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On Species' Boundaries in Zausodes-Complex Species

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ON SPECIES' BOUNDARIES IN *ZAUSODES*-COMPLEX SPECIES

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

18S	eukaryotic small ribosomal subunit DNA, has molecular weight of 18 Sverdrup
BP	bootstrap proportions
cyt*b*	cytochrome apoenzyme *b*
DNA	deoxyribonucleic acid
EtOH	ethanol
ML	maximum likelihood
mt	mitochondrial
NJ	neighbor-joining
nuc	nuclear
PCR	polymerase chain reaction
PP	posterior probability
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
S	Sverdrup

ABSTRACT

Prior to 1999, the harpacticoid copepod species *Zausodes arenicolus* had been considered to be very useful for ecological studies because its unusual shape made it easy to recognize. Bouck *et al.*'s (1999) taxonomic revision based on an evaluation of the morphology of specimens from the northern Gulf of Mexico near the Florida State University Coastal and Marine Laboratory split *Z. arenicolus* into three species, some of which co-occur. If Bouck *et al.* are correct, then the previous ecological results would be suspect, and *Z. arenicolus* would be much less useful in ecological work. It is therefore important to evaluate the validity of the species' boundaries they erected. Because convergent evolution and morphological stasis are known in harpacticoid copepods, morphologically based approaches such as those of Bouck *et al.* may not be sufficient. I decided to use gene-sequencing methods instead, but I saved voucher material from each specimen so I could search for new morphological characters if the initial morphological identifications and those indicated by gene sequences did not agree. A 393-base-pair region of the mitochondrial cytochrome *b* gene and a 478-base-pair region of the 18S rRNA gene were separately analyzed from individual *Zausodes*-complex specimens found at five sites within 30 km of the Florida State University Coastal and Marine Laboratory. *Zausodes septimus* individuals formed a single cytochrome *b* gene-sequence clade, whereas *Z. arenicolus* individuals formed five clades. The uncorrected genetic divergences among these clades were ten times greater than the divergences within them, a degree of differences that suggests that each clade is a different species. The 18S rDNA results support those from cytochrome *b*. Subsequent analysis of the vouchers revealed morphological differences that will allow two of the *Z. arenicolus* clades to be described as new species. Further analysis may reveal morphological differentiation among the other three clades. In sum, Bouck *et al.* (1999) perceived some but not all of the species present.

ON SPECIES' BOUNDARIES IN *ZAUSODES*-COMPLEX SPECIES

1. Introduction

The *Zausodes* complex consists of all those species that were assigned to the genus *Zausodes* C. B. Wilson (Copepoda: Harpacticoida: Harpacticidae) prior to 1999. These species live in seagrass meadows (Bell *et al.* 1987), sandy beaches, and shallow subtidal sands to at least 20 m depth (Bouck *et al.* 1999). Most Zausodes-complex species have been found in subtropical regions; but *Zausodes arenicolus* C. B. Wilson is known from Massachusetts (Wilson 1932), and *Z. septimus* Lang occurs off central California (Lang 1965).

Before 1999, *Zausodes* species were considered very useful for ecological studies (see e.g., Kern and Bell 1984, Varon and Thistle 1988) because their distinctive body shape, which is dorsal-ventrally flattened and ovoid in outline, made them easy to identify; and only a single species was thought to be present in most locations. After finding several individuals of *Zausodes* in the northern Gulf of Mexico in the vicinity of the Florida State University Coastal and Marine Laboratory (FSUCML) that could not be assigned to *Zausodes arenicolus*, the only species thought to be present in the region, Bouck *et al.* (1999) used morphological techniques to perform a thorough taxonomic analysis of the *Zausodes*-like individuals in this region.

Bouck *et al.* (1999) concluded that populations that had been identified as *Zausodes arenicolus* in the vicinity of the FSUCML consisted of *Z. arenicolus*; *Mucropedia cookorum* Bouck, Thistle, and Huys; *M. kirstenae* Bouck, Thistle, and Huys; and *Neozausodes shulenbergeri* Bouck, Thistle, and Huys. In addition to describing three new species, they clarified the species' description of *Z. arenicolus*. Further, their work showed that two or more species in this complex can co-occur on scales of <100 cm^2. For example, *M. kirstenae* and *M. cookorum* were routinely collected in the same 15.5 cm^2 core samples (Bouck *et al.* 1999). If Bouck *et al.* (1999) are correct, then (1) the conclusions of some ecological studies (*e.g.,* Varon and Thistle 1988) would be suspect, (2) *Zausodes* species would become much less attractive for ecological studies, and (3) the new morphological characters they used to define the species' boundaries would be additional tools for use in taxonomic discrimination. It is therefore worthwhile to test the validity of Bouck *et al.*'s (1999) species' boundaries.

Such a test could be done by repeating and expanding the traditional morphological analysis Bouck *et al.* (1999) used to erect species' boundaries. However, morphological stasis and convergent evolution are known to occur in harpacticoid copepod evolution (see, *e.g.*, Ganz and Burton 1995, Rocha-Olivares *et al.* 2001), so species may be overlooked when morphology alone is used for species' delimitation. For example, recent gene-sequencing studies of harpacticoid copepods revealed evidence of cryptic speciation (Schizas *et al.* 1999, Rocha-Olivares *et al*. 2001), pseudo-sibling species (Rocha-Olivares *et al.* 2001; Schizas *et al.* 1999, 2002; Staton *et al.* 2005), and incipient speciation (Schizas *et al.* 1999, 2002). I therefore chose to use gene-sequencing methods to verify the species' boundaries defined by Bouck *et al.* (1999).

2. Materials and Methods

2.1. Sample Collection and Processing

Samples were collected from five sites in the northern Gulf of Mexico (Figure 1 and Table 1). Approximately the top centimeter of sediment was scraped into a plastic bag. The samples were stored on ice (up to 24 h) until the sediment could be sieved. Otherwise, the samples were emptied into a 30-µm mesh sieve to remove excess water, preserved in 95% ethanol (EtOH), and stored at -20°C. Each sample was separated into size classes with nested sieves of the following mesh apertures: 300, 250, 177, 150, 105, 63, and 30 µm. The contents of the 300- and 30-µm mesh sieves were discarded because adult specimens pass through a 300-µm sieve and are retained on a 63-µm sieve. The contents of the other sieves were stored separately in 95% EtOH at -20ºC.

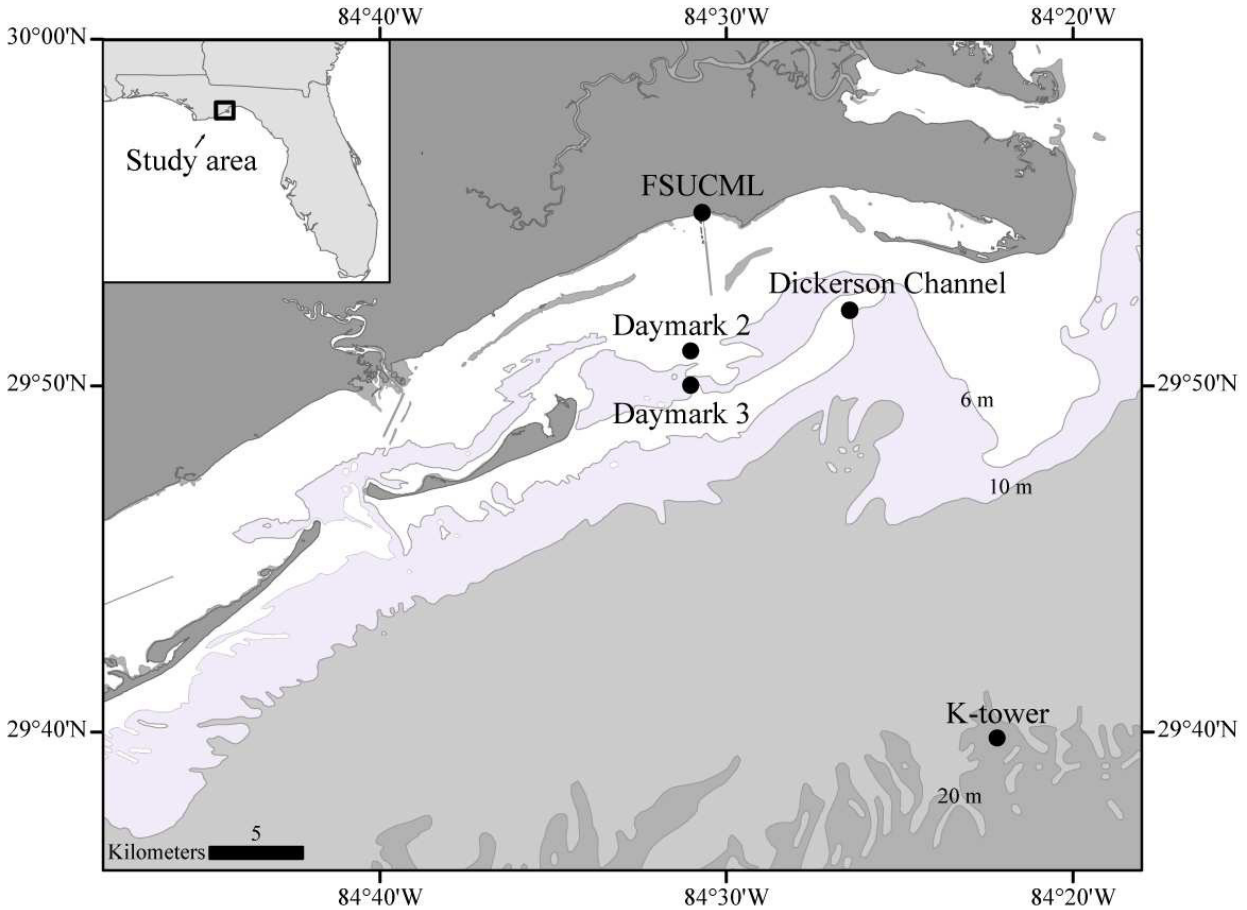

Figure. 1. A chart of the northern Gulf of Mexico off the panhandle coast of Florida showing the locations of the study sites (filled circles). Depth contours are in meters. FSUCML = Florida State University Coastal and Marine Laboratory.

Harpacticoids were separated from the sediment with Ludox® HS-40 (E.I. du Pont de Nemours & Co., Inc.) following Burgess (2001). Ludox specific gravity was 1.15 – 1.31 g cm^{-3}, so the harpacticoids, which have a lower specific gravity, float while the denser sediment particles sink. The harpacticoids were collected on a 30-µm aperture sieve and stained overnight in a solution of 200 ml of 95% EtOH and 0.25 g of rose Bengal. *Zausodes*-complex individuals were separated from other harpacticoids with the aid of a dissecting microscope. As explained above, specimens were recognized by their unusual body shape. Dr. David Thistle (Department of Oceanography, Florida State University) used the key in Bouck *et al.* (1999) to identify adult females to nominal species on the basis of their morphology. Of the 12 described *Zausodes*-complex species, *Zausodes arenicolus*, *Z. septimus*, *Neozausodes shulenbergeri*, and *Mucropedia cookorum* were found (Tables 1 and 2).

Table 1. The latitude, longitude, depth, and sampling dates of sites sampled for this study. The specimen identification (ID) numbers used in this study are also listed.

Site name	Latitude	Longitude	Depth (m)	Collection date	Specimen ID numbers
FSUCML	29° 54.995'N	84° 30.694'W	0.5	30 September 2006	ML.Z.1-9, 19
				25 October 2007	M.1
				29 January 2008	M.2-6
				10 March 2008	M0.1, 3-5
				28 March 2008	M1.1-3
Dickerson Channel	29° 52.169'N	84° 26.428'W	5	30 May 2007	DC.64, 67-72, 75-79, 82, 83, 86-90
Daymark 2	29° 50.992'N	84° 31.010'W	5.5	28 March 2008	D0.3, 5, 25
Daymark 3	29° 50.003'N	84° 31.016'W	5.5	30 May 2007	D2.16, 17, 39, 41, 49, 50
K-tower	29° 39.811'N	84° 22.177'W	16	8 August 2007	K.4, 7, 9-15, 20
				23 January 2008	K0.1, 5, 6, 8, 11, 12, 14

Table 2. The distribution of Bouck *et al.*'s taxa among my sites. Y = present. N = absent.

	Sites				
Species	Daymark 2	Daymark 3	Dickerson Channel	FSUCML	K-tower
Z. arenicolus	Y	Y	Y	Y	Y
Z. septimus	N	Y	Y	N	Y
N. shulenbergeri	N	Y	N	N	N
M. cookorum	N	N	N	N	Y

Adult females in good condition were processed (described below) for further morphological analysis and gene sequencing. The procedure changed during the study. At first, the entire specimen was used for gene sequencing, so either no voucher was retained or the male of a clasped male-female pair was retained. Although gene-sequencing was usually successful, further morphological analysis could not be done, so the method was discontinued. In the second version, the specimen was cut in two portions as shown in Figure 2. This method resulted in decreased gene-sequencing success. In the third version, the specimen's maxillipeds (Mxp), antennules (A1), antennae (A2), and the five pairs of pereiopods (P1-P5) were removed and stored in a glycerin drop on a wax slide as vouchers. The remainder of the specimen was used for gene-sequence analysis. Unfortunately, the stored body parts could not always be found, so further morphological analyses were often incomplete. In the final version (detailed below), key body parts needed for additional morphological analysis were cut from the specimen (Figure 3) and permanently mounted. In all versions, the egg sacs were included in the extractions of gravid females to increase the yield of DNA.

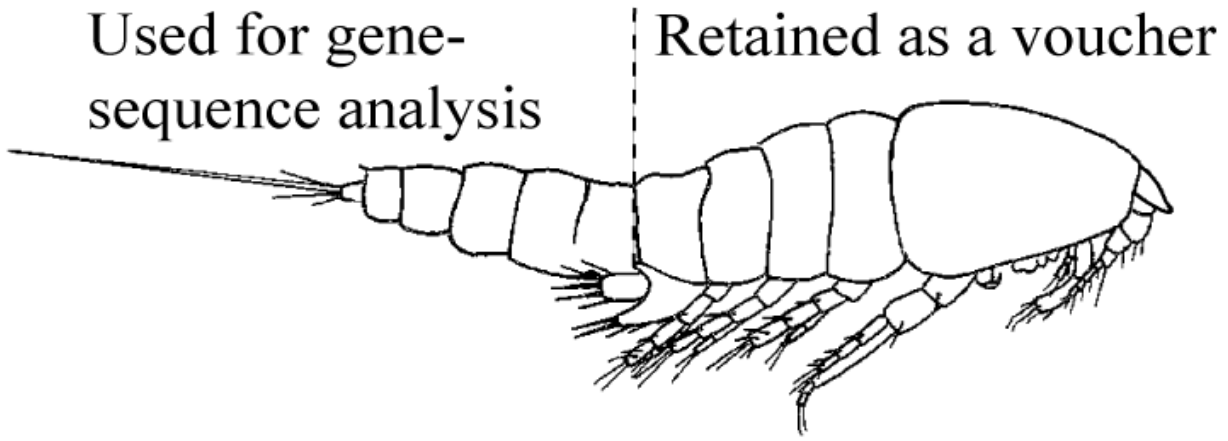

Figure 2. A harpacticoid in lateral view showing how it was divided (dashed line) for the second version of the method and the fate of each portion.

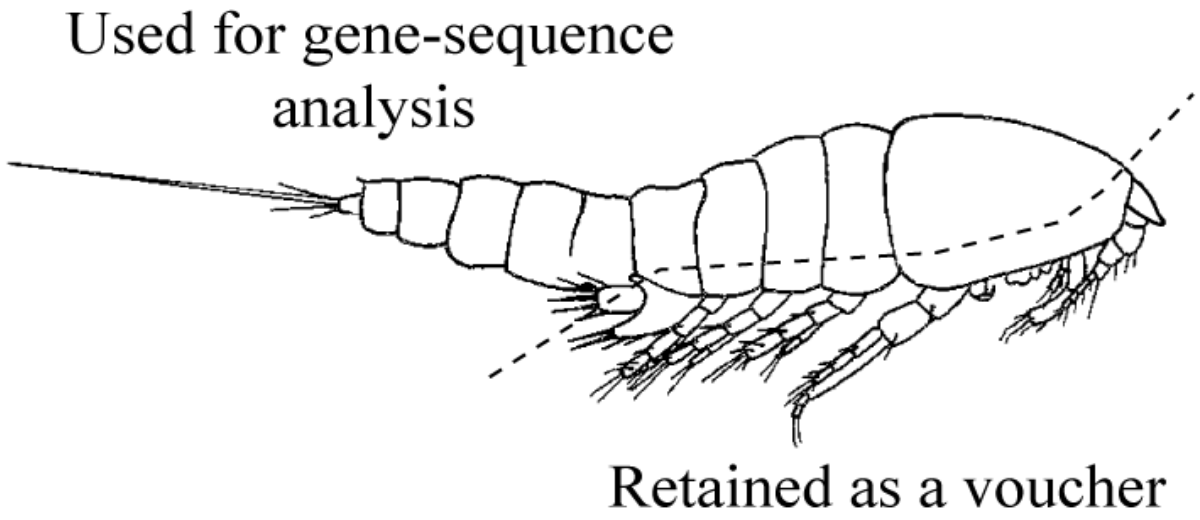

Figure 3. A harpacticoid in lateral view showing how it was divided (dashed line) for the fourth version of the method and the fate of each portion.

The process of identifying, dividing, and storing the specimens for subsequent analyses with the fourth version consisted of the following steps: (1) transferring the specimen from EtOH to a drop of glycerin on a depression slide, (2) positioning it on its right side under a cover slip, (3) examining it with a compound microscope at 1050× magnification, (4) identifying the specimen to nominal species, (5) transferring the specimen to another glycerin drop on a flat slide, (6) removing the urosome with tungsten needles, (7) placing the urosome in a labeled well of 10× PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, Invitrogen, Carlsbad, California) in a depression-well plate, (8) removing the P5, (9) mounting the P5 with Hoyer's mounting medium (Pfannkuche and Thiel, 1988) under a cover slip on a labeled slide, (10) repeating the previous two steps for P1-P4, Mxp, A2, and A1, and (11) transferring the remainder of the specimen to the same well as in step 7. The mounted appendages were retained as voucher material. All pieces in the 10× PCR buffer were transferred to a second labeled well of 10× PCR buffer to remove excess glycerine and EtOH and then to a sterile 0.5-ml microcentrifuge tube with 10 µl of 10× PCR buffer for DNA extraction, following a GeneReleaser™ (Bioventures, Inc., Murphreeboro, Tennessee) protocol (Schizas *et al.* 1997) as modified by Staton *et al.* (2005). Two protocol changes were made: (1) the extraction was incubated in a 55°C water bath overnight before placing it in a 100°C heating block, and (2) the GeneReleaser™ residue, after removal of the supernatant, was retained at 4°C until it was used in the first polymerase chain reaction (PCR, Saiki *et al*. 1988) amplification of the mitochondrial (mt) cytochrome apoenzyme *b* (cyt*b*) gene.

2.2. Choice of Genetic Markers

The mt cyt*b* gene has been used in successful studies of cryptic species and of population-genetic structure for harpacticoids (Schizas *et al*. 1999, 2002; Staton *et al*. 2005) because its rate of nucleotide substitution is appropriate for such questions. I identified DNA primers (see below) that amplify and sequence an approximately 450-base-pair (bp) region of mt cyt*b* in species of the *Zausodes* complex.

The nuclear (nuc) 18S ribosomal RNA (rRNA) gene has been successfully used in previous studies of Harpacticidae species (e.g., Burton and Lee 1994, Burton *et al.* 2005). This gene mutates at a slower rate, so species-level genetic divergences will be lower than for cyt*b*. In addition, the nuclear genome is not linked to the mitochondrial genome, so these genes provide independent assessments of genetic diversity. DNA primers (see below) were used to amplify and sequence an approximately 530-bp region of the nuc 18S gene.

2.3. DNA Amplification and Sequencing

A 449-bp portion of the mt cyt*b* gene was PCR-amplified with protocols and thermal-cycling parameters modified from Staton *et al.* (2005) in a Bio-Rad icycler (Bio-Rad Laboratories, Hercules, California). The primers cytb424z (5' GGHTAYGTDYTWCCYTGAGGACAAAT 3') and cytb876z (5' GCATATGCAAATAAAAAATATCAYTCAGG 3'), modified from Staton *et al.* (2005), were used in the PCR reactions. The first PCR amplification of an individual extraction was performed by adding 50 µl of the following PCR reaction mix to the GeneReleaser™ residue of a DNA extraction: 1:5 dilution of 5× iProof™ HF Buffer (20 mM Tris-HCl pH 7.4 at 25°C, 0.1 mM EDTA, 1mM DTT, 100mM KCl, 0.5% Tween 20, 0.5% Nonidet P 40, 200 µg/ml BSA, 50% Glycerol; Bio-Rad Laboratories), 200 µM of each deoxyribonucleotide triphosphate (USB Corporation, Cleveland, Ohio), 1.0 µM of each primer, and 1 unit iProof™ High-Fidelity DNA polymerase (Bio-Rad Laboratories). If this amplification protocol was unsuccessful, then 6-8 µl of the extraction supernatant were amplified with the above PCR reaction mix. DNA was initially denatured at 98°C for 3 min, followed by 10 cycles of 98°C denaturing for 30 s, 47°C annealing for 30 s, and 72°C extension for 45 s. This step was immediately followed by 30 cycles of 98°C denaturing for 30 s, 53°C annealing for 30 s, and 72°C extension for 45 s. The amplification ended with a final extension of 7 min at 72°C. PCR products were stored at 4°C until visualized by agarose-gel electrophoresis and subsequent PCR product purification.

A 528-bp portion of the nuc 18S rRNA gene was PCR amplified with protocols and thermal-cycling parameters modified from Spears *et al.* (1994). Only specimens that were successfully amplified and sequenced for the mt cyt*b* gene were amplified and sequenced for the 18S rRNA gene. Two microliters of a 1:10 dilution of the extraction supernatant was used as the DNA template. The primers HI− (5' GTGCATGGCCGTTCTTAGTTG 3') and 329 (5' TAATGATCCTTCCGCAGGTTCACCTACGG3') provided by Spears (pers. comm.) were used in the PCR reactions. The PCR reaction mix described above was used to amplify this gene. DNA was initially denatured at 95°C for 3 min, followed by 40 cycles of 95°C denaturing for 40 s, 50°C annealing for 1 min, and 72°C extension for 3 min. The thermal-cycler protocol was complete after a final extension of 15 min at 72°C. PCR products were stored at 4°C until visualized by agarose-gel electrophoresis and then product purification.

Ten µl of the PCR product and 2 µl of a pGem 3Zf(-) DNA ladder (Molecular Cloning Facility, Florida State University, Tallahassee, Florida) were visualized with a ultraviolet transilluminator after electrophoresis in either a 0.8% agarose/1× tris-borate-EDTA gel stained with ethidium bromide and run at 80 v or a 1.2% agarose/1× sodium borate acid gel (Brody and Kern 2004) stained with Gelstar® nucleic acid gel stain (Lonza Rockland, Inc., Rockland, Maine) and run at 200 v. PCR products were purified with the CONCERT™ Rapid Purification System (Life Technologies, Germany) per manufacture's protocol, and both strands of DNA templates (~30 ng) were cycle-sequenced with a PRISM® Big Dye™ Terminator Ready Reaction Kit (Applied Biosystems, Inc., Foster City, California). Electrophoresis of reaction mixtures was done on an Applied Biosystems PRISM® 3100 Genetic Analyzer. Templates were sequenced with the amplification primers at the Florida State University Sequencing Facility.

2.4. Sequence Analyses

Sequencher™ 4.6 (GeneCodes, Ann Arbor, Michigan) was used to assemble and edit a consensus sequence from double-stranded PCR products. The authenticity of the *Zausodes*-complex sequences was assessed by a BLAST search of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) and by a EMBOSS Transeq (European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom) search for the presence of stop and nonsense codons. At least one representative from the sister genus *Tigriopus* was the outgroup taxon in subsequent phylogenetic analyses. The program CLUSTAL X 2.0.9 (Thompson *et al*. 1997) was used to align separately the cyt*b* and 18S rRNA gene sequences. Sequences from the primer regions were removed before further analyses, resulting in cyt*b* and 18S rDNA gene fragments of 393 and 479 bp respectively. Multiple-sequence alignment of cyt*b* gene data with CLUSTAL's default alignment parameters (gap opening = 10, gap extension = 0.2, delay divergent sequences = - 30%, DNA transition weight = 0.5, negative matrix off, Gonnet series protein weight matrix, and IUB DNA weight matrix) required no adjustment. Alignment of 18S data with CLUSTAL's default parameters also required no adjustment. The sequence from each haplotype was submitted to GenBank (accession numbers FJ668333-FJ668367).

Basic sequence statistics were obtained from PAUP* 4.0b10 (Swofford 2003). Separate neighbor-joining (NJ, Saitou and Nei 1987) analyses were performed on the aligned cyt*b* and 18S rRNA gene sequences from specimens collected at all sites. The sequences of the two genes were not combined because the genes mutate independently and have different rates of nucleotide substitution. Modeltest 3.7 (Posada and Crandall 1998), MrMTgui 1.0 (http://www.genedrift.org, Paulo Nuin), and PAUP* were used to select a model of nucleotide evolution with an initial NJ topology inferred from Jukes and Cantor (1969) genetic distances. A heuristic search with the selected model was performed with PAUP* to infer a maximum likelihood (ML, Felsenstein 1981) phylogenetic tree. If this tree did not match the Jukes-Cantor tree, PAUP* was used to estimate new model parameters, and the heuristic search was repeated with these new parameters to optimize the ML phylogenetic tree. When consecutive inferred ML trees had the same topology, the later model parameters were used to infer the optimized ML phylogenetic tree. The model selected by the hierarchical-likelihood-ratio-test method (Huelsenbeck and Crandall 1997) was used for subsequent analyses after confirming that the phylogenetic tree obtained with the Akaike information criterion method (Akaike 1973) had the

same topology. Confidence in nominal-species clades was assessed with nonparametric bootstrap proportions (BP; Felsenstein 1985) for 100 pseudoreplicates. ML methods were also implemented with a Bayesian approach as executed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with a double run of 4 Markov chains for 4 million generations. Markov chains were sampled every 100 generations, and these subsamples were used to create a 50% majority-rule consensus tree in PAUP* with the first 25% of the trees discarded so confidence in the nominal-species clades could be evaluated by Bayesian posterior probabilities (PP). One representative of each cyt*b* haplotype was used to calculate uncorrected pairwise divergences and ML-inferred pairwise divergences with PAUP*. These divergences were calculated for the entire cyt*b* sequence as well as separately for the first, second, and third codon position and were used to develop mutation-saturation curves.

2.5. Additional morphological analysis

The morphological species identifications were superimposed on clades of the ML trees to check whether nominal-species clades were congruent with the initial species identifications. Congruence between morphological species identifications and a single nominal-species clade with strong BP and PP support signified a good identification that required no further morphological analysis to identify additional morphological characters for species identification. If the morphological identifications were not congruent with nominal-species clades, the vouchers were studied by David Thistle. He focused on the P4 and P5 because these appendages had been observed to be variable during the initial identifications (Thistle, pers. comm.). With the aid of a compound microscope at 1050× magnification and a drawing tube, Thistle made detailed line drawings of the P4 and P5 of each specimen. The drawings were compared to detect differences among specimens. For each character that differed, character states were identified and assigned. These newly recognized character states were then compared to cyt*b* gene-sequence clades from the ML phylogenetic tree. When a character state corresponded to a single-sequence clade, that state was considered useful for delimitation of that clade. When a state corresponded to more than one sequence clade, specimens in these clades needed additional morphological analysis to identify a character or characters to delimit these clades from one another. A sequence clade whose individuals had multiple states of a given character revealed morphological characters that are phenotypically variable.

3. Results

3.1. Cyt*b*

The cyt*b* gene was successfully amplified and sequenced for 68 *Zausodes*-complex specimens. Initial morphological identification of these specimens revealed one *Mucropedia cookorum*, two *Neozausodes shulenbergeri*, 14 *Zausodes septimus*, and 51 *Z. arenicolus*. No stop codons were found in any of the sequences. No insertions or deletions (indels) were observed in a multiple alignment of the 393-bp fragment of the 68 specimens and the outgroup taxa *Tigriopus californicus* (GenBank accession number NC008831) and *Tigriopus japonicas* (GenBank accession number NC003979). Including the outgroup specimens, 30 haplotypes

(Table 3) were present in the multiple alignment. One representative from each haplotype was used to calculate the average base frequencies of 0.2976, 0.1333, 0.1543, and 0.4148 (A, C, G, and T). A χ^2 test of homogeneity revealed the haplotype base frequencies to be significantly different ($p = 2 \times 10^{-8}$, df = 87, $\chi^2 = 180.2963$). The GC content of *Tigriopus* was noticeably higher, so the base frequency analysis was rerun without these sequences (Table 3). This χ^2 test was not significant ($p = 0.3705$, df = 81, $\chi^2 = 84.5954$). The average base frequencies of this data set were 0.3048, 0.1295, 0.1478, and 0.4179 (A, C, G, and T). The average GC content of *N. shulenbergeri* was the highest at 0.3440 and that of *Z. septimus* was lowest at 0.2427 (Table 3). The average transition-transversion (Ti/Tv) ratios were calculated from pairwise comparisons of these haplotypes with and without *Tigriopus* (Table 4). Ti/Tv ratios for each of the three sites of the codon are also reported. After excluding undefined values (Tv = 0), the entire 393-bp sequence had the highest average value, whereas the first position of the codon had the lowest.

Table 3. The average base frequencies of mt cyt*b* haplotypes. Clade designations correspond to cyt*b* clades as labeled in Figure 4. The *Tigriopus* clade consists of one haplotype each from *T. californicus* and *T. japonicus*.

	Number of haplotypes	A	C	G	T	GC
Tigriopus	2	0.1972	0.1858	0.2456	0.3715	0.4313
M. cookorum	1	0.2901	0.1221	0.1578	0.4300	0.2799
N. shulenbergeri	2	0.2912	0.2061	0.1379	0.3648	0.3440
Z. arenicolus clade 1	2	0.3104	0.1361	0.1476	0.4059	0.2837
Z. arenicolus clade 2	3	0.3028	0.1323	0.1247	0.4402	0.2570
Z. arenicolus clade 3	1	0.2850	0.1374	0.1705	0.4071	0.3079
Z. arenicolus clade 4	11	0.3102	0.1264	0.1508	0.4126	0.2772
Z. arenicolus clade 5	3	0.2850	0.1476	0.1484	0.4190	0.2960
Z. septimus	5	0.3160	0.0906	0.1522	0.4412	0.2427
Zausodes-complex	28	0.3048	0.1295	0.1478	0.4179	0.2773

Table 4. Ti/Tv ratios of pairwise comparisons of mt cyt*b* haplotypes for the entire sequence as well as separately for the first, second, and third positions of the codons. When no transversions occurred, the data were excluded because the value was undefined.

	Entire sequence	First position	Second position	Third position
Average of all haplotypes	1.0446	0.8408	0.9893	1.0191
Average of Zausodes-complex	1.0612	0.8362	1.0499	0.9896
Maximum	7	2	1.57	7
Minimum	0	0.42	0	0.36

The following TVM+I+G (GTR+G+I in PAUP*) model parameters, selected by procedures described above, were used to infer a ML phylogenetic tree (Figure 4) of cyt*b* sequences with PAUP*: base frequencies = (0.2954, 0.1302, 0.1417), number of substitutions = 6, rate matrix = (0.8418, 6.9769, 0.9388, 2.9706, 6.9769), rates = gamma, shape = 0.9966, and proportion of invariable sites = 0.2991. Strongly supported (BP ≥ 90, PP = 100) single nominal species clades corresponded to the individuals identified morphologically as *M. cookorum*, *Z. septimus*, and *N. shulenbergeri* (Figure 4). In contrast, specimens morphologically identified as *Z. arenicolus* were divided into five nominal-species clades. The *Z. arenicolus* clade numbers given in Figure 4 are the same numbers used throughout the remainder of this study. Each of these clades was strongly supported (BP ≥ 97, PP = 100) and had low within-clade genetic divergence (≤ 0.023, uncorrected; Table 5). The within-clade divergences of *N. shulenbergeri* and *Z. septimus* are respectively 0.008 and ≤ 0.036. Among the *Zausodes*-complex clades, the between-clade divergences (0.219 – 0.342) were approximately 10-fold higher than the within clade divergences. *T. californicus* and *T. japonicas* were 0.257 divergent from each other, but each was ~1.5 × more divergent from the *Zausodes*-complex species.

Figure 4 and Table 6 show the number of specimens I analyzed from each clade at each site. *Zausodes arenicolus* clade 5 occurred at Daymark 2, Daymark 3, and Dickerson Channel, which are about equal distance from the coast (~7 km, Figure 1) and at similar depths (~5 m, Table 1). In contrast, *Z. septimus* was found at Daymark 3, Dickerson Channel, and K-tower, which is deeper and further from the coast. *Zausodes arenicolus* clades 1 and 4 were each found at two sites. *Mucropedia cookorum*, *N. shulenbergeri*, *Z. arenicolus* clade 2, and *Z. arenicolus* clade 3 were each found at a single site. The FSUCML site, which was shallowest and closest to the coast, had specimens from only *Z. arenicolus* clades 3 and 4; the clades that are the least divergent from one another (0.219 – 0.229, Table 5). The K-tower and Dickerson Channel sites each had specimens from only a single *Z. arenicolus* clade (2 and 5 respectively). In contrast, two *Z. arenicolus* clades were found at Daymark 2 and three were found at Daymark 3 (Table 6).

Saturation curves of *Zausodes*-complex cyt*b* haplotypes in Figure 5 reveal that much of the sequence data is saturated or near saturation, so relationships among the clades cannot be inferred with confidence. The saturation curves begin to level off when uncorrected pairwise divergences stay relatively constant as the pairwise ML-inferred divergences continue to increase (indicating saturation). Saturation curves for the entire 393-bp fragment (Figure 5A, D) and for the 131 bp that are in the third position of the codon (Figure 5E, F) begin to level off at 0.30 and 0.40 uncorrected divergence, respectively. The saturation curve for the second codon position (Figure 5C,D) has not begun to level off (not saturated), and the pairwise divergences do not exceed 0.18, whereas the saturation curve for the first codon position (Figure 5B, D) is just beginning to level off at 0.30 uncorrected divergence (nearing saturation).

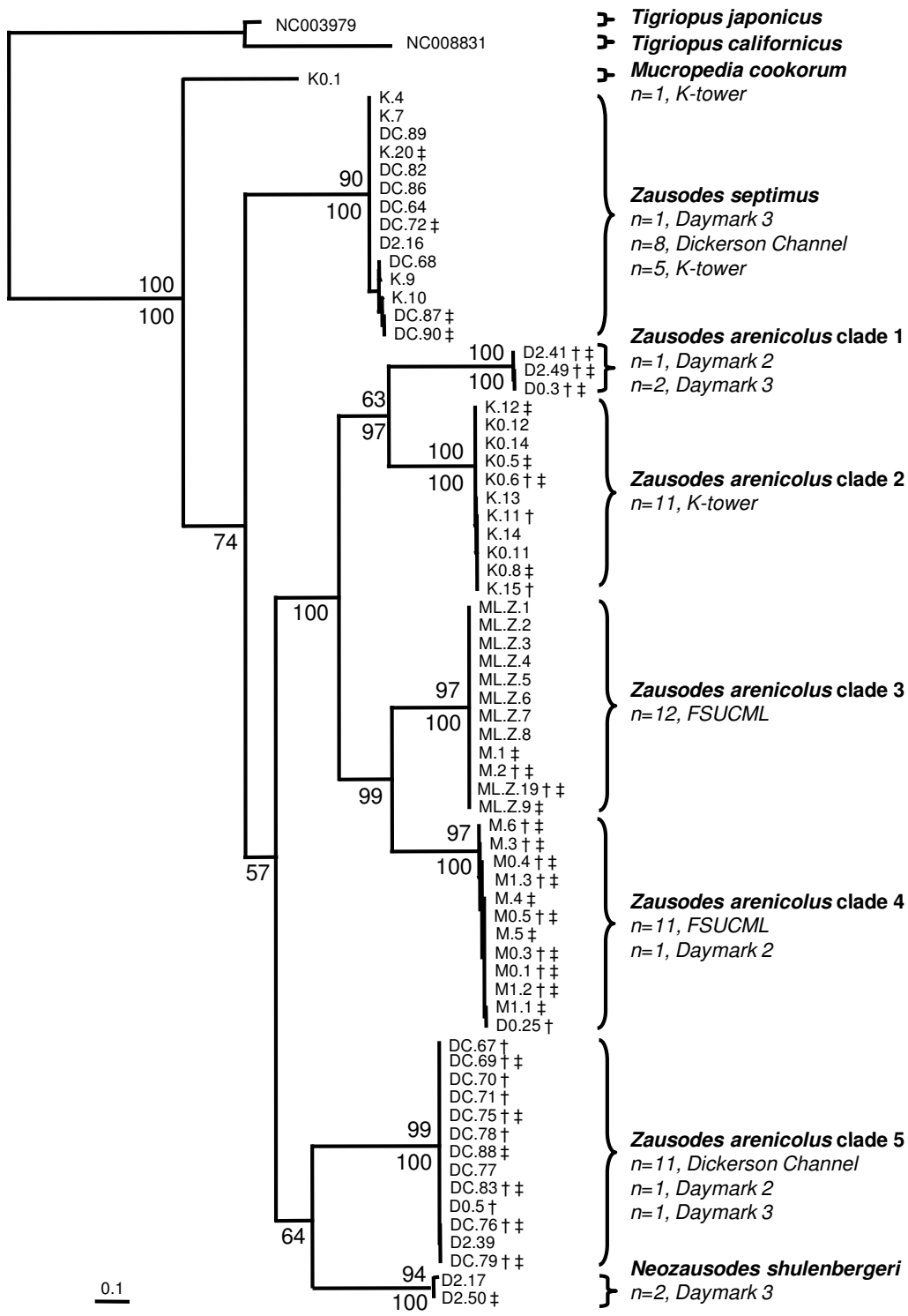

Figure 4. Maximum-likelihood phylogenetic tree inferred from cyt*b* sequences. Each sequence is labeled with the sample identification number given in Table 1 or the GenBank accession number. n = the number of specimens. The numbers at nodes are the bootstrap proportions (top) and posterior probabilities (bottom) for values ≥50. † = specimens with vouchers that were used for additional morphological analysis. ‡ = specimens for which partial 18S rDNA sequence data were obtained. Scale bar denotes inferred divergences.

Table 5. Pairwise cyt*b* uncorrected divergences. Top values are the averages; bottom values are the minimum and the maximum. – denotes single-sequence clades, so no within-clade pairwise comparison could be made.

	T. japonicus	*T. californicus*	*M. cookorum*	*Z. septimus*	*Z. arenicolus* clade 1	*Z. arenicolus* clade 2	*Z. arenicolus* clade 3	*Z. arenicolus* clade 4	*Z. arenicolus* clade 5	*N. shulenbergeri*
T. japonicus	–									
T. californicus	0.257 (0.257,0.257)	–								
M. cookorum	0.366 (0.366, 0.366)	0.394 (0.394, 0.394)	–							
Z. septimus	0.363 (0.361, 0.369)	0.398 (0.394, 0.402)	0.261 (0.257, 0.270)	0.017 (0.000, 0.036)						
Z. arenicolus clade 1	0.398 (0.397, 0.399)	0.412 (0.412, 0.412)	0.314 (0.313, 0.316)	0.270 (0.267, 0.277)	0.002 (0.000, 0.003)					
Z. arenicolus clade 2	0.397 (0.397, 0.397)	0.434 (0.433, 0.435)	0.298 (0.298, 0.298)	0.264 (0.257, 0.267)	0.230 (0.229, 0.232)	0.003 (0.000, 0.005)				
Z. arenicolus clade 3	0.410 (0.410, 0.410)	0.433 (0.433, 0.433)	0.321 (0.321, 0.321)	0.299 (0.295, 0.300)	0.285 (0.285, 0.285)	0.274 (0.272, 0.275)	0.000 (0.000, 0.000)			
Z. arenicolus clade 4	0.406 (0.400, 0.415)	0.445 (0.440, 0.450)	0.333 (0.326, 0.336)	0.274 (0.265, 0.282)	0.284 (0.280, 0.290)	0.247 (0.242, 0.252)	0.224 (0.219, 0.229)	0.012 (0.000, 0.023)		
Z. arenicolus clade 5	0.390 (0.389, 0.392)	0.423 (0.422, 0.425)	0.300 (0.300, 0.303)	0.284 (0.280, 0.293)	0.334 (0.333, 0.336)	0.298 (0.298, 0.300)	0.296 (0.295, 0.298)	0.286 (0.280, 0.295)	0.001 (0.000, 0.005)	
N. shulenbergeri	0.411 (0.410, 0.411)	0.422 (0.421, 0.423)	0.309 (0.305, 0.313)	0.312 (0.305, 0.316)	0.320 (0.318, 0.324)	0.320 (0.318, 0.321)	0.282 (0.280, 0.283)	0.336 (0.331, 0.342)	0.280 (0.277, 0.285)	0.008 (0.008, 0.008)

Table 6. The number of specimens from *Zausodes septimus* and *Z. arenicolus* clades that were found at sites. – denotes no specimens were found.

	Sites				
Species	FSUCML	Dickerson Channel	Daymark 2	Daymark 3	K-tower
Z. septimus	–	8	–	1	5
Z. arenicolus					
Clade 1	–	–	1	2	–
Clade 2	–	–	–	–	11
Clade 3	12	–	–	–	–
Clade 4	11	–	1	–	–
Clade 5	–	11	1	1	–

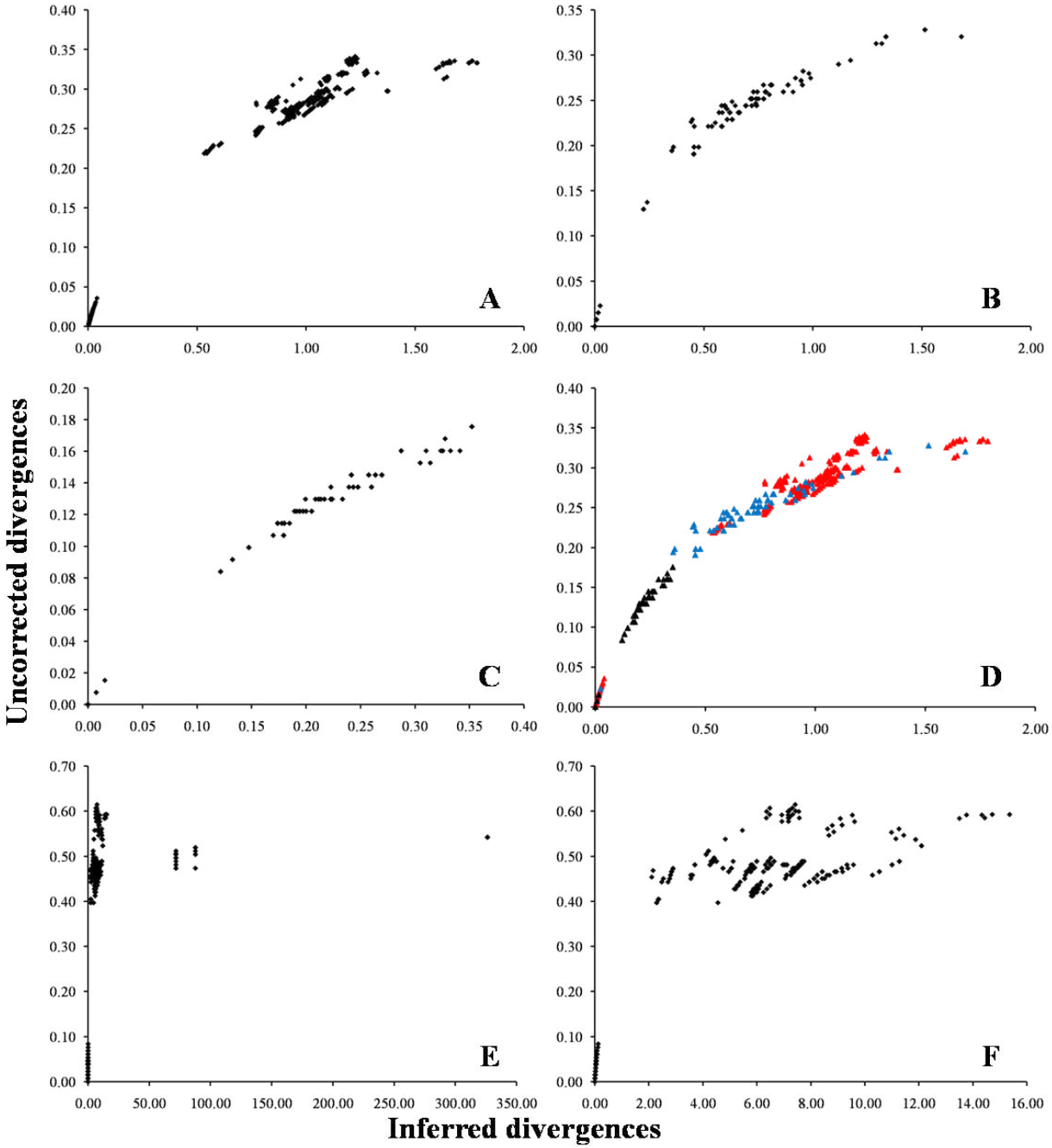

Figure 5. Saturation curves for pairwise comparisons of *Zausodes*-complex species: (A) entire 393-bp fragment of cyt*b*; (B) only first position of codon; (C) only second position of codon; (D) combination of A (red), B (black), and C (blue); (E) only third position of codon; and (F) only third position of codon with inferred divergences ≥ 50 removed. Inferred divergences were calculated from the ML model of nucleotide evolution used to infer the cyt*b* phylogenetic tree in Figure 4. Note that the X-axis and Y-axis values differ between graphs.

3.2. 18S

A 478-bp portion of the 18S rRNA gene was successfully amplified and sequenced for 33 *Zausodes*-complex specimens. Based on initial morphological identification, these specimens consisted of one *Neozausodes shulenbergeri*, four *Zausodes septimus*, and 28 *Z. arenicolus*. A multiple alignment of these sequences and the outgroup taxon *T. californicus* (479 bp, GenBank accession number AY599492) had 484 sites to account for indels. Including the outgroup specimen, 7 haplotypes (Table 7) were present in the multiple alignment. One representative from each haplotype was used to calculate average base frequencies of 0.2758, 0.2638, 0.2378, and 0.2226 (A, C, G, and T). A χ^2 test of homogeneity revealed the base frequencies were not significantly different among haplotypes ($p = 1$, $df = 18$, $\chi^2 = 0.508255$). The average GC content of *N. shulenbergeri* was the lowest (0.4958) and *Z. septimus* was the highest (0.2427, Table 7). The average transition-transversion (Ti/Tv) ratios from pairwise comparisons of these haplotypes with and without *Tigriopus* were respectively 3.4147 and 4.5189.

Table 7. The base frequencies for 18S rDNA haplotype.

	Number of haplotypes	A	C	G	T	GC
Tigriopus	1	0.2714	0.2631	0.2359	0.2297	0.4990
N. shulenbergeri	1	0.2803	0.2636	0.2322	0.2239	0.4958
Z. arenicolus clade 1	1	0.2720	0.2636	0.2427	0.2218	0.5063
Z. arenicolus clade 2	1	0.2782	0.2615	0.2364	0.2239	0.4979
Z. arenicolus clade 3, 4	1	0.2762	0.2657	0.2385	0.2197	0.5042
Z. arenicolus clade 5	1	0.2782	0.2636	0.2364	0.2218	0.5000
Z. septimus	1	0.2741	0.2657	0.2427	0.2176	0.5084

The following TRNef+G (GTR+G in PAUP*) model parameters, selected by procedures described above, were used to infer a ML phylogenetic tree (Figure 6) for the 18S rDNA sequences with PAUP*: equal base frequencies, number of substitutions = 6, rate matrix = (1.424471, 9.041368, 1.543252, $4.0289e^{-11}$, 2.034255), rates = gamma, shape = 31.6334, and proportion of invariable sites = 0.799566. The 18S rDNA tree supported those clades defined by the cyt*b* data, except that *Z. arenicolus* clades 3 and 4 formed a single 18S clade. The outgroup differed from the other clades by 31-35 base changes (Table 8). The *Zausodes*-complex clades differed by only one to 12 bases. Specimens in a clade consisted of a single haplotype. Few clades had strong BP or PP support (Figure 6), which is not surprising when so few changes occurred between sequences. Of the 33 18S rDNA sequences, 17 were obtained from extractions of gravid females (egg sac included). The initial plan for this study was to use only a mitochondrial gene, which is passed down maternally, so mother and offspring would have identical sequences. Because half of the nuclear DNA comes from the mother, I might have found polymorphic sequences if the parents had different 18S sequences, but I did not.

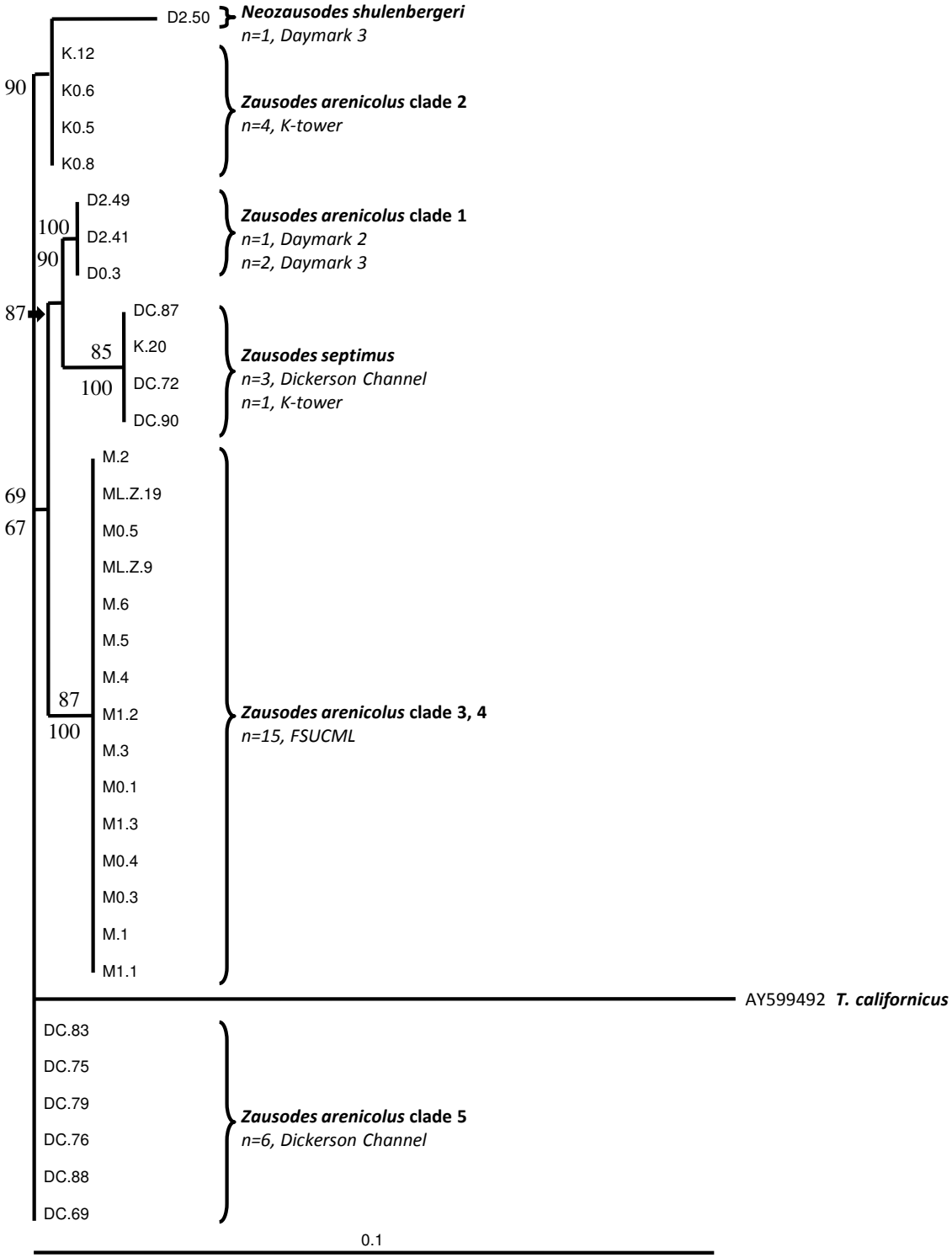

Figure 6. Maximum-likelihood phylogenetic tree of 18S rDNA sequences. Each sequence is labeled with the sample ID from Table 1 or the GenBank accession number AY599492. n = the number of sequences. Clade numbers assigned to *Z. arenicolus* lineages are the same as those in Figure 4. The numbers at nodes are the bootstrap proportions (top) and posterior probabilities (bottom) for values ≥50. Scale bar denotes inferred genetic divergence.

Table 8. Average number of base changes for pairwise comparisons of 18S rDNA sequences. − denotes single-sequence clades, so no pairwise comparison could be made.

	Z. arenicolus clade 1	*Z. septimus*	*N. shulenbergeri*	*Z. arenicolus* clade 2	*Z. arenicolus* clade 5	*Z. arenicolus* clade 3, 4	*T. californicus*
Z. arenicolus clade 1	0						
Z. septimus	5	0					
N. shulenbergeri	9	10	−				
Z. arenicolus clade 2	4	7	7	0			
Z. arenicolus clade 5	3	6	8	1	0		
Z. arenicolus clade 3, 4	5	8	12	5	4	0	
T. californicus	33	34	35	32	31	33	−

3.3. Morphological Reanalysis

The *Z. arenicolus* specimens were the only ones requiring further morphological examination because the initial morphological identifications of the other species agreed with the nominal-sequence clades in the inferred ML phylogenetic trees. Because voucher material was not available, twenty-seven of the 54 specimens could not be re-examined for morphological differences. The P4 and P5 (Figure 7 arrows) of the other 27 specimens were re-examined to search for finer-scale morphological differences. Variation in the degree of fusion of the second and third segments of the P4 endopod was found (Figure 8). In some specimens, the fusion was complete; in most, the two segments were completely separate. In a few specimens, I observed partial fusion. All specimens from all *Z. arenicolus* clades, except clade 4, had a 3-segmented (*i.e.* unfused) P4 endopod. In contrast, clade 4 specimens had all three P4 states. Therefore, this character was not useful for species delimitation.

In contrast, P5 character states could be used. Three character states (A, B, C) were observed for the P5 endopodal lobe (Figure 9). State A has 4 setae; states B and C have 5 setae along the inner margin edge (lower right side in the illustration). The setae of state A are all comparable in size. In contrast, two setae are notably smaller than the other three in state B. In state C, one seta is notably smaller than the others (Figure 9 arrow). State A corresponds to clade 2, C to 5, and B to the other three clades (Figure 10).

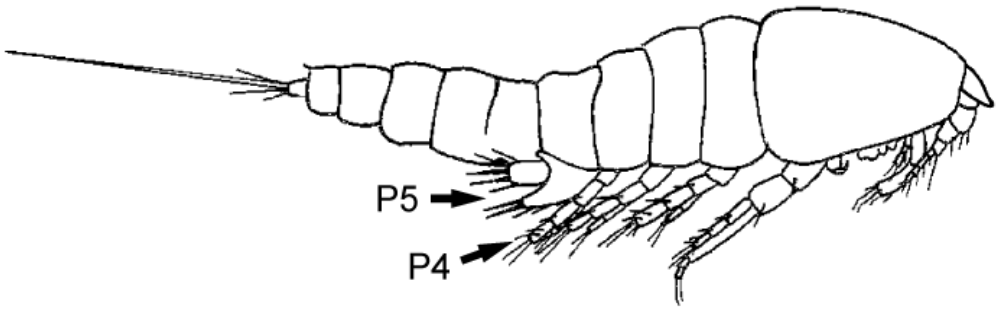

Figure 7. A harpacticoid in lateral view showing the location of the P4 and P5.

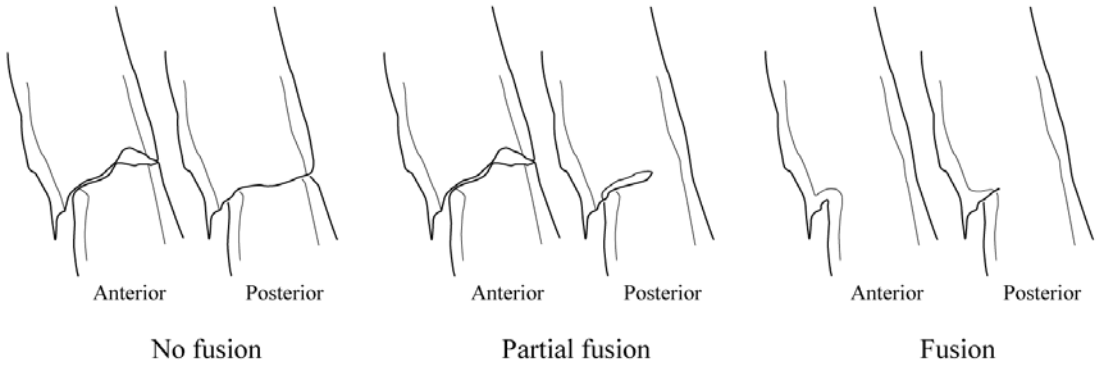

Figure 8. P4 character states as viewed from the front and the back. Only the joint between the second and third segments is illustrated.

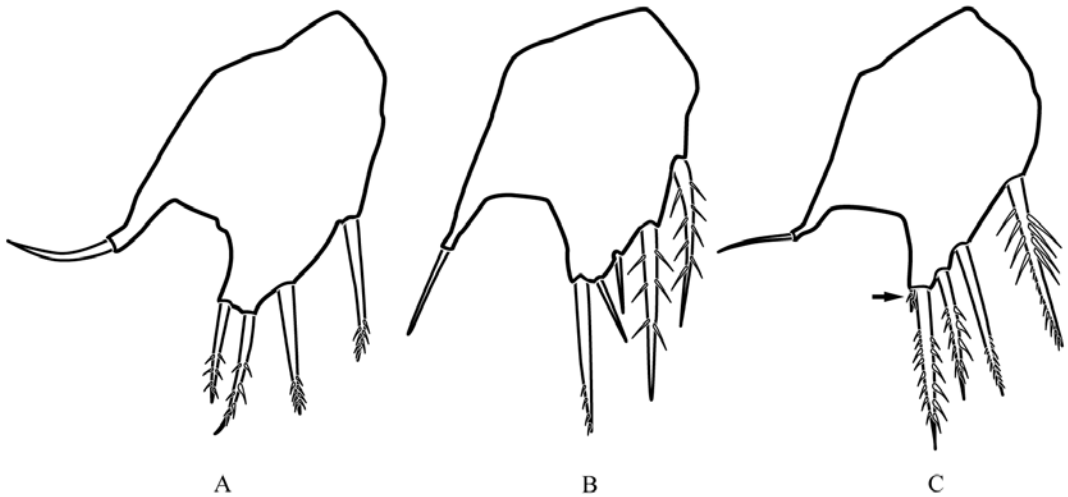

Figure 9. P5 character states. The exopod has been removed to more clearly illustrate the changes. Not all accessory setae and setules are illustrated. The arrow indicates a small, but taxonomically significant, seta.

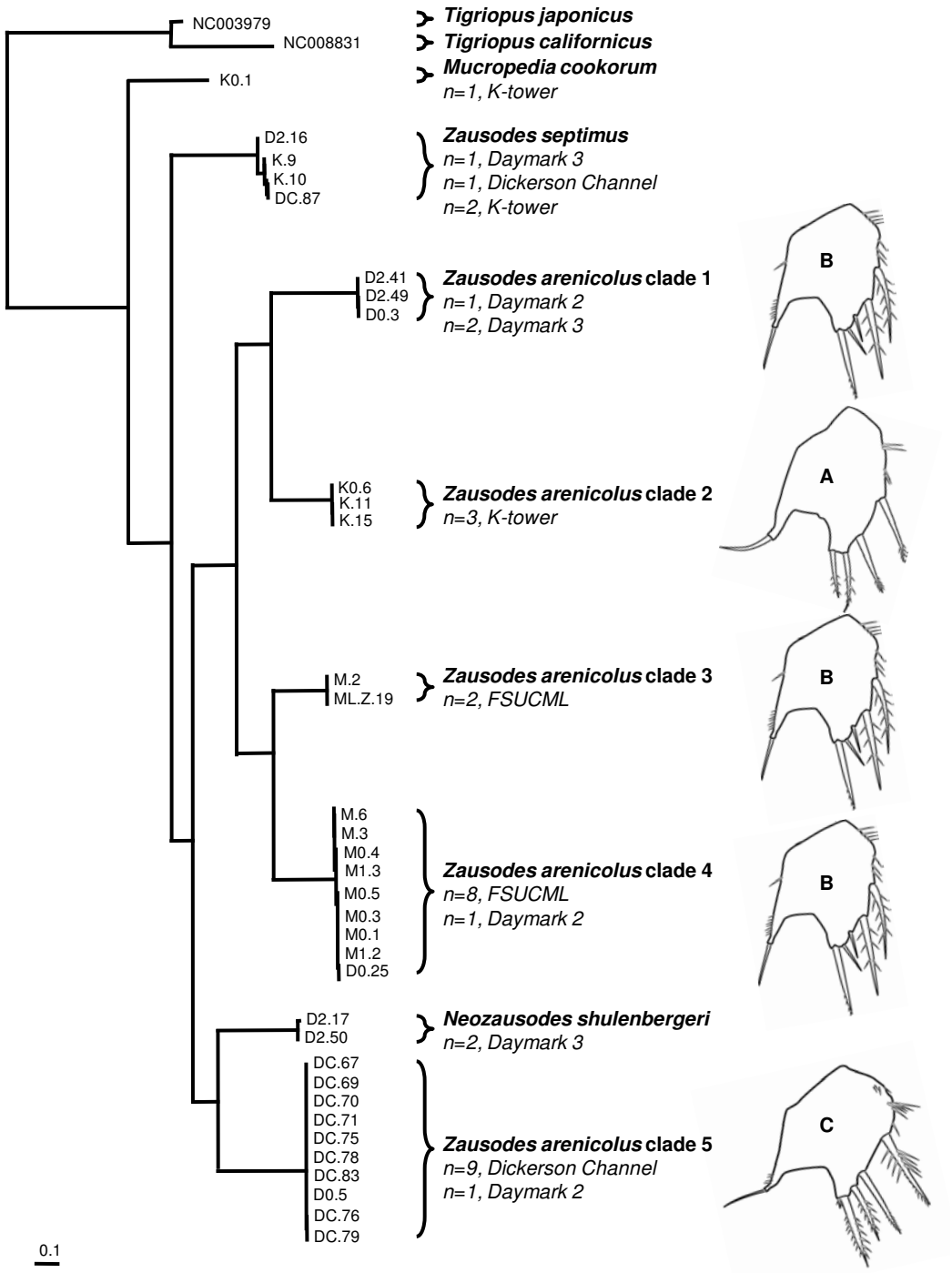

Figure 10. A modification of the cyt*b* ML phylogeny in Figure 4 that shows which P5 character states correspond to *Z. arenicolus* clades. As shown in Figure 4, clades were strongly supported (bootstrap proportions ≥94 and posterior probabilities =100). Only *Z. arenicolus* specimens that underwent further morphological examination are shown as well as one representative of each haplotype from the other species clades. Clade numbers and topology are the same as in Figure 4. P5 character states corresponding to cyt*b* sequence clades are next to clade names. n = the number of specimens from each sampling site in the clade. Sample identification or GenBank accession numbers are listed at the branch tips.

4. Discussion

4.1. Gene-sequence Analyses

My gene-sequence analyses suggest that the characters used by Bouck *et al.* to define species' boundaries were informative but that Bouck *et al.* failed to recognize all the species present. In particular, the cyt*b* gene-sequence data revealed high genetic divergences among *Z. arenicolus* specimens from the five northern Gulf of Mexico sampling sites, suggesting that this taxon needs revision. In contrast, *Z. septimus* had low genetic divergence among specimens, providing support for the current suite of morphological characters defining it. Too few specimens of *M. cookorum* and *N. shulenbergeri* were collected and sequenced for meaningful gene-sequence analyses. *Zausodes*-complex clades inferred from the ML phylogenetic tree (Figure 1) were 0.224-0.336 divergent, which is comparable to the average cyt*b* divergence of 0.280 that supported the pseudo-sibling species recognized in Staton *et al*. (2005). Similarly, the average inter-clade cyt*b* divergences for clades recognized by Thistle as morphological species prior to genetic analyses were at least 0.257, which is comparable to the average inter-clade divergences for *Z. arenicolus* (0.224-0.336). High divergence values, such as these, can sometimes indicate that a pseudo-gene was amplified and sequenced. This possibility is unlikely in this study because the sequences lacked stop codons and the cyt*b* clades were congruent with 18S rDNA clades, an independent assessment of genetic diversity.

Divergence values for delimiting species are not typically standardized because the rate of mutation is not uniform across all taxa, but mutation rates are usually similar for closely related species (see, *e.g.*, Lefébure *et al.* 2006). For the *Z. arenicolus* specimens I studied, the inter-clade cyt*b* divergences were approximately ten times the intra-clade divergences. According to Hebert et al. (2004), this degree of divergence could indicate that the clades are separate species. The use of a threshold to delineate species has been criticized (see Lefébure *et al.* 2006). Never the less, these divergence values between pairs of *Z. arenicolus* clades suggest that *Z. arenicolus* sensu Bouck *et al.* (1999) needs to be split into five species, but to do so will require additional evidence.

The congruence of mt cyt*b* and nuc 18S rDNA sequence data supports my finding that *Z. arenicolus* requires taxonomic revision. *Zausodes arenicolus* 18S rDNA sequences formed four clades instead of the five formed from cyt*b* sequences. The two least divergent cyt*b* clades (3 and 4) formed one 18S clade, which is not surprising because of the lower rate of mutation of the 18S rRNA gene (see, *e.g.,* Burton and Lee 1994, Burton *et al.* 2005).

Additional analyses of cyt*b* and 18S sequences did not reveal information useful for interpreting phylogenetic relationships among the *Zausodes*-complex clades. Because cyt*b* data was saturated, phylogenetic relationships cannot be revealed by the ML phylogenetic tree. No unusual patterns were observed for any of the basic sequence statistics (*i.e*. base frequencies and Ti/Tv ratio) within the *Zausodes* complex, but *Tigriopus* species have significantly different base frequencies for the cyt*b* gene. Too few harpacticoid cyt*b* sequences are currently available to determine whether this difference is atypical.

Gene-sequence evidence for cryptic species or pseudo-sibling species is considered stronger when genetically distinct clades are found in sympatry rather than in allopatry. In sympatry, distinct clades imply that a reproductive barrier exists because a reproductive barrier allows the accumulation of genetic differences (Knowlton and Weigt 1997). Only *Z. arenicolus*

clade 2 was not found in sympatry with other *Z. arenicolus* clades, but this clade is supported by morphological data (P5 character state A). Sympatry provides additional support for the other four clades being newly recognized species.

4.2. Implications from Gene-sequence Data

Whether or not the gene-sequencing and geographic data are interpreted to support the existence of previously unrecognized species, the data do highlight previously unknown genetic diversity within *Z. arenicolus*. Taxonomy aside, this diversity is important for the understanding of harpacticoid copepod ecology and conservation. When species have large ranges with little genetic diversity, the loss of a local population may have little impact because specimens from neighboring populations would recolonize the area. In contrast, if species have small ranges, the loss of a population could result in the loss of the species or at least the loss of an important amount of its genetic diversity. Loss of a species from an environment could potentially have detrimental effects for the community ecology if the species plays an important role in the community that is not filled by another species. The species may not be able to recover from loss of genetic diversity (see, *e.g.*, Soulé 1986, Avise 1994), especially if the remaining haplotypes are less fit (*e.g.*, more susceptible to disease).

None of the *Z. arenicolus* clades were found at sites more than 8 km apart, whereas *Z. septimus* was found at sites ~25 km apart (Table 6, Figure 1). This pattern suggests *Z. septimus* has gene flow among more distant sites in the region near the FSUCML than the *Z. arenicolus* clades. Harpacticoids do not have a planktonic dispersal stage, so dispersal is expected to be on a small scale during the life cycle of an individual. The difference in apparent geographic range between species in the *Zausodes*-complex implies that dispersal differs between clades or that the clades thrive in different environments. Harpacticoid species are known to differ in onshore-offshore distribution in response to differences in environmental parameters such as grain size, wave action, currents, predation, food quality, and food quantity (see, *e.g.*, Harris 1972a, b, c). Because my sites were at different depths, nominal species identified by *Z. arenicolus* cyt*b* clades may be distributed among sampling sites according to their environmental preferences. Further research will be required to determine whether the clades have larger horizontal ranges than onshore-offshore ranges and what environmental variables differ between the environments among the species' ranges. To understand the ecological and biological differences that contribute to these differences, species must be identified properly, which may require gene-sequence analysis.

4.3. Morphological Reanalysis

Recent harpacticoid studies have been prompted by the authors' observations of morphological differences in populations thought to consist of a single species. For example, Staton *et al*. (2005) and Rocha-Olivares *et al*. (2001) used gene-sequence data to evaluate whether such differences were just phenotypic variation or evidence for pseudo-sibling species for *Nannopus palustris* and *Cletocamptus deitersi* respectively. Staton *et al.* found thin-bodied and fat-bodied females with a straight basal region of the terminal seta of the caudal ramus were not genetically distinct for a 281-bp region of the mt cyt*b* gene. In contrast, they found the fat-

bodied specimens with a notched basal region of the terminal caudal ramus seta were genetically distinct by an average 0.28 uncorrected genetic distance. They concluded that these genetically distinct clades that occurred in sympatry were pseudo-sibling species. Similarly, Rocha-Olivares *et al.* found differences in the setal formula of the P3 exopod that were congruent with the gene-sequence clades they obtained. Gene-sequences can contribute to our understanding of species, but they do not define a species (Knowlton 2000), so it is important to return to traditional taxonomic methods to look for congruence with gene sequences before new species are recognized. Unlike the Staton *et al.* and Rocha-Olivares *et al.* studies, morphological differences were not observed within *Z. arenicolus* prior to this study.

Zausodes arenicolus is one of three *Zausodes*-complex species that has retained the ancestral condition of a 3-segmented P4 endopod (Bouck *et al.* 1999). When the P4 vouchers were re-examined, specimens from four of the five cyt*b* clades had 3 segments. In contrast, the second and third segments of most specimens in clade 4 were fused, and the other specimens either had partial fusion or no fusion (Figure 8). Because this character is labile, it cannot be used for delimitation of clade 4. Bouck *et al.* (1999) suggested that P4 segmentation is an evolutionary labile character, so it is possible that this clade may be undergoing a shift toward two segments (see also Lang 1965). Remnants of the 3-segmented state can be found in *N. sextus* and *N. areolatus, i.e.,* a dentiform notch on the outer margin (Bouck *et al.* 1999) and an incomplete surface suture (Lang 1965), respectively. Similarly, all specimens in clade 4 have a dentiform notch, and those with fusion or partial fusion of these segments have an incomplete surface suture.

The ancestral state for the *Zausodes*-complex species has five well-developed setae on the P5 endopodal lobe (Bouck *et al*., 1999). Three *Z. arenicolus* character states for the P5 endopodal lobe were identified in this study. In *Z. arenicolus* as described in Bouck *et al.* (1999) the 3rd and 4th setae are reduced (my character state B). Clades 1, 3, and 4 have this character state. In contrast, clade 2 has only four well-defined setae (character state A). The only other *Zausodes*-complex species to have only four setae is *Z. septimus*. Character state C has four well-developed setae and a rudimentary seta in the distal corner (Figure 9). This arrangement occurs in the *Mucropedia* species and is difficult to see unless the specimen is dissected (Thistle pers. com). Because states C and A each correspond to a single *Z. arenicolus* clade, this character appears to be suitable for delineation of clades 2 and 5. Clades 2 and 5 are likely pseudo-sibling species to the other clades. Although state B corresponds to three clades, this character is still suitable for describing these nominal-species clades because setal formula is constant for a species and sex (Huys *et al.* 1996). Complete evaluation of the remaining vouchers may reveal additional differences for species delimitation.

4.4. Implications from Methods

When a specimen needs to be identified to species, the typical worker is unlikely to have the time for a complete comparison of all features to those in the original description. As a result, keys are used that are thought to reliably separate species based on a subset of the potential characters, if possible, those easily observed. Thus, when using a key, important morphological differences can be overlooked. Even when morphological differences are recognized, the differences are often attributed to phenotypic plasticity (see, *e.g.*, Rocha-Olivares *et al.* 2001, Staton *et al.* 2005). These problems with traditional methods have resulted in

unrecognized harpacticoid species (see, *e.g.*, Bouck *et al.* 1999). If the relationship between character differences and species' boundaries were better understood, then (1) fewer morphological differences would be overlooked, (2) better keys could be developed, (3) more species would be recognized, and (4) a better estimate of harpacticoid diversity could be obtained, which is important to understanding harpacticoid ecology and to conservation.

I have no reason to believe *Zausodes*-complex species are unusual among harpacticoid copepods, so I expect many unrecognized harpacticoid species exist, even in well-studied groups in well-studied areas. My work has demonstrated that gene-sequences can be used to recognize harpacticoid taxa requiring taxonomic revision (*i.e., Z. arenicolus*) even when morphological differences were overlooked by traditional methods of species identification. In addition, the results demonstrate the usefulness of retaining vouchers for additional morphological analysis after gene sequencing. If vouchers had not been retained, all of the *Z. arenicolus* clades would be considered as potentially cryptic species. Instead, I could show that some of the clades were pseudo-sibling species. These new character states can now be used to identify additional specimens relatively quickly.

Therefore, the method used in this study can aid harpacticoid taxonomy by (1) identifying unrecognized species, (2) verifying species' boundaries, (3) identifying character differences that have been overlooked with traditional species' identification methods (*i.e.,* P5 endopodal lobe setation), (4) potentially determining whether new informative characters can be identified for use in taxonomy, and (5) identifying whether characters are variable for a taxon or taxa (*i.e.,* P4 exopod segmentation). In turn, better taxonomic keys will be developed, and a better estimate of harpacticoid diversity can be obtained.

5. Conclusion

I found that the species' boundaries defined in Bouck *et al.* (1999) were accurate, but that Bouck *et al.* failed to recognize all the species present locally. The cyt*b* gene-sequence data revealed high genetic divergences among *Z. arenicolus* specimens from my sites, suggesting *Z. arenicolus* could consist of five species. In contrast, *Z. septimus* had low genetic divergence among specimens from the region, providing support for the species' boundary defined by Bouck *et al.* (1999). Congruence of mt cyt*b* gene sequences, 18S rRNA gene sequences, and morphology reanalysis provided strong evidence that two *Z. arenicolus* clades were pseudo-sibling species to the other clades. Because they occur in sympatry, the other three *Z. arenicolus* clades could be cryptic species or pseudo-sibling species (if further morphological analysis reveals morphological differences). The method I used could aid harpacticoid taxonomists in the identification of new species and the validation of characters used to define species' boundaries.

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