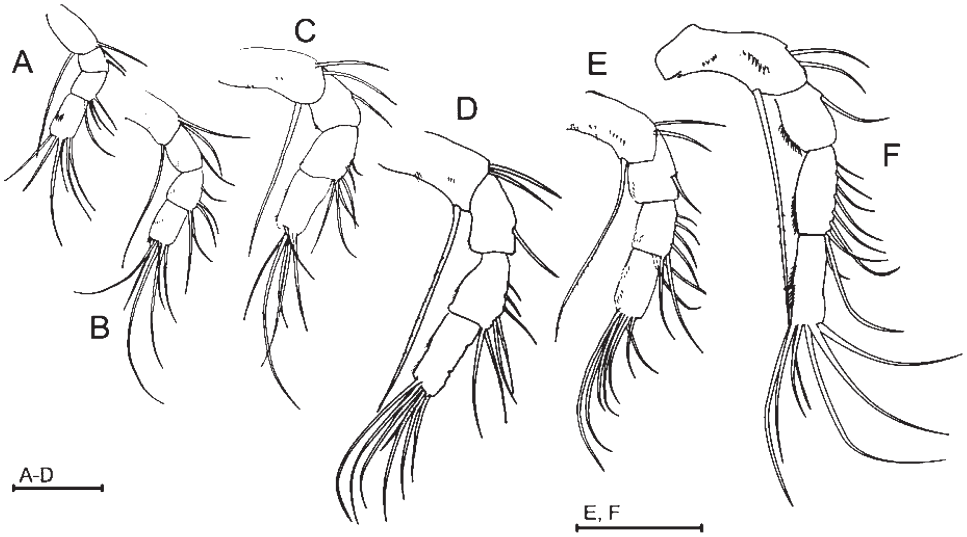

Fig. 3. *Acanthocyclops trajani.* Development of antenna from C1 to adult. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀ Scale bar: 50 µm.

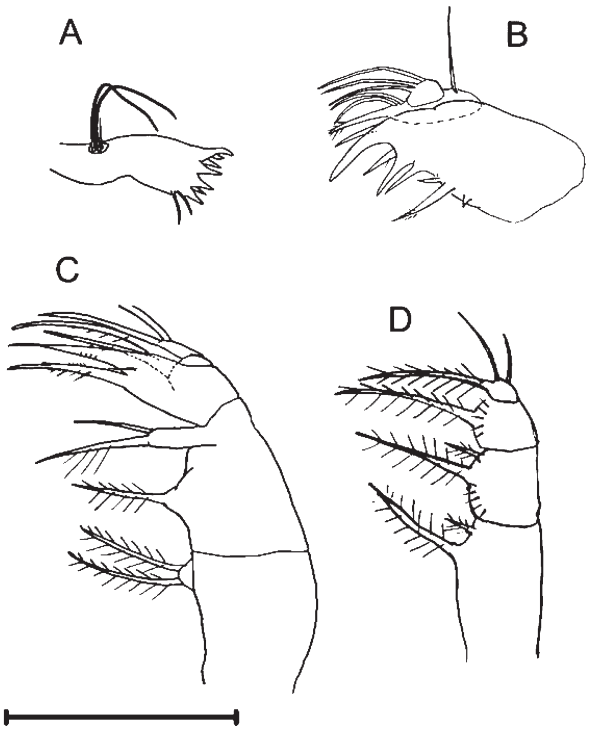

Fig. 4. *Acanthocyclops trajani*. Mouth parts of copepodid 1. **A** – mandible; **B** – maxillula; **C** – maxilla; **D** – maxilliped. Scale bar: 50 µm.

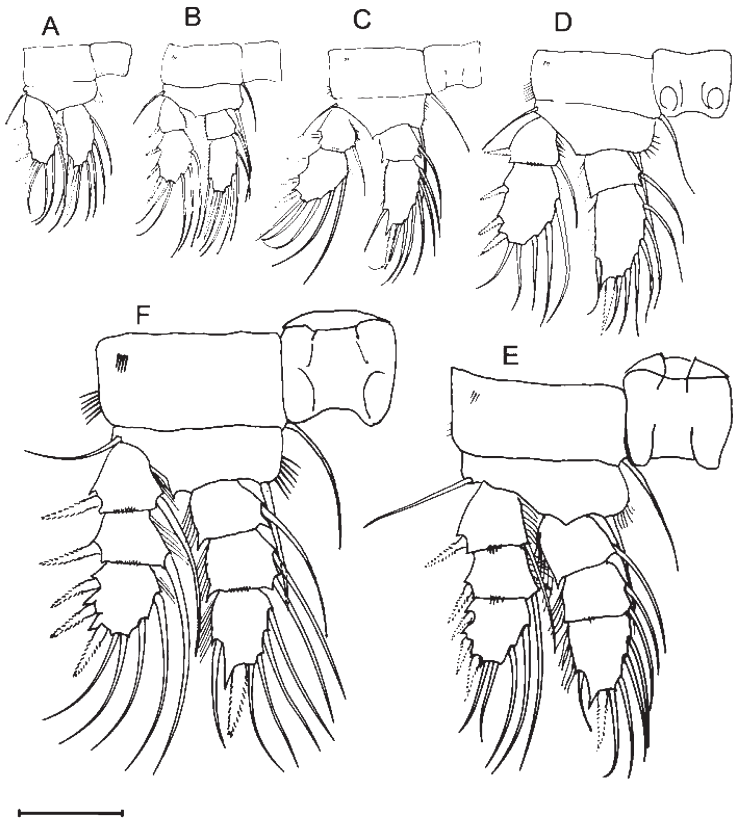

Fig. 5. *Acanthocyclops trajani*. Development of P1, swimming leg in posterior view. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀. Scale bar: 50 µm.

Mandible with well developed gnathobase bearing a row of six or seven sharp teeth and two setae at inner distal angle. Vestigial mandibular palp with two long setae and one naked distal seta (Fig. 4A).

Maxillule composed of strong praecoxa, coxa, and reduced two-segmented palp (Fig. 4B). Praecoxa with four spines and four setae articulating at base. Proximal segment of palp bears two naked setae and one spinulose seta on inner margin, plus outer seta representing exopod. Distal segment of palp represents endopod, armed with three naked setae.

Maxilla (Fig. 4C) comprises praecoxa, coxa, basipodid, and two-segmented endopodid. Praecoxa bears single endite with two spinulose setae. Coxa bears a long spinulose seta and a distal endite bearing subdistal long spinulose seta and distal claw-like seta. Basopodid is extended into a large distal endite bearing two claw-like setae and one fine seta subdistally. First endopodid bears two strong spinulose setae; second endopodid has one strong spinulose seta and two shorter setae.

Maxilliped (Fig. 4D) is well developed and distinctly four-segmented, comprising syncoxa, basipodid, and a two-segmented endopodid. Syncoxa and basipodid are each armed with two spinulose setae, the proximal much longer than the distal. Basipodid with row of 5 spinules near inner proximal angle. First endopodid armed with a spinulose seta and a row of 5 spinules along its inner margin. Second endopodid bears one long spinulose seta and two shorter apical setae. The length of the longer of the two shortest apical setae is twice that of the shortest seta.

Swimming legs P1 (Fig. 5A) and P2 (Fig. 6A) each with a two-segmented protopod. Intercoxal sclerite and coxopodid of P1 and P2 unornamented. Basipodid of both legs armed with fine spinules on inner margin,

Table 1. Armature and articulation of copepodid stages C1 – C3 of *A. trajani* and *A. einslei*.

Stage		C1	C2	C3
body somites		5	6	7
A1 segments		6	7	9
A2 (enp)		1; 4; 6	1; 5; 6	1; 6; 6
P1		one-segmented	two-segmented	two-segmented
	cox	0 – 0	0 – 1	0 – 1
	bas	1 – 0	1 – 1	1 – 1
	exp	IV, 2, 2	I–0; III, 2, 3	I–1; III,2,3
	enp	1, I–1, 4	0–1; 1, I–1, 4	0–1; 1, I–1,5
P2		one-segmented	two-segmented	two-segmented
	cox	0 – 0	0 – 1	0 – 1
	bas	1 – 0	1 – 0	1 – 0
	exp	III, I–1, 2	I–0; II,I–1, 3	I–1; III,I–1,4
	enp	1, I–1, 3	0–1; 1,I–1, 3	0–1; 1,I–1,4
P3		leg bud	one-segmented	two-segmented
	cox		0 – 0	0 – 1
	bas		1 – 0	1 – 0
	exp		III ,I–1,2	I – 0; II,I–1,3
	enp		1,I–1,3	0 – 1; 1,I–1,3
P4			leg bud	one-segmented
	cox			0 – 0
	bas			1 – 0
	exp			III,I–1,2
	enp			1,II,3

Spines are denoted by Roman numerals, setae by Arabic numerals. The armature formulae of the segments within a ramus are separated by semicolons. Pattern of description is based on Sewel (1949) ex. Huys & Boxshall (1991). cox – coxopodid; bas – basipodid; exp – exopodid; enp – endopodid.

and with one seta on external margin. Both legs composed of single segment rami. Row of long spinules at inner margin of exopodid and outer margin of endopodid. Ornamentation of swimming legs as in Table 1.

Copepodid 2

Mean body length 497 ± 64 µm (n = 10). Differing from C1 as follows: body six-segmented, comprising cephalothorax, three prosome somites and two urosome somites (Fig. 1B). Cephalothorax and next two urosome somites bear swimming legs.

Antennule seven-segmented with ornamentation as follows: 6, 2, 2, 2+ae, 2, 2+ae, 7+ae (Fig. 2B).

Antenna four-segmented (Fig. 3B). Basipodid armed with two short rows of small spines near the outer margin. Inner setae set close together at distal angle. Exopodid is represented by spinulose seta. First endopodal segment with small spinular row in inner margin. Second endopodal segment armed with five setae along inner margin and small spinular row near apical end of endopodid. Third endopodal segment has six setae distally, armed with two spinular rows, one on outer distal margin and second in the centre of the endopodal segment.

Coxopodid of P1–P3 bears small spinular row at outer proximal angle of caudal side, and spinulose seta at inner distal angle present in P1 and P2 (Figs 5B, 6B). Basipodid of P1–P3 bears row of fine spinules on apical inner edge and seta in outer margin as well. Basipodid of P1 armed with long spinulose seta at inner apical edge reaching half the length of second endopodid.

P1 and P2 with two-segmented exopodid and endopodid. Row of long setules presented along inner margin of exopodid and outer margin of endopodid. First endopodal and exopodal segment of P2 bear a posterior row of 5–6 spinules. Basipodid of P2 and P3 has spinular row on outer margin. P3 composed of two-segmented protopod, which bears a row of long setules along inner and outer margins of outer and inner segment, respectively (Fig. 7A). Ornamentation of swimming legs as in Table 1.

Copepodid 3

Mean body length 609 ± 78 µm (n = 10). Differing from C2 as follows: body seven-segmented, composed of five-segmented prosome bearing swimming legs 1 to 4, fifth leg P5 and two-segmented urosome (Fig. 1C).

Antennule nine-segmented with ornamentation as follows: 3, 3, 2, 2, 3, 2+ae, 2, 2+ae, 7+ae (Fig. 2C). First segment bears spinular row near outer margin.

Antenna four-segmented (Fig. 3C). Second endopodal segment armed with six setae along distal margin.

P1: coxopodid bears row of long spinules along outer margin. New seta present on inner margin of first expodal and second endopodal segment. First exopodal segment bears posterior spinular row (Fig. 5C). P2: exp1–2 and enp2 with new seta on inner margin, and exp2 with new spine on outer margin, (Fig. 6C). P3: exopodid and endopodid two-segmented. Exp1 and enp1 each bear a posterior spinular row. New seta appears at inner margin of coxopodid. Enp2 P2 and P3 outer apical spines are 0.81 ± 0.04 and 1.04 ± 0.04 times as long as segment, respectively (Fig. 7B). P4: with two-segmented protopod. In distal endopodid, ratio of inner apical spine to outer is 1.26 ± 0.07:1 and segment length to width ratio is 3.1:1. Coxopodid bears rows of shorter spinules near distal and proximal margins, and row of longer spinules at outer proximal angle on caudal side (Fig. 7F). P5 consisting of a single segment with outer long seta and inner spine (Fig. 8A). Ornamentation of swimming legs as in Table 1.

Copepodid 4

Mean body length 716 ± 88 µm (n = 10). Differing from C3 as follows: body eight segmented, composed

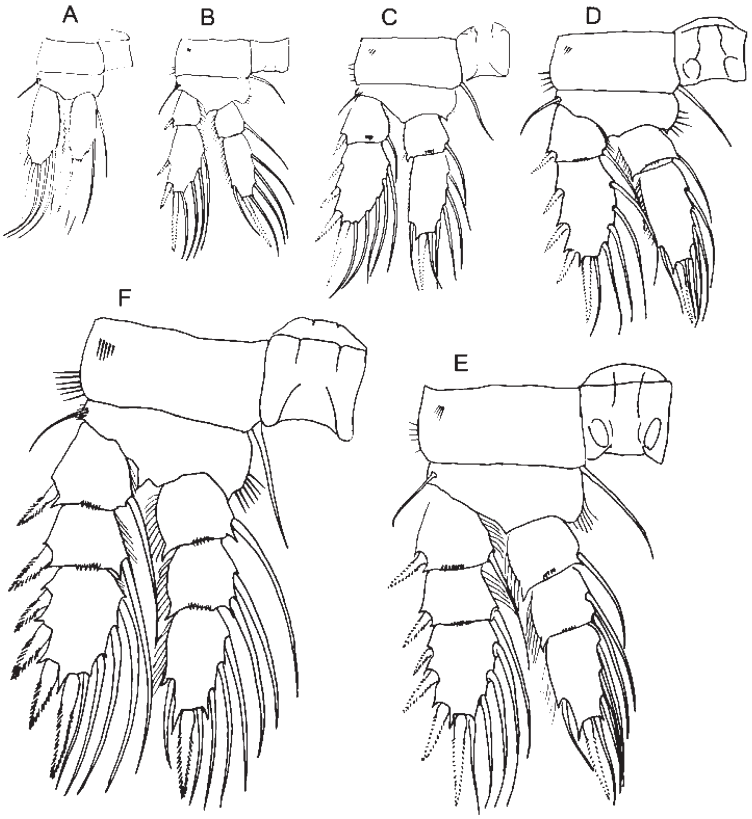

Fig. 6. *Acanthocyclops trajani*. Development of P2, swimming leg in posterior view. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀. Scale bar: 50 µm.

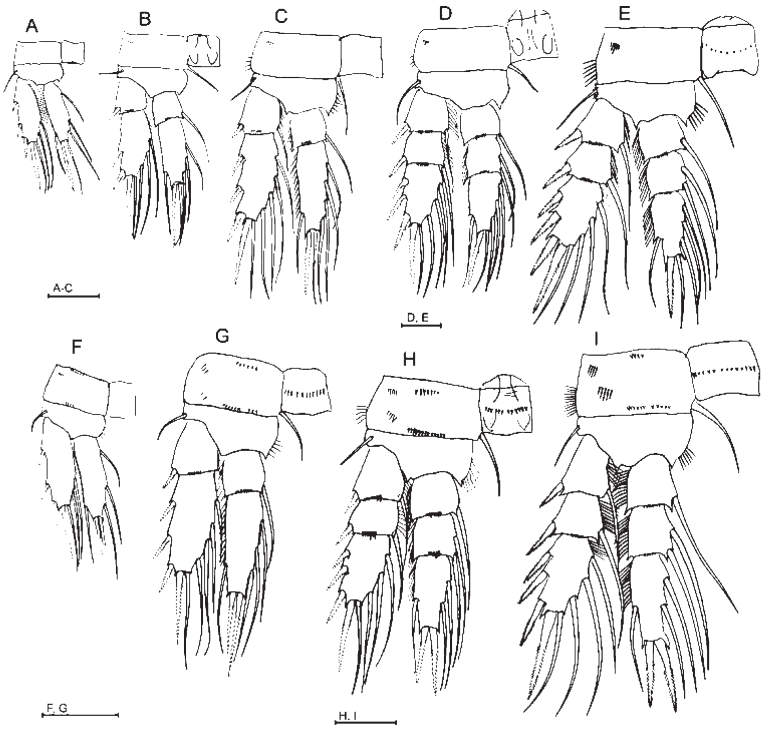

Fig. 7. *Acanthocyclops trajani*. Development of P3 (**A** – **E**) and P4 (**F** – **I**), swimming legs in posterior view. **A** – C2; **B** – C3; **C** – C4; **D** – C5 ♀; **E** – adult ♀; **F** – C3; **G** – C4; **H** – C5 ♀; **I** – adult ♀. Scale bar: 50 µm.

of five-segmented prosome bearing swimming legs 1 to 4, fifth leg P5, and three-segmented urosome (Fig. 1D).

Antennule ten-segmented with ornamentation as follows: 5, 6, 2, 2, 2, 3, 2+ae, 2, 2+ae, 7+ae (Fig. 2D). First segment bears spinular row near outer margin. Proximal, central and distal setae in sixth segment not reaching distal end of seventh, eighth and tenth segment, respectively. Outer distal seta in eighth segment reach distal end of tenth segment.

Antenna four-segmented (Fig. 3D). Both second and third endopodal segment armed with seven setae.

P1–P4 composed of two-segmented rami. P1 and P2 with the same ornamentation as in C3 (Figs 5D, 6D). P3: new seta appears in exp1–2 and enp2 on inner margin. New spine present on outer margin of exp2 segment. Coxopodid bears row of long spinules along outer margin (Fig. 7C). P4: coxopodid with new seta present at inner angle, and with rows of shorter spinules near distal and proximal margins. Two rows of thinner and longer spinules near lateral margin on caudal side. Intercoxal sclerite bears row of spinules on caudal side. First exopodal and endopodal segment bear posterior spinular row. Enp2 P4 inner apical spine is 1.26 ± 0.03 times as long as outer, and segment length to width ratio is 2.66 ± 0.11:1 (Fig. 7G). P5 two-segmented (Fig. 8B). Basal segment armed with long outer seta. Free segment armed with long apical seta and short subapical spine. P6 represented by outer long seta and inner spine reaching almost half of seta length (Fig. 8J). Ornamentation of swimming legs as in Table 1.

Copepodid 5 female

Mean body length 941 ± 137 µm (n = 10). Differing from copepodid 4 as follows: body nine-segmented, composed of five-segmented prosome bearing

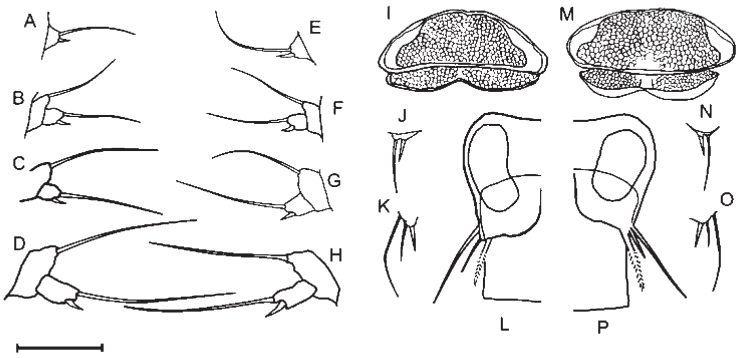

Fig. 8. *Acanthocyclops trajani* (**A** – **D**, **I** – **L**) and *A. einslei* (**E** – **H**, **M** – **P**). Receptaculum seminis, P5 and P6. **A**, **E** – P5 of C3; **B**, **F** – P5 of C4; **C**, **G** – P5 of C5 ♀; **D**, **H** – P5 of adult ♀; **I**, **M** – receptaculum seminis of adult ♀; **J**, **N** – P6 of C4; **K**, **O** – P6 of C5♂; **L**, **P** – two first abdominal somites of adult ♂. Scale bar: 50 µm.

swimming legs 1 to 4, and four-segmented urosome (Fig. 1E). Ratio of first abdominal segment width to height is 2:1.

Antennule 11-segmented with ornamentation as follows: 7, 4, 8, 3, 2, 2, 3, 2+ae, 2, 2+ae, 7+ae (Fig. 2E).

Antenna four-segmented (Fig. 3E). Basipodid bears four rows of small spines along outer margin. Second endopodal segment armed with eight setae along distal margin.

P1–P4 composed of three-segmented rami. P1: posterior spinular row is present in first two exopodal segments (Fig. 5E). P2–P4: enp1–2 and exp1–2 bear posterior spinular row (Figs 6E, 7D and H). P4: enp3 P4 inner apical spine length is 1.18 ± 0.02 times that of outer, and segment length to width ratio is 2.35 ± 0.15:1. Outer lateral seta inserted at 0.63 ± 0.03 of the segment length (Fig. 7H). P5 has the same pattern as in C4 (Fig. 8C). Ornamentation of swimming legs as in Table 2.

Copepodid 5 male

Mean body length 813 ± 121 µm (n = 10). Differed from female C5 as follows: First abdominal segment square, P6 at distal margin (Fig. 1F).

Antennule ten-segmented with ornamentation as follows: 7, 4, 8, 5, 3 + spine, 1 + ae + 2 spines, 2, 2+ae, 7+ae. Fourth segment is half divided (Fig. 2G).

Antenna four-segmented same as in female C5.

Ornamentation of appendages is as in female C5. P6 lateral, near distal margin of first urosomite dorsally, formed by longer seta, shorter seta, and short spine (Fig. 8K).

Description of *Acanthocyclops trajani* adults

Adult female

Mean body length 1194 ± 117 µm (n = 10). Body nine-segmented, comprising four-segmented prosome and five-segmented urosome (Fig. 1G).

Antennule 17-segmented reaching distal margin of first thoracic somite with setae ornamentation as follows: 8, 4, 2, 6, 4, 1+spine, 2, 1, 1, 0, 1, 1+ae, 0, 1, 2, 2+ae, 7+ae (Fig. 2F). Aesthetascs in twelfth segment pass through distal end of fourteenth segment.

Antenna four-segmented (Fig. 3F). Basipodid bears four rows of small spines along outer margin. Second endopodal segment bears nine setae along distal margin. All endopodal segments bear small spinular row along inner margin. Terminal endopodal segment armed with seven setae.

Labrum with 12 teeth. Maxillule as in Fig. 9A. Mandible as in Fig. 9B.

Maxilla (Fig. 9C) composed of praecoxa, coxa, basipodid, and two-segmented endopodid. Praecoxa bears single endite with two spinulose setae. Coxa composed of two endites, proximal with long spinulose seta and distal with claw-like seta distal and subdistal long spinulose seta. Basipodid is extended into a large distal endite bearing two claw-like setae and one fine seta subdistally. First endopoditd armed with two strong spinulose setae, second endopodid armed with one strong spinulose seta and two shorter setae.

Maxilliped (Fig. 9D) is well developed, composed of four segments comprising syncoxa, basipodid, and two-segmented endopodid. Syncoxa and basipodid armed with three and two spinulose setae, respective-

Table 2. Armature and articulation of copepodid stages C4, C5 and adult *A. trajani* and *A. einslei*.

Stage	C4		C5		Adult	
body somites	8		10♂ 9♀		10♂ 9♀	
A1 segments	10		10♂ / 11♀		17♀	
A2 (enp)	1; 7; 7		1; 8; 7		1; 8; 7	
P1	two-segmented		three-segmented		three-segmented	
cox	0 – 1		0 – 1		0 – 1	
bas	1 – 1		1 – 1		1 – 1	
exp	I–1; III,2,3		I–1; I–1; III,2,2		I–1; I–1; III,2,2	
enp	0–1; 1, I–1,5		0–1; 0–2; 1,I–1,3		0–1; 0–2; 1,I–1,3	
P2	two-segmented		three-segmented		Three-segmented	
cox	0 – 1		0 – 1		0 – 1	
bas	1 – 0		1 – 0		1 – 0	
exp	I–1; III,I–1,4		I–1; I–1; III,I–1,3		I–1; I–1; III,I–1,3	
enp	0–1; 1,I–1,4		0–1; 0–2; 1,I–1,3		0–1; 0–2; 1,I–1,3	
P3	two-segmented		three-segmented		Three-segmented	
cox	0 – 1		0 – 1		0 – 1	
bas	1 – 0		1 – 0		1 – 0	
exp	I – 1;III,I-1,4		I–1; I–1; III,I–1,3		I–1; I–1; III,I–1,3	
enp	0–1; 1,I–1,4	0–1; I,I–1,4	0–1; 0–2; 1,I–1,3	0–1; 0–2; I,I–1,3	0–1; 0–2; 1,I–1,3	0–1; 0–2; I,I–1,3
P4	two-segmented		three-segmented		three-segmented	
cox	0 – 1		0 – 1		0 – 1	
bas	1 – 0		1 – 0		1 – 0	
exp	I–0; III,I–1,4		I–1; I–1; III,I–1,3		I–1; I–1; III,I–1,3	
enp	0–1; 1,II,3	0–1; I,II,3	0–1; 0–2; 1,II,2	0–1; 0–2; I,II,2	0–1; 0–2; 1,II,2	0–1; 0–2; I,II,2
Species	*A. trajani*	*A. einslei*	*A. trajani*	*A. einslei*	*A. trajani*	*A. einslei*

Spines are denoted by Roman numerals, setae by Arabic numerals. The armature formulae of the segments within a ramus are separated by semicolons. Pattern of description is based on Sewel (1949) ex. Huys & Boxshall (1991). cox – coxopodid; bas – basipodid; exp – exopodid; enp – endopodid.

ly. Basipodid bears spinular row of five strong spinules near inner proximal angle. First endopodid armed with spinulose seta and row of four spinules along inner margin. Second endopodid armed with one long spinulose seta and two shorter apical setae. The length of the longer of the two shorter apical setae is twice that of the shortest seta.

Genital segment broadly rounded in anterior half, and cylindrical in posterior half. Receptaculum seminis is concave anteriorly, narrower posteriorly, notched in middle and joined to the anterior part (Fig. 8I).

P1–P4 composed of three-segmented rami. Coxopodid of P1–P4 bear small spinular row at outer proximal angle of caudal side, and are armed with spinulose seta at inner distal angle (Figs 5F, 6F, 7E and I). Coxopodid of P4 bears row of shorter spinules along distal and proximal margins and two rows of longer spinules near outer margin. Intercoxal sclerite of P3 and P4 bear row of spinules on caudal side. Basipodid of P1–P4 bear row of fine spinules in apical inner edge. Basipodid of P1 armed with long spinulose seta in inner apical edge and reaching one quarter of third endopodid. Enp3 P4: ratio of length to width 2.72 ± 0.29:1, ratio of inner apical spine to outer 1.18 ± 0.09:1 and 0.87 ± 0.07 the length of segment. Lateral seta/spine inserted at 0.61 ± 0.01 of the segment length. Ornamentation of swimming legs as in the Table 2. P5 two-segmented (Fig. 8C).

Furcal rami parallel, without hairs on the inner margin, 4.85:1 length to width ratio. Small spine present in first quarter, and lateral seta inserted in last quarter of furcal rami.

Adult male

Mean body length 921 ± 95 µm (n = 10). Body ten-segmented, composed of four-segmented prosome and six-segmented urosome (Fig. 1H).

Antennule as in Fig. 2H, number of segments is difficult to estimate due to medial segments which are only partly divided. Three aesthetascs present in first segment, one in fourth, ninth, fourteenth and distal segments. All aesthetascs have a chitinized base.

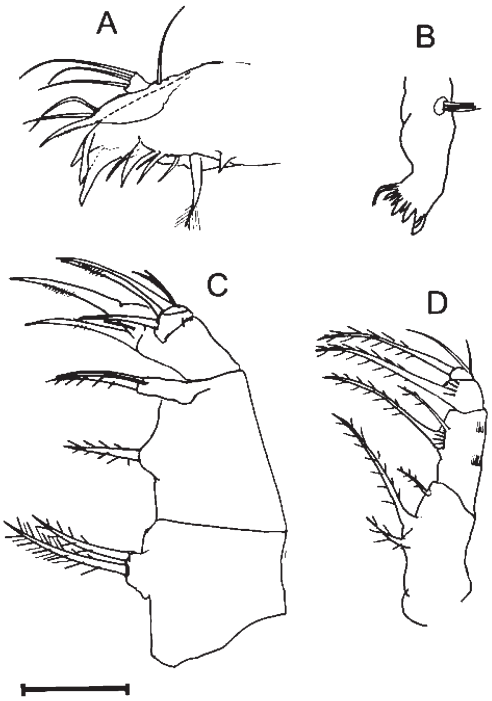

Fig. 9. *Acanthocyclops trajani*. Mouth parts of adult ♀. **A** – maxillula; **B** – mandible; **C** – maxilla; **D** – maxilliped. Scale bar: 50 µm.

Antenna four-segmented with the same pattern as in adult female.

Ornamentation of appendages is as in adult female. P6 as in Fig. 8 L.

Description of *Acanthocyclops einslei* copepodids

Copepodid 1

Mean body length 401 ± 69 µm (n = 10). Body is five-segmented, composed of four-segmented prosome and one segment urosome. Cephalothorax bears antennule, antenna, mandible, maxillule, maxilla, maxilliped and first pair of swimming legs. Second segment of prosome bears second pair of swimming legs. Third prosome bears outer long seta and inner short spine at posterolateral angle representing third swimming leg. Length to width ratio of caudal rami 2.8:1. Each ramus is armed with six setae. Innermost and outermost setae are plumose, two middle setae naked. Lateral seta near centre of caudal rami, dorsal seta in the distal two thirds of caudal rami.

Antennule six-segmented (Fig. 10A), ornamentation as follows: 3, 3, 2 + ae, 1, 2 + ae, 7 + ae.

Antenna four-segmented (Fig. 11A) with same shape as *A. trajani*.

Mandible, maxillule, maxilliped, and maxilla have same shape as *A. trajani* (Fig. 12A–D). The length of the shortest seta in distal endopodid of maxiliped is less than half that of the second longest apical seta.

Swimming legs P1 (Fig. 13A) and P2 (Fig. 14A) have the same pattern as in *A. trajani*. Ornamentation of swimming legs as in Table 1.

Copepodid 2

Mean body length 504 ± 85 µm (n = 10). Differing from C1 as follows: body six-segmented, composed of cephalothorax and five trunk somites. Cephalothorax and first two trunk somites bear swimming legs.

Antennule seven-segmented with ornamentation as follows: 6, 2, 2, 2+ae, 2, 2+ae, 7+ae (Fig. 10B).

Four-segmented antenna has same shape as *A. trajani* (Fig. 11B).

Coxopodid and basipodid of P1–P3 have the same pattern as *A. trajani*. Exopodid and endopodid of P1 and P2 two-segmented bearing row of long setules along inner and outer margins (Figs 13B, 14B). P2 enp1 and exp1 bear posterior spinular row. Basipodid of all legs have spinular row around the outer seta insertion. P3 composed of two-segmented protopod. Row of long setules present along inner margin of exopodite and outer margin of endopodid (Fig 15A). Ornamentation of swimming legs as in the Table 1.

Copepodid 3

Mean body length 621 ± 83 µm (n = 10). Differing from C2 as follows: body seven-segmented, composed of five-segmented prosome bearing swimming legs 1 to 4, fifth leg P5 and two-segmented urosome.

Antennule nine-segmented with ornamentation as follows: 3, 3, 2, 2, 3, 2+ae, 2, 2+ae, 7+ae (Fig. 10C).

Antenna four-segmented (Fig. 11C) has the same pattern as *A. trajani*.

P1, P2 and P3 have the same pattern as *A. trajani* (Figs 13C, 14C, 15B). Enp2 P2 and P3 outer apical spine are 0.64 ± 0.03 and 0.81 ± 0.07 length of segment, respectively. P4 and P5 have the same pattern as *A. trajani*, except: Ratio of enp P4 inner apical spine to outer is 1.12 ± 0.04:1, and length to width ratio of segment is 2.81 ± 0.16:1 (Fig. 15F). P5 one segmented with outer long seta and inner short spine (Fig. 8E). Ornamentation of swimming legs as in the Table 1.

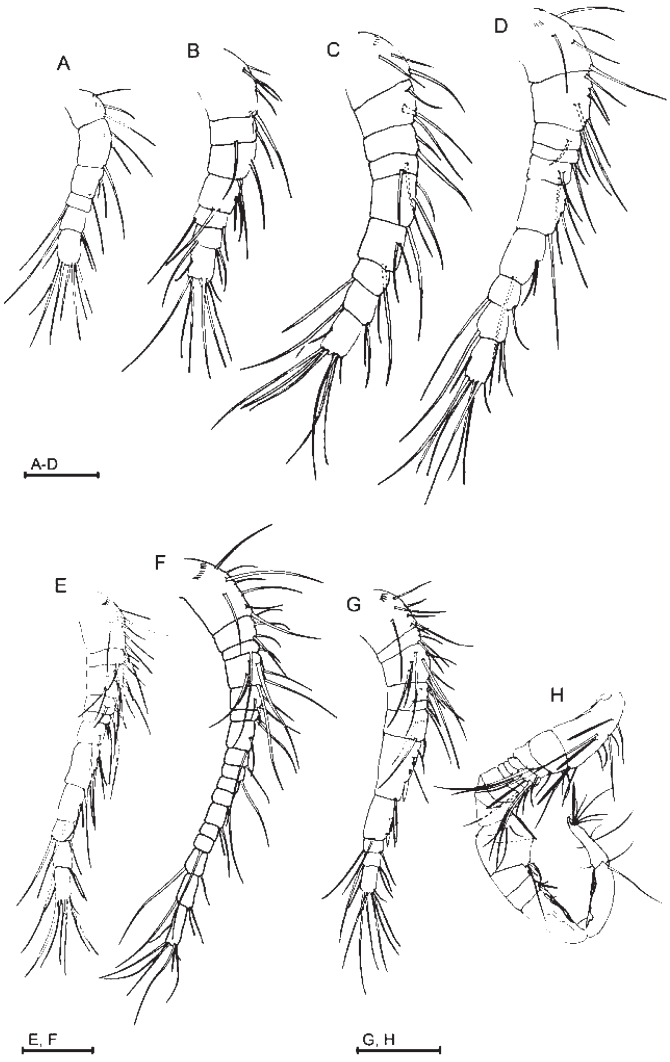

Fig. 10. *Acanthocyclops einslei*. Antennular development from C1 to adult. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀; **G** – C5 ♂; **H** – adult ♂. Scale bar: 50 µm (A – D), 100 µm (E – H).

Copepodid 4

Mean body length 792 ± 133 µm (n = 10). Differing from C3 as follows: body eight-segmented, composed of five-segmented prosome bearing swimming legs 1 to 4, and three-segmented urosome.

Antennule ten-segmented with ornamentation as follows: 5, 6, 2, 2, 2, 3, 2+ae, 2, 2+ae, 7+ae (Fig. 10D). Outer setae in sixth segment reach distal end of seventh, eighth, and tenth segment, respectively. Outer seta in eighth segment extends past distal end of tenth segment.

Antenna four-segmented (Fig. 11D)

P1–P4 have the same pattern as in *A. trajani* (Figs 13D, 14D, 15C, G), except: ratio of inner apical spine to outer is 1.12 ± 0.01:1 and distal segment is 2.70 ± 0.12 times as long as the distal segment (Fig. 15G). Outer lateral spine present in P3 and P4 distal segment. P5 two-segmented as in Fig. 8F. P6 represented by outer long seta and inner spine reaching one third of seta length (Fig. 8N). Ornamentation of swimming legs as in Table 2.

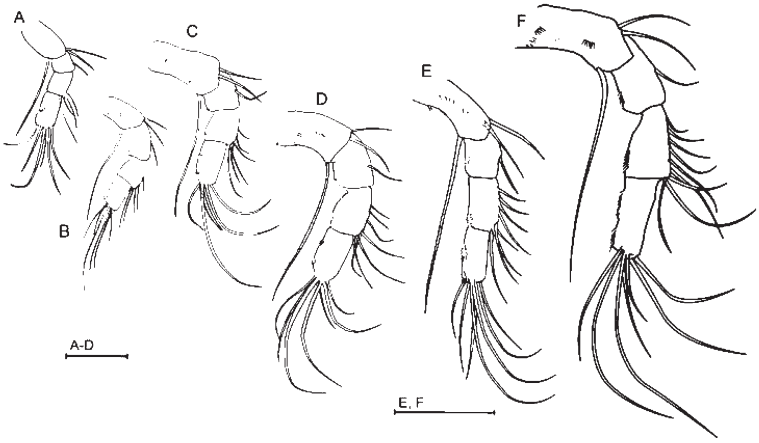

Fig. 11. *Acanthocyclops einslei.* Development of antenna from C1 to adult. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀ Scale bar: 50 µm.

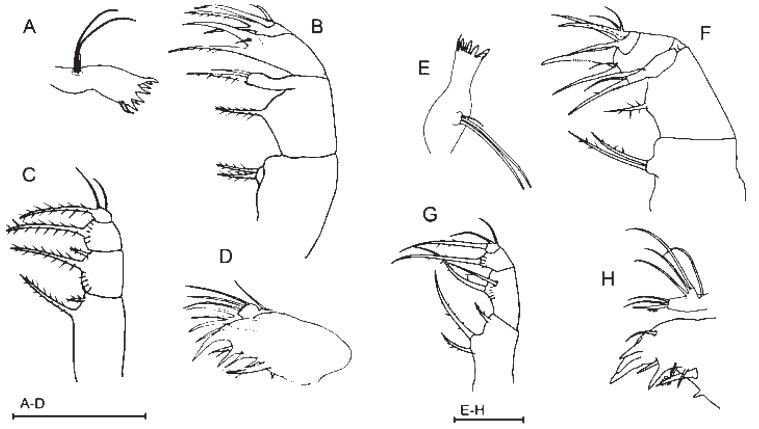

Fig. 12. *Acanthocyclops einslei*. Mouth parts of C1 (**A** – **D**) and adult ♀ (**E** – **H**). **A** – mandible; **B** – maxilla; **C** – maxilliped; **D** – maxillula ; **E** – mandible ; **F** – maxilla ; **G** – maxilliped; **H** – maxillula and maxillular palp. Scale bar: 50 µm.

Copepodid 5 female

Mean body length 1026 ± 138 µm (n = 10). Differing from copepodid 4 as follows: body nine-segmented, comprising five-segmented prosome bearing swimming legs 1 to 4 and four-segmented urosome. Width to height ratio of first abdominal segment 2:1. P6 near distal margin.

Antennule eleven-segmented with ornamentation as follows: 7, 4, 8, 3, 2, 2, 3, 2+ae, 2, 2+ae, 7+ae. In contrast to *A. trajani,* two most proximal setae in third segment do not reach distal end of sixth segment. The central seta and the most distal seta in the seventh segment extend beyond the distal end of ninth and eleventh segments, respectively (Fig. 10E).

Antenna four-segmented (Fig. 11E) has similar shape to *A. trajani*.

P1–P4 have the same pattern as *A. trajani* (Figs 13E, 14E, 15D and H), except the following: ratio of enp3 P4 inner apical spine to outer is 1.10 ± 0.05:1and length to width ratio of segment is 1.96 ± 0.02:1. Outer lateral spine inserted at 0.71 ± 0.01 of distal segment length. P5 has the same pattern as in C4 (Fig. 8G). Ornamentation of swimming legs as in Table 2.

Copepodid 5 male

Mean body length 913 ± 162 µm (n = 10). Differing from female copepodid 5 as follows: First abdominal segment square, P6 placed at distal margin.

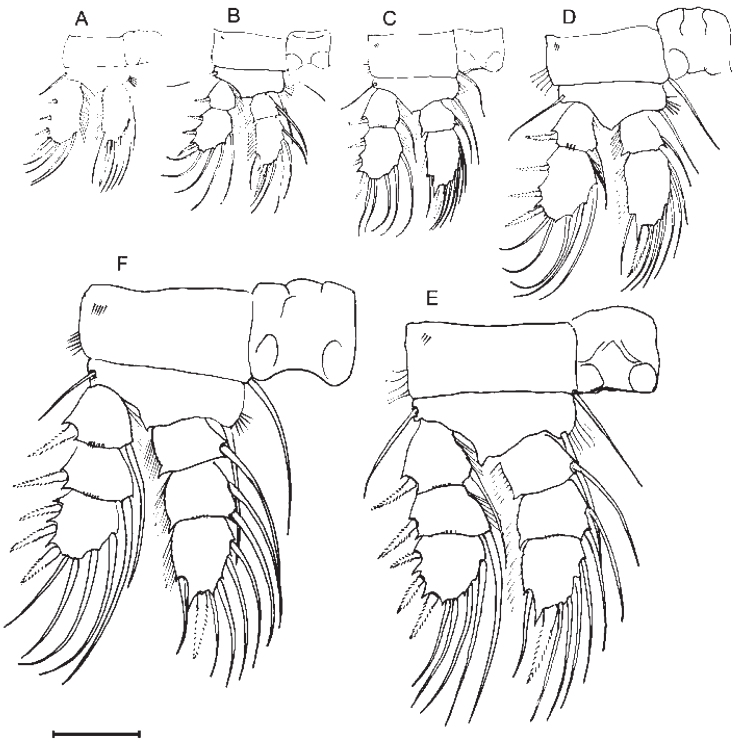

Fig. 13. *Acanthocyclops einslei*. Development of P1, swimming leg in posterior view. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀. Scale bar: 50 µm.

Antennule ten-segmented with ornamentation as follows: 7, 4, 8, 5, 3 + 3 spines, 1 + ae + 2 spines, 2, 2+ae, 7+ae (Fig. 10G).

Antenna four-segmented, has the same pattern as in female C5.

Appendages have the same pattern as in female C5. P6 as in Fig. 8O, central seta not longer than inner spine.

Description of *Acanthocyclops einslei* adult

Adult female

Mean body length 1353 ± 139 µm (n = 10). Body nine-segmented, composed of four-segmented prosome and five-segmented urosome.

Antennule 17-segmented, reaching distal margin of first thoracic somite with setae ornamentation as follows: 8, 4, 2, 6, 4, 1+spine, 2, 1, 1, 0, 1, 1+ae, 0, 1, 2, 2+ae, 7+ae. Aesthetascs in 12th segment reach distal end of 14th segment (Fig. 10F).

Four-segmented antenna (Fig. 11F) has the same shape as *A. trajani*.

Labrum has 12 teeth. Maxillule, mandible, maxilla, and maxilliped (Figs 12E–H) are as in *A. trajani*, except in the two most apical setae in maxilliped distal endopodid, the ratio of the longest naked seta to the shortest is 1.7:1 (Fig. 12G).

Genital segment broadly rounded in anterior half, and cylindrical in posterior half. Receptaculum seminis is more oval anteriorly than in *A. trajani* (Fig. 8M). Posteriorly narrower, notched in middle and tapered to blunt tip, not jointed to anterior.

P1–P4 as *A. trajani* (Figs 13F, 14F, 15E and I), with exception of P1–P3 intercoxal sclerites, which do not bear spinular row on caudal side. Enp3 P4: ratio of inner apical spine to outer is 1.03 ± 0.04:1, and length to width ratio of the segment is 2.50 ± 0.22:1. Lateral spine insertion at 0.76 ± 0.03 of segment length. Ornamentation of swimming legs as in the Table 1. P5 two-segmented (Fig. 8H).

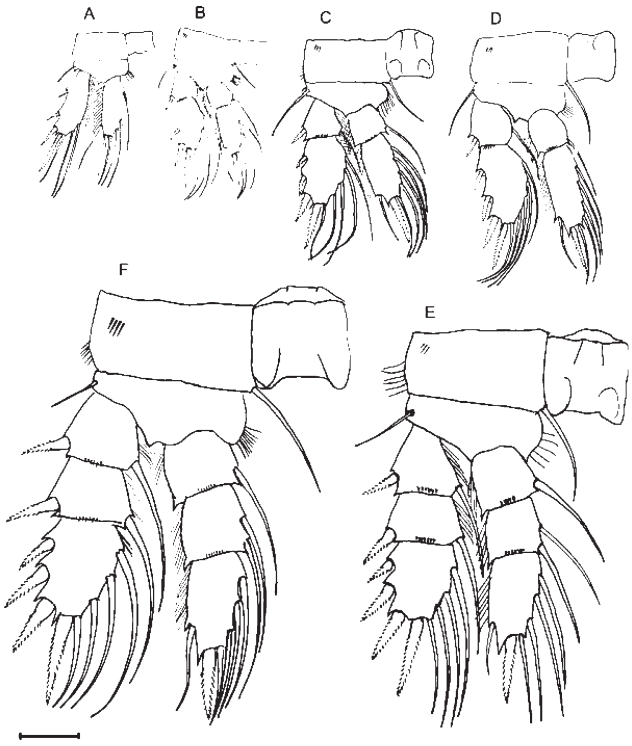

Fig. 14. *Acanthocyclops einslei*. Development of P2, swimming leg in posterior view. **A** – C1; **B** – C2; **C** – C3; **D** – C4; **E** – C5 ♀; **F** – adult ♀. Scale bar: 50 µm.

Furcal rami parallel without hairs on the inner margin, length/width ratio 5.18 ± 0.57:1. Small spine present in first quarter, and lateral seta inserted in last quarter of furcal rami. Ornamentation of swimming legs is shown in Table 2.

Adult male

Mean body length 1104 ± 94 µm (n = 10). Body ten-segmented, composed of four-segmented prosome and six-segmented urosome.

Antennule has the same pattern as *A. trajani* (Fig 10H.).

Antenna four-segmented with the same pattern as in adult female.

Ornamentation of appendages is as in adult female. P6, central seta reach mid length of inner spine (Fig. 8P)

Differential diagnoses

The main discriminating characters between both species studied are attributed to distal endopodid of P4. The ratio of inner and outer apical spine as well as length/width ratio of this segment is in *A. trajani* always higher than 1.1 and 2.3 (Fig. 8G–I), respectively. In contrast, in *A. einslei* this ratio is always lower than 1.1 and 2.5 (Fig. 12G–I), respectively. The site of outer lateral spine in distal endopodid of P4 is more distal in *A. einslei* (Fig. 12G–I) than that in *A. trajani* (Fig. 8G–I), where the seta or spine-like seta is placed more proximally, near the centre of the segment. An additional discriminating trait which is useful in males is the pattern of P6. *A. trajani* has middle seta in C5 which is always longer than the inner spine (Fig. 8K), whereas in *A. einslei* this seta is never longer than inner spine (Fig. 8O). In adult males of *A. trajani* the middle seta reaches approximately 80 % of outer seta (Fig. 8 L), whereas in *A. einslei* this seta reaches half of the length of inner spine (Fig. 8P).

Other differences can be found in the length of particular setae on the antennules. Proximal, central and distal setae in the antennular sixth segment doesn't reach the distal end of the seventh, eighth and tenth segment in C4 of *A. trajani*, whereas in *A. einslei*, the setae reach the distal end of these segments. Similarly, the outer distal seta in the eighth segment reaches the

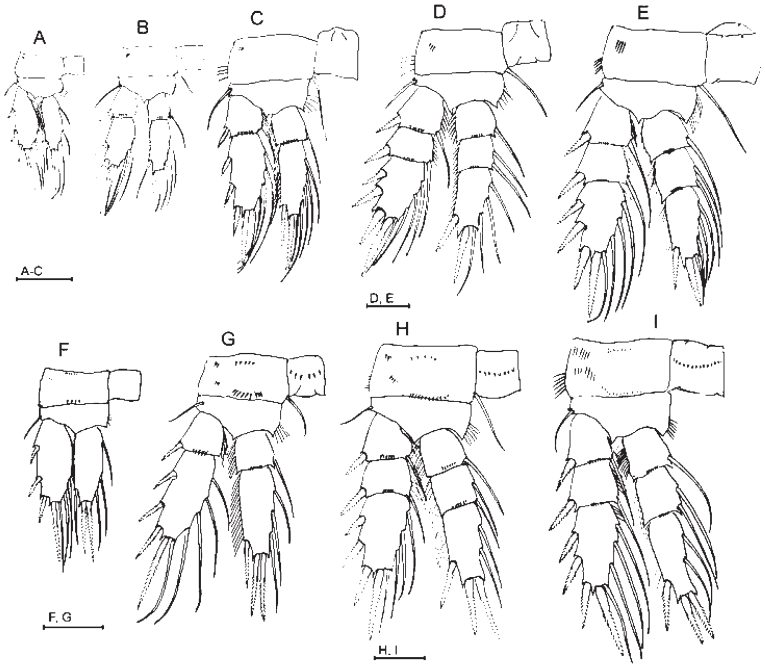

Fig. 15. *Acanthocyclops einslei*. Development of P3 (**A** – **E**) and P4 (**F** – **I**), swimming legs in posterior view. **A** – C2; **B** – C3; **C** – C4; **D** – C5 ♀; **E** – adult ♀; **F** – C3; **G** – C4; **H** – C5 ♀; **I** – adult ♀. Scale bar: 50 µm.

distal end of the tenth segment in *A. trajani*, whereas in *A. einslei* this seta extends to the distal end of the tenth segment (Figs 5D and 11D). In contrast to the antennule in the fifth copepodid stage of *A. trajani, A. einslei* has two most proximal setae in the third segment not reaching the distal end of the sixth segment. Similarly, in contrast to *A. trajani*, the central seta and the most distal seta in the seventh segment of *A. einslei* C5 female extend beyond the distal end of the ninth and eleventh segments, respectively (Figs 2E and 10E).

Discussion

Acanthocyclops Kiefer, 1927 is characterized by high intraspecific morphological, genetic and karyotype variability (e.g. Dodson et al. 2003, Grishanin et al. 2005, 2006, Yang et al. 2009, Blaha et al. 2010). In the present study I reported the detailed copepodid morphology of two recently described species, *A. trajani* and *A. einslei*. My morphological analysis showed the species to have similar patterns in articulation and ornamentation of appendages and antennules. However, several morphological characteristics may be used as discrimination markers. Specifically, major differences were seen in ratios of inner to outer apical spine in enp3 P4 as well as in site of lateral seta/spine insertion and length/width ratio of this segment in the final copepodid stages which correspond with patterns in adults of the species.

In adult females of *A. trajani*, aesthetascs in the twelfth segment of the antennule pass the distal end of the fourteenth segment, whereas, in *A. einslei*, they reach only to the distal end of the fourteenth segment. Similar observations were reported by Mirabdullayev & Defaye (2002, 2004). In contrast to the setae presence in laboratory cultured individuals, spine-like setae are frequently found in final copepodid stages and adults of *A. trajani* and *A. einslei* from wild populations. The spine-like setae are usually located in endopodites and/or exopodites of P3 and P4, as was reported for *A. robustus* (in fact *A. trajani*) by Lescher-Motoue (1996) and Dodson et al. (2003), and explained as consequences of environmental factors.

My observations, along with those of previous authors (e.g. Einsle 1989, Czaika 1982, Alekseev 2000), show that the discrimination of later *Acanthocyclops*

copepodid stages from other common pond or lake planktonic copepodids, at least to the correct genus, is feasible, usually according to segmentation of P5 and patterns of furcal ornamentation as well as ratios of furcal setae, and the size of copepodid stages of particular species. However, the size of copepods is primarily determined by the temperature and type of food available (Abdullahi 1992).

The morphology of copepodid phases of *A. trajani* has been partially described by Caramujo & Boavida (1998) and by Turki et al. (2002). However, in both studies the description was done under the synonym of *A. robustus*. The study by Turki et al. (2002) is more detailed. However, some differences in the description with the present study exist. My description diverges from Turki's as follows: copepodid 1 has four not three inner lateral setae in enp1 P1. Copepodid 2 has three not four setae in enp2 P2. Copepodid 3 has two not one apical seta in exp1 P1 and six not five setae in enp2 P1 (Fig.13C). Copepodid 4 has five not six setae in enp2 P2 (Fig. 14D). Some of these differences may have been caused by damage to setae during handling, since sites of missing seta insertion are recognizable. Additional differences were found in antennulary articulation and ornamentation, especially in male C5. Turki et al. (2002) described antennule to be composed of 11-segments, whereas in the present study, 10 segments were observed with fourth segments only half divided. Furthermore, I found spines localized in the sixth and seventh segments (Fig. 2G), which were not mentioned or depicted by Turki et al. (2002). On the other hand, the descriptions published by Caramujo & Boavida (1998) agree in most respects with mine. In addition, Caramujo & Boavida (1998) described the formation of P4 in the three latest copepodid stages as well as in the adult female. A similar formation of P4 development and ornamentation was observed in the present study.

The patterns of antennule segmentation and ornamentation were studied in 35 copepod species by Schutze et al. (2000). Their results showed that the main pattern investigated for the subfamily Cyclopinae was represented by *Cyclops* sp. However, remarkable differences in setation were observed for several species including *Acanthocyclops* having the same pattern, where five setae in the first segment of A1 in C3 are located. In contrast, the present study and the study of Turki et al. (2002) found in *A. trajani* only three setae there.

The general morphological pattern of copepodid swimming leg development is in accordance with the common pattern exhibited by 20 genera of cyclopoid copepods (Ferrari 1988), where the complete number of leg segments as well as ornamentation is present from C5 stage. The sexual dimorphism illustrated in this study is apparent from the same stage in size, ornamentation of antennula, number of abdominal segments, and shape of the first segment.

To conclude, my morphological analysis showed that copepodid morphology in the analysed species is remarkably similar overall and shows an identical pattern in articulation and ornamentation of appendages and antennules. However, visible differences in enp3 P4 and antennular ornamentation were found in the later stage copepodids.

Acknowledgements

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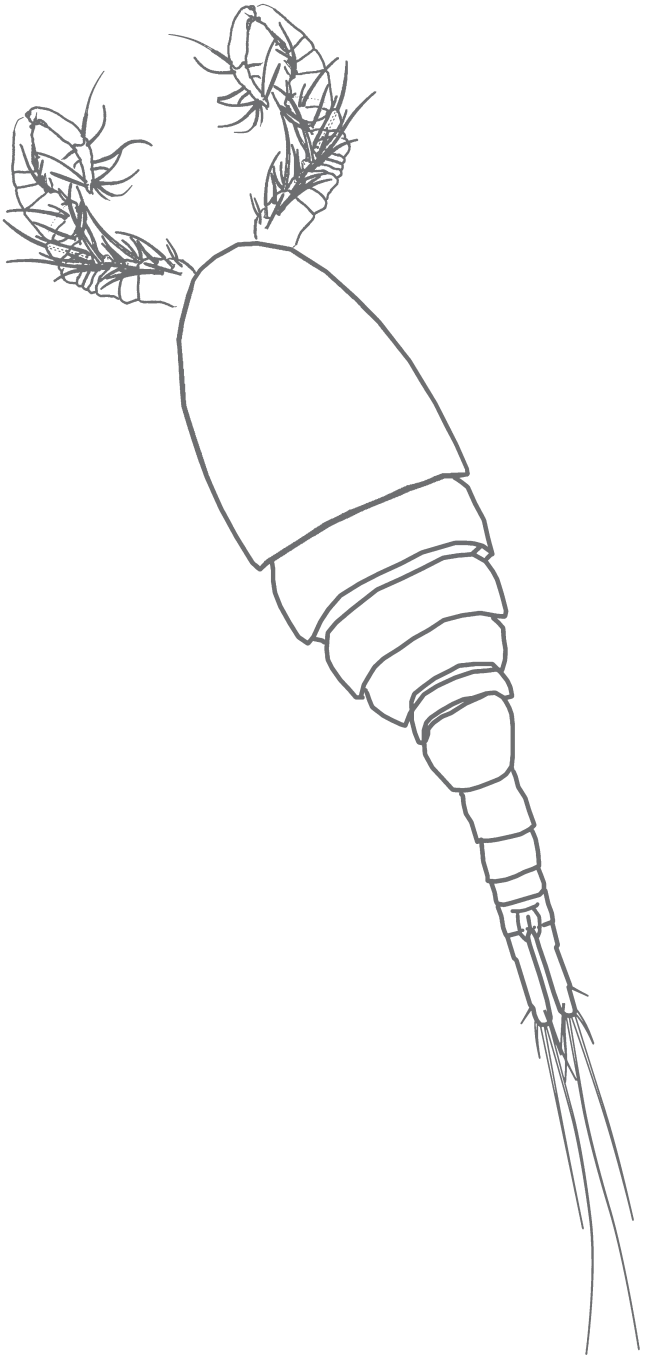
CHAPTER 4

GENERAL DISCUSSION • ENGLISH SUMMARY • CZECH SUMMARY • ACKNOWLEDGEMENTS • LIST OF PUBLICATIONS • TRAINING AND SUPERVISION PLAN DURING STUDY • CURRICULUM VITAE



GENERAL DISCUSSION

Naturally, copepod species determination is still based on morphology, applying different methods of analyses, from simple description to advanced statistical methods. Moreover, species determination is based on morphology of adult stages, mainly females however developmental stages prevail in environment through the year. However, advanced methods are highly desirable for analyses of morphologically complicated species complex as *Acanthocyclops vernalis-robustus* definitely is (Dodson et al., 2003; Bláha et al., 2010).

In the present thesis the precise morphological description of copepodid stages was realized. The morphological analyses of copepodid stages of *A. trajani* and *A. einslei* showed that copepodid morphology in the analysed species is remarkably similar overall and shows an identical pattern in articulation and armature of appendages and antennules. However, visible differences in enp3 P4 and antennal armature were found in the later stage copepodids. These differences correspond with morphology of adults, but due to high morphological plasticity it's difficult to determine them. But based on differences in enp3 P4, the proper determination is still possible.

The general morphological pattern of copepodid swimming leg development is in accordance with the common pattern exhibited by majority of cyclopoid copepods genera (Ferrari, 1988). The sexual dimorphism illustrated in this study is apparent from the same stage in size, armature of antennula, number of abdominal segments, and shape of the first segment. Although the morphology of copepodid phases of *A. trajani* has been partially described by Caramujo & Boavida (1998) and by Turki et al. (2002), in both studies the description was done under the synonym of *A. robustus*. The differences between present study and Turki's study may have been caused by damages of setae during handling, since sites of missing seta insertion are recognizable. Additional differences were also found in antennulary articulation and armature, especially in male C5. On the other hand, the descriptions published by Caramujo & Boavida (1998) are in good agreement with present study.

The discrimination of later *Acanthocyclops* copepodid stages from other common pond or lake planktonic copepodids, as well as assignment of concrete species to correct genus, is feasible as implying by other authors (e.g. Einslei, 1989; Czaika, 1982; Alekseev, 2000). Additionally, the short key provided in this study could be a helpful tool for basic ecological studies of zooplankton communities that is not dependent on adult presence and will deepen knowledge of relationships among copepod species during their development. Discrimination of *Acanthocyclops* species based on adult morphology is still confounded because of an apparent phenotypic plasticity of many traits. Therefore, in the study Bláha et al. (2010) we focused on characteristics of the distal endopodite of the fourth swimming leg (enp3 P4), proposed by Mirabdullayev and Defaye (2002, 2004) which seems to be most useful as an proper identification marker in *Acanthocyclops* taxonomy. Moreover these characteristics were for a long time neglected. So far, copepodologists focused mainly on count of setae or spines in exopodites or endopodites (Dodson, 1994; Dodson et al., 2003). In the study Bláha et al. (2010) the relative size of body parts was applied to eliminate the effect of body size, which is mainly influenced by environmental factors with remarkable consequences on intraspecific morphological variability among populations (Coker, 1932; Dodson et al., 2003). To test the independence of relative length (indices) to body size we have made regression analysis of indices to body length and analysis of simple length traits to body length (not shown in our study). Although we cannot say that indices are fully independent on body length, by using this relative size of body parts the influence of body size is markedly eliminated.

Because of it in the study Bláha et al. (2010) the combination of morphological and molecular markers was used to clarify whether the the phenotypic subdivision and morphological variability is related to genetic divergence. The data set obtained from the measurement of the fourth swimming leg (enp3 P4) were statistically processed using partial principal component analysis (pPCA). The pPCA analysis

was realised on data set gathered from 179 individuals that represent specimens from 22 European populations of three copepod species as follow: *A. trajani*, *A. einslei* and *A. vernalis*. The pPCA depicted the all specimens into three distinct clusters corresponding with the taxonomic status of the species analysed. In addition, pPCA analysis showed that the most species specific distinguishing characteristics (indices) are as followed: site of lateral seta insertion (Lo:L enp3 P4) unambiguously differentiates *A. einslei* from other *Acanthocyclops* species, and the ratio of the two apical spines in enp3 P4 (IAS:EAS) differentiates *A. trajani* from *A. vernalis*. Another useful trait discriminating *A. vernalis* from *A. trajani* and *A. einslei* is shape of genital double-segment. In *A. trajani* and *A. einslei* is broadly rounded in its anterior part whereas in *A. vernalis* extended into "blunt lobe" on either side as reported also by Kiefer and Fryer (1978) and Dodson (1994).

Analysis of mitochondrial 12S rRNA was firstly used for detection of phylogenetic relationships among analysed species especially cyclopoid copepod in general (Bláha et al., 2010). Other commonly used mitochondrial genes (16S rRNA, COI) however didn't provide sufficient results, that could be useful for population studies in calanoid copepods (Bucklin et al., 1999, 2003; Lee, 2000; Machida et al., 2004; Thums and Harrison, 2009), and other crustaceans (Adamowicz et al., 2008; Seidel et al., 2009; Filipová et al., 2010).

Taken together, the the taxonomy status of *A. trajani* and *A. einslei* was also supported by sequence analysis of mitochondrial DNA (mtDNA), where subsequent construction of phylogenetic trees using Bayesian Inference (BI) clearly depicted two major phylogenetic clades corresponding with the taxonomic status of both species. On the other hand the specimens morphologically determined as *A. vernalis*, were divided into two deeply divergent clades, based on mtDNA sequence divergences. The degree of interspecies sequence differences represent 20% between *A. trajani* and *A. einslei*. Moreover, the degree of intraspecific diversity observed for both species (3.3% and 2.5%, respectively) is similar to that of other crustaceans (King and Hanner, 1998; Petrusek et al., 2007; Thielsch et al., 2009). Molecular variance between *A. trajani* and *A. einslei* approached the minimum interspecific distances reported for other crustacean taxa (5.6–19.4%) (e.g. Petrusek et al., 2004; Parmakelis et al., 2008; Petrusek et al., 2008). Thus the observed sequence differences are clearly within the range of interspecific differences, while the sequence differences within lineages A (3.3%) and B (2.5%) were in the range of intraspecific variation. Moreover, the sequence divergence (20%) between *A. trajani* and *A. einslei* might arguably be substantial enough to indicate divergence into two biological species. Moreover, the sequence divergence (26%) between two clades in case of specimens morphologicaly determined as a *A. vernalis* indicated the existence of another species that may not be a sister taxon of *A. vernalis* s.s. Determining whether the lineages of the *A. vernalis* morphotype identified in this work represent full species or intraspecific units will require additional work that considers mating compatibility, gene flow at nuclear loci, and ecological and physiological divergence.

It is worth to be mentioned that, mating compatibility was also used to solve *Acanthocyclops* species complex several times (Lowndes, 1928; Price, 1958; Smith, 1981; Dodson et al., 2003; Grishanin et al., 2006). Because of it in the study Bláha et al. (2010) we have also compared partial sequence of one nuclear gene (18S rDNA) gathered from European representatives of *A. trajani* and *A. vernalis* with those sequences of 18S rDNA provided by Grishanin et al. (2005). Interestingly, our analysis clearly assigned Grishanins' sequences to European ones. In addition, Grishanin et al. (2006) reported one successful mating between populations S102 and S115, i.e. populations assigned by Bláha et al. (2010) to different species (*A. vernalis*, *A. trajani*, respectively) based on nuclear rDNA. Moreover, these populations have even different chromosome number, but still survive in laboratory culture for sixty generations. This is probably the only one recorded case of interspecific hybridization supported by rDNA sequences and chromosome number, although Smith (1981) reported also successful mating between "limnetica" female and "brevispinosus" male producing fertile F1, when inbred having "limnetica" phenotype.

Nevertheless, without precise determination of analysed species, results are difficult to interpret.

The situation in America, where took place all studies mentioned above, is different from European simply because of misidentification and confusion of several *Acanthocyclops* species (Smith, 1981; Dodson, 1994; Dodson et al., 2003; Grishanin et al., 2005, 2006) or due to existing reproductively incompatible populations representing really different species, undergoing morphological stasis, and possible to reveal only by molecular methods with proper markers. It is probably only question of time to reveal similar situation in Europe, depending on analyses of sufficient amount of populations and specimens such as apparent from study of Bláha et al. (2010) revealed new cryptic lineage within *A. vernalis*.

CONCLUSIONS

We can conclude that characteristics of the distal endopodite of the fourth swimming leg seems to be most useful as an proper identification marker in *Acanthocyclops* taxonomy, useful for distinguishing of older copepodid stages as well. Based on molecular markers the taxonomic status of two recently described species (*A. trajani* and *A. einslei*) was corroborated, however showed hidden diversity within species determined as *A. vernalis* and so far undistinguishable from each other morphologicaly.

The situation within *Acanthocyclops vernalis-robustus* species complex was for a long time full of dark corners, which time to time revealed difficultly its secrets. Luckily, the effort was not useless and copepodologists such as S.I. Dodson, I.M. Mirabdullayev and D. Defaye revealed new species and improve morphological discrimination within such a complicated genus. Additionally, with using molecular and cytogenetic methods, the knowledge about particular species was further deepened mainly due to A.K. Grishanin. Author of this thesis believes that also his contribution into *Acanthocyclops* problems was not useless and brings interesting remarks, which can provoke for further work within such a fascinating animals group.

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ENGLISH SUMMARY

Molecular and morphological aspects within *Acanthocyclops* Kiefer, 1927

Martin Bláha

This study used basic description as well as advanced methods in morphological analyses of newly described species of copepod genus *Acanthocyclops* Kiefer, 1927. Together with advanced morphological methods, molecular study was consider as a very useful tool for analysing specimens from different populations of three European *Acanthocyclops* species, namely *A. trajani*, *A. einslei* and *A. vernalis*.

The copepodid phases and adults of *Acanthocyclops trajani* and *Acanthocyclops einslei* were studied to record their distinguishing characteristics. Detailed morphological examination showed that copepodids of both species were very similar, and showed an identical pattern of articulation and armature of appendages and antennules. Differences in the distal endopodid of the fourth swimming leg as well as in antennal armature were found in later stage copepodids of both species. *A. trajani* had a higher ratio of apical spines on the distal endopodid of the fourth swimming leg compared to *A. einslei*, as well as a higher segment length/width ratio. Based on morphological descriptions of the copepodids of *A. trajani* and *A. einslei*, reported in this study, and also on published descriptions of other common pond and lake copepod species (i.e. *Megacyclops*, *Cyclops*, *Mesocyclops*, *Macrocyclops*), discrimination by genus of later stage copepodids as feasible and may serves as a tool for basic ecological studies of zooplankton communities that is not dependent on the presence of adult specimens.

Morphology in andults of *Acanthocyclops* species is confounded by an apparent morphological plasticity and in general, morphological traits are highly variable, and morphology is too constrained to give complete information of phylogenetic relationships. Our study combined morphological and molecular techniques to investigate the taxonomic and phylogenetic relationships of three species of *Acanthocyclops* (*Acanthocyclops trajani*, *Acanthocyclops einslei* and *Acanthocyclops vernalis*) inhabiting continental Europe. Morphological indices subjected to partial principal component analysis (pPCA) separated sample populations into three distinct clusters corresponding with the taxonomic status of the species analysed. In addition, the taxonomy status of *A. trajani* and *A. einslei* was in agreement with molecular data; however, the intraspecific variation in sequences of 12S rRNA was lower in contrast to specimens morphologically determined as *A. vernalis*, which were divided into two deeply divergent clades, based on mtDNA sequence divergences. Moreover, high sequence divergence (26%) between these clades indicated the existence of another species that may not be a sister taxon of *A. vernalis* s.s.

Results in our study point to the need for further taxonomic work on *Acanthocyclops*, considering detailed morphology of sister species of *A. vernalis* as well as extension of molecular analyses to other not only European *Acanthocyclops* species.

CZECH SUMMARY

Molekulární a morfologické aspekty v rámci rodu *Acanthocyclops* Kiefer, 1927

Martin Bláha

Předkládaná práce využívá ke studiu buchanek rodu *Acanthocyclops* Kiefer, 1927 základní morfologické metody popisu, ale také pokročilé metody morfologické analýzy. Ty, společně s molekulárními metodami představují velmi užitečné nástroje při analýze jedinců z rozdílných populací tří evropských druhů buchanek rodu *Acanthocyclops*, jmenovitě *A. trajani*, *A. einslei* and *A. vernalis*.

Morfologický popis kopepoditových stádií a dospělců druhů *Acanthocyclops trajani* a *Acanthocyclops einslei* byl realizován za účelem nalézt druhově specifické rozdíly u vývojových stádií. Detailní morfologická analýza ukázala, že morfologie kopepoditů obou dvou druhů je velmi podobná. I přesto byly nalezeny rozdíly u starších kopepoditů, a to v otrnění a obrvení antenuly, stejně tak i v morfologii distálního endopoditu čtvrté plovací nožky. Druh *A. trajani* vykazuje vyšší poměr apikálních trnů na tomto článku, stejně tak i poměr šířky a délky je u tohoto druhu vyšší než u druhu *A. einslei*. Na základě prezentovaného popisu kopepoditových stádií obou druhů, a také na základě již publikovaných dat o morfologii vývojových stádií dalších druhů buchanek (rody *Megacyclops*, *Cyclops*, *Mesocyclops*, *Macrocyclops*), je možné od sebe starší kopepoditová stádia odlišit. To by mělo napomoci při ekologických studiích planktonních společenstev, které nemusí být odkázány pouze na přítomnost dospělých stádií, tedy stádií na které jsou všechny determinační klíče orientovány.

Morfologie dospělců buchanek v rámci rodu *Acanthocyclops* je zastřena značnou morfologickou plasticitou a variabilitou jednotlivých znaků. Tím pádem je na základě morfologie složité usuzovat na phylogenetické vztahy mezi jednotlivými druhy. Naše studie kombinovala morfologické a molekulární metody za účelem popsání taxonomických a fylogenetických vztahů tří druhů v rámci rodu, druhů A*canthocyclops trajani*, *A. einslei* a *A. vernalis* obývajích evropské kontinentální vody. Vybrané morfologické indexy byly analyzovány pomocí diskriminační analýzy (pPCA), která rozdělila analyzované populace tří jmenovaných druhů do tří klastrů. Tyto klastry odpovídaly jejich současnému taxonomickému statusu. Navíc, taxonomický status odpovídal také výstupům z molekulární analýzy mitochondriálního genu pro ribozomální subjednotku 12S rRNA. Variabilita sekvencí byla u druhů *A. trajani* a *A. einslei* výrazně nižší než u druhu určeného jako *A. vernalis*. Populace tohoto druhu byly na základě molekulární analýzy rozděleny do dvou kládů, které se od sebe lišili ve 26 % analyzovaných sekvencí. Takto vysoká divergence naznačuje existenci dalšího druhu, který by nemusel být dokonce ani sesterským druhem druhu *A. vernalis*.

Výsledky předkládané v této práci naznačují potřebu další intenzivní taxonomické práce v rámci tohoto rodu, zahrnující jak detailní morfologické analýzy nově zjištěného sesterského druhu *A. vernalis*, tak také rozšíření molekulárních analýz na další druhy rodu *Acanthocyclops*.

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4. 047/2010/Z – Breeding and environmental aspects of aquaculture and hydrocoenoses. (2010–2012, leader Assoc. Prof. Dipl.-Ing. Martin Flajšhans, Dr.rer.agr.)

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TRAINING AND SUPERVISION PLAN DURING STUDY

Name	Martin Bláha		
Research department	Ethology and Nutrition of Fish and Crayfish		
Supervisor	Prof. MSc. Zdeněk Brandl, CSc.		
Period	1st October 2006 until 24th of September 2010		
Ph.D. courses			**Year**
Biostatistics			2007
English			2008
Applied hydrobiology			2008
Fish genetics			2008
Fish ecology			2010
Technical English			2010
Scientific seminars			**Year**
Seminar days of RIFCH			2007–10
UNESCO International postgradual Training Course on Limnology			2008–9
International conferences			**Year**
15. konference České Limnologické Společnosti a Slovenskej Limnologickéj Spoločnosti, Třeboň, 22.–26. června, 2009.			2009
Diversification in Inland Finfish Aquaculture, Písek, 16th-18th May 2011.			2011
Foreign stays during Ph.D. study at RIFCH		**Term**	**Year**
Kenyan Marine Fishery Research Institute, Kisumu, Kenya, Sustainability of fishery in Turkana Lake, Kalokol and Longalani, Kenya.		3 weeks	2008
Dr. Jose Martin Gallardo, Universidad de Extremadura, Badajoz, Spain. (Plankton diversity study, teaching assistance)		1 month	2008

CURRICULUM VITAE

PERSONAL INFORMATION

Surname:	**Bláha**
First name:	**Martin**
Title:	**Dipl.-Ing.**
Born:	15th April, 1980 in Strakonice
Nationality:	Czech
Marital Status:	Single

EDUCATION

1998–2001	DiS. – College of Water Management and Ecology, Vodňany
2001–2006	Dipl.-Ing. – University of South Bohemia, Agricultural Faculty, České Budějovice, specialization fishery

PROFESSIONAL EXPERIENCE

2006–2010	Ph.D. student USB RIFCH, Laboratory of Ethology and Nutrition of Fish and Crayfish

PH.D. COURSES

Biostatistic, Applied hydrobiology, Fish genetics, Fish ecology, English language, Technical English

SPECIALIZATION

Hydrobiology, fish feeding biology

KNOWLEDGE OF LANGUAGES

English (fluent), Spanish (well)

FOREIGN STAYS DURING PH.D. STUDY AT RIFCH

2008	Cooperation with Kenyan Marine Fishery Research Institute, Kisumu, Kenya, Sustainability of fishery in Turkana Lake
2008	University of Extramadura, Badajoz, Spain

PEDAGOGICAL EXPERIENCES

Consultant of one diploma thesis, leader of two bachelor thesis, Seminar days at USB RIFCH (2007, 2008, 2009, 2010), seminars on the topic applied hydrobiology for the Summer school 2010

Leading of students in UNESCO International postgradual Training Course on Limnology, Třeboň, Botanical Institute, ASCR (2008, 2009)

Teaching of subject Fishery hydrobiology (2009, 2010) and Zoology (2011)

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