Use of the mitochondrial 16S rRNA gene for phylogenetic and population studies of Crustacea

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ABSTRACT

The mitochondrial 16S DNA is a structural, non-coding gene. Its transcript is the large subunit ribosomal RNA (16S rRNA) that in a conserved secondary structure, and in association with proteins, forms the large subunit of mitochondrial ribosomes. This gene is used frequently for molecular systematics of several animal taxa, including Crustacea. The complete 16S mtDNA sequence is so far known for only one crustacean species, the anostracan *Artemia franciscana*. However, a single region of approximately 520 base pairs has become one of the most commonly used sequences for molecular studies of crustaceans during this decade. Thereby, evolutionary questions of very different temporal and spatial scales have been addressed. To demonstrate this wide range of applications we review previous studies based on the 16S rRNA gene and present new results that resolve some of the phylogenetic relationships between brachyuran families but also allow detection of geographic variation in an intertidal crab species.

1 INTRODUCTION

At the end of the last decade, sequence data for portions of animal (and human) mitochondrial genomes (mtDNA) and their subsequent use for reconstructing phylogenetic relationships revolutionized evolutionary biology (e.g. Avise et al. 1987, Cann et al. 1987, Moritz et al. 1987). MtDNA is maternally inherited, haploid, and present in multiple copies in most cells. These features facilitate its amplification and the interpretation of sequencing results. DNA from crustacean mitochondria was first obtained by Batuecas et al. (1988) in a study of the genome organization of this organelle in *Artemia*. Four years later it was used for the first time in a crustacean molecular phylogeny (Cunningham et al. 1992). So far, the anostracan *Artemia franciscana* is the only crustacean for which the complete mitochondrial genome has been sequenced (Valverde et al. 1994). In this species, it consists of 15,822 basepairs and the typical 37 genes of which 13 are protein coding, 22 are tRNAs and 2 rRNAs.

Most of the presently published molecular phylogenies of crustaceans rely on single genes to reconstruct species relationships. Gene trees are thereby assumed to re-

flect species trees. Besides 16S rRNA, other commonly used mitochondrial genes are the cytochrome oxidase I (COI), cytochrome b (cyt-b), and the small subunit ribosomal RNA (12S rRNA). While COI and cyt-b are mitochondrial coding genes, the 12S and 16S mtDNAs are structural, non-coding genes. Their rRNA transcripts form part of the mitochondrial ribosomes where they play important catalytic roles during translation of mRNAs into proteins. The rRNAs have a secondary structure with stems and loops, and some regions of the molecules are active in biochemical processes and thus more conserved in their sequence. The combination of variable and conserved regions within the same gene is probably one of the reasons why 16S rRNA has become one of the most popular genes for reconstructing animal phylogenies. If properly applied, it allows the study of old phylogenetic relationships as well as fairly recent separation events. On the other hand, the popularity of this gene is probably also due to its successful use by previous workers and the limited number of alternative primers available for invertebrate molecular comparisons.

Here we summarize all previous studies that used 16S rRNA for crustacean systematics or population comparisons, present new sequences for brachyuran crustaceans, and discuss alignment procedures as well as the use of this gene as a molecular clock. To demonstrate the broad range of applications for the 16S rRNA gene, we present two extremes: a phylogeny of the Brachyura based on conserved regions of this gene and an intraspecific analysis of the grapsid marsh crab species *Sesarma reticulatum*.

2 MATERIALS & METHODS

All published information on 16S mtDNA of Crustacea was gathered by means of a literature and genetic database research. We generated additional 16S mtDNA sequences of several brachyuran taxa for phylogenetic and biogeographical comparisons. American specimens were identified by DLF and CDS, material from New Caledonia by P. Castro, and from Spain by I. López de la Rosa. All specimens from which sequences were obtained are deposited in biological collections as vouchers (see Table 1). New sequences have been deposited in the EMBL database (AJ130799-AJ130817).

All specimens used for DNA sequencing were preserved in 75-90% ethanol. Genomic DNA was isolated from the muscle tissue of walking legs or claws using a phenol-chloroform extraction (Kocher et al. 1989). Selective amplification of a ~565 basepair product from the mitochondrial 16S rRNA gene was carried out by a polymerase-chain-reaction (PCR) (35-40 cycles; 1 min 94°/1 min 50-55°/2 min 72° denaturing/annealing/extension temperatures) using primers 16Sar (5'–CGCCTGTTTATCAAAAACAT–3') and 16Sbr (5'–CCGGTCTGAACTCAGATCACGT–3') from Palumbi et al. (1991). PCR products were purified with 100,000–MW Millipore or Microcon 100 filters and sequenced with the ABI BigDye terminator mix in an ABI Prism 310 Genetic Analyzer.

Brachyuran sequences were aligned manually with the multisequence editing program ESEE (Cabot & Beckenbach 1989), with special consideration of the secondary structure of the gene (Machado et al. 1993, Schneider-Broussard & Neigel 1997). For our phylogenetic analysis, we used only sequences that included the complete length

Table 1. List of species for which 16S mtDNA was sequenced and used for phylogenetic analyses of brachyuran families. MNHN-B: Muséum National d'History Naturelle, Paris; R: Colllection Rudolf Diesel, Starnberg; SMF: Senckenberg Museum, Frankfurt a.M.; USLZ: University of Southwestern Louisiana Zoological Collection, Lafayette.

Species	Family	Locality of collection	Collection number
Petrolisthes armatus	Porcellanidae	Louisiana: Grande Isle	ULLZ 3779
Xantho poressa	Xanthidae	Spain: Cádiz	ULLZ 3808
Panopeus herbstii	Panopeidae	South Carolina: Charleston	ULLZ 3778
Menippe nodifrons	Menippidae	Mexico: Veracruz	ULLZ 3720
Trapezia cymodoce	Trapeziidae	New Caledonia	MNHN-B 24961
Cancer irroratus	Cancridae	Maine: Portland	ULLZ 3843
Carcinus maenas	Portunidae	New Hampshire: Hampton B.	ULLZ 3840
Callinectes sapidus	Portunidae	Louisiana: Isles Dernieres	ULLZ 3895
Epilobocera sinuatifrons	Pseudothelphusidae	Puerto Rico: Guajataca	R 199
Eudaniela garmani	Pseudothelphusidae	Trinidad	R 269
Palicus obesus	Palicidae	Gulf of Mexico	ULLZ 3852
Crossotonotus compressipes	Palicidae	New Caledonia	MNHN-B 6215
Pinnixa retineus	Pinnotheridae	Texas: Corpus Christi	ULLZ 3870
Pachygrapsus transverus	Grapsidae	Louisiana: Grande Isle	ULLZ 3782
Percnon gebbesi	Grapsidae	Puerto Rico: North coast	R 153
Sesarma reticulatum	Grapsidae	Delaware: Woodland Beach	ULLZ 3835
Cyrograpsus altimanus	Grapsidae	Argentina: Santa Clara	SMF 24545
Cardisoma crassum	Gecarcinidae	Costa Rica: Rincón	SMF 24543
Gecarcinus lateralis	Gecarcinidae	Mexico: Veracruz	ULLZ 3722

between the 16Sar and 16Sbr primers (~ 520 basepairs). The following sequences from molecular databases were included: *Scopimera globosa* (accession number AB002124), *Leipocten trigranulum* (AB002129), *Uca lactea* (AB002130), *Macrophthalmus banzai* (AB002132) (all Ocypodidae), *Mictyris brevidactylus* (AB002133) (Mictyridae), *Menippe mercenaria* (U20737), and *M. mercenaria* nuclear copy (U20738) (Menippidae). Distance matrices of sequence divergence were analyzed using Kimura 2-parameter distances and neighbor-joining (NJ) (Saitou & Nei 1987) with the program MEGA (Kumar et al. 1993). Statistical significance of groups within inferred trees was evaluated by the interior branch method (Rzhetsky & Nei 1992) and by bootstrapping the maximum parsimony (MP) analysis with 1000 replicates using the program PAUP (Swofford 1993). For the MP analysis, transversions were weighted three times greater than transitions and gaps were treated as missings.

3 RESULTS

Table 2 summarizes all presently published studies that use 16S mtDNA for either phylogenetic or population studies of crustaceans. As can be seen from this table and Table 3, most primer combinations have been used to amplify a DNA region of approximately 520 basepair length (here termed '16Sar-br region' after the corresponding universal primers designed by T.D. Kocher and S.R. Palumbi). Palumbi et al. (1991) showed variations of these primers for some animal taxa, including the arthropod *Drosophila* (Table 3). Since then, the 16Sar-br region has been clearly the most commonly sequenced region of 16S mtDNA. In few cases (not including the *Artemia* complete mtDNA sequence), crustacean workers have sequenced beyond this region, which would determine the actual sequence in the primer regions. These cases are the recent study by Kitaura et al. (1998) that revealed the sequence (5'–YGCCTGTTTATYAAAAACAT–3') for the 16Sar region in ocypodid crabs and a study by Crandall & Fitzpatrick (1996) showing the sequence (5'–CCGGTCTGAACTCAAATCATGT–3') for the 16Sbr region in cambarid crayfish.

Table 2. List of studies that use 16S mtDNA sequence for crustacean phylogenies or population genetics, with the number of individuals (ind.), species (sp.), genera (gen.), and families (fam.) included, as well as the sequence or alignment lengths in basepairs (bp) and primers used.

Taxonomic groups	Length	Primer combination	References
Cladocera			
Daphniidae: *Daphnia* (4 sp./13ind.)	491 bp	ar & br	Taylor et al. 1998
Copepoda			
Calanidae, Metridinidae: *Calanus, Metridia* (10sp/14ind.)	387 bp	ar-Dr & br-Dy	Bucklin et al. 1995
Calanidae: *Pseudocalanus* (2 sp./19 ind.)	250 bp	167 & br-Dy	Bucklin et al. 1998a
Calanidae: *Nannocalamus minor* (2 sp.?/155 ind	440 bp	ar-Dr & br-Dy	Bucklin et al. 1996a
Calanidae: *Calanus* (3 sp./20 ind.)	430 bp	ar-Dr & br-Dy	Bucklin et al. 1992
Calanidae: *Calanus pacificus* (27 ind.)	449 bp	ar-Dr & br-Dy	Bucklin & Lajeunesse 1994
Calanidae: *Calanus finmarchicus* (182 ind.)	350 bp	ar-Dr & br-Dy	Bucklin & Kocher 1996, Bucklin et al. 1996b
Calanidae: review of above data on Calanidae		ar-Dr & br-Dy	Bucklin et al. 1998b
Isopoda			
Serolidae: (11 gen./15 sp.)	503 bp	ar & br	Held, in press
Amphipoda			
Lysianassidae: (4 gen./7sp./32 ind.)	179 bp	Amph1 & Amph2	France & Kocher 1996a
Lysianassidae: *Eurythenes gryllus* (95 ind.)	437 bp	ar & br-Dr, Amph1 & Amph2	France & Kocher 1996b
Euphausiacea			
Euphausiidae: (3gen./sp./6 ind.)	524 bp	ar & br	Patarnello et al. 1996
Decapoda			
Penaeidae: *Penaeus* (2 sp./7 ind.)	438 bp	ar & br	Machado et al. 1993
Penaeidae: *Penaeus* (11 sp./=40 ind.)	472 bp	ar & br	Chu et al. 1998
Penaeidae: *Metapenaeopsis* (7 sp./16 ind.)	475 bp	ar & br	Tong et al., in press

Table 2. Continued.

Taxonomic groups	Length	Primer combination	References
Palinuridae: *Palinurus* (4 sp./13 ind.)	491 bp	ar & br	Sarver et al. 1998
Nephropidae: (5 gen./7 sp.)	350 of 474 bp	ar & SB	Tam & Kornfield 1998
Cambaridae: (3 gen./38 sp./72 ind.)	554 bp	1471 & 1472	Crandall & Fitzpatrick 1996; Crandall 1998
Cambaridae & Parastacidae: (9 gen./12 sp.)	535 bp	1471 & 1472	Crandall et al. 1995
Parastacidae: (3 gen./9 sp)	~550 bp	1471 & 1472	Lawler & Crandall 1998
Parastacidae: *Euastacus* (10 sp.)	461 bp	ar & br	Ponniah & Hughes 1998
Paguridae, Lithodidae et al.: (4 fam./7 gen./10 sp./12 ind.)	420 bp	ar & br	Cunningham et al. 1992
Hippidae: *Emerita* (6 sp./9 ind.)	~400 bp	ar & 16SB	Tam et al. 1996
Menippidae: *Menippe* (2 sp./ 9 ind.)	525 bp	ar & br, ar-M & br-I	Schneider-Broussard & Neigel 1997, Schneider-Broussard et al. 1998
Portunidae: *Carcinus, Callinectes* (5 sp./47 ind.)	395 bp	Dar &Dbr	Geller et al. 1997
Grapsidae & Gecarcinidae: (26 gen.)	531 of 589 bp	ar & br, 1472	Schubart et al., in press
Grapsidae: Sesarminae (4 gen./21 sp.)	522 bp	ar & br, L12 & H16	Schubart et al. 1998
Grapsidae: *Sesarma* (2 sp./16 ind.)	526 bp	ar & br, L12 & H16	Schubart et al., 1998b
Grapsidae: *Pachygrapsus* (2 sp./ 5 ind.)	510 bp	ar & br, L12 & H16	Cuesta & Schubart, 1998
Ocypodidae et al.: (7 gen./30 sp. 24 ind.)	491	ar & br	Levinton et al. 1996
Ocypodidae: *Uca* (24 sp./27 ind.)	491	ar & br	Sturmbauer et al. 1996
Ocypodidae: (10 gen./ 20 sp.)	~1170	br, L2482, L2510, H2492, H2716, H3058, H3062	Kitaura et al. 1998

Alignment of 16S mtDNA corresponding to the 16Sar-br region from several brachyuran families confirmed a marked heterogeneity in sequence conservation among portions of this region. Even after applying secondary structure models to this alignment (Machado et al. 1993, Schneider-Broussard & Neigel 1997), the most variable regions could not be aligned with enough certainty to assume that homologous positions were being compared. We therefore excluded these regions and positions with compensatory mutations (see Discussion) from the phylogenetic analyses. Our initial alignment of 572 positions was thereby reduced to 426 basepairs, of which 215 were variable and 170 parsimony-informative. A phylogenetic analysis of brachyuran families based on these conserved regions of the 16S mtDNA was carried out with NJ and MP. The resulting tree is presented in Figure 1. Only confidence / bootstrap values above 50% of the interior-branch method (NJ) and of the bootstrap analysis (MP) are shown.

In another case study, the 16Sar-br region was sequenced for several specimens of

Table 3. List of primers used for amplifying 16S mtDNA in crustaceans.

Name	Primer sequence (5' -> 3')	Designation	References
Forward primers (L-strand)			
1. 16Sar primer and modifications			
ar:	CGCCTGTTTATCAAAAACAT	universal	Palumbi et al. 1991
ar-Dr:	CGCCTGTTTAACAAAAACAT	*Drosophila*	Palumbi et al. 1991
1471:	CCTGTTTANCAAAAACAT	Evertebrata	Crandall & Fitzpatrick 1996
Dar:	CGCCTGTTTAHYAAAAACAT	universal	Geller et al. 1997
L2510:	CGCCTGTTTAACAAAGACAT	Evertebrata	Kitaura et al. 1998
2. Internal primers to 16Sar-br region			
167:	GACGAGAAGACCCTATGAAG	Calanoida	Bucklin et al 1998a
Amph1:	GACGACAAGACCCTAAAAGG	Amphipoda	France & Kocher 1996
ar-M:	ATAAGACCCTATAAAGC	*Menippe*	Schneider-Broussard & Neigel 1997
L12:	TGACCGTGCAAAGGTAGCATAA	Grapsoidea	Schubart et al. 1998a
3. External primers to 16Sar-br region			
L2482:	GAAGGAACTCGGCAA	universal?	Kitaura et al. 1998
Reverse primers (H-strand)			
1. 16Sbr primer and modificications			
br:	CCGGTCTGAACTCAGATCACGT	universal	Palumbi et al. 1991
br-Dr:	CCGGTTTGAACTCAGATCATG	*Drosophila*	Palumbi et al. 1991
br-Dy	CCGGTTTGAACTCAGATCACGT	*Drosophila yakuba*	Bucklin et al. 1995
SB:	CTCCGGTTTGAACTCAGATC	Arthropoda	Xiong & Kocher 1991
Dbr:	CCGGTCTGAACTCAGMTCAYGT	universal	Geller et al. 1997
H3062:	CCGGTCTGAACTCAGATCA	universal	Kitaura et al. 1998
H3058	TCCGGTCTGAACTCAGATCACGTA	universal	Kitaura et al. 1998
H2492:	CAGACATGTTTTTAATAAACAGGC	~reverse of ar	Kitaura et al. 1998
2. Internal primers to 16Sar-br region			
Amph1	CGCTGTTATCCCTAAAGTA	Amphipoda	France & Kocher 1996
br-I:	CCGCCCCAGCAAAATAAA	*Menippe*	Schneider-Broussard & Neigel 1997
H16:	TTATCRCCCCAATAAAATA	Grapsoidea	Schubart et al. 1998a
H2716i	AAGTTTTATAGGGTCTTATCGTC	Ocypodoidea	Kitaura et al. 1998
3. External primers to 16Sar-br region			
1472:	AGATAGAAACCAACCTGG	Evertebrata	Crandall & Fitzpatrick 1996

the grapsid marsh crab *Sesarma reticulatum* to determine intraspecific variability and to test the hypothesis that populations from the Gulf of Mexico are distinct from populations of the American Atlantic between Massachusetts and Florida (here called Atlantic) and might represent a distinct species (Zimmerman & Felder 1991, Staton & Felder 1992, Felder & Staton 1994). The comparison of 532 basepairs of mtDNA from eight crab specimens collected at six localities between Delaware and Texas revealed the existence of five different haplotypes. From their genetic divergence, these haplotypes can be clearly separated into two distinct groups, corresponding to the Gulf of Mexico and the Atlantic (Table 4). Haplotypes from the Gulf of Mexico differed by at most three nucleotide substitutions, with the most divergent haplotype found in western Florida. Likewise, there was also little divergence among haplotypes from the Atlantic; the same haplotype was found in three individuals and the second haplotype differed by only one transition. In contrast, eight diagnostic sequence differences (2-3 transversions, 4-5 transitions and 1 indel) separate Gulf of Mexico from Atlantic haplotypes. The extent of this sequence divergence suggests a relatively long period of isolation.

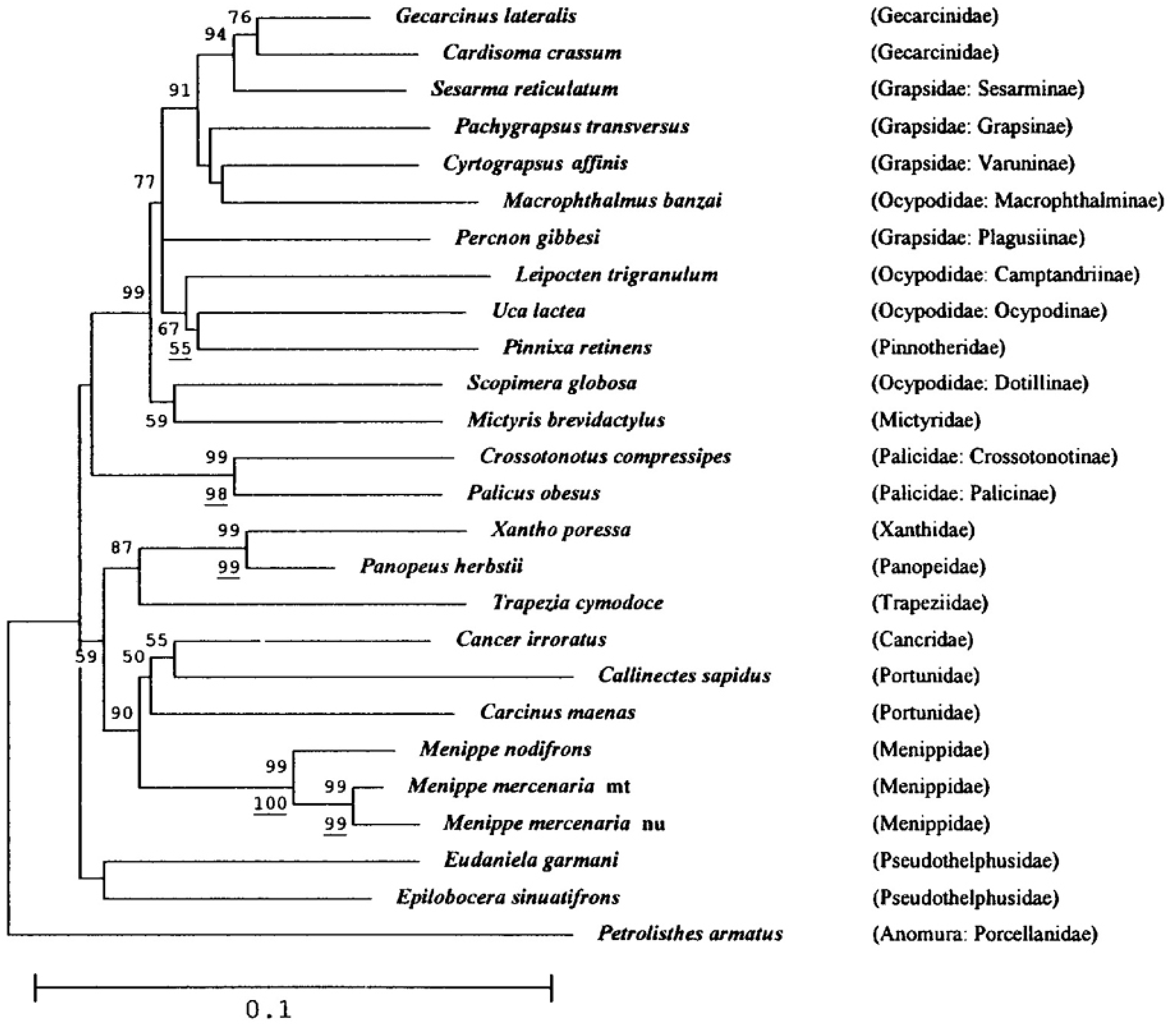

Figure 1. Phylogenetic relationships among several brachyuran families as inferred from 426 co-served basepairs of the 16S rRNA gene. The tree topology is based on a neighbor-joining analysis using Kimura 2-parameter distances. Confidence values are from an internal node test and a bootstrap maximum parsimony analysis (underlined) (only values > 50 shown); mt: mitochondrial gene, nu: nuclear copy.

Table 4. Percent genetic divergence and number of differences between 532-basepair of the 16S mt-DNA of 8 specimens of *Sesarma rerticulatum* from the Gulf of Mexico (GM) and the northwestern Atlantic (ATL); (s: Transition, v: Tranverion, i: Indel).

	GM-1	GM-2	GM-3	GM-4	ATL-1	ATL-2, 3, 4
GM-1 (Sabine Pass, TX)	–	0.2	0	0.4	1.5	1.7
GM-2 (Calcasieu Pass, LA)	1s	–	0.2	0.6	1.5	1.7
GM-3 (Cocodrie, LA)	0	1s	–	0.4	1.5	1.7
GM-4 (Alligator Point, FL)	1v, 1s	1v, 2s	1v, 1s	–	1.5	1.9
ATL-1 (Brunswick, GA)	3v, 4s, 1i	3v, 4s, 1i	3v, 4s, 1i	2v, 6s, 1i	–	0.2
ATL-2 (Brunswick, GA)	3v, 5s, 1i	3v, 5s, 1i	3v, 5s, 1i	2v, 7s, 1i	1s	–
ATL-3, 4 (Woodland Beach, DE)	3v, 5s, 1i	3v, 5s, 1i	3v, 5s, 1i	2v, 7s, 1i	1s	–

4 DISCUSSION

The broad range of applications for mitochondrial 16S rRNA gene sequences in phylogenetic and population studies of crustaceans has been demonstrated (Table 2, present results). However, certain characteristics of this gene should be taken into account when using it for molecular systematics. While not subject to the constraints of protein-coding sequences, the rRNA product is subject to specific secondary structure constraints, and its sequence is heterogeneous in the degree of conservation. Occasionally, copies of the 16S rRNA gene are translocated into the nuclear genome.

4.1 *Secondary structure*

Molecules of rRNA have conserved secondary structures that consist of base-paired stems and unpaired loops, as well as regions that play important roles in essential biochemical processes (binding of protein, mRNA, tRNA, and termination suppression). This configuration of localized structural and functional constraints places regions of high sequence conservation adjacent to highly variable ones, and is one of the reasons this gene has such a wide range of application in evolutionary studies. On the other hand, the relevance of the secondary structure should not be overlooked. Alignments of divergent rRNA sequences should begin with the underlying secondary structure, which should then be used to 'anchor' the alignment (see Kjer 1995). The secondary structure is certainly the most conserved character of this gene and therefore the alignment of individual nucleotides should be subordinated to the conservation of the overall structure to determine homologous positions. For four crustacean species, hypothetical models of secondary structures are available: *Artemia* sp. (see Palmero et al. 1988), *Daphnia* sp. (see Taylor et al. 1998), *Farfantepenaeus* (formerly *Penaeus*) *notialis* (see Machado et al. 1993) and *Menippe* sp. (see Schneider-Broussard & Neigel 1997). Even though these reconstructions are not entirely consistent with each other (probably none of them reflects the final truth, for which one would need the entire rRNA molecule to consider all potential stems), they represent the most important stem and loop regions within the 16Sar-br region. The addition of gaps to alignments (to compensate for insertions or deletions) should preferably occur in loop regions (see Kjer 1995).

Another characteristic of the secondary structure is that mutations in stem regions are normally followed by compensatory mutations on the other branch of the stem to re-establish C-G, A-U, or G-U base pairs. Such compensatory mutations help to verify stem regions and after being recognized should be regarded as single mutation events or character states (Kjer 1995).

4.2 *Different forms of mutations*

Three forms of single-base mutations must be taken into account when aligning and analyzing DNA sequences: transitions, transversions, and indels (insertions and deletions). Transitions are fairly common in comparison to transversions. They represent the change from one pyrimidine to the other, or one purine to the other and thus do not involve much structural change. In some phylogenetic analyses, transversions may therefore be weighted more than transitions.

Unlike in protein-coding genes, there is no selective pressure against shifts in the reading frame throughout an rRNA sequence. A deletion or insertion of a single base makes a coding gene non-functional, while it might not have any effect on an rRNA molecule. As a consequence, indels accumulate in rRNAs over time and result in considerable length variation of the same gene across taxa. The difficulty for molecular systematists consists in determining where to place gaps when aligning homologous sequences to ensure comparison of homologous bases. As discussed above, the alignment should be based first on secondary structure. Once the secondary structure is determined and ambiguous positions are confined to variable loop regions, gaps should be placed in a way that transitions become more common than transversions, especially if transversions are given stronger weight in subsequent phylogenetic analyses. If uncertainty about the exact alignment of variable regions remains, it is advisable not to include these regions in phylogenetic analyses.

4.3 *Molecular dating*

In three studies, the rate of molecular change has been estimated independently for the 16Sar-br region of the 16S rRNA gene in crustaceans. Cunningham et al. (1992) related the divergence of left and right-handed hermit crabs (73-78 Myr), the vicariance of Atlantic and Pacific cold-water marine faunas (35-40 Myr), and the vicariance of the amphi-Atlantic boreal marine province (2.5-3.1 Myr) to their modified Kimura distances in order to calculate the separation of king and hermit crabs. On the basis of their data, we calculated a divergence rate of 0.38% per Myr (not 2.2% per Myr as erroneously cited in Schubart et al. 1998a) for anomuran crabs. The other two available calibrations are for brachyuran crabs and are based on genetic distances of transisthmian species pairs across the Panama Isthmus (closure approximately 3.1 Myr ago) resulting in a divergence rate of 0.9% per Myr (Sturmbauer et al. 1996) for *Uca* and 0.65-0.88% per Myr (Schubart et al. 1998a) for *Sesarma*. The anomuran rate is strikingly lower, but more recent results from this taxon indicate that this may be artifactual (Cunningham, pers. comm.). The brachyuran rates, in contrast, are fairly similar and their use is therefore recommended for molecular dating in brachyuran crabs, especially when looking at relatively recent time periods (e.g. Schneider-Broussard et al. 1998).

Several crustacean species are characterized by a large number of different haplotypes for the 16S rRNA gene. This is especially true for planktonic species: 68 haplotypes in *Nannocalanus minor* Type I (see Bucklin et al. 1996a), 30 in *Calanus finmarchicus* (see Bucklin et al. 1996b) 13 in *Pseudocalanus moultoni* (see Bucklin et al. 1998a). In contrast, single haplotypes have been documented for two species of the green crab genus *Carcinus* (see Geller et al. 1997) and along entire coastlines for the littoral grapsid crab species *Pachygrapsus transversus* (unpubl. data). Marked intraspecific variation can possibly blur calculations of molecular clocks when single representatives are used for each species. To increase the accuracy of the clock, several individuals per population/species should be sequenced and only invariable positions used for the molecular calibration.

Caution also needs to be taken when comparing rates of sequence divergence, if regions of different length and location within the 16S rRNA gene have been used for calibration. As stated above, this gene contains highly conserved and more variable

regions. Often the areas adjacent to the primers are rather conserved. Studies that do not include the entire region, most often missing the regions closest to the primers, might therefore result in an upwardly biased estimate of divergence. In some analyses of sequence data, the most variable regions are excluded, because of the difficulty of a precise alignment (e.g. this study). In this case, molecular dating cannot be performed using calibrations from other studies, unless the restricted data set is recalibrated. Molecular dating of older events often suffers from convergences and multiple substitutions that accumulate in the sequences, while dating of very recent events must take into account sequence polymorphism (see above). In all cases, molecular clocks should ideally be calibrated with geological events from a similar time period and using estimates that allow for multiple substitutions.

4.4 *Pseudogenes*

A potential risk in the use of the 16S rRNA gene for phylogenetic analyses are translocated copies of this gene in the nuclear genome. In the *Menippe mercenaria* – *M. adina* complex, the presence of such a pseudogene has been recently shown and discussed by Schneider-Broussard & Neigel (1997) and Schneider-Broussard et al. (1998). Similar findings with 16S mtDNA of ghost shrimp (Bilodeau, pers. comm.) and another mitochondrial gene in crabs (unpubl. observ.) suggest that the occurrence of pseudogenes is not an unusual phenomenon and is a potential source of artifacts. In the case of the *Menippe mercenaria* – *M. adina* complex, the closest relative, *M. nodifrons*, constitutes an outgroup to both the mitochondrial gene and the nuclear copy (Fig. 1). The pseudogene thus evolved after separation of these species. Accidental sequencing of the pseudogene in this case would not alter the phylogeny. Nevertheless, it can severely confuse results in studies of lower systematic levels or biogeographic analyses. The fact that pseudogenes in the nuclear genome are nonfunctional makes it likely that mutations occur also within normally conserved regions, and thus primers should not amplify these translocated copies after prolonged evolutionary times.

4.5 *Present results and outlook*

The two studies here presented exemplify scenarios of very different evolutionary time scales and consist of preliminary results. The geological record of brachyuran crabs suggests that most of the divergence into what we consider modern families took place 30-40 Myr ago (Glaessner 1969, Schram 1986). Our tree of a few selected brachyuran families suggests one very early split into two main groups. On one branch we find the Grapsidae, Gecarcinidae, Ocypodidae, Mictyridae and Pinnotheridae (all Thoracotremata sensu Guinot 1978), on the other we have the Xanthoidea, Portunidae, Cancridae (all Heterotremata) thus rendering support to the classification of Guinot (1978). The position of the Palicidae (monophyletic) and Pseudothelphusidae (early split between South and Central American representatives) is unresolved, which is also the case in morphological taxonomy. More ancestral Brachyura (Podotremata) were not included (see Spears et al. 1992). The phylogenetic tree furthermore suggests a paraphyly of the Grapsidae (inclusion of the Gecarcinidae and Macrophthalminae), a polyphyly of the Ocypodidae (different subfamilies paired with

several other families like Pinnotheridae and Grapsidae) and a polyphyly of the Xanthoidea, justifying the splitting into different families (sensu Guinot 1978). The internal node method (NJ) is based on a standard error test and confidence values reflect statistical significance levels. Excessive weight should therefore not be placed on groupings with levels under 95%. This is corroborated by the MP bootstrap analysis that results in a large consensus for most of the tree with exception of some of the more recent separations (Xanthidae-Panopeidae, subfamilies of the Palicidae, *Menippe* sp.) (see Fig. 1). The lack of resolution with the parsimony method is possibly due to genetic saturation in the variable regions (increased numbers of homoplasies) and an insufficient substitution rate in the conserved ones. The time of 30 to 40 Myr thus seems to represent a limit for the use of the 16S rRNA gene in phylogenies. Sequencing of additional genes and representatives from other families, will potentially help to better resolve some of the suggested branching patterns in brachyuran phylogeny.

In the molecular biogeographic study of *Sesarma reticulatum*, sequences from additional specimens need to be obtained in order to determine the approximate number of haplotypes present. If the pattern of molecular divergence between the Gulf of Mexico and the Atlantic continues to hold, recognition of a new species will be supported by 16S mtDNA, confirming previous distinctions based on allozymes, color, and physiology (see Zimmerman & Felder 1991, Staton & Felder 1992, Felder & Staton 1994, Mangum & McKenney 1996). Also in other studies, 16S mtDNA clearly separates populations of crustacean species (Bucklin & Lajeunesse 1994, Bucklin et al. 1996a, France & Kocher 1996b, Sarver et al. 1998, Cuesta & Schubart 1998, Schubart et al. 1998b), the last study including the description of a new species.

Repeated new discoveries of cryptic species among plant and animal groups by molecular methods, raises the question of whether genetically distinct populations that are morphologically indistinguishable should be described as separate species. This would involve some assumptions and methodological disadvantages (identification only possible after DNA extraction). On the other hand, molecular evolution is a continuous process and isolated populations will always diverge over time. However, morphology does not necessarily change over extended time periods if ecological factors remain constant, as shown by comparison of several trans-isthmian marine invertebrate species. Along with the molecular changes there may be physiological, ecological or behavioral changes that are not readily evident in morphology, as for *Sesarma reticulatum* in studies to date (Zimmerman & Felder 1991, Staton & Felder 1992). Molecular findings can thus 'alert' systematists to potentially distinct populations and instigate studies that might reveal divergence in additional non-molecular characters. However, even without those additional findings, molecular systematics should be viewed as a highly effective tool for documenting and classifying the biological diversity of this world.

ACKNOWLEDGEMENTS

This paper was presented as an oral contribution at the Fourth International Crustacean Congress in Amsterdam in July 1998 and we wish to thank the organizers of this

meeting. The present study was funded by the U.S. Department of Energy (grant no. DE-FG02-97ER12220). We extend our gratitude to Lanie Bilodeau, Pedro Castro, Keith Crandall, José Antonio Cuesta, Rudolf Diesel, Danièle Guinot, Michelle Harrison, Carol Lee, Inmaculada López de la Rosa, Harriet Perry, Eduardo Spivak, and Kit Tam for providing information, material, or sequences, assisting during collections, or identifying specimens. Jun Kitaura, Mutsumi Nishida, and Keiji Wada kindly made several complete 16Sar-br sequences available for the brachyuran phylogeny. Some sequences were generated in the laboratory of Blair Hedges (Pennsylvania State University), when CDS was funded by the Deutsche Forschungsgemeinschaft (grant Di 479-2). Danièle Guinot, Christoph Held, and one anonymous referee assisted with valuable comments on the manuscript. This is contribution no. 65 from the USL Laboratory for Crustacean Research.

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[In: The Biodiversity Crisis and Crustacea. Proceedings of the Fourth International Crustacean Congress, Amsterdam, Netherlands, 20-24 July 1998, vol. 2]

The page proofs of this manuscript contained several serious mistakes. Unfortunately the editors of Crustacean Issues only considered some of the corrections that we made. The important corrections that were not considered are being repeated here and the references in press updated.

p. 819, Table 1 (see attached original version)

- heading: "ULLZ: University of Louisiana Lafayette Zoological Collection, Lafayette" (not USLZ)
- taxonomic authorities see original version (omitted in printed version)
- *Pinnixa retinens* (not *P. retineus*)
- *Pachygrapsus transversus* (not *P. transverus*)
- *Cyrtograpsus affinis* from Argentina: Río de la Plata (not *C. altimanus* from Santa Clara)

p. 820-821, Table 2 (see attached original version)

- Penaeidae: *Penaeus* (11 sp./~40 ind.) (not = 40 ind.)

p. 822, Table 3 (see attached original version)

- Amph1: GACGACAAGACCCTAAAAGC (not GACGACAAGACCCTAAAAGG)

p. 823, Table 4 (see attached original version)

- heading: "s: transition, v: transversion, i: indel" (not s: Transition, v: Tranverion, i: Indel)

p. 829, References update:

Held, C. 2000. Phylogeny and biogeography of serolid isopods (Crustacea, Isopoda, Serolidae) and the use of ribosomal expansion segments in molecular systematics. Mol. Phylogenet. Evol. 15(2): -178.

Schubart, C.D., J.A. Cuesta, R. Diesel & D.L. Felder, 2000. Molecular phylogeny, taxonomy, and evolution of non-marine lineages within the American Grapsoidea (Crustacea: Brachyura). Mol. Phylogenet. Evol. 15(2): 179-190.

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Table 1. List of species for which 16S mtDNA was sequenced and used for phylogenetic analyses of brachyuran families. MNHN-B: Muséum National d'Histoire Naturelle, Paris; R: Collection Rudolf Diesel, Starnberg; SMF: Senckenberg Museum, Frankfurt a.M.; USLZ: University of Southwestern Louisiana Zoological Collection, Lafayette.

Species	Family	Locality of collection	Collection number
Petrolisthes armatus (Gibbes, 1850)	Porcellanidae	Lousiana: Grande Isle	USLZ 3779
Xantho poressa (Olivi, 1792)	Xanthidae	Spain: Cádiz	USLZ 3808
Panopeus herbstii H. Milne Edwards, 1834	Panopeidae	South Carolina: Charleston	USLZ 3778
Menippe nodifrons Stimpson, 1859	Menippidae	Mexico: Veracruz	USLZ 3720
Trapezia cymodoce (Herbst, 1801)	Trapeziidae	New Caledonia	MNHN-B 24961
Cancer irroratus Say, 1817	Cancridae	Maine: Portland	USLZ 3843
Carcinus maenas (Linnaeus, 1758)	Portunidae	New Hampshire: Hampton B.	USLZ 3840
Callinectes sapidus Rathbun, 1896	Portunidae	Louisiana: Isles Dernieres	USLZ 3896
Epilobocera sinuatifrons (A. Milne Edwards, 1866)	Pseudothelphusidae	Puerto Rico: Guajataca	R 199
Eudaniela garmani (Rathbun, 1898)	Pseudothelphusidae	Trinidad	R 269
Palicus obesus (A. Milne Edwards, 1880)	Palicidae	Gulf of Mexico	USLZ 3852
Crossotonotus spinipes (de Man, 1888)	Palicidae	New Caledonia	MNHN-B 26215
Pinnixa retinens Rathbun, 1918	Pinnotheridae	Texas: Corpus Christi	USLZ 3870
Pachygrapsus transversus (Gibbes, 1850)	Grapsidae	Lousiana: Grande Isle	USLZ 3782
Percnon gibbesi (H. Milne Edwards, 1853)	Grapsidae	Puerto Rico: north coast	R 153
Sesarma reticulatum (Say, 1817)	Grapsidae	Delaware: Woodland Beach	USLZ 3835
Cyrtograpsus altimanus Rathbun, 1898	Grapsidae	Argentina: Santa Clara	SMF 24544
Cardisoma crassum Smith, 1870	Gecarcinidae	Costa Rica: Rincón	SMF 24543
Gecarcinus lateralis (Freminville, 1835)	Gecarcinidae	Mexico: Veracruz	USLZ 3722

Table 2. List of studies that use 16S mtDNA sequence for crustacean phylogenies or population genetics, with the number of individuals (ind.), species (sp.), genera (gen.), and families (fam.) included, as well as the sequence or alignment lengths in basepairs (bp) and primers used.

Taxonomic groups	Length	Primer combination	Reference
Cladocera			
Daphniidae: *Daphnia* (4 sp./13 ind.)	491 bp	ar & br	Taylor et al., in press
Copepoda			
Calanidae, Metridinidae: *Calanus*, *Metridia* (10 sp./14 ind.)	387 bp	ar-Dr & br-Dy	Bucklin et al. 1995
Calanidae: *Pseudocalanus* (2 sp./19 ind.)	250 bp	167 & br-Dy	Bucklin et al. 1998a
Calanidae: *Nannocalanus minor* (2 sp.?/155 ind.)	440 bp	ar-Dr & br-Dy	Bucklin et al. 1996a
Calanidae: *Calanus* (3 sp./20 ind.)	430 bp	ar-Dr & br-Dy	Bucklin et al. 1992
Calanidae: *Calanus pacificus* (27 ind.)	449 bp	ar-Dr & br-Dy	Bucklin & Lajeunesse 1994
Calanidae: *Calanus finmarchicus* (182 ind.)	350 bp	ar-Dr & br-Dy	Bucklin & Kocher 1996, Bucklin et al. 1996b
Calanidae: review of above data on Calanidae		ar-Dr & br-Dy	Bucklin et al. 1998b
Isopoda			
Serolidae (11 gen./15 sp.)	503 bp	ar & br	Held, submitted
Amphipoda			
Lysianassidae (4 gen./7 sp./32 ind.)	179 bp	Amph1 & Amph2	France & Kocher 1996a
Lysianassidae: *Eurythenes gryllus* (95 ind.)	437 bp	ar & br-Dr, Amph1 & Amph2	France & Kocher 1996b
Euphausiacea			
Euphausiidae (3 gen./5 sp./6 ind.)	524 bp	ar & br	Patarnello et al. 1996
Decapoda			
Penaeidae: *Penaeus* (2 sp./7 ind.)	438 bp	ar & br	Machado et al. 1993
Penaeidae: *Penaeus* (11 sp./=40 ind.)	472 bp	ar & br	Chu et al. 1998
Penaeidae: *Metapenaeus* (7 sp./16 ind.)	475 bp	ar & br	Tong et al., submitted
Palinuridae: *Palinurus* (4 sp./13 ind.)	491 bp	ar & br	Sarver et al. 1998
Nephropidae (5 gen./7 sp.)	350 of 474 bp	ar & SB	Tam & Kornfield 1998

Table 2. Continued

Cambaridae (3 gen./38 sp./72 ind.)	554 bp	1471 & 1472	Crandall & Fitzpatrick Jr. 1996; Crandall 1998
Cambaridae & Parastacidae (9 gen./12 sp.)	535 bp	1471 & 1472	Crandall et al. 1995
Parastacidae: (3 gen./9 sp.)	~ 550 bp	1471 & 1472	Lawler & Crandall 1998
Parastacidae: *Euastacus* (10 sp.)	461 bp	ar & br	Ponniah & Hughes 1998
Paguridae, Lithodidae et al. (4 fam./7 gen./10 sp./12 ind.)	420 bp	ar & br	Cunningham et al. 1992
Hippidae: *Emerita* (6 sp./9 ind.)	~ 400 bp	ar & 16SB	Tam et al. 1996
Menippidae: *Menippe* (2 sp./9 ind.)	525 bp	ar & br, ar-M & br-I	Schneider-Broussard & Neigel 1997, Schneider-Broussard et al., 1998
Portunidae: *Carcinus*, *Callinectes* (5 sp./47 ind.)	395 bp	Dar & Dbr	Geller et al. 1997
Grapsidae & Gecarcinidae (26 gen.)	593 bp	ar & br, 1472	Schubart et al., submitted
Grapsidae: Sesarminae (4 gen./21 sp.)	522 bp	ar & br, L12 & H16	Schubart et al. 1998
Grapsidae: *Sesarma* (2 sp./16 ind.)	526 bp	ar & br, L12 & H16	Schubart et al., in press
Grapsidae: *Pachygrapsus* (2 sp./5 ind.)	510 bp	ar & br, L12 & H16	Cuesta & Schubart, in press
Ocypodidae et al. (7 gen./30 sp./34 ind.)	491 bp	ar & br	Levinton et al. 1996
Ocypodidae: *Uca* (24 sp./27 ind.)	491 bp	ar & br	Sturmbauer et al. 1996
Ocypodidae (10 gen./ 20 sp.)	~ 1 170 bp	br, L2482, L2510, H2492, H2716, H3058, H3062	Kitaura et al. 1998

Table 3. List of primers used for amplifying 16S mtDNA in crustaceans.

Name	Primer sequence (5' -> 3')	Designation	Reference
Forward primers (L-strand)			
1) 16Sar primer and modifications			
ar:	CGCCTGTTTATCAAAAACAT	universal	Palumbi et al. 1991
ar-Dr:	CGCCTGTTTAACAAAAACAT	*Drosophila*	Palumbi et al. 1991
1471:	CCTGTTTANCAAAAACAT	Evertebrata	Crandall & Fitzpatrick 1996
Dar:	CGCCTGTTTAHYAAAAACAT	universal	Geller et al. 1997
L2510:	CGCCTGTTTAACAAAGACAT	Evertebrata	Kitaura et al. 1998
2) Internal primers to 16Sar-br region			
167:	GACGAGAAGACCCTATGAAG	Calanoida	Bucklin et al. 1998a
Amph1:	GACGACAAGACCCTAAAAGC	Amphipoda	France & Kocher 1996
ar-M:	ATAAGACCCTATAAAGC	*Menippe*	Schneider-Broussard & Neigel 1997
L12:	TGACCGTGCAAAGGTAGCATAA	Grapsoidea	Schubart et al. 1998
3) External primers to 16Sar-br region			
L2482:	GAAGGAACTCGGCAA	universal?	Kitaura et al. 1998
Reverse primers (H-strand)			
1) 16Sbr primer and modifications			
br:	CCGGTCTGAACTCAGATCACGT	universal	Palumbi et al. 1991
br-Dr:	CCGGTTTGAACTCAGATCATG	*Drosophila*	Palumbi et al. 1991
br-Dy:	CCGGTTTGAACTCAGATCACGT	*Drosophila yakuba*	Bucklin et al. 1995
SB:	CTCCGGTTTGAACTCAGATC	Arthropoda	Xiong & Kocher 1991
Dbr:	CCGGTCTGAACTCAGMTCAYGT	universal	Geller et al. 1997
H3062:	CCGGTCTGAACTCAGATCA	universal	Kitaura et al. 1998
H3058:	TCCGGTCTGAACTCAGATCACGTA	universal	Kitaura et al. 1998
H2492:	CAGACATGTTTTTAATAAACAGGC	~ reverse of ar	Kitaura et al. 1998
2) Internal primers to 16Sar-br region			
AMPH1:	CGCTGTTATCCCTAAAGTA	Amphipoda	France & Kocher 1996
br-I:	CCGCCCCAGCAAAATAAA	*Menippe*	Schneider-Broussard & Neigel 1997
H16:	TTATCRCCCCAATAAAATA	Grapsoidea	Schubart et al. 1998
H2716i:	AAGTTTTATAGGGTCTTATCGTC	Ocypodoidea	Kitaura et al. 1998
3) External primers to 16Sar-br region			
1472:	AGATAGAAACCAACCTGG	Evertebrata	Crandall & Fitzpatrick 1996

Table 4. Percent genetic divergence and number of differences between 532-basepair sequences of the 16S mtDNA of 8 specimens of *Sesarma reticulatum* from the Gulf of Mexico (GM) and the northwestern Atlantic (ATL); (s: transition, v: transversion, i: indel).

	GM-1	GM-2	GM-3	GM-4	ATL-1	ATL-2,3,4
GM-1 (Sabine Pass, TX)	-	0.2	0	0.4	1.5	1.7
GM-2 (Calcasieu Pass, LA)	1s	-	0.2	0.6	1.5	1.7
GM-3 (Cocodrie, LA)	0	1s	-	0.4	1.5	1.7
GM-4 (Alligator Point, FL)	1v,1s	1v,2s	1v,1s	-	1.5	1.9
ATL-1 (Brunswick, GA)	3v,4s,1i	3v,4s,1i	3v,4s,1i	2v,6s,1i	-	0.2
ATL-2 (Brunswick, GA)	3v,5s,1i	3v,5s,1i	3v,5s,1i	2v,7s,1i	1s	-
ATL-3,4 (Woodland Beach, DE)	3v,5s,1i	3v,5s,1i	3v,5s,1i	2v,7s,1i	1s	-