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Phylogeny of the freshwater copepod *Mesocyclops* (Crustacea: Cyclopidae) based on combined molecular and morphological data, with notes on biogeography

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ABSTRACT

We combined molecular and morphological characters in a copepod taxon for which obtaining a sufficiently high number of characters that evolve at different rates is a challenge. Few molecular markers are known to resolve evolutionary relationships in the copepods, and thus there is potential for morphology to contribute substantially to phylogenetic reconstruction. We used a morphology based tree of the entire *Mesocyclops* genus to guide our taxon sampling of 10 species for molecular and combined analyses. Morphology including polymorphic characters, 18S rDNA, and ITS2 sequences were analyzed using parsimony, ML, and Bayesian methods. Strong similarities among topologies were observed regardless of the character type or algorithm, with higher levels of support obtained in combined data analyses. In combined analyses Old World species formed a monophyletic group and New World species formed a paraphyletic group in this freshwater, predominantly (sub)tropical genus. *Mesocyclops darwini* was the single taxon whose relationships showed conflict among the previous reconstructions using only morphological characters and the tree inferred from the combined data set. Support for these alternative positions of *M. darwini* were compared using constraint tests, with the result supporting monophyly of Old World taxa.

1. Introduction

The Cyclopidae is the most species-rich family (ca. 830 species) among copepod lineages invading continental waters and comprises the largest group within the subclass Copepoda (Defaye and Dussart, 2006; Boxshall and Defaye, 2008). Few published works (Kiefer, 1927; Gurney, 1933; Ferrari, 1998) discuss the inter- or intra-generic relationships within the Cyclopidae. *Mesocyclops* G.O. Sars, 1914, with 73 nominal species, is one of the largest cyclopid genera. The genus is cosmopolitan in distribution but the overwhelming majority of taxa occur in (sub)tropical waters (Hołyńska et al., 2003). These medium-sized (0.5–2.0 mm) copepods are common in small pools, marshes and as plankton in lakes of variable size and age. A few taxa have invaded subterranean habitats. Some *Mesocyclops* species can serve as intermediate hosts of the guinea worm (*Dracunculus medinensis*), a nematode parasite that infests humans mainly in Sub-Saharan Africa (Molyneux, 1998). Other *Mesocyclops* have been successfully used in the biological control of the disease-vector *Aedes* mosquitoes (e.g. Marten et al., 1994; Vu et al., 2000).

Almost all we know about the systematics of *Mesocyclops* is based on morphological characters of the adult female of extant species; no fossil record exists. With the exception of a few comparative investigations (Dahms and Fernando, 1992, 1993a,b, Dahms and Fernando, 1995) the larval and male morphology is poorly understood. A turning point in *Mesocyclops* taxonomy was Van de Velde's revision (1984a,b) of the African fauna, which revealed the diagnostic value of several surface microstructures ('microcharacters'), and resulted in a significant increase in the number of described species. This led to a complete taxonomic revision of the *Mesocyclops* fauna of the world (Hołyńska et al., 2003) and subsequent reconstruction of morphologically based phylogenies of the entire genus (Hołyńska, 2006). The *Mesocyclops* genus is the only cyclopid taxon for which a phylogenetic hypothesis based on complete taxon sampling has been published and thus is the most feasible group for designing taxon sampling for molecule based phylogenies and exploring the contribution of molecular markers to phylogenetic reconstruction of copepods.

Currently only a few nuclear and mitochondrial markers have been employed to successfully resolve phylogenetic relationships in copepods. The *18S* and *28S* nuclear ribosomal DNA genes have been used to resolve relationships at the ordinal, familial, or generic levels (Bucklin et al., 2003; Braga et al., 1999; Thum, 2004; Huys et al., 2006, 2007), while the internal transcribed spacer region II (ITS2) has been used to resolve relationships at the species and

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population levels (Goetze, 2005; Ki et al., 2009; Bucklin and Frost, 2009; Thum and Harrison, 2009). Mitochondrial *COI* and *16S* genes appear to contain less phylogenetic signal in copepods relative to other taxa and may be more informative for questions related to population differentiation or cryptic speciation (Edmands, 2001; Lee, 2000; Hebert et al., 2003; Caudill and Bucklin, 2004; Eyun et al., 2007; Goetze, 2005; Adamowicz et al., 2007; Thum and Harrison, 2009). Mitochondrial genes evolve unusually rapidly in some copepods compared to other arthropods (Shao and Baker, 2007), with some closely related copepod species exhibiting unexpected gene order rearrangements (Machida et al., 2002, 2004; Burton et al., 2006, 2007). Although the awareness of the importance of screening for presence of *COI* pseudogenes is increasing (Song et al., 2008; Buhay, 2009), the difficulty in amplifying or sequencing functional gene copies in some copepod taxa sometimes causes investigators to resort to using additional markers or procuring their COI sequences from cDNA libraries (Thum and Derry, 2008; Thum and Harrison, 2009; Nonomura et al., 2008; Machida et al., 2009). When *COI* and 16S mitochondrial DNA sequences are combined with those of other mitochondrial genes (*lrRNA*, *ND4L*, *ND6*), resolution of interspecific relationships can be achieved within the marine calanoid *Neocalanus* (Machida et al., 2006). On the other hand, when combined with cytochrome *b* sequences, many relationships among freshwater, morphologically similar calanoids of *Skistodiaptomus* remain unresolved (Thum and Harrison, 2009*).* The search for additional parsimony informative molecular characters, such as *EF-1*$\propto$, has not yet met with success in resolving relationships within copepods, (Goetze, 2006). However, this molecule, EF-2$\propto$, and *Pol II*, among other proteins, are informative in resolving relationships of copepods to other arthropod lineages (Regier et al., 2008).

In the present paper we combine 81 morphological characters and 1000 nucleotides derived from ITS2 and partial 18S rDNA sequences of 15 *Mesocyclops* and *Thermocyclops* taxa. Though widely known to be most informative in resolving deep relationships, the 18S rDNA sequences were available from a larger taxon sampling study (Wyngaard and da Rocha, unpublished data) and so it seemed reasonable to include them here. COI sequences evolved too rapidly (even at the codon level) to include them in the present study (McClellan and Wyngaard, unpublished data). By themselves, the molecular and morphological data sets in *Mesocyclops* possess few phylogenetically informative characters. Combined data analyses are likely to be a sensible solution to the problem of obtaining the sufficiently large number of characters and variety of evolutionary rates necessary to achieve strong statistical support (de Queiroz and Gatesy, 2006). To our knowledge, the only phylogenetic analysis based on combined data of a copepod is that of *Clausocalanus*, a marine calanoid genus (Bucklin and Frost, 2009). This effort yielded mixed success, due in part to the challenge of identifying phylogenetically informative characters discussed above and the especially complex and challenging systematics of this genus. A second study used both morphology and molecules to infer the ordinal status of the copepod Monstrilloida, but did not combine these data in their analyses (Huys et al., 2007). It is commonly expected that molecular characters will swamp the phylogenetic signal of morphological data, but a recent review suggests that partitioned data sets comprised of as few as 5% morphological characters may still yield valuable contributions by morphology (Nylander et al., 2004). Comparisons of trees built using different types of characters may serve to inform us as to the particular phylogenetic utility of each kind of character, an objective that is useful when studying a taxon for which phylogenetic analysis is considered to be in its infancy.

There is very little known of the historical biogeography of Cyclopidae, and this mainly is the consequence of an almost complete lack of paleontological records and the extremely few phylogenetic analyses constructed of the group. Morphology based phylogenetic reconstructions in *Mesocyclops* (Hołyńska, 2006) indicated monophyly of a large group comprising all Old World (Africa, Eurasia and Australia) taxa, except for the Paleotropical (tropical Old World) *rarus*-group. The New World (North and South America) species formed a monophyletic or paraphyletic group in these analyses, depending upon the position of the *rarus* clade, which appeared either as first offshoot of the genus or the sister of a Neotropical (South American) clade. Different topologies of the deep relationships in *Mesocyclops* are tested in the present study by combined use of morphological and molecular characters. Competing hypotheses of the geographic origin and age of *Mesocyclops* that are consistent with our preferred phylogeny, are discussed.

2. Materials and methods

2.1. Taxon sampling

The morphology based tree (Fig. 1A, after Fig. 7A in Hołyńska, 2006) containing 71 species of *Mesocyclops* served as a guide to sample the major clades for the present study (Table 1). This guide tree (Fig. 1A) included all *Mesocyclops* species, used the parsimony criterion, contained the largest set of outgroups, and minimized the cells for missing characters. The combined data analyses comprised 10 of the 73 described species of *Mesocyclops* and two species of *Thermocyclops,* which were chosen as outgroups. The choice of outgroups was based on the above phylogenetic hypothesis as well as broader taxon sampling of 43 species in Cyclopidae, comprising all four subfamilies and using partial sequence of the *18S* rDNA gene (Wyngaard and da Rocha, unpublished data). *Thermocyclops* appeared as a close relative in all these analyses. Kiefer (1927) proposed *Thermocyclops* as a subgenus of *Mesocyclops*.

Four additional taxa were explored for their utility as outgroups, but each resulted in exclusion of more nucleotides of ITS2 in the multiple sequence alignment than when *Thermocyclops* was used. These four taxa were *Diacyclops bernardi* (Petkovski, 1986), a species initially allocated to *Mesocyclops* and later transferred to *Diacyclops* (Reid, 1993), *Macrocyclops albidus, Diacyclops uruguayensis,* and *Cyclops strenuus.* Multiple populations of *Mesocyclops edax* and *Mesocyclops longisetus* afforded the opportunity to examine population variability in morphological and molecular characters.

2.2. Morphological characters

The morphological character matrix is based on collections (at least 2–7 locales) that are independent from the sources used to obtain the molecular data, with the exception of *M. edax* (Appendix A). Details of the collecting sites are given in Hołyńska (2006) and references therein.

In cases in which polymorphic characters were scored, additional specimens from additional locales were examined, the references of which also are given in Hołyńska (2006). Polymorphic characters were treated according to the scaled and unordered methods (Campbell and Frost, 1993; Wiens, 2000). The descriptions of the morphological characters used in the present study are identical to those used by Hołyńska (2006) to reconstruct the entire genus (Fig. 1A), with the exception of the minor modification of a character state of character 25. This modified character state (0) is long, reaching about middle of the terminal segment. The coding of the 81 morphological characters is given in Appendix B (https//:xxxxxxx, for review purposes, see WORD filename: Appendix B Morphology).

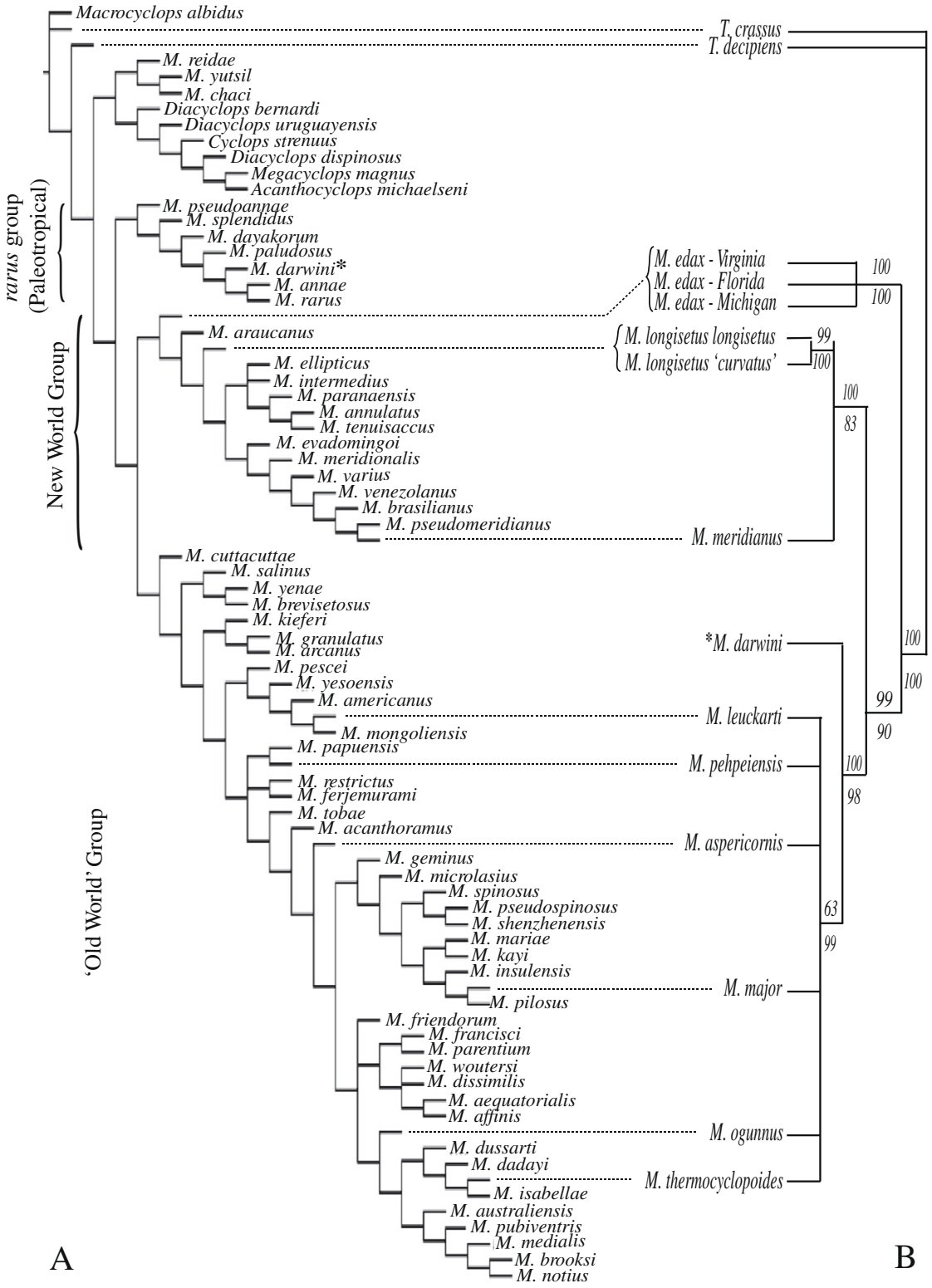

Fig. 1. Phylogenetic relationships of *Mesocyclops*. (A) Morphology based, majority consensus of MP analyzes, which includes all *Mesocyclops* species described before 2006 (after Fig. 7A in Hołyńska, 2006). (B) Combined morphology and 18S rDNA and ITS2 sequences of MP and Bayesian analyzes. Numbers above each node correspond to bootstrap proportions and numbers below each node correspond to Bayesian credibility values.

Two separate analyses of the morphology matrix were run treating polymorphic states in different ways: (1) unordered and (2) scaled. Both methods recognize polymorphic states as separate states. In the unordered run all characters are coded as unordered.

Table 1
Pairwise uncorrected genetic distances among *Mesocyclops* and *Thermocyclops* as identified by phylogenetic analysis. Distances based on ITS2 sequences are shown in the top half and those based on partial 18S rDNA sequences, in the bottom half. Included in the ITS2 sequences are partial sequences of the *5.8S* and *28S* rDNA genes which contributed to the sequence variability.

#	Species name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*T. crassus*		0.048	0.051	0.048	0.046	0.062	0.056	0.062	0.077	0.090	0.072	0.074	0.074	0.079	0.082
2	*T. decipiens*	0.005		0.056	0.056	0.059	0.072	0.066	0.064	0.082	0.095	0.069	0.072	0.079	0.077	0.087
3	*M. edax* (VA)	0.013	0.011		0.000	0.002	0.056	0.056	0.054	0.069	0.082	0.066	0.069	0.071	0.074	0.074
4	*M. edax* (MI)	0.013	0.012	0.000		0.002	0.054	0.054	0.051	0.066	0.079	0.064	0.066	0.069	0.071	0.071
5	*M. edax* (FL)	0.013	0.012	0.000	0.000		0.056	0.056	0.054	0.064	0.076	0.066	0.069	0.066	0.074	0.069
6	*M. longisetus*	0.020	0.015	0.010	0.010	0.010		0.005	0.023	0.064	0.077	0.056	0.059	0.069	0.064	0.069
7	*M. longisetus 'curvatus'*	0.020	0.015	0.010	0.010	0.010	0.000		0.018	0.066	0.079	0.051	0.054	0.064	0.059	0.071
8	*M. meridianus*	0.022	0.017	0.012	0.012	0.012	0.008	0.008		0.071	0.084	0.058	0.056	0.071	0.066	0.076
9	*M. darwini*	0.013	0.008	0.017	0.017	0.017	0.015	0.015	0.016		0.018	0.020	0.023	0.018	0.023	0.005
10	*M. major*	0.017	0.015	0.012	0.012	0.012	0.017	0.017	0.018	0.017		0.033	0.036	0.030	0.036	0.023
11	*M. leuckarti*	0.017	0.015	0.012	0.012	0.012	0.017	0.017	0.018	0.017	0.000		0.002	0.018	0.008	0.025
12	*M. pehpeiensis*	0.018	0.017	0.013	0.013	0.013	0.017	0.017	0.018	0.015	0.002	0.002		0.020	0.010	0.028
13	*M. aspericornis*	0.020	0.015	0.015	0.015	0.015	0.015	0.015	0.017	0.013	0.003	0.003	0.002		0.020	0.023
14	*M. ogunnus*	0.022	0.020	0.015	0.015	0.015	0.020	0.020	0.022	0.022	0.005	0.005	0.007	0.008		0.028
15	*M. thermocyclopoides*	0.018	0.017	0.012	0.012	0.012	0.017	0.017	0.018	0.018	0.002	0.002	0.003	0.005	0.003	

Any transformation between fixed present, fixed absent and polymorphic states has equal cost. In the scaled run characters 6, 8–11, 14, 17, 23, 50, 53, 54, 63 and 79 were coded as unordered, and all others were coded as ordered; the fixed characters 4, 6–12 14, 17, 21–23, 25, 28, 37, 38, 41–45, 47, 50, 53–55, 57, 59–63, 68, 74, 75, 77, 79 and 81 are given a weight of 2, and polymorphic characters are given a weight of one. In the scaled method the states fixed present, polymorphic, and fixed absent are ordered. When polymorphic states are not found, it is assumed that they were present but unobserved and the cost of the transformation from fixed absent to fixed present is 2.

2.3. Molecular characters

Genomic DNA was extracted from single adult female copepods that had been frozen in liquid nitrogen or fixed in undenatured 95% alcohol. Genomic DNA was obtained using the PROMEGA Total RNA isolation kit, omitting the DNAse step, with a resultant volume of 50 μl. Genomic DNA (5 μl) was used in the polymerase chain reaction (50 μl). Amplification reactions of the 3′ end of the 18S rRNA gene were carried out using the primers 18s329 [5′: TAATGATCCTTCCGCAGGTT:3′] and 18sI- [5′: AACT(C,T)AAAGGAAT TGACGG:3′] given in Spears et al. (1992). Amplification conditions consisted of 5 min at 95 °C, and then 40 s at 95 °C, 25 s at 50 °C, 3 min at 72 °C for 40 cycles, and extension for 15 min at 72 °C. Amplification reactions of the ITS2 region were carried out using the primer ITS3F [5′: GCATCGATGAAGAACGCAGC:3′] which is anchored in the 5.8S ribosomal gene and given in White et al. (1990) and ITS10R [5′: TACGGGCCTATCACCCTCTACG:3′], which amplifies about 110 bp of the 28S rDNA gene. Amplification conditions consisted of 50 s at 95 °C, and then 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C for 35 cycles, and extension for 7 min at 72 °C.

DNA sequencing was carried out on an ABI 3730xl automated DNA sequencer. Proofreading, editing, and alignments were performed using the program Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Multiple sequence alignment of the 18s rDNA was accomplished by eye and did not require exclusion of any sites. Multiple sequence alignment of the ITS2 was accomplished using the default in ClustalW (Thompson et al., 1994) followed by modification by eye and resulted in 135 ambiguous nucleotide sites being eliminated. Pairwise percentage differences in sequences were computed as uncorrected distances.

All species except *Mesocyclops leuckarti* were reared in the laboratory for one or more generations, using sister–brother matings to generate isofemale lines. The female used to establish each isofemale line or her offspring were identified by M. Hołyńska or J. Reid using Hołyńska et al. (2003) and these vouchers were deposited in the National Museum of Natural History, Smithsonian Institution. A single female offspring of each isofemale line was used to obtain a genomic DNA extract. For each population, a second individual was sequenced to check for intrapopulation variability. Collection numbers and localities for voucher specimens and GenBank accession numbers are given in Appendix A.

2.4. Phylogenetic analyses

Phylogenetic trees were estimated using both parsimony and probabilistic methods. Five parsimony analyses using different data combinations and character weighting were conducted using PAUP* beta version 4.0b10 (Swofford, 2002): (1) morphological characters only, unordered with equal weights; (2) morphological characters only, with some characters ordered, and state transformation costs weighted by 2 in non-polymorphic characters (see Section 2, and Appendix B for weighted characters); (3) combined molecular analysis of both ITS2 and 18S rDNA regions; (4) combined molecular data and morphological characters unordered with equal weights; (5) combined molecular data and morphological characters with some characters ordered and state transformation costs weighted by 2 in non-polymorphic characters.

Optimal trees were estimated using heuristic searches with 1000 replicates of random stepwise addition and tree bisection and reconnection (TBR) branch swapping. Bootstrap resampling (Felsenstein, 1985a) was applied to assess support for individual nodes using 1000 bootstrap replicates and full heuristic searches with 100 replicates of random stepwise addition and TBR branch swapping.

Maximum-likelihood (ML) analyses also were performed using PAUP* but only using the combined *ITS2* and 18S molecular data (MP search #3 above). Searches were conducted using a successive approximations approach (Swofford et al., 1996; Sullivan et al., 2005). Both hierarchical likelihood ratio tests and the Akaike Information Criterion as implemented in Modeltest v3.7 (Posada and Crandall, 1998) were used to find the best fitting model of sequence evolution for a tree estimated using neighbor joining (NJ). Parameter values estimated on the NJ tree were fixed in initial heuristic searches with starting trees obtained via NJ and TBR branch swapping. Optimal tree(s) obtained from this search were used to estimate new parameter values under an identical model. These new parameter values were then fixed in a second search with the same conditions as the initial run. This process was repeated until the same tree and parameter values were obtained in two successive searches. Bootstrap resampling was applied using ML with 500

pseudoreplicates and heuristic searches as above except that successive approximations were not conducted for each pseudoreplicate. Parameter estimates for bootstrap analyses were fixed at the parameter values obtained from the final iteration of the corresponding successive approximations analysis. In our evaluation of branch support strength, we considered a bootstrap value of ⩾95% as strongly supported (Felsenstein and Kishino, 1993), <95% to ⩾70% as moderately supported, and <70% as weakly supported.

Bayesian analyses on the combined morphological and molecular data set were performed using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using a general time-reversible (GTR) model of sequence evolution for molecular data. Morphological characters were unordered, equally weighted, and treated as "standard" assuming the Mk evolutionary model (Lewis, 2001), which allows for *k* number of discrete character states and the rate of character change to be equal or to vary. Rate variation among all sites was estimated with a gamma distribution, as well as the parameter that estimated the proportion of invariant sites. Two independent runs were performed in MrBayes with 20×10^6 generations and four Markov chains (3 heated, 1 cold) using default heating values. Parameter values for the model were estimated from the data and most were initiated with default uniform priors except that branch lengths were unconstrained (no molecular clock) with default exponential priors. Trees and parameter values were sampled every 1000 generations resulting in 20,000 saved trees per analysis, of which 10,000 were discarded as "burn-in". Stationarity was assessed by plotting the $-\ln L$ per generation in the program Tracer 1.4 (Rambaut and Drummond, 2005) and using the average standard deviation of split frequencies available in MrBayes. If the two simultaneous runs are converging on the same tree, the average standard deviation of split frequencies are expected to approach zero. After confirming that the analysis appeared to reach stationarity, the resultant 20,000 trees (10,000 from each simultaneous run) were used to calculate Bayesian credibility values (BC) for each branch in a 50% majority-rule consensus tree. Clades with BC ⩾ 95% were considered strongly supported with the caveat that BC may overestimate support for reasons discussed by Alfaro et al. (2003), Douady et al. (2003), and Lewis et al. (2005).

2.5. Deep relationships within Mesocyclops – Hypothesis testing

Significance tests were used to examine support for the optimal tree(s) relative to alternative hypotheses of deep relationships of *Mesocyclops* taxa. Constraint tests tested the optimal (parsimony or likelihood) topology in the absence of a constraint (Fig. 1B) against the optimal tree under a topological constraint (Topologies 1–3 of Fig. 3).

To find the optimal tree(s) compatible with a particular phylogenetic hypothesis, topologies containing the minimal number of groups necessary to describe the hypothesis of interest were constructed using MacClade 4.08 (Maddison and Maddison, 2003). These partially resolved topologies were loaded as constraints in PAUP*, which was then used to analyze the sequence (and morphological) data under the parsimony criterion using heuristic searches with the same settings used to estimate the most parsimonious trees in the absence of topological constraints. Phylogenetic hypotheses were tested similarly under likelihood using constraint trees and a successive approximations approach using the same search strategies as described for searches in the absence of topological constraints.

Wilcoxon signed-ranks (WSR) (Felsenstein, 1985b; Templeton, 1983) and Kishino–Hasegawa (KH) (Kishino and Hasegawa, 1989) tests were used with the parsimony trees. The three alternative hypotheses each were tested using MP data treatment strategies 3, 4, and 5 as described above. They were conducted as two-tailed tests using PAUP*, which incorporates a correction for tied ranks. Goldman et al. (2000) criticized the application of the KH and WSR tests as applied in many studies because this test assumes that phylogenetic hypotheses are selected post-search. In any case, Shimodaira–Hasegawa (SH) (Shimodaira and Hasegawa, 1999) tests also were performed to test the optimal ML tree using the combined molecular dataset only in the absence of topological constraints (which corresponded to one of the a priori hypotheses) independently of the data analysis (a priori). However, as the optimal trees used in all of our tests corresponded with one of the three alternative hypotheses identified in previous studies (Figs. 7A and B, 15 in Hołyńska, 2006), they may be considered to have been chosen a priori against the optimal ML trees under each of the alternative hypotheses (see Fig. 3 for constraint trees). SH tests were conducted in PAUP* using 10,000 resampling estimated ln-likelihoods of sites (RELL).

3. Results

3.1. Morphology based phylogenies and the effect of taxon sampling

Hołyńska (2006) used different outgroup combinations and two algorithms in her analyses and obtained conflicting results concerning the deep relationships of *Mesocyclops*, namely between North American *M. edax*, the Neotropical species (e.g., *M. longisetus, Mesocyclops meridianus*), 'Old World' group (e.g., *M. leuckarti, Mesocyclops pehpeiensis, Mesocyclops major, Mesocyclops aspericornis, Mesocyclops ogunnus, Mesocyclops thermocyclopoides*), and the Paleotropical *rarus* clade (e.g., *Mesocyclops darwini*). On the tree that we chose as a guide for our taxon sampling, the *rarus*-group is the first offshoot of the monophyletic *Mesocyclops*, and the 'Old World' group is the sister of a clade that includes *M. edax* and the Neotropical species. The topology of our 15 taxa, MP tree based on unordered morphology is very similar to the guide tree based on Hołyńska (2006, Fig. 7A), except for the positions of *M. edax* and *M. darwini* (Figs. 1A and 2A). Of the 81 morphological characters used in the analyses, 50 were parsimony informative.

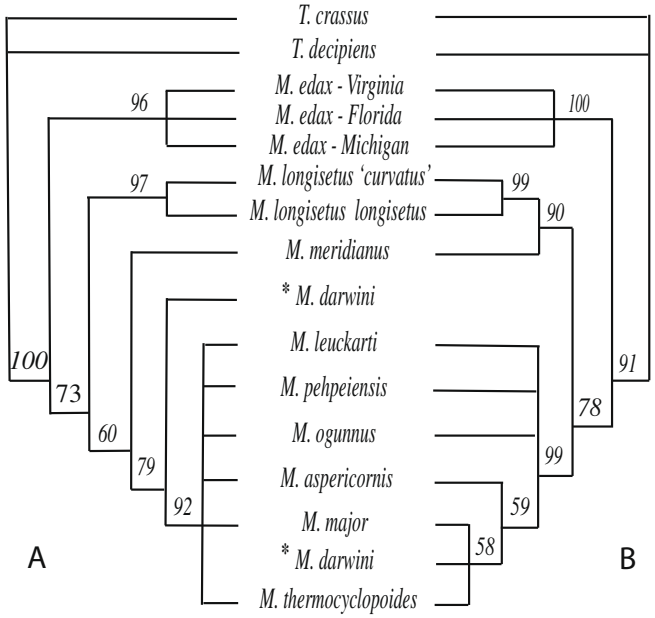

Fig. 2. Phylogenetic relationships of *Mesocyclops* based on morphology or molecules and using *Thermocyclops* as outgroups. Numbers at nodes refer to bootstrap values. (A) Unordered morphology, MP. (B) Combined 18S rDNA and ITS2 sequences, ML.

Four additional nodes, (*leuckarti*, (*pehpeiensis*, (*aspericornis*, (*major*, (*ogunnus*, *thermocyclopoides*))))), were resolved in a strict consensus tree of the MP analyses using ordered morphological characters in the 15 taxa trees. Bootstrap support values of these clades were weak (tree not shown). The tree topologies in the unordered and ordered setting were otherwise consistent.

3.2. Molecule based trees with limited taxon sampling

The 603 bp region of the 18S rDNA molecule aligned unambiguously, with only a single gap shared by the Old World group sensu Hołyńska (2006). Pairwise distances among the *Mesocyclops* ingroup species were typically 0.1–0.2% (Table 1). No sequence variation among the Michigan, Virginia, and Florida *M. edax* populations, nor between the Louisiana population and Brazilian *curvatus* form of *M. longisetus*, was detected. The average nucleotide composition among the 15 sequences was A, 25.8% (range 25.7–25.9%), C, 28.5% (range 28.4–28.9%), G, 24.1% (range 23.7–24.4%), T, 21.6% (range 21.6–22.0%). Nucleotide base composition did not differ significantly across taxa ($X^2 = 0.42$, d.f. = 42, $P > 0.999$).

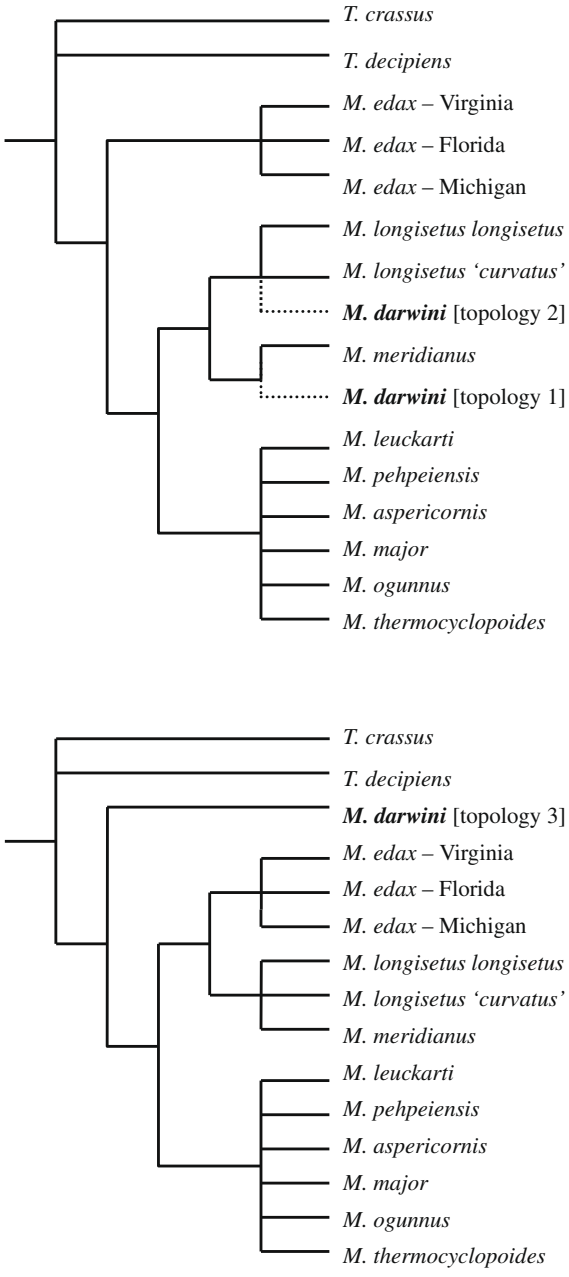

Fig. 3. Three alternative hypotheses of *Mesocyclops* phylogeny based on morphology that were tested against the phylogeny based on both morphology and molecules shown in Fig. 1B. The constraint tests compared the position of *M. darwini* and thus evaluated the support for alternative hypotheses about deep relationships in the genus *Mesocyclops*. All topologies are from Hołyńska (2006): Topology 1 (Figs. 7B and 20C), topology 2 (Figs. 15 and 20C), and topology 3 (Figs. 7A and 20B).

The amplified regions for which ITS2 sequences of all 15 taxa could be unambiguously aligned at the 5′ and 3′ ends yielded fragments that varied in length from 487 nts in *Thermocyclops decipiens* to 501 nts in *M. thermocyclopoides*. Only 397 of these sites could be unambiguously aligned and thus used in the phylogenetic analyses. Pairwise distances among the *Mesocyclops* ingroup species based on these 397 sites varied from 0.2% to 8.4% (Table 1). Intraspecific variation among the Michigan, Virginia, and Florida populations of *M. edax* of a 533 bp long amplified sequence contained only 11 (0.02%) variable sites. About twice as many sites, 20, varied between the 533 bp amplified fragments of the US *M. longisetus longisetus* and Brazilian *M. longisetus 'curvatus'* populations. The average nucleotide composition among the 15 sequences was A, 20.7% (range 19.9–21.3%), C, 28.2% (range 27.5–29.2%), G, 29.8 (range 29.5–30.3%), T, 21.2% (range 20.6–22.2%). Nucleotide base composition did not differ significantly across taxa ($X^2 = 2.58$, d.f. = 42, $P > 0.999$).

The molecule based trees constructed using ML and MP (not shown) optimality criteria were similar to the morphological tree based on complete taxon sampling, with the exception that *M. darwini* was nested in the 'Old World' group (Fig. 2B), rather than being sister to a clade including both Old World and New World species (Fig. 1A). Together the 18S rDNA and ITS2 sequences comprised 1000 nucleotides that were used in the phylogenetic analyses of the 15 taxa tree. Of these, 54 characters were parsimony informative. The model of evolution that best fit the molecular data when subjected to ModelTest using the AIC criterion was TrN + I + G for the combined 18s rDNA and ITS2 sequences. Gamma distribution shape parameter alpha = 0.7332; I = 0.7919.

Similar topologies and levels of support at most nodes were obtained for the morphology based MP trees and the molecule based ML and Bayesian (not shown) trees when only 15 taxa were sampled (Fig. 2A and B). The number of parsimony informative characters is also similar: 54 molecular characters and 50 morphological characters. In the morphology based tree, *M. darwini* is a sister group to the 'Old World' *Mesocyclops* (*M. leuckarti, M. pehpeiensis, M. ogunnus, M. aspericornis, M. major,* and *M. thermocyclopoides*), although this relationship is supported at the moderate bootstrap value of 79. In the molecule based tree, *M. darwini* is not a sister to all other Old World taxa, but is nested in the 'Old World' clade, where it forms a monophyletic group with *M. major* and *M. thermocyclopoides*.

3.3. Combined data tree with limited taxon sampling

The topology produced by combined data and limited taxon sampling (Fig. 1B) is strikingly similar to the morphology based tree with complete taxon sampling (Fig. 1A) and published in Hołyńska (Fig. 7A in 2006). As observed in the comparison of the morphology based tree with the molecular based tree of 15 taxa (Figs. 1A and 2B), the distinctive disparity between the trees lies in the position of *M. darwini,* and thus of the clade containing *M. major, M. leuckarti, M. pehpeiensis, M. aspericornis, M. ogunnus,* and *M. thermocyclopoides* (Fig. 1B). These differences were observed whether the combined data analyses were performed with MP or Bayesian analyses. The combined use of morphological and molecular characters to create the 15 taxa tree (Fig. 1B) resulted in a tree whose topology of the New World clade was congruent with

the molecule based tree (Fig. 2B) but whose topology of the Old World clade was congruent with the morphology based tree (Fig. 2A).

3.4. Tests of alternative hypotheses of deep relationships in Mesocyclops

In the trees constructed in the present study *M. darwini* is either nested in or the sister of a clade containing *M. leuckarti, M. pehpeiensis, M. aspericornis, M. major, M. ogunnus,* and *M. thermocyclopoides*. The trees based on combined data of morphology (whether unordered – Fig. 1B or ordered – not shown) and molecules were tested against each of the three alternative hypotheses with different placements of *M. darwini* (Fig. 3, Topologies 1–3,). Parsimony-based statistical tests (WSR and KH tests) rejected all three alternative topologies (Table 2) in both combined data sets differing only in treatment of morphological characters. When the SH test is applied only to the molecular data, this test also rejected all three alternative topologies. Bayesian hypothesis tests applied to the combined data of unordered morphology and molecules rejected all three alternative hypotheses for the placement of *M. darwini* (Fig. 3). Using a 95% credible interval cutoff, there were no compatible trees in the post- burn-in sample compatible with any of the three topologies.

Overall, the results of the phylogenetic analyses presented here reveal largely similar topologies, whether based on only molecules or morphology or a combined data set. There was a trend toward increased support of the clades as the number of characters increased from the 81 morphological characters to 1068 combined characters.

4. Discussion

4.1. Mesocyclops phylogeny

Mesocyclops is the first cyclopoid genus for which a molecular phylogeny has been constructed and the first cyclopoid taxon to be subjected to phylogenetic analyses that combine both morphology and molecules (Figs. 2B and 1B). *Mesocyclops* is also the only cyclopoid genus for which a phylogeny based on complete taxon sampling has been published (Hołyńska, 2006) and this has allowed tests of whether limited taxon sampling based on a combined set of characters could be used to refine phylogenetic hypotheses.

Sampling less than 20% of the genus and using either molecules or morphology yielded topologies very similar to those obtained with complete taxon sampling and morphology only (Figs. 1 and 2). Yet, in contrast with the reconstructions using full taxon sampling (Hołyńska, 2006), our analyses, regardless of the type of data (morphology, molecule, or combined) consistently support monophyly of all Old World taxa and paraphyly of the New World species (Figs 1B and 2A and B; Table 2). This finding was unexpected, as in the previous reconstructions the 'Old World' group (defined by, among others, the lack of medial spine on P1 basipodite) did not form a clade with the Paleotropical *rarus*-group (P1 basipodite with medial spine) here represented by *M. darwini*. Rather, the *rarus*-group either appeared as the sister of the large clade including both New World and Old World taxa (Fig. 7a in Hołyńska, 2006; Fig. 3: topology 3) or the sister clade of a Neotropical clade (Fig. 7B, 15 in Hołyńska, 2006; Fig. 3: topologies 1 and 2).

The discovery of new species of *Mesocyclops* can be expected from any part of the tropics; nevertheless, incomplete species discovery is unlikely to have a significant effect on the phylogenetic reconstructions presented here. Comprehensive taxonomic and zoogeographic revisions were made relatively recently in all major tropical regions (tropical America: Gutiérrez-Aguirre and Suárez-Morales, 2001; Gutiérrez-Aguirre et al., 2006; Africa: Van de Velde, 1984; Southeast Asia: Hołyńska, 2000; tropical Australia: Hołyńska and Brown, 2003). The New Zealand *Mesocyclops* are completely unknown, but this predominantly tropical genus may have just a few representatives in this temperate climate region.

4.2. Molecule based phylogenies

As expected the 18S rDNA molecule evolved slowly within *Mesocyclops*, containing species pairwise genetic distances less than 2% (Table 1). This molecule has proven to be among the most useful of molecular markers in eukaryotes, and its use in resolving generic and species level relationships (Thum, 2004) and higher order analyses of copepod phylogeny (Bucklin et al., 2003; Huys et al., 2006, 2007) has been demonstrated. No interpopulation variability in the 3′ end fragment of the 18S rDNA molecule was observed among the Michigan, Virginia, and Florida *M. edax* populations. Similarly, no variability was detected between the U.S. and Brazilian populations of *M. longisetus*.

The ITS2 region apparently evolves at a rate that is very informative at the population and species levels. The large proportion of ambiguously aligned sites is likely due to relaxed selection on the loops of this spacer molecule, as discussed by Wesson et al.

Table 2
Results of Wilcoxon signed-ranks (WSR), Kishino-Hasegawa, and Shimodaira-Hasegawa (SH) tests comparing the topology based on the combined data set with three alternative phylogenetic hypotheses (topologies) based on morphology and presented in Holynska (2006). For each hypothesis and data set, the score of the best tree conforming to the specified hypothesis, its difference from the score of the best tree overall, and the *P*-values are given. MU refers to the combined data set using unordered morphological characters and MO refers to the combined data set using ordered morphological characters. Significant values ($P \leqslant 0.05$), indicating rejection of the hypothesis, are indicated with an asterisk.

Tree	WSR tests			KH tests		SH tests	
	Length	*N*	*P*	Length diff.	*P*	δ-ln *L*	*P*
Topology 1							
Combined (MU)	313	33	0.001*	23	0.001*	N/A	N/A
Combined (MO)	356	33	0.001*	25	0.001*	N/A	N/A
Molecular	154	32	0.001*	21	0.001*	44.786	0.000*
Topology 2							
Combined (MU)	313	33	0.001*	23	0.001*	N/A	N/A
Combined (MO)	357	33	0.001*	26	0.001*	N/A	N/A
Molecular	154	32	0.0004*	21	0.0004*	44.786	0.000*
Topology 3							
Combined (MU)	305	35	0.011*	15	0.011*	N/A	N/A
Combined (MO)	350	35	0.015*	19	0.013*	N/A	N/A
Molecular	145	31	0.0396*	12	0.0395*	27.285	0.009*

(1992). The considerable variability in the ITS2 sequences among the freshwater cyclopoids studied here makes the multiple alignment especially sensitive to choice of outgroup. Multiple sequence alignments using *D. bernardi*, which was once placed within the *Mesocyclops*, required the exclusion of more sites than those using *Thermocyclops*. Future analyses should examine if species occupying alternative phylogenetic levels in the *Thermocyclop*s group would necessitate exclusion of fewer characters than when using *Thermocyclops decipiens* or *Thermocyclops crassus*. Hampering this endeavor is the lack of a complete taxonomy of *Thermocyclops* on which to base a phylogeny and infer deep relationships. Interpopulational differences in ITS2 sequences were detectable and repeatable, but not so large that they obscured species level relationships. Rocha-Olivares et al. (2001) found such high levels of sequence variability in this spacer region that they could not align populations of the marine harpacticoid copepod *Cletocamptus deitersi* species complex. In contrast, Goetze (2005) used the ITS2 region to successfully resolve relationships among cryptic species of the marine calanoid *Eucalanus*.

The only study with which we can compare the utility of our combined data approach is that of Bucklin and Frost (2009) on *Clausocalanus*. Though their study (which utilized ITS2, and portions of the 5.8S rDNA and mtCOI sequences) and the present study examined a comparable number of nucleotide sites, *Mesocyclops* possesses more morphological characters amenable to phylogenetic analysis (81) than does the *Clausocalanus* (16), which may account for the higher degree of resolution realized for most nodes in the reconstructions of *Mesocyclops* phylogenies. The cryptic species or recently diverged populations that are likely more prevalent in *Clausocalanus* requires, as does the present study, the necessity to sample more nucleotide sites in molecules that are phylogenetically informative at levels of divergence of species. Both ITS1 and ITS2 regions hold promise for providing more phylogenetically informative characters if models of secondary structure become available for increasing the ability to align larger regions of these molecules, thus reducing the number of ambiguously aligned characters and enabling the inclusion of additional outgroups in the tree reconstruction. Improvements in inferring homologies, and thereby improving alignments, may do more to increase the resolution of nodes than improvements in computational methods for phylogenetic reconstruction (Kjer et al., 2009). The current difficulties of amplifying or sequencing functional copies of the COI molecule in some copepods (Nonomura et al., 2008) will likely be overcome as its unusual variability in some copepods (Machida et al., 2002, 2004; Burton et al., 2007; Ki et al., 2009) becomes better understood.

4.3. Combined molecular and morphological based phylogenies, alternative topologies

The reconstruction of *Mesocyclops* phylogeny using the combined character set and limited taxon sampling generally yielded higher levels of support when compared with analyses based on limited taxon sampling and only one type of character. Remaining unresolved, and of particular interest, is the position of *M. darwini*, a representative of the *rarus*-group. The relationships of the *rarus* clade to the 'Old World' and New World clades are needed to inform us about the deep relationships of *Mesocyclops.* The position of the *rarus* group, a clade with seven species distributed in Africa, Madagascar, South Asia, Southeast Asia, New Guinea, and Australia varied according to the outgroups and algorithm when the *Mesocyclops* genus was sampled completely (Hołyńska, 2006). The combined data analysis, similar to the reconstructions based on morphological characters only (Figs 1B and 2A), placed *M. darwini* (Australia, New Guinea) as a sister to all other Old World species. The ML analyses based on molecules only grouped the *M. darwini* within the 'Old World' clade (Fig. 2B). Alternative topologies were compared using constraint tests (Fig. 3, Table 2), and a close relationship of *M. darwini* (nested in, or sister) with the Old World species was supported.

In the combined (molecule and morphology) parsimony analyses (both 'unordered' and 'scaled' run), three unambiguous morphological character transformations (char 36: 0–>1; char 44: 0–>1; char 45: 0–>1) define the clade including *M. darwini* and 'Old World' group sensu Hołyńska, 2006. All the three apomorphic features are 'gains' and concern the spinule ornamentation on the frontal surface of the antenna and mandible. The apomorphies on the mandible (chars 44 and 45) in the Old World clade (*rarus*-group + 'Old World' group) are unique, because the spinule groups in question are missing in all New World *Mesocyclops* species and several outgroup taxa (data in Hołyńska, 2006). Among the Old World taxa there is only a single known exception, *Mesocyclops cuttacuttae* a North Australian subterranean species, in which the ancestral states (spinule groups are missing) appear. As to the third character (char 36) a comparison extended to all representatives of the genus and large outgroup (data in Hołyńska, 2006) leaves little doubt about the derived state of this feature in the Old World clade: while in most Old World species there is a longer row with many spinules along the lateral margin of the antennal coxobasis, in all New World species and all outgroup taxa this row is short consisting of few spinules (Hołyńska, 2006). Yet, it is not fully clear when the derived character state arose: the 'many spinules' state appear only in three representatives (*Mesocyclops darwini, Mesocyclops rarus*, and *Mesocyclops paludosus*) of the *rarus*-group, while in four other species (*Mesocyclops annae*, *Mesocyclops pseudoannae*, *Mesocyclops splendidus* and *Mesocyclops dayakorum*) of the *rarus*-group the ancestral 'few spinules' state is present. This might suggest that derived 'many spinules' state appeared after the Old World clade had taken its origin.

Clearly, addition of more taxa of the *rarus*-group and other species closer to the root of the genus would lend more confidence to our results.

4.4. Biogeography of Mesocyclops

The lack of fossils, the most credible source of information from which we can try to infer the geographical origin and age of this (sub)tropical genus, requires that we rely on information gleaned from extant fauna: phylogeny, zoogeographic distribution, and taxonomic richness of the zoogeographic regions. Furthermore, a few fossils are known only for some distantly related copepod taxa and exceptionally rapid evolution of the mitochondrial genome in *Mesocyclops* preclude using the molecular clock to estimate the ages of the lineages (Palmer, 1960; Cressey and Patterson, 1973; Cressey and Boxshall, 1989; McClellan and Wyngaard, unpublished data).

In the tree based on the largest set of the characters (combined molecules and morphology; Fig. 1B) the New World species form a paraphyletic group and the Old World species (*M. darwini* and members of the 'Old World group' sensu Hołyńska, 2006) constitute a monophyletic group. This single topology (Fig. 1B), however, can be compatible with different hypotheses of the historical biogeography of the genus, depending on what significance is given to dispersal events in the evolution of the genus. And, even when vicariance is favoured against dispersal, various processes acting in different times and/or space could result in the same vicariant pattern. Below, we discuss some possible scenarios with the intent of stimulating further studies on the systematics and zoogeography of *Mesocyclops* and other tropical cyclopid genera that would contribute to a more informed view of the evolutionary history of this genus.

4.4.1. Dispersal – New World origin?

The topology of the combined tree (Fig. 1B) might suggest that *Mesocyclops* originated in the New World, and the Old World was

later invaded (Fig. 4A). A post-Gondwanan, late Eocene/Oligocene (ca 35 MYA) dispersal from Africa to South America was hypothesized by Schrago and Russo (2003) to explain the origin of New World monkeys. The transatlantic dispersal route from east to west could act partly because of the favorable water and wind currents, and partly because of the chains of islands in the Sierra Leone Rise and Walvis Ridge, which appeared as the sea level lowered at the Eocene–Oligocene transition. These stepping stone islands might facilitate passive dispersal of the freshwater cyclopids in the opposite direction as well, from South America to Africa. Wide intercontinental distribution of some *Mesocyclops* taxa and their not uncommon occurrences in the insular faunas do indicate good dispersal abilities in certain representatives of the genus (e.g., *M. aspericornis, M. thermocyclopoides, M. ogunnus, M. longisetus*, etc.). Asia may have been invaded from Africa mainly after the Miocene contact of the two continents (Bănărescu, 1995), and Australia and may have been subsequently invaded by fauna from Southeast Asia.

Nonetheless, the great taxonomic diversity of the Old World *Mesocyclops* argues against a New World origin of the genus and

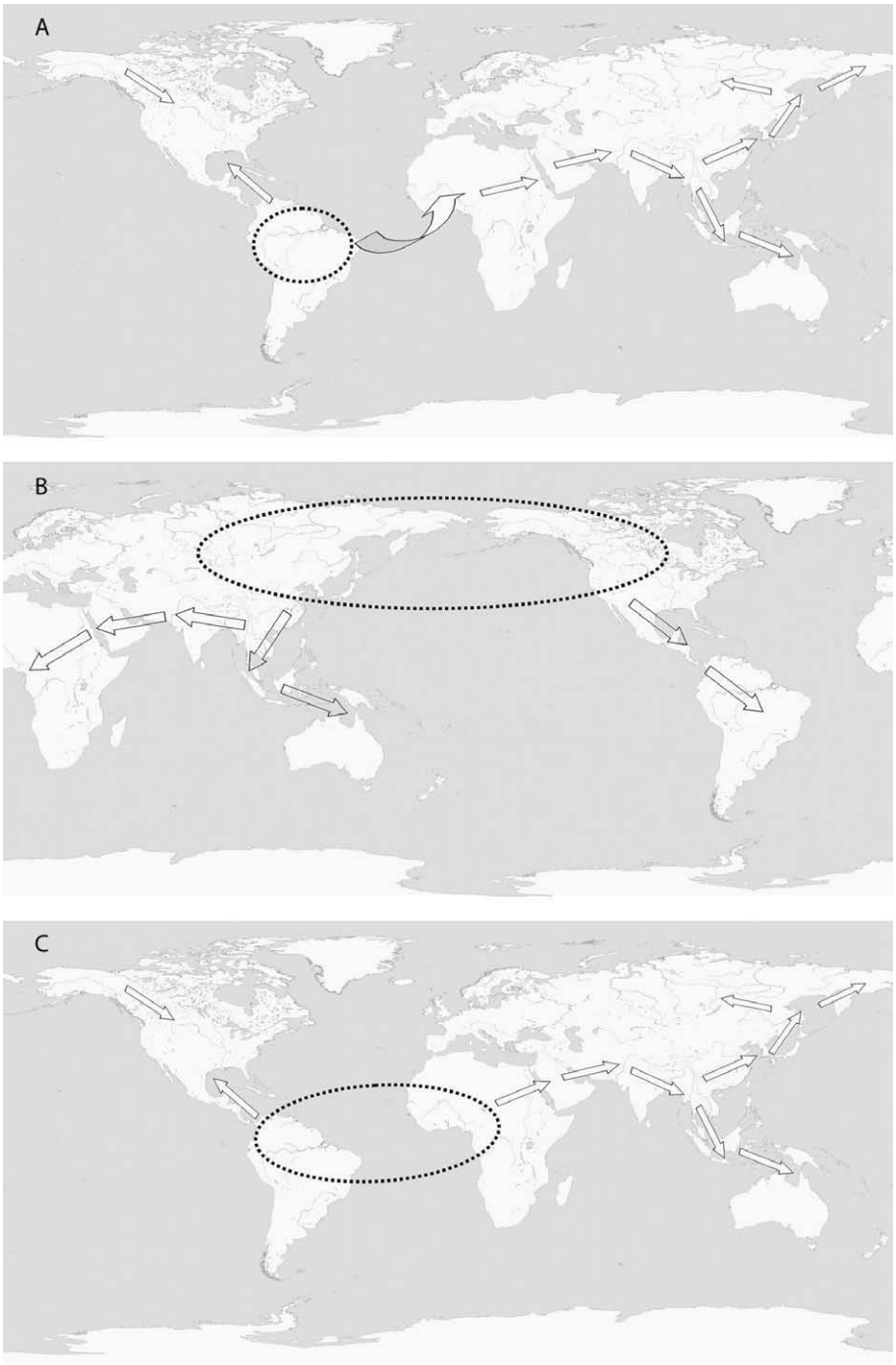

Fig. 4. Alternative hypotheses of geographical origin of the *Mesocyclops* genus. (A) New World origin hypothesis. Area indicated by dotted line shows presumed ancestral area. Half-gray arrow shows long-distance dispersal event. Open arrows show the main routes of dispersal in the genus. (B) Laurasian origin hypothesis. Area indicated by dotted line shows presumed ancestral area. Open arrows show the main routes of dispersal in the genus. (C) West Gondwanan origin hypothesis. Area indicated by dotted line shows presumed ancestral area. Open arrows show the main routes of dispersal in the genus.

younger age of the Old World group. The Old World continents (Africa, Eurasia, Australia) harbor about three times as many species as the New World (North and South America). A minimum of ten morphologically distinct groups occur in the Old World, whereas there are four or five groups in the Americas, depending upon whether the *Mesocyclops reidae – Mesocyclops chaci – Mesocyclops yutsil* clade, a group excluded from 'true *Mesocyclops*' in Hołyńska (2006), is also counted here. The use of divergence dating analyses also would be highly informative to test these ages proposed here, but those data are currently unavailable. Support also would be gleaned for a New World origin, if the lineages close to the root in putative close relatives of *Mesocyclops*, such as the *Thermocyclops*, are South American in distribution.

4.4.2. Vicariance – Laurasian or West Gondwanan origin?

Also to be considered is that our combined tree (Fig. 1B) and most morphology based reconstructions with full taxon sampling (Hołyńska, 2006) show separation of the Old World lineage at a very early phase of *Mesocyclops* evolution, which renders a vicariant scenario equally plausible. In this case one speciation event (*M. edax)* within the large ancestral area, and one vicariance event separating the formerly connected New World and Old World land masses, could explain the present distribution patterns (Fig. 4B and C). There are at least two explanations as to where and when a splitting into Old World and New World lineages could arise:

Laurasian origin – Land bridge connections between East Asia, North America and Europe existed until the Early Eocene, when the climate was much warmer and more humid than present day. Tropical rainforests reached the 50th latitudinal degree, and paratropical rainforest extended to 60–65 latitudinal degrees (Wolfe, 1985). The Thulean bridge connecting North America with Europe, and the Beringian bridge connecting America with East Asia facilitated the interchange of (sub)tropical fauna (Sanmartín et al., 2001). The Thulean bridge broke off in the Early Eocene. The Beringian existed even in the Pleistocene; yet, the drastic cooling that started in the Eocene–Oligocene boundary may have inhibited exchange of warm-adapted forms between North America and Asia. The deep divergence of the Old World and New World lineages of *Mesocyclops* could be connected to a Late Eocene–Oligocene event, when the Beringian bridge became impermeable for tropical/subtropical animals. According to this scenario (Fig. 4B) *Mesocyclops* had its origin in Laurasia, and the global cooling caused shifts of the distributional areas of the (sub)tropical forms southwards, similar to those observed for instance in tapirs and several tropical plants (Ashley et al., 1996; Morley and Dick, 2003).

We cannot, however, find any clade in *Mesocyclops* that would show the tropical amphi-Pacific distribution (present in the Neotropics and Southeast Asia, but absent in Africa) common in tropical plants with Laurasian origin (Morley and Dick, 2003). Close contact between East Asia and North America can be inferred from the geographic distribution of the members of the *leuckarti*-clade (Fig. 1 A, Hołyńska, 2006) [*M. leuckarti*: whole Palearctic; *Mesocyclops mongoliensis*: Mongolia China; *Mesocyclops americanus*: North America; *Mesocyclops yesoensis*: Hokkaido (Japan); *Mesocyclops pescei*: Bahamas and Mexico], yet the *leuckarti*-clade being a temperate zone group cannot be conclusive of Laurasian origin of the tropical *Mesocyclops*.

West Gondwanan origin – Worldwide distribution of the genus suggests an old origin. The more ancestral lineages of the morphology based trees with full taxon sampling (Hołyńska, 2006; see Fig. 1A) occur in Gondwanan continents: i.e. *rarus*-group in Africa, Madagascar, India, Southeast Asia, New Guinea, Australia; *Mesocyclops cuttacuttae* in Australia; *Mesocyclops salinus*: in Africa; *Mesocyclops yenae – Mesocyclops brevisetosus*: in Southeast Asia, New Guinea and Australia; and the Neotropical clade. The basal New World – Old World split in *Mesocyclops* can be explained if the genus originated in the part of Gondwana that included northern (tropical) South America and Africa, and the divergence of the New and Old World clades could be connected to mid-late Cretaceous (110-95 MYA) separation of the landmasses (Fig. 4C). Tropical Asia would be populated from Africa after the Miocene contact of the continents, yet the Australian fauna, similarly to that in the "dispersal scenario", might have its origin in Southeast Asia. Evidently, inclusion of more taxa, especially from the more basal representatives of the 'Old World' clade, the *rarus* group, and the Neotropical species, is highly recommended in the future analyses to relate phylogeny with the sequential break up of Gondwana.

5. Conclusion

Trees based on different types of characters, algorithms and extent of taxon sampling all yielded similar topologies, with the highest support recovered when morphological and molecular data are combined. The paucity of well studied molecules for phylogenetic analyses of copepods is such that morphology can contribute substantial information toward constructing topologies, as well as provide guidance in designing molecular studies. For the specific problem explored in the present paper, perhaps it will be more important to increase the sampling in the *rarus*-group, the New World taxa, and more basal representatives of the 'Old World' group rather than to add new characters.

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Appendix A

Species, locale, museum specimen numbers, and GenBank accession numbers of specimens from which 18S rDNA and ITS2 sequences were obtained. All specimens are deposited in the National Museum of Natural History (USNM), Smithsonian Institution, Washington, District of Columbia.

Outgroups: *Thermocyclops crassus* Haiphong City, Nam Ha Province, North Vietnam (USNM1121776, GQ848503, GQ848489); *Thermocyclops decipiens*, Barra Bonita Reservoir, São Carlos, SP, Brazil, (USNM1121777, GQ848504, GQ848490).

Ingroup: *Mesocyclops aspericornis*, Barra Bonita Reservoir, Brazil (USNM1121763, GQ848515, GQ848501); *Mesocyclops darwini*, North Queensland, Australia, (USNM1121764, GQ848511, GQ848497); *Mesocyclops edax*, Lake Thonotosassa, Hillsborough Co., Florida, USA, (USNM1121765, GQ848507, GQ848493); *Mesocyclops edax*, Lake Shenandoah, Rockingham Co., Virginia USA, (USNM1121766, GQ848505, GQ848491); *Mesocyclops edax*, Douglas Lake, Cheybogan Co., Michigan, USA, (USNM11217677, GQ848506, GQ848492); *Mesocyclops leuckarti*, Schöhsee, Ploen, Germany, (USNM1121768, GQ848513, GQ848499); *Mesocyclops longisetus*, Joe Brown Lagoon, Orleans Parish, Louisiana, USA, (USNM1121769, GQ848508, GQ848494); *Mesocyclops longisetus* '*curvatus*', University São Carlos, São Paulo, Brazil, (USNM1121770, GQ848509, GQ848495); *Mesocyclops major*, Lake Otjikoto, Tsumeb, Namíbia, (USNM1121771, GQ848512, GQ848498); *M. meridianus*, Barra Bonita Reservoir, Barra Bonita City, Brazil, (USNM1121772,

GQ848510, GQ848496); *Mesocyclops ogunnus*, Barra Bonita Reservoir, Barra Bonita City, Brazil, (USNM1121773, GQ848516, GQ848502); *Mesocyclops pehpeiensis*, Washington, DC, USA, (USNM1121774, GQ848514, GQ848500); *Mesocyclops thermocyclopoides*, Bao-Shan Reservoir, Hsin-chu Co., Taiwan, (USNM-1083794, EF581894, EF581983).

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2010.02.029.

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