Decoupling of Molecular and Morphological Evolution in Deep Lineages of a Meiobenthic Harpacticoid Copepod

Axayácatl Rocha-Olivares, John W. Fleeger, and David W. Foltz

Department of Biological Sciences, Louisiana State University

Molecular and biochemical genetic analyses have revealed that many marine invertebrate taxa, including some well-studied and presumably cosmopolitan species, are actually complexes of sibling species. When morphological differences are slight and estimated divergence times are old, data suggest either unusually high rates of sequence evolution or long-term morphological stasis. Here, five gene regions (mitochondrial cytochrome oxidase subunit I and large-subunit ribosomal 16S rDNA and nuclear ITS1, 5.8S rDNA, and ITS2) were analyzed in four geographic samples of the meiobenthic harpacticoid copepod *Cletocamptus deitersi*. Molecular sequences revealed four extremely differentiated molecular lineages with unalignable nuclear intergenic spacers and mitochondrial uncorrected divergences reaching 25% (cytochrome oxidase) and 36% (16S rDNA). These levels of divergence are greater than those reported previously for congeneric species in diverse invertebrate taxa, including crustaceans. The nominally intraspecific divergence matches or exceeds the corresponding divergence from a known congener (*Cletocamptus helobius*). A molecular clock applied to the cytochrome oxidase subunit I data suggests that these lineages split in the Miocene, consistent with the fossil record of a North American Cletocamptus from the same period. Morphological differences among the major lineages are subtle but congruent with the patterns of genetic differentiation. Our conclusion, based on concordant patterns of variation in two mitochondrial and three nuclear gene regions, as well as morphological observations, is that *C. deitersi* in North America is composed of at least four separate species by the genealogical concordance, phylogenetic, and morphological-species criteria. Alternative explanations for the deep phylogenetic nodes and apparent morphological stasis, including high rates of sequence evolution, balancing selection, and genetic signatures of historical events, are considered unlikely.

Introduction

When morphological stasis persists after speciation events, resulting species may continue to diverge genetically in the absence of morphological differentiation, producing cryptic species. An inability to recognize cryptic or sibling species using traditional morphological analyses has hindered our understanding of ecological and evolutionary processes, with negative consequences such as underestimation of true levels of species-richness, overestimation of potential for long-distance dispersal in putatively cosmopolitan species, failure to recognize cryptic biological invasions, and misinterpretation of ecological and paleoecological data (e.g., Knowlton et al. 1992; Geller et al. 1997).

Cryptic species of small metazoan invertebrates (<2 mm body length and <5 µg dry weight) are particularly difficult to identify using morphological criteria alone. Small body size generally reduces the number of taxonomic characters available, and many groups have soft bodies that preserve poorly. With improved microscopic techniques and more consistent use of an advanced conceptual framework of systematics methods, many invertebrates once supposed cosmopolitan are now considered to have more limited geographic ranges.

For example, the fraction of cyclopoid copepods that are putatively Holarctic has decreased from 68% to 28% as the proportion of Nearctic species shared with the Palearctic region has steadily declined (Reid 1998). Cryptic species are also known to occur among harpacticoid copepods (e.g., Ganz and Burton 1995); however, improved taxonomic techniques and inclusion of characters such as body ornamentation suggest that harpacticoid cryptic species can frequently be distinguished based on subtle morphological characters (Huys et al. 1996), thereby representing "pseudosibling species" *sensu* Knowlton (1993).

Cletocamptus deitersi (Richard 1897) is a canthocamptid meiobenthic copepod with a highly cosmopolitan distribution reported from inland brines (seeps, streams, and lakes), as well as coastal estuaries and mangroves on all continents except Europe (Mielke 2000). This species has been shown to be morphologically polymorphic within and between populations (e.g., Fleeger 1980; Mielke 2000). *Cletocamptus deitersi* lacks a planktonic dispersing larval stage, since free-living larval stages develop benthically, and adults and juveniles are capable of only short-distance (meter-scale) dispersal through the water column (Sun and Fleeger 1994). The geographical isolation of inland brines suggests that long-distance migration and colonization events are rare and that gene flow should be limited among inland populations. Morphological variability and the potential for geographic differentiation make *C. deitersi* a good candidate for analyses of geographic genetic variation aimed at determining the existence of cryptic species and understanding how rates of morphological and molecular evolution are related.

Using a multilocus approach, we analyzed *Cletocamptus deitersi* from four localities in North America

to determine if separate populations were represented by morphologically and/or genetically differentiated species. We also obtained genetic data from *Cletocamptus helobius,* a readily identifiable and morphologically differentiated congeneric species, to establish a reference level of indisputably interspecific genetic differentiation. An undescribed harpacticoid (Canuellidae: *Coullana* sp.) and a planktonic calanoid (*Calanus pacificus* Brodsky 1948) were used as outgroups in phylogenetic reconstructions.

Materials and Methods
Copepod Samples and DNA Extraction

Samples were collected at an inland brine seep in Alabama (June 1998, 31°23′N, 87°53′W) and at two locations on the coast of Louisiana (Port Fourchon, December 1998, 29°05.4′N 90°05.8′W, and Cocodrie, January 1999, 29°15.2′N, 90°39.8′W). *Cletocamptus deitersi* was also obtained from laboratory cultures established from collections made in the Salton Sea, California (July 1997, 33°13′N, 115°52′W) and at the mouth of the Estero del Yugo estuary (January 2000, 23°18.14′N, 106°29′W) in Mazatlán, Sinaloa, Mexico. These environments are very different. For example, salinity at the Alabama brine seep reaches 45 ppt close to the source, and the stream is located in a bottomland forest subject to annual flood and drought cycles some 83 km from the coast. The Louisiana salt marsh is tidally flooded, and salinity ranges from 0 to 28 ppt. The Salton Sea, where salinity has been increasing to over 44 ppt in recent times, was created in 1905 and is more than 100 km inland from the Pacific Ocean and from the Colorado River Delta in the upper Gulf of California.

Field-collected copepods were removed from sieved sediment and fixed in 95% ethanol. All harpacticoids were carefully identified by one of us (J.W.F.). We extracted total genomic DNA from individual copepods based on Schizas et al. (1997). Ethanol-preserved specimens of *C. pacificus* from southern California (32°25′N, 119°58′W) were provided and identified by Annie Townsend (Scripps Institution of Oceanography). DNA extraction for these copepods was done by standard proteinase-K digestion and phenol-chloroform-isoamyl organic extractions, followed by ethanol precipitation.

PCR Amplification and Sequencing

All copepods were subject to an initial PCR amplification of the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (COX-I) gene using universal primers (Folmer et al. 1994; 0.72 mM total dNTP, 2.5 mM MgCl$_2$, 1 U Promega *Taq* in manufacturer's ''A'' buffer; conditions for Perkin Elmer 480 thermal cycling were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 40°C, and 60 s at 72°C, and a final extension time of 5 min at 72°C). We sequenced two additional gene regions in copepods representative of the major lineages identified on the basis of COX-I sequences (except for the Mexican copepods, for which all gene regions were sequenced

in all individuals): (1) part of the mitochondrial large-subunit ribosomal DNA (LSU rDNA 16S), amplified using Palumbi's (1996) 16SAR and 16SBR universal primers, and (2) part of the nuclear rDNA, comprising all of ITS1, ITS2, and the intervening 5.8S rDNA, amplified using Heath, Rawson, and Hilbish's (1995) ITS primers (cf. table 1 in Heath, Rawson, and Hilbish 1995). We designed Cletocamptus-specific internal primers for reamplification of weak initial reactions (COX-I: CLEintF [TTTTGATTTTCTYATCCAGC] and CLEintR [CCTAGTAANGARGAAATTCC]), for amplification when universal primers failed (16S: 16SciF [YTAAGGTAGCATAGTAA] and 16SciR [TTAATTCAACATCGANGTC]), and for sequencing of long PCR products (nuclear rDNA: 5.8SciF [GGGGTCGATGAAGAACG] and 5.8SciR [CCCTGA-GCCAGACATGG]). Target PCR products were electrophoresed, excised from gels (2% agarose), purified using columns (QiaQuick, Promega), and sequenced using Applied BioSystems ABI-Prism Big-Dye terminator chemistry (Perkin-Elmer) in scaled-down reactions and run on an ABI-377 Gene Analyzer. Representative nucleotide sequences have been submitted to GenBank (AF315001–AF315033).

Multiplex Haplotype-Specific PCR

We designed a rapid, cost-effective method of large-scale genotype screening of individual copepods, using a multiplex haplotype-specific PCR reaction (MHS-PCR; Rocha-Olivares 1998), to assign copepods to one of the first two COX-I mtDNA major lineages. Initial LCOI-HCOI amplicons were used as templates in the MHS-PCR, in which the size of the products, visualized in an agarose gel, could be used for PCR-based genotyping. Multiplexing with nested primers produced a positive control amplicon in all haplotypes and a second, haplotype-specific, amplicon, whose size would be diagnostic (fig. 1). All MHS-PCR batches included experimental positive and negative controls. We sequenced a number of copepods genotyped with MHS-PCR to verify the accuracy of the method.

Data Analyses

Orthologous nucleotide sequences were aligned with the CLUSTAL W algorithm (Thompson, Higgins, and Gibson 1994) with default parameters. In mitochondrial and nuclear rDNAs, inferred indels were further adjusted by eye to minimize those separated by one or very few bases. Aligned nucleotide sequences, with the exception of internal transcribed spacer (ITS) regions, were used to reconstruct gene trees using maximum parsimony (MP) and neighbor joining (NJ) with the program PAUP*, version 4.0b4a (Swofford 1998). NJ searches were made with distances computed using a best-fit model of nucleotide substitution. Models were fit to each data set (COX-I, 16S, and 5.8S) under a maximum-likelihood framework and tested for significance (overall $\alpha = 0.05$ after Bonferroni correction) with likelihood ratio tests (LRTs) using the program Modeltest, version 3.04 (Posada and Crandall 1998). Branch sup-

Table 1
Number of Copepods (*n*), Number of Distinct mtDNA Haplotypes or Nuclear Gene Copies (*h*), and Accession Numbers of Representative Sequences Submitted to GenBank (GB) of Each Copepod Species Genotyped by Direct Sequencing (COX-I, 16S, n rDNA) or Multiplex Haplotype-specific PCR of the COX-I Gene (MHS-PCR)

SPECIES (sample)[a]	COX-I[b]			16S[c]			N rDNA[d]			MHS-PCR
	n	*h*	GB	*n*	*h*	GB	*n*	*h*[e]	GB	*n*
Cletocamptus deitersi (AL)[c]	42	10	AF315009	12	2	AF315001	5	5/2/5	AF315021–AF315025	55
C. deitersi (LA)	17	6	AF315012	20	5	AF315002	3	3/2/3	AF315026–AF315028	126
C. deitersi (CA)	33	4	AF315011	4	3	AF315003	4	4/1/4	AF315029–AF315031	
C. deitersi (MX)	11	1	AF315010	11	1	AF315004	11	2/1/1	AF315033–AF315034	
Cletocamptus helobius	6	1	AF315014	3	1	AF315005	4	4/2/4	AF315017–AF315020	
Coullana sp.	2	1	AF315015	1	1	AF315007	0	—	—	
Calanus pacificus	3	3	AF315013	4	3	AF315006	1	1	AF315016	

[a] AL = Alabama; LA = Louisiana; CA = California; MX = Mazatlán, Mexico.
[b] Mitochondrial cytochrome oxidase subunit I.
[c] Mitochondrial LSU rDNA (16S).
[d] Nuclear rDNA consisting of ITS1, the 5.8S rRNA gene, and ITS2.
[e] Number of distinct ITS1/5.8S rRNA/ITS2 gene copies.

port was assessed by 1,000 nonparametric bootstrap replicates. Phylogenetic analyses of the mtDNA 16S rDNA were performed on the data set bounded by the 16SciF-16SciR primers (313 bp), not the entire 16SAR-16SBR alignment (466 bp), which was incomplete for the Mexican *C. deitersi* and *C. helobius.* Competing phylogenetic hypotheses and the existence of a molecular clock were tested under a likelihood framework with LRTs. The program Seq-Gen (Rambaut and Grassly 1997) was used for parametric bootstrapping of data sets. Rates of synonymous and nonsynonymous substitutions were computed following Yang and Nielsen (1998) with the program PAML, version 3.0b (Yang 1997).

Morphological/Genetic Comparisons

For both sexes, the number of inner setae on the distal segment of the third-swimming-leg exopod is known to differ within and among *C. deitersi* populations (Fleeger 1980) and in related species (Mielke 2000). To determine if morphological and genetic patterns correlated in *C. deitersi,* 51 specimens from Louisiana and 23 from Alabama were examined morphologically and genetically. The third leg was removed with a fine probe, mounted on a microscope slide, and examined with phase microscopy. The remaining copepod tissue was used for DNA extraction and genotyping by MHS-PCR. Specimens were given identification numbers upon dissection, and genetic analysis was conducted without knowledge of their geographic origins. In addition, the third-leg exopods of several specimens from California and Mexico were examined and compared with specimens from the Gulf of Mexico.

Results
Genotypic Variability

A total of 135 copepods (121 *C. deitersi,* 7 *C. helobius,* 2 *Coullana* sp., and 5 *C. pacificus*) were initially sequenced for one or more gene regions. The number of distinct COX-I haplotypes per locality ranged from 1 (Mazatlán) to 10 (Alabama) (table 1). COX-I sequences fell into one of three major types, which will be hereinafter referred to as "major types" or "major lineages." Type I was found among copepods from Alabama, and type II was found predominantly among organisms from Louisiana but also in Alabama, whereas type III was exclusively present in all copepods from California (IIIC) and Mazatlán (IIIM). A small fraction of copepods from Louisiana also harbored type I sequences.

Of all the *C. deitersi* of types I and II genotyped (sequencing + MHS-PCR, total *N* = 240), 11 (7.7%) from Louisiana harbored type I, and 132 (92.3%) harbored type II COX-I sequences. Conversely, 90 (92.8%) *C. deitersi* from Alabama had type I, whereas only 7 (7.2%) had type II. All COX-I sequences obtained from 15 haphazardly selected copepods genotyped via MHS-PCR (both type I and type II) corroborated the identifications obtained with the PCR-based method.

Eleven distinct 16S sequences were found among 47 *C. deitersi* analyzed (table 1). Overall, the 16S rDNA
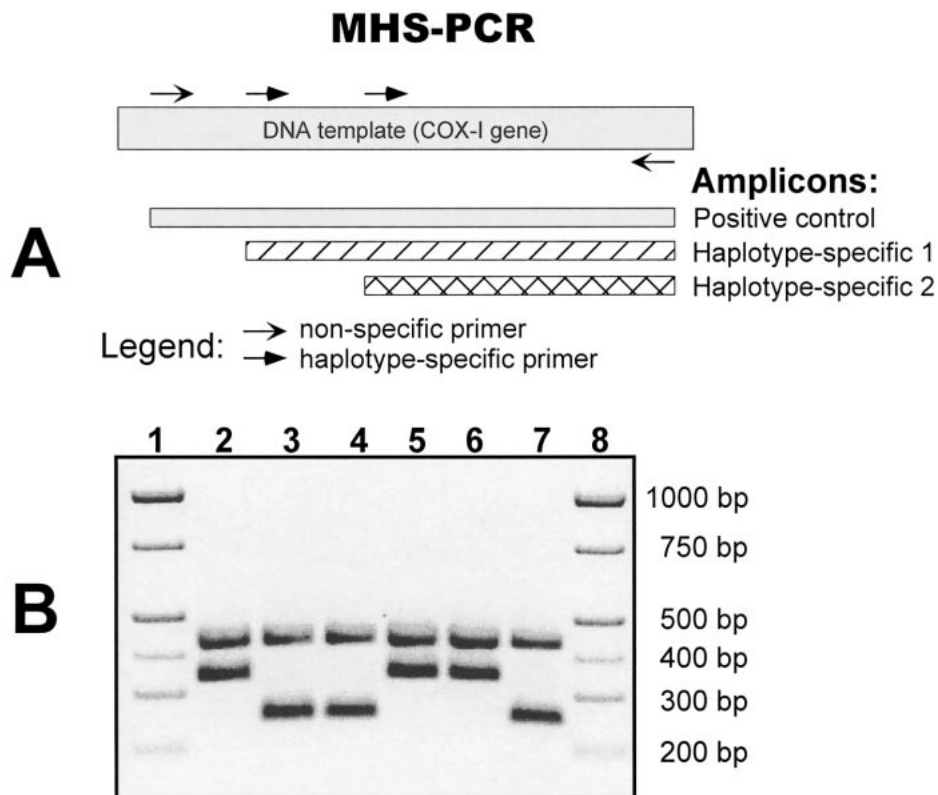
MHS-PCR

FIG. 1.—Multiple haplotype-specific PCR (MHS-PCR) used to genotype individual *Cletocamptus deitersi* based on cytochrome oxidase subunit I amplification. *A,* A pair of nonspecific primers is used as a positive control, and an additional pair of haplotype-specific primers yields amplicons of diagnostic size used for genotyping. *B,* Negative gel image of the results obtained with *C. deitersi.* Lanes 1 and 8 show molecular markers; lane 2 shows a type I copepod verified by direct sequencing (427- and 349-bp bands); lanes 3 and 4 show unknown copepods from Louisiana; lanes 5 and 6 show unknown copepods from Alabama; and lane 7 shows a type II copepod verified by direct sequencing (427- and 253-bp bands).

followed the same pattern as COX-I, in that sequences fell into major types analogous to those identified earlier.

We obtained nuclear rDNA sequences from 23 *C. deitersi,* 4 *C. helobius,* and 1 *C. pacificus* (table 1). The nuclear gene regions showed different levels of variation, with ITS having the highest and 5.8S having the lowest. Except for the Mexican copepods that shared two distinct ITS1 alleles but only one ITS2, the remaining copepods had the same number of ITS1 and ITS2 distinct alleles. In sharp contrast, the 5.8S rDNA showed very low variability, with only four distinct alleles among the 23 *C. deitersi* and two among the 4 *C. helobius* (table 1). However, no nuclear alleles were shared between the two congeneric species. In both ITS regions, alleles assorted into three extremely divergent groups, each matching the major mitochondrial types (I, II, and III) previously identified on the basis of the mtDNA sequences. On the other hand, one of the four 5.8S alleles was shared by individuals with different mtDNA types, notwithstanding the fact that in each organism the same 5.8S allele was flanked by drastically different ITS alleles falling into the divergent groups described above.

Intraspecific and Interspecific Levels of Genetic Differentiation

Levels of genetic differentiation within each major type of *C. deitersi* were small and typical of those found among conspecific organisms, leading to the assumption that each type represents a putative species. Mitochondrial genes were less variable (0.2%–1.7%; diagonal of table 2) than noncoding nuclear ITS regions (2.7%–8.5% in faster-evolving ITS1 and 0.7%–2.6% in ITS2; table 3). As expected, intralineage variation in the 5.8S rRNA gene was virtually absent. Although copepods from Mazatlán were unequivocally most closely related to those from California, they were quite differentiated in both mitochondrial (11% in COX-I and 5% in 16S rRNA) and nuclear (12% in ITS1 and 2.8% in ITS2) genes (table 3). Thus, four species may be represented within our three major lineages of *C. deitersi.*

Further evidence that our nominal *C. deitersi* is composed of more than one species came from a comparison with its morphologically differentiable congener *C. helobius.* Organisms of the three major lineages of *C. deitersi* were as genetically differentiated from each other as they were from *C. helobius.* Corrected levels of mtDNA sequence divergence among the three major lineages were at least one, if not two, orders of magnitude higher than intralineage divergences, with values ranging from 48.7% to 88.3% (20.8%–26.4% uncorrected) in the COX-I gene and from 29.4% to 82.5% (20.3%–36.0% uncorrected) in the 16S rRNA (table 2). The corresponding values of indisputably interspecific (i.e., *C. deitersi* vs. *C. helobius*) differentiation were 71.9%–

Table 2
Mean Pairwise Genetic Distances in the COX-I (above diagonal) and 16S (below diagonal) Mitochondrial Genes in Four Lineages of *Cletocamptus deitersi* (types I, II, IIIC, and IIIM) and in *Cletocamptus helobius* (CH)

	Type I	Type II	Type IIIC	Type IIIM	CH
Type I.......	0.27 (0.2–0.5), $n = 28$ 0.35, $n = 1$	23.15 (22.5–25.3) 61.59 (58.5–74.4) $n = 64$	24.68 (24.0–26.1) 68.44 (62.2–82.0) $n = 32$	26.27 (26.1–26.4) 76.70 (73.9–88.3) $n = 8$	26.77 (24.5–26.8) 79.67 (77.3–91.7) $n = 8$
Type II.....	32.15 (31.4–32.4) 58.03 (56.0–58.9) $n = 10$	0.33 (0.2–0.6), $n = 28$ 0.69 (0.3–1.0), $n = 10$	21.49 (20.8–22.0) 51.28 (48.7–53.4) $n = 32$	22.13 (22.0–22.3) 52.02 (51.2–53.0) $n = 8$	23.82 (23.7–24.0) 72.80 (71.9–74.1) $n = 8$
Type IIIC...	35.77 (35.6–36.0) 80.76 (79.1–82.5) $n = 6$	20.92 (20.3–21.3) 30.54 (29.4–31.2) $n = 15$	1.19 (0.2–1.7), $n = 6$ 0.69 (0.3–1.0), $n = 3$	8.66 (8.4–8.8) 11.04 (10.6–11.3) $n = 4$	26.37 (26.1–26.6) 88.45 (80.5–82.0) $n = 4$
Type IIIM...	35.68 (35.7–35.7) 82.16 (81.8–82.5) $n = 2$	21.07 (20.7–21.1) 31.67 (30.9–32.2) $n = 5$	4.48 (4.1–4.8) 4.64 (4.3–5.0) $n = 3$	NA	25.84 79.26 $n = 1$
CH	35.77 (35.8–35.8) 81.12 (80.8–81.4) $n = 2$	31.96 (31.7–32.1) 63.57 (63.1–63.9) $n = 5$	32.89 (32.6–33.0) 70.17 (69.9–70.5) $n = 3$	32.28 68.51 $n = 1$	NA

NOTE.—16S distances were computed on a 16SAR-16SBR fragment corrected for missing data for type IIIM and CH. On the diagonal, line 1 = COX-I, line 2 = 16S (mean uncorrected percent nucleotide site differences [%p], n = number of comparisons). Off the diagonal, line 1 = uncorrected %p, line 2 = %K81uf+I+G (COX-I), and %TIM+I+G (16S) (range in parentheses), line 3 = number of comparisons. See text for explanation of genetic distance measures. NA = not applicable.

91.7% (23.7%–26.8% uncorrected) for the COX-I gene and 63.1%–81.4% (31.7%–35.8% uncorrected) for the 16S rRNA (table 2). Data from nuclear genes were consistent with mitochondrial results. For the widely divergent noncoding ITS regions, nucleotide sequences of alleles from different lineages were so different that their alignment was dubious (fig. 2). Conspicuous size differences (ITS1 lengths were 359–376 bp in type I, 292–300 bp in type II, 288–289 bp in type IIIC, and 284 bp in type IIIM; whereas ITS2 lengths were 221–228 bp in type I, 204–206 bp in type II, 215–219 bp in type IIIC, and 216 bp in type IIIM) and a large degree of sequence differentiation are indicative of a thorough randomization of the nucleotide sequences via mutation and recombination, such that statements of sequence homology can no longer be established confidently by align-

ment. Thus, no quantitative appraisal of divergence is given (table 3); instead, we present the imposed multiple alignment as qualitative evidence of the extent of differentiation (fig. 2). The alignment shows that aside from the well conserved 18S and 5.8S rDNAs, the alignability of the sequences decays rapidly in the ITS regions, but not to the same extent among the lineages of Cletocamptus, since types II and III share more structural elements. Finally, in the slowly evolving 5.8S rRNA gene, mean levels of intraspecific variation in the nominal *C. deitersi* were smaller (0.6%–1.5%) than those of interspecific variation (3.7%–4.6%).

Molecular Evolution and Phylogenetic Analyses

The 16S amplicons in *C. deitersi* were either 462 bp (type I) or 467–468 bp (types II and III) in length.

Table 3
Mean Uncorrected Genetic Distances (%) in the ITS1, 5.8S, and ITS2 Gene Regions in Four Lineages of *Cletocamptus deitersi* (types I, II, IIIC, and IIIM) and in *Cletocamptus helobius* (CH)

Gene	Comparison	Mean	n	Range
ITS-1	Type I × type I	7.63	10	0.6–12.6
	Type II × type II	2.73	3	2.1–3.4
	Type IIIC × type IIIC	4.06	6	2.4–5.9
	Type IIIM × type IIIM	8.50	1	NA
	Type IIIC × type IIIM	11.95	8	8.5–15.7
ITS-2	Type I × type I	0.73	10	0.0–1.4
	Type II × type II	0.65	3	0.5–1.0
	Type IIIC × type IIIC	2.60	3	1.8–3.2
	Type IIIM × type IIIM	NA	0	NA
	Type IIIC × type IIIM	2.80	3	1.4–4.6
5.8S	Type I × type I	0.61	1	NA
	Type II × type II	0.61	1	NA
	Type III × type III	NA	0	NA
	Type I × type II	1.53	2	1.2–1.8
	Type I × type III	0.92	2	0.6–1.2
	Type II × type III	0.61	1	NA
	CH × type I	3.70	4	3.1–4.3
	CH × type II	4.62	2	4.3–4.9
	CH × type III	3.99	2	3.7–4.3

NOTE.—n = number of pairwise comparisons averaged. NA = not applicable. All type III copepods from California (IIIC) and Mazatlán (IIIM) shared the same 5.8S gene copy.

The amplified mitochondrial COX-I fragment was 658 bp long in all organisms. Almost a third of the nucleotides were variable (228 total variable sites; the ratio of variable 1:2:3 codon positions was 43:8:177). Inferred COX-I amino acid sequences revealed that all nucleotide substitutions within types I and II were silent (details not shown). Most amino acid replacements were conservative with respect to hydrophobicity (20 out of 23; see supplementary data at http://www.molbiolevol.org). Rates of synonymous and nonsynonymous substitutions along the lineages leading to types I, II, and III were d_S = 11.19, d_N = 0.04 (I); d_S = 1.56, d_N = 0.006 (II); and d_S = 1.87, d_N = 0.007 (III).

Different models of nucleotide substitution were fitted to each data set. For the COX-I gene, the best-fit model was K81 (Kimura 1981) with unequal base frequencies corrected for proportion of invariable sites (I) and for rate heterogeneity among sites with a Gamma distribution (G; Yang 1993). For the 16S rRNA gene, the best-fit model was TIM (TIM+I+G; Rodriguez et al. 1990), whereas for the 5.8S rRNA gene, the best-fit model was Jukes-Cantor (Jukes and Cantor 1969) (table 4).

The COX-I and 16S rDNA alignments including all taxa had, respectively, 309 and 215 variable sites (treating indels as missing data), of which 275 and 180 were parsimony-informative. The strict-consensus tree of the eight COX-I most-parsimonious reconstructions obtained via branch-and-bound (length = 670 steps, consistency index [CI] = 0.72, retention index [RI] = 0.91) was completely congruent with the NJ tree (fig. 3). For the 16S, the NJ tree differed from the three branch-and-bound most-parsimonious trees (length = 419 steps, CI = 0.79, RI = 0.87) in that Cletocamptus was not monophyletic. Instead, *C. helobius* was sister to type I sequences and both were a sister group to the rest of the *C. deitersi* sequences (fig. 3). An NJ tree constrained by monophyly of *C. deitersi* was not significantly different from the unconstrained tree (LRT; δ = 2.48, *P* = 0.43). In the MP analyses, the monophyly of *C. deitersi* had a nonparametric bootstrap branch support of 64%. Trees featured extensive unresolved polytomies within each major type (I, II, and IIIC) but were otherwise completely resolved. Both mtDNA gene trees featured long branches, in excess of 30% corrected sequence divergence, separating *C. deitersi* types I, II, and III and the other congeneric and noncongeneric species. All bootstrap replicates recovered the monophyly of the three major mitochondrial types in both genes. Within type III sequences, the Californian haplotypes formed a reciprocally monophyletic assemblage relative to the Mexican haplotypes with very high bootstrap support. Despite overall topological congruence of the gene trees, considering that a 16S NJ tree constrained by the monophyly of *C. deitersi* is not significantly worse than the one shown (fig. 3), the two mtDNA genes were not equally powerful in resolving the relationships of the major lineages within Cletocamptus. For example, the sister taxon relationship between types II and III was very strongly supported by the NJ analysis of the 16S data set but less so by the corresponding MP analysis

or by the COX-I data set (bootstrap 55%–57%). Monophyly of Cletocamptus and Harpacticoida, however, was very well supported by the mtDNA data (bootstrap > 93%).

The nuclear 5.8S rDNA alignment was 166 bp long with 32 variable sites, of which only 6 were parsimony-informative. An exhaustive search produced a single most-parsimonious reconstruction (length = 33, CI = 1.0, RI = 1.0), and the NJ reconstruction was completely congruent (fig. 4). Unlike the mitochondrial gene trees, the nuclear 5.8S rDNA proved more powerful for supporting the monophyly of *C. deitersi* (fig. 4). Variation within *C. deitersi* was too small to gain any insight into the phylogenetic relationships among mitochondrial lineages.

Branch-and-bound maximum-likelihood analyses using best-fit models of substitution and one representative sequence of each Cletocamptus lineage, including *C. helobius,* indicated that the existence of a molecular clock could not be rejected in the mtDNA genes (COX-I: χ^2 = 2.89, *P* = 0.41; 16S: χ^2 = 6.04, *P* = 0.11).

Morphological/Genetic Comparisons

All specimens of *C. deitersi* examined in this study had indistinguishable body plans (i.e., body length and width, rostrum shape, caudal rami shape, and leg segmentation were essentially identical). Small variations in leg setation and body ornamentation have been described in species related to *C. deitersi*. For example, the number of inner setae on the distal segment of the third-leg exopod of *Cletocamptus axi* (one seta) differs from that of *Cletocamptus schmidti* (two setae) in both sexes (Mielke 2000). Specimens from Louisiana, California, and Mexico all had one inner seta, whereas those from Alabama had two. MHS-PCR was successful for 70 of the 74 specimens. Copepods with one inner seta on the third-leg exopod, all from Louisiana, were of mtDNA COX-I major type II, whereas those with two inner setae, all from Alabama, were of type I.

Discussion
Genetic Diagnosis of Species Boundaries

Species criteria are not necessarily mutually exclusive, and alternate criteria typically capture different aspects of the biological information present in a particular data set. We suggest that rather than use a single criterion specified a priori for diagnosing species boundaries, using multiple criteria simultaneously can produce species identifications that are more robust to the assumptions employed by different investigators. Therefore, where appropriate, we will make specific reference below to the genealogical concordance, phylogenetic, and morphological species criteria (see de Queiroz [1998] for terminology).

The nominally intraspecific genetic differentiation found in mitochondrial and nuclear gene regions among major lineages of *C. deitersi* represents the largest yet reported for a marine invertebrate and strongly suggests that North American lineages are composed of cryptic species. The likelihood that the large genetic differen-

```
                 10         20         30         40         50         60         70         80         90        100
consensus   GTTTCCGTAG GTGAACCTGC GGAAGGATCA TTATCGATTG CTTCTTTTTC TTTTCACTTG ACTGTGATTT GAAAATTGGC AGGCAAGCTG AAACAAACTG
AF315017    .......... .......... ......-... ..C...--- ---------- ---------- ---------- ---------- ---------- ----------
AF315018    .......... .......... ......-... ..C...--- ---------- ---------- ---------- ---------- ---------- ----------
AF315019    .......... .......... ......-... ..C...--- ---------- ---------- ---------- ---------- ---------- ----------
AF315020    .......... .......... ......-... ..C...--- ---------- ---------- ---------- ---------- ---------- ----------
AF315021    .......... .......... .......... ....G... .......... .......... .......... .......-.. .......... ..........
AF315022    .......... .......... .......... ....G... .......... .......... .......... .......... .......... ..........
AF315023    .......... .......... .......... ....G... .......... .......... .......... .......... .A........ ....----..
AF315024    .......... .......... .......... ....G... .......... .......... .......... .......-.. .......... ....TG..C.
AF315025    .......... .......... .......... ....G... .......... .......... .......... .......-.. .A........ ....---..
AF315026    .......... .......... .......... ....G--- .......... .......... .......... .......... .......... ..........
AF315027    .......... .......... .......... ....G--- ---------- ---------- ---------- ---------- ---------- ----------
AF315028    .......... .......... .......... ....G--- ---------- ---------- ---------- ---------- ---------- ----------
AF315029    .......... .......... .......... .....--- ---------- ---------- ---------- ---------- ---------- ----------
AF315030    .......... .......... .......... .....--- ---------- ---------- ---------- ---------- ---------- ----------
AF315031    .......... .......... .......... .....--- ---------- ---------- ---------- ---------- ---------- ----------
AF315008    .......... .......... .......... .....--- ---------- ---------- ---------- ---------- ---------- ----------
AF315032    .......... .......... .......... .....--- ---------- ---------- ---------- ---------- ---------- ----------
AF315033    .......... .......... .......... .....--- ---------- ---------- ---------- ---------- ---------- ----------
            >>>>>>>>>> >>>>>>>>>| |||||||||| |||/////// ////////// ////////// ////////// ////////// ////////// //////////

                110        120        130        140        150        160        170        180        190        200
consensus   AACTTGGTTT TGACGGCCTA GAGAGAGTAA GTCTWKTTGR GTTGAGCTTT TGTAAAGTCT YGGGTGGSTT GGTAGATGCG AGTTTTCCTT GCCATCTTTY
AF315017    ---------- ---------- ---------- ---------- ...A.AGG.. .T..TCTCGG C.TT.T.TG. .T.G.T..GC ..CCC.GGGA AG.C.G.C.C
AF315018    ---------- ---------- ---------- ---------- ...A.AG... .T..TCTCGG C.TT.T.TA. .T.G.T..GC ..CCC.GGGA AG.C.G.C.C
AF315019    ---------- ---------- ---------- ---------- ...A.AG... .T..TCTCGG C.TT.T.TG. .T.G.T..GC ..CCC.GGGA AG.C.G.C.C
AF315020    ---------- ---------- ---------- ---------- ...A.AG... .T..TCTCGG C.TT.T.TG. .T.G.T..GC ..CCC.GGGA AG.C.G.C.C
AF315021    .......... .....C... A......AG. ...GAG.C.A ..C.T..GCC ..GC.G-CAA AC.AGT.T.G CA.TCCCTT. ..-GGCT.CA TT.TCT---C
AF315022    .......... .......... ........AG ...GGG.C.A ..C.T.GGCG ..GC.G-.GA GT.AGTTG.. .A.TCCCTT. ...GG..T..C TT.T.TC..C
AF315023    .......... .......... ........AG ...GGG.C.A ..C.T.GGCG ..GC.G-.GA GT.AGTTG.G .A.TCCCTT. ...GAGGGG. TT.TCT---C
AF315024    .......G.. .......... ........AG ...GCG.C.A ..C.T..ACG CAGTG---AA TT.AG.TA.. .A.TCCCTT. ...GG..T..C TT.T.TC.CC
AF315025    .......... .......... ........AG ...GGG.C.A ..C.T.GGCG ..GC.G-.GA GT.AGTTG.G .A.TCCCTT. ...GAGGGGG TT.TCT---C
AF315026    ---------- ---------- ---T.CT.C. T.G.TT.GAT .A.....A.. CA..T..... C...A..GC. T.G.----- TT..C..... ..-----..T
AF315027    ---------- ---------- ---T.CT.C. T.G.TT.GAT .A.....A.. CA..T..... C...A..GC. T.G.----- TT..C..... ..-----..T
AF315028    ---------- ---------- ---T.CT.C. T.G.TT.GAT .A.....A.. CA..T..... C...A..GC. T.G.----- TT..C..... ..-----..T
AF315029    ---------- ---------- -------..T .G..AAA.-G C..T....AA ..AT...... T...C..... ..G....... .AA..C.... ..T.....T
AF315030    ---------- ---------- -------..T .G..AAA.-G C..T....AA ..AT......C T...C..CC. ..G.A..... .AA..C.C.. ..T.....T
AF315031    ---------- ---------- -------..T .G..AAA.-G C..T....AA ..AT...... T....C.... ..G....... .AA..C.... ..T...T..T
AF315008    ---------- ---------- -------..T .G..AAA.-G C..T....AA ..AT...... T....C.... ..G....... .AA..C.... ..T.....T
AF315032    ---------- ---------- -------..T .CT.TTA.AG CAATT.A.AA ACA..-.... T..T-..C.. ..G....... .A...C.... ..T....--T
AF315033    ---------- ---------- -------..T .CT.TTA..C C..AGCAAAA AC.CCGGCC. T....C.... ..A....... .A...C.... ..T.....T
            ////////// ////////// ////////// ////////// ////////// ////////// ////////// ////////// ////////// //////////

                210        220        230        240        250        260        270        280        290        300
consensus   TTTCTTAGWC TGGGAGSGTCT TGGTTCCYKT TGCATGGTTC CTCGCCGSYT GMGGTTKGSS TGGTTCTTKT TTTGCCTCTT TTTGTTGGGG RAGGAGGACA
AF315017    ....A.G.TG CT..AC.G.G .CA...TCT. .A..C..AGT GA.....GC. .A.TC.T.GG ..CAGG.CTC ..CTT..TC. .C.C.....A GGA.T.A..T
AF315018    ....A.G.TG CT..AC.G.G .C....TCT. .A..C..AGT GA.....GC. .A.TC.T.GG .CAGGCCTC ..CTT..TC. .C.C.....A GGA.T.A..T
AF315019    ....A.G.TG CT..AC.G.G .CA...TCT. .A..C..AGT GA.....GC. .A.TC.T.GG ..CAG...CTC ..CTT..TC. .C.C.....A GGA.T.A..T
AF315020    ....A.G.TG CT..AC.G.G .C....TCT. .A..C..AGT GA.....GC. .A.TC.T.GG ..CAG...CTC ..CTT..TC. .C.C.....A GGA.T.A..T
AF315021    ......-CTT .CTTCC...C G.C....CTC C-.T.CTC.G ..G....GCC .A.C..T.GG .C..C.C.T. CAAAAGAT.. ......TTTT G.TTG.C...
AF315022    ......GCTT CCT.CC.... ..C....CT. C-.T.CTC.G ..G....GCC .A.C..T.GG .C..C.C.T. CAAAAGA... ......TTTT G.TTG.C...
AF315023    ......-CTT .CTTCC.... ..C....CT. C-.T.CTC.G ..G....GCC .A.C..T.GG .C..C.C.T. CAAAAGAA.. .C.T..TTTT G.TTG.C...
AF315024    ......GCTT CCC.CC.... ..C....CT. .T.T.CTC.G ..G....GCC .A.C..T.GG .C..C.C.T. CAAAAGA... .A....TTCT G.TTG.C...
AF315025    ......-CTT .CTTCC.... ..C....CT. C-.T.CTC.G ..G....GCC .A.C..T.GG .C..C.C.T. CAAAAGAA.. .C.....TTTT G.TTG.C...
AF315026    --G..G..A. ..CT.GC... ..T...TGC .TT...A... ....TT.CTC ACC.AC.CC ..AG...G.. .........C. ...TA.TTT. A....T..AG
AF315027    --G..G.TA. ..CT.GC... ..T...TGC ..T...A... ...TT.CTC ACC.A.GACC ..A.....G. .........C. ...TA.TTT. A....T..AG
AF315028    --G..G.TA. ..CT.GC... ..T...TGC .T...A... ....TT.CTC ACC.ACG.CC ..A.....G. .........C. ...TA.TTT. A....T..AG
AF315029    ...T.-..A. .....GC... .......TGG ....G..... ...T.GG.CT. TC...GCG.CC ..TG....GG .........C ....A-...A A..A.A..G.
AF315030    C..T.-..A. .....GC... .......TGG ....G..... .CT.GG.CT. TC...CG.CC ...G....GG G.........C ....A-.... A..A.A..G.
AF315031    ...T.....A. ...GT.T. .......TGG ....G..... ...T.GG.CT. TC...CG.CC .......GG G.........C ....A-...A A..A.A.GG.
AF315008    ...T.-..A. .....GC... .......TGG ....G..... ...T.GG.CT. TC...CG.CC ...G....GG G.........C ....A-.... A..A.A..G.
AF315032    ...T.-..A. .....GC... .......TG. ....G..... ...T.TG.CT. TC...CG.CC ..TG....GG G.........C ....A-.... AT.A.A..G.
AF315033    ...T---..A. .....GC... .......TG ....G..... ...T.TG.CT. TC...CG.CC ..TG....GG G.........C ....G-.... AT.A..AA.G.
            ////////// ////////// ////////// ////////// ////////// ////////// ////////// ////////// ////////// //////////

                310        320        330        340        350        360        370        380        390        400
consensus   RGTTCWCTTC GCTTCCGCCC TGGTTGGGTT TGTTTATCGT GCCTCGGAAG AGCTGGGGAA AAAAAAGTTT GTCTTTTTTT CATTTTCTTT TCTAKTAAAA
AF315017    GC.CAAAG.G AT...AC..T ..C.CA...A .A......TG CTTCTTCG.T GCG.T.T.TT .GCTTTT... T.....GAAA AG...GAAC. .G.TTC...T
AF315018    GC.CAAAG.G AT...AC..T ..C.CA...A .A......TG CTTCTTCG.T GCG.T.T.TT .GCTTTT... T.....GAAA AG...GAAC. .G.TTC...T
AF315019    GC.CAAAG.G AT...AC..T ..C.CA...A .A......TG CTTCTTCG.T GCG.T.T.TT .GCTTTT... T.....GAAA AG...GAAC. .G.TTC...T
AF315020    GC.CAAAG.G AT...AC..T ..C.CA...A .A......TG CTTCTTCG.T GCG.T.T.TT .GCTTTT... T.....GAAA AG...GAAC. .GCTTC...T
AF315021    GAAA.AG... C...G.T.GG GTT...CC.. ..CGC-.... ...GA.TCT. CAAA.AA... .G....A... ...GCA.... .--------- --A.TGC.T.
AF315022    GAAA.AG... C...G.T.GG GTT...CC.. ..CGC-.... ...GA.TTT. CAAA.AA..- --...A... ...GCA.... TG...C.AAC GAA.CA..T.
AF315023    GAAA.AG... C...G.T.GG GTT...CC.. ..CGC-.... ...GA.TCT. CAAA.AA... .G....A... ...GCA.... .--------- --A.TGC.T.
AF315024    GAAA.AG... C...G.T.GG G.C.CC.... ..CGC-.... ...GA.TTT. CAGA.AA..- --..G..A... ...GCA.... TG...C.AAC G.A.CG..T.
AF315025    GAAA.AG... C...G.T.GG GTT...CC.. ..CGC-.... ...GA.TCT. CAAA.AA... .G....A... ...GCA.... .--------- --A.TGC.T.
AF315026    A.AG.TTC.T ....T..... .T.C.C..G. .T...T.T.. ..........A .....T.A.. ..-----... .AT......C ......C.... G.CTT.GGGC
AF315027    A.AG.TTC.T ....T..... .T.C.C..G. .T...T.-.. ..........A .....T.A.. ..-----... .AT......C ......-.... G.CTG.GCGC
AF315028    A.AG.TTC.T ....T..... .T.C.C..G. .T...T.-.. ..........A .....T.A.. ..-----... .AT......C ......-.... G.CTG.GCGC
AF315029    A...TT.... ..CC.T.... A.....-T.. ..G..-----. ..........A .....T.T.. ..------.-- ----...... ...........  G.....T
AF315030    A...TT.... ..CC.T.... A.....-T.. ..G..-----. .............. .....T.T.. ..-----.-- ----...... ...........  G.....T
AF315031    A...TT.... ..CC.T.... A.....TAT. ..........A .....T.T.. ..------.-- ----...... ...........  G.....T
AF315008    A...TT.... ..CC.T.... A.....-T.. ..G..-----. .............. .....T.T.. ..-.-.-.-- ----...... ...........  G.....T
AF315032    A...TT.... ..CCTT..G. A.....TAT. ..........  .....T.T.. ..-.-.-.-- ----...... ...........  G.....T
AF315033    A...TT.... T.CCTT..G. A.....TTT. ......-----  ......T.T.T. ..-- ----...... ...........  G.....T
            ////////// ////////// /////////// ////////// ////////// ////////// ////////// ////////// ////////// //////////

                410        420        430        440        450        460        470        480        490        500
consensus   AAGAAWGAAA GAGAAATAAC AACACTATAC AGTGGATCAC TTGGCTCGGG GGTCGATGAA GAACGCAGCY AACTGCGCGT CGGAATGTGA ACTGCAGGAC
AF315017    ..TC.T..G. ..A...A.-. T......... .......... .......... .......... .......T.. .......... .......... ..........
AF315018    ..TC.T..G. ..A...A.-. T......... .......... .......... .......... .......T.. .......... .......... ..........
AF315019    ..TC.T..G. ..A...A.-. T......... .......... .......... .......... .......T.. .......... .......... ..........
AF315020    ..TC.T..G. ..A...A.-. T......... .......... .......... .......... .......T.. .......... .......... ..........
AF315021    .TC..T.T.- ---------T .G........ .......... .......... .......... .......T.. .......... .......... ..........
AF315022    .TC..T.T.- ---------T .......... .......... .......... .......... .......T.. .......... .......... ..........
AF315023    .TC..T.T.- ---------T .......... .......... .......... .......... .......T.. .......... .......... ..........
AF315024    .TC..T.T.- ---------T .......... .......... .......... .......... .......T.. .......... .......... ..........
AF315025    .TC..T.T.- ---------T .......... .......... .......... .......... .......T.. .......... .......... ..........
AF315026    ..A.GAAT.. .........G. .......... .......... .......... .......... .......C.. .......... .......... ..........
AF315027    ..A.GAAT.. .........G. .......... ......C... .......... .......... .......C.. .......... .......... ..........
AF315028    ..A.GAAT.T .........G. .......... ......C... .......... .......... .......C.. .......... .......... ..........
AF315029    T....A.... .........-. .......... .......... .......... .......... .......C.. .......... .......... ..........
AF315030    T....A.... .........-. .......... .......... .......... .......... .......C.. .......... .......... ..........
AF315031    T....A.... .........-. .......... .......... .......... .......... .......C.. .......... .......... ..........
AF315008    T....A.... .........-. .......... .......... .......... .......... .......C.. .......... .......... ..........
AF315032    T....A.... .........-. .......... .......... .......... .......... .......C.. .......... .......... ..........
AF315033    T....A.... .........-. .......... .......... .......... .......... .......C.. .......... .......... ..........
            ////////// ////////// /////==== ========== ========== ========== ========== ========== ========== ==========
```
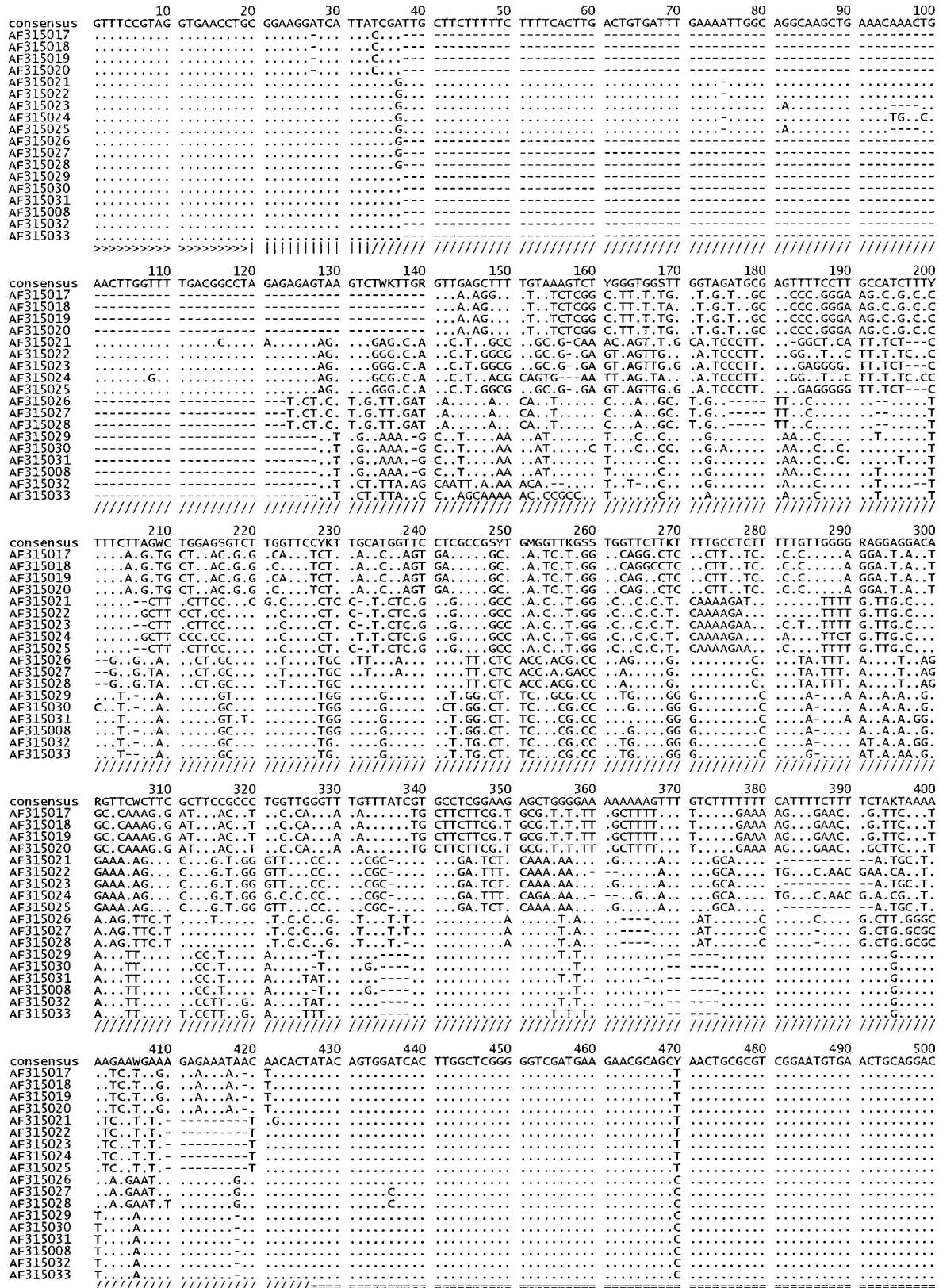
FIG. 2.—CLUSTAL W DNA multiple alignment of part of the nuclear rDNA comprising the entire internal transcribed spacers (ITS1 and ITS2) and the intervening 5.8S ribosomal RNA gene. Gene regions are indicated at the bottom of the alignment. Labels refer to GenBank accession numbers presented in table 1. Dots represent identity with the majority-rule consensus sequence on top, and dashes represent inferred indels.

```
                510        520        530        540        550        560        570        580        590        600
consensus  ACATGAACAT CGACACTTTG AACGCACATT GCGGACTGTG GCTTGTCCAC AGTCCATGTC TGGCTCAGGG TCGGTTGAAA AGCATTCATC CAATGTGCAT
AF315017   .......... .....T.... .......... ......C... .......... T......... .......... A...AA.... .......... A.T....G..
AF315018   .......... .....T.... .......... ......C... .......... T......... .......... A...A..... .......... A.T....G..
AF315019   .......... .....T.... .......... ......C... .......... T......... .......... A...A..... .......... A.T....G..
AF315020   .......... .....T.... .......... ......C... .......... T......... .......... A...AA.... .......... A.T....G..
AF315021   .......... .......... .......... .......... .......... .......... .......... -  --........ ....T..A.G
AF315022   .......... .......... .......... .......... .......... .......... .......... -  --........ ....T..A.G
AF315023   .......... .......... .......... .......... .......... .......... .......... -  --........ ....T..A.G
AF315024   .......... .......... .......... .......... .......... .......... .......... -  --........ ....T..A.G
AF315025   .......... .......... .......... .......... .......... .......... .......... -  --........ ....T..A.G
AF315026   .......... .......... .......... .......... .......... .......... .......... -  --........ ...-.GA.T.
AF315027   .......... .......... .......... .......... .......... .......... .......... -  --........ ...-.GA.T.
AF315028   .......... .......... .......... .......... .......... .......... .......... -  --........ ...-.GA.T.
AF315029   .......... .......... .......... .......... .......... .......... .......... -  --........ ...A..T...
AF315030   .......... .......... .......... .......... .......... .......... .......... -  --........ ...A..T...
AF315031   .......... .......... .......... .......... .......... .......... .......... -  --........ ...A..T...
AF315008   .......... .......... .......... .......... .......... .......... .......... -  --........ ...A..T...
AF315032   .......... .......... .......... .......... .......... .......... .......... -  --........ ...A..T...
AF315033   .......... .......... .......... .......... .......... .......... .......... -  --....... ...A..T...
           ========== ========== ========== ========== ========== ========== ========== ========== ========== ==\\\\\\\\

                610        620        630        640        650        660        670        680        690        700
consensus  TTGTTGAGCT TTGGCATTGG SMTTKSKSYT TTCTGAGAGR GAAAGWCATT CTCTGTTTCT TTTGCAGTGG CTTTAAGRTG CAWAAATGGT TMGGGAGCAC
AF315017   CGT..TTCGA ..T...A.T. CAC.GGGCT. .-.......A ..G.TT.TC. ..TCTC.... ..CT...... ......AA.. ..TGT.-.A. GA........
AF315018   CGT..TTCGA ..T...A.T. CAC.GGGCT. .-.......A ..G.TT.... .AA.TC.... ..CT...... ......AA.. ..TGT.-.A. GA........
AF315019   CGT..TTCGA ..T...A.T. CAC.GGGCT. .-.......A ..G..AG..A ..T.AC.... ..CT...... ......AA.. ..TGT.-.A. GA......G.
AF315020   CGT..TTCGA ..T...A.T. CAC.GGGCT. .-.......A ..G..A.... .AT.TC.... ..CT...... ......AA.. ..TGC.-.A. GA........
AF315021   AAA------- ------.... CA..GGGCT. .....C...A ....A----- -......... .......... ...C...A.T TTT.TT.C.. AA.A..T..A
AF315022   AAA------- ------.... CA..GGGCT. .....C...A ....A----- -......... .......... ...C...A.T TTT.TT.C.. AA.A..T..A
AF315023   AAA------- ------.... CA..GGGCT. .....C...A ....A----- -......... .......... ...C...A.T TTT.TT.C.. AA.A..T..A
AF315024   AAA------- ------.... CA..GGGCT. .....C...A ....A----- -......... .......... ...C...A.T TTT.TT.C.. AA.A..T..A
AF315025   AAA------- ------.... CA..GGGCT. .....C...A ....A----- -......... .......... ...C...A.T TTT.TT.C.. AA.A..T..A
AF315026   ...----.. ...... GC..TCTGC. ....C.---G T...----- ---------- ----...... ...C..G.T T.AT.--.A. .C-----AG.
AF315027   ...----.. ...... GC..TCTGC. ....C.---G T...----- ---------- ----...... ...C..G.T T.AT.--.A. .C-----AG.
AF315028   ...----.. ...... GC..TCTGC. ....C.---G T...----- ---------- ----...... ...C..G.T TGAT.--.A. .C-----AG.
AF315029   .......... .......... GC.GTCTGC. ...GTTA.AG -...----- ---------- ----...... ......G... .GA..-... .CT.CT.A.G
AF315030   .......... .......... GC.GTCTGC. ...GTTA.AG -...----- ---------- ----...... ......G... .GA..-... .CT.A..G--
AF315031   .......... .......... GC.GTCTGC. ...GTTT.AG -...----- ---------- ----...... ......G... ..A..-... .CA.CTTACG
AF315008   .......... .......... GC.GTCTGC. ...GTTA.AG -...----- ---------- ----...... ......G... .GA..-... .CT.A..G--
AF315032   .......... .......... GC.GTCTGC. ...GTTA.AG -...----- ---------- ----...... ......G... .GA..-... .CT.A..G--
AF315033   .......... .......... GC.GTCTGC. ...GTTA.AG -...----- ---------- ----...... ......G.. .GA..-... .CT.A..G--
           \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\

                710        720        730        740        750        760        770        780        790        800
consensus  TCTCTRAATC TCTGTTGACK CTTRSWSKCG KGATTAAAAG CTCYCTGGGT CTTCMKCGAA TGAGGTGGCC TCTAACGTTT TGAGSWGAGW ATGGTTCTCT
AF315017   .G.T.A.--- -GA.C.C..G GCAACTCT.. G.C.A.GTTA G..CTGA.T. GCCACTGA.C .TG.TGTTT- ---------- ----CT..AT C.CC.AAGAG
AF315018   .G.T.A.--- -GA.C.C..G GCAACTCT.. G.C.A.GTTA G..CTGA.T. GCCACTGA.C .TG.TGTTT- ---------- ----CT..AT C.CC.AAGAG
AF315019   .G.T.A.--- -GA.C.C..G GCAACTCT.. G.C.A.GTTA G..CTGA.T. GCCACTGA.C .TG.TGTTT- ---------- ----CT..AT C.CC.AAGAG
AF315020   .G.T.A.--- -GA.C.C..G GCAACTCT.. G.C.A.GTTA G..CTGA.T. GCCACTGA.C .TG.TGTTT- ---------- ----CT..AT C.CC.AAGAG
AF315021   .TCACAGT.G G..T.CTTAG AAGACTCTT. G....T...C .ATC...... ...TCTT.T- -..A...A.- G.---T.GA. ...ACT..TT GGAAG.T.G.
AF315022   .TCACAGT.G G..C.CTTAG AAGACTCTT. G....T...C .ATC...... ...TCTT.T- -.-...A. G.---T.GA. ...ACT..TT GGAAG.T.G.
AF315023   .TCACAGT.G G..C.CTTAG AAGACTCTT. G....T...C .ATC...... ...TCTT.T- -.-...A. G.---T.GA. ...ACT..TT GAAAG.T.G.
AF315024   .TCACAGT.G G..C.CTTAG AAGACTCTT. G....T...C .ATC...... ...TCTT.T- -.-...A. G.---T.GA. ...ACT..TT GAAAG.T.G.
AF315025   .TCACAGT.G G..C.CTTAG AAGACTCTT. G....T...C .ATC...... ...TCTT.T- -.-...A. G.---T.GA. ...ACT..TT GAAAG.T.G.
AF315026   A....G.... .........T ...GGAGGAA T--GA...TT .C.T...... ....AG...- -.-.----- ..A.GAT.C. GT-.GA...A .........T-
AF315027   A....G.... .........T ...GGAGGAA T--GA...TT .C.T...... ....AG...- -.-.----- ..A.GA..C. GT-.GA...A .........T-
AF315028   A....G.... .........T ...GGAGGAA T--GA...TT .C.T...... ....AG...- -.-.----- ..A.GA..C. GT-.GA...A .........T.
AF315029   G....G.... .........T ...GGAGG.T T-...GCG.. T..T...... ....AG.... -.-....-- ......C..- ---.GA...A .....C.C.-
AF315030   -....G.... .........T ...GGAGG.T T-...GCG.. T..T...... ....AG.... -.-....-- ......C..- ---.GA...A .....C.C.-
AF315031   G....G.... .........T ...GGAGG.T T-...GCG.. T..T...... ....AG.... -.-....-- ......C..- ---.GA...A .....C.C.-
AF315008   -....G.... .........T ...GGAGG.T T-...GCG.. T..T...... ....AG.... -.-....-- ......C..- ---.GA...A .....C.C.-
AF315032   -....G.... .........T ...GGAGG.T T-...GCG.. ...T...... ....AG.... -.-....-- ......C..- ---.GA...A .....C...-
AF315033   -....G.... .........T ...GGAGG.T TT-..GCG.. ...T...... ....AG.... -.-....-- ......C..- ---.GA...A .....C...-
           \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\

                810        820        830        840        850        859
consensus  TTGWYTTTGC CCTCTCTCCT CWCATTATTG ATCTTTCTTT CGACCTCAGA TCAGACCAG
AF315017   ...TTGC.-T .A.G.TG.AG .TT...GC.- --.....G.CA .......... .........
AF315018   ...TTGC.-T .A.G.TG.AG .TT...GC.- --.....G.CA .......... .........
AF315019   ...TTGC.-T .A.G.TG.AG .TT...GC.- --.....G.CA .......... .........
AF315020   ...TTGC.-T .A.G.TG.AG .TT...GC.- --.....G.CA .......... .........
AF315021   ..ATT...TG -..T..AATC TTG...---- --TCA.TGG. .......... .........
AF315022   ..ATT...TG -..T..AATC TTG...---- --TCA.TGG. .......... .........
AF315023   ..ATT...TT T..T..AATC TTG...A... ..TCA.TGG. .......... .........
AF315024   ..ATT...TG -..T..AATC TTG...---- --TCA.TGG. .......... .........
AF315025   ..ATT...TT -..T..AATC TTG...---- --TCA.TGG.C .......... .........
AF315026   -AAAC..... .......... .AA.AACG-- --T....G.C .......... .........
AF315027   -AAAC..... .......... .AA.AACG-- --T....G.C .......... .........
AF315028   .AAAC..... .......... .AA.AACG-- --T....G.C .......... .........
AF315029   G..AC..... .......... .A.T...... --.A.A.... .......... .........
AF315030   G..AC..... .......... .A.T.A...- --.A.A.... .......... .........
AF315031   G..AC..... .......... .A.T.A...- --.A.A.... .......... .........
AF315008   G..AC..... .......... .A.T.A...- --.A.A.... .......... .........
AF315032   G..AC..... .......... .A.T...... --.A.A.... .......... .........
AF315033   G..AC..... .......... .A.T...... --.A.A.... .......... .........
           \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ \\\\\\\\\\ <<<<<<<<<< <<<<<<<<<
```

Legend:

>>>> <<<<	Forward and reverse primer
|||||||||||||||||||	18S SSU rRNA gene
/////////////////	ITS1
========	5.8S rRNA gene
\\\\\\\\\\\\\\\\\\	ITS2

FIG. 2 *(Continued)*

Table 4
Parameters of the Models of Nucleotide Substitution Fit to Mitochondrial and Nuclear Genes of *Cletocamptus deitersi*

Gene	Model[a]	Base Frequencies (ACG)	Rate Matrix[b]	I[c]	G[d]
COX-I.	K 81	0.2689, 0.1688, 0.2051	r(A→G) = 9.778 r(A→T) = 2.014	0.435	1.141
16S rDNA	TIM	0.4024, 0.1518, 0.1443	r(A→G) = 8.327 r(A→T) = 3.047 r(C→T) = 11.053	0.222	4.235
5.8S rDNA.	JC	0.25, 0.25, 0.25	r(N→notN) = 1.0	NA[e]	NA

[a] K 81 (Kimura 1981), TIM (Rodriguez et al. 1990), and JC (Jukes and Cantor 1969).
[b] Relative rates to r(A→C) fixed at 1.0; only distinct elements of the matrix are shown.
[c] Proportion of invariable sites.
[d] Shape parameter of the Gamma distribution assumed to describe rate heterogeneity among sites.
[e] Not applicable.

tiation is due to sequences derived from nonfunctional paralogous copies (e.g., pseudogenes), as reported for other crustaceans (Schubart, Neigel, and Felder 2000*b*), is negligible. This assertion is supported by patterns of nucleotide substitutions, in relation to reading frames and rates of synonymous and nonsynonymous substitutions per synonymous and nonsynonymous sites (d_S/d_N) (COX-I), coding versus noncoding regions (5.8S rDNA), and secondary structures (16S, 5.8S rDNA) observed in the gene regions examined (details not shown). Also, our sequence data sets are large (table 1), so the apparent absence of intermediate nodes in our phylogenetic reconstructions is not an artifact of inadequate sampling within our four collecting sites (e.g., Funk 1999). Instead, the three major types (I, II, and III) can be characterized as separate species using the phylogenetic-species and the genealogical-concordance criteria. In addition, our data and preliminary morphological observations (S. Gómez, personal communication) suggest that type III may be composed of two species. Admittedly, we analyzed samples from only four localities in one subcontinent of the almost cosmopolitan *C. deitersi.* Because our phylogenetic trees are based on a limited geographic sampling, it is possible that analysis of additional *C. deitersi* from other locations would uncover nodes that are intermediate in depth between those sep-
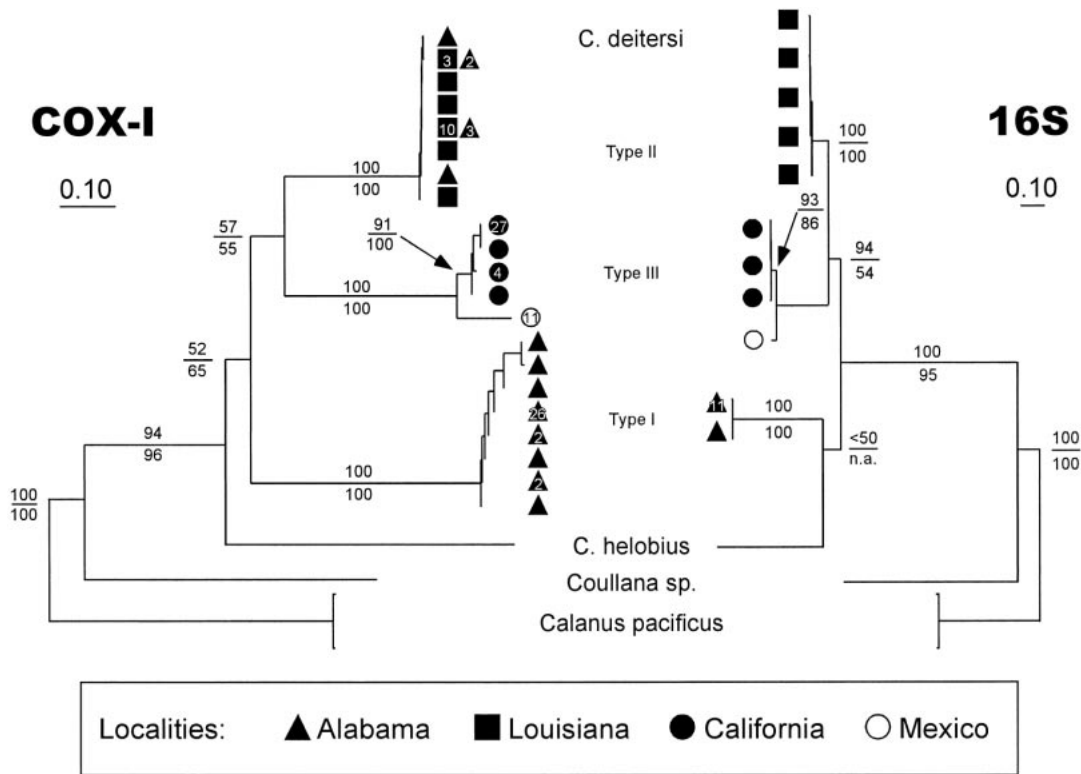
Fig. 3.—Neighbor-joining (NJ) phylogenetic reconstructions based on genetic distances of distinct mitochondrial cytochrome oxidase subunit I (COX-I, K81uf+I+G distances) and 16S ribosomal RNA (16S, TIM+I+G distances) haplotypes of the harpacticoid copepods *Cletocamptus deitersi, Cletocamptus helobius,* and *Coullana* sp. and the calanoid copepod *Calanus pacificus.* Branch lengths are proportional to genetic distance according to scales shown. Numbers on branches are nonparametric bootstrap support values (1,000 replicates) obtained with NJ (above) and with maximum parsimony (below). Localities where haplotypes were found are indicated by symbols; numbers in symbols are the numbers of copepods sharing a particular haplotype. See text for explanation of genetic distance measures. n.a. = not applicable.
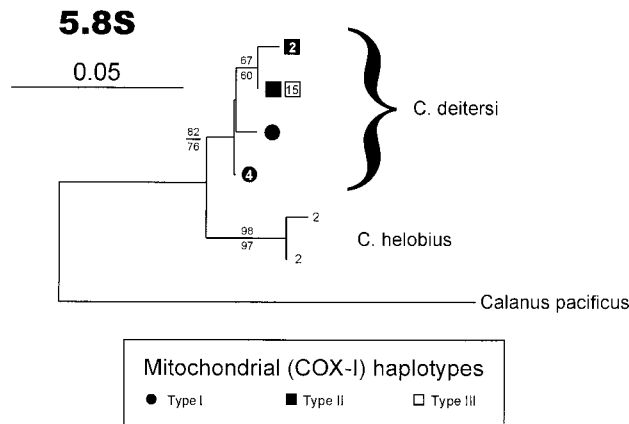
FIG. 4.—Neighbor-joining (NJ) phylogenetic reconstruction based on Jukes-Cantor genetic distances of distinct nuclear 5.8S ribosomal RNA alleles of the harpacticoid copepods *Cletocamptus deitersi* and *Cletocamptus helobius* and the calanoid copepod *Calanus pacificus.* Branch lengths represent genetic distances according to the scale shown. Numbers on branches represent nonparametric bootstrap support values (1,000 replicates) obtained with NJ (above) and with maximum parsimony (below). The corresponding mitochondrial haplotype found in the same copepods is indicated by the symbols. Numbers in symbols represent the individuals sharing the same 5.8S rRNA gene copy.

arating the major lineages and those separating individual sequences within lineages. Although such a potential result would complicate the use of sequence data for systematic purposes within Cletocamptus, it would not invalidate our major conclusions concerning the existence of deep lineages and morphological stasis.

Additional support for the assertion that our collections are composed of highly divergent species comes from comparison with orthologous sequences of a bona fide congeneric species (tables 2 and 3) and from comparisons with patterns of genetic differentiation found in more than 200 invertebrate taxa (table 5). These patterns show that levels of mtDNA differentiation among the major types in *C. deitersi* are comparable to those found at several supraspecific and often suprageneric levels (table 5). The divergence between Mexican (IIIM) and Californian (IIIC) copepods is dwarfed by that found among the major mtDNA types (I, II, and III), but it is comparable to that separating morphologically differentiated and uncontested species in other congeneric arthropods (table 5). In contrast, intraspecific differentiation in divergent lineages suspected of harboring cryptic or sibling species are generally considerably smaller than those found in *C. deitersi* (table 5). Levels of mtDNA divergence comparable to those in *C. deitersi* have been found in the freshwater amphipod *Hyalella azteca* and the marine harpacticoid copepod *Tigriopus californicus.* In *H. azteca,* seven mitochondrial lineages of presumed cryptic species sampled within central glaciated North America showed extreme levels of divergence (COX-I: 20.2%–27.6% corrected; Witt and Hebert 2000). In *T. californicus,* allopatric populations at one end of the species range in southern Baja California exhibit reproductive isolation (Ganz and Burton 1995) and very large levels of divergence (COX-I: 18%–23% un-

corrected; S. Edmands, personal communication) from the rest.

We have not found in the literature a degree of ITS differentiation as extreme (i.e., unalignable sequences) as the one found among the putative cryptic species in *C. deitersi* in conspecifics and in members of closely related complexes of cryptic or sibling species. Congeneric invertebrates can sometimes show significant differentiation in the ITSs of nuclear ribosomal genes (e.g., up to 23% across taxa in Acropora; Odorico and Miller 1997). More often, however, ITS sequences show incipient levels of differentiation (<5%) among subspecific organisms or others belonging to complexes of cryptic or sibling species (e.g., black flies in the genus Simulium; Tang et al. 1996). In the ITS1 of *Microarthridion littorale,* the only harpacticoid ITS sequence reported to date, the largest divergence (only 10.8%) was found between organisms bearing extreme cytochrome *b* differentiation (ca. 30%, computed from data in Schizas et al. [1999]).

The comparisons cited above strongly suggest that genetic differentiation among the major lineages of the nominal harpacticoid *C. deitersi* would be reconcilable with intraspecific polymorphism only in the presence of (1) extraordinarily high rates of molecular evolution in both mitochondrial and nuclear genomes, (2) strong balancing selection, or (3) historical vicariant events that would maintain the polymorphisms. We argue that additional evidence contradicts these alternative interpretations.

First, there appears to be a strong correspondence between morphology (third-leg setation) and molecular lineage (nuclear and mitochondrial) in 70 nominal *C. deitersi* COX-I's examined from Louisiana and Alabama. All copepods with one inner seta on the third-leg exopod were from Louisiana and all were of type II, whereas those with two inner setae, all from Alabama, were of type I. Our molecular studies show that both type I and type II lineages are sympatric in Louisiana and Alabama, with both locations exhibiting one dominant and one rare lineage. Even though leg setation could be environmentally determined (e.g., by salinity, which is much higher at the brine stream), Fleeger (1980) found that both morphotypes (one or two inner setae on the third-leg exopod) co-occur in Louisiana salt marshes. This suggests that leg setation is not a morphologically plastic nongenetic response or an adaptive character under strong habitat-specific selection that has evolved rapidly relative to the timing of cladogenetic events. This trait thus provides more evidence of concordant patterns of molecular and morphological variation, consistent with the interpretation that types I and II constitute separate species on multiple criteria. Concordance of molecular and morphological variation would not be expected if the deep phylogenetic nodes separating the major types were an artifact of high mutation rates in the gene regions examined.

Second, the suggestion that gene regions from two genomes would all be under strong balancing selection appears unlikely. Selection can maintain ancient polymorphisms within, and even between, species (Hughes

Table 5
Comparison of Genetic Divergence in Mitochondrial Genes Among Lineages of *Cletocamptus deitersi* and Among Other Invertebrate Taxa of Different Taxonomic Levels

Taxa[a]	Distance mtDNA		Gene	Source Reference
	Range[b]	Metric[c]		
Among supraspecific or suprageneric taxa				
C. deitersi (among major lineages)	20.8–26.8	p	COX-I	This study
Penaeid shrimps: *Penaeus* (3)	8.6–15.2	p	COX-I	Palumbi and Benzi (1991)
C. deitersi (among major lineages)	24.7–34.1	K2P	COX-I	This study
Xiphosura: *Tachypleus* (2), *Carcinoscorpius* (1), and *Limulus* (1)	5.6–20.0	K2P	COX-I	Avise, Nelson, and Sugita (1994)
Mole crabs: *Emerita* (6)	12.0–24.0	K2P	COX-I	Tam, Kornfield, and Ojeda (1996)
C. deitersi (among major lineages)	20.3–36.0	p	16S	This study
NW Atlantic noncongeneric calanoids: *Calanus* (4), *Nannocalanus* (1), *Meridia* (2), *Centropages* (2), *Pseudocalanus* (2), and *Temora* (1)	20.0–30.0	p	16S	Bucklin, Caudill, and Guarnieri (1998)
Marine gastropods: *Littorina* (21), *Nodilittorina* (6), *Tectarius* (5), and *Turritella* (10)	7.5–25.0	p	16S	Etter et al. (1999)
Marine bivalves: *Crassostrea* (3) and *Quadrula* (4)	0.31–16.0	p	16S	Etter et al. (1999)
Notostracans: *Lepidurus* (4)	4.0–12.6	p	12S	King and Hanner (1998)
Calanoids: *Euchaeta* (3)	12.5–28.5	p	16S	Braga et al. (1999)
Calanoids: *Paraeuchaeta* (4)	4.2–24.9	p	16S	Braga et al. (1999)
Distant copepod genera (suprafamily divergences): Calanoida (7 genera in 6 families in 3 superfamilies)	24.0–36.3	p	16S	Braga et al. (1999)
C. deitersi (among major lineages)	23.2–49.3	K2P	16S	This study
Xiphosura: *Tachypleus* (2), *Carcinoscorpius* (1), and *Limulus* (1)	9.3–22.0	K2P	16S	Avise, Nelson, and Sugita (1994)
Mole crabs: *Emerita* (6)	3.9–17.0	K2P	16S	Tam, Kornfield, and Ojeda (1996)
Mud crabs: Panopeidae/Xanthidae (9 genera) vs. Trapezidae/Menippidae (2 genera)	16.8–24.9	K2P	16S	Schubart, Neigel, and Felder (2000a)
Anomurans: Diogenidae (2 spp. in 2 genera), Paguridae (8 spp. in 3 genera), Lithoidae (2 spp. in 2 genera)	25.0–30.9	K2P	16S	Cunningham, Blackstone, and Buss (1992)
Grapsoid crabs: 24 genera (1 sp. each) in 10 families	4.4–24.9	K2P	16S	Schubart et al. (2000)
Arthropod subphyla: krill (Euphausiidae, 5 spp. in 3 genera) vs. *Drosophila pseudoobscura*	29.0–31.0	K2P	16S	Patarnello et al. (1996)
C. deitersi (among major lineages)	34.0–120.2	HKY-Γ	16S	This study
Anaspideans: *Anaspides* (1) vs. *Allanaspides* (1)	25.8	HKY-Γ	16S	Jarman and Elliott (2000)
Among uncontested closely related taxa				
C. deitersi (between type III lineages)	8.7	p	COX-I	This study
Sea skaters: *Halobates* (6)	4.5–8.1	p	COX-I	Andersen et al. (2000)
C. deitersi (between type III lineages)	9.4	K2P	COX-I	This study
Geminate snapping shrimps: *Alpheus* (8)	7.7–8.5	K2P	COX-I	Knowlton et al. (1993)
C. deitersi (between type III lineages)	4.7	K2P	COX-I	This study
Mud crabs: Panopeidae (17 spp. in 4 genera)	0.0–9.2	K2P	16S	Schubart, Neigel, and Felder (2000a)
Krill: *Euphausia* (2)	3.5	K2P	16S	Patarnello et al. (1996)
Hermit crabs: Paguridae (4 spp. in 3 genera)	2.7–6.9	K2P	16S	Cunningham, Blackstone, and Buss (1992)
C. deitersi (between type III lineages)	5.1	HKY-Γ	16S	This study
Grapsid crabs: *Sesarma* (2)	3.2	HKY-Γ	16S	Schubart, Diesel, and Hedges (1998)
Within cryptic species complexes				
C. deitersi (among major lineages)	20.8–26.8	p	COX-I	This study
Maximum worldwide in calanoid *Eurytemora affinis* (5)	17	p	COX-I	Lee (1999)
Harpacticoid copepod: *Tigriopus californicus* (2)	18.2–21.1	p	COX-I	Burton and Lee (1994)
C. deitersi (among major lineages)	24.7–34.1	K2P	COX-I	This study
Sea-star: *Leptasterias* sp. (2)	2.3–7.8	K2P	COX-I	Foltz (1997)
Pseudoscorpion: *Cordylochernes scorpioides* (5)	2.6–13.8	K2P	COX-I	Wilcox et al. (1997)

Table 5
Continued

Taxa[a]	Distance mtDNA			Source Reference
	Range[b]	Metric[c]	Gene	
Mole crab: *Emerita talpoida* (3)	5.0–5.7	K2P	COX-I	Tam, Kornfield, and Ojeda (1996)
Mole crab: *Emerita analoga* (2)	2.6–4.5	K2P	COX-I	Tam, Kornfield, and Ojeda (1996)
Sympatric snapping shrimp: *Alpheus* sp. (5)	6.3–16.4	K2P	COX-I	Knowlton et al. (1993)
Freshwater amphipod: *Hyalella azteca* (7)	20.2–27.6	K2P	COX-I	Witt and Hebert (2000)
C. deitersi (among major lineages)	20.3–36.0	p	16S	This study
Oligochaete: *Tubifex tubifex* (4)	5.9–13.0	p	16S	Sturmbauer et al. (1999)
NW Atlantic calanoid copepods: *Pseudocalanus* sp. (2) and *Nannocalanus minor* (2)	5.0–10.0	p	16S	Bucklin, Caudill, and Guarnieri (1998)
C. deitersi (among major lineages)	23.2–49.3	K2P	16S	This study
Brittle star: *Ophiotrix* sp. (2)	9.0–12.0	K2P	16S	Baric and Sturmbauer (1999)

[a] Numbers in parentheses are numbers of species or taxa compared.

[b] A single number indicates a unique comparison.

[c] p = percentage of sequence divergence; K2P = Kimura two-parameter model (Kimura 1980), HKY-Γ = Hasegawa, Kishino, and Yano (1985) model with gamma correction for rate heterogeneity among sites (Yang 1993). Several distance metrics are reported for *C. deitersi* for ease of comparison with earlier studies.

and Yeager 1998). The only mechanism for maintenance of ancient alleles in mtDNA that has been reported in invertebrates is male- and female-specific mtDNA genomes associated with sex determination in some bivalves (e.g., Quesada, Wenne, and Skibinski 1999). However, this is unlikely here, since the skewed relative frequencies of type I and type II sequences between Alabama and Louisiana (i.e., type I is rare in Louisiana, but type II is rare in Alabama) suggests no connection with sex determination.

Third, it is possible that *C. deitersi* shows genetic signatures of historical vicariant events in the form of high levels of genetic differentiation. Such patterns have been documented for a number of coastal marine invertebrates in different regions of the world, including the harpacticoid copepod *T. californicus* (Burton and Lee 1994; but see Burton 1998). In these continuously distributed species, a deep genetic break coincides with a documented faunal break, reflecting the influence of historical vicariant events (phylogeographic category I of Avise [2000]). Limited geographic sampling prevents determination of whether any of our deep phylogenetic nodes correspond to known phylogeographic break points in other species. However, this scenario does not involve mechanisms preventing conspecific organisms from hybridizing in sympatry. Although present samples are limited, we did not detect heterozygosity for nuclear rDNA sequences characteristic of types I and II in either the Alabama or the Louisiana samples. This inferred lack of hybridization provides evidence against a vicariant explanation of deep phylogenetic nodes and corroborates the genealogical-concordance results. Additional nuclear genes are needed to determine the extent of reproductive isolation of sympatric types I and II, which would satisfy the biological-species criterion.

Temporal Perspective

Extensive genetic differentiation among the genetic lineages of *C. deitersi* suggests long times since initial divergence. Assuming that the rate of molecular evolution in the COX-I gene calibrated in other crustaceans can be applied to *C. deitersi,* copepods from the Salton Sea and Mazatlán appear to have shared a common ancestor between the late Miocene and the early Pliocene (table 6). The molecular ancestor of lineages II and III dates back to the early and middle Miocene, and the oldest divergence between lineages I and II and lineage III dates back to a time between the late Eocene and the early Miocene (table 6). These dates are in remarkable agreement with the fossil record. Fossilized specimens assignable to Cletocamptus have been found in sedimentary deposits dating to the middle and late Miocene (Palmer 1960). The fossils are similar to an extant species, *Cletocamptus albuquerquensis* (Herrick) but cannot be identified due to lack of preservation of key characters. However, close resemblance between the fossil and recent Cletocamptus suggests a morphological conservatism that has lasted for more than 10 Myr, a timescale comparable to the one suggested by the molecular data of the seemingly old cryptic species of *C. deitersi.*

Table 6
Ranges of Divergence Since Common Ancestor Based on Corrected Sequence Divergence and Crustacean Molecular-Clock Calibrations

	16S		COX-I	
	Mean Corrected Distance		Mean Corrected Distance	
Common Ancestor of	(%)	Divergence Range (Myr)	(%)	Divergence Range (Myr)
IIIM and IIIC..........	4.66K	5.1K–12.3K	9.35K–11.1H	3.9K–7.9H
II and III.............	24.52K	27.2K–64.5K	26.20K–40.67H	10.9H–29.1H
I and II, III............	46.83K	52.0K–123.2K	30.56K–51.17H	12.7K–36.6H

Note.—Rates of molecular evolution used for the 16S rRNA gene include 0.38% Kimura two-parameter/Myr for anomurans (Cunningham, Blackstone, and Buss 1992) and 0.90% Kimura two-parameter/Myr for fiddler crabs (Sturmbauer, Levinton, and Christy 1996). A second calibration of 0.65%–0.88% Kimura two-parameter/Myr (Schubart, Diesel, and Hedges 1998) obtained from grapsid crabs gave intermediate values. COX-I calibrations of geminate species of alpheid shrimps include 1.4% maximum-likelihood-Γ/Myr (Knowlton and Weigt 1998) and 2.4% Kimura two-parameter/Myr (Knowlton et al. 1993). A third calibration of 1.66% Kimura two-parameter/Myr (Schubart, Diesel, and Hedges 1998) obtained from grapsid crabs also gave intermediate values. Letters following values refer to the correction using the HKY85-Γ (H) or the Kimura two-parameter model (K). These models were used in order to match those used in the calibrations; best-fit models would produce older estimates.

The mitochondrial 16S rDNA distances and rates yield larger estimates of divergence than the COX-I gene data (table 6). The discrepancy may be accounted for by a larger correction imposed by the Hasegawa, Kishino, and Yano (1985) model with gamma correction for rate heterogeneity among sites (Yang 1993) on the 16S rRNA gene, particularly with respect to the rate heterogeneity among sites, and, more importantly, by the relative rates of evolution of both genes in *C. deitersi*, where it appears that the 16S rRNA gene evolves faster than the COX-I gene (table 2). This difference is likely the result of the inclusion of hypervariable regions in the molecule (i.e., loops) that are much less variable in the taxa and timescales involved in rate calibrations. Rates previously reported (cf. table 6) reflect that the COX-I gene evolves twice to almost six times as fast as the 16S gene. Even if the present COX-I gene sequences showed uncorrected saturation and the "true" rate of evolution was closer to the one shown by the 16S rRNA gene, this would only increase the estimates of time since divergence, making the morphological conservatism of *C. deitersi* even more remarkable.

Morphological Stasis

Two types of analyses have generally been conducted to address the question of whether evolutionary processes such as drift, mutation, and selection result in a correlation between rates of morphological and molecular evolution over diverse time intervals. One approach is to collect DNA sequence or other molecular data on morphologically conservative taxa and compare the amounts of molecular divergence at particular taxonomic levels to amounts of divergence characterizing the same taxonomic levels in less conservative taxa (e.g., Wilson, Carlson, and White 1977; Avise, Nelson, and Sugita 1994; King and Hanner 1998; Jarman and Elliott 2000). These studies have provided clear evidence of a decoupling between the two rates of evolution in selected taxa. When molecular divergence times can be dated with geological evidence, the results are consistent with morphological stasis. The generality of these studies has been questioned, both on the grounds that taxa known a priori to be morphologically conser-

vative were chosen for analysis (Omland 1997) and on the grounds that nonmorphological characters such as physiological, ecological, or behavioral traits might show changes that are correlated with molecular differences (Schubart, Neigel, and Felder 2000b). A second approach is to compare rates of morphological and molecular evolution in taxa chosen without bias regarding expectations about these rates. For example, Omland (1997) found significant positive correlations between inferred morphological and molecular branch lengths in a phylogenetic study of eight taxa. Our study is somewhat different from both of these approaches in that we had no prior expectation of ancient divergence times in the presence of morphological conservatism within the nominal *C. deitersi*. This result may, in part, reflect developing taxonomic resolution within Cletocamptus, and retrospective analysis of our four major types (I, II, IIIC, and IIIM) has revealed subtle morphological differences among them (S. Gómez, personal communication). However, even the most genetically divergent lineages in our data set share identical body plans, and they cannot be differentiated by casual observation at magnifications of less than 100× (J.W.F., unpublished data). We conclude that the morphological stasis of these types over a time interval of at least 10 Myr is real. Given the challenge of studying evolutionary and ecological processes in these small benthic copepods, one way to increase our understanding of the mechanisms and patterns of morphological stasis from a comparative perspective would be to examine additional meiobenthic invertebrate taxa for the occurrence of cryptic species and deep evolutionary lineages.

Supplementary Material

The nucleotide alignment of the COX-I sequences submitted to GenBank is available at http://www.molbiolevol.org.

Acknowledgments

LITERATURE CITED

ANDERSEN, N. M., L. CHENG, J. DAMGAARD, and F. A. H. SPERLING. 2000. Mitochondrial DNA sequence variation and phylogeography of oceanic insects (Hemiptera: Gerridae: *Halobates* spp.). Mar. Biol. **136**:421–430.

AVISE, J. C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge, Mass.

AVISE, J. C., W. S. NELSON, and H. SUGITA. 1994. A speciational history of ''living fossils'': molecular evolutionary patterns in horseshoe crabs. Evolution **48**:1986–2001.

BARIC, S., and C. STURMBAUER. 1999. Ecological parallelism and cryptic species in the genus *Ophiothrix* derived from mitochondrial DNA sequences. Mol. Phylogenet. Evol. **11**: 157–162.

BRAGA, E., R. ZARDOYA, A. MEYER, and J. YEN. 1999. Mitochondrial and nuclear rRNA based copepod phylogeny with emphasis on the Euchaetidae (Calanoida). Mar. Biol. **133**:79–90.

BUCKLIN, A., C. C. CAUDILL, and M. GUARNIERI. 1998. Population genetics and phylogeny of planktonic copepods. Pp. 303–318 *in* K. E. COOKSEY, ed. Molecular approaches to the study of the ocean. Chapman and Hall, London.

BURTON, R. S. 1998. Intraspecific phylogeography across the Point Conception biogeographic boundary. Evolution **52**: 734–745.

BURTON, R. S., and B. N. LEE. 1994. Nuclear and mitochondrial gene genealogies and allozyme polymorphism across a major phylogeographic break in the copepod *Tigriopus californicus.* Proc. Natl. Acad. Sci. USA **91**:5197–5201.

CUNNINGHAM, C. W., N. W. BLACKSTONE, and L. W. BUSS. 1992. Evolution of king crabs from hermit crab ancestors. Nature (Lond.) **355**:539–542.

DE QUEIROZ, K. 1998. The general lineage concepts of species, species criteria, and the process of speciation. Pp. 57–75 *in* D. J. HOWARD and S. H. BERLOCHER, eds. Endless forms: species and speciation. Oxford University Press, New York.

ETTER, R. J., M. A. REX, M. C. CHASE, and J. M. QUATTRO. 1999. A genetic dimension to deep-sea biodiversity. Deep Sea Res. I Oceanogr. Res. Pap. **46**:1095–1099.

FLEEGER, J. W. 1980. Morphological variation in *Cletocamptus* (Copepoda: Harpacticoida), with description of a new species from Louisiana salt marshes. Trans. Am. Microsc. Soc. **99**:25–31.

FOLMER, O., M. BLACK, W. HOEH, R. LUTZ, and R. VRIJENHOEK. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. **3**:294–299.

FOLTZ, D. W. 1997. Hybridization frequency is negatively correlated with divergence time of mitochondrial DNA haplotypes in a sea star (*Leptasterias* spp.) species complex. Evolution **51**:283–288.

FUNK, D. J. 1999. Molecular systematics of cytochrome oxidase I and 16S from *Neochlamisus* leaf beetles and the importance of sampling. Mol. Biol. Evol. **16**:67–82.

GANZ, H. H., and R. S. BURTON. 1995. Genetic differentiation and reproductive incompatibility among Baja California populations of the copepod *Tigriopus californicus.* Mar. Biol. **123**:821–827.

GELLER, J. B., E. D. WALTON, E. D. GROSHOLZ, and G. M. RUIZ. 1997. Cryptic invasions of the crab *Carcinus* detected by molecular phylogeography. Mol. Ecol. **6**:901–906.

HASEGAWA, M., H. KISHINO, and T. YANO. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **21**:160–174.

HEATH, D. D., P. D. RAWSON, and T. J. HILBISH. 1995. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Can. J. Fish. Aquat. Sci. **52**:2621–2627.

HUGHES, A. L., and M. YEAGER. 1998. Natural selection at major histocompatibility complex loci of vertebrates. Annu. Rev. Genet. **32**:415–435.

HUYS, R., J. M. GEE, C. G. MOORE, and R. HAMOND. 1996. Marine and brackish water harpacticoid copepods. Part 1. Linnean Society of London, Dorchester, England.

JARMAN, S. N., and N. G. ELLIOTT. 2000. DNA evidence for morphological and cryptic Cenozoic speciations in the Anaspididae, 'living fossils' from the Triassic. J. Evol. Biol. **13**:624–633.

JUKES, T. H., and C. R. CANTOR. 1969. Evolution of protein molecules. Pp. 21–132 *in* H. N. MUNRO, ed. Mammalian protein metabolism. Academic Press, New York.

KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**:111–120.

———. 1981. Estimation of evolutionary distances between homologous nucleotide sequences. Proc. Natl. Acad. Sci. USA **78**:454–458.

KING, J. L., and R. HANNER. 1998. Cryptic species in a ''living fossil'' lineage: taxonomic and phylogenetic relationships within the genus *Lepidurus* (Crustacea: Notostraca) in North America. Mol. Phylogenet. Evol. **10**:23–36.

KNOWLTON, N. 1993. Sibling species in the sea. Annu. Rev. Ecol. Syst. **24**:189–216.

KNOWLTON, N., and L. A. WEIGT. 1998. New dates and new rates for divergence across the Isthmus of Panama. Proc. R. Soc. Lond. B Biol. Sci. **265**:2257–2263.

KNOWLTON, N., L. A. WEIGT, L. A. SOLORZANO, D. K. MILLS, and E. BERMINGHAM. 1993. Divergence in proteins, mitochondrial DNA, and reproductive compatibility across the Isthmus of Panama. Science **260**:1629–1632.

KNOWLTON, N., E. WEIL, L. A. WEIGT, and H. M. GUZMAN. 1992. Sibling species in *Montastraea annularis,* coral bleaching, and the coral climate record. Science **255**:330–333.

LEE, C. E. 1999. Rapid and repeated invasions of fresh water by the copepod *Eurytemora affinis.* Evolution **53**:1423–1434.

MIELKE, W. 2000. Two new species of *Cletocamptus* (Copepoda: Harpacticoida) from Galapagos, closely related to the cosmopolitan *C. deitersi.* J. Crustac. Biol. **20**:273–284.

ODORICO, D. M., and D. J. MILLER. 1997. Variation in the ribosomal internal transcribed spacers and 5.8S rDNA among five species of *Acropora* (Cnidaria; Scleractinia): patterns of variation consistent with reticulate evolution. Mol. Biol. Evol. **14**:465–473.

OMLAND, K. E. 1997. Correlated rates of molecular and morphological evolution. Evolution **51**:1381–1393.

PALMER, A. R. 1960. Miocene copepods from the Mojave Desert, California. J. Paleontol. **34**:447–452.

PALUMBI, S. R. 1996. Nucleic acids II: the polymerase chain reaction. Pp. 205–247 *in* D. M. HILLIS, C. MORITZ, and B. K. MABLE, eds. Molecular systematics. Sinauer, Sunderland, Mass.

PALUMBI, S. R., and J. BENZIE. 1991. Large mitochondrial DNA differences between morphologically similar penaeid shrimp. Mol. Mar. Biol. Biotechnol. **1**:27–34.

PATARNELLO, T., L. BARGELLONI, V. VAROTTO, and B. BATTAGLIA. 1996. Krill evolution and the Antarctic Ocean currents: evidence of vicariant speciation as inferred by molecular data. Mar. Biol. **126**:603–608.

POSADA, D., and K. A. CRANDALL. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14**:817–818.

QUESADA, H., R. WENNE, and D. O. SKIBINSKI. 1999. Interspecies transfer of female mitochondrial DNA is coupled with role-reversals and departure from neutrality in the mussel *Mytilus trossulus*. Mol. Biol. Evol. **16**:655–665.

RAMBAUT, A., and N. C. GRASSLY. 1997. Seq-Gen: an application for the Monte Carlo simulation of DNA sequence evolution along phylogenetic trees. Comput. Appl. Biosci. **13**:235–238.

REID, J. W. 1998. How "cosmopolitan" are the continental cyclopoid copepods? Comparison of the North American and Eurasian faunas, with description of *Acanthocyclops parasensitivus* sp.n. (Copepoda: Cyclopoida) from the U.S.A. Zool. Anz. **236**:109–118.

ROCHA-OLIVARES, A. 1998. Multiplex haplotype-specific PCR: a new approach for species identification of the early life stages of rockfishes of the species-rich genus *Sebastes* Cuvier. J. Exp. Mar. Biol. Ecol. **231**:279–290.

RODRIGUEZ, F., J. F. OLIVER, A. MARIN, and J. R. MEDINA. 1990. The general stochastic model of nucleotide substitution. J. Theor. Biol. **142**:485–501.

SCHIZAS, N. V., G. T. STREET, B. C. COULL, G. T. CHANDLER, and J. M. QUATTRO. 1997. An efficient DNA extraction method for small metazoans. Mol. Mar. Biol. Biotechnol. **6**:381–383.

———. 1999. Molecular population structure of the marine benthic copepod *Microarthridion littorale* along the southeastern and Gulf coasts of the USA. Mar. Biol. **135**:399–405.

SCHUBART, C. D., J. A. CUESTA, R. DIESEL, and D. L. FELDER. 2000. Molecular phylogeny, taxonomy, and evolution of nonmarine lineages within the American grapsoid crabs (Crustacea: Brachyura). Mol. Phylogenet. Evol. **15**:179–190.

SCHUBART, C. D., R. DIESEL, and S. B. HEDGES. 1998. Rapid evolution to terrestrial life in Jamaican crabs. Nature **393**:363–365.

SCHUBART, C. D., J. E. NEIGEL, and D. L. FELDER. 2000*a*. Molecular phylogeny of mud crabs (Brachyura: Panopei-

dae) from the northwestern Atlantic and the role of morphological stasis and convergence. Mar. Biol. **137**:11–18.

———. 2000*b*. Use of the mitochondrial 16S rRNA gene for phylogenetic and population studies of Crustacea. Pp. 817–830 *in* J. C. VON VAUPEL KLEIN and F. R. SCHRAM, eds. The diversity crisis and Crustacea. A. A. Balkema, Rotterdam, The Netherlands.

STURMBAUER, C., J. S. LEVINTON, and J. CHRISTY. 1996. Molecular phylogeny analysis of fiddler crabs: test of the hypothesis of increasing behavioral complexity in evolution. Proc. Natl. Acad. Sci. USA **93**:10855–10857.

STURMBAUER, C., G. B. OPADIYA, H. NIEDERSTATTER, A. RIEDMANN, and R. DALLINGER. 1999. Mitochondrial DNA reveals cryptic oligochaete species differing in cadmium resistance. Mol. Biol. Evol. **16**:967–974.

SUN, B., and J. W. FLEEGER. 1994. Field experiments on the colonization of meiofauna into sediment depressions. Mar. Ecol. Prog. Ser. **110**:167–175.

SWOFFORD, D. L. 1998. PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0. Sinauer, Sunderland, Mass.

TAM, Y. K., I. KORNFIELD, and F. P. OJEDA. 1996. Divergence and zoogeography of mole crabs, *Emerita* spp. (Decapoda: Hippidae), in the Americas. Mar. Biol. **125**:489–497.

TANG, J., L. TOE, C. BACK, and T. R. UNNASCH. 1996. Intraspecific heterogeneity of the rDNA internal transcribed spacer in the *Simulium damnosum* (Diptera: Simuliidae) complex. Mol. Biol. Evol. **13**:244–252.

THOMPSON, J. D., D. G. HIGGINS, and T. J. GIBSON. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.

WILCOX, T. P., L. HUGG, J. A. ZEH, and D. W. ZEH. 1997. Mitochondrial DNA sequencing reveals extreme genetic differentiation in a cryptic species complex of neotropical pseudoscorpions. Mol. Phylogenet. Evol. **7**:208–216.

WILSON, A. C., S. S. CARLSON, and T. J. WHITE. 1977. Biochemical evolution. Annu. Rev. Biochem. **46**:573–639.

WITT, J. D. S., and P. D. N. HEBERT. 2000. Cryptic species diversity and evolution in the amphipod genus *Hyalella* within central glaciated North America: a molecular phylogenetic approach. Can. J. Fish. Aquat. Sci. **57**:687–698.

YANG, Z. 1993. Maximum-likelihood estimation of phylogeny from DNA sequences when substitution rates differ over sites. Mol. Biol. Evol. **10**:1396–1401.

———. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput. Appl. Biosci. **13**:555–556.

YANG, Z., and R. NIELSEN. 1998. Synonymous and nonsynonymous rate variation in nuclear genes of mammals. J. Mol. Evol. **46**:409–418.