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

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

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

Introduction

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

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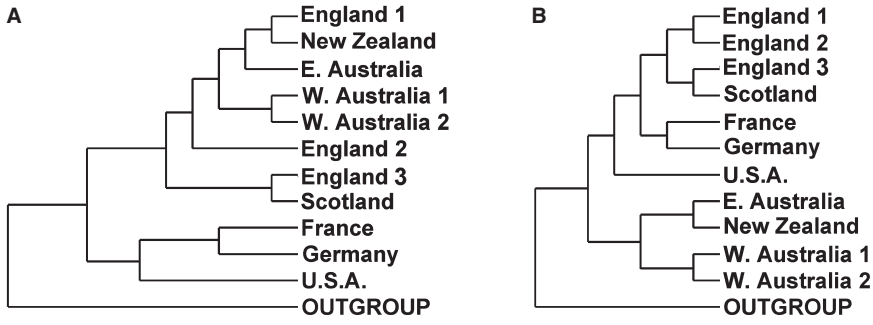

Fig. 1. Two competing hypothetical phylogenies of *M. albidus* (Jurine, 1820) that may explain its cosmopolitan distribution, and especially the presence of disjunct populations in Australia and New Zealand: (A) anthropogenic translocation associated with early shipping activities by European settlers;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

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

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

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

Material and methods

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

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

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

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

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

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

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

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

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

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

Results

Molecular analyses

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

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

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

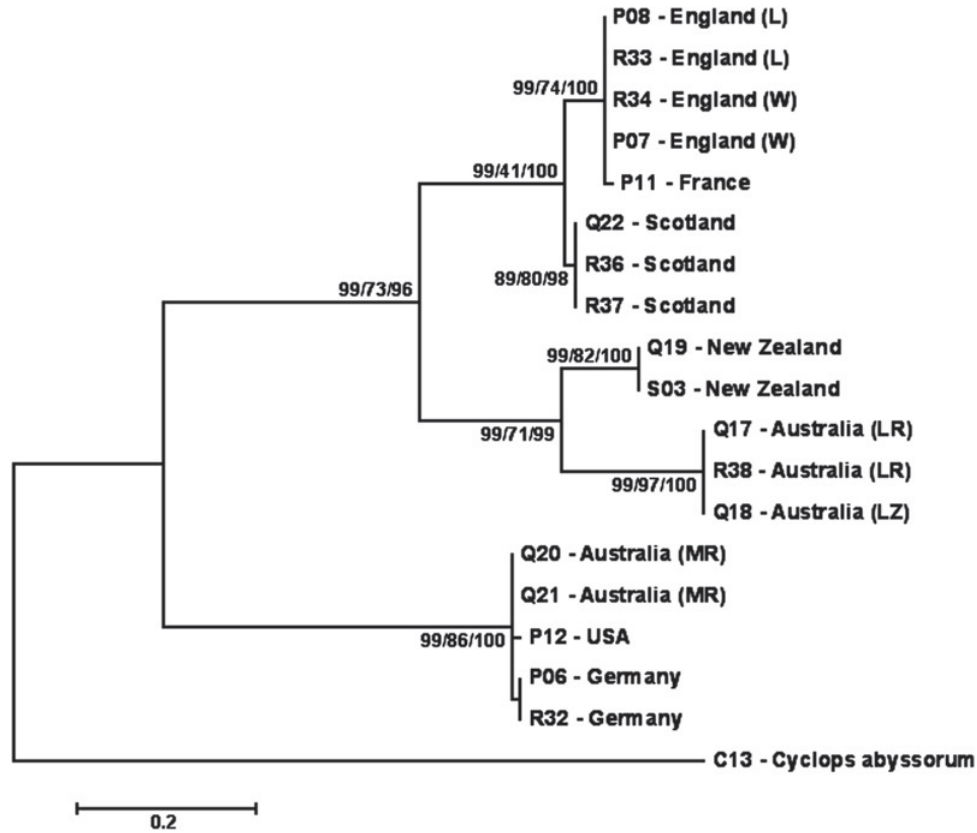

Fig. 2. One of six equally parsimonious trees based on 12S sequence data (CI = 0.921; RI = 0.964) for 18 specimens from 10 different locations of *M. albidus* (Jurine, 1820). The numbers on the branches representing bootstrap values for three different methods (MP/ML/NJ). The outgroup in this analysis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

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

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

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

Comparative morphology

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

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

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

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

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

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

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

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

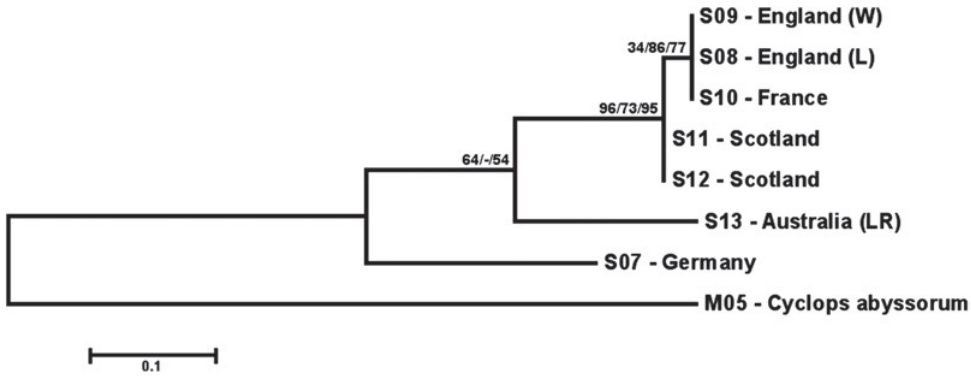

Fig. 3. One of 18 equally parsimonious trees based on 16S sequence data (CI = 0.937; RI = 0.666) for seven specimens from six different locations of *M. albidus* (Jurine, 1820). The numbers on the branches representing bootstrap values for three different methods (MP/ML/NJ). The outgroup in this analysis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

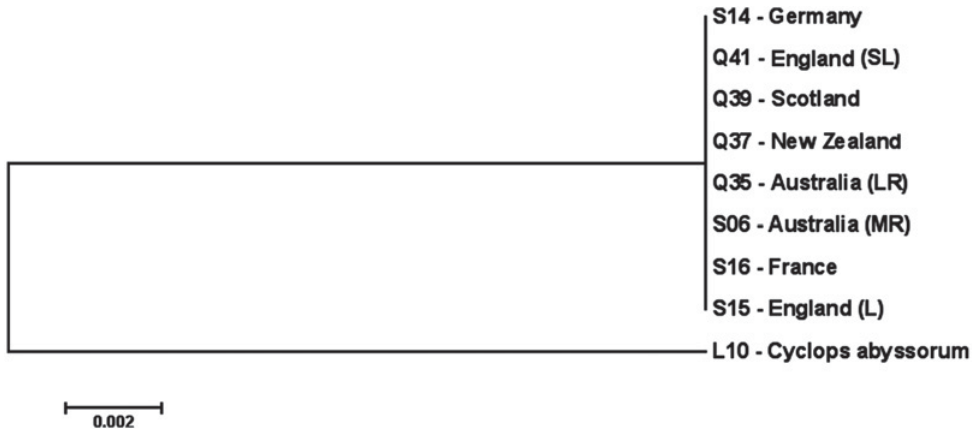

Fig. 4. NJ tree based on the 18S sequence data for eight specimens from as many different locations of *M. albidus* (Jurine, 1820). The outgroup in this analysis was *C. abyssorum* G.O. Sars, 1863, collected in Vysne Temnosmrecinske, Slovakia. The cladogram is drawn to scale and specimen numbers correspond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

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

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

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

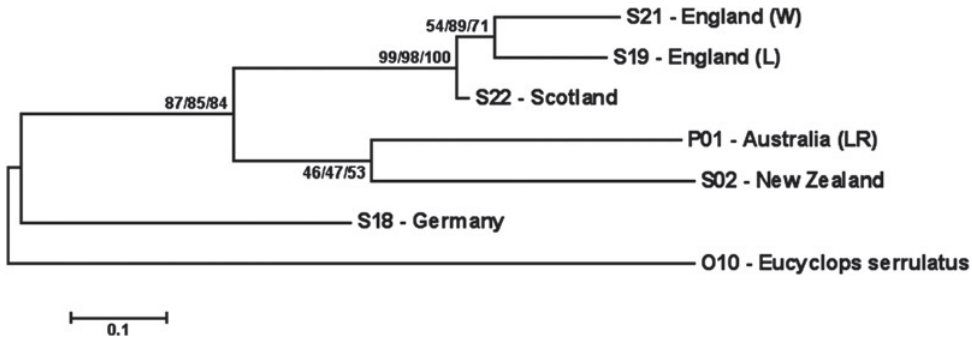

Fig. 5. Single most parsimonious tree based on cytB sequence data (CI = 0.859; RI = 0.642) for six specimens from as many different locations of *M. albidus* (Jurine, 1820). The numbers on the branches representing bootstrap values for three different methods (MP/ML/NJ). The outgroup in this analysis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

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

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

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

Discussion

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

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

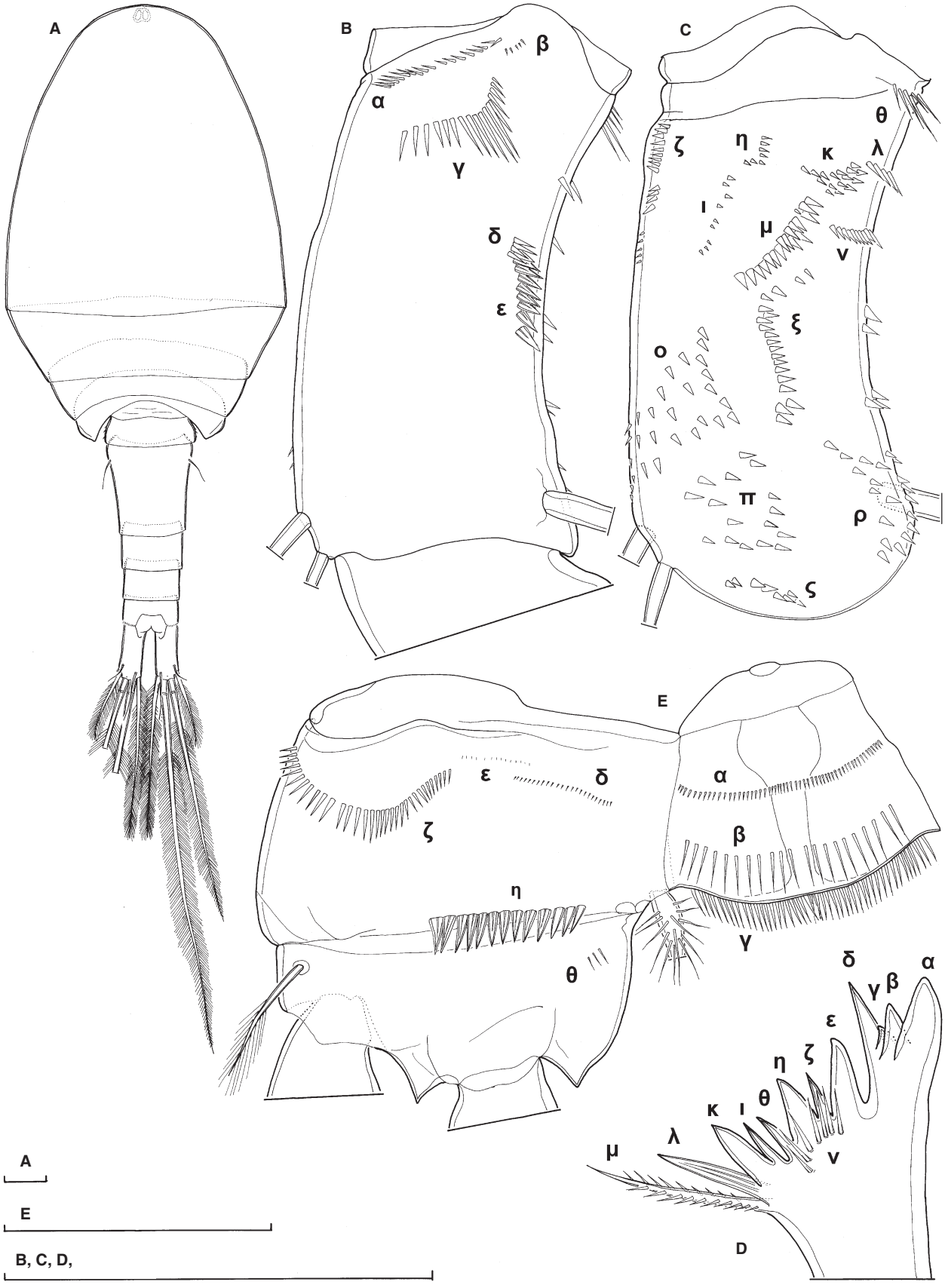

Fig. 6. *M. albidus s.l.* (Jurine, 1820), USA, adult female (NIBRIV0000232633): (A) habitus dorsal view; (B) basis of antenna, anterior view; (C) basis of antenna, posterior view; (D) cutting edge of mandibula, anterior view; (E) intercoxal plate, coxa, and basis of fourth swimming leg, posterior view.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

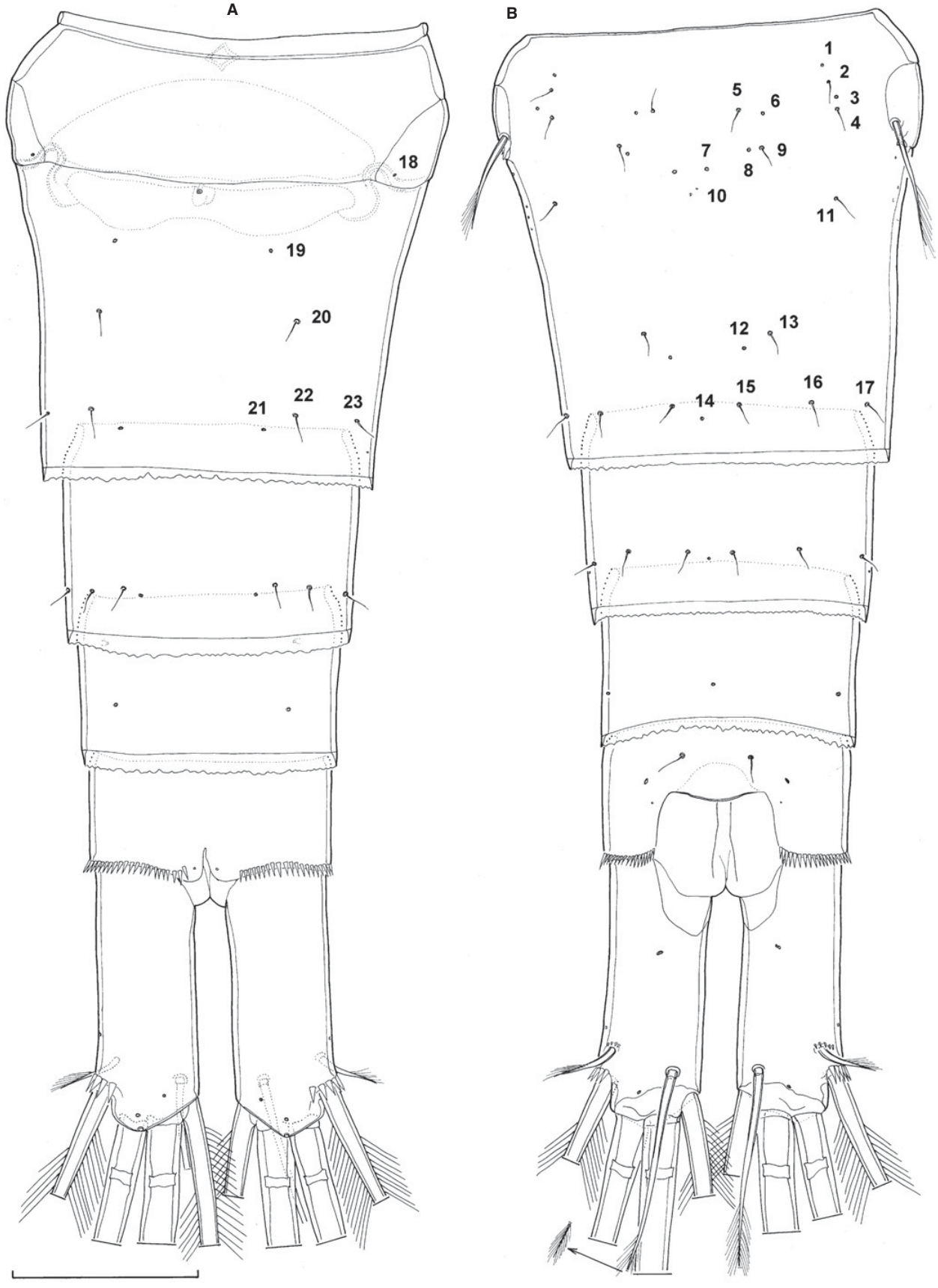

Fig. 7. *M. albidus s.l.* (Jurine, 1820), USA, adult female (NIBRIV0000232633): (A) urosome, ventral view; (B) urosome, dorsal view. Numbers identify different pores and sensilla on genital double somite. Scale: 100 μm.

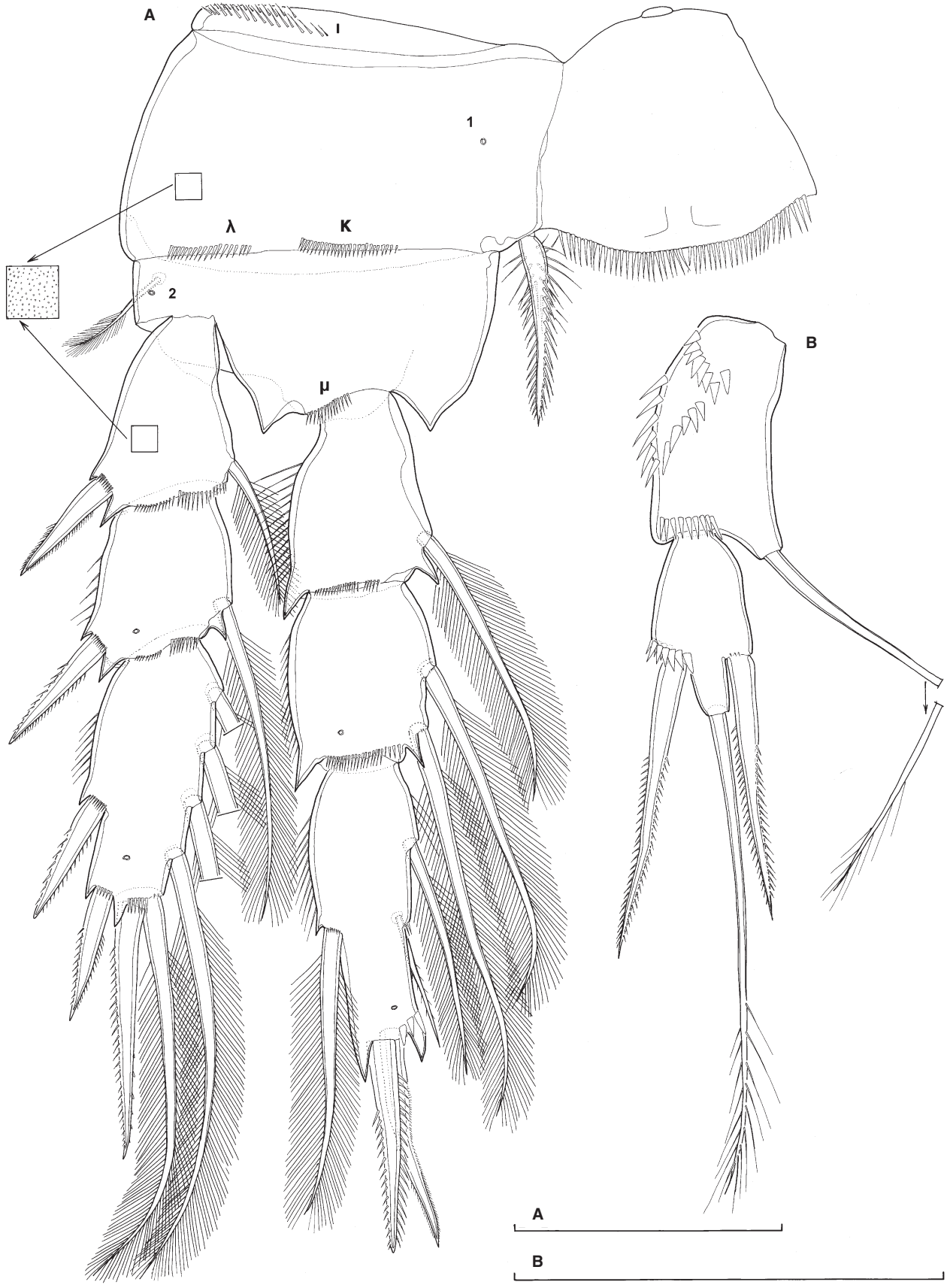

Fig. 8. *M. albidus s.l.* (Jurine, 1820), USA, adult female (NIBRIV0000232633): (A) fourth swimming leg, anterior view; (B) fifth leg, anterior view. Greek letters identify different rows of spinules and numbers identify pores on coxa and basis. Both scales 100 μm.

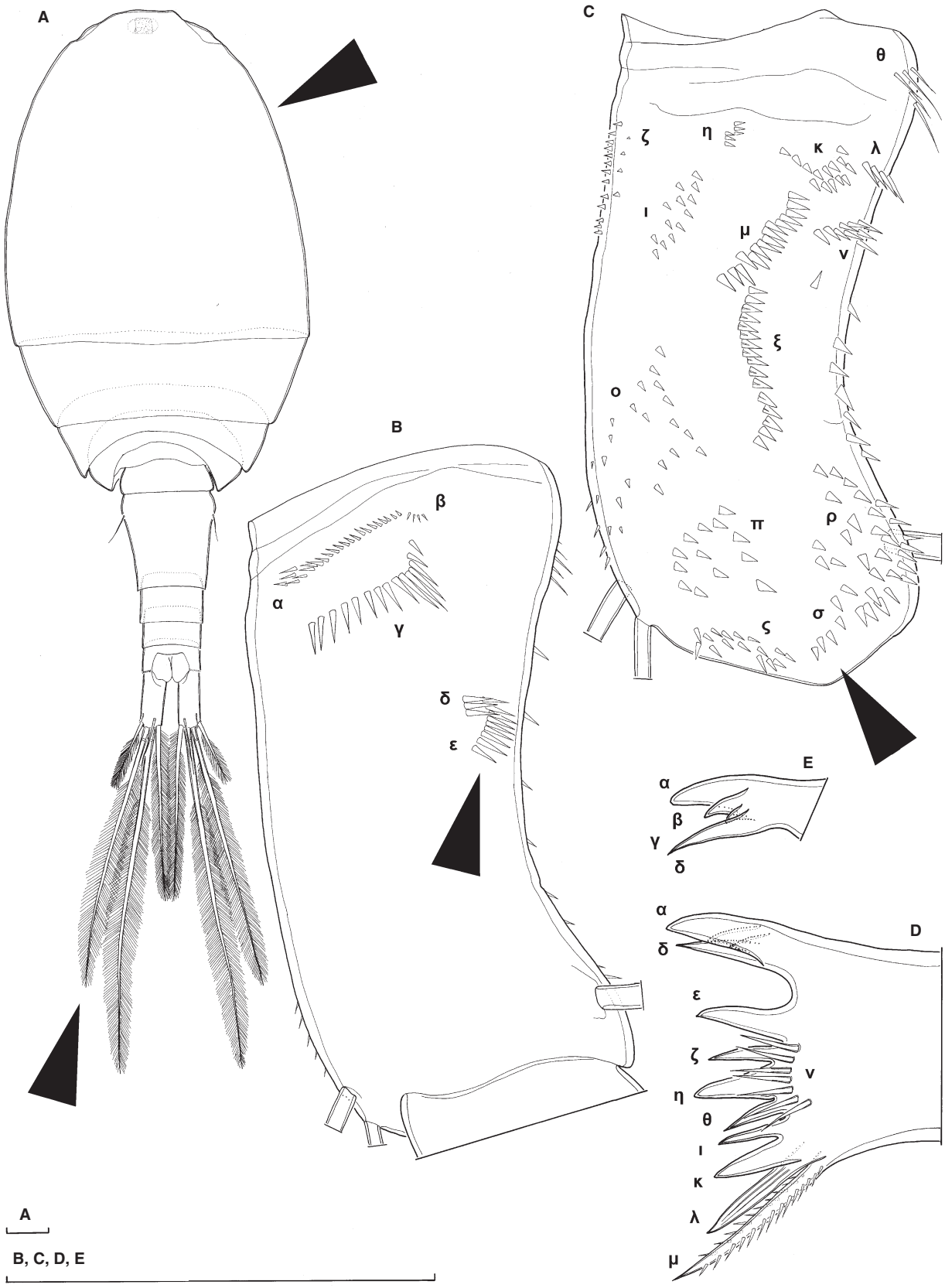

Fig. 9. *M. albidus s.l.* (Jurine, 1820), Scotland, adult female (NIBRIV0000232634): (A) habitus dorsal view; (B) basis of antenna, anterior view; (C) basis of antenna, posterior view; (D) cutting edge of mandibula, anterior view; (E) quadricuspidate ventral spine on cutting edge of mandibula, posterior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

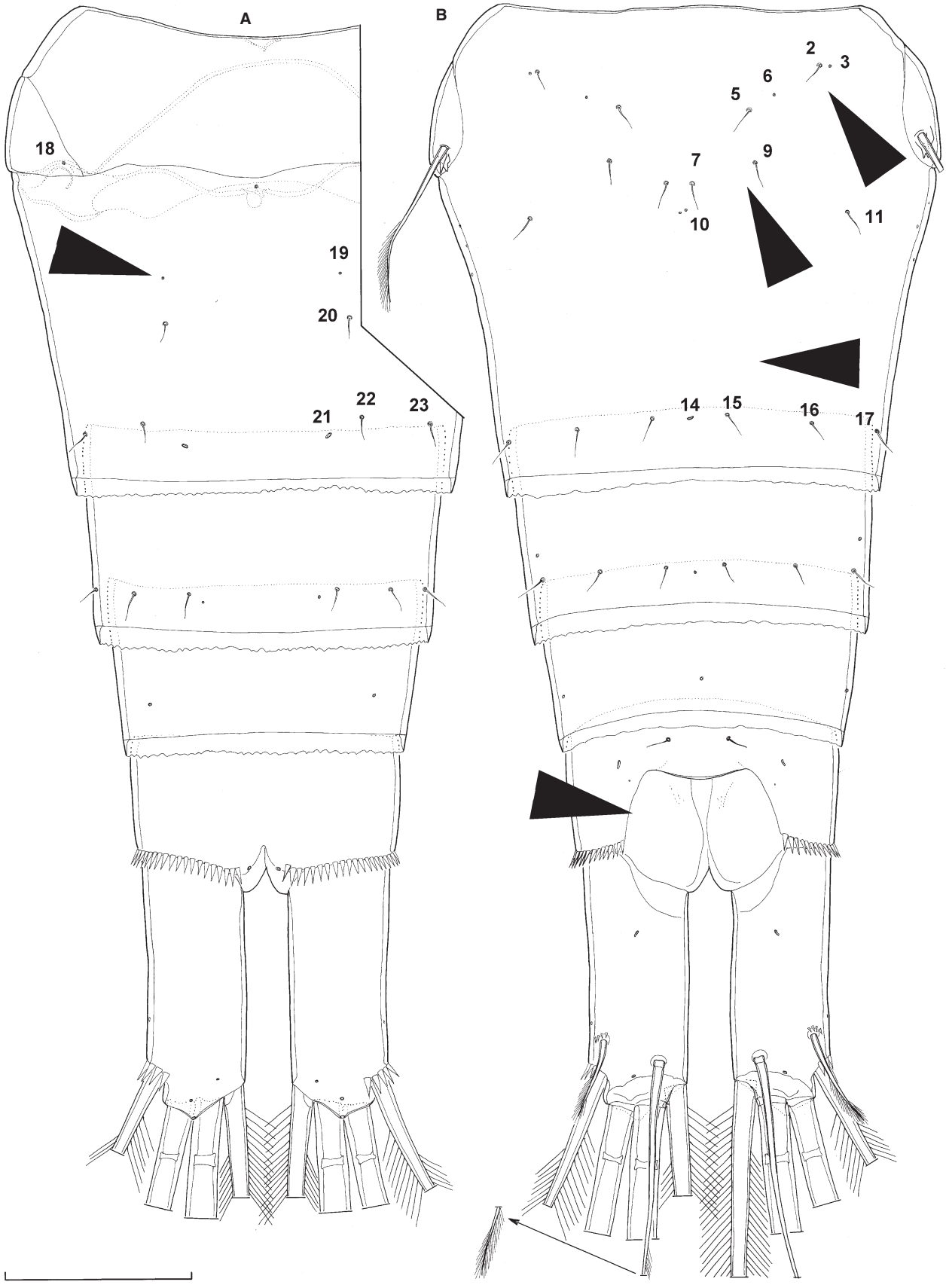

Fig. 10. *M. albidus s.l.* (Jurine, 1820), Scotland, adult female (NIBRIV0000232634): (A) urosome, ventral view; (B) urosome, dorsal view. Numbers identify different pores and sensilla on genital double somite and arrows point out most distinguishing features. Scale 100 μm.

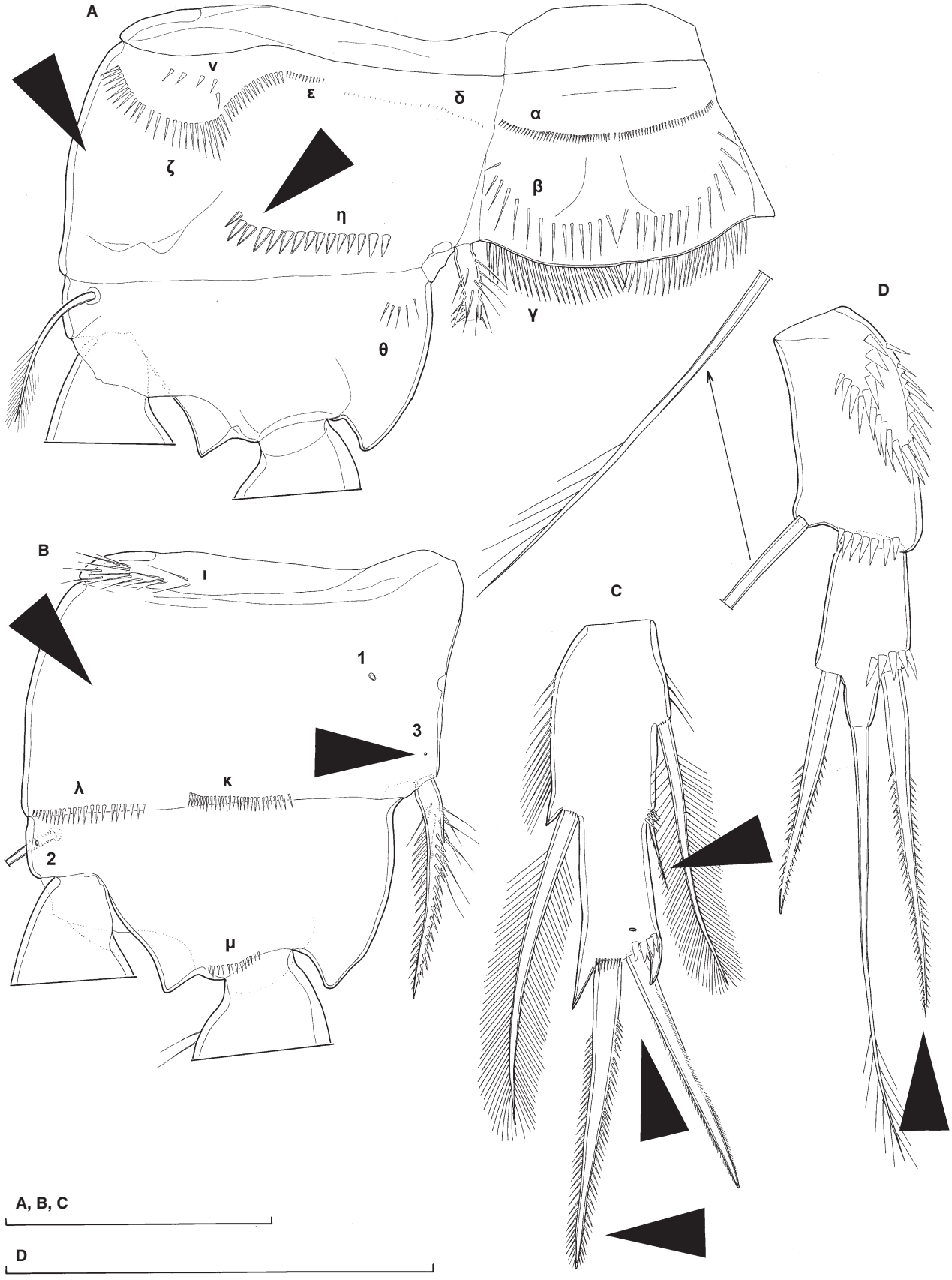

Fig. 11. *M. albidus s.l.* (Jurine, 1820), Scotland, adult female (NIBRIV0000232634): (A) intercoxal plate, coxa and basis of fourth swimming leg, posterior view; (B) intercoxal plate, coxa and basis of fourth swimming leg, anterior view; (C) third endopodal segment of fourth swimming leg, anterior view;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

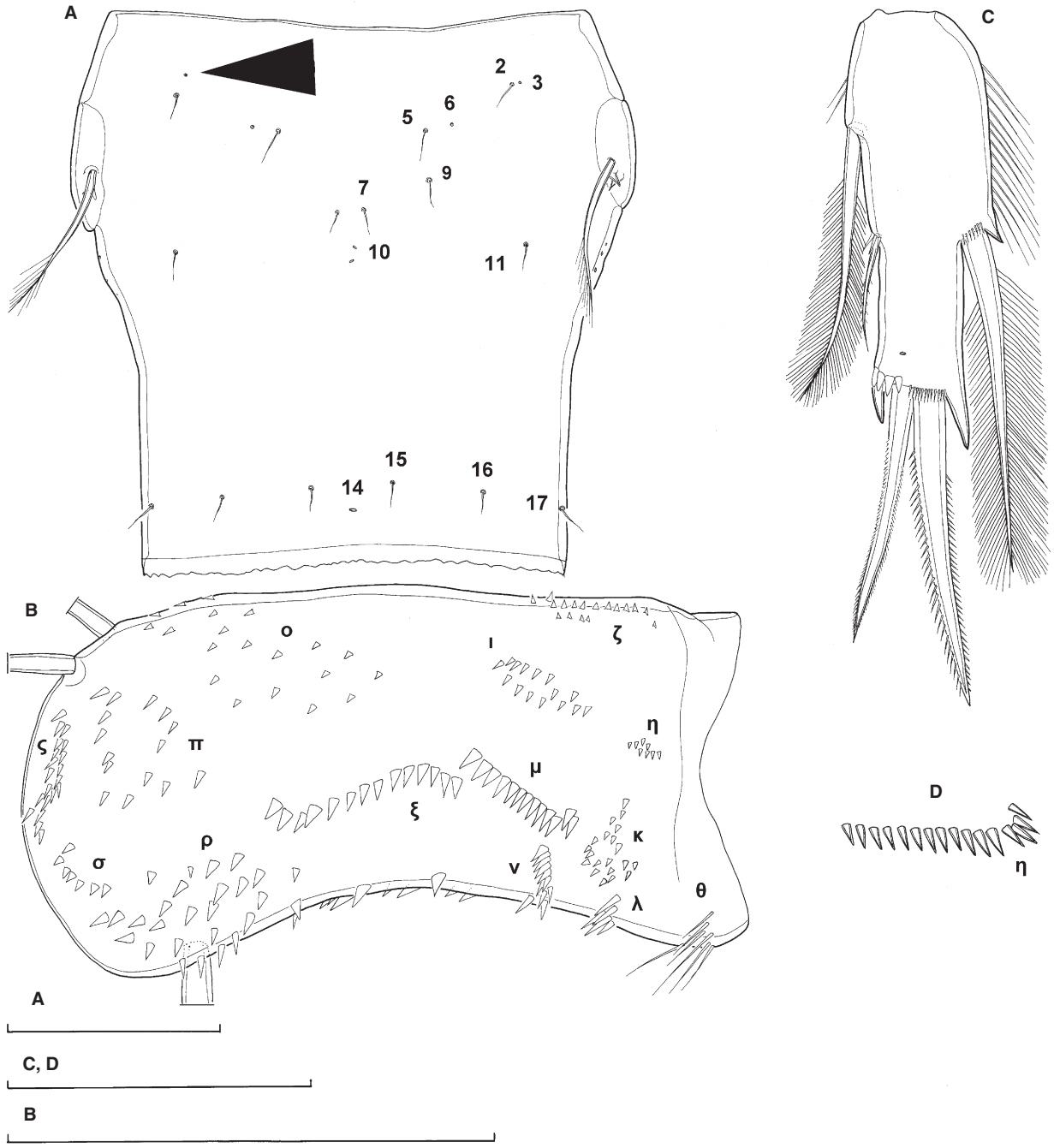

Fig. 12. *M. albidus s.l.* (Jurine, 1820), England (W), adult female (NIBRIV0000232635): (A) genital double-somite, dorsal view; (B) basis of antenna, posterior view; (C) third endopodal segment of fourth swimming leg, anterior view; (D) distal row of spinules on posterior surface of fourth swimming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

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

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

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

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

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

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

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