MOLECULAR GENETIC VARIATION OF *CALANUS PACIFICUS* (COPEPODA: CALANOIDA): PRELIMINARY EVALUATION OF GENETIC STRUCTURE AND SUBSPECIFIC DIFFERENTIATION BASED ON MTDNA SEQUENCES

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ABSTRACT

Molecular genetic data can reveal the systematic relationships among biological organisms and may be particularly useful for taxa that are morphologically indistinguishable. The copepod *Calanus pacificus* may include several subspecies, although no morphological characters diagnostic of each subspecies have been found. We use DNA sequence variation of a mitochondrial gene to investigate the degree of genetic differentiation associated with these systematic groupings and to begin a study of the evolutionary significance of geographic variation in the species. The DNA sequence of a 449 base pair portion of the mitochondrial 16S rRNA gene was determined for 27 individuals of *C. pacificus* collected from five sites in the North Pacific Ocean (Dabob Bay, Puget Sound, Wash.; three sites in the California Current; and the former Ocean Weather Station Papa). The molecular genetic differentiation between individuals of *C. p. oceanicus* and *C. p. californicus* (0.9% to 1.0% sequence difference) was greater than that between individuals of *C. p. californicus* from different geographic regions (0.2% to 0.5% difference), but less than that among species of *Calanus* (12% to 18% difference). The strongest evidence of subspecific differentiation is that the *C. p. oceanicus* individuals were grouped together and separate from *C. p. californicus* on a tree constructed by neighbor joining with 1000 bootstrapped subreplicates. Determination of the systematic significance of the molecular divergence will require further analysis of geographic and systematic patterns of intraspecific variation within *C. pacificus*, and analysis of additional genes.

RESUMEN

Las relaciones sistemáticas entre los organismos pueden ser reveladas con datos de genética molecular, que además pueden ser particularmente útiles para revelar taxa que son indistinguibles morfológicamente. El copépodo *Calanus pacificus* podría incluir varias subespecies, a pesar de que no se han encontrado caracteres diagnóstico para cada una de éstas. Usamos variación en la secuencia de ADN de un gen de la mitocondria para investigar el grado de diferenciación genética asociado con estos agrupamientos sistemáticos y para empezar a estudiar el significado evolutivo de la variación geográfica en la especie. Se determinó la secuencia de ADN en una porción de 449 pares de bases del gen del mitocondria 16S ARNr en 27 individuos de *C. pacificus* colectados en 5 sitios del Oceáno Pacífico Norte (Bahía Dabob, Puget Sound, Washington, tres sitios en la Corriente de California, y la antigua estación climatológica oceánica Papa). La diferenciación genética molecular entre los individuos de *C. p. oceanicus* y *C. p. californicus* (0.9% a 1.0% de diferencia en la secuencia) fué mayor que entre individuos de *C. p. californicus* de diferentes regiones (0.2% a 0.5% de diferencia), pero menor que entre especies de *Calanus* (12% a 18% de diferencia). La evidencia más fuerte de diferenciación subespecífica es que el análisis agrupó a los individuos de *C. p. oceanicus* y separó a los de *C. p. californicus* en un árbol construido por el método de unión de vecinos con 1000 muestras obtenidas por muestreo repetitivo automatizado ("bootstrapping"). La determinación del significado sistemático de la divergencia molecular requerirá más análisis de los patrones geográficos y sistemáticos de la variación intraespecífica en *C. pacificus* y análisis de más genes.

INTRODUCTION

Molecular Taxonomy

Where morphological or physiological evidence of systematic relationships is unclear, genetic characters may provide accurate and unambiguous indicators of the systematics of a group. Molecular characteristics have been used to identify and discriminate species (Wilson et al. 1985). But although genetic characteristics can frequently show patterns of relationship, they frequently do not indicate the level of taxonomic divergence between taxa. For example, there is no benchmark for differentiation at the genus, species, or subspecies level. Fixed differences between taxa for traits encoded by nuclear genes (especially allozymic variants of enzymes) are frequently considered to constitute sufficient evidence of distinction at the species level. It is essential that both intra- and interspecific variation of genetic characteristics are quantified and compared in order to understand the taxonomic significance of genetic differences.

Questions of the systematic significance of genetic differences within a species are especially difficult to re-

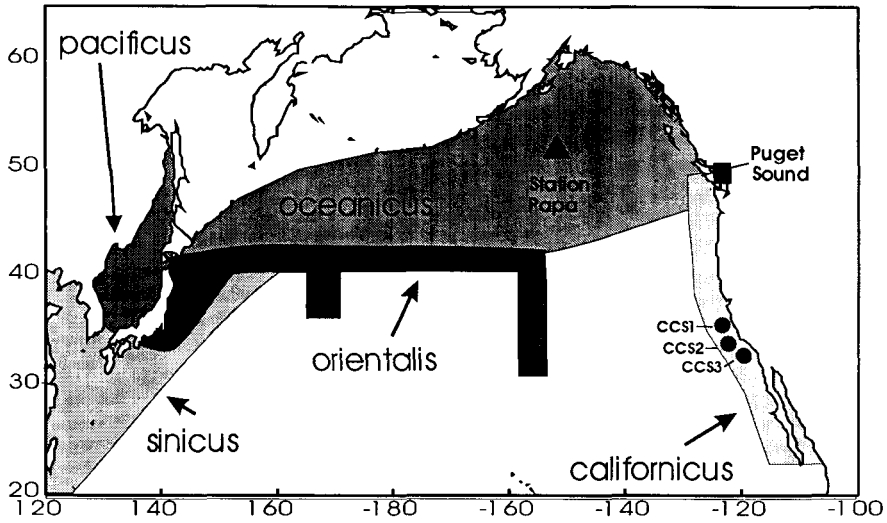

Figure 1. Geographic distribution of the subspecies of *Calanus pacificus* (also showing *C. sinicus*). Collection sites are shown as symbols. There are four sites within the range of *C. p. californicus*: Puget Sound (*square*) and CCS1, CCS2, and CCS3 (*circles*); one site, Station Papa (*triangle*), is within the range of *C. p. oceanicus*. (Distributions are derived from unpublished work by A. Fleminger.)

solve, since these groups may be so recently reproductively isolated that there may be no detectable genetic divergence. There are currently no established levels of genetic divergence associated with subspecies, sibling species, and semispecies. Intuitively, one might expect that these levels of genetic differentiation would be less than that between species and greater than that between conspecific populations.

Numerous researchers have quantified the genetic variation among conspecific populations by using mitochondrial DNA (mtDNA) (see review by Avise et al. 1987). The primary advantage of mtDNA for these studies is that it is inherited maternally; mtDNA may be expected to exhibit greater differences than nuclear DNA between lineages (Birky et al. 1989). Among marine invertebrates, several species have been shown to exhibit significant differentiation among conspecific populations (i.e., significant population genetic structure). The following species have been shown to be genetically structured on mesoscales to large scales (i.e., 100s to 1000s of km) by restriction fragment length polymorphisms of mtDNA: the horseshoe crab, *Limulus polyphemus* (Saunders et al. 1986); the oyster *Crassostrea virginica* (Reeb and Avise 1990); and the mussel, *Mytilus* spp. (Edwards and Skibinski 1987). Restriction fragment polymorphisms have also revealed significant genetic differentiation among geographic populations of marine fish, including herring (Kornfield and Bogdanowicz 1987), red drum (Gold and Richardson 1991), haddock (Zwanenburg et al. 1992), and plaice (Stott et al. 1992).

More recently, the nucleotide base sequence variation of mtDNA has been used to examine population structure in marine fish and invertebrates. The base sequence of a portion of cytochrome b discriminated populations of cod (Carr and Marshall 1991a, b) and blue marlin (Finnerty and Block 1992). Mitochondrial genes have revealed considerable intraspecific sequence variation in the sea urchins *Strongylocentrotus pallidus* (Palumbi and Kessing 1991) and *Heliocidaris tuberculata* (McMillan et al. 1992), and in the penaeid shrimp, *Penaeus stylirostris* (Palumbi and Benzie 1991); however, little or no geographic structuring was observed for these species. Two general principles emerge from the many studies: first, that both marine fish and invertebrates are quite variable at the protein and molecular level, and second, that this variability is resolved into genetically distinguishable, geographic populations in only some of the species (see Ovenden 1990 for a review of marine stock assessment using mtDNA).

Taxonomy of *Calanus* Species

Three subspecies of the calanoid copepod *Calanus pacificus* have been distinguished on the basis of the relative position of the spermathecae of females and the number of denticles and endopodite length of the fifth leg of males (Brodsky 1965). Intermediate forms for these characters exist, and neither the position of urosomal pores nor the shape of the fifth thoracic leg differs diagnostically among the subspecies (Fleminger and Hulsemann, unpublished data). Although the systematic significance of the morphological variation is in some doubt, the subspecies have been referred to in print repeatedly (Alldredge et al. 1984; Fleminger 1985; Fleminger and Hulsemann 1987).

The subspecies have distinct geographic distributions (figure 1). *C. p. californicus* is the only species of *Calanus*

found in the California Current between central California and the Gulf of California (Fleminger 1964). This subspecies exhibits complex behavioral reactions to hydrographic conditions (Cox et al. 1983) and marked migratory behavior (Alldredge et al. 1984) that is adaptive for life in the coastal upwelling region of the California Current. Another subspecies, *C. p. oceanicus*, has an oceanic distribution and may occur across the entire northern North Pacific (Brodsky 1965). The third subspecies, *C. p. pacificus*, is found in the temperate western Pacific, where it may overlap extensively with *C. sinicus*, from which it is morphologically distinguishable (Brodsky 1965).

In this preliminary analysis, genetic differences among samples of *C. pacificus* were determined in order to reveal the geographic and systematic patterns of molecular variation within the species. We present results based on sequencing a portion of the mitochondrial 16S rRNA gene. We chose this gene because it has been shown to vary sufficiently to make it possible to discriminate closely related species (e.g., Cunningham et al. 1992; Xiong and Kocher 1991). Also, previous studies have shown that there is considerable variation within several *Calanus* species and some evidence of population genetic structure (Bucklin and Kann 1991; Bucklin and Kocher, unpublished data).

MATERIALS AND METHODS

Sample Collection

Samples of each species were collected by net tow and preserved in 95% ethyl alcohol. One sample of *C. pacificus* from the California Current (CCS1) was collected in June 1992. Two other California Current samples of *C. pacificus* were collected during California Cooperative Fisheries Investigation (CalCOFI) surveys: one (CCS2) in November 1989 from CalCOFI station number 83.42, and the other (CCS3) in April 1992 from CalCOFI station 93.40. The locations of the sample sites were: CCS1 (34°54' N; 123°01' W); CCS2 (33°75' N; 121.9°9' W); and CCS3 (32°25' N; 118°10' W). The Ocean Weather Station Papa (PAPA) sample of *C. pacificus* was collected at 50° N; 145° W; the sample from Puget Sound, Wash. (PUGET) was collected at 47°45.5' N; 122°49.5' W (figure 1).

DNA Amplification

Mitochondrial DNA amplifications were performed on specimens preserved in 95% ethyl alcohol. The copepods were rehydrated in 0.4 ml of distilled deionized H_2O for 24 hrs prior to amplification. Each individual was then homogenized in a PCR buffer containing 77 µl dH_2O, 8 µl of 25 mg/ml $MgCl_2$, and 10 µl 10X PCR buffer (Promega Corp., Madison, Wis.) and refrigerated for 24 hrs. After incubation, the remainder of the PCR reaction mixture was added: 10 µl dNTP (Perkin-Elmer Corp., Norwalk, Conn.) 1 µl each of the 16SAR and 16SBR primers (100 µM concentration), and 1 µl Taq polymerase enzyme (Promega Corp.). The reaction volume was 100 µl; 2 drops of mineral oil were added on top to prevent evaporation.

The amplification primers used were 16SAR and 16SBR (Palumbi et al. 1991) based on the *Drosophila yakuba* sequence (Clary and Wolstenholme 1985). The sequences are:

16SAR 5' = CGCCTGTTTAACAAAAACAT = 3'
16SBR 5' = CCGGTTTGAACTCAGATCACGT = 3'

Amplification was carried out in a Perkin-Elmer thermal cycler, model 480. The amplification protocol was denaturation at 94°C for 1 min; annealing at 45°C for 2 min; and extension at 72°C for 3 min. The amplification was carried through 35 cycles and maintained at 4°C.

Amplification products to be sequenced were checked for size and purity by loading 10 µl on a 1% agarose gel containing ethidium bromide. Only amplification products showing bright, sharp bands were selected for sequencing. These products were purified by loading 45 µl onto a 1% Nusieve gel containing ethidium bromide, and electrophoresed at 50 volts. Product bands were cut from the gel and melted by heating to 65°C in 1.7 ml Eppendorf tubes. The temperature was lowered to 37°C, and 5–10 units of agarase (Sigma Chemical Corp., Chicago) were added. The samples were incubated overnight to ensure complete digestion of the agarose.

DNA Sequencing

The sequencing reaction was done in a Perkin Elmer thermal cycler, model 480, using a cycle-sequencing kit (Applied Biosystems, Inc., Foster City, Calif.). Fluorescently labeled dideoxynucleotides were incorporated during an asymmetrical amplification using the 16SBR primer. This primer was selected because it consistently produced good sequence data.

Nucleotide sequencing was carried out in an Applied Biosystems, Inc., automated DNA sequencer. The automated sequencer relies on an amplification reaction to produce strands terminated with fluorescently labeled dideoxynucleotides (Smith et al. 1986). The sequencer uses a 6% acrylamide gel; gels are electrophoresed for 11 hrs. The sequences are shown as fluorescent emission spectra for each base, resulting in a 4-color chromatogram. The sequence chromatogram is read by the computer

[1]Palumbi, S., A. Martin, S. Romano, W. O. McMillan, L. Stice, and G. Grabowski. 1991. The simple fool's guide to PCR (ver. 2). Unpublished manuscript.

software (SeqEd, version 2.0) and checked thoroughly for accurate machine reading.

Sequence Alignment and Data Analysis

The Genetics Computer Group (GCG) Sequence Analysis Software Package was used for alignments and preliminary analyses; the programs are based on those by Smithies et al. (1981) and are now commercially available as a package. The multiple-sequence alignment program PileUp (Devereaux et al. 1984) was used to align the sequences for each individual; PileUp is a simplification of the progressive alignment method of Feng and Doolittle (1987). Although the program has limitations, it is a safeguard against human subjectivity in the alignment process. Several parameters of the PileUp program can be altered: gap penalties used ranged from 0.5 to 5.0, and gap length penalties ranged from 0 to 0.5; the definitive alignment was done with a gap penalty of 5.0 and a gap length penalty of 0.3, which maximized the sequence identities.

A difference matrix was calculated for all pairwise comparisons of individual sequences. The mean and the standard error of the percent differences between individuals were determined for comparisons within and between samples.

Phylogeny Reconstruction

A tree was constructed by means of neighbor joining (Saitou and Nei 1987) in the software package MEGA (Molecular Evolutionary Genetics Analysis; Kumar et al. 1993) to show the molecular relationships among the 27 individuals. The neighbor-joining protocol first determines a distance matrix and then reconstructs the phylogeny. Tamura-Nei distances were used (Tamura and Nei 1993), based on both transitions and transversions. The resultant tree was tested for statistical significance by 1000 bootstrap replications.

RESULTS

Nucleotide base sequences for a 440-base-pair region of the mitochondrial 16S rRNA gene of *C. pacificus* was determined for 27 individuals (figure 2). There were 16 haplotypes among the 27 individuals. We found that 11 individuals were unique; 5 individuals shared the same haplotype; four other haplotypes were shared by 2–4 individuals.

Among the three samples of *C. pacificus* collected in the California Current (CCS1, CCS2, and CCS3), individuals differed by three or fewer bases among the 449 (≤0.7%). Among individuals within one sample, differences averaged considerably less than 1%: the mean difference between individuals within the PUGET sample was 0.20% and between individuals within the PAPA sample was 0.14% (figure 3). For comparisons between

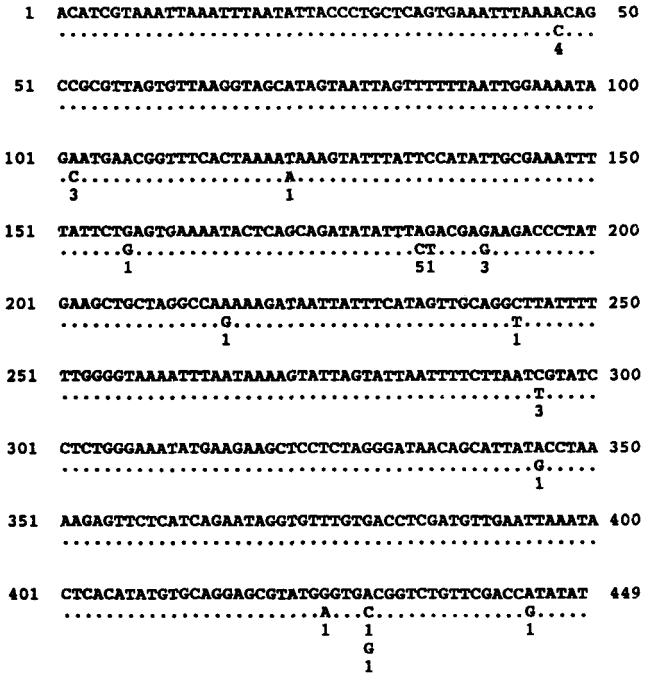

Figure 2. DNA base sequence for a 449 base pair region of the mitochondrial 16S rRNA gene for samples of geographic populations of *Calanus pacificus*. Only the sequence for the most abundant haplotype, shared by 5 of the 27 individuals sequenced, is shown. Substitutions are indicated just below each variable site; the number of haplotypes that exhibit each substitution is shown below the base substituted. The sequence alignment was done by the PileUp program of the GCG Software Package (Devereaux et al. 1984). Alignment was done with a gap weight of 5.0 and gap length weight of 0.3.

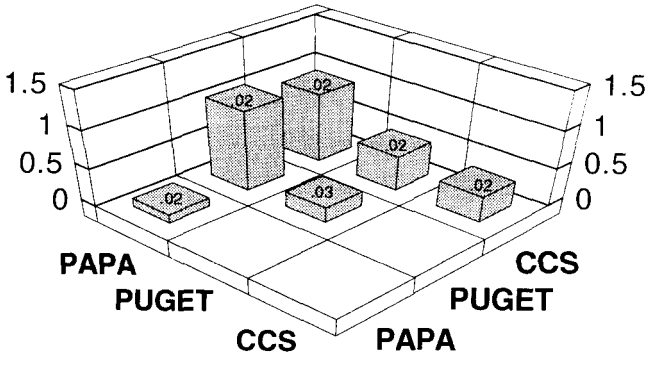

Figure 3. Percent DNA base sequence differences among samples of *C. pacificus*. The three samples collected in the California Current (CCS) have been pooled for comparison with the Puget Sound samples (PUGET) and the sample collected at Ocean Weather Station Papa (PAPA). Collection locations are given in figure 1 and the text. The bar height indicates the percent differences for pairwise comparisons within and between samples (or pools of samples). Numbers shown on top of the bars are the standard error; all bar heights differ significantly, except those of the CCS vs PAPA and PUGET vs PAPA comparisons, which are not different.

individuals in the CCS and PUGET samples (i.e., between individuals of *C. p. californicus*) the mean difference was 0.46%. In contrast, comparisons between *C. p. californicus* and *C. p. oceanicus* were 0.92% (for CCS vs PAPA samples) and 1.06% (for PUGET vs PAPA samples). The differences between *C. p. oceanicus* and *C. p.*

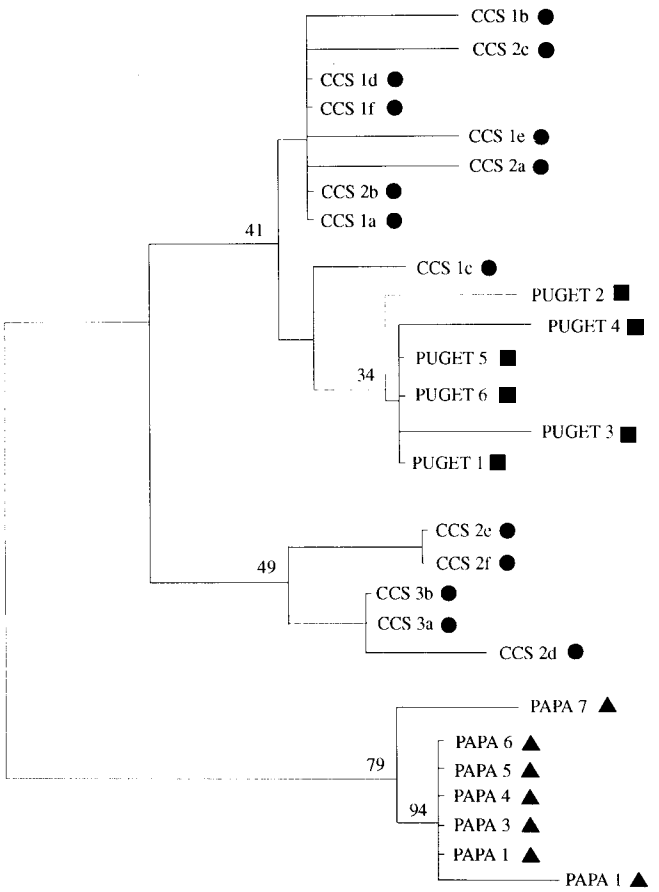

Figure 4. Neighbor-joining tree showing genetic differences among the 27 individuals of *Calanus pacificus*. Abbreviations are: California Current Collections (CCS), Puget Sound collection (PUGET), and O.W.S. Papa collection (PAPA). The symbols correspond to those used for the collection locations in figure 1. The tree is based on the neighbor-joining method, using Tamura-Jukes distances and considering both transitions and tranversions. Numbers on the branches are branch lengths; numbers in italics at branchpoints are the percentage of trees that show that branchpoint among 1000 bootstrapped replicates.

californicus individuals were significantly greater than the differences among individuals of the same subspecies (figure 3).

A phylogenetic analysis of the individuals of the five samples of *C. pacificus* further suggested that the *C. p. oceanicus* sample collected from Ocean Weather Station Papa was distinctive (figure 4). The tree topology was robust to the method of analysis: all trees indicated the distinctiveness of the individuals in the PAPA sample. The neighbor-joining tree (figure 4) has the *C. p. oceanicus* individuals clustered together and significantly distinct in 1000 bootstrapped subreplicates (Saitou and Nei 1987).

DISCUSSION

Previous studies of DNA sequence variation of the mitochondrial 16S rRNA gene have revealed differences in the sequences of a 450 bp region between species of *Calanus* on the order of 12% to 18% (Bucklin et al. 1992). Other studies comparing sequence data of the mitochondrial 16S rRNA gene between crustacean species have shown similar results. Between species of the shrimp *Penaeus*, 11% sequence differences were found by Machado et al. (1992) for this same region of the mitochondrial 16S rRNA gene. Palumbi and Benzie (1991) estimated interspecific differences between two *Penaeus* species at 11% (based on sequence data) and 9% (based on RFLP data). Brasher et al. (1992) showed that the mean mtDNA sequence divergence between three species of the rock lobster *Jasus* was 6.2%, whereas these species differed from a fourth by 14.9% to 16.7%. These divergences, and those of *Calanus* species, are typical of the congeneric species that have been studied (Avise et al. 1987).

Levels of mtDNA sequence variation within species of *Calanus* for the 16S rRNA gene ranged between 1.3% (between *C. helgolandicus* samples) and 2.6% (between *C. finmarchicus* samples) (Bucklin and Kocher, unpublished data). The characteristic pattern of intraspecific variation is that one haplotype is very common (approaching 50% of individuals sequenced), while there are many rare or unique haplotypes in each population. Intraspecific differences among individuals of *C. pacificus* are generally less than 1% (figure 3). Within the same subspecies, differences are on the order to 0.2%; between subspecies, the differences are approximately 1%.

Using the mitochondrial 16S rRNA gene, Xiong and Kocher (1993) measured differentiation within species of the black fly genus, *Simulium*, to be 0.47%, and within "complexes" (sibling species groups) to be 1.09%. For crustaceans, Silberman et al. (1994) found mtDNA sequence differences among conspecific populations to be between 1.2% and 1.7%, based on RFLP analysis. Another study by McLean et al. (1983) used restriction fragment length polymorphisms of mtDNA to establish levels of intraspecific variation of *Panulirus*, which they found to be similar to humans, deer mice, and *Drosophila*. These levels of intraspecific variation for the mitochondrial 16S rRNA gene may prove low in comparison with other mitochondrial and nuclear genes. Analysis of intraspecific variation of addition genes is important to determine whether the patterns described here are typical of all genes.

Although the sequence difference between the two subspecies of *C. pacificus* is far less than that between species, and similar to that between conspecific populations, these data cannot be said to "prove" that the geographic forms of *C. pacificus* do or do not deserve subspecific status. It is very difficult to determine the level of systematic distinctiveness using molecular characteristics, especially mtDNA. One problem is that no threshold levels of divergence of mtDNA sequences have

been associated with speciation events or subspecific differentiation, as has been done in some cases for nuclear genes. Rates of divergence of mtDNA appear to be rather variable from taxon to taxon. Also, recent divergences may not be reflected in the mtDNA characteristics, and newer taxa may show little or no sequence divergence.

In addition to the measures of sequence divergence, phylogenetic approaches are useful for identifying genetically distinct groups and for recognizing geographic structure (this approach has been called phylogeography; Avise et al. 1987). For example, despite little sequence differentiation between individuals of the different subspecies of *C. pacificus*, all individuals of *C. p. oceanicus* cluster together in the neighbor-joining tree (figure 4). This distinctive grouping is statistically significant according to the bootstrap test performed: 6 of the 7 individuals cluster 94% of the time, and the seventh individual is grouped with these 79% of the time. The 20 individuals of *C. p. californicus* are variable among themselves, but there are no statistically distinct groupings within the subspecies (other than a slight tendency of the individuals from Puget Sound to group together; figure 4). As found in an earlier study (Bucklin and Kocher, unpublished data), there was more intraspecific variation among 87 individuals of *C. finmarchicus* than among the *C. pacificus* individuals (the most divergent *C. finmarchicus* individuals were 2.7% different in sequence for this same gene region), but there was no distinctive clustering of individuals by geographic region or taxonomic subgroup.

In summary, mtDNA sequence data for 27 individuals yields some evidence of the differentiation of the open-ocean sample (O.W.S. Papa) of *C. pacificus*, which occurs within the geographic range of *C. p. oceanicus*. In contrast, there is no significant differentiation of any subgroup across the large latitudinal range of *C. p. californicus* (Puget Sound and throughout the California Current). The genetic differentiation of the *C. p. oceanicus* sample is apparent in the tree constructed by neighbor joining and is statistically sound according to a bootstrap test (figure 4). The tree construction provides the best evidence of the genetic distinctiveness of the subspecies of *C. pacificus*. The amount of sequence difference between individuals of the different subspecies (approximately 1%) is similar to that between conspecific individuals of other crustacean species (McLean et al. 1983; Silberman 1994), including other species of *Calanus* (Bucklin and Kocher, unpublished data). If the subspecies are reproductively isolated and thus genetically distinct, the divergence has been a relatively recent one. These subspecies may be too young to exhibit significant genetic divergence.

Molecular genetic traits of organisms may be a useful means for resolving long-standing, fundamental questions about the systematics and evolution of planktonic organisms. Questions having to do with the taxonomic significance of intraspecific variation are particularly problematical for species that show little intra- or interspecific variation, and may be particularly amenable to molecular analysis.

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