Contributions to Zoology, 70 (1) 23-39 (2001)
SPB Academic Publishing bv, The Hague

Hierarchical analysis of mtDNA variation and the use of mtDNA for isopod (Crustacea: Peracarida: Isopoda) systematics

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Keywords: 12S rRNA, 16S rRNA, COI, mitochondrial DNA, isopod, Crustacea, molecular

Abstract

Carefully collected molecular data and rigorous analyses are revolutionizing today's phylogenetic studies. Although molecular data have been used to estimate various invertebrate phylogenies for more than a decade, this study is the first survey of different regions of mitochondrial DNA in isopod crustaceans assessing sequence divergence and hence the usefulness of these regions to infer phylogeny at different hierarchical levels. I evaluate three loci from the mitochondrial genome (two ribosomal RNAs (12S, 16S) and one protein-coding (COI)) for their appropriateness in inferring isopod phylogeny at the suborder level and below. The patterns are similar for all three loci with the most speciose suborders of isopods also having the most divergent mitochondrial nucleotide sequences. Recommendations for designing an order- or suborder-level molecular study in previously unstudied groups of Crustacea would include: (1) collecting a minimum of two-four species or genera thought to be most divergent, (2) sampling across the group of interest as equally as possible in terms of taxonomic representation and the distribution of species, (3) surveying several genes, and (4) carrying out preliminary alignments, checking data for nucleotide bias, transition/transversion ratios, and saturation levels before committing to a large-scale sequencing effort.

Contents

Introduction

The crustacean order Isopoda is important and interesting because it has a broad geographic distribution and is morphologically diverse. There are more than 10,000 described marine, freshwater, and terrestrial species, ranging in length from 0.5 mm to 440 mm. They are common inhabitants of nearly all environments, and most groups are free-living. Many are scavengers or grazers, although some are temporary or obligatory parasites of fishes and other crustaceans. Many species are shallow water inhabitants, but some taxa are well adapted to life in the deep sea, subterranean groundwater, and thermal springs. Isopods are members of the superorder Peracarida, and a synapomorphy of the superorder is a brooding life style (there are no free-living larvae; development is direct with young emerging with the adult morphology) and thus there is purported poor dispersal ability.

The first isopod was described in 1764 (*Asellus* Geoffroy), and the group's systematics and taxonomy has been bantered about ever since. Present workers recognize ten suborders, and over the past twenty years isopods have received considerable

morphological systematic attention, with ordinal summaries provided by Bowman and Abele 1982, Brusca and Iverson 1985, Schram 1986, Wägele 1989, and Brusca and Wilson 1991. Morphological character-based, cladistic analyses have been carried out for several isopod taxa (e.g., idoteid and arcturid valviferans, Brusca 1984, Poore 1995; corallanid flabelliferans, Delaney 1989; phreatoicids, Wägele 1989; janirid asellotans, Wilson 1994; serolids, Brandt 1988, 1992).

Molecular techniques have invigorated crustacean systematics over the last dozen years with primary contributions stemming from higher-level systematics. A comprehensive list of molecular phylogenetic studies carried out to date for the Crustacea at the species level and higher appears in Table 1. No published studies exist within the Isopoda, although several mitochondrial (mt) DNA studies based on the 16S ribosomal RNA (rRNA) gene are in progress (suborders of Isopoda, Dreyer, Ph.D. dissertation, Ruhr-Universität Bochum; species of *Thermosphaeroma*, Davis et al., in review; and genera and families of Oniscidea, Michel, Université de Poitiers).

As researchers turn to molecular methods, mtDNA is being used to address both higher-level systematics and population-level questions. However, there are pitfalls when using inappropriate sequence data for phylogenetic inference. Selecting a gene for phylogenetic analysis requires matching the level of sequence variation to the desired taxonomic level of study. Several recent papers have focused on the identification of genes that are useful for phylogenetic analysis at different taxonomic levels (Brower and DeSalle 1994, Friedlander et al. 1994, Graybeal 1994, Simon et al. 1994, Sullivan et al. 1995). Mitochondrial 12S- and 16S rRNA genes and the protein-coding cytochrome oxidase c subunit I (COI) gene have been studied extensively within recently diverged lineages of arthropods (<5 million years ago, mya). Sea urchins and butterflies exhibit similar divergence rates for a given gene, with the rate linear with time and 1.8-2.3% divergence per million years (Bermingham and Lessios 1993, Brower 1994). However, when more ancient lineages (>75 mya) of vertebrates are compared, different mtDNA genes vary considerably with respect to divergence rate, i.e., some genes are more conserved than others (Cummings et al. 1995). Understanding basic parameters such as patterns of nucleotide substitution and rate variation among sites is important for proper application of DNA sequence data to molecular systematic studies (Yang 1994, Yang and Kumar 1996, Blouin et al. 1998, Whitfield and Cameron 1998).

Mitochondrial 12S rRNA, 16S rRNA, and COI genes are attractive to crustacean evolutionary biologists because universal and crustacean-specific primers are readily available for polymerase chain reaction (PCR; Saiki et al. 1988) amplification, and amplified gene fragment sizes are amenable to manual and automated sequencing techniques. Comparative arthropod sequences are known for these genes and extracting sufficient and adequate quality DNA from ethanol-preserved specimens of highly variable preservation are attainable goals for organisms with a considerable range in body size. By describing patterns of sequence divergence within and among populations, species, genera, families, and suborders of isopods, the appropriateness of three mitochondrial genes for evolutionary questions at various taxonomic levels can be determined.

Material and methods

Sources of specimens and DNA preservation

The taxa used at each taxonomic (hierarchical) level of comparison are shown in Table 2. The suborder Flabellifera may not be a monophyletic taxon (Kussakin 1979, Bruce 1981, Wägele 1989, Brusca and Wilson 1991), and relationships of the families included within the Flabellifera have also been controversial. In this study flabelliferan families are considered separate taxonomic entities and in figures are referred to by the family name followed by "(Flabellifera)." Most specimens were collected by the author; additional specimens were donated by colleagues (see Acknowledgements). Most specimens were fixed and preserved in 95% ethanol, and in some instances DNA was extracted from specimens fixed in 70-75% ethanol. The latter specimens had body sizes >10 mm.

Table 1. Molecular phylogenetic studies within Crustacea at the species level and higher with studies grouped by taxa. Genes studied include nuclear 18S rRNA, mitochondrial 12S- and 16S rRNAs, and protein-coding mitochondrial cytochrome oxidase c subunit I (COI) gene fragments.

Taxon	Hierarchical Level	Reference	Description	Gene
Crustacea	class	Abele et al. 1989	Pentastomidia are Crustacea	18S rRNA
Crustacea	class	Spears and Abele 1997	phylogeny of Crustacea	18S rRNA
Crustacea	class	Spears and Abele 1999	foliaceous limbs: Branchiopoda, Cephalocarida, and Phyllocarida	18S rRNA
Branchiopoda	class	Hanner and Fugate 1997	phylogeny of branchiopods	12S rRNA
Branchiopoda	class	Spears and Abele 2000	phylogeny of branchiopods	18S rRNA
Branchiopoda: Cladocera	order	Lehman et al. 1995	phylogeny of *Daphnia*	12S rRNA
Branchiopoda: Cladocera	subgenus/species	Colbourne and Hebert 1996	*Daphnia*	12S rRNA
Branchiopoda: Cladocera	species	Taylor et al. 1998	cryptic endemism of *Daphnia*	16S rRNA
Maxillopoda	class	Abele et al. 1992	class relationships	18S rRNA
Maxillopoda: Cirripedia	suborder	Spears et al. 1994	thecostracan relationships	18S rRNA
Maxillopoda: Cirripedia	species	vanSyoc 1995	*Pollicipes* diversity	COI
Maxillopoda: Cirripedia	genus	Mizrahi et al. 1998	phylogenetic position of *Ibla*	18S rRNA
Maxillopoda: Cirripedia	family/genus	Harris et al. 2000	select thoracican barnacles	18S rRNA
Maxillopoda: Cirripedia	genus	Perl-Treves et al. 2000	thecostracans: *Verruca, Paralepas,* and *Dendrogaster*	18S rRNA
Maxillopoda: Copepoda	species	Bucklin et al. 1992	intraspecific and interspecific patterns (Calanoida)	16S rRNA
Maxillopoda: Copepoda	species	Bucklin et al. 1995	species of *Calanus* (Calanoida)	16S rRNA
Malacostraca: Decapoda	order	Kim and Abele 1990	ordinal relationships	18S rRNA
Malacostraca: Decapoda	order	Abele 1991	morphology and molecular data	18S rRNA
Malacostraca: Brachyura	infraorder	Spears et al. 1992	monophyly of brachyuran crabs	18S rRNA
Malacostraca: Brachyura	family	Schubart et al. 2000a	phylogeny of brachyuran families	16S rRNA
Malacostraca: Anomura	infraorder	Cunningham et al. 1992	king crabs and hermit crabs	16S rRNA
Malacostraca: Decapoda	genus	Crandall et al. 1995	Australian crayfish (Parastacidae)	16S rRNA
Malacostraca: Decapoda	genus	Lawler and Crandall 1998	*Euastacus and Astacopsis*	16S rRNA
Malacostraca: Decapoda	species	Ponniah and Hughes 1998	*Euastacus* relationships (Parastacidae)	16S rRNA
Malacostraca: Decapoda	subgenus	Crandall and Fitzpatrick 1996	crayfish (Cambaridae)	16S rRNA
Malacostraca: Decapoda	species	Crandall 1998	Ozark crayfishes (Cambaridae)	16S rRNA
Malacostraca: Decapoda	species	Tam et al. 1996	divergence and zoogeography of mole crabs (Hippidae)	16S rRNA
Malacostraca: Decapoda	genus/subgenus	Sturmbauer et al. 1996	fiddler crabs (Ocypodidae)	16S rRNA
Malacostraca: Decapoda	species	Geller et al. 1997	cryptic invasion of *Carcinus* (Carcinidae)	16S rRNA
Malacostraca: Decapoda	genus	Tam and Kornfield 1998	phylogeny of clawed lobsters (Nephropidae)	16S rRNA
Malacostraca: Decapoda	species/subspecies	Sarver et al. 1998	species/subspecies differentiation of *Panulirus argus* (Palinuridae)	16S rRNA
Malacostraca: Euphausiacea	species	Patarnello et al. 1996	relationships of krill	16S rRNA
Malacostraca: Brachyura	species	Schneider-Boussard et al. 1998	sequence variation in stone crabs *Menippe adina* and *M. mercernaria*	16S rRNA
Malacostraca: Brachyura and 16S rRNA	subfamily/genus	Kitaura et al. 1998	relationships of Ocypodidae	12S rRNA
Malacostraca: Decapoda	species	Schubart et al. 1998	species of *Sesarma* (Grapsidae)	16S rRNA
Malacostraca: Decapoda and COI	species	Schubart et al. 1998	Jamaican grapsid crabs (Grapsidae)	16S rRNA
Malacostraca: Decapoda	subfamily/genus	Schubart et al. 2000b	phylogeny of Grapsoidea	16S rRNA
Malacostraca: Amphipoda	genus/species	France and Kocher 1996	deepsea Lysianassidae	16S rRNA
Malacostraca: Mysidacea	family	Casanova, J.-P. et al. 1998	Lophogastrida	16S rRNA
Malacostraca: Isopoda	genus/species	Michel-Salzat and Bouchon 2000	phylogenetic relationship among oniscids	16S rRNA
Malacostraca: Isopoda	genus/species	Held 2000	phylogeny and biogeography of serolids	16S rRNA and 18S rRNA

DNA extraction, primers, PCR amplification, and sequencing

Debris and ectoparasites were shaken off specimens by submerging them in deionized water and exposing them to ultrasound waves for 5-10 seconds. Specimens were then rinsed 3-4 times in deionized water. Since isopods vary considerably in body size, two different extraction protocols were used. DNA from specimens less than 3 mm in length was extracted using a standard phenol-chloroform protocol (Cunningham and Buss 1993). Appendages (antennae, pereopods, or pleopods) were dissected off specimens larger than 5 mm and tissue similarly extracted. Alternatively, 25 mg of tissue (entire specimen, anterior, or posterior half of specimen) were extracted using the QIAamp Tissue Kit (Qiagen, Inc., Valencia, CA). One to four µl of DNA template were used in 50-µl PCR reactions.

The COI sequence was amplified using the Folmer et al. (1994) universal primers (LCO1490 and HCO2198, ~442 base pairs, bp), and Palumbi et al. (1991) universal 16Sar and 16Sbr primers were used for the 16S rRNA fragment (~378 bp). A ~275 bp region of the 12S rRNA gene was amplified using peracarid specific primers (12SCRF: 5'-GAG AGT GAC GGG CGA TAT GT-3'; 12SCRR: 5'-AAA CCA GGA TTA GAT ACC CTA TTA T-3').

For the PCR reaction, Perkin Elmer (Foster City, CA) or Promega (Madison, WI) 10X buffer and the manufacturer's respective *Taq* DNA polymerase (2.5 units) were used with an initial denaturation period of 3 minutes at 95°C, followed by 35 cycles at 94°C for 15 seconds and extension for 1.5 minutes at 72°C. Annealing temperatures ranged from 48°C (COI) to 52°C (12S rRNA and 16S rRNA) for 1 minute. PCR amplification product (3-6 µl) was electrophoresed through an ethidium bromide-stained 1-2% agarose gel, and the product was checked for proper size. Remaining PCR product was purified with polyethylene glycol (PEG) or with Sephadex G-50 (Sigma Chemical, Inc.) and Centrisep columns (Princeton Separations, Adelphia, NJ), or if necessary, gel purified using the Qiagen Gel Purification Kit. DNA was then cycle sequenced with ABI (Applied Biosystems Inc., Foster City, CA) Big Dye terminators, and both strands were sequenced on an ABI 377 automated sequencer. Nucleotide sequences were edited using the Sequencher software package (ver. 3.1, GeneCodes Corp., Ann Arbor, MI), and sequences were searched for similarity to other arthropods using BLAST (Basic Local Alignment Search Tool, URL: http://www.ncbi.nlm.nih.gov/BLAST/index.html). Additionally, the accuracy of COI sequences was verified by translating nucleotides to amino acids with MacClade 3.06 (Maddison and Maddison 1996), and all sequences were verified for proper reading frame.

Sequence alignment strategy

Thirty-three COI sequences were aligned by hand since there were no insertions or deletions of nucleotides or amino acids. One data set was prepared for amino acid and a second data set for nucleotide analyses. The multiple-sequence-alignment program CLUSTAL W 1.74 (Gibson et al. 1996) was set to default settings (slow/accurate gap open penalty = 15.00, gap extension penalty = 6.66, *k*-tuple size = 2, transitions not weighted) and used to align 49 and 18 16S rRNA and 12S rRNA sequences, respectively.

Determining nucleotide composition, sequence divergence, and transition/transversion bias

The essential component of genomic structure are the two linear polynucleotide chains composed of two purines (adenine [A] and guanine [G]) that hydrogen-bond to two pyrimidines (thymine [T] and cytosine [C], respectively). Nucleotide frequency can result in taxon- and gene-specific patterns of nucleotide composition. The phylogenetic analysis program PAUP* version 4.062 (Swofford 1999) was used to determine nucleotide composition.

A method of summarizing the relationship between two sequences is by their fraction (or percentage) of similarity or dissimilarity. In its simplest form, the similarity is equal to the number of aligned sequence positions containing identical residues (bases or amino acids) divided by the number of

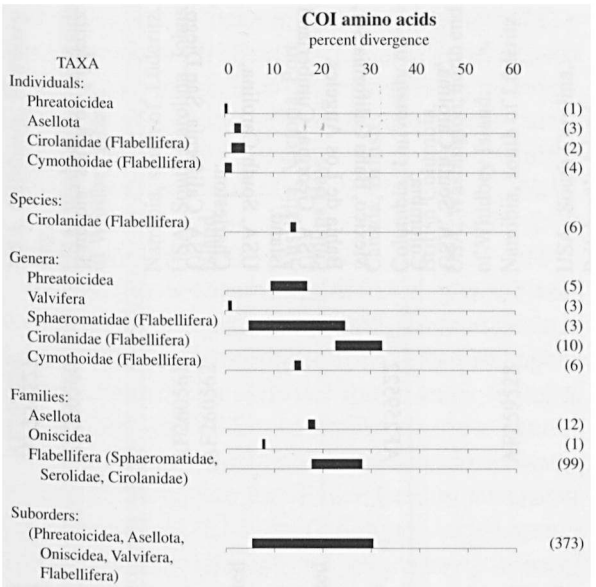

Fig. 1. Percent divergence ("uncorrected p") for cytochrome oxidase c subunit I (147 amino acids, 33 taxa) plotted against taxonomic level. Minimum and maximum divergence measure expressed as range (bar). Number of pairwise comparisons indicated in parentheses on right.

sequence positions being compared. Dissimilarity is merely the proportion (p) of nucleotide sites (n) at which the two sequences being compared are different (n_d), $p = n_d/n$. For example, if two taxa are represented by 20 aligned nucleotides and they differ at 5 sites, the "uncorrected p" value is 0.25. These two sequences can also be said to have 75% similarity.

Percent-sequence divergence ("uncorrected p") values obtained using PAUP* are summarized in Figs. 1-4 for COI amino acids, COI nucleotides, 16S rRNA, and 12S rRNA within *individuals, species, genera, families,* and *suborders*. Sequence comparisons were made at the lowest taxonomic level possible. For example, for COI amino acids (Fig. 1), the two sphaeromatids *Sphaeramene* and *Sphaeroma* were compared at the genus level. Since only one species in each genus was sequenced, this was the only comparison possible at this hierarchial level. Plots in all instances represent sequences of individuals from a single specimen lot, i.e., a single population, except *Caecidotea* (Asellota) which comes from two populations a few kilometers apart. All other collection localities ranged in area from 0.5 to 3 m^2. In the case of the fish ectoparasite *Lironeca vulgaris* (Cymothoidae), the data are from two ovigerous females from which two individual young (mancas) were removed and for this analysis treated as "individuals."

Transition/transversion (ti/tv) tables were generated from the same data sets as above with PAUP* and plotted with a program written by N. D. Pentcheff (unpublished) (Fig. 5). The number of transitions versus the number of transversions in all pairwise comparisons of COI, 16S rRNA, and 12S rRNA sequences are corrected for sequence length variation by dividing the ti/tv ratio by the number of nucleotides in each sequence.

GenBank submission

GenBank accession numbers for sequences listed in Table 2 are as follows: COI sequences AF255775-AF255791, AF260834-AF260846, 16S rRNA sequences AF259531-AF259547, AF260847-AF260870, and 12S rRNA sequences AF259521-AF259530, AF260558-AF260562, AF260564.

Results

Nucleotide composition

Nucleotide composition of COI, 16S rRNA, and 12S rRNA sequences are provided in Table 3. COI nucleotide composition is reported for all three codon positions: first and second positions only, and third positions alone. The G statistic for the log-likelihood ratio goodness-of-fit test was used to determine whether nucleotide composition was equal within a given gene. A nucleotide bias ($p < 0.001$) was found for all genes. For COI (all positions) there is roughly a 7% A+T bias. The A+T bias nearly disappears when third positions are removed, yet Ts are favored over As. COI third positions alone are ca. 68% A+T. Both ribosomal RNA genes have nearly equal A+T composition (ca. 58% and 62% A+T for 16S- and 12S rRNA, respectively).

Table 2. Isopod taxa examined in analyses, current taxonomy, authors' reference numbers, GenBank accession numbers for genes sequenced, and sources of material.

Suborder/Family	Genus	Species	Reference No.	Genes COI	16S rRNA	12S rRNA	Locality
Phreatoicidea							
Phreatoicidae	*Colubotelson*	*thompson*	288/357	AF255775	AF259531	AF259525	Australia, Tasmania.
	Colubotelson	*thompsoni*	398		AF260869		
	Crenoicus	*buntiae*	328			AF260564	Australia, New South Wales,
	Crenoicus	*buntiae*	393		not submitted		Boyds National Park.
	Crenoicus	*buntiae*	286	AF255776	AF259532	AF259524	
	Crenoicus	*buntiae*	345		AF260870		
	Paramphisopus	*palustris*	329	AF255777	AF259533	AF259523	Australia, Western Australia, Perth.
	Paramphisopus	*palustris*	389		not submited		
Asellota							
Asellidae	*Caecidotea*	*sp.*	181	AF255778	AF259534		USA, Washington, D.C., Rock Creek National Park.
	Caecidotea	*sp.*	184	AF260834		AF259529	USA, Maryland, Rock Creek Park.
Janiridae	*Ianiropsis*	*epilittoralis*	199		AF260858		USA, California, SIO MBRD
	Ianiropsis	*epilittoralis*	207		AF260859		wet table.
	Ianiropsis	*epilittoralis*	380	AF260835			
	Ianiropsis	*epilittoralis*	381	AF260836			
Joeropsidae	*Joeropsis*	*dubia*	173		AF260860		
	Joeropsis	*dubia*	382	AF260837			
	Joeropsis	*dubia*	383	AF260837			
Oniscidea							
Armadillidiidae	*Armadillidium*	*vulgare*	347		AF260847		USA, South Carolina, Columbia.
	Armadillidium	*vulgare*	390	AF255779	AF259535	AF259522	
Tylidae	*Tylos*	*punctatus*	384		AF260865		Mexico, Baha California Sur, Bahía de Los Angeles.
Ligiidae	*Ligia*	*exotica*	195		AF260861		USA, Georgia, Cumberland Island.
	Ligia	*exotica*	387		AF260863		USA, South Carolina, Charleston.
	Ligia	*occidentalis*	196		AF260862		USA, California, San Diego.
	Ligia	*occidentalis*	219	AF255780	AF259536		
Valvifera							
Idoteidae	*Glyptoidotea*	*lichtensteini*	180		AF260853		Namibia, south of Lüderitz.
	Glyptoidotea	*lichtensteini*	290	AF255781			
	Glyptoidotea	*lichtensteini*	394		AF259537	AF259527	

	Idotea	*resecata*	182	AF255782	AF259538	AF259526	USA, California, Monterey Bay.
	Idotea	*resecata*	420		AF260854		
	Idotea	*wosnesenskii*	354		AF260855	AF260560	USA, Washington, west side of Whidbey Island.
	Idotea	*wosnesenskii*	363		AF260856		
	Idotea	*wosnesenskii*	395		AF260857		
	Paridotea	*ungulata*	215	AF255783	AF259539		Namibia, south of Lüderitz.
	Synidotea	*laticauda*	331			AF260561	USA, South Carolina, Johns Island.
	Synidotea	*laticauda*	358			AF260562	
	Synidotea	*laticauda*	386		not submitted		
Anthuridea							
Anthuridae	*Apanthura*	*sp.*	335	AF255789	AF259545		Australia, Victoria, Port Philips Bay.
	Apanthura	*sp.*	400		not submitted		
Flabellifera							
Sphaeromatidae	*Gnorimosphaeroma*	*oregonensis*	324		AF260866		Canada, British Columbia, University of British Columbia.
	Gnorimosphaeroma	*oregonensis*	360		AF260867		USA, Washington, north end of Whidbey Island.
	Gnorimosphaeroma	*oregonensis*	391	AF260845	AF260868		
	Sphaeramene	*polytylotos*	183	AF255784	AF259540	AF259528	Namibia, south of Lüderitz.
	Sphaeramene	*polytylotos*	216	AF260846			
	Sphaeroma	*quadridentata*	284	AF255785			USA, South Carolina, Pritchard's Island.
	Sphaeroma	*quadridentata*	392		AF259541		
Serolidae	*Serolina*	*bakeri*	287	AF255786			Australia, Tasmania, Tasmania Peninsula.
	Serolina	*bakeri*	336		AF260864		
	Serolina	*bakeri*	349		AF259542		
Cirolanidae	*Cirolana*	*harfordi*	169			AF259521	USA, California, Monterey Peninsula.
	Cirolana	*harfordi*	210	AF260838	AF259543		
	Cirolana	*harfordi*	289	AF255787			
	Cirolana	*harfordi*	403		not submitted	not submitted	
	Cirolana	*rugicauda*	179	AF255788	AF259544	AF260558	Namibia, south of Lüderitz.
	Cirolana	*rugicauda*	330	AF260839		AF260559	
Cirolanidae	*Cirolana*	*rugicauda*	388	AF260840	AF260848	AF259530	Namibia, south of Lüderitz
	Excirolana	*chiltoni*	198		AF260849		USA, California, San Diego County.
	Excirolana	*chiltoni*	211	AF260841			
	Excirolana	*chiltoni*	402		AF260850		
	Excirolana	*mayana*	385		AF260851		Mexico, Baja California Sur, Bahía de Los Angeles.
Cymothoidae	*Lironeca*	*vulgaris*	200	AF260842			California, San Diego County.
	Lironeca	*vulgaris*	218	AF260843	AF260852		
	Lironeca	*vulgaris*	401	AF255790	AF259546		
	Olencira	*praegustator*	213	AF259547	AF259547		USA, South Carolina, Charleston Harbor.
	Olencira	*praegustator*	396	AF260869			

Table 3. Nucleotide composition and sequence length (total number of nucleotides) for all isopods surveyed. A = adenine, T = thymine, C = cytosine, G = guanine. Numeric values in parentheses following COI represent codon positions included in calculations. Values for nucleotides expressed as percent of total. The log-likelihood ratio goodness-of-fit test (G stat) was significant for all gene sequences ($p < 0.001$), df = 3.

Gene sequence	A	T	C	G	Total No. Nucleotides	G stat
COI (1, 2, 3)	23.6	37.3	19.5	19.6	442	1141
COI (1, 2)	19.6	35.1	22.4	22.9	295	521
COI (3)	31.6	41.5	13.7	13.2	147	1142
16S rRNA	35.8	29.9	16.3	18.0	378	1997
12S rRNA	32.8	32.7	14.8	19.7	275	521

Sequence divergence

Figures 1-4 summarize the pairwise sequence divergence for COI amino acids and nucleotides, and 16S- and 12S rRNA sequences. These data are arranged in taxonomic (hierarchical) fashion with minimum and maximum sequence divergences observed for each taxonomic ranking.

The COI comparisons for 33 taxa are shown in Figs. 1 and 2. This data set is based on 442 bases, i.e., 147 amino acids. Note all sequences were truncated to the length of the shortest sequence to eliminate spurious values due to unequal sequence length. Figure 1 summarizes the amino acid comparisons. For *individuals* from the same population pairwise sequence divergence ranged from 0-4.1%. Phreatoicid sequences were identical, and comparisons of Asellota ranged from 0.7-2.0%. Comparisons among *individuals* from populations of Flabellifera are reported separately (see Methods, for discussion of taxonomic treatment of Flabellifera taxa) for the members of the family Cirolanidae (1.4-4.1%) and Cymothoidae (0-1.4%). Comparisons of *species* of the flabelliferan family Cirolanidae were observed to have 13.6-14.7% sequence divergences. At the *genus* level, five hierarchical comparisons could be made with values ranging between 9.5-32% for all comparisons. At the *family* level, three hierarchical comparisons could be made with the flabelliferan families exhibiting the largest range, 17.7-33.6%. The largest range was clearly exhibited by the flabelliferan family Sphaeromatidae.

The same 33 taxa were used in the nucleotide comparisons (Fig. 2). Overall, these comparisons were comparable to amino acid divergence patterns. Here values for comparisons of *individuals* ranged from 0-3.2%, *species* 32.9-34.9%, *genera* 15.8-37.6%, *families* 22.9-34.5%, and across *suborders* 20.7-35.5%.

The 16S rRNA data set (Fig. 3) contained 49 taxa for which 429 aligned bases were compared. Among *individuals*, pairwise sequence divergences ranged from 0-2.7%. Specimens of Oniscidea (*Ligia exotica*) from South Carolina and Georgia populations were separated by ~240 km, and the sequences obtained are identical. Sequence divergences for oniscid, valviferan, and cirolanid *species* comparisons ranged from 14.5-21.3%. Across-*genera* divergences for phreatoicids, asellotans, valviferans, sphaeromatids, cirolanids, and cymothoids ranged from 8.9-49.1%. Of these comparisons, the Cirolanidae exhibit the greatest divergences (38.9-49.1%). *Familial* comparisons ranged from 34.4-49.1%. The *subordinal* comparisons were 28.2-49.1%.

The 12S rRNA data set (Fig. 4) contained 18 taxa for which 312 aligned bases were used in comparisons. Three comparisons of *individuals* from single populations were possible. The two phreatoicid sequences were identical, the two valviferan sequences were 4.3% dissimilar, and the six cirolanid sequence comparisons were 47-52% dissimilar. The trend toward increasing sequence divergences is maintained for taxonomic levels from *species* to *genera* to *families* to *suborder*, with the greatest value reaching 50.2%.

Transition/transversion bias

A frequently used measure of substitutions is the calculation of transitions (ti) and transversions (tv). Transitions are substitutions between A and G (purines) or between C and T (pyrimidines). Transversions are substitutions between a purine and a pyrimidine. Generally, transitions occur more frequently than transversions, even though for any given nucleotide position twice as many possible transversions may occur as transitions. Figure 5 illustrates the transition/transversion (ti/tv) values

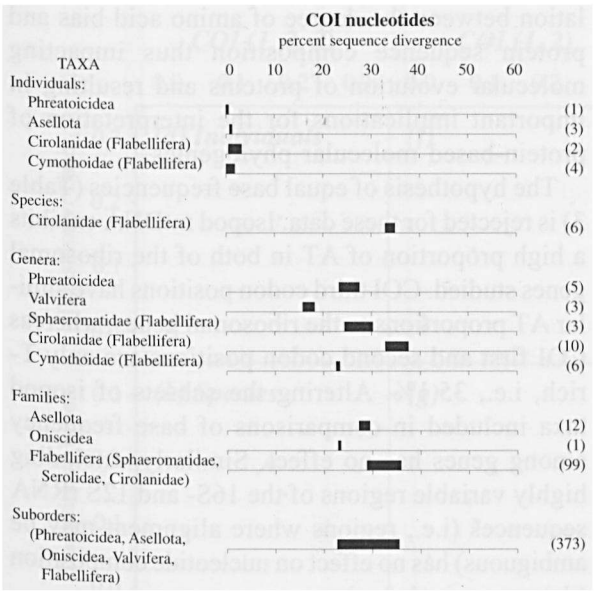

Fig. 2. Percent sequence divergence ("uncorrected p") for cytochrome oxidase c subunit I (442 bases, 33 taxa) plotted against taxonomic level. Minimum and maximum divergence measure expressed as range (bar). Number of pairwise comparisons indicated in parentheses on right.

corrected for sequence length for each hierarchical comparison (*individual*, *species*, *genus*, *family*, and *suborder*) as discussed previously. During the preparation of Fig. 5, the importance of correcting ti/tv ratios for sequence length became obvious when comparing multiple genes, and although some studies have depicted ti/tv plots with regression lines, the non-independence of transitions and transversions make such depictions inappropriate (Purvis and Bromham 1997).

Excluding third codon positions (Fig. 5f-j)[1] reduces ti/tv ratios (compare Fig. 5a-e to 5f-j). These results suggest that for *suborder-*, *family-*, and possibly *genus*-level comparisons, the third positions are saturated. In the ti/tv ratio comparisons for *individuals*, *species*, and *genera* (Fig. 5a, f, k, l, m, p, q, and r), the data points fall into roughly two clusters. This is in part the result of the number and kinds of taxonomic comparisons possible with this data set and reflects the larger divergences observed for the flabelliferan families Cirolanidae and Sphaeromatidae relative to all other isopods.

[1]note sequence comparisons are identical in Figure 5a-e and 5f-j

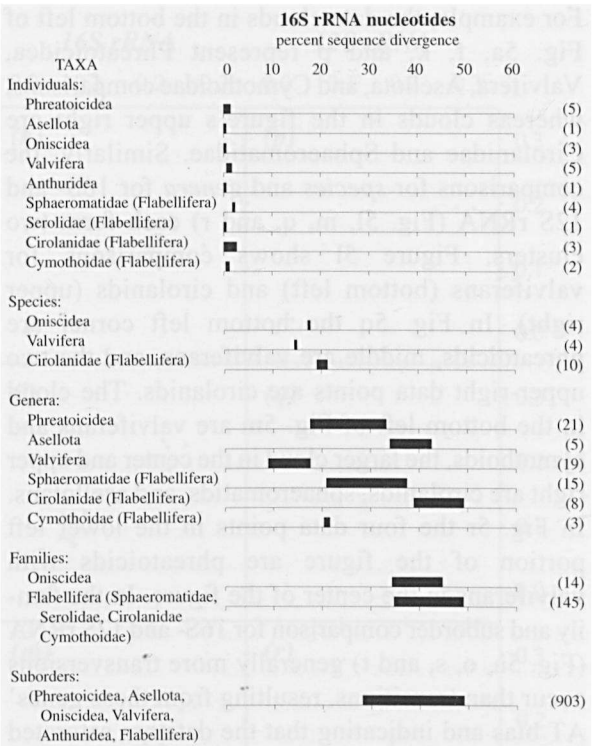

Fig. 3. Percent sequence divergence ("uncorrected p") for 16S rRNA (429 aligned bases, 49 taxa) plotted against taxonomic level. Minimum and maximum divergence measure expressed as range (bar). Number of pairwise comparisons indicated in parentheses on right.

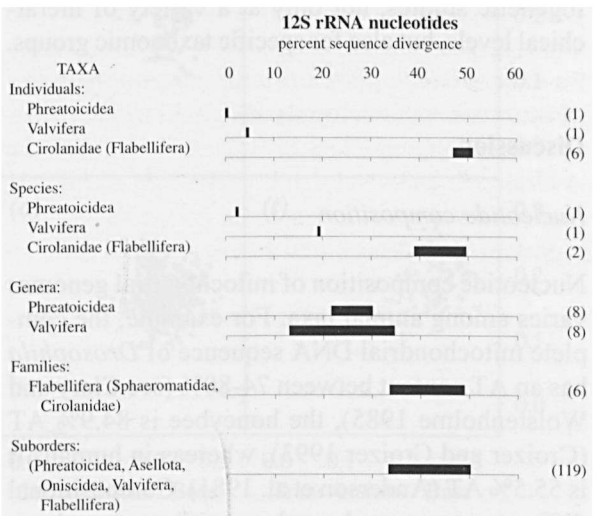

Fig. 4. Percent sequence divergence ("uncorrected p") for 12S rRNA (312 aligned bases, 18 taxa) plotted against taxonomic level. Minimum and maximum divergence measure expressed as range (bar). Number of pairwise comparisons indicated in parentheses on right.

For example, the data clouds in the bottom left of Fig. 5a, f, k, and p represent Phreatoicidea, Valvifera, Asellota, and Cymothoidae comparisons, whereas clouds in the figure's upper right are Cirolanidae and Sphaeromatidae. Similarly, the comparisons for *species* and *genera* for 16S- and 12S rRNA (Fig. 5l, m, q, and r) each form two clusters. Figure 5l shows comparisons for valviferans (bottom left) and cirolanids (upper right). In Fig. 5q the bottom left corner are phreatoicids, middle are valviferans, and the two upper-right data points are cirolanids. The cloud in the bottom left of Fig. 5m are valviferans and cymothoids, the larger cloud in the center and upper right are cirolanids, sphaeromatids, and asellotans. In Fig. 5r the four data points in the lower left portion of the figure are phreatoicids with valviferans in the center of the figure. In the family and suborder comparison for 16S- and 12S rRNA (Fig. 5n, o, s, and t) generally more transversions occur than transitions, resulting from these genes' AT bias and indicating that the data are saturated (i.e., increasing homoplasy masks phylogenetic signal) at these hierarchical levels. In Fig. 5t, the lone data point at the bottom left is a comparison of a cirolanid to an oniscid, clearly two distant taxa whose low ti/tv value reflects saturation. These findings have broad implications for isopod phylogenetic studies, not only at a variety of hierarchical levels, but also for specific taxonomic groups.

Discussion

Nucleotide composition

Nucleotide composition of mitochondrial genomes varies among animal taxa. For example, the complete mitochondrial DNA sequence of *Drosophila* has an AT content between 74-80% (see Clary and Wolstenholme 1985), the honeybee is 84.9% AT (Croizer and Croizer 1993), whereas in humans it is 55.5% AT (Anderson et al. 1981). Compositional differences among homologous sequences have been attributed to both variation in selective constraints and changes in mutation patterns during evolutionary divergence (Perna and Kocher 1995). Singer and Hickey (2000) found a postitive correlation between the degree of amino acid bias and protein sequence composition thus impacting molecular evolution of proteins and resulting in important implications for the interpretation of protein-based molecular phylogenies.

The hypothesis of equal base frequencies (Table 3) is rejected for these data. Isopod mtDNA exhibits a high proportion of AT in both of the ribosomal genes studied. COI third codon positions have similar AT proportions to the ribosomal genes, whereas COI first and second codon positions are only T-rich, i.e., 35.1%. Altering the subsets of isopod taxa included in comparisons of base frequency among genes has no effect. Similarly, excluding highly variable regions of the 16S- and 12S rRNA sequences (i.e., regions where alignment may be ambiguous) has no effect on nucleotide composition (data are not shown).

Overall these data suggest that isopod crustaceans examined here have a smaller AT bias compared to insects. Still, isopod AT bias is more similar to insects and other molting organisms (Ecdysozoa) (e.g., nematodes, Blouin et al. 1998) than to humans. An artifact of AT-rich mtDNA is that taxa have a tendency to group in phylogenetic analyses based more on shared nucleotide composition than on shared history (Hasegawa et al. 1993, Steel et al. 1993), underscoring the importance of appropriate substitution models when estimating phylogenetic relationships. The findings herein are congruent with the 16S rRNA data for Australian freshwater crayfish genera (Lawler and Crandall 1998), which also found an AT bias: A=32.2%, T=35.3%, C=10.8%, and G=21.7%. Similarly, Hanner and Fugate's (1997) study found the 12S rRNA gene of branchiopod crustacean orders to have an average AT bias of A=34.3% and T=31.9%. The findings of Funk et al. (1995) for phytophagus beetles revealed not only a stronger AT bias compared to what has been found in crustaceans, but the AT bias was greater for the 16S rRNA gene than for the COI gene (AT bias for 16S rRNA: A=37.3%, T=41.2%; COI: A=28.9%, T=37.1%). Whitfield and Cameron's (1998) study of hymenopteran taxa exhibited the greatest proportions of AT nucleotides of any organism yet measured. For the 16S rRNA gene, these workers found the mean percent A+T to be 82.2% with the AT content highest in groups

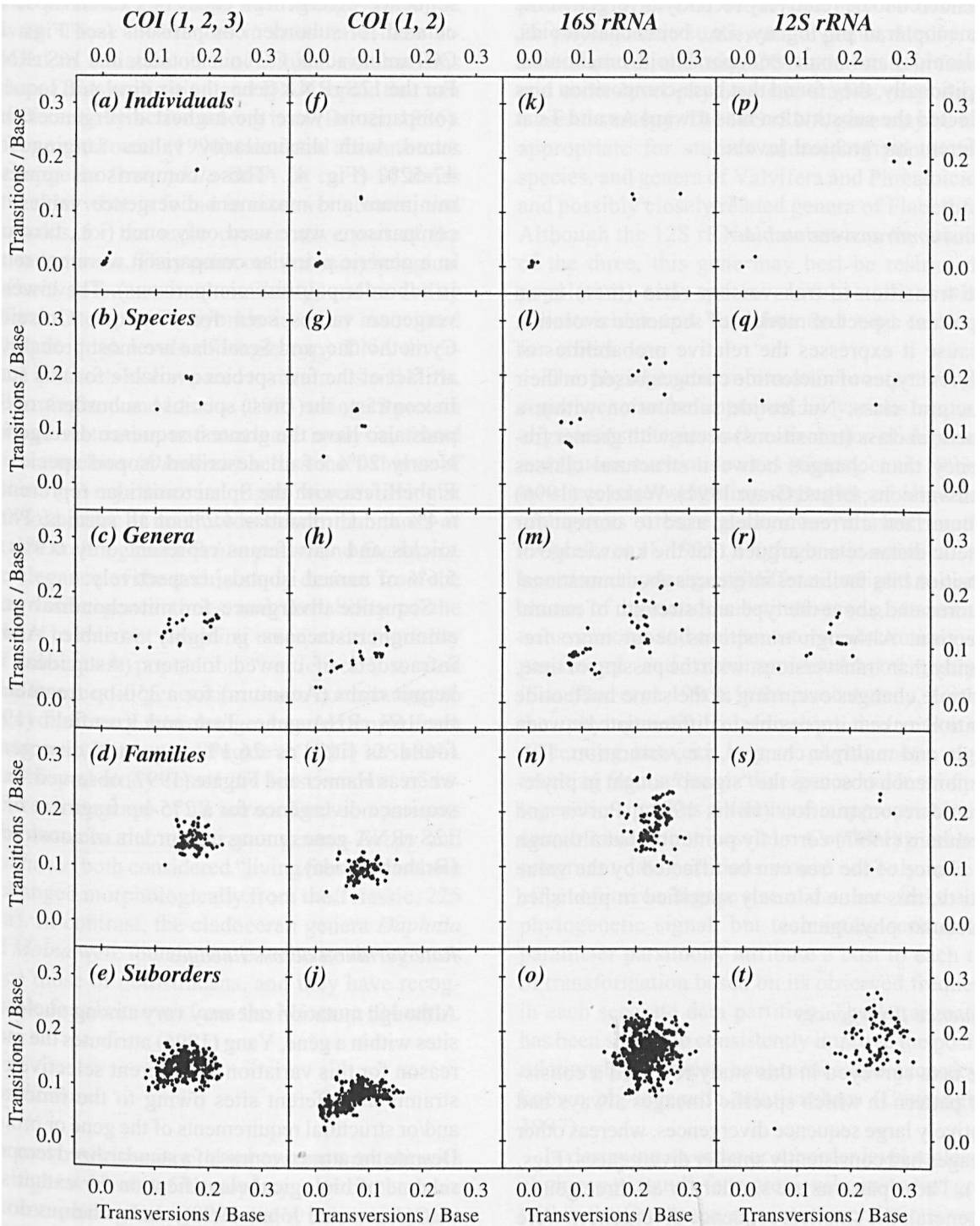

Fig. 5. Number of transitions versus number of transversions in all pairwise comparisons of COI, 16S rRNA, and 12S rRNA sequences corrected for sequence-length variation (Pentcheff, unpublished). COI first, second, and third codon positions (COI 1,2,3) are summarized in the first column (a-e). COI first and second codon positions (COI 1,2) are represented in the second column (f-j). 16S rRNA and 12S rRNA are summarized in the third and fourth columns, k-o and p-t, respectively. This figure gives an indication of the extent of transition/transversion bias and the extent of saturation in substitutions among different hierarchical levels. Some points overlap one another.

considered to be relatively recently diverged in the hymenopteran phylogeny, i.e., bees, chalcidoids, scelionids, and some endoparasitoid brachonids. Additionally, they found that base-composition bias reflected the substitution bias toward As and Ts at different hierarchical levels.

Transition/transversion bias

The transition-to-transversion ratio (ti/tv) is an important aspect of models of sequence evolution because it expresses the relative probabilities of different types of nucleotide changes based on their structural class. Nucleotide substitution within a structural class (transitions) occur with greater frequency than changes between structural classes (transversions; Li and Graur 1991). Wakeley (1996) summarized current models used to correct for genetic distance and argued that the knowledge of transition bias facilitates inferences about mutational patterns and about the type and strength of natural selection. Although transitions occur more frequently than transversions, with the passing of time, multiple changes occurring at the same nucleotide position make it impossible to differentiate between single and multiple changes, i.e., saturation. This phenomenon obscures the "signal" sought in phylogenetic reconstruction (Hillis 1991). Purvis and Bromham (1997) correctly point out that although the choice of the tree can be affected by the value of ti/tv, this value is rarely specified in published molecular phylogenies.

Sequence divergences

The taxa surveyed in this study revealed a consistent pattern in which specific lineages always had relatively large sequence divergences, whereas other lineages had consistently smaller divergences (Figs. 1-4). These patterns are similar for all three genes. In general, the smallest sequence divergences were observed for members of the suborders Phreatoicidea and Valvifera, and the largest divergences observed among members of the flabelliferan families Sphaeromatidae and Cirolanidae. For example, in some generic comparisons of Cirolanidae the sequence divergences equal or exceed those calculated for suborder comparisons (see Figs. 1-3; COI amino acids, COI nucleotides, and 16S rRNA). For the 12S rRNA gene, the six cirolanid sequence comparisons were the highest divergences measured, with dissimilarity values ranging from 47-52% (Fig. 4). These comparisons represent minimum and maximum divergence values, and comparisons were used only once (i.e., taxa used in a generic pairwise comparison were not reused in suborder pairwise comparisons). The lower divergence values seen for Asellota, Oniscidea, Cymothoidae, and Serolidae are most probably an artifact of the few species available for this study. In contrast, the most speciose suborders of isopods also have the greatest sequence divergences. Nearly 20% of all described isopod species are Flabellifera with the Sphaeromatidae representing 6.4% and Cirolanidae 4.2% of all species. Phreatoicids and valviferans represent only 0.9% and 5.6% of named isopods, respectively.

Sequence divergence for mitochondrial genes among crustaceans is highly variable. Among infraorders of clawed lobsters (Astacidea) and hermit crabs (Anomura) for a 350-bp fragment of the 16S rRNA gene, Tam and Kornfield (1998) found as little as 26.1% sequence divergence, whereas Hanner and Fugate (1997) observed 46.8% sequence divergence for a 275-bp fragment of the 12S rRNA gene among infraorders of anostracans (Branchiopoda).

Rate variation across lineages

Although mutation rate may vary among nucleotide sites within a gene, Yang (1996) attributes the major reason for this variation to different selective constraints at different sites owing to the functional and/or structural requirements of the gene or protein. Despite the attractiveness of a standardized temporal scheme of biological classification for extant species (Avise and Johns 1999), such schemes do not account for highly unequal evolutionary rates among lineages. For example, Britten (1986) measured a 5-fold rate change between different vertebrate and invertebrate groups. Rodents, sea urchins, and *Drosophila* have the fastest evolving DNA, whereas

higher primates and some bird lineages have the slowest. These differences in rates have been attributed to variation in biochemical mechanisms such as DNA replication and DNA repair that can be differentially active among taxa (Britten 1986). Caccone and Powell (1990) calculated absolute rates of change in *Drosophila* DNA as ca. 5-10 times faster than what is found in most vertebrates, and this holds for the more conservative part of the nuclear genome. They point out that morphological similarity, chromosomal similarity, and/or ability to form interspecific hybrids is often associated with quite high levels of single-copy DNA divergence in insects as compared to mammals and birds.

Bermingham and Lessios (1993) using 23 protein loci and restriction endonuclease analysis, found that sea urchins (*Diadema*) across the Isthmus of Panama (~3 mya since its origin) were evolving at a 10-fold order of magnitude slower than those of the two urchin genera *Echinometra* and *Eucidaris*. The elegance of the urchin study derives from the known divergence times and the ability of the authors to rule out sampling error, mass mortality and subsequent population "bottleneck", as well as differences in generation times contributing to this evolutionary rate change. In an example from crustaceans, Hanner and Fugate's (1997) 12S rRNA branchiopod study found the least amount of sequence divergence of all intraordinal comparisons to be between the notostracan genera *Triops* and *Lepidurus* (both considered "living fossils" and both unchanged morphologically from the Triassic, 225 mya). In contrast, the cladoceran genera *Daphnia* and *Moina* were found to have sequence divergences twice those of notostracans, and they have recognizable fossils known from the Miocene (24 mya).

Conclusions

The most speciose isopod subordérs, wrought with the highest levels of homoplasy, can be the bane of a morphological systematist's existence. On a molecular level, these same groups may also have the most divergent mitochondrial nucleotide sequences. For all three genes, members of the suborder Flabellifera have the most divergent DNA. Based on the ti/tv ratios (Fig. 5), eliminating third positions from COI sequences may be desirable for phylogenetic studies at the genus, family, and suborder level, and using COI amino acids in family- and order-level phylogenetic studies may also be a useful strategy. The 16S rRNA gene may be most appropriate for studies addressing populations, species, and genera of Valvifera and Phreatoicidea, and possibly closely related genera of Flabellifera. Although the 12S rRNA data set was the smallest of the three, this gene may best be restricted to population and species level studies within Phreatoicidea and Valvifera and used cautiously for others.

The phylogenetic information content of gene sequences can be improved by various sequence alignment strategies such as exclusion of alignment-ambiguous nucleotide sites (Gatesy et al. 1993) or by successive weighting strategies of alignment-ambiguous sites, e.g., by the "elision" method (Wheeler et al. 1995). Phylogenetic noise can also be reduced by assigning different weights to certain classes of nucleotide substitutions (e.g., transitions, transversions, and compensatory substitutions) into parsimony and maximum-likelihood analyses (Swofford et al. 1996). In the present study, in which the phylogenetic relationships are unknown, discerning between loss of phylogenetic signal resulting from sequence divergence and loss of phylogenetic signal as a result of inappropriate taxonomic rank is not possible. Combining multiple congruent data partitions would not only have the effect of increasing the size of data sets and thereby phylogenetic signal, but techniques such as six-parameter parsimony attribute a cost to each type of transformation based on its observed frequency in each separate data partition. The latter method has been shown to consistently improve the positive relationship between congruence and accuracy of known phylogenetic relationships (Cunningham 1997).

In designing an order or suborder level molecular phylogenetic study for a previously unstudied group within the Crustacea, my recommendations would include: (1) collection of a minimum two to four species or genera thought to be most divergent, i.e., most distantly related (this may include taxa which are the most speciose, have unusual lifestyles, morphology, and/or have had a tumultuous taxo-

nomic history); (2) obtain as equal a representation of taxa across the group as possible; (3) survey two to three genes, if possible, (alignment problems are greatly reduced by using protein-coding genes; single copy nuclear genes); (4) carry out preliminary alignments, check data for nucleotide bias, ti/tv ratios, and saturation levels before committing to a large-scale sequencing effort.

Acknowledgements

I thank members of my dissertation committee Bruce Coull, Joe Quattro, Sally Woodin, and Jon Ahlquist for their helpful suggestions and careful reviews of the manuscript. I am especially grateful to Travis Glenn, Trisha Spears, Scott France, and Bob vanSyoc for their extremely helpful technical advice, both at the bench and with the analyses, as well as their invaluable discussions on molecular techniques and phylogenetics. I thank Dean Pentcheff for writing computer programs which immensely aided in data presentation, and for his thoughtful and clear-minded suggestions from which this work has benefited. This research was supported by the National Science Foundation, Dissertation Improvement Grant DEB-9701524; University South of Carolina, American Museum of Natural History, Theodore Roosevelt Memorial Fund; Abele/Spears Laboratory, Florida State University, Tallahassee; Slocum-Lunz Foundation; Sigma Xi, Grants-in-Aid of Research; and by grants from the Environmental Protection Agency and Department of Energy to Bruce Coull. I thank Todd Haney and two anonymous reviewers for their invaluable comments on the manuscript.

Colleagues who contributed specimens are gratefully acknowledged: A. Richardson, University of Tasmania, Hobart, Australia; G. Wilson, Australian Museum, Sydney, Australia; T. Spears and S. Boyce, Florida State University, Tallahassee, Florida; D. Sanger, Marine Resources, Charleston, South Carolina; T. J. Hilbish, University of South Carolina, Columbia, South Carolina; G. C. B. Poore, Museum of Victoria, Melbourne, Australia; T. Stebbins, City of San Diego, San Diego, California; R. Wiseman and C. Biernbaum, College of Charleston, Charleston, South Carolina.

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Received: 20 April 2000