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PHYLOGENETIC RELATIONSHIPS OF CLAWED LOBSTER GENERA (DECAPODA: NEPHROPIDAE) BASED ON MITOCHONDRIAL 16S rRNA GENE SEQUENCES

Yan Kit Tam and Irv Kornfield

A B S T R A C T

Approximately 350 base pairs (bp) of the mitochondrial 16S rRNA gene were used to study the phylogenetic relationships among 5 genera of the clawed lobster family Nephropidae (infraorder Astacidea), including *Homarus*, *Homarinus*, *Metanephrops*, *Nephrops*, and *Nephropsis*. Maximum-parsimony analysis, using a hermit crab, *Pagurus pollicaris* (infraorder Anomura), as an outgroup, produced a tree topology in which *Homarus* and *Nephrops* formed a well-supported clade that excluded *Homarinus*. The same tree topology was obtained from both neighbor-joining and maximum-likelihood analyses. Some morphological characters that appear synapomorphic for *Nephrops* and *Metanephrops* may be due to convergence rather than symplesiomorphy. The current taxonomy, therefore, does not reflect the phylogeny of this group as suggested by the molecular data. More molecular data and studies using homologous morphological characters are needed to reach a better understanding of the phylogenetic history of clawed lobsters.

Clawed lobsters are marine decapods belonging to the superfamily Nephropoidea (Decapoda: Astacidea). The Nephropidae Dana, 1852, comprising three subfamilies and eleven genera, contains most of the clawed lobsters in this superfamily (Holthuis, 1974). Among them are the commercially important genera *Homarus* Weber, 1795, *Nephrops* Leach, 1814, and *Metanephrops* Jenkins, 1972. Holthuis (1991) presented a comprehensive review of clawed lobsters, emphasizing those that are of interest to global fisheries.

The Nephropidae is an old family which has a fossil record extending from the Middle Jurassic to the Recent (Glaessner, 1969). Ornamentations on the carapace of fossils are well preserved; these patterns of grooves and eminences may provide clues to the evolution of lobster lineages. Based on carapace morphology, Glaessner (1969) proposed a hypothetical phylogeny of astacideans. In this, the fossil genus *Palaeophoberus* Glaessner, 1932, gave rise to the genus *Hoploparia* McCoy, 1849, one line of which developed into crayfish (*Astacus*), and another line into *Nephrops*-like and *Homarus*-like lobsters. It had been proposed that fossil and Recent nephropids were composed of two subfamilies (Mertin, 1941), the Nephropinae and the Homarinae. However, based on the morphology of living lobsters, Holthuis (1974) did not accept the idea.

The current taxonomy of clawed lobsters is based on morphological features (Glaessner, 1969; Holthuis, 1991). Although morpholog-

ical characters may provide valuable information for taxonomic classification, the characters used in distinguishing different taxa may not be homologous structures and thus may not contain phylogenetic signals. Thus, the taxonomic classification of clawed lobsters may not reflect the phylogenetic relationships of the group. Homologous structures need to be defined and applied in making phylogenetic inferences (Tshudy, 1993); homoplasy of characters, such as convergence and parallelism, produce noise and mislead data analysis. Depending on the degree that some morphological characters are convergent, phylogenetic inferences may be compromised. This concern applies to inferences about fossil nephropids as well.

Molecular characters, particularly DNA sequence data, provide an independent data set with which to construct phylogenetic hypotheses (Hillis *et al.*, 1996). Molecular studies of phylogenetic relationships among clawed lobsters are limited. Chu *et al.* (1990) studied enzyme polymorphism in three species of *Metanephrops* in Taiwan. Hedgecock *et al.* (1977) examined the genetic variation between *Homarus americanus* H. Milne Edwards, 1837, and *H. gammarus* (Linnaeus, 1758) by using allozyme data. The present study, using molecular characters as an alternative approach to morphology, provides independent clues about the phylogeny of the clawed lobsters. By using universal primers within a conservative region in the mito-

Table 1. Species studied and the sampling localities.

| Species | Infraorder | Abbreviations | Sampling locality |
|--|-------------|---------------|-----------------------------|
| <i>Homarus americanus</i> H. Milne Edwards, 1837 | Astacidea | HA | Gulf of Maine, U.S.A. |
| <i>Homarus gammarus</i> (Linnaeus, 1758) | Astacidea | HG | Guernsey, U.K. |
| <i>Homarinus capensis</i> (Herbst, 1792) | Astacidea | HC | Cape Province, South Africa |
| <i>Metanephrops mozambicus</i> Macpherson, 1990 | Astacidea | MM | Natal, South Africa |
| <i>Nephrops norvegicus</i> (Linnaeus, 1758) | Astacidea | NN | Celtic Sea, Ireland |
| <i>Nephropsis aculeata</i> Smith, 1881 | Astacidea | NA | Massachusetts, U.S.A. |
| <i>Nephropsis stewarti</i> Wood-Mason, 1872 | Astacidea | NS | Natal, South Africa |
| <i>Panulirus longipes</i> (A. Milne Edwards, 1868) | Palinuridea | PL | South China Sea, Hong Kong |
| <i>Scyllarides nodifer</i> (Stimpson, 1866) | Palinuridea | SN | Gulf of Mexico, U.S.A. |
| <i>Pagurus pollicaris</i> Say, 1817 | Anomura | PP | Massachusetts, U.S.A. |

chondrial genome, a homologous DNA segment from all the taxa can be amplified and compared.

A now conventional source for DNA sequences is the mitochondrion (Avice, 1994), an organelle that contains multiple copies of a small, maternally inherited genome, mitochondrial DNA (mtdna). MtDNA sequences were used to define taxonomic relationships among species of *Homarus* and distinguish from them the genus *Homarinus* Kornfield, 1995, the Cape lobster of South Africa (Kornfield *et al.*, 1995). The 16S ribosomal RNA (rRNA) gene in the mitochondrial genome contains conservative regions which have been used in many phylogenetic studies at generic and higher taxonomic levels for a wide variety of organisms (Hillis *et al.*, 1996). Recently, Crandall and Fitzpatrick (1996) used a mitochondrial 16S rRNA gene sequence to study the relationships of crayfishes. The present study examined relationships among five of the 11 extant genera of the family Nephropidae by comparing partial sequences of the mt16S rRNA gene. Our objective was to compare phylogenetic hypotheses based on this molecular data set to the current taxonomy based on morphological features.

MATERIALS AND METHODS

Seven species in 5 genera of the clawed lobster family Nephropidae were studied: *Homarus americanus*, *Homarus gammarus*, *Homarinus capensis* (Herbst, 1792), *Metanephrops mozambicus* Macpherson, 1990, *Nephrops norvegicus* (Linnaeus, 1758), *Nephropsis aculeata* Smith, 1881, and *Nephropsis stewarti* Wood-Mason, 1872 (Table 1). One individual from each species was used for analysis. A spiny lobster, *Panulirus longipes* (A. Milne Edwards, 1868), and a slipper lobster, *Scyllarides nodifer* (Stimpson, 1866), both belonging to the infraorder Palinuridea, were also included in the analysis. The hermit crab

Pagurus pollicaris Say, 1817 (infraorder Anomura), was used as an outgroup for parsimony analysis.

DNA was prepared from samples of muscle from the abdomen or pereopods. MtDNA was prepared by phenol/chloroform extraction of proteinase-K-digested tissues (Ausubel *et al.*, 1989). The DNA templates were then subjected to polymerase chain reaction (PCR) (Saiki *et al.*, 1988), using standard protocols (Palumbi *et al.*, 1991). A 570-base-pair (bp) region within the mt16S rRNA (16S) gene was amplified using primers 16SA (5'CGC-CTGTTTATCAAAAACAT3') and 16SB (5'CTCCG-GTTTGAAGCTCAGATC3') (Xiong and Kocher, 1991). PCR amplification was performed using 30 cycles of 94°C, 30 s / 50°C, 60 s / 72°C, 90 s; the initial denaturation step at 94°C lasted 5 min and the final extension step at 72°C lasted 10 min. Double-stranded PCR products were subjected to asymmetric PCR from both 5'-direction to generate templates for dideoxy sequencing (Sanger *et al.*, 1977), using 35 cycles of the same PCR conditions. Prior to sequencing, PCR products were purified by filtration through Millipore Ultrafree-MC regenerated cellulose membrane filters of 30,000 nominal molecular weight limit (NMWL) (Millipore Corporation).

Sequences were aligned using ESEE (Cabot and Beckenbach, 1989). Secondary structures of the partial mt16S rRNA gene sequences were inferred using Mulford (Jaeger *et al.*, 1989) to assure homologous sequence alignment. Sequences without 2 highly variable regions (Parker and Kornfield, 1996) were subjected to all data analyses (Fig. 1). A matrix of sequence divergence using Kimura's two-parameter method (Kimura, 1980) was generated and subjected to neighbor-joining analysis using MEGA (Kumar *et al.*, 1993). Five hundred bootstrap replicates were performed to access the confidence level at each branch (Felsenstein, 1985). Maximum-likelihood trees were constructed using the program DNAML in PHYLIP, Version 3.5 (Felsenstein, 1993). Parsimony analysis was conducted using the exhaustive search option in PAUP Version 3.1.1 (Swofford, 1993); alignment gaps were included as characters and only phylogenetically informative characters (Hillis *et al.*, 1996) were used. To assess the heuristic confidence in the parsimony trees generated by PAUP, 2,000 bootstrap replicates were performed. In order to compare the effects of including and excluding the 2 highly variable regions, all available data (i.e., 474 bp) were also subjected to maximum-parsimony analysis using PAUP as described above. Different tree topologies were compared by using the user-defined tree function in MacClade, Version 3 (Maddison and Maddison, 1992).

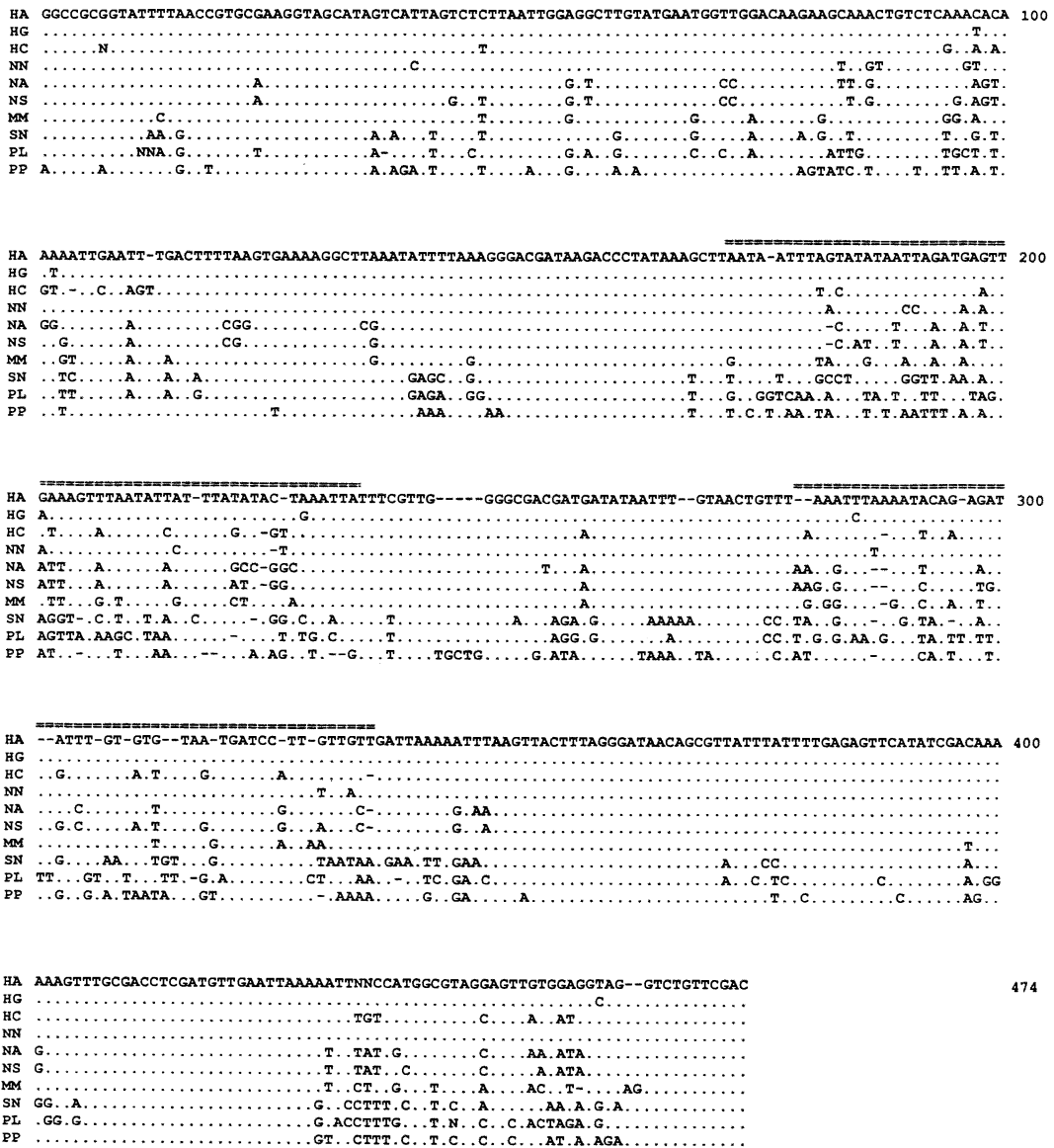


Fig. 1. Sequence data for the partial mitochondrial 16S rRNA gene for all taxa in the study. Abbreviations for taxa are defined in Table 1. Sequences over-shadowed by “=” represent highly variable regions with ambiguous homologous sequence alignment which were excluded from all data analyses. “.” indicates identity with the reference sequence of *Homarus americanus*; “-” indicates gap; N indicates an undetermined nucleotide.

RESULTS

Approximately 450 (bp) of the mt16S rRNA gene were sequenced from all individuals (Fig. 1). All sequences have been deposited in GenBank (accession numbers U11238, U11247, U55843, U96083–U96089). In Fig. 1, the over-scored regions represent two highly variable segments within the amplified sequences. Since the secondary structures of

these variable regions indicated ambiguous sequence alignment, these two regions were excluded from all subsequent data analyses. In all, about 350 nucleotides were used for data analysis. Table 2 presents estimates of pairwise sequence divergence of all taxa using the two-parameter method of Kimura (1980). Among the clawed lobsters, *Nephropsis* and *Metanephrops* were quite divergent genetically (>7%) from the other taxa. However, the extent of

Table 2. Two-parameter estimates of sequence divergence (Kimura, 1980) based on partial mitochondrial 16S rRNA gene sequences. Estimates are expressed as percentages. Abbreviations refer to Table 1.

| | HA | HG | HC | NN | NA | NS | MM | SN | PL | PP |
|----|----|------|------|------|-------|-------|-------|-------|-------|-------|
| HA | — | 0.88 | 4.54 | 1.78 | 10.76 | 9.38 | 7.44 | 22.40 | 26.00 | 23.98 |
| HG | | — | 4.54 | 2.08 | 11.09 | 10.05 | 8.10 | 22.78 | 25.95 | 24.37 |
| HC | | | — | 6.13 | 9.38 | 9.04 | 7.75 | 23.95 | 26.72 | 25.97 |
| NN | | | | — | 11.12 | 10.41 | 9.14 | 24.50 | 26.46 | 24.40 |
| NA | | | | | — | 4.21 | 12.54 | 29.08 | 27.39 | 30.33 |
| NS | | | | | | — | 10.40 | 25.88 | 26.93 | 26.71 |
| MM | | | | | | | — | 21.97 | 22.77 | 26.84 |
| SN | | | | | | | | — | 16.34 | 21.46 |
| PL | | | | | | | | | — | 27.49 |
| PP | | | | | | | | | | — |

divergence of *Nephrops norvegicus* was unexpected. This species exhibited much less divergence ($1.93\% \pm 0.2\%$) from the two species of *Homarus* than was observed between species of *Homarus* and *Homarinus capensis* ($4.54\% \pm 0\%$), a taxon until recently congeneric with *Homarus*. The slipper lobster *Scyllarides nodifer* and the spiny lobster *Panulirus longipes*, both in the infraorder Palinu-

ridea, had an average genetic divergence of $25.2\% \pm 0.60\%$ from the clawed lobsters (infraorder Astacidea), while the hermit crab *Pagurus pollicaris* (infraorder Anomura) had an average genetic divergence of $26.1\% \pm 0.9\%$ from the clawed lobsters. This similar level of genetic divergence among infraorders indicates that mutations in the mt16S rRNA gene are saturated at this taxonomic level.

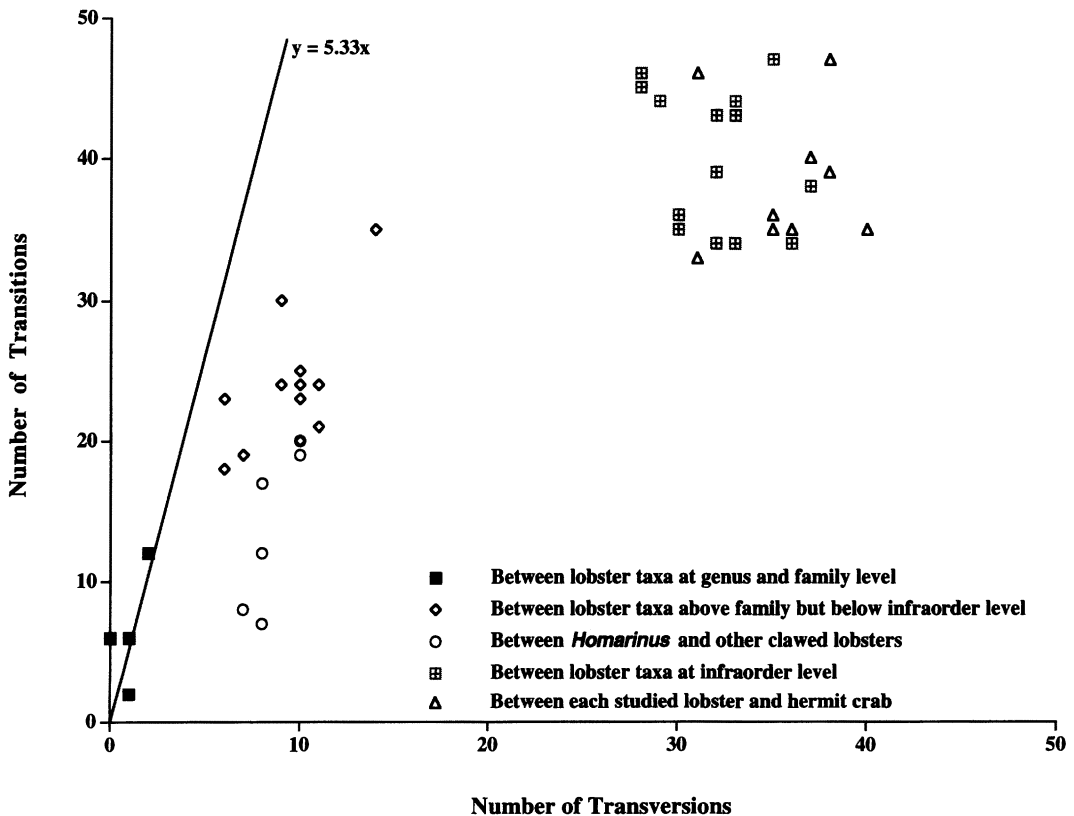


Fig. 2. Number of transitions versus number of transversions in all pairwise comparisons of partial mitochondrial 16S rRNA gene sequences. This figure gives an indication of the extent of transitional bias and the extent of saturation in substitutions.

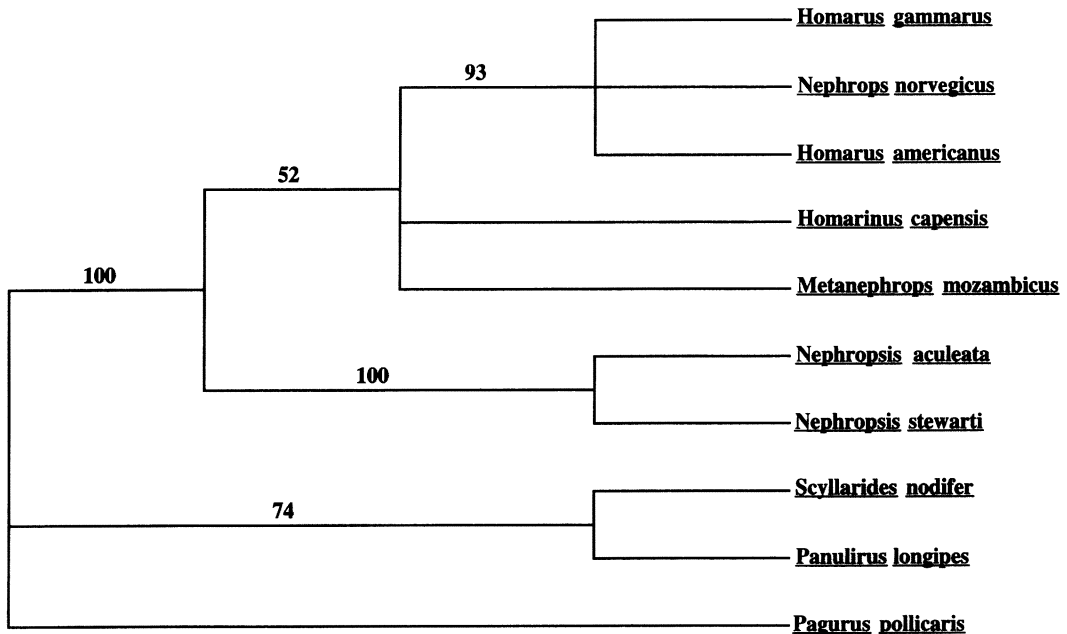


Fig. 3. 50% majority-rule consensus of nine most-parsimonious trees based on maximum-parsimony analysis of partial mitochondrial 16S rRNA gene sequences for lobsters. Tree was rooted using *Pagurus pollicaris*. Bootstrap values (2,000 replicates) are shown on the branches. Each of the nine most-parsimonious trees required 164 steps and had a consistency index of 0.707.

Figure 2 depicts the extent of transitional bias and also the level of saturation in mutated sites by plotting the number of transitions against the number of transversions in all pairwise comparisons of taxa. The slope showed in Fig. 2 gives a rough indication of the initial transition-to-transversion ratio among closely related species. A slope of 5.0 indicates a 10:1 transition-to-transversion bias. The figure illustrates partial saturation among genera within an infraorder, while saturation is close to complete between infraorders.

Figure 3 presents a 50% majority rule consensus of nine most parsimonious trees resulting from a maximum-parsimony analysis of the mt16S rRNA gene sequences. The weighing of transition to transversion in the analysis is 1 to 5. Parsimony analysis based on transversions alone gave a topology consistent to that using both transitions and transversions. Maximum-parsimony, using the hermit crab as an outgroup, yielded nine shortest trees with tree length of 164 and consistency index of 0.707. The next six shortest trees have a tree length of 165 and a consistency index of 0.703. The differences in topology among all these trees were the relative positions of taxa within a clade containing all clawed lobsters. However, in this larger clade, the spe-

cies of *Homarus* and *Nephrops* consistently grouped together. *Homarinus* was always excluded from the *Homarus-Nephrops* clade. Another strongly supported clade was formed by the two species of *Nephropsis*. The phylogenetic positions of *Homarinus* or *Metanephrops* could not be resolved with confidence (but see below). When the tree topology was constrained (by using MacClade V.3) so that *Homarus* and *Homarinus* formed a clade while *Nephrops* and *Metanephrops* formed another clade, the total tree length is eight steps (i.e., tree length = 172) more than the total tree length in Fig. 3, while the consistency index is smaller (=0.67). Figure 4 shows a neighbor-joining tree based on a distance matrix calculated by using the two-parameter method of Kimura (1980). Five hundred bootstrap replications of the neighbor-joining analysis gave a topology similar to that of Fig. 3 when branches with confidence levels less than 50% were collapsed. The bootstrapped neighbor-joining analysis indicates strong support (Bootstrap Proportion [BP] = 84%) for *Homarinus* being a sister taxon to the *Homarus-Nephrops* clade. In the maximum-parsimony analysis, the two species of *Nephropsis* formed a sister clade with low confidence level (BP = 52%, Fig. 3) to

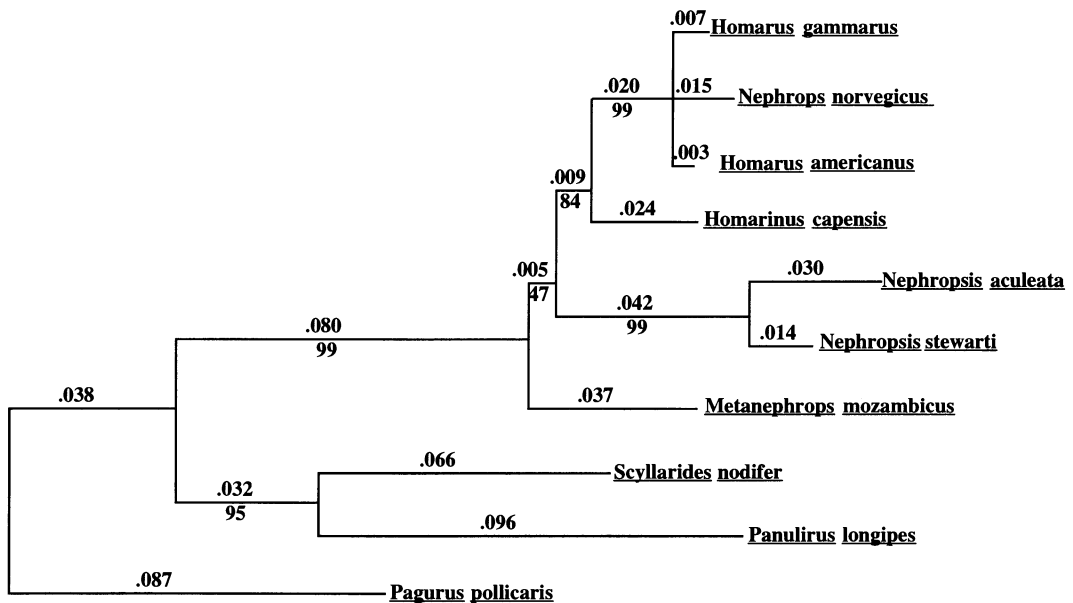


Fig. 4. Neighbor-joining tree for lobsters based on partial mitochondrial 16S rRNA gene sequences. Numbers above branches are branch lengths. Numbers below branches indicate confidence levels (%) for branch length.

all other clawed lobsters. This relationship was not resolved in the neighbor-joining analysis (BP = 47%). Maximum-likelihood analysis also yielded a consistent tree topology as did both maximum-parsimony and neighbor-joining analyses, after collapsing branches whose confidence limits overlapped zero.

In order to see the effect of including the two highly variable regions on the outcome of the cladistic analysis, all available data (i.e., 474 bp of the mtDNA 16S gene) were subjected to maximum-parsimony analysis using PAUP under the same settings as the analysis without the highly variable regions. The maximum-parsimony analysis yielded four shortest trees of tree length and consistency index equal to 316 and 0.668, respectively. The next four shortest trees have tree lengths of 317. The consensus tree is identical to the consensus tree which resulted from the analysis without the highly variable regions. However, the inclusion of these highly variable regions did not improve the resolution of the cladogram.

In summary, the various data analyses yielded identical tree topologies in which a strong (BP = 93–99, Figs. 3, 4, respectively) clade was formed by the two species of *Homarus* and *Nephrops*, although suggested relationships among these three taxa within

the clade (Table 2) could not be resolved with confidence. Another strong clade was formed by the two species of *Nephropsis*. *Homarinus* was always excluded from the *Homarus-Nephrops* clade, but its position as sister to this clade, supported by neighbor-joining analysis, was ambiguous under maximum-parsimony. All clawed lobsters formed a significant (BP = 100, Fig. 3; BP = 99, Fig. 4) clade relative to both spiny and slipper lobsters, which together formed a clade with a moderate bootstrap value under parsimony (BP = 74, Fig. 3), and with a higher support (BP = 95, Fig. 4) under neighbor-joining.

DISCUSSION

Ornamentation, such as grooves, spines, and carinae of the carapace of both fossil and extant clawed lobsters, has been used by carcinologists as a clue to the phylogenetic relationships among lobsters. In the hypothetical evolutionary scheme of astacideans of Glaessner (1969), based on carapace morphology, both *Nephrops*-like and *Homarus*-like lobsters diverged separately from the *Hoploparia* lineage during the Middle or Late Cretaceous. As described in the species catalogue of lobsters (Holthuis, 1991), *Homarus* has a smooth abdomen lacking grooves and spines and smooth first chelipeds without

ridges, while *Nephrops* has a grooved abdomen and grooved first chelipeds with spines. In addition, the first chelipeds of *Homarus* are wide and thick, while those of *Nephrops* are slender and much longer than wide. In addition to having a small body size, *Homarinus capensis* has a smooth body with first chelipeds that are fully covered with hairs. The shape of its body and the smooth morphology of its abdomen and chelipeds have been the basis for placing it in the genus *Homarus*. However, additional distinct morphological characters, such as the presence of a dense coat of setae on the outer surface of the first chelipeds and scattered setae distributed over other body parts, and extensive genetic divergence (Kornfield *et al.*, 1995) suggest that this species constitutes a separate genus. The exact phylogenetic position of *Homarinus capensis* remains enigmatic, although the distinction of this taxon from species of *Homarus* is clear and significant. All species of *Metanephrops* have previously been regarded as belonging to *Nephrops*. Based on the relative size of the left and right first chelipeds, the size and abundance of spines on the carapace, the number of ridges on the carapace, and the margins of the rostrum, Jenkins (1972) removed all except the European species from *Nephrops* to form the genus *Metanephrops*. Both the study of *Homarinus* and the study of *Metanephrops* indicate that taxonomy may change as more specimens become available and more acute examinations of the comparative morphology of clawed lobsters are undertaken. This supports the idea that morphological characters used in taxonomic classification may or may not contain phylogenetic signals. Furthermore, such changes in taxonomic ranking do not automatically support previous or prevailing hypotheses of phylogenetic relationships, for example, that *Homarinus* must be closely related to *Homarus*, or that *Metanephrops* is a sister genus to *Nephrops*. In the case of *Nephrops norvegicus*, the inequality in size of the first chelipeds is a character shared also with both *Homarus* and *Homarinus*, but not with either *Metanephrops* or *Nephropsis*. On the other hand, the distinct grooves and ridges on the chelipeds, carapace, and abdomen in *Nephrops* are also found in *Metanephrops*, but not in *Homarus* or *Homarinus*.

In cladistic analysis, homology is a criti-

cal requirement. Tshudy (1993) examined 40 morphological characters for cladistic analysis of the clawed lobster families Chileno-phoberidae and Nephropidae. Twenty-nine of the 40 characters were informative phylogenetically. Among these 29 informative characters, only nine were reliable indicators of phylogeny (i.e., were not homoplastic). Tshudy's (1993) cladistic analysis suggests that the existing, intuitive suprageneric classification of clawed lobsters is phylogenetically incorrect. The present study, which used molecular characters as an alternative approach to morphological characters, provides independent clues about the phylogeny of clawed lobsters.

The most parsimonious molecular trees (Fig. 3) reveal a significant clade formed by all clawed lobsters in the present study. Within this group, *Nephrops* and *Homarus* form a well-supported internal clade (bp = 93, Fig. 3) that excludes *Homarinus*. The exact phylogenetic position of *Homarinus* is ambiguous, although the neighbor-joining tree suggests (bp = 84%) that *Homarinus* is a sister taxon to the *Homarus-Nephrops* clade. In general habitus, *Homarinus* is more *Homarus*-like rather than *Nephrops*-like. If the molecular phylogeny presented here reflects the "true" phylogeny of clawed lobsters, the similarities between *Homarus* and *Homarinus*, such as wide and thick claw with a smooth palm and smooth abdomen, would be due to convergence or symplesiomorphy, not synapomorphy. The disparity in genetic and morphological divergence between *Homarus* and *Homarinus* illustrates that taxa that are genetically divergent need not present obvious morphological autapomorphies. It would appear that the grooved palms with spines and ridges shared by *Nephrops* and *Metanephrops* are due to convergence rather than symplesiomorphy. The former explanation requires two independent character-state changes, while the latter requires three. This hypothesis assumes that the plesiomorphic state is smooth palms lacking ornamentation. Our results support the decision by Holthuis (1974) to retain *Homarus*-like lobster taxa in the Nephropinae, instead of erecting a separate subfamily Homarinae. The phylogenetic affinities of *Homarinus* remain tentative, since the present study cannot completely resolve the phylogenetic relationships among *Homarinus*, *Metanephrops*, and the *Homarus-Nephrops* clade. While *Homarinus* was not

included in the study by Tshudy (1993), *Nephrops* and *Metanephrops* were grouped together in the same clade which excluded *Homarus* in his study. Thus, there is incongruence between morphological and molecular data in inferring clawed-lobster phylogeny, although both suggested that the current taxonomy lacks phylogenetic basis. In order to confirm and to improve the resolution of the phylogenetic relationships among clawed-lobster genera, further studies involving other morphological or genetic characters, including longer sequences of the mt16S rRNA gene or other genes, are necessary.

Paleontologists use morphological characters and stratigraphic records of extinct and extant taxa to construct phylogeny. However, there are many limitations on the use of fossils, including limited number of specimens, the degree of preservation or completeness of the fossils, and the difficulty in identifying homologous characters for comparison. Therefore, additional approaches are needed. While molecular approaches may not be generally applicable to fossil crustaceans, both morphological and molecular approaches should be applied, if possible, when constructing phylogenies. Parker (1997) gave an enlightening discussion of the advantages and disadvantages of using either morphological or molecular approaches to phylogenetic studies, as well as the utility of the combined approach. De Queiroz *et al.* (1995) gave a comprehensive review of arguments in favor of each of these views. The "total evidence" approach is currently debated, and it is unclear whether different sets of data should be analyzed separately or combined and analyzed simultaneously. It has been argued that combining data sets can enhance detection of real phylogenetic groups. However, combined analyses may also give misleading results when there is heterogeneity among data sets.

In conclusion, it appears that *Nephrops* is closely related to *Homarus*, while *Homarinus* is outside the clade containing these two genera. The superficial morphological resemblance of *Homarinus* to *Homarus*, and *Nephrops* to *Metanephrops*, is due to convergence or symplesiomorphy and does not reflect the pattern of relationships revealed by the mt16S rDNA data presented herein. These results also illustrate that taxa which are genetically divergent, such as *Homarinus* and *Homarus*,

need not present extensive morphological autapomorphies. Taxonomic classification of clawed lobsters based solely on superficial morphological characters does not reflect phylogenetic relationships, and studies on fossilized specimens can give only hypothetical results that need to be tested or complemented by other approaches. Both morphological and molecular approaches complement each other and are needed to infer phylogeny.

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