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The genus *Cyclops* (Copepoda, Cyclopoida) in Europe

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Although copepods of the genus *Cyclops* are among the most common and dominant plankton taxa of lakes in the northern temperate zone, their taxonomy is still unclear. We analysed an extensive array of *Cyclops* populations from Europe by means of molecular methods and evaluated morphological characters. Altogether, 68 populations of *Cyclops* species were sampled, assigned to morphospecies and sequenced for the 12S rRNA gene. Selected populations of each morphospecies were additionally sequenced for three mitochondrial (16S rRNA, cytochrome b, COI) and two nuclear genes (18S rRNA, ITS1) and analysed for micromorphological traits. Our analysis revealed fifteen lineages that can be regarded as separate species. Thirteen of these match currently accepted species, while the remaining two lineages were distinct from the other described species. Thus, their taxonomic status is open to further studies. Besides taxonomy, our study gives new insights into the ecology, distribution and phylogenetic relationships of these species. Finally, a set of morphological traits was selected to facilitate identification.

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Introduction

Of the currently recognized species of the genus *Cyclops* O.F. Müller, 1776 (Crustacea, Copepoda), seven were described in the second half of the 19th century: *Cyclops strenuus* Fischer, 1851, *Cyclops furcifer* Claus, 1857, *Cyclops insignis* Claus, 1857, *Cyclops abyssorum* G.O. Sars, 1863, *Cyclops lacustris* G.O. Sars, 1863, *Cyclops scutifer* G.O. Sars, 1863 and *Cyclops vicinus* Uljanin, 1875. Since then, the number of recognized species has fluctuated considerably – from a 'lumping' together by considering *C. furcifer*, *C. scutifer*, *C. abyssorum* and *C. vicinus* to be synonyms of *C. strenuus* (Schmeil 1892), to an 'oversplitting' of up to 52 species and subspecies (Lindberg 1957). Most recently, the last major revision of the genus (Einsle 1996b) resulted in 22 species worldwide.

Like in most invertebrates, species descriptions of cyclopoid copepods are based mainly on morphological traits. The range of these traits has extended since the era of early investigators from large distinct structures (antennule segmentation, thoracic legs and their armature, furca) to microstructures (spinulation and setation, minute pits, pore configuration), which have often been shown to be species-specific. A good example of such microstructures is the configuration of spinules on the coxopodite of the fourth

leg. These structures were earlier just depicted (Schmeil 1892; Guerney 1933) without being mentioned in the text. Einsle (1985) pointed out their taxonomical importance and later on (Einsle 1996a,b) he included them in the diagnoses of *Cyclops* species. Other microcharacters used in the taxonomy of *Cyclops* were described for the antennules, antennae, maxillules and maxillipeds (Einsle 1996b; Dussart & Defaye 2001; Hołyńska & Dahms 2004; Hołyńska 2008). Microcharacters were also extensively used in revisions of the genera *Paracyclops* and *Ochridacyclops* (Karaytug 1999), *Mesocyclops* (Hołyńska *et al.* 2003) and *Thermocyclops* (Mirabdullayev *et al.* 2003).

While the morphological delimitation of *Cyclops* species advanced, analyses of other non-morphological traits began to be used as well. Einsle (1962) was the first to extend species diagnoses in *Cyclops* using observable cellular structures that are independent of external morphology, namely chromatin diminution at the beginning of egg cleavage. This technique, however, has not become widely used in cyclopoid taxonomy because of the need to have live ovigerous females in high quantity and because of the absence of chromatin diminution in many genera or even inside some genera of Cyclopinae (Dussart & Defaye 2001). Moreover, the patterns of excised DNA during the diminution process are in fact also evaluated as morphological traits, although at the cellular level. Another method independent of external morphology was based on allozyme analysis using cellulose acetate electrophoresis. With this method, it was possible to discriminate among the morphologically similar species *Cyclops vicinus–C. kikuchii* Smirnov, 1932 (Einsle 1994) and *C. heberti* Einsle, 1996–*C. divergens* Lindberg, 1936 (syn.: *C. singularis* Einsle, 1996)–*C. furcifer* (Einsle 1996a).

Since the 1990s, the molecular genetic analysis of selected segments of DNA has become a standard method for species delimitation. This approach has been well established in the taxonomy of Cladocera (Petrusek *et al.* 2008) and Calanoida (Bucklin *et al.* 1995; Lindeque *et al.* 1999; Adamowicz *et al.* 2007; Scheihing *et al.* 2010). However, molecular genetic studies on Cyclopoida are scarce (Bláha *et al.* 2010; Wyngaard *et al.* 2010; Hamrová *et al.* 2012), and a broad genetic study on the genus *Cyclops* has not yet been carried out. Therefore, our main objective was to assess whether lineages emerging from a phylogenetic analysis correspond to currently accepted morphospecies. Another goal was to identify groups of related species within the genus and search for their phylogenetic relations.

Material and methods

Study sites, sampling and species assignment

Our study focuses on European species of the genus *Cyclops* that were sampled from 64 localities between 2004 and 2013. The samples came from Albania, Austria, Bulgaria, the Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Montenegro, Norway (incl. Svalbard), Poland, Romania, Russia (Lake Baikal), Slovakia, Spain, Sweden and Switzerland (Table S1) and included various habitats such as glacial lakes, rift lakes, a karst lake, reservoirs, fishponds, mining lakes, riverine, and ephemeral and rock pools. Zooplankton were collected by plankton net tows from the shore or from an inflatable boat. Shallow pools were sampled by a plankton net attached to a pole, or water was scooped up with a plastic jug and filtered through the plankton net. All samples for subsequent molecular analysis were preserved in 96% ethanol and stored in a refrigerator.

Identification was performed according to Einsle (1996b). Specimens were assigned to established morphospecies by comparison with detailed descriptions and figures, but not by the usage of the dichotomic key. The identification was subsequently checked using microcharacters on cephalothoracic appendages described by Hołyńska & Dahms (2004). For species not included in their study (*Cyclops bohater* Kozminski, 1933, *Cyclops ochridanus* Kiefer, 1932, *Cyclops kikuchii*, *C. vicinus*), these traits were newly established.

Morphological analysis

For the detailed study of morphological traits, the techniques of observation and dissection recommended by Dussart & Defaye (2001) were followed, but in addition copepods were treated in hot potassium hydroxide. About 5 ethanol-preserved specimens were transferred into a small glass beaker, and 1 mL of 10% KOH was added. The beaker was covered with a large coverslip and heated at 80 °C for 20 min (formalin-preserved specimens need to be heated at 90 °C for 30 min; the optimal variant for different samples must be tested) to dissolve the soft tissues of the specimens until only translucent chitinous envelopes remained. Washing off the hydroxide was performed using a chamber made of a small plastic test tube, the bottom of which was replaced by a nylon netting of 40 μm mesh size. The washed copepods were then stained overnight in water with a few drops of chlorazol black in ethanol. The stained copepods were transferred into a 1:1 mixture of 70% ethanol and glycerol and heated at 80 °C until the water and ethanol evaporated, which made them ready for dissection. The dissection was performed in a drop of glycerol under a stereomicroscope. Dissection needles consisted of short, well-sharpened tungsten wires of 0.3 mm diameter, attached to inoculating loop holders.

The following copepod parts were isolated and mounted individually in a series of permanent mounts: antennules (A1), maxillules (Mxl), maxillipeds (Mxp) and the first and

fourth pair of swimming legs (P1, P4). These appendages were chosen due to their likelihood of bearing important qualitative characters. Among the dissected appendages the antenna was also included. The ornamentation of its coxobasis was extensively documented, but is not included in this study because the patterns were, for the time being, unclear.

The dissected parts were transferred from the glycerol, one at a time, on the tip of a needle into a drop of the synthetic mounting medium Hydro-Matrix (Micro-Tech-Lab) onto a slide. After proper orientation of the transferred part (e.g. the caudal side of P4 upwards), they were covered by a coverslip. If the drop of Hydro-Matrix was small enough, parts such as the antenna, maxilliped and swimming legs were pressed by the coverslip into a single plane, which allowed all important features to remain in focus at once. After mounting, the slides were placed horizontally until the Hydro-Matrix medium solidified.

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

Molecular analysis

DNA from individual adult females or from egg sacs was extracted in 50 μL of proteinase K solution, following the protocol of Schwenk *et al.* (1998) and in some cases by the HotSHOT method (Montero-Pau *et al.* 2008). The number of analysed individuals per population differed, ranging from one to six (Table S1). For some populations, only a single specimen was analysed because of the scarcity of animals in the samples or because of failure during DNA isolation. For all 68 populations (sampled from 64 localities), a 430-bp-long fragment of the mitochondrial gene for the small ribosomal subunit (12S) rRNA was amplified. Additionally, for further phylogenetic analyses one population of each species was chosen and parts of the following three mitochondrial and two nuclear genes were amplified: the small ribosomal subunit (16S) rRNA (350 bp), cytochrome b (Cytb; 360 bp), cytochrome c oxidase subunit I (COI; 658 bp), the small ribosomal subunit (18S) rRNA (630 bp) and internal transcribed spacer 1 (ITS-1; 420–540 bp). These additional genes were examined to increase the reliability of the phylogenetic tree.

PCR reactions were run in 20 μL containing 1 × PCR Dream Taq buffer (Fermentas), 0.2 mM dNTPs, 4 mM $MgCl_2$, 0.4 mM of each primer, 0.6 U Dream Taq polymerase (Fermentas) and 4 μL of the DNA template. For the amplifications of specific sequences, published primer pairs were used: 12S (Machida *et al.* 2004), 16S (Palumbi & Benzie 1991; Braga *et al.* 1999), 18S (Spears *et al.* 1992), COI (Folmer *et al.* 1994), Cytb (Merritt *et al.* 1998) and ITS-1 (Chu *et al.* 2001); all are listed in Table S2. The PCR cycle consisted of the following steps: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 94 °C for 45 s, annealing for 45 s at 60 °C (for 12S, 16S, 18S, ITS-1) or at 48 °C (COI and Cytb), and elongation at 72 °C for 1.5 min, with final elongation at 72 °C for 6 min. PCR products were purified by ethanol precipitation or with a QIAquick Gel Extraction Kit (QIAGEN) and sequenced by dideoxynucleotide termination (using the primers marked in Table S2) at Macrogen, Inc., the Faculty of Science, Charles University in Prague, or the University of Valencia using Applied Biosystems PRISM 3730XL and 3130XL DNA Analyzer capillary sequencers. Specimens of *Acanthocyclops americanus* Marsh, 1893, *Macrocyclops albidus* Jurine, 1820, *Megacyclops viridis* Jurine, 1820 from Germany and the Czech Republic, respectively, were used as outgroup.

Data analysis

All sequences were checked twice manually in MEGA v5 (Tamura *et al.* 2011). The 12S sequence data set was analysed first. The sequences were aligned by the PRANK algorithm (Löytynoja & Goldman 2005), a phylogeny-aware method specifically developed to deal with alignments containing gaps, using the Prankster interface (http://www.ebi.ac.uk/goldman-srv/prank/prankster/).
Afterwards, the data set was tested for poorly alignable regions using Gblocks Server 0.91b (Castresana 2000; Talavera & Castresana 2007), with default settings but allowing gaps, and the ambiguously aligned positions detected were subsequently excluded. This process resulted in a 350-bp-long alignment. The TrN+I+G model of evolution was chosen by corrected Akaike Information Criterion (AICc) in jModelTest 2 (Guindon & Gascuel 2003; Darriba *et al.* 2012). A maximum-likelihood (ML) tree was constructed in GARLI v2.0 (Zwickl 2006) with 20 replicates, and bootstrapping was performed with 1000 replicates. Bootstrap results were summarized using the SumTrees script of the DendroPy package v3.12.0 (Sukumaran & Holder 2010). The ML tree obtained was visualized using FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and used as the basis for further species identification.

Next, the sequence data of other genes were analysed in selected populations (Table S1). The sequences for the protein coding genes (COI and Cytb) were aligned separately using ClustalW (Thompson *et al.* 1994) and

checked by translating into protein sequences in MEGA. The alignments of non-coding sequences (12S, 16S, 18S and ITS-1) were performed using Prankster and Gblocks Server, as described above. Evolutionary divergences for each marker were calculated under Kimura's 2-parameter (K2P) model with pairwise deletion of missing data in MEGA. Substitution saturation was tested for each gene in DAMBE v5.3 (Xia 2013) to inspect any loss in phylogenetic signal. SequenceMatrix (Vaidya *et al.* 2011) was used to concatenate the sequences from the six loci, resulting in a 2531-bp-long alignment.

Optimal partitioning schemes and substitution models of molecular evolution were selected with PartitionFinder v1.1.1 (Lanfear *et al.* 2012, 2014). The data set was divided into 10 partitions (12S, 16S, 18S, ITS-1, the three COI and the three Cytb codon positions) and all combinations were tested. For subsequent maximum-likelihood (ML) inference in GARLI, the following five-partition scheme was selected under the AICc: 12S and 16S with the GTR+I+G model; first codon positions of COI and Cytb with TrN+I+G; second positions of COI and Cytb with K81uf+I+G; 18S and third positions of COI and Cytb with HKY+G; and ITS1 with GTR+G. For Bayesian inference (BI) in MrBayes v3.2.2 (Ronquist *et al.* 2012), the three-partition scheme was selected under the Bayesian Information Criterion (BIC): 12S, 16S, first and second positions of COI and Cytb with the HKY+I+G model; 18S and ITS-1 with K80 + I+G; and third positions of COI and Cytb with HKY+G. Model parameters and branch lengths were unlinked between partitions.

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

Results

Morphological analysis

Permanent slides were prepared from all 15 morphospecies under study. In total, 122 individual copepods were dissected and observed for morphological characters. We used a selection of the most informative traits that would be able to discriminate among the different species. Ten traits were selected that were helpful in confirming identification, and are displayed in Fig. 1. The distribution of those traits in the 15 species clustered into three clades is shown in Table 1. Whereas some traits were fixed (no polymorphy), others showed intraspecific variation in particular species. Two traits (F and I, Table 1) occurred in only one species each, while one trait (B) occurred (with one exception) exclusively in clade 1.

Molecular analysis

In total, 540 *Cyclops* individuals were analysed with molecular markers and 371 DNA sequences were obtained (Table S1). The amplification success rates varied for different molecular markers. First, we tried to amplify the sequences of COI, but this worked for just a few populations (Table S1). The genes for 16S and Cytb were amplified in 53% and 73% of the species under study, respectively. Amplification of the nuclear genes for 18S and ITS-1 was successful in most cases (Table S1), except for two populations of *C. strenuus*, where the DNA of epibiont ciliates (*Vorticella* sp.) was amplified instead. In contrast, amplification of the 12S gene was the most successful (95% of the species). All newly obtained Cyclopidae sequences were deposited in GenBank under accession numbers KP772854–KP772911, KP772914–KP773019, KP773022–KP773041, KP773043–KP773046, KP773049–KP773224 and KP940578–KP940579.

The substitution saturation detected by DAMBE was low; thus, all six studied gene partitions had a sufficiently high phylogenetic signal. Evolutionary divergences among *Cyclops* species were 0–0.7% in 18S, 0.1–39.4% in ITS-1, 3.7–20.7% in 16S, 16.0–31.7% in COI, 20.1–39.8% in Cytb and 1.3–43.1% in 12S, while those within species were always lower (Tables S3–S8).

The final phylogenetic tree of the concatenated data set (Fig. 2) was based on the ML topology obtained from GARLI, with the bootstrap support values of BI and the analysis of SATé. The ML and BI topologies were identical, while the SATé topology differed just in the position of one branch (*C. scutifer*). All 15 *Cyclops* species (including two lineages not conspecific with any species examined here, see below) were well separated, supported by high bootstrap values and their independent species status confirmed. Three main clades were distinguished on the phylograms: clade 1 comprising *C. lacustris*, *C. bo-*

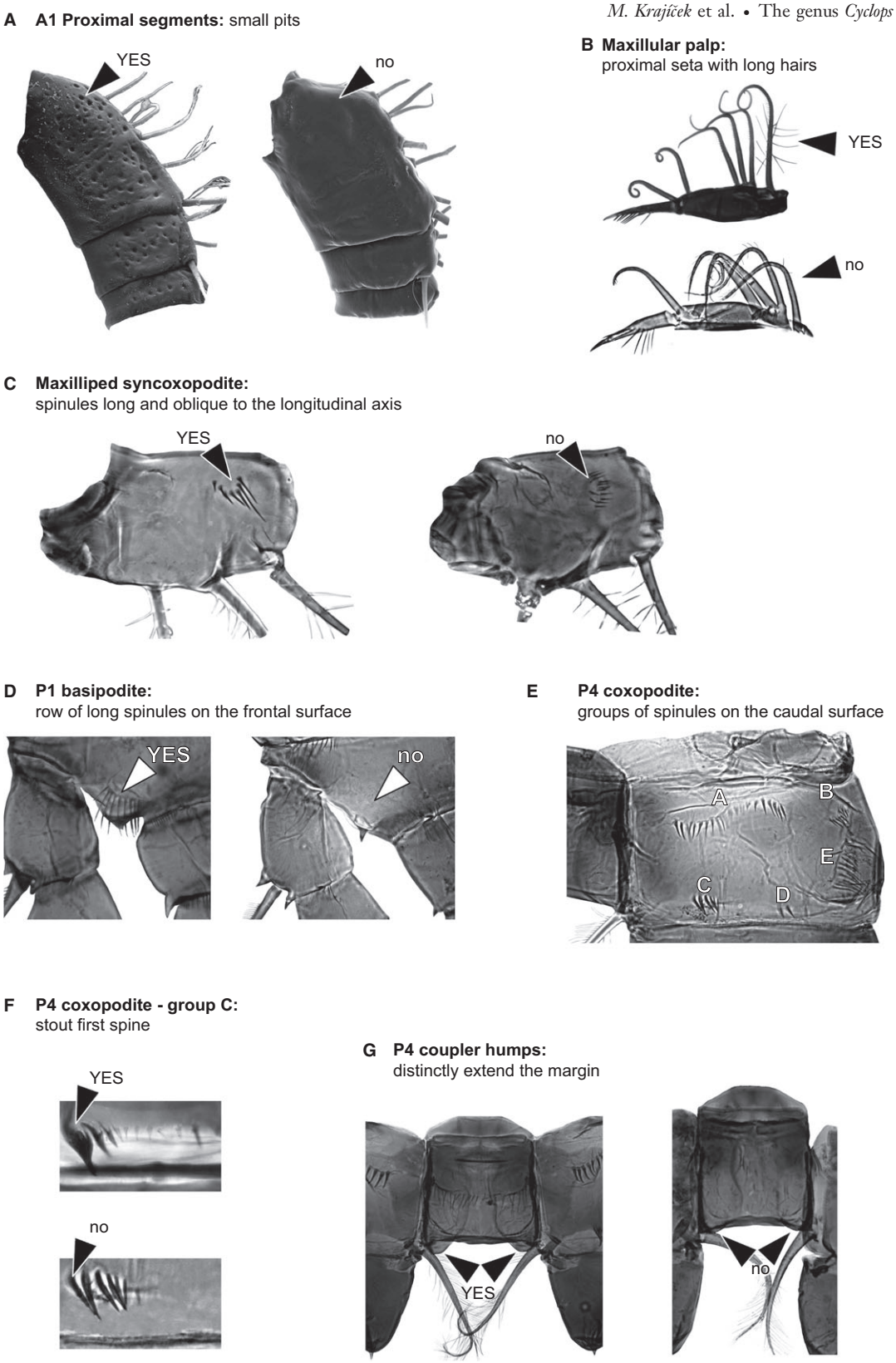

Fig. 1 A-J. Ten morphological characters of *Cyclops* species. — A. Illustrated by scanning electron microscope images.— B–J. Illustrated by photographs from an optical microscope. Usually, both character states are displayed and marked. Abbreviations: A1, antennule; P1, first pair of swimming legs; P4, fourth pair of swimming legs; Th4, fourth thoracic segment.

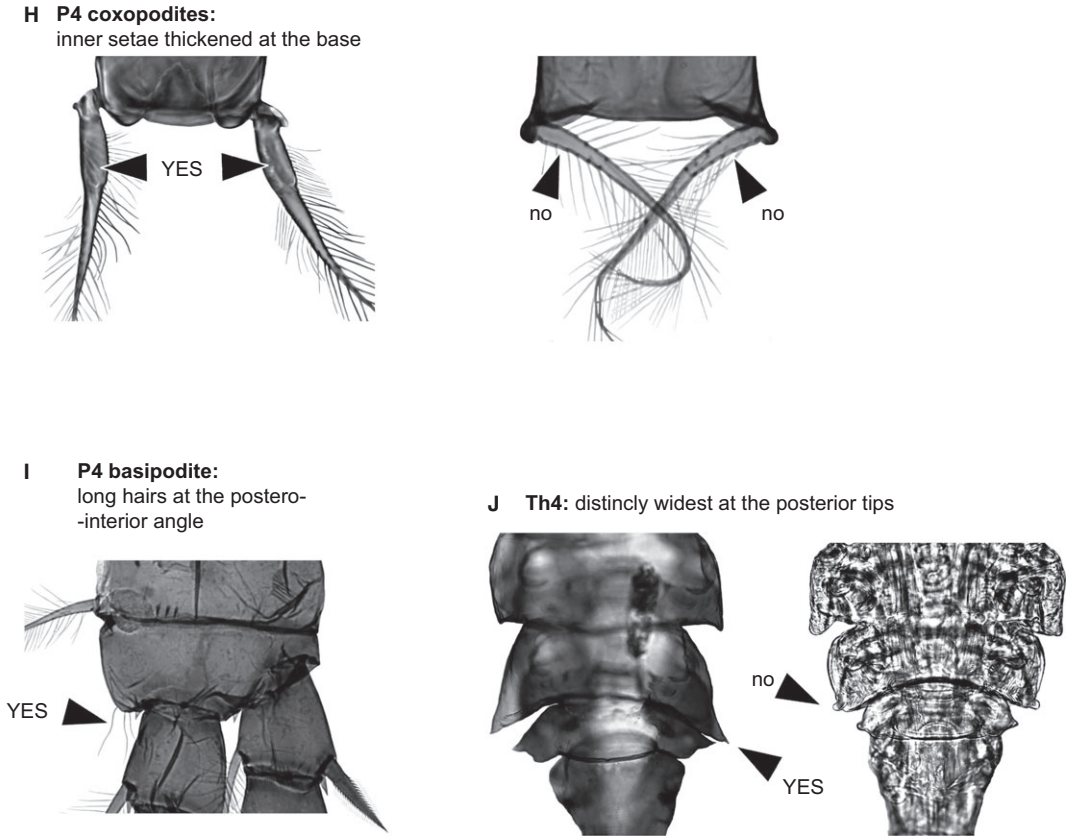

Fig. 1 Continued

hater, *C.* sp. Y, *Cyclops heberti*, *C. ochridanus*, *C. divergens*, *C. abyssorum* and *C.* sp. X; clade 2 which is a sister to clade 1 and comprises *Cyclops kolensis* Lilljeborg, 1901, *C. strenuus*, *C. scutifer*, *C. furcifer* and *C. insignis*; and clade 3 which is a sister to clades 1 and 2 and comprises *C. vicinus* and *C. kikuchii*. The outgroup species were resolved as well, with *A. americanus* being the most closely related and *M. albidus* the most distant. The phylogenetic relationships revealed from 12S sequences (Fig. S1) and from the concatenated data set (Fig. 2) are very similar.

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

Discussion

Morphology

A thorough discussion of morphological traits is outside the scope of this study. Here only we compare our findings (Table 1; Fig. 1) with those of previous authors.

The small cuticular pits on the surface of the proximal segments of antennules (A1) were mentioned by Einsle (1996a,b) in *C. heberti* and *C. divergens* (syn.: *C. singularis* Einsle, 1996) as 'small deepened circles (tiny craters)' on the dorsal surface of the first four segments. Our scanning electron microscope images also confirmed this surface ornamentation on the dorsal parts of the thoracic segments and on the genital double segment. When using an optical microscope, this ornamentation is readily observable on the dorsal surface of the three proximal segments of the antennule, which can therefore be used as a standard for routine examination of the presence or absence of this trait.

The ornamentation of the coxopodite of the fourth swimming legs (P4) is an important character for species identification, firstly emphasized by Einsle (1985, 1996b). Groups of spinules on the caudal surface are labelled as A,

Table 1 Phylogenetic relationships of *Cyclops* species and a table of some morphological characters

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

A1, antennule; P1, first pair of swimming legs; P4, fourth pair of swimming legs; Th4, fourth thoracic segment; Y, character is present; n, character is absent; var, variable character; ?, character not examined. Parentheses in the column P4 coxopodite ornamentation indicate that the relevant group of spinules can be present or absent. The three main clades are indicated by the numbers 1, 2 and 3. Sources of information: E, Einsle (1996b); H&D, Hołyńska & Dahms (2004); H, Hołyńska (2008); F, ascertained or confirmed by J. Fott (this study). Details of characters are explained in Fig. 1 and in the text.

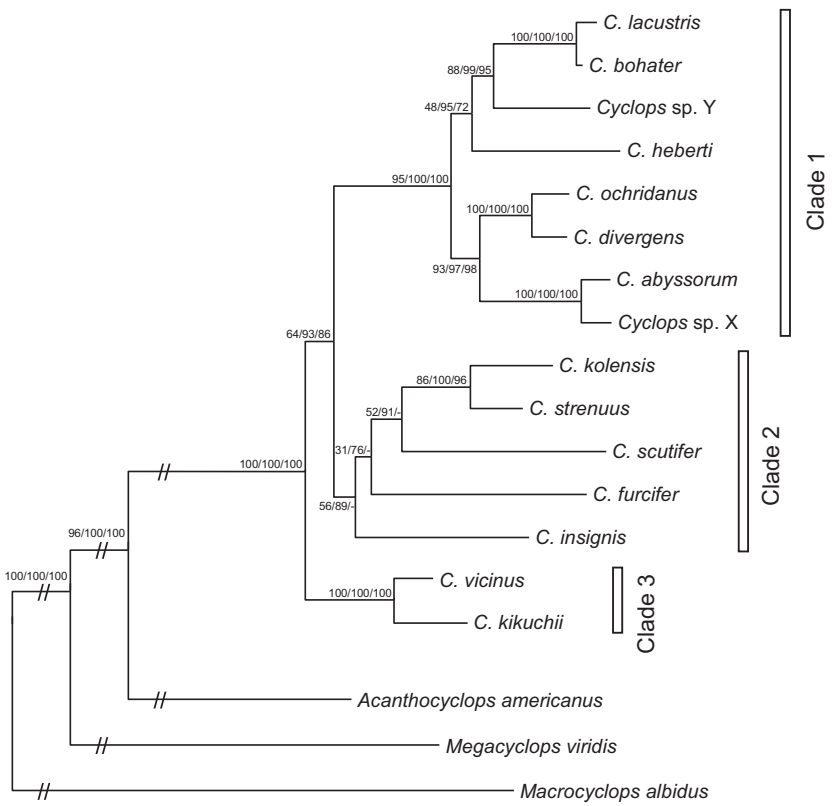

Fig. 2 Phylogenetic relationships of *Cyclops* species. The maximum-likelihood tree was based on the 2531-bp-long alignment consisting of fragments of the mitochondrial markers for 12S rRNA, 16S rRNA, cytochrome b and cytochrome c oxidase I, and the nuclear markers for 18S rRNA and internal transcribed spacer 1. Numbers at nodes indicate branch supports (as percents) assessed by maximum-likelihood (GARLI), Bayesian inference (MrBayes) and approximately-maximum-likelihood (SATé). The three main clades are marked with the numbers 1, 2 and 3.

B, C, D, E. Groups A and C are always present, while the presence of groups B, D and E may be species-specific. A peculiar microcharacter to *Cyclops heberti* is the first spine of group C, which is remarkably stout (Fig. 1F), while the following spines in this line are smaller. This trait of *C. heberti* was well illustrated by Einsle (1996a).

We can confirm the usefulness of the following microcharacters described by Hołyńska & Dahms (2004) and Hołyńska (2008) for species identification: (i) the presence or absence of long setules on the proximalmost seta of the maxillular (Mxl) palp. (ii) The maxilliped syncoxopodite may be ornamented on the frontal surface with long spinules oriented obliquely to the longitudinal axis of the segment. Alternatively, the spinules are parallel to the axis or they are very short with no apparent orientation. (iii) The basipodite of the first swimming legs (P1) may bear a row of long spinules on the frontal surface, above the large spine between the insertion of the endo- and exopodite. A single lineage (*Cyclops* sp. Y) is distinguishable by the presence of long hairs at the posterio-interior angle of the P4 basipodite (Fig. 1I). This trait has not been recorded in any other *Cyclops* species so far. Similar thin setules on the P4 basipodite are present in some *Megacyclops* and *Acanthocyclops* species (Einsle 1996b), but they are more numerous and much shorter.

To trace the distribution of the studied morphological traits in the main clades, we mapped the characters of each species onto the phylogenetic tree (Table 1). Unexpectedly, trait B (Fig. 1; Table 1) – long hairs on the proximalmost seta of the maxillular palp – is present in all species of clade 1 (*C. lacustris*, *C. bohater*, C. sp. Y, *C. heberti*, *C. ochridanus*, *C. divergens*, *C. abyssorum*, *C.* sp. X) but is absent (with the single exception of *C. furcifer*) in all other *Cyclops* species included in our analyses. The spine formula in species of clade 1 is exclusively 3-4-3-3, while in the rest of phylogram the formula is 2-3-3-3 (*C. kolensis*, *C. insignis*, *C. vicinus*, *C. kikuchii*) or variable (*C. furcifer*), with two exceptions: *C. strenuus* (3-4-3-3) and *C. scutifer* (3-4-3-3, rarely 3-3-3-3) (Einsle 1996b). Thus, the morphological characterization of clades 1, 2 and 3 appears to be rather weak so far.

Taxonomic status of the current Cyclops lineages

Accepting that morphospecies are hypotheses that should be tested by different approaches (Dayrat 2005), we tested the present 'morphological' state-of-the-art in *Cyclops* taxonomy by analysing the DNA sequences of particular genes. In this process, the delineation of cohesive lineages was the primary task, which was then followed by assignment of the lineages to the presently recognized morphospecies. This process led to 13 successful matches, solving some problems in the taxonomy of *C. abyssorum* and the discovery of two species not yet identified, which altogether considerably clarifies the current taxonomy in *Cyclops*.

One of the most problematic species of the genus is *C. abyssorum*, with several described subunits perceived as morphotypes or subspecies (Kiefer 1978; Einsle 1980, 1996b; Hołyńska 2008). Our molecular data on populations of *C. abyssorum* covered several Scandinavian populations from lakes and rock pools, a population from a large and deep subalpine lake (Lugano – '*praealpinus*'-type) and several high mountain lakes (Pyrenees, Alps, Tatras, Dinarids – '*tatricus*'-type), but no substantial variation in 12S or 16S sequences was observed. Among these populations there is also one from Lake Ulvenvann lying in the *terra typica*. This lake is situated close to the type locality, Lake Maridal, where *Cyclops abyssorum* does not occur any more (J. P. Nilssen, personal communication). The sequence variation of these populations is low: values for Kimura 2-parameter distances did not exceed 1.6% in 12S and 1.3% in 16S, which is within the normal range of intraspecific variation (Bláha *et al.* 2010). Regarding this species, all populations of *C. abyssorum* confirmed by molecular analysis came from glacial lakes or reservoirs in high mountains or in northern latitudes. They were mainly from lakes but also from rock pools in coastal areas of Norway (Hvaler) and Finland (Tvärmine). Any findings of *C. abyssorum* from waters in lowlands from central and southern Europe are thus suspect and need revision.

A species morphologically most similar to *C. abyssorum* is *C. divergens*. Since 2003, we have collected *C. divergens* repeatedly in the plankton of Slapy Reservoir (Czech Republic), and molecular analysis undoubtedly confirmed this. Later, it was also identified from the plankton of other deep canyon-shaped reservoirs in the Czech Republic. Our molecular analyses of *Cyclops* from reservoirs or karst lakes in Spain (Banyoles, Ebro, González Lacasa, Siurana) also confirm *C. divergens*. This is in accordance with the suggestion of Hołyńska (2008) that the distribution of *C. divergens* (*C. abyssorum divergens* in her interpretation) is not restricted to shallow, often astatic waters, but includes deep water bodies as well.

Cyclops strenuus, the first described species of the genus, is an ecologically variable species that can be found in different types of pools, fishponds, reservoirs and lakes. According to our observations, it often dominates brown-water pools in woodlands or on the edges of forests, while pools in meadows and fields are inhabited by other species (*C. divergens*, *C. heberti*, *C. furcifer* and *C. vicinus*).

The most frequent species in the Czech Republic, from where we have the most data, is *Cyclops vicinus*. This species is common in fishponds and reservoirs, as well as in small astatic waters. We found it in reservoirs and lakes in Spain,

Switzerland, Austria, Bulgaria and Greece, as well as in the glacial mountain lake Šiško, Montenegro (1660 m).

Cyclops kikuchii and *C. vicinus* are the only species pair that cannot be distinguished by any of the morphological traits mentioned in Table 1, but they are well separated on both phylograms. The main morphological character used to differentiate them is the relative length of the terminal furcal setae (Einsle 1996b).

An interesting pair of species is that of *C. bohater* and *C. lacustris*. They seem to be very closely related genetically but they are easily distinguishable by their morphology – for example body size, shape of thoracic segments, pattern of spinules on the antennal coxobasis, length proportions of the fourth endopodite distal segment and its terminal spines (Einsle 1996b). Admittedly, our molecular analysis was based only on two specimens of *C. bohater* from Schöhsee (Germany) and two specimens of *C. lacustris* from Lake Mjøsa (Norway). This discrepancy between genetic and morphological similarity is worthy of further attention.

Cyclops sp. X seems to be widely distributed in Europe, but until now it has not been recognized as a distinct species. It has been reported either as *C. strenuus* (Bosselmann 1974) or *C. abyssorum*. A full description and naming as a new species is beyond the scope of this study. *Cyclops* sp. Y bears some resemblance (size, furca, genital double segment) to *Cyclops ricae* Monchenko, 1977 (Einsle 1996b) known from Lake Ritza, Great Caucasus.

It may seem that because of their universality, molecular methods are more feasible than morphological ones in copepods, which need cumbersome dissection and special expertise. However, the two approaches are complementary and one cannot stand without the other. The negative reputation of copepod dissection comes from insufficient know-how, as the time-tested technique has rarely been described in detail. By applying the method described here, dissection of even small structures is relatively easy and can be mastered in several hours.

Phylogeny and historical biogeography

Results from conservative markers such as the nuclear ribosomal 18S support the monophyly of the European species of the genus *Cyclops* (Table S7). Mitochondrial markers and ITS-1 grouped the studied species into three different clades, with high genetic distances (Fig. 2; Tables S3–S6 and S8). Divergence time estimates from standard evolutionary rates for crustacean mitochondrial DNA (ranging from 2.6–0.9% K2P distances per My; Ketmaier *et al.* 2003; Milligan *et al.* 2011 and references therein) indicated that divergence among the three clades is old, probably corresponding to climate changes occurring from the mid-Miocene to the latest Miocene. The increase in cooling and seasonality during that epoch are considered responsible for the retreat of tropical species and the diversification of many groups of organisms able to survive in cool as well as in dry conditions (Potter & Szatmari 2009). Later, the advance and retreat of ice during glacial episodes in the Pleistocene had a major impact on species distributions, with northern expansion during interglacial periods and southward retreats during subsequent cooling (Hewit 1996; Taberlet *et al.* 1998; Schmitt & Varga 2012). This likely promoted further divergence and speciation within clades.

The distribution of *Cyclops* species within the three clades (Fig. 2) has some bearing on their geographical distribution as stated by Kiefer (1978), Einsle (1996b) and Hołyńska (2008). Clade 1 is composed of eight species that can be further divided into two subgroups. The first subgroup consists of *C. divergens*, *C. ochridanus*, *C. abyssorum* and *C.* sp. X., whereas the second one includes *C. bohater*, *C. lacustris*, *C. sp. Y* and *C. heberti*. Of all these species, *C. divergens* has the widest geographical distribution within clade 1. It extends from its farthest northern locations in the southern parts of the UK and Poland to its southernmost ones in Lebanon and southern Iran, and from Portugal in the west to Uzbekistan in the east (Hołyńska 2008). *Cyclops divergens* inhabits small astatic puddles, riverine pools and small ponds, but also lives in the plankton of lakes and reservoirs. The remaining species, except for *C. heberti*, are lake plankton dwellers with more restricted geographical distribution. *Cyclops ochridanus*, which is genetically very closely related to *C. divergens* (Fig. 2; Tables S3–S5 and S8), is an endemic species from the plankton of the ancient Lake Ohrid. We hypothesize that the ancestor of clade 1 was a widely distributed species similar in its ecological plasticity to the extant *C. divergens*. From this ancestor, an array of species could have been separated due to Pleistocene climatic fluctuations (Schmitt 2007; Schmitt & Varga 2012), each of them having more restricted geographical and ecological ranges.

Clades 2 and 3 (Fig. 2) consist of species with a wide northern geographical distribution regardless of their ecological plasticity. *Cyclops kolensis* (northern Palaearctic) and *C. scutifer* (northern Holarctic) are typical plankton species inhabiting lakes, *C. strenuus* (Palaearctic) has wide ecological range from large lakes to temporary ponds and pools, and *C. furcifer* (Holarctic) and *C. insignis* (Europe) live in temporary ponds and pools. *Cyclops vicinus* is distributed in Europe and Asia, inhabiting eutrophic and mesotrophic lakes, permanent ponds and temporary pools; references to North America are questionable (Einsle 1996b). *Cyclops kikuchii* was described from Japan, but most records are from Europe. Its ecological preferences are poorly known.

According to our molecular phylogenetic tree (Fig. 2), we hypothesize that the diversity and distribution of *Cyclops* species resulted from several events:

1. An older speciation, indicated by separation of the three distinct clades.
2. The Pleistocene glaciation events, which brought about the separation of species within clades. This is well illustrated by the ultimate split of the divergens–abyssorum group. Currently *C. abyssorum* shows a clear boreoalpine distribution, whereas *C. divergens* is widespread over the Mediterranean and Middle East and in the lowlands of Central Europe.
3. The isolation of the endemic *C. ochridanus* in Lake Ohrid.
4. The recent dispersions caused by man (translocation due to fish introductions, eutrophication) indicated by the molecular uniformity shown by *C. vicinus*.

A shortcoming of the present study is its restriction to Europe (except for the *C. kolensis* population from Lake Baikal, which was found to be genetically similar to *C. kolensis* from Scandinavia). The challenge for future studies are genetic analyses of *Cyclops* populations outside the area explored so far, namely Mediterranean islands and North Africa, the Caucasus, the Middle and Far East, the Himalayas, Mongolia and North America). Broadening our knowledge in this direction would help in better understanding the phylogeny and ecology of this genus.

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References

Adamowicz, S. J., Menu-Marque, S., Hebert, P. D. N. & Purvis, A. (2007). Molecular systematics and patterns of morphological evolution in the Centropagidae (Copepoda: Calanoida) of Argentina. *Biological Journal of the Linnean Society*, *90*, 279–292.

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

Bosselmann, S. (1974). The crustacean plankton of Lake Esrom. *Archiv für Hydrobiologie*, *74*, 18–31.

Braga, E., Zardoya, R., Meyer, A. & Yen, J. (1999). Mitochondrial and nuclear rRNA based copepod phylogeny with emphasis on the Euchaetidae (Calanoida). *Marine Biology*, *133*, 79–90.

Bucklin, A., Frost, B. W. & Kocher, T. D. (1995). Molecular systematics of six *Calanus* and three *Metridia* species (Calanoida: Copepoda). *Marine Biology*, *121*, 655–664.

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, *17*, 540–552.

Chu, K. H., Li, C. P. & Ho, H. Y. (2001). The first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in crustacea. *Marine Biotechnology*, *3*, 355–361.

Darriba, D., Taboada, G., Doallo, R. & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, *9*, 772.

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, *85*, 407–415.

Dussart, B. H. & Defaye, D. (2001). Introduction to the Copepoda. In Dumont, H. J. (ed.). *Guides to the Identification of the Macroinvertebrates of the Continental Waters of the World*. (p. 344, Leiden: Backhuys Publishers BV.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

Einsle, U. (1962). Die Bedeutung der Chromatin- Diminution für die Systematik der Gattung *Cyclops* s.str. *Naturwiss*, *49*, 90.

Einsle, U. (1980). Systematic problems and zoogeography in cyclopoids. In W. C. Kerfoot (Ed.) *Evolution and Ecology of Zooplankton Communities* (pp. 679–684). Lebanon: University Press of New England.

Einsle, U. (1985). A further criterion for the identification of species in the genus *Cyclops* s. str. *(Copepoda, Cyclopoida)*. *Crustaceana*, *49*, 299–309.

Einsle, U. (1994). *Cyclops kikuchii* Smirnov, 1932 (Copepoda, Cyclopoida), eine selbständige Art aus süddeutschen Gewässern. *Crustaceana*, *66*, 240–246.

Einsle, U. (1996a). *Cyclops heberti* n.sp. and *Cyclops singularis* n.sp., two new species within the genus *Cyclops* ('strenuus-subgroup') (Crust Copepoda) from ephemeral ponds in southern Germany. *Hydrobiologia*, *319*, 167–177.

Einsle, U. (1996b). Copepoda: Cyclopoida. Genera *Cyclops*, *Megacyclops*, *Acanthocyclops*. In H. J. F. Dumont (Ed.) *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World*, *10* (pp. 82). Amsterdam: SPB Academic Publishing.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*, 294–229.

Guerney, R. (1933). British fresh-water Copepoda, *vol.3*. (p. 384). London: The Ray Society.

Guindon, S. & Gascuel, O. (2003). A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology*, *52*, 696–704.

Hamrová, E., Krajíček, M., Karanovic, T., Černý, M. & Petrusek, A. (2012). Congruent patterns of lineage diversity in two species complexes of planktonic crustaceans, *Daphnia longispina* (Cladocera) and *Eucyclops serrulatus* (Copepoda), in East European mountain lakes. *Zoological Journal of the Linnean Society*, *166*, 754–767.

Hewit, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, *58*, 247–276.

Hołyńska, M. (2008). On the morphology and geographical distribution of some problematic South Palaearctic *Cyclops* (Copepoda: Cyclopidae). *Journal of Natural History*, *42*, 2011–2039.

Hołyńska, M. & Dahms, H. U. (2004). New diagnostic microcharacters of the cephalothoracic appendages in *Cyclops* OF Müller, 1776 (Crustacea, Copepoda, Cyclopoida). *Zoosystema*, *26*, 175–198.

Holynska, M., Reid, J. W. & Ueda, H. (2003). Genus *Mesocyclops* Sars, 1914. pp 12–213. In: Ueda, H. & Reid J. W. (Eds), *Copepoda: Cyclopoida. Genera Mesocyclops and Thermocyclops*. vol. *20*, in H. J. F. Dumont (Ed.), Guides to the Identification of the Microinvertebrates of the Continental Waters of the World. Leiden: Backhuys Publishers.

Karaytug, S. (1999). Copepoda: Cyclopoida. Genera *Paracyclops*, *Ochridacyclops* and key to the Eucyclopinae. In H. J. F. Dumont (Ed.), *Guides to the Identification of the Microinvertebrates of the Continental Waters of the World*, *14* (p. 217). Leiden: Backhuys Publishers

Ketmaier, V., Argano, V. & Caccone, A. (2003). Phylogeography and molecular rates of subterranean aquatic Stenasellid Isopods with a peri-Tyrrhenian distribution. *Molecular Ecology*, *12*, 547–555.

Kiefer, F. (1978). Freilebende Copepoda. In F. Kiefer & G. Fryer (Eds), *Das Zooplankton der Binnengewässer*, 2. Teil (Die Binnengewässer, pp. 1–343). Stuttgart: E. Schweizerbartsche Verlagsbuchhandlung.

Lanfear, R., Calcott, B., Ho, S. Y. W. & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*, 1695–1701.

Lanfear, R., Calcott, B., Kainer, D., Mayer, C. & Stamatakis, A. (2014). Selecting optimal partitioning schemes for phylogenomic datasets. *BMC Evolutionary Biology*, *14*, 82.

Lindberg, K. (1957). *Le groupe Cyclops rubens (syn. Cyclops strenuus): Revision du genre Cyclops s. str. (O. F. Müller 1770)(Crustacés copépodes)*. (p. 335). Lund: Sweden: C. W. K. Gleerup.

Lindeque, P. K., Harris, R. P., Jones, M. B. & Smerdon, G. R. (1999). Simple molecular method to distinguish the identity of *Calanus* species (Copepoda: Calanoida) at any developmental stage. *Marine Biology*, *133*, 91–96.

Liu, K., Warnow, T. J., Holder, M. T., Nelesen, S. M., Yu, J., Stamatakis, A. P. & Linder, C. R. (2012). SATe-II: very fast and accurate simultaneous estimation of multiple sequence alignments and phylogenetic trees. *Systematic Biology*, *61*, 90–106.

Löytynoja, A. & Goldman, N. (2005). An algorithm for progressive multiple alignment of sequences with insertions. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 10557–10562.

Machida, R. J., Miya, M. U., Nishida, M. & Nishida, S. (2004). Large-scale gene rearrangements in the mitochondrial genomes of two calanoid copepods *Eucalanus bungii* and *Neocalanus cristatus* (Crustacea), with notes on new versatile primers for the srRNA and COI genes. *Gene*, *332*, 71–78.

Merritt, T. J., Shi, L., Chase, M. C., Rex, M. a, Etter, R. J. & Quattro, J. M. (1998). Universal cytochrome b primers facilitate intraspecific studies in molluscan taxa. *Molecular Marine Biology and Biotechnology*, *7*, 7–11.

Milligan, P. J., Stahl, E. A., Schizas, N. V. & Turner, J. T. (2011). Phylogeography of the copepod *Acartia hudsonica* in estuaries of the northeastern United States. *Hydrobiologia*, *666*, 155–165.

Mirabdullayev, I., Reid, J. W. & Ueda, H. (2003). Genus *Thermocyclops* Kiefer, 1927. pp 214–302. In: Ueda H. & Reid J. W. (Eds), *Copepoda: Cyclopoida. Genera Mesocyclops and Thermocyclops*. vol. *20*, in H. J. F. Dumont(Ed.), Guides to the Identification of the Microinvertebrates of the Continental Waters of the World. Leiden: Backhuys Publishers.

Montero-Pau, J., Gómez, A. & Muñoz, J. (2008). Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs. *Limnology and Oceanography: Methods*, *6*, 218–222.

Palumbi, S. R. & Benzie, J. (1991). Large mitochondrial DNA differences between morphologically similar Penaeid shrimp. *Molecular Marine Biology and Biotechnology*, *1*, 27–34.

Petrusek, A., Hobaek, A., Nilssen, J. P., Skage, M., Cerny, M., Brede, N. & Schwenk, K. (2008). A taxonomic reappraisal of the European *Daphnia longispina* complex (Crustacea, Cladocera, Anomopoda). *Zoologica Scripta*, *37*, 507–519.

Potter, E. A. & Szatmari, P. (2009). Global Miocene tectonics and the modern Word. *Earth-Science Reviews*, *96*, 279–295.

Price, M. N., Dehal, P. S. & Arkin, A. P. (2010). FastTree 2 - approximately maximum-likelihood trees for large alignments. *PLoS ONE*, *5*, e9490.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*, 539–542.

Scheihing, R., Cardenas, L., Nespolo, F. R.Krall, P., Walz, K., Kohshima, S. & Labarca, P. (2010). Morphological and molecular analysis of centropagids from the high Andean plateau (Copepoda: Calanoidea). *Hydrobiologia*, *637*, 45–52.

Schmeil, O. (1892). *Deutschlands freilebende Süßwasser-Copepoden, I Teil: Cyclopidae* (p. 191). Stuttgart: Theodor Fischer.

Schmitt, T. (2007). Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. *Frontiers in Zoology*, *4*, 11.

Schmitt, T. & Varga, Z. (2012). xtra-Mediterranean refugia: The rule and not the exception? *Frontiers in Zoology*, *9*, 22.

Schwenk, K., Sand, A., Boersma, M., Brehm, M., Mader, E., Offerhaus, D. & Spaak, P. (1998). Genetic markers, genealogies and biogeographic patterns in the cladocera. *Aquatic Ecology*, *32*, 37–51.

Spears, T., Abele, L. G. & Kim, W. (1992). The Monophyly of Brachyuran Crabs: A Phylogenetic Study Based on 18S rRNA. *Systematic Biology*, *41*, 446–461.

Sukumaran, J. & Holder, M. T. (2010). DendroPy: a Python library for phylogenetic computing. *Bioinformatics*, *26*, 1569–1571.

Taberlet, P., Fumagalli, L., Wust-Saucy, A. G. & Cossons, J. F. (1998). Comparative phylogeography and postglacial colonization routs in Europe. *Molecular Ecology*, *7*, 453–464.

Talavera, G. & Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology*, *56*, 564–577.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*, 2731–2739.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*, 4673–4680.

Vaidya, G., Lohman, D. J. & Meier, R. (2011). SequenceMatrix: concatenation software for the fast assembly of multi-gene datasets with character set and codon information. *Cladistics*, *27*, 171–180.

Wyngaard, G. A., Hołyńska, M. & Schulte, J. A. (2010). Phylogeny of the freshwater copepod *Mesocyclops* (Crustacea: Cyclopidae) based on combined molecular and morphological data, with notes on biogeography. *Molecular Phylogenetics and Evolution*, *55*, 753–764.

Xia, X. (2013). DAMBE5: a comprehensive software package for data analysis in molecular biology and evolution. *Molecular Biology and Evolution*, *30*, 1720–1728.

Zwickl, D. J. (2006). *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion*. PhD Thesis. The University of Texas at Austin.

Supporting Information

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

Table S1. List of analysed species and populations including numbers of individuals examined morphologically and genetically.

Table S2. List of primers used in PCR reactions.

Tables S3–S8. Estimates of evolutionary divergence within and between *Cyclops* species for each of the six molecular markers (12S rRNA, 16S rRNA, COI, Cytb, 18S rRNA and ITS-1) used in this study. The analysis was conducted using Kimura's 2-parameter (K2P) model in MEGA v5.

Fig. S1. Sequence variation and relationships of *Cyclops* populations based on the mitochondrial 12S rRNA gene shown on a maximum-likelihood tree.