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Confidential

Review of PhD Thesis

Name of the PhD student: Ing. Martin Bláha	Name of supervisor: **prof. Zdeněk Brandl CSc.**
Title of PhD thesis: Molecular and morphological aspects within *Acanthocyclops* Kiefer, 1927	

REVIEWER:

Surname: **Jan**	Institution: Dept. of Ecology, Faculty of Science, Charles University in Prague
Name: **Fott**	
Titles: RNDr, CSc.	E-mail: **fott@natur.cuni.cz**
Please describe your professional relationship to the PhD student: No professional relationship	Please describe your field of expertise: Limnology, Plankton, Copepoda

QUESTIONNAIRE

Originality, scientific importance, prospects of the PhD thesis and benefits for basic or applied research

Evaluate its competitiveness in the international context and compare its level with the current state of the art in the field:

While molecular methods have been used recently in taxonomy of planktonic Crustacea (e.g. cladocerans, marine copepods), freshwater copepods of the family Cyclopidae make an exception in this field. *Acanthocyclops* are common and sometimes dominant elements of plankton, littoral and even astatic waters in the northern temperate zone. Morphological divergence among morphotypes described so far is low and opinions on their taxonomical position have been conflicting. The present dissertation is, to my knowledge, the first attempt to delimit species within *Acanthocyclops* by combination of a cladistic approach based on mitochondrial gene analyses with a morphological approach. The study on discrimination of copepodid stages is novel as well.

The outputs of the study contribute to basic research in the fields of taxonomy and phylogeny of Copepoda, and ecology of freshwater communities.

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Preparation of the PhD thesis, targets of the work and deliverables
Evaluate the overall level of preparation of the PhD thesis and the originality of the selected approaches; evaluate publications and whether the targets set in the PhD thesis correspond with the declared purpose of the thesis:

The thesis consists of general introduction, two publications representing the main part of the thesis, general discussion and conclusions. Each part is accompanied by a list of references. The publiations are:

(1) 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.
By means of analyzing mitochondrial DNA the authors were able to distinguish four main clades in the *Acanthocyclops* species complex. The clades A and B correspond well to the morphological species *A. trajani* and *A. einslei*, respectively. The clades C and D correspond both to the present morphotype *A. vernalis*, as it is defined by the shape of the genital double-somite, by length proportions of the furca, and length proportions of the distal segment of the fourth endopodite. The morphological differences among the morphotypes *A. trajani*, *A. einslei* and *A. vernalis* are well documented in the Fig. 2 and Table 2.
The study brings an independent confirmation of validity of the species *A. trajani* and *A. einslei*.
The question, whether the clades C and D represent two distinct species within the *Acanthocyclops* complex or not, is open to further studies based on morphology, cytology, molecular genetics and cross-breeding.
Being not an expert in molecular biology, I evaluate here rather the conclusions and their importance for taxonomy, than the methods used in the study. According to my opinion the paper brings a new insight into phylogeny and taxonomy of the cyclopoid genus *Acanthocyclops*.

(2) Bláha M: Description of copepodid and adult *Acanthocyclops traiani* (Mirabdullaev & Defaye 2002) and *A. einslei* (Mirabdullaev & Defaye 2004) (Copepoda: Cyclopoida) with notes on their discrimination. – Submitted to Fundamental and Applied Limnology.
Acanthocyclops traiani and *A. einslei* are two species of very similar morphology. Previous investigators (e.g. Brandl, unpublished determination key) distinguished them, using the names *A. americanus* and *A. robustus*. In the Einsle's monograph (1996) they are considered as one species, under the name *A. robustus*. Later on Mirabdullaev and Defaye (2002, 2004) confirmed them as distinct species and solved the mess in nomenclature by giving them new names. The present author has gone further by finding out discriminatory traits not only for adults, but for copepodid stages as well.
The author presents drawings of antennula, antenna and four swimming legs of five copepodid stages and adult in female of both species. The fifth (copulatory) leg is presented in copepodid 2,4,5 and adult. Other drawings show the mouth parts in adults and some characters of adult males as well. The figures are accompanied by detailed descriptions, measurements and differential diagnoses. This makes possible distinguishing the two species in samples where adult specimens are absent, at least in later copepodids, and distinguishing them from copepodids of other cyclopoid genera.
All the figures were drawn with use of printed micrographs. This technique, not yet commonly used, makes possible to point out the desired characters without sacrifice of authenticity. Moreover, the stored digital images may serve as documentation.

General Introduction, General Discussion, Conclusions
These parts of the thesis put the topics into broader framemework than in the published papers. They are well written to the point, but they lack a thorough language correction.
The objectives of the thesis as declared on the page 11 were achieved.

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OVERALL COMMENTARY ON THE PhD THESIS

Please write comments:

I have only some minor suggestions to the second manuscript.

p. 37, Abstract: copepodid phases – better: copepodid stages
distal endopodid (here and further in the text) – should be: distal segment of the endopodite

p. 38, Material and Methods: .... immersed in a drop of glycerine on a cover slip – should be: .... immersed in a drop of glycerine and observed under a cover slip

p. 38, Results
Cephalothorax with antennule, antenna..etc. – should be: Cephalothorax with pairs of appendages (antennula, antenna... etc.)

p. 48, Table 2, row: body somites – under C5 should be: 9(male), 9(female)

FINAL RECOMMENDATION

X **can be recommended for defence of PhD Thesis**
☐ **can be recommended with reservations for defence of PhD Thesis**
☐ **can not be recommended for defence of PhD Thesis**

Prague, July 9th 2010
.....
Date and place

Jan Fott
.....
Surname and signature

Confidential

Review of FFPW USB PhD Thesis

Name of the PhD student: Ing. **Martin Bláha**	Name of supervisor: **Prof. RNDr. Zdeněk Brandl CSc.**
Title of PhD thesis:Molecular and morphological aspects within *Acanthocyclops* Kiefer, 1927	

REVIEWER:

Surname: **Hołyńska**	Institution: **Museum and Institute of Zoology Polish Academy of Sciences** **Wilcza 64** **00-679 Warszawa, Poland**
Given name: **Maria, Katalin**	
Titles: Docent dr. hab.	E-mail: **mariahol@miiz.waw.pl**
Please describe your professional relationship to the PhD student: **I was in correspondence with Mr. Bláha about the ecology and morphology of *Acanthocyclops robustus* species-complex**.	Please describe your field of expertise: freshwater cyclopoids (Crustacea, Copepoda) evolution, systematics, zoogeography

QUESTIONNAIRE

Originality, scientific importance, prospects of the PhD thesis and benefits for basic or applied research

Evaluate its competitiveness in the international context and compare its level with the current state of the art in the field:

Acanthocyclops belongs to the most species-rich genera in Cyclopidae, one of the largest crustacean families in the continental waters. The genus is distributed mainly in the northern temperate zone, and more than half of the taxa is subterranean. The large-bodied, less-oligomerized, surface-water representatives of the genus, which are common in the Holarctis but also occur in tropical Americas and the southern temperate zone, were often assigned to the so-called „*vernalis-robustus* complex". We do not know whether this group is *natural*, or the *vernalis-robustus* complex is in fact a term without strict phylogenetic meaning, and the member taxa do not form a natural (monophyletic) group.

The representatives of the *vernalis-robustus* complex belong to the most common elements of the lake/pond fauna in Europe, and they can be the intermediate hosts of various nematod and flatworm parasites that infect fishes and sometimes humans. Traditional, morphology-based taxonomy has made certain progress in resolving the taxonomic relationships within the *vernalis-robustus* complex. Among others two new species, very often misidentified by the European limnologists as *A. robustus*, have been recently described, and separated from other members of the *vernalis-robustus* complex by morphometric traits and fine surface structures. Morphologists (Mirabdullayev and Defaye, 2004) warned, however, that other criteria (e.g. cytogenetic, or molecular) would be desirable „to test" the morphological definition of the new taxa.

Martin Bláha not only applied new molecular criteria (mitochondrial and nuclear genes), but also compared the postnaupliar development and adult morphology, and tested separation of the taxa in the morphometric characters by an advanced technique, principal component analysis. First use of the mitochondrial 12S rRNA gene in the phylogeny of these cyclopid taxa, further increases the value of Martin Bláha's researches. The molecular markers supported monophyly of *A. trajani* and *A. einslei*, and also pointed to a relatively large molecular distance between these taxa.

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Comparison of the 18S rDNA sequences from the European populations of *Acanthocyclop vernalis* and *A. trajani* with genbank data of some Nearctic *Acanthocyclops*, revealed the identity of these North American forms. Martin Bláha's molecular analyses (12S rRNA and 18S rDNA) showed that the Holarctic populations assigned by the available morphological criteria to *A. vernalis*, comprise genetically distant populations, and under the name „*vernalis*" eventually more than one species may be hidden.

Preparation of the PhD thesis, targets of the work and deliverables
Evaluate the overall level of preparation of the PhD thesis and the originality of the selected approaches; evaluate publications and whether the targets set in the PhD thesis correspond with the declared purpose of the thesis:

In the analyses of taxonomic/phylogenetic relationships, Martin Bláha compared both molecular (mitochondrial 12S rRNA and nuclear 18S rDNA) and morphological characters (adult and larval morphology) in 9 populations of *A. einslei* from the Czech Republic and Slovakia, 19 populations of *A. trajani* from the Czech Republic, Greece, Spain and Portugal, and 12 populations of *A. vernalis* from the Czech Republic, Slovakia, Bulgaria, Montenegro, and Switzerland. Martin Bláha used different methods (Bayesian, maximum parsimony, and neighbour joining) in the phylogenetic tree reconstructions, and the tree topologies gained by these methods proved to be consistent.

Traditional morhological characterizations applied in the descriptions of the copepodid and adult stages of *A. trajani* and *A. einslei,* were supplemented with principal component analysis of the morphometric traits in *A. trajani*, *A. einslei* and *A. vernalis*.

The results have been presented in **2 peer-reviewed papers published in prestigious international journals with high impact factors** [Zoologica Scripta (2010), Fundamental and Applied Limnology (2010)], and also at the Conference of Czech Limnological Society (2009). During his PhD studies Martin Bláha (as co-author) also published three other papers of the field of limnology and fishery, not closely connected to the title of the PhD thesis, and he is also a co-author of a fishery patent.

Martin Bláha's approach to cyclopid systematics deserves praise, as he trained himself in both morphology and molecular systematics.

Many people (like me) insist on one approach and co-operate with the specialists of other fields, or do the molecular researches after he/she had become familiar with copepod morphology. It is very hard to do both in the same time equally well. It is like someone would like to learn Hungarian and Polish language simultaneously. Most of my comments (see below) just concern the very difficult nature of this ambitious task.

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OVERALL COMMENTARY ON THE PhD THESIS

The title of PhD thesis is somewhat misleading, as it suggests this work deals with the morphology and molecular systematics of the whole *Acanthocyclops* genus [64 (sub)species], although Mr. Bláha analyzed these traits in 3 taxa of the *vernalis-robustus* species-group. Similar inaccuracies also show up in other parts, for instance in the Zoologica Scripta paper (Blaha et al. 2010) and the General Discussion of the thesis, where instead of the *vernalis-robustus* species-complex, „*Acanthocyclops* species complex" is mentioned. If the Author wanted to refer to all the taxa in *Acanthocyclops*, he should simply write *Acanthocyclops* genus, but if he worked with a subset of closely related species within the genus, he should use the specific name (*vernalis*, or *robustus*, or *vernalis-robustus*), under which this group of species was most often referred to in the literature. Accordingly, the aim of the thesis was to resolve the taxonomic relationships in the *vernalis-robustus* group rather, than „within *Acanthocyclops* species complex" (see p. 11 General Introduction). Of course, **the thesis which used morphology (both adult and larval) and molecules in the revision of the *vernalis-robustus* species-group, fully deserves the attention of all specialists interested in freshwater copepod evolution,** and there is no need to use the more general but equivocal term, „*Acanthocyclops* species complex".

In the General Introduction it would be worth mentioning the great progress that cyclopid taxonomy has made in the last 30-40 years. The surface microstructures, so-called „micro-characters", proved to be very useful tools to resolve the taxonomic (e.g. in *Paracyclops*, *Cyclops*, *Diacyclops* and *Mesocyclops)* and phylogenetic relationships (e.g. in *Mesocyclops*). That morphology seems to have little to say to the systematics in some cyclopid groups, is mainly due to the fact that the number of the well-trained and active morphologists is rapidly decreasing in the last decades.

The General Introduction conveys the most important information of the state-of-art of the field, and a comprehensive bibliography is very helpful for both the specialists and beginners. It is a little bit pity that a few errors remained in the citation of the publications:

- Papers cited in the text of General Introduction but missing in the References: Avise, 1994, 2000; Bucklin et al., 1992, 1995; Coker, 1934a; Einsle, 1977; Gonzáles et al., 2008; Hill et al., 2001; Hopp and Maier, 2005; Hořická et al., 2006; Jersabek et al., 2001; Lee and Frost, 2002; Lefébure et al., 2006; Lescher- Moutoué, 1996; Lindeque et al., 1999; Mayr, 1941; Morton, 1985; Nilssen and Wærvågen, 2003; Ponyi, 1967; Purasjoki and Viljamaa, 1982; Roche, 1990; Takahashi et al., 2008; Trochine et al., 2006; Vijverberg, 1977; Vijverberg and Richter, 1982.
- Papers listed in the References but not mentioned in the text of the General Introduction: Avise and Ball 1991; Boxshall and Halsey, 2004; Fryer, 1954; Kiefer and Fryer, 1978; Lowndes 1932; Machida et al., 2004; Petrusek et al., 2004; Pfenninger and Schwenk, 2007; Price, 1958; Reid, 1993; Trontelj et al., 2005; Yang et al., 2009.

Chapter 2: Molecular and morphological patterns across *Acanthocyclops vernalis-robustus* species complex (Copepoda: Cyclopoida) [Zoologica Scripta, 2010]

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This publication is an important contribution to the taxonomy of the *vernalis-robustus* group, and my comments mainly concern our different approaches how Mr. Bláha and I look at the problems and data in systematics.

First of all, because of the partial taxon sampling of the analysis, I would be cautious about the extent this paper resolved the phylogenetic relationships between *A. trajani, A. einslei* and *A. vernalis.* The analyses included 3 European representatives of the *vernalis-robustus* species-group. The European *A. robustus*, as well as all the other taxa living outside of Europe [e.g *.A. brevispinosus* wich is considered by Mirabdullayev and Defaye (2004) as the closest relative of *M. einslei*] and assigned to *vernalis-robustus* complex were not analyzed here. Neither this nor previous studies showed that *vernalis-robustus* complex is a monophyletic group, although monophyly of the complex, defined by shared derived characters, is a necessary assumption to any further analysis of the relationships between the member taxa of the *vernalis-robustus* complex. It is quite possible that what we call *vernalis-robustus* complex is an assemblage of less-oligomerized taxa that form a paraphpyletic group at the best, and could only be defined by ancestral character states in *Acanthocyclops*. Outgroup: Outgroup choice often has strong effect on the tree topology, especially when character evolution is fast — therefore I would also add closer taxa, some *Acanthocyclops* species, to the outgroups.

To inadequacy of the morphological characters in *Acanthocyclops* phylogeny has been referred to in a few places in the Zoologica Scripta paper and also in the General Introduction and the Conclusion of the thesis. We should tell about this, because the misunderstandings around this question (which unfortunately are not uncommon also among the decisision makers in science) cause much loss for systematics.

To say in general that morphology is not so (or less) informative as the molecular traits, because certain morphological characters change too slowly (or some others evolve too fast), is the same mistake as someone would state, that because COI changes too fast in Cyclopoida the molecular characters *en bloc* have less value in cyclopoid phylogeny. Both molecular taxonomists/phylogeneticists and morphologists search for characters that evolve at a rate that is proper to disclose the evolutionary relationships in the group in question. A character can be informative for a particular group, and does not work in another taxa. This is why, the wording „The fourth swimming leg seems to be most useful as an proper identification marker in **Acanthocyclops** taxonomy" [see Conclusion of the thesis] is not correct. The fourth swimming leg provides useful characters in the *vernalis-robustus* group but not in all *Acanthocyclops* species!

The fact is, when morphologists start studying a cyclopid group in detail (see papers of Mirabdullayev and Defaye, or the works of I. Van de Velde on African *Mesocyclops*, or the articles of S. Karaytug on *Paracyclops*), they do find diagnostic (often fine, but stable) characters. Such a thorough investigation unfortunately is still missing in *Acanthocyclops vernalis*.

The morphological characters also seem to be phylogenetically informative – problems arise when you study parasitic copepods, but even then some of the morphological traits tell the true story.

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I could not find any information in the paper whether voucher specimens of the taxa studied (= reference specimens from the samples used for molecular investigations, which can be used in future comparisons) are preserved, and where they were deposited.

Mr. Bláha showed the separation of *A. trajani*, *M. einslei* and *A. vernalis* in several morphometric traits. It was good that beyond the figure with the PCA analysis, the Authors also added a Table with the raw data (mean, range and SD). It can be my fault, yet it is much easier for me to read table 2 and find the biological meaning of the data, than to interpret the distribution pattern along the principal component axes.

I am curious if the Authors also found stable differences between taxa in such characters as: the spinulation of the basal seta inserted in front of the large medial claw of the maxillar basipodite (such differences were already mentioned by Mirabdulayev and Defaye (2002, 2004), and in shape of the seminal receptacle (the posterior part is narrower in *A. einslei* than in *A. trajani*).

In lack of fossils the estimation of the divergence time seems to be a very risky enterprise. An alternative approach could be perhaps comparing the present zoogeographic distributional areas in these *Acanthocyclops* species with the distributional patterns in those taxa (not necessarily animals) where paleobiogeography can rely on rich fossil records.

Let's hope, however, that soon new fosssils will support our hypotheses of cyclopid evolution!

Chapter 3: Description of copepodid and adult *Acanthocyclops trajani* (Mirabdullayev & Defaye, 2002) and *A. enslei* (Mirabdullayev & Defaye, 2004) (Copepoda: Cyclopoida) with notes on their discrimination [Fundamental and Applied Limnology, 2010]

Comparison of the ontogenetic development is the most time-consuming work, albeit it often provides very important information (e.g. to the character polarization) for future phylogenetic studies. These sorts of investigations, as Mr Bláha also mentioned, have practical implications as well: in most samples the larval instars dominate the adults, yet in majority of the taxa no identification keys to the naupliar and copepodid stages are available. Mr Bláha found several morphological characters that make species identification also in the copepodid stages of *A. trajani* and *A. einslei* possible. The morphological descriptions and illustrations meet the international standards of cyclopid taxonomy. My few comments mainly concern some minor inaccuracies in the description.

The last prosomal somite in CII to adult is the fourth leg bearing somite, thence there are 4 prosomal somites from CII to adult, and 2, 3, 4, 5 and 5/6 urosomal somites in CII, CIII, CIV, CV, and CVI (adult female/male) instars, respectively. The fifth leg bearing somite is never part of the prosome in Cyclopoida. In CI there are 3 prosomal somites bearing P1, P2 and the bud of P3, which are followed by 2 urosomal somites. The female genital double-somite (second urosomal somite) is composed of the last thoracic segment (which bears P6) and the first abdominal segment. In male the so called genital-segment is the last thoracic segment (P6 are inserted on this segment)!

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There is a slip of pen in the Abstract, which also pops up in the English Summary of the thesis: Differences were found in the antennular (not the antennal that is A2) ornamentation between the copepodids of *A. trajani* and *A. einslei*.

In the characterization of the copepodid 2 of *A. trajani* the Author wrote as follows: „P3 composed of two-segmented protopod, which bears a row of long setules along inner and outer margins of outer and inner segment, respectively" What does Mr. Bláha mean under „outer and inner segment"? It would be rather odd to call the coxopodite and basipodite this way.

Armature formula of the antennule in the copepodid 5 males of *A. trajani* and *A. einslei*: the Author wrote and illustrated a ten-segmented antennule, but the armature formulae refer to 9 segments only!

The correct spelling is Sewell (Sewel in the text).

It would have been good to explain, why the Author thinks that *Acanthocyclops* identified by Turki et al. (2002) as *A. robustus*, was in fact conspecific with *A. trajani* Mirabdullayev et Defaye, 2002 – you can be right, but an argumentation is missing.

These minor failings however do not change the fact that Martin Bláha successfully applied both molecular and morphological criteria in the delimitation of the taxa in the *vernalis-robustus* species complex, and laid the foundations of further investigations that use synthetic approach in the study of *Acanthocyclops* evolution. Congratulations, and I wish Martin could get support for his future researches!

05 June, Warsaw
2010

Maria Łapińska

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FINAL RECOMMENDATION

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- [ ] **can not be recommended for defence of PhD Thesis**

05 July 2010, Warsaw
Date and place

HOKYŃSKA Marie Małgorzata
Surname and signature