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Molecular-genetic-based contribution to the taxonomy of the *Acanthocyclops robustus* group

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Long-standing taxonomic problems involving the *Acanthocyclops robustus–vernalis* complex of freshwater cyclopoids have not been resolved. After Kiefer's designation of *A. robustus* Sars as an older synonym of *Acanthocycops americanus* Marsh, a lot of data indicating their differentiation have been accumulated. To handle this taxonomical problem, representative populations from type localities of the respective taxa and from other European and US sites were analyzed morphologically and genetically using mitochondrial COI and 12S rRNA. Molecular-genetic analysis revealed that the three species described at the end of the nineteenth century: *A. robustus*, *A. americanus* and *A. vernalis* are well-separated species with genetic distances between them of around 20% for both genes. Within *A. vernalis* we also found substantial genetic variability (5–10%). The recently described species *A. trajani* corresponds to *A. americanus*, and *A. einslei* to *A. robustus*. *A. americanus* is re-described and its re-establishment as a valid species is suggested.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:68FC2C52-54FF-417D-9057-7B822BC7F15A

Keywords: *Acanthocyclops americanus*; *Acanthocyclops robustus*; taxonomy; phenotypic plasticity; COI; 12S rRNA

Introduction

At the end of the nineteenth century three species of the *Acanthocyclops robustus* group were described: *Acanthocyclops vernalis* by Fisher (1853) from the St Petersburg area (Russia); *Acanthocyclops robustus* by Sars (1892) from the Oslo area (Norway); and *Acanthocyclops americanus* by Marsh (1892) from Wisconsin (USA). Since the first finding of *A. americanus* in Europe by Lowndes (1926, 1928a,b), this species has been found in other European countries (Dussart 1967, 1971; Alekseev and Kossova 1976) and the form *A. americanus* f. *spinosa* was described by Monchenko (1961) in Ukraine. The main European freshwater copepod fauna identification keys, such as Gurney (1933), Rylov (1948), Dussart (1969), Monchenko (1974) and Alekseev (1998), recognized *A. americanus* as a distinct taxon.

All these species show high variability; the spine formula, once used as a taxonomic character for the differentiation of *vernalis* and *robustus* is not stable but shows inter- and intra-population variability, including bilateral asymmetry in the same individual.

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Another character used that was also not stable was the conversion into spine of the outer seta of the distal segment of the Leg 4 endopodite. Because of this, Petkovski (1975) in his revision of "*vernalis*" group in Yugoslavia, summarized the chaotic state of the taxonomy of the species of this group and described *A. robustus* f. *limnetica*, which corresponded to *A. americanus*. Then, Kiefer (1976, 1978), trying to solve this same taxonomic problem, replaced the name *A. americanus* with *A. robustus* and merged the two taxa. Kiefer came to this conclusion after comparing specimens from Marsh's and Sars' collections, but not original type materials (collected respectively in Wisconsin, USA and Lake Maridaslvan, Oslo vicinity) because in both cases the type material was lost. Figures and descriptions of Kiefer's *A. robustus* from Lake Mjosa near Oslo (but not from the type locality) corresponded to *A. americanus*, possibly owing to examination of more pelagic samples and/or invasion of *A. americanus* at that site. Kiefer's synonymy was accepted by most authors and *A. americanus* almost disappeared from distribution and ecological studies of planktonic organisms, and also from identification keys, such as the world identification guide of Einsle (1996) for this genus of cyclopoids.

However, there was too much variability within one species and *A. robustus* has been recently split again. For related North American taxa, Dahms and Fernando (1997) re-described *A. brevispinosus* (Herrick) and separated it from *A. robustus* and confirmed its validity following Dodson's (1994) earlier proposition. Alekseev et al. (2002) confirmed that *A. robustus sensu* Sars really exists and it was found in Belgium together with *A. vernalis* and *A. americanus*. All three "species" could be easily separated one from one another. These authors raised the question of re-establishment of *A. americanus* as a valid species and its separation from *A. robustus*. On the other hand, Mirabdullayev and Defaye (2002) re-described *A. robustus* from Kiefer's samples of Lake Mjosa, but since there were only females in those samples, to describe the male they used specimens from Lappland (Sweden) from the Kiefer collection. However, the male they described corresponded to *A. americanus* according to the infallible character of the sixth leg. In the same work, a new species is described, *A. trajani*, and among the listed synonyms for this species are *A. americanus* (Marsh, 1893) and *A. americanus* f. *spinosa* (Monchenko, 1961). Another species of the same group, *A. einslei*, was also later described by the same authors (Mirabdullayev and Defaye 2004).

Besides morphology, Kiefer (1978) as well as others (Petkovski 1975; Fryer 1985) recognized differences in the ecology of *A. vernalis* and "*A. robustus*" *sensu* Kiefer (defined by the round shape of the female abdominal segment); the latter was considered pelagic or limnetic whereas *A. vernalis* was considered littoral–benthic. Similar observations but with other species nomenclature were pointed out by Alekseev et al. (2002), who indicated that *A. vernalis* and *A. robustus* inhabit the near-shore area and/or near-bottom zone in lakes, and are very rarely collected mixed with truly planktonic species, while *A. americanus* is a pelagic species inhabiting eutrophic water bodies. Alekseev (1986) also documented differences in behaviour between nauplii of *A. vernalis* and *A. americanus* that reflect the ecological preferences of each species; nauplii of *A. vernalis* react by sinking down and staying in the bottom when disturbed, but not the nauplii of *A. americanus*, which remain wandering randomly.

Just recently a molecular-genetic approach (Bláha et al. 2010) has been undertaken on this species complex, which has shown the necessity of molecular studies in species with morphological stasis, i.e. species with very slow rates of morphological change relative to molecular evolution. The present article is mainly devoted to testing, by

means of mitochondrial DNA variation (COI; 12S rRNA), the taxonomic status of the European species of the *vernalis–americanus–robustus* complex and the validity of new designations, in recent times, of members of this complex. To be able to draw reliable taxonomical conclusions we sequenced specimens from type localities. We also tested whether forms that differed in a plastic character, used as a taxonomic character in the past, diverged or not in these mitochondrial genes.

Materials and methods

Selection of populations

To obtain relevant results from the taxonomic point of view we sampled type localities (type regions when a specific water body was not given in the description). Specimens used were collected with a sweep net from: (1) Peterhoff, St Pertersburg, Russia, type locality for *A. vernalis* (Fisher, 1853); (2) Lake Maridalsvann, Oslo, Norway, type locality for *A. robustus* (Sars, 1892); (3) two ponds in Dane County, Wisconsin, USA, *terra typica* for *A. americanus*; and (4) Etang de Noes, France, type locality for *A. trajani* (Mirabdullayev and Defaye, 2001). Individuals of the *A. robustus* complex from several other localities in eastern Spain (Tarragona–Valencia), France (three localities near Paris) and the USA (near Washington DC and Arizona) were also collected in the same way, to be compared using both molecular-genetic and morphological approaches. From eastern Spain we included samples from localities with different ecologies (a pond in a city park, two reservoirs, a coastal lagoon, two rice paddies and a quarry pond).

Genetic analysis

Genetic analysis was performed on pre-identified species and forms selected from ethanol-preserved samples. DNA was extracted from single whole individuals following the standard protocol procedure from InstaGene Matrix (Bio-Rad). Fragments of two different genes (COI and 12S rRNA) were amplified from Chelex nucleic acid extraction by polymerase chain reaction (PCR). A COI region was amplified using forward and reverse primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198. (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). The PCR conditions were: initial denaturing step at 94°C (2 min) followed by five cycles of denaturing, annealing and extension of 94°C (40 s), 45°C (40 s), 72°C (60 s), and 35 cycles of 94°C (40 s) denaturing, 51°C (40 s) annealing and 72°C (60 s) of extension with final extension step at 72°C (5 min).

A 12S region was amplified using forward and reverse primers L13337-12S (5′-YCTACTWTGYTACGACTTATCTC-3′) and H13842-12S (5′-TGTGCCAG CASCTGCGGTTAKAC-3′) (Machida et al. 2004). The PCR conditions were: initial denaturing step at 95°C (4 min) followed by 40 cycles of denaturing, annealing and extension of 94°C (45 s), 60°C (45 s), 72°C (90 s), final extension step at 72°C (6 min).

The PCR products were purified by the "High Pure PCR Product Purification Kit" (Roche). Sequencing was carried out by the ABI PRISM BigDye Terminator version 3.1 system (Applied Biosystems) on an ABI 3730 automated sequencer. Almost all PCR products were sequenced in both directions by the amplified primers and were overlapped to obtain COI (658 bp) and 12S rRNA (426 bp) gene sequences. Altogether, 52 individuals (accession numbers KC016141-192 and KC130335-353 for the COI and

12S genes, respectively) representing the European species of the complex were used in sequencing and phylogenetic analysis. For comparative purposes, the set of 12S rRNA sequences from GenBank (accession numbers presented in Table 1) corresponding to the work of Bláha et al. (2010) were included in the phylogenetic studies. This set of GenBank sequences had only 343 bp, thus phylogenetic relationships were restricted to this number, but the longer 426 bp 12 rRNA sequences from our specimens are available on GenBank.

Obtained sequences were verified, corrected and assembled with STANDEN software and aligned by ClustalW algorithm in MEGA 5 software (Tamura et al. 2011). Phylogenetic relationships were evaluated by several approaches conducted separately for mitochondrial genes (MP, ML and BI). For maximum likelihood analysis, the Hasegawa–Kishino–Yano+G (HKY+G) and Tamura 3-parameter (T92+G) were selected as best-fit models of nucleotide substitution by MEGA 5 software (Tamura et al. 2011) using the Bayesian Information Criterion (BIC) for COI and 12S, respectively. Maximum likelihood trees were constructed with MEGA 5 software (Tamura et al. 2011) using the previously determined models of nucleotide substitution; gamma distribution was approximated using five rate categories and nearest-neighbour interchange (NNI) was used as heuristic method for tree inference. Nodal support for the resulting branches was estimated with 1000 bootstrap replications. Maximum parsimony (MP) trees were constructed using MEGA 5 software (Tamura et al. 2011) with heuristic search based in close-neighbour interchange (CNI) as branch-swapping algorithm and support for the nodes was evaluated by bootstrapping with 1000 pseudoreplicates. BI phylogeny was obtained using MrBayes 3.2 (Ronquist et al. 2012) under the best evolutionary models described and two runs of four chains during 10 million generations. The first 10% of trees were discarded as burn-in and a majority rule consensus tree was obtained from the remaining samples after the two runs were combined. Genetic divergence was estimated by the Kimura 2-parameter (K2P) distance between haplotypes.

Results

Morphological results

Several morphological differences between the specimens from type localities were found to assign the specimens unambiguously to the mentioned species of *Acanthocyclops*. *A. vernalis* (Figure 1) from two sites in the St Petersburg area could be easily differentiated from all the others by the shape of the genital somite and the receptaculum seminis (RS). The three main characters for the diagnosis of this species were: (1) the upper part of the female genital somite wide and angular with sharpening lobes, its maximum width approximately equal to its length, RS with an anterior depression; (2) two apical spines of endopodite 3 of Leg 4 of unequal length, internal shorter than external; (3) male rudimental Leg 6 with strong inner spine and two setae, middle seta significantly shorter than spine, outer seta about 1.3–2.3 times as long as inner spine.

A. robustus (Figure 2) from Lake Maridalsvann had: (1) a more round genital segment without sharpening lobes, although somewhat triangular; (2) apical spines of end3P4 sub-equal, but internal spine usually longer than external one, and outer lateral spine of end3P4 inserted much more distally than inner seta; (3) male Leg 6 with a very

Table 1. List of analyzed *Acanthocyclops* individuals, indicating collection localities and dates, as well as specimens' haplotypes and accession numbers of COI and 12S rRNA sequences. Specimens from type localities are shown in bold and set apart with lines. Localities and other data for identically coincident haplotypes from GenBank are also indicated.

Taxon	Locality	Country	Habitat	Collection date	Analyzed genes	Haplotypes	COI Accession nº	12S Accession nº
A. vernalis	**Peterhof, S.Petersburg**	Russia	pond littoral	2009/09	COI, 12S	COIv1, 12Sv1	KC016192	KC130353
A. vernalis	**Peterhof, S.Petersburg**	Russia	pond littoral	2009/09	COI, 12S	COIv2, 12Sv1	KC016188	KC130350
A. vernalis	**Peterhof, S.Petersburg**	Russia	pond littoral	2009/09	COI, 12S	COIv3, 12Sv2	KC016189	KC130351
A. vernalis	**Peterhof, S.Petersburg**	Russia	pond littoral	2009/09	COI, 12S	COIv4, 12Sv2	KC016190	KC130352
A. vernalis	**S. Petersburg area**	Russia	brook	2008/09	COI	COIv5	KC016191	-
A. robustus/ enslei	**L. Maridalsvann, Oslo**	Norway	lake littoral	2009/06	COI	COIr1	KC106182-83	-
A. robustus/ enslei	**L. Maridalsvann, Oslo**	Norway	lake littoral	2009/06	COI, 12S	COIr1, 12Sr1	KC016184-87	KC130348-49
A. americanus*	**Madison, Wisconsin**	USA	pond	2005/07	COI	COIa1	KC016157-8	-
A. americanus	Washington, DC	USA	pond	2009/05	COI	COIa1	KC016152	-
*A. americanus**	Kino Springs, Arizona	USA	Spring pond	2009/03	COI	COIa1	KC016154	-
*A. americanus**	Kino Springs, Arizona	USA	Spring pond	2009/03	COI, 12S	COIa1, 12Sa1	KC016155-56	KC130339-40
A. trajani/americanus *	**Noes, near Paris**	France	pond	2009/03	COI	COIa1	KC016164-69	-
*A. americanus**	L. Creteil, near Paris	France	lake	2009/03	COI	COIa1	KC016161	-
*A. americanus **	Corteza, Sinarcas	Spain	pond	2006/03	COI	COIa1	KC016178	-
*A. americanus**	Turia park, Valencia	Spain	pond	2009/04	COI, 12S	COIa2, 12Sa1	KC016180-81	KC13046-47
*A. americanus**	L. Creteil, near Paris	France	lake	2009/03	COI	COIa3	KC016163	-
*A. americanus**	Bois Boulogne, Paris	France	pond	2009/03	COI	COIa3	KC16159-60	-
*A. americanus**	L. Creteil, near Paris	France	lake	2009/11	COI, 12S	COIa4, 12Sa2	KC016162	KC130341
A. americanus	Guiamets, Tarragona	Spain	reservoir	2010/07	COI	COIa3	KC016169-70	-
A. americanus	Guiamets, Tarragona	Spain	reservoir	2010/07	COI, 12S	COIa3, 12Sa2	KC016173	KC130343

(Continued)

Table 1. (Continued).

Taxon	Locality	Country	Habitat	Collection date	Analyzed genes	Haplotypes	COI Accession nº	12S Accession nº
A. americanus	Riba-Roja, Ebro river	Spain	reservoir	2010/09	COI	COIa3	KC016174	-
A. americanus	Riba-Roja, Ebro river	Spain	reservoir	2010/09	COI, 12S	COIa3, 12Sa2	KC016176	KC130345
A. americanus	Guiamets, Tarragona	Spain	reservoir	2010/07	COI, 12S	COIa5, 12Sa3	KC016172	KC130342
A. americanus	Riba-Roja, Ebro river	Spain	reservoir	2010/09	COI, 12S	COIa5, 12Sa3	KC016175	KC130344
A. americanus	Albufera Valencia	Spain	coastal lagoon	2009/04	COI, 12S	COIa5, 12Sa3	KC016146	KC130338
A. americanus *	Albufera Valencia	Spain	coastal lagoon	2010/01	COI	COIa6	KC016145	-
A. americanus *	Albufera Valencia	Spain	coastal lagoon	2010/01	COI, 12S	COIa6, 12Sa4	KC016144	KC139337
A. americanus	Albufera Valencia	Spain	lagoon littoral	2009/04	COI	COIa6	KC016147-51	-
A. americanus *	Albufera Valencia	Spain	lagoon littoral	2010/01	COI, 12S	COIa6, 12Sa4	KC016143	KC130336
A. americanus	Albufera Valencia	Spain	rice field	2010/01	COI	COIa6	KC016142	-
A. americanus *	Albufera Valencia	Spain	rice field	2010/01	COI, 12S	COIa6, 12Sa4	KC016141	KC130335
A. americanus	Kino Springs, Arizona	USA	spring pond	2009/03	COI	COIa7	KC016153	-
A. americanus	Guiamets, Tarragona	Spain	reservoir	2010/07	COI	COIa8	KC016171	-
A. americanus *	Corteza, Sinarcas	Spain	quarry pond	2006/03	COI	COIa9	KC016179	-
A. americanus *	Corteza, Sinarcas	Spain	quarry pond	2006/03	COI	COI10	KC016177	-

Bláha et al. 2010 : 12S haplotypes coincident with haplotypes of this study

Taxon	Locality	Country	Habitat		Analysed genes	Haplotype Bláha et al.	Coincident with	12S Accession nº
A. vernalis	Volynka river, Strakonice	Czechia	river littoral	·	12S	V1	12Sv2 (2 ind)	FN821976
A. einslei	Skolov	Czechia	pool	·	12S	E2	12Sr1 (2 ind)	FN821967
A. trajani	Doiranis	Greece	lake	·	12S	T5	12Sa2 (3 ind)	FN821974
A. trajani	Petron	Greece	lake	·	12S	T6	12Sa3 (3 ind)	FN821975

*indicates *A. americanus f. spinosus*

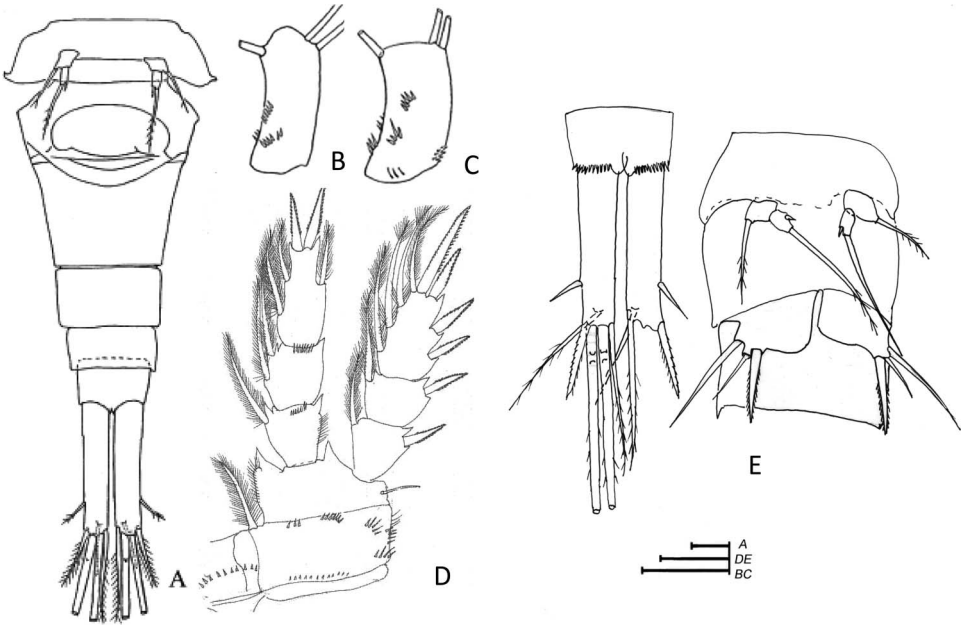

Figure 1. *Acanthocyclops vernalis* (Fisher) female (A–D) and male (E) from the type locality, Peterhof, St Petersburg, Russia. (A) Abdomen ventral view; (B) antennal basipod frontal side; (C) antennal basipod caudal side; (D) Leg 4 with coxa and intercoxal sclerite; (E) furca, and Legs 5 and 6.

Notes: D and E are from the same samples used for molecular genetics, registered in Table 1.

strong inner spine longer than middle seta and slightly shorter than outer seta. The discriminative character, specifically, the group of spinules in antennal basepodite, near exopodite seta, used by Mirabdullayev and Defaye (2002, 2004) to separate *A. robustus* from the two other species was not present in the specimens from this site (Figure 2). Microcharacters of the antennal basipodite are good taxonomic characters for species discrimination in some genus of cyclopoids such as *Eucyclops* or *Mesocyclops*, but are too variable at population level in the genus *Acanthocyclops* to be used for species separation. In a studied *A. robustus* population from one sampling point in River Jucar (Antella, Spain), only 30% of individuals had this group of spinules. This character was also found to be subject to variability, even within a small localized *Acanthocyclops* population in the Missouri River and an intermittent river in the Great Lakes area (Lewis et al. 2004).

A. americanus (Figures 3–6) from Wisconsin and *A. trajani* from Etàng de Noes were morphologically alike and also equal to the other specimens from the USA, Spain and France (Table 1) used for molecular-genetic analyses that are grouped together in one clade (see "Molecular-genetic results"). In this species, the main discriminative characters were: (1) genital somite rounded and wider in its anterior part; (2) length of apical spines of end3P4 usually unequal, internal longer than external, position of lateral outer seta/spine of end3P4 approximately at the same distance as lateral inner second seta; (3) male rudimental Leg 6 consists of a weak inner spine and two setae, the middle one practically equal in length to spine and the outer seta more than 2.0–2.5 times length of inner spine.

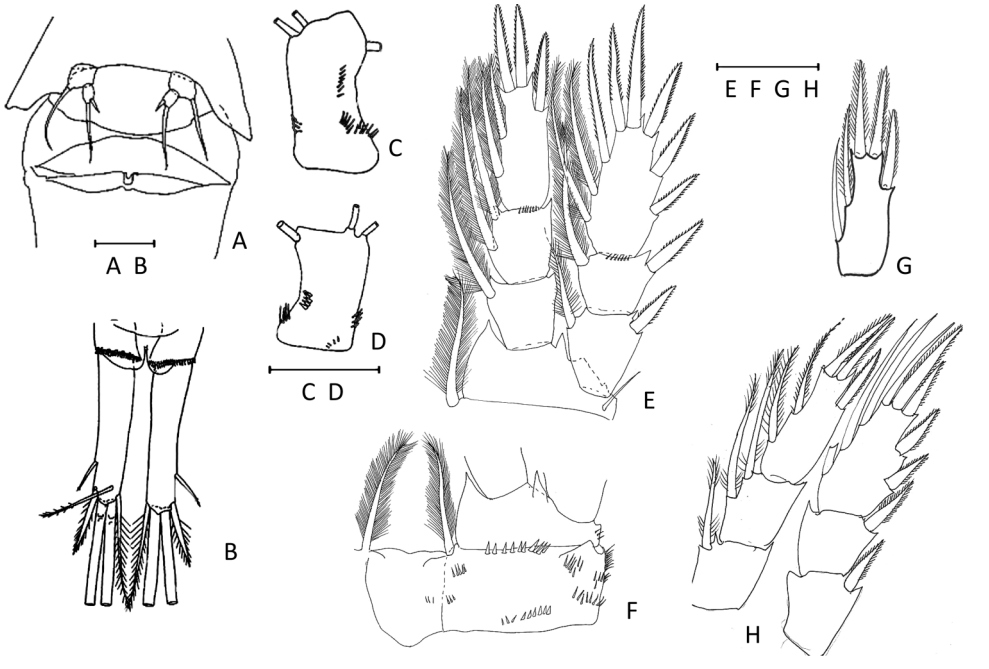

Figure 2. *Acanthocyclops robustus* (Sars) female (A–F) and male (G–H) from the type locality, Lake Maridalsvann Oslo, Norway. (A) Leg 5 and genital segment in ventral view; (B) furca; (C) antennal basipod frontal side (D) antennal basipod caudal side; (E) Leg 4; (G) Leg4 distal segment of endopod; (H) Leg 4 endopod and exopod.

Notes: All from the same samples used for molecular genetics, registered in Table 1.

None of the three original descriptions or illustrations of *A. americanus–vernalis–robustus* is adequate according to present-day standards. We do not see any reason to discriminate the name *americanus* and we consider it as an older synonym than *trajani.* For this reason we include a description of this species with neotypes from *terra typica*, also used in molecular-genetic analyses.

Description of A. americanus

A holotype of *A. americanus* (Marsh) was not selected by the author and a sample from the Marsh collection in the US National Museum in Washington labelled as containing *A. americanus*, according to Mirabdullayev and Defaye (2004), contained a mixture of several *Acanthocyclops* species. To solve the complicated situation with species of the *robustus* group a morphological re-description of *A. americanus* needs to be made from the same material used in the molecular-genetic study. This cannot be done for old alcohol-preserved copepods. In accordance with paragraph 74A ICZN in the case when the old *type seria* contains several closely related species a neotype can be selected from another group but collected in *terra typica*. For this study we selected an adult female as the neotype of *A. americanus* (Marsh) and a male as the allotype from a pond in Madison, Wisconsin, USA (near

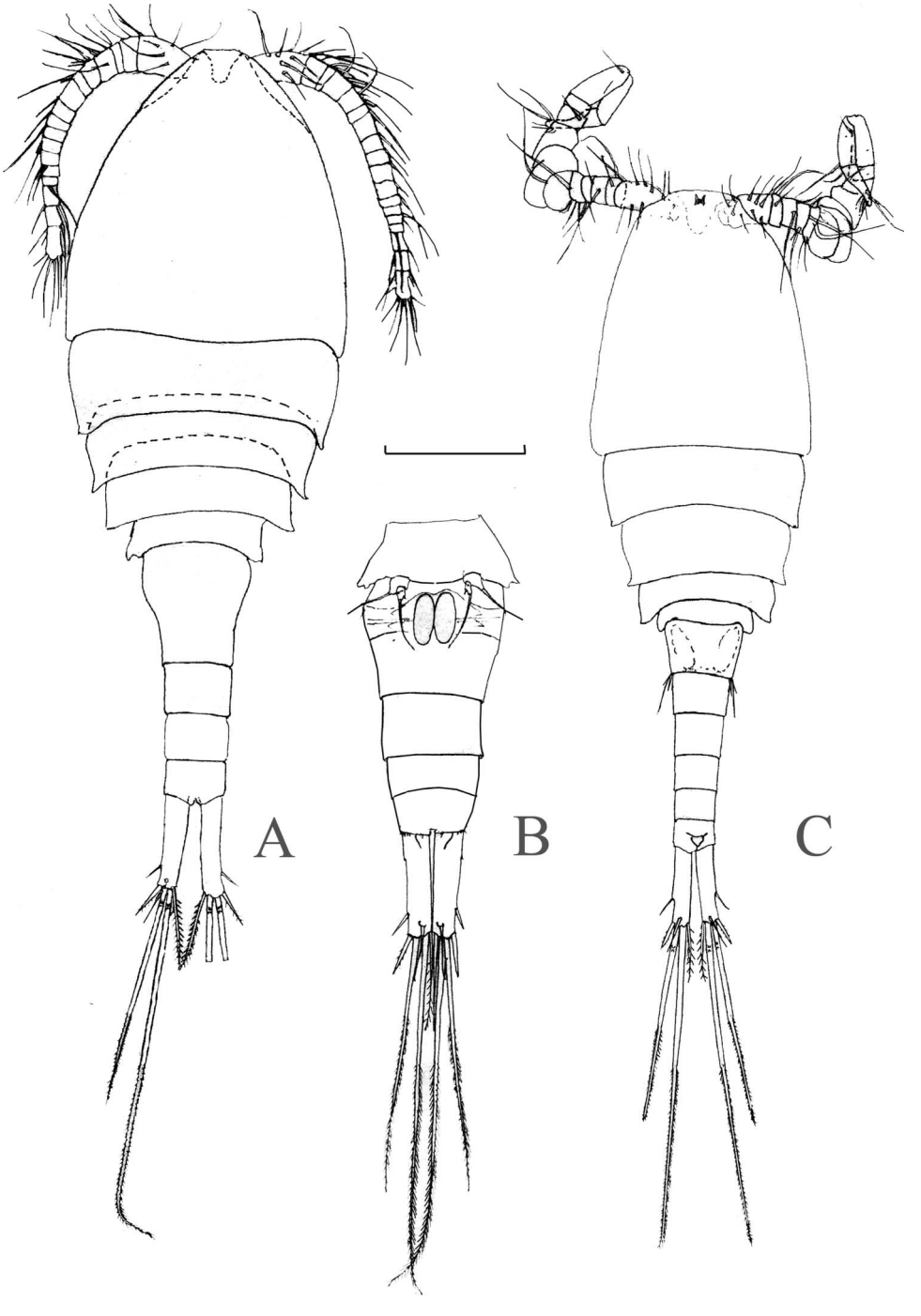

Figure 3. *Acanthocyclops americanus* (Marsh) from the *terra typica*, Madison, Wisconsin, USA. (A–B) Female neotype; (C) male allotype.

Notes: Scale bar = 200 μm; neotype and allotype are from same samples used for molecular genetics, registered in Table 1, as are those in Figures 4–6).

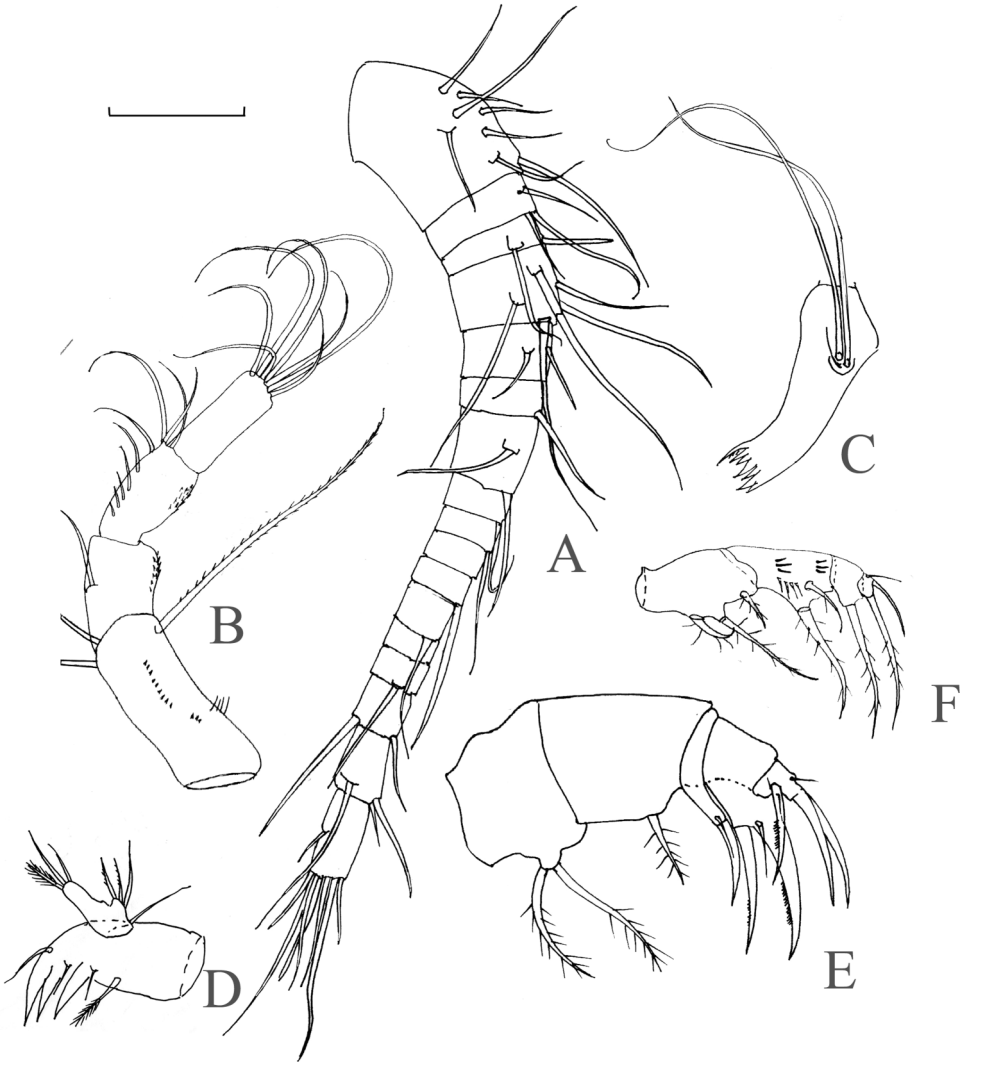

Figure 4. *Acanthocyclops americanus* (Marsh) female neotype from the *terra typica*, Madison, Wisconsin, USA. (A) Antennule; (B) antenna; (C) mandible; (D) maxillule; (E) maxilla; (F) maxilliped.

Note: Scale bar = 50 μm.

James Madison Memorial High School) collected in July 2005 by Dr Stanley Dodson. Once identified, specimens were divided in two sub-groups: one for molecular-genetic analysis and the other to provide the neotype, allotype and descriptive material. The neotype and allotype have been deposited in the Zoological Institute, St Petersburg, Russia. Hereafter a re-description of *A. americanus* (Marsh, 1893) is provided.

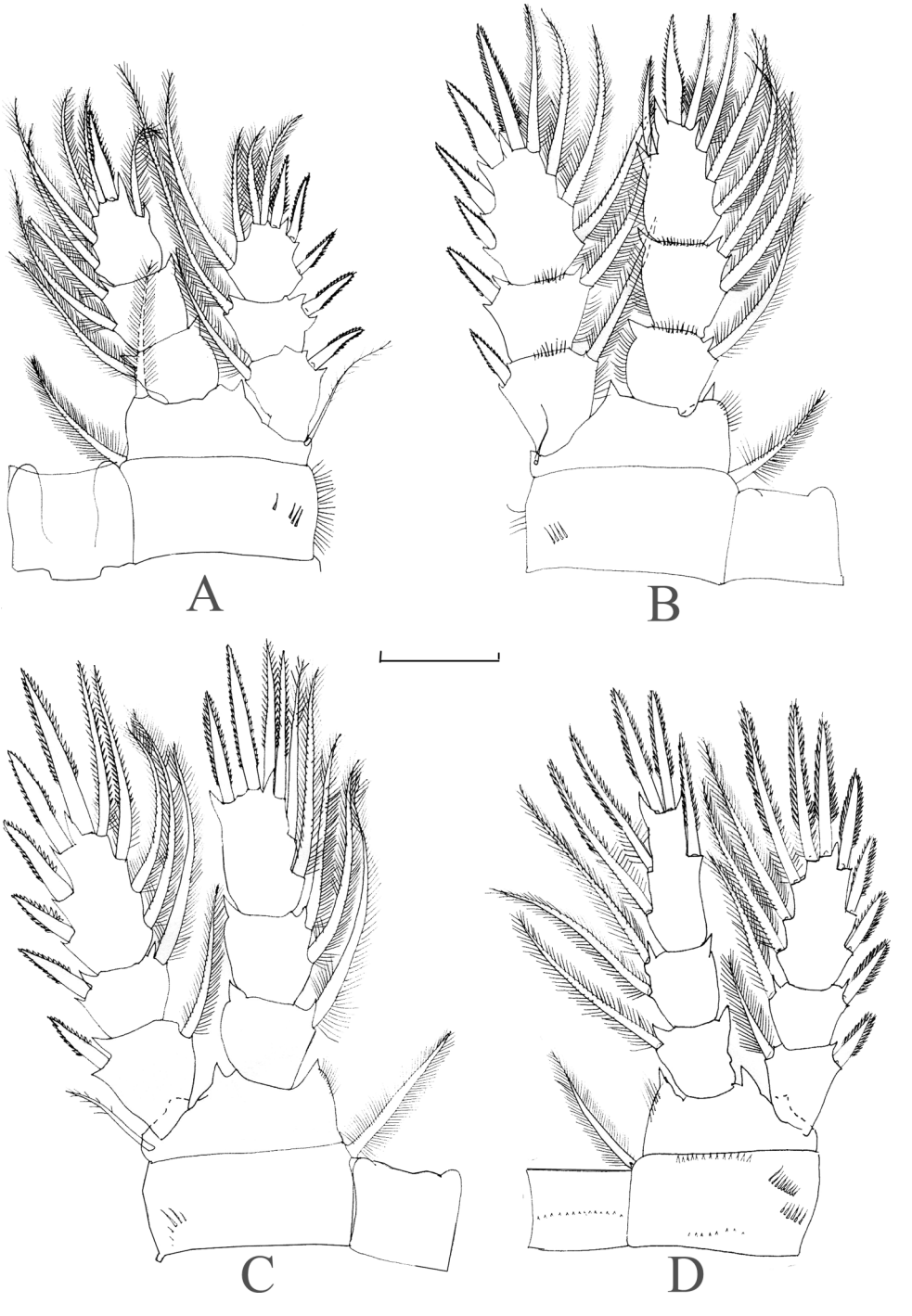

Figure 5. *Acanthocyclops americanus* (Marsh) female neotype from the *terra typica*, Madison, Wisconsin, USA. (A–D) Swimming legs 1–4, in order.

Note: Scale bar = 50 μm.

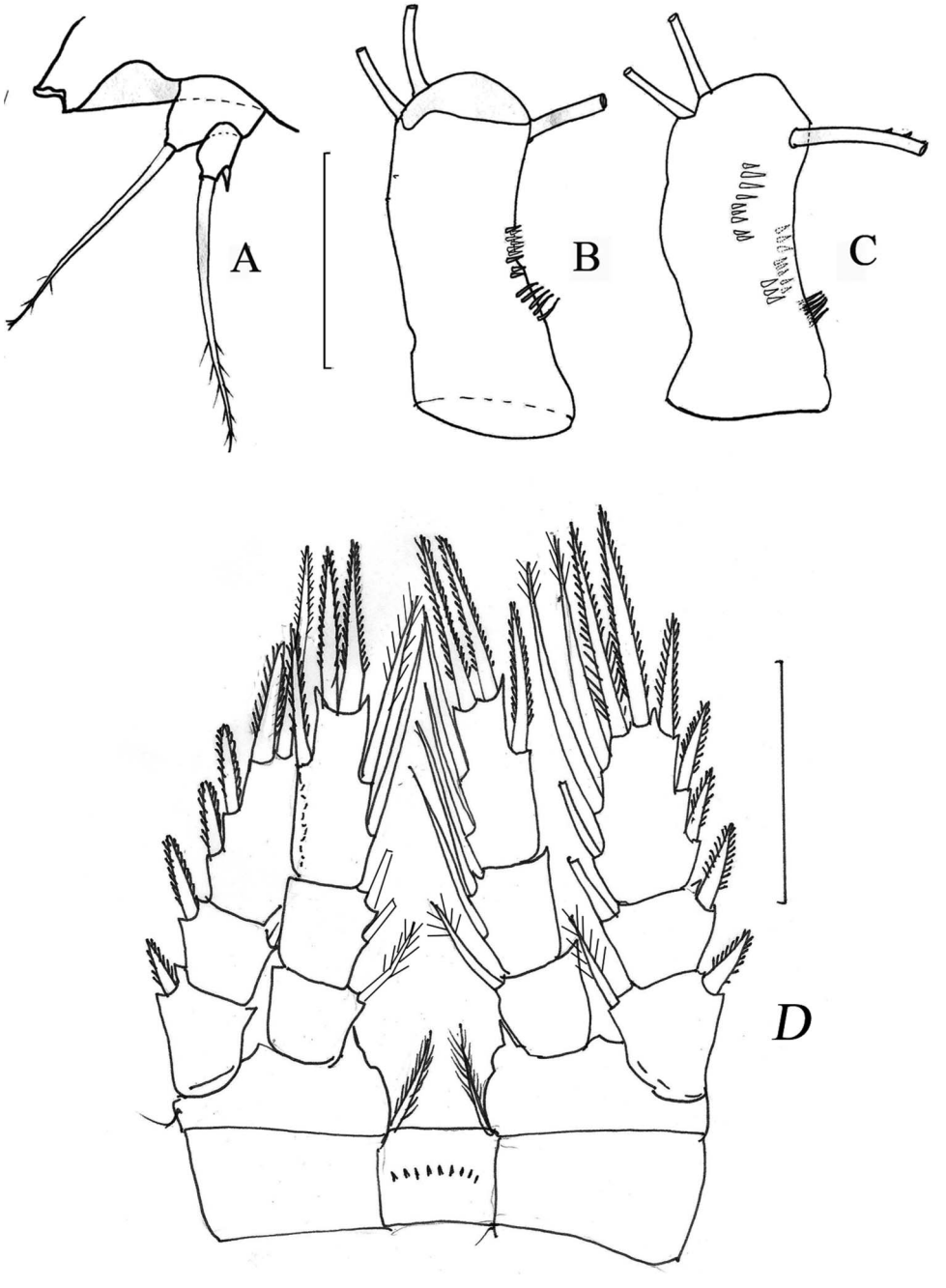

Figure 6. *Acanthocyclops americanus* (Marsh) female neotype (A–C) and male allotype (4) from the *terra typica*, Madison, Wisconsin, USA. (A) Female Leg 5; (B) female antennal basipod frontal side; (C) female antennal basipod caudal side; (D) male Leg 4.

Note: Scale bar 50 μm.

Acanthocyclops americanus (Marsh, 1893)

Acanthocyclops robustus f. *limnetica* Petkovski, 1975.

Acanthocyclops robustus Kiefer (1976) part., Fryer (1985), Dodson (1994), Lescher-Moutoué (1996), Einsle (1996).

Acanthocyclops trajani Mirabdullayev and Defaye 2002.

Material examined. All populations of this species used in genetic analyses are registered in Table 1, and include material from *americanus terra typica* localities (previously mentioned pond in Madison, Wisconsin, leg. Dr S. Dodson and a pond in city park, Washington DC, USA, leg. Dr V. Ivanenko) and the *A. trajani* type locality (Etang de Noes, vicinity of Paris, coll. V. Alekseev).

Additional material. USA: pond in California, leg. Dr B. Kuperman; Canada: pond in Waterloo, Ontario, leg. Dr H. Fernando; Canada: Lake Pin-Rouge, Montreal University field station, Quebec, 9 October 1998, coll. V. Alekseev; Mexico: pond in Mexico City, 12 October 2009 coll. V. Alekseev; Russia: small river in the River Volga delta, May 1984, coll. V. Alekseev; Russia: small lake in northeast Tchukotka, June 1985, leg. Dr P. Krylov; Russia: the River Ob delta, western Syberia, leg, Dr V. Kuzikova; Russia: Posolsky Bay, Baikal, central Siberia, 2 July 1986, coll. V. Alekseev; Russia: rice fields, Krasnodar vicinity, July 1987, leg, O. Feraponova; Russia: lake in tundra, Tchaun, Magadan vicinity, 15 July 1982, leg, Dr E. Streletzkaya; Russia: pond in Pushkin Town, Leningrad district, 28 June 1997, coll. V. Alekseev; Latvia: Riga Bay, 22 July 1997, coll. V. Alekseev; Armenia: Lake Sevan, 4 July 1990, coll. V. Alekseev; Ukraine: pond in Krymia peninsula, Simferopol vicinity, 2 May 1990, coll. V. Alekseev; Ukraine: pond in Kiev vicinity, August 2011, coll. V. Monchenko; Norway: pond in City Park, Oslo, 23 June 2009, coll. V. Alekseev; Germany: canal in Oldenburg, July 1997, coll. V. Alekseev; Belgium: pond in university botanical garden, Ghent, 23 June 1989, coll. V. Alekseev; France: pond in botanical garden, Boulogne-sur-Mer, 20 July 2007, coll. V. Alekseev; Spain: Ebro River delta, June 2003, coll. M.R. Miracle; Spain: Guadalquivir River, Sevilla, 4 January 2009, coll. V. Alekseev; Egypt: River Nile, Cairo, 12 April 2003, coll. V. Alekseev.

Redescription of the neotype female and allotype male. Female neotype dissected on slide N 56736 and placed into the type collection of Zoological Institute of the Russian Academy of Sciences, St Petersburg, Russia. The type series also includes two females and one male preserved in pure glycerol.

Female neotype. Body transparent, colourless. Full body length without furcal seta 1217 μm. Egg sacs with 35–40 eggs each. Cephalosome as long as wide, with maximum width close to its end (Figure 3A).

Genital double somite 1.1 times as long as wide, with seminal receptacle with wide transparent zone in its frontal part (Figures 3A–B). Anal segment with row of small dense dents, proctodeum with single row of setules on both sides. Furcal rami parallel without hairs on inner margin, five times as long as wide. Length proportions of distal setae, beginning from outer terminal seta: 1.0/5.3/9.0/2.1. Inner seta sub-equal

to furca length. Dorsal seta about 1.2 times as long as inner setae and lateral setae about half length of outer setae.

Antennule of 17 segments, not reaching distal margin of first thoracic somite. Setation of antennular segments (aesthetascs in Roman numbers) beginning from first: 8/4/2/6/3+I/1/2/1/1/0/1+I/2/0/1/2/2+I/7+I. Shortest setae of distal segment less than length of distal segment (Figure 4A). Antennal basipodite at caudal side with three lateral groups of hair-setae in middle (Figures 4B and 6B). At frontal side this segment with two groups (9+3) of strong denticules on top, long line of 15–17 relatively small spinules in the central part and two groups of several spinules as shown in Figure 6C. Third segment bearing nine seta and last segment bearing seven seta.

Gnathobase of mandible with six teeth, rudiment of endopodial segment with two long setae and short seta (Figures 4C). Maxillula with three strong and three small teeth, two strong setae; maxillar palp with seven setae, different in length (Figure 4D). Maxilla of five segments, praecoxa with two strong setae in its middle part; coxa with a strong seta in the middle and an endite bearing two claw-like setae; basal endite with two very strong claw-like spines, both with a row of spinules and small setae near the place of fusion of the rudimentary endopod. Endopodite 1 with three claw-like setae and endopodite 2 bearing distally two long setae (Figure 4E). Maxilliped of four segments, praecoxa + coxa with two strong setae in the middle part and small setae at their distal end; basis with two setae of different length and three groups of strong spinules near the insertion of the setae; first segment of endopod with strong spine and rudimentary endopod, bearing a strong spine and two hairless setae (Figure 4F).

Swimming legs 1–4 with three-segmented rami (Figure 5A–D). Distal segments of exopod of legs 1/2/3/4 with 3/4/4/4 spines, respectively. Distal segments of endopod of Legs 1–2 with one spine and five setae. Distal segment of endopod Legs 3–4 elongated, with two strong spines at its end. In Leg 4 this segment 2.6 times as long as wide and inner apical spine 1.1 times as long as outer spine. Insertion of the lateral outer seta/spine approximately at the same distance from the end of the segment as the second lateral inner seta. Intercoxal sclerite Leg 1 with high hills, and without hairs on its free edge. Intercoxal sclerites Legs 2–3 also with small hills on free edge and without hairs on its free edge (Figure 5A–C). Intercoxal sclerite Leg 4 with small hills and row of small denticules in middle part. Coxa Leg 4 with four groups of dents typical for several species of *robustus* group (Figure 5D). Rudimentary Leg 5 two-segmented, basal segment with long outer seta. Distal segment with long apical seta and short inner spine less than half of segment length (Figure 6A).

Male allotype. Dissected and mounted in glycerol surrounded with Canadian balsam on slide N 56737. One male from the type series is preserved in pure glycerol (N) and placed in slide covered with cover glass with plasticine tombs and not compressed. All material is in the collection of the Zoological Institute of the Russian Academy of Sciences (Reg. N 56736–56738). Body length without furcal seta 1030 μm.

Cephalosome 1.3 times as long as wide, with maximal width close to end of its length (Figure 3C). Caudal rami 4.3 times as long as wide, slender inner seta more than twice length of outer spine-like seta. Lateral seta placed without spinules in its base. Dorsal seta placed near inner seta insertion, about 1.2 times as long as outer seta.

Antennule 12-segmented. Setation of antennular segments (aesthetascs in Roman numbers) beginning from first: 7(III)/3/1/6(I)/1/1/1/4(I)/0/1/0/7. Antennal

basopodite ornamentation as in female. Morphology of mouth appendages and Leg 1–3 basically as in female.

Distal segment of endopod Leg 4 about three times as long as wide, with inner apical spine about as long as the segment and 1.25 times as long as outer apical spine (Figure 6D). Insertion of lateral seta/spine in this segment as in female. Inner edge of basis of Leg 4 with short hair-setae, coxa of Leg 4 with strong spine. Coxa Leg 4 on its caudal side, with same groups of dents as in female but fewer in number. Intercoxal sclerite Leg 4 without hills and hair-setae on its free edge, but with short row of small spinules in middle. Rudimentary Leg 5 two-segmented, with setae and spine of similar proportion to female. Rudimentary Leg 6 with inner spine, middle seta slightly shorter than spine and a very long outer seta (relative lengths 1.0/0.75/2.1) that clearly separates it from other species of this group.

Ecology. Planktonic, often dominating in eutrophic water bodies.

Distribution. Described from North America, it is widely distributed in North and Central America, in the tropics mainly in mountain lakes. In Europe it was first found in the beginning of the twentieth century in England, but since then it has been frequently found in all European countries (named *A. robustus*, after Kiefer's revision). Its area seems to be expanding occurring mainly in coastal water bodies, fish ponds and reservoirs. It has been found also in Africa, Asia and South America.

Phenotypic plasticity. Swimming legs spine formula is seasonally variable as well as the conversion to spine of the lateral seta of endopodite 3P4 (this gave rise to the name *A. americanus spinosus* Monchenco, 1961).

Collections from the Natural Park of Albufera of Valencia have been reanalyzed to look for phenotypic variability. This Natural Park is a coastal wetland near the town of Valencia, consisting mainly of a coastal lagoon surrounded by a marshland now transformed into rice fields and irrigation channels where the dominant species is *A. americanus*, previously referred to as *A. robustus* (Oltra and Miracle 1992; Alfonso and Miracle 1990). As in other localities, *A. americanus* in Albufera of Valencia showed a significant variability in size: females, 850–1300 μm and males, 670–990 μm, according to summer–winter development. Spine formula of exopods was mostly 3444/3444, and only a small fraction of individuals showed some variation. In summer a few individuals had the formula 3443/3443 and in winter we found individuals with 3444/4444, although other combinations were also possible, always with an important degree of asymmetry, such as 3344/3444. Dodson (1994) described a similar morphological seasonality in his "*A. robustus*" specimens and separated them into two forms, "cold-water" and "warm-water", based on the number of spiniform versus setiform setae on the terminal segment of the Leg 4 exopod.

In Albufera of Valencia Natural Park there are also seasonal variations in the dominance of forms of the *typicus* and *spinosus* type. The seasonal spinosus character of P4 endopodite seems to be related to factors, other than temperature, affecting diapause and development. In the lagoon and channels, which are permanent water bodies, the typical form of *A. americanus* is the most abundant and common one and only from November to February, a small proportion of the population was *A. americanus spinosus*. Whereas in rice fields, which are temporary habitats, the dominant form is *A. americanus spinosus* present also in summer. From these results we

relate the presence of the *spinosus* form with a population coming from diapause. The rice fields are dry during two periods: late summer–autumn and late winter–early spring, so their *A. americanus* population may have in summer as well as in winter individuals coming from diapause stages. In temporary intradunal ponds of this park the form *spinosus* is also the most frequent type found. The spine in the P4 endopodite may be of help when they move out of the sediment. In the genetic analyses we have included both forms *A. americanus* from Albufera of Valencia, with seta and with spine.

Molecular-genetic results

Diversity in the COI gene

In total, 52 individuals from 3 species and 1 form variant (Table 1) were sequenced for 658 nucleotides giving 15 haplotypes, which contained 460 variable sites and 139 parsimony informative sites. All phylogenetic methods (ML, Bayesian and parsimony) resulted in the same topology. The ML topology is presented in Figure 7. Our data

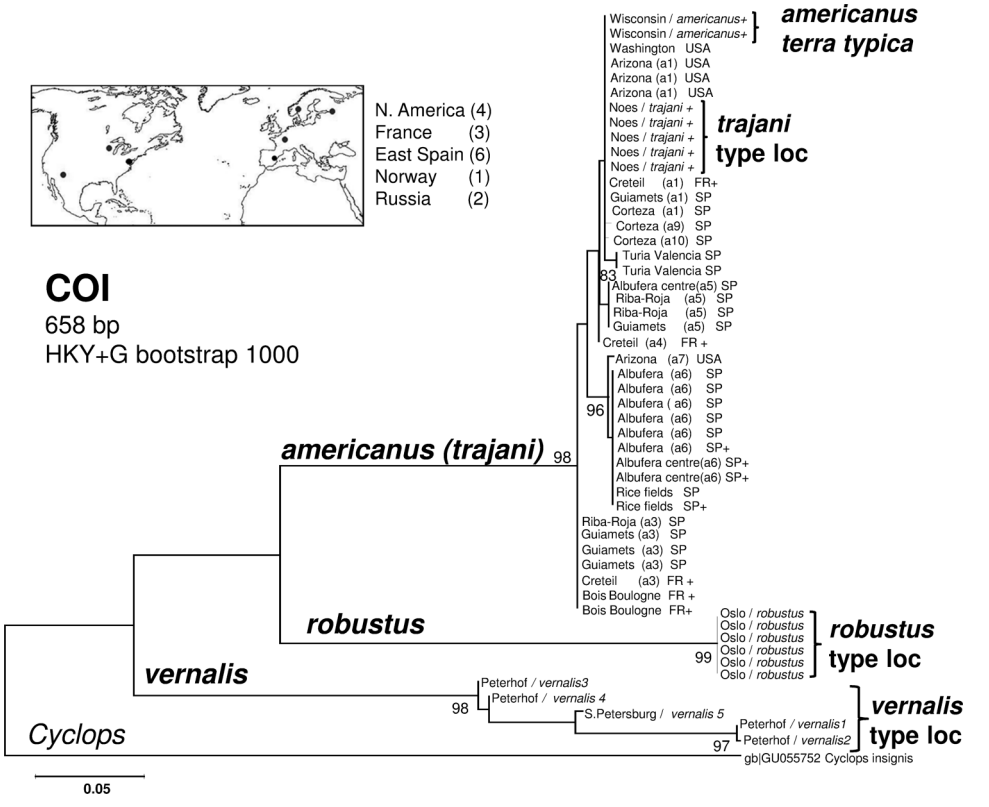

Figure 7. Phylogenetic relationships based on maximum-likelihood analysis of mitochondrial COI sequences. Numbers beside nodes indicate bootstrap support values >70%. Individuals are identified by locality and the haplotype name is added if there more than one haplotype in the same locality.

Notes: Haplotype names a1-a10 correpond to COIa1-COIa10 and *vernalis* 1–5 to COIv1-COIv5 from Table 1 and Figure 9; a map showing the geographical position of sampled areas and the number of localities in each area is shown in the top left part of the figure.

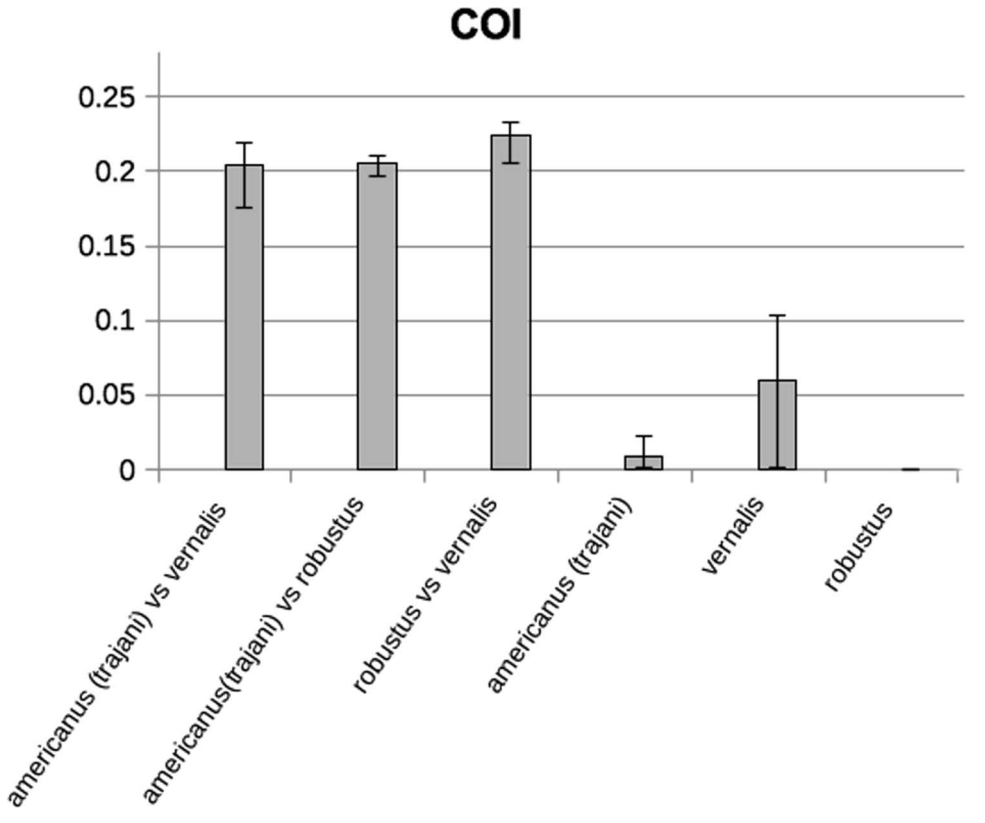

Figure 8. Pairwise COI sequence divergence within the *americanus–robustus–vernalis* complex. Genetic distance (Kimura 2-parameter) is compared between and within the three clades depicted in the tree of Figure 7.

Note: Columns indicate mean values and bars indicate range (min.–max.).

show three clearly differentiated well-supported lineages, corresponding to the three species described at the end of the nineteenth century: *robustus*, *vernalis* and *americanus*. Genetic sequence divergence (K2P distance for haplotypes) between these three lineages was very high – around 20% (Figure 8). Using minimum–maximum pairwise K2P distances, divergence times among these species may be estimated at 10–14 My, assuming a strict molecular clock hypothesis and a substitution rate of 1.66% per My, taken from COI calibrations for Decapoda, owing to the absence of specific calibrations for the rate of substitutions in Copepoda. The general rate of COI evolution was estimated at 1.66–2.30% per My for shore-crabs by Schubart et al. (1998) and at 1.40% per My for mangrove alpheid shrimps by Knowlton and Weigt (1998).

K2P genetic distances were also calculated taking into account only non–synonymous substitutions (codons 1 and 2), giving average distances of 0.032, 0.015 and 0.029 between *americanus–vernalis*, *americanus–robustus* and *robustus–vernalis*, respectively. These distances are also quite high.

The six individuals of *A. robustus* from the type locality (two samples taken on the same day) were grouped together without differences between them as a totally

separate clade. The five individuals of *A. vernalis* from the type locality (four samples from Peterhof, and one sample from a nearby area) also resolved as a totally separate clade, but with a relatively high diversity among the four haplotypes (up to 10%; Figure 8). All the other 41 individuals clustered together in 1 clade, corresponding to the species *A. americanus* (recently renamed *A. trajani*), with very small genetic divergence within them. *A. americanus* individuals from the type region of the first description, Wisconsin, USA, had exactly the same sequence as the individuals from Etang de Noes, France, the type locality of *A. trajani*. Furthermore, most specimens from other USA localities as far apart as Washington and Arizona also had this exact same sequence, together with specimens from a Spanish reservoir and Lake Creteil in France. Within one site, it is common to have two or three haplotypes which are also found at other sites. The other haplotype from the USA was almost coincident with haplotypes from the coastal lagoon Albufera of Valencia. The analyzed individuals within this clade have 11 haplotypes in total, but the maximum sequence divergence between them was 2.2% (Figure 8). Distances taking into account only non-synonymous substitutions within species were 0.001 for *americanus* and 0.007 for *vernalis*, confirming the homogeneity of *americanus* haplotypes and the higher divergence within the *vernalis* lineage.

The specimens of the form *spinosus* (inner spine in end3P4) within the *americanus* clade had exactly the same sequence as the individuals without this character (typical form with seta instead of spine in inner side of end3P4) from the same site. Individuals from the Albufera coastal lagoon taken on the shore and in the centre, as well as the individuals from the surrounding rice fields, had the same sequence regardless of whether they were the form *spinosus* or the typical form with seta.

Diversity in 12S rRNA gene

PCR-amplification efficiency was much higher for the 12S gene than for COI. Most of the individuals sequenced for COI were sequenced for 12S, but since 12S is slightly less variable than COI some redundant individuals were not used for the analysis; therefore, a total of 19 individuals were sequenced from 3 species and 1 form variant (Table 1) for 426 sites. For this 12S gene, there were 22 existing haplotype sequences (343 sites) of this species complex in GeneBank, all derived from the work of Bláha et al. (2010) and they have been included in the phylogenetic tree for comparison. Thus, we worked with a total of 441 sequences, corresponding to 25 haplotypes that contained 107 variable and 88 parsimony informative sites. As for COI, the different phylogenetic methods produced congruent trees; all predicted the existence of four well-differentiated lineages. The topology of the ML tree is presented in Figure 9. The reconstructed phylogeny shows the same three clades corresponding to the same three species separated by COI plus a new clade represented only by GenBank haplotype sequences. This corresponds to an apparently new species of this group, not found in our samples. Genetic divergence (evaluated with K2P [Figure 10]) between these clades, as in COI, was very high – around 20% (ranging from 16 to 25%). The two individuals from the type locality of *A. robustus* were clustered together with one haplotype from GenBank resulting from individuals collected in the Czech Republic and in Slovakia and identified as *A. einslei* by Bláha et al. (2010). This indicates that the newly described species *A. einslei* is a synonym of *A. robustus*. The four individuals from the type locality of *A. vernalis* clustered together with *A. vernalis* haplotypes

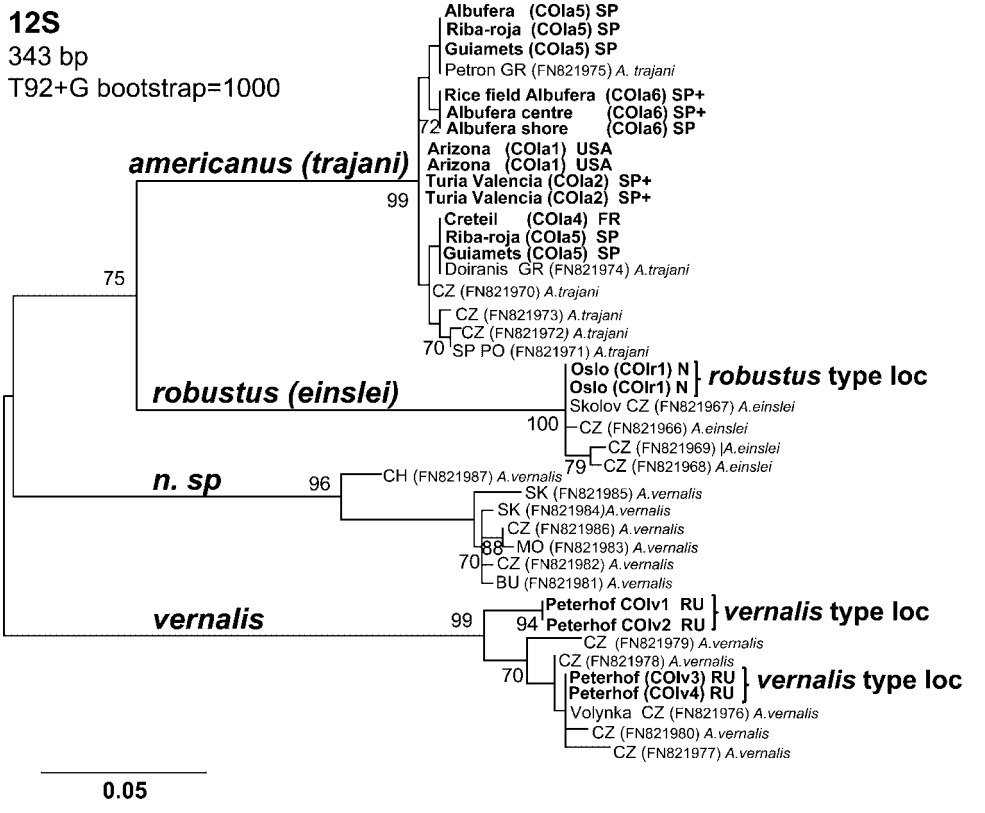

Figure 9. Phylogenetic relationships based on maximum-likelihood analysis of mitochondrial 12S rRNA sequences. Numbers beside nodes indicate bootstrap support values >70%. Individuals for which nucleotide sequences are determined in this work are shown in bold; they are identified by locality and the corresponding COI haplotype name registered in Table 1 and shown abbreviated in Figure 7. The set of added existing 12S sequences for comparative purposes are identified by country, locality (when coincident with our sequences), GenBank accession number and assigned taxonomcal nomenclature; they are all taken from Bláha et al. (2010).

from GenBank from individuals collected in the Czech Republic and identified by Bláha et al. (2010). Two had exactly the same sequence as the most frequent GenBank haplotype found in this clade. Variability within this clade was also relatively high, up to 5.2%, but less than within the clade resulting from COI (with only our specimens from the St Petersburg area). The individuals identified as *A. americanus* in the present study from several localities and from the type locality of *A. trajani* clustered together with the so-called *A. trajani* from GenBank collected in several localities of the Czech Republic, two in Greece, one in Portugal and one in western Spain. Altogether, the mitochondrial gene 12S was slightly less variable than COI with six haplotypes and very limited variability between them, ranging from 0.3 to 1.9%. The haplotype from the type locality for *A. trajani* was identical to the USA haplotype and to one of the haplotypes from eastern Spain. The two Greek haplotypes from GenBank were identical to two eastern Spain haplotypes and one from Lake Creteil, whereas those from

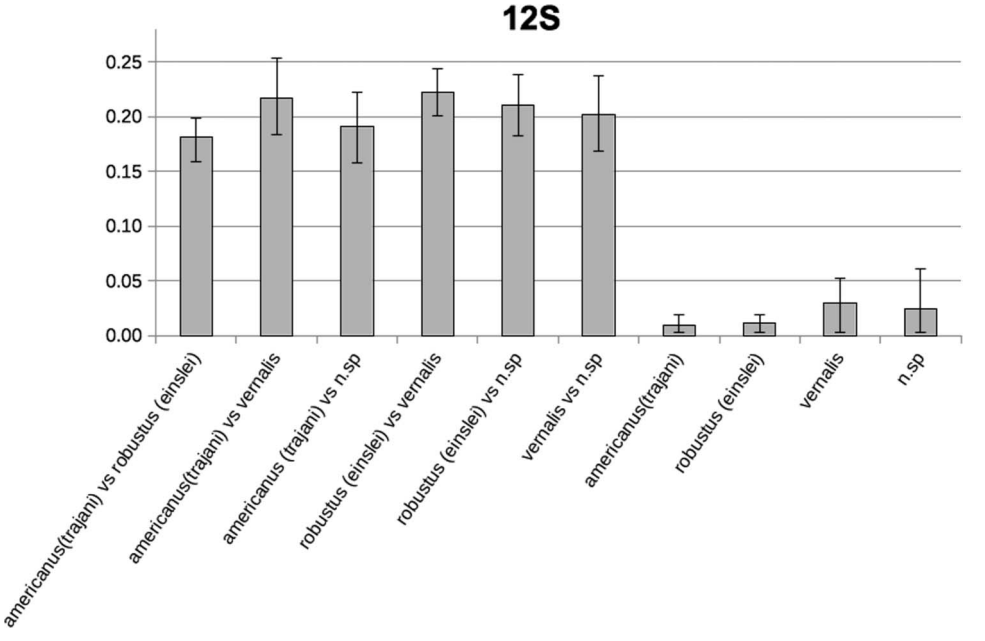

Figure 10. Pairwise 12S rRNA sequence divergence within the *americanus–robustus–vernalis* complex. Genetic distance (Kimura 2-parameter) is compared between and within the four clades depicted in the tree of Figure 9.

Note: Columns indicate mean values and bars indicate range (min.–max.).

the Czech Republic, Portugal and western Spain from GenBank were very similar but not identical to the ones analyzed in the present study.

As found with COI, the *spinosus* form of *A. americanus* from the Albufera area has the exact same sequence as the typical form from the same area.

Discussion

Both mitochondrial genes COI and 12S rRNA confirm the existence of distinct lineages corresponding to the three *Acanthocyclops* species described at the end of the nineteenth century: *vernalis* Fisher; *robustus* Sars; and *americanus* Marsh. They are morphologically quite similar and Kiefer (1976), in his revision of the genus, synonymized the species *americanus* and *robustus* because they were probably found together in the same site (Mjosa Lake, near Oslo). However, the degree of genetic isolation between these two species is high, according to our genetic divergence data (approximately 20%). This divergence is clearly in the range of inter-specific variation. Similarly, *A. vernalis* sequences differ from those of the aforementioned species by the same magnitude. Bláha et al. (2010) gave a preliminary divergence estimation of 10–15 My for this species according to sequence divergence among the four lineages they found in 12S, taking into account the mentioned general rate of evolution of the COI gene in Decapoda. Owing to the absence of specific calibration points to apply methods of divergence times for copepods, this is a very rough estimation. Nevertheless, the obtained COI divergences, reported in the present results,

gave the same Miocenic rough age estimation using the COI clock-calibration for Decapoda. Furthermore, the obtained COI divergences, taking into account only non-synonymous substitutions (codons 1 and 2, see "Results"), are also similar to the distances found by Mayor et al. (2010) between species of *Diacyclops* of Lake Baikal (0.016–0.026) with a group age estimated by these authors of 20–25 My. The important genetic divergence found by Bláha et al. (2010) in the sequences of the much more stable nuclear 18S rRNA gene also confirms without any doubt that *A. americanus* is a species well separated from *A. vernalis*.

The estimated old age of separation of the *Acanthocyclops* species of this complex is not unexpected, taking into account that freshwater harpacticoids (Canthocamptidae) have been found in carboniferous bitumen (Selden et al. 2010), that Boxshall and Jaume (2000), based on biogeographical data, suppose that the family Cyclopidae were also in the first wave of copepods colonizing Pangaea freshwater habitats and that Palmer (1960) found paleontological records of cyclopoids in Miocenic sediments. Furthermore, it is not unlikely that other genetically distinct but morphologically uniform lineages will also be found within this species complex when more populations are analyzed. Bláha et al. (2010) found the existence of a cryptic species inside the *vernalis* morphotype, living in alpine acid lakes.

Great variability among *A. vernalis* from St Petersburg was found within the very low number of individuals studied, which indicates the old colonization of this region by this species. In contrast, the very low variability within *A. americanus* (*trajani*), resulting from a higher number of localities in Spain, France and USA, indicates the invasive character of this species that seems to be expanding with great success in a changing environment. Although several localities with different ecologies were sampled in Spain, sequence variation was very low (2.2% divergence), with some haplotypes identical to US populations in both COI and 12S genes. We found also matching COI sequences in the Barcode of Life Database (BOLD Systems), from the project Crustacea of Bio bus 2009 (BCRUS031,032,037,069,098,099, JMCRUS 037, 185), consisting of six haplotypes identified as Cyclopoida that were very close and within the small variability of our *A. americanus* group of COI haplotypes. The BOLD Systems specimens came from several places in the USA (backwaters of Lake Texoma, and Willis Biological Station in Oklahoma and Willow Creek reservoir in Arizona). This, together with the low variability found in the US populations sequenced in the present study, indicates that it is a widespread species in North America with little genetic variation. All this confirms that North American populations described as *americanus* and later named *robustus* (following synonymy of Kiefer), are the same as European populations identified first as *americanus*, later as *robustus* (following Kiefer's synonymy) and recently as *trajani*.

Populations of *A. americanus* with identical haplotypes in distant countries, such as those from the USA, Spain, France and Greece, might reflect a recent common history of dispersal. This wide distribution of identical haplotypes and its area of expansion in reservoirs or coastal lagoons in Spain (present results) and fishponds in the Czech Republic (*A. trajani* [Bláha et al. 2010]) subject to eutrophication or hydrological transformations indicate the recent invasive character of this species. Furthermore, Locke et al. (1993) reported *A. americanus* from ballast water and considered it to be a potential invader of the Great Lakes; however, this is just a hypothesis, since they could in fact be Great Lake natives. Also, recent expansion of this species in Europe, before Kiefer synonymized it with *A. robustus*, has been documented in Europe by Lowndes

(1926), Monchenko (1961), Dussart (1967; 1971) and Alekseev and Kossova (1976). Therefore, this leads us to hypothesize that *A. americanus* is originally an American species which has recently spread owing to human-derived translocation, but at the same time the ongoing expansion of its range is a result of man's impacts on water body hydrology and the raising of trophic levels.

Another taxonomic contribution from the present study is that the species of this group are morphologically very plastic to the extent that taxonomic characters used in the past are not good to differentiate these species. We have shown here that the transformation of the inner seta of endP4 into a spine does not imply any genetic differentiation in the studied population and may be coincident with identical COI or 12S sequences, even if specimens were collected from different seasons or from temporary environments. Seta converted to spines are probably a kind of cyclomorphosis as an adaptation of the species to seasonal variability, as other authors have concluded (Mastrantuono 1980; Lescher-Moutoué 1996).

The analyses of individuals originating from type localities allow us to suggest a nomenclature revision of the species complex. Ecological and distributional literature have commonly used the misleading name *A. robustus* to designate what is the most frequent species of the group: *A. americanus*, erroneously synonymized with the former species. This is especially the case in publications on planktonic "*A. robustus*". These should be now correctly renamed, by synonymizing the younger name *A. trajani* with the first description name *A. americanus.* Marsh's (1892) description of *americanus* may not be very precise, but the round genital segment was figured and since we have been keeping the names of the first descriptions from old times that in many cases are no better than the description of Marsh, there is no reason to consider it as invalid. The ICZN code gives Marsh's name priority and, therefore, it is the valid name in this case.

Another problem is also the recently described species *A. einslei*, whose morphological description corresponds to *A. robustus.* As Bláha et al. (2010) found within the *A. vernalis* morphotype, it is also probable that cryptic species will be found within the *A. robustus* morphotype when an increasing number of populations are screened for genetic variation. However, with the morphological and genetic information that we have now, *A. einslei* is a synonym of *A. robustus* and *A. robustus* is the valid name according to the rules of nomenclature.

Taxonomy should work for ecology, and vice versa. There are now an increasing number of studies of applied ecology that rely on the identification keys of organisms. In our opinion, characters used to identify species should be unambiguous and easy to look at. While waiting for further studies, we propose the following traditional discriminatory characters to differentiate European *Acanthocyclops* species of 17-segmented antennula:

(1) Genital segment in female wide (ratio length/width about 1 or less) with upper part of genital segment in female angular with sharpening lobes (Figure 1A). External apical spine of end3P4 slightly longer than internal. Insertion of lateral outer seta/spine of end3P4 at the same level as the second seta of inner site (Figure 1D). Male P6 with strong inner spine and shorter middle seta (Figure 1E). Littoral and benthic zone of ponds, lakes and rivers .... *A. vernalis* (Fischer).

(Existence of other species, similar in morphology, in small shallow acid water bodies according to Bláha et al. [2010].)

(2) Upper part of genital segment in female without sharpening lobes, sometimes more roundish but usually triangularly shaped (Figure 2A). External apical spine of end3P4 equal or slightly shorter than internal. Insertion of lateral outer seta/spine of end3P4 much more distal than seta of inner site (Figure 2E–H). Male P6 with a very strong and long inner spine that can almost reach the length of the outer seta and very short middle seta. In live/fresh conserved animals, chitin usually with brownish colour. Littoral and benthic zone of rivers, ponds and lakes ..... *A. robustus* (Sars).

(3) Genital segment in female elongated (ratio length/width 1.1–1.3 [Figures 3A–B]), rounded in its first part. End3P4 (Figure 5D): external apical spine of end3P4 equal or slightly shorter than internal. Insertion of lateral outer seta/spine of end3P4 at same level as seta of inner site. Male P6 with slender inner spine of similar size to middle seta and long outer seta, about twice the inner spine. Body transparent, pale or with greenish colour. Planktonic in large lakes, reservoirs also common in shallow water bodies with high pH, dominant crustacean in highly eutrophic waters .... *A. americanus* (March). *A. americanus spinosus* (Monchenko) is a seasonal form of *A. americanus* (March) with the lateral outer seta of distal segment endopodite P4 transformed into spine. This form is not genetically different in the studied genes and is more common in the cold season.

Conclusions

(1) Molecular-genetic analysis revealed that the three species described at the end of the nineteenth century: *A. robustus*, *A. americanus* and *A. vernalis* from the type localities are well-separated species with genetic distances between them of around 20% for COI and 12S rRNA sequences.

(2) The recently described European planktonic form *A. trajani* is a junior synonym of *A. americanus* that is re-described and re-established as a valid species.

(3) *A. americanus spinosus* is a seasonal variation, probably adapted to the regularly drying-up environment, and the name has no nomenclatural validity.

(4) *A. einslei* form central Europe is not genetically different from *A. robustus* from Oslo.

(5) More studies are needed because this *robustus–vernalis* group of benthic cyclopoid species in Europe is not homogeneous and possibly includes several other sibling species, as revealed by Bláha et al. (2010).

Acknowledgements

The special role of Prof. S. Dodson in the success of this study should be mentioned. He collected very important type material for molecular-genetic analyses from the *terra typica* and other USA localities and sent these to us a few days before his tragic passing away. We are also grateful to several colleagues who provided us with material: Dr V. Ivanenko, Prof. H.C. Fernando,

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