

## Molecular phylogeny of the spiny lobster genus *Panulirus* (Decapoda: Palinuridae)

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**Abstract.** Phylogenetic relationships among all described species and four subspecies (total of 21 taxa) of the spiny lobster genus *Panulirus* White, 1847, were examined with nucleotide sequence data from portions of two mitochondrial genes, large-subunit ribosomal RNA (16S) and cytochrome oxidase subunit I (COI). Multiple sequence alignments were subjected to maximum-parsimony, neighbour-joining, and maximum-likelihood analysis with *Jasus edwardsii* as the outgroup. Two major lineages within *Panulirus* were recovered by all three methods for both the 16S and COI alignments analysed separately and for the combined alignment. The first lineage included all species of *Panulirus* classified as Groups I and II by previous morphologically based definitions. The second included all species classified as Groups III and IV. Relationships within major lineages were not well resolved; the molecular phylogeny did not support separation of Group I from Group II or of Group III from Group IV. The degree of sequence divergence between different pairs of species was higher in pairwise comparisons between species in Group I/II (16S: 2.8–19.4%; COI: 12.4–31.8%) than in those between species in Group III/IV (16S: 5.3–13.2%; COI: 12.6–19.6%). This pattern suggests that the Group I/II lineage may represent an earlier radiation of species within *Panulirus*.

### Introduction

The spiny lobster genus *Panulirus* White, 1847, has long been of interest to evolutionary biologists because of its high level of species diversity, its wide geographic distribution, and the importance of many species to commercial fisheries. Among genera in the family Palinuridae, *Panulirus* has been the most successful in terms of species diversity; 19 species have been described to date, three of which are divided into seven recognized subspecies (Holthuis 1991; George 1997; Sarver *et al.* 1998). The key to this successful radiation is thought to be the invasion by species of *Panulirus* of shallow-water, tropical environments, which permits the occupation of varied habitats not accessible to congeners (George and Main 1967; George 1997). Because of morphological, ecological, and behavioural diversity, both within and among species of *Panulirus*, the phylogenetic history of this group has not been well understood.

The group has undergone a number of taxonomic divisions based on interspecific diversity in morphological traits. George and Main (1967) proposed the first of these, in which they divided *Panulirus* into four informal groups (I–IV) without giving these groups specific taxonomic

rankings. These four groups were distinguished by comparative morphology of the exopods associated with the second and third maxillipeds of adults (Table 1). Species of *Panulirus* differ in the degree of development of these exopods. Within the genus *Panulirus*, species with the most reduced exopods (Group IV) are regarded as the most recently evolved, whereas the presence of exopods with flagella on either the second or third maxillipeds (or both) is considered the ancestral condition (Groups I and II; George and Main 1967). All other palinurid genera have fully developed exopods.

More recently McWilliam (1995) proposed a revised taxonomic scheme based on interspecific diversity in morphological traits associated with phyllosoma larval and puerulus stages. This revised scheme is largely concordant with George and Main's (1967) classification, except in the placements of *P. interruptus* and *P. guttatus*. Comparative morphological data indicated two basic phyllosoma and puerulus forms. Members of the more recent radiation of tropical species (Groups III and IV) share a more specialized form of phyllosoma and puerulus than those of the earlier, subtropical radiation (Groups I and II). The phyllosoma and the puerulus of *P. interruptus* are of intermediate form, and the phyllosoma of *P. guttatus* shows

**Table 1. Arrangement of species of *Panulirus* based on morphological and geographic differences adapted from George and Main (1967) and McWilliam (1995)**

	Exopod Condition		<i>Panulirus</i> sp.			
	3rd maxilliped	2nd maxilliped	Indo-West Pacific	East Pacific	West Atlantic	East Atlantic
I	Present with flagellum	Present with flagellum	<i>cygnus</i> <i>japonicus</i> <i>longipes</i> <i>marginatus</i> <i>pascuensis</i>	<i>interruptus</i>	<i>argus</i>	
II	Present no flagellum	Present with flagellum	<i>penicillatus</i>	<i>penicillatus</i>	<i>echinatus</i> <i>guttatus</i>	<i>echinatus</i>
III	Absent	Present with flagellum	<i>polyphagus</i>	<i>gracilis</i> <i>inflatus</i>	<i>laevicauda</i>	<i>regius</i>
IV	Absent	Present no flagellum <sup>1</sup>	<i>homarus</i> <i>ornatus</i> <i>stimpsoni</i> <i>versicolor</i>			

<sup>1</sup>Except where it is reduced in *P. homarus*

the more derived state of the specialized, spatulate antennae characteristic of species in Groups III and IV.

The biogeographic history within the genus has been debated, mostly with respect to the timing of divergence among the four species groups (George and Main 1967; Baisre and Ruiz de Quevedo 1982; Pollock 1992, 1993; McWilliam 1995; Sekiguchi 1995; George 1997), but it is generally agreed that Groups I and II represent an early radiation of *Panulirus* species from an Indo-Pacific ancestor followed by a later radiation, Groups III and IV, also from an Indo-Pacific ancestor (Table 1). This historical interpretation is based on the retention of pleisiomorphic morphological traits by species in Groups I and II (fully developed exopods of the second and third maxillipeds in adults; George and Main 1967) and less specialized forms of phyllosoma and puerulus stages (McWilliam 1995). First attempts at dating the initial radiation of Groups I and II suggested an association with Pleistocene glaciation periods (George and Main 1967; Pollock 1992, 1993; Sekiguchi 1995). More recently, George (1997) has argued that genetic divergences of a subset of *Panulirus* species based on allozyme data (Chan and Chu 1996) suggest speciation of Groups I and II as early as the late Miocene. That geologic time is associated with the formation of new habitats after collisions of India and Australia with the Asian plate and associated alteration of circulation patterns and restricted larval transport between the Pacific and Indian Oceans, which favoured divergence between some western Pacific and Indian Ocean species (George 1997).

Few studies have used a phylogenetic approach to test these biogeographic hypotheses or to examine the pattern of speciation among members of *Panulirus*. Sarver *et al.* (1998) examined a subset of species within *Panulirus* in

their description of a new Brazilian subspecies, *P. argus westonii*, based on nucleotide sequence divergence in two mitochondrial genes. Their analyses provided strong support for the genetic divergence of *P. a. argus* from *P. a. westonii*, but did not include enough of the remaining species of *Panulirus* to reveal phylogenetic relationships within the genus.

Here, we report results of phylogenetic analyses based on nucleotide sequence data from two regions of the mitochondrial genome, the large-subunit ribosomal RNA (16S) and the cytochrome oxidase I subunit (COI). We included sequences of these gene regions for all species and four subspecies (total of 21 taxa) within the genus *Panulirus* in our phylogenetic analyses. Our specific aims in recovering the molecular phylogeny were to determine the phylogenetic relationships among all members of the genus and to test previously proposed taxonomic groupings and biogeographic hypotheses. The mitochondrial gene regions used in this study have proven useful for reconstructing detailed phylogenies that are independent of morphological characters and are free from the environmental influence that may bias morphologically based topologies (Simon *et al.* 1994).

## Methods

### *Specimens included in the phylogeny*

We examined 19 species of *Panulirus* for sequence variation at both the 16S and COI gene regions. For two species, *P. homarus* and *P. longipes*, two recognized subspecies of each, *P. homarus homarus* and *P. h. megasculpta* (Berry 1974) and *P. longipes longipes* and *P. l. femoristriga*, were sequenced for both gene regions. The *P. l. femoristriga* used in this analysis was the 'cross-banded' form and has also been described as *P. femoristriga* (Chan and Chu 1996), *P. longipes* 'shirahige' (George 1997), and most recently as *P. l.*

*bispinosus* (Chan and Ng 2001). The red rock lobster, *Jasus edwardsii*, a member of *Silentes*, the sister taxon to *Stridentes* (which includes *Panulirus*) (George and Main 1967), was used as the outgroup taxon in

these analyses. Locality information for each of the specimens included in the phylogenetic analyses is given in Table 2. A single individual of each species was sequenced.

**Table 2. Sample locations and GenBank accession numbers for species used in COI and 16S sequence analyses**  
Species abbreviations are included for data presented in Table 3

Gene	Species	Abbreviation	Location	Submitted by	GenBank Accession No.	
COI	<i>Jasus edwardsii</i>	jas	Fiordland, New Zealand	R. W. George	AF339473	
	<i>P. argus argus</i>	arg	Long Key, Florida, USA	M. J. Childress	AF339452	
	<i>P. cygnus</i>	cyg	Western Australia	J. Silberman	AF339453	
	<i>P. echinatus</i>	ech	Brazil	J. Silberman	AF339454	
	<i>P. gracilis</i>	gra	Mazatlán, Mexico	R. W. George	AF339455	
	<i>P. guttatus</i>	gut	Long Key, Florida, USA	M. J. Childress	AF339456	
	<i>P. homarus homarus</i>	hoh	Marquesas Is.	R. W. George	AF339457	
	<i>P. homarus megasculpta</i>	hom	Sadh, Oman	R. Mohan	AF339458	
	<i>P. inflatus</i>	inf	Mazatlán, Mexico	R. W. George	AF339459	
	<i>P. interruptus</i>	int	Goleta Bay, Calif., USA	S. Anderson	AF339460	
	<i>P. japonicus</i>	jap	Shikoki Is., Japan	R. W. George	AF339461	
	<i>P. laevicauda</i>	lae	Brazil	J. Silberman	AF339462	
	<i>P. longipes femoristriga</i> <sup>1</sup>	lof	Torres Strait, Australia	C. R. Pitcher	AF339463	
	<i>P. longipes longipes</i>	lol	Philippines		AF339464	
	<i>P. marginatus</i>	mar	Hawaii	F. Parrish	AF339465	
	<i>P. ornatus</i>	orn	Torres Strait, Australia	C. R. Pitcher	AF339467	
	<i>P. pascuensis</i>	pas	Easter Is.	R. W. George	AF339466	
	<i>P. penicillatus</i>	pen	Palau Is.	P. & L. Colin	AF339468	
	<i>P. polyphagus</i>	pol	Singapore	P. Ng	AF339469	
	<i>P. regius</i>	reg	Pointe-Noire, Congo	Dr. Crosnier	AF339470	
	<i>P. stimpsoni</i>	sti	Hong Kong	Ka Hou Chu	AF339471	
	<i>P. versicolor</i>	ver	Palau Is.	P. & L. Colin	AF339472	
	16S	<i>Jasus edwardsii</i>	jas	Fiordland, New Zealand	R. W. George	AF337979
		<i>P. argus argus</i>	arg	Miami, Florida, USA	J. Silberman	AF337966
		<i>P. cygnus</i>	cyg	Western Australia	J. Silberman	AF337967
		<i>P. echinatus</i>	ech	Brazil	J. Silberman	AF337965
<i>P. gracilis</i>		gra	Mazatlán, Mexico	M. Hendrikkx	AF337964	
<i>P. guttatus</i>		gut	Miami, Florida, USA	J. Silberman	AF337963	
<i>P. homarus homarus</i>		hoh	Singapore	P. Ng	AF337962	
<i>P. homarus megasculpta</i>		hom	Sadh, Oman	R. Mohan	AF337961	
<i>P. inflatus</i>		inf	Mazatlán, Mexico	M. Hendrikkx	AF337960	
<i>P. interruptus</i>		int	Southern California, USA	J. Silberman	AF337959	
<i>P. japonicus</i>		jap	Japan	M. Katoh	AF337968	
<i>P. laevicauda</i>		lae	Brazil	J. Silberman	AF337969	
<i>P. longipes femoristriga</i>		lof	Singapore	P. Ng	AF339156	
<i>P. longipes longipes</i>		lol	Philippines		AF337970	
<i>P. marginatus</i>		mar	Hawaii	F. Parrish	AF337972	
<i>P. ornatus</i>		orn	Singapore	P. Ng	AF337971	
<i>P. pascuensis</i>		pas	Easter Is.	R. W. George	AF337973	
<i>P. penicillatus</i>		pen	Singapore	P. Ng	AF337974	
<i>P. polyphagus</i>		pol	Singapore	P. Ng	AF337975	
<i>P. regius</i>		reg	Pointe-Noire, Congo	Dr. Crosnier	AF337976	
<i>P. stimpsoni</i>		sti	Hong Kong	Ka Hou Chu	AF337977	
<i>P. versicolor</i>		ver	Philippines		AF337978	

<sup>1</sup>'Cross-banded' form called *P. femoristriga* by Chan and Chu (1996), *P. longipes* 'shirahige' by George (1997) and *P. longipes bispinosus* by Chan and Ng (2001).

### Molecular Techniques

Genomic DNA was extracted from muscle tissue of each individual with the G'NOME DNA extraction kit (BIO 101, Vista, California) for polymerase chain reaction (PCR; Saiki *et al.* 1988) amplification of the COI gene region. Standard phenol/chloroform extraction techniques were used for DNA isolation from specimens used to amplify the 16S region (Hillis *et al.* 1990). Portions of these two mitochondrial genes (550 base pairs (bp) of 16S and 650 bp of COI) were amplified by PCR with conserved primers: 16Sar-L and 16Sbr-H for 16S (Palumbi *et al.* 1991), L-CO1490 and H-CO2198 for COI (Folmer *et al.* 1994). Conditions for symmetric PCR amplification of each gene region followed standard conditions (Palumbi 1996). Each set of primers was used for both PCR amplification and sequencing.

A ~650-bp segment of COI was amplified, and PCR products were purified with Qiaquick PCR purification columns (Qiagen, Inc., Valencia, California). This purified product was used as template in sequencing reactions with the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, California). These were run on the Applied Biosystems Model 377 Automated DNA Sequencing System located in the Molecular Core Research Facility at Idaho State University. Sequences were determined for both DNA strands for all individuals.

A ~550-bp segment of 16S was amplified, and PCR products were purified as indicated above. The purified double-stranded amplification products were used as templates for DNA sequencing with the  $\Delta$  Taq cycle sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio). Cycle sequencing reactions used  $\gamma$ <sup>33</sup>P-dATP end-labelled primers and approximately 0.1  $\mu$ g of DNA template, followed by manual sequencing. Sequences were determined for both DNA strands for all individuals.

### Alignment and Phylogenetic Analyses

Alignments of sequences for both gene regions were first generated with Clustal V (Higgins and Sharp 1988) and then adjusted by eye to produce the final alignments. Two ambiguous regions containing multiple indels (total of 27 characters) were detected in the 16S alignment and were deleted before phylogenetic analyses. The 22 taxa for the 16S and COI alignments were analysed by maximum parsimony, neighbour joining (Saitou and Nei 1987), and maximum likelihood (Felsenstein 1981) as implemented in version 4.0b4a of PAUP\* (Swofford 1998).

The data from the two gene regions were analysed in a two-step process. First, the two sets of data were separately subjected to maximum parsimony, neighbour-joining, and maximum-likelihood analyses. The maximum-parsimony analysis employed a heuristic search, with the following options: keep minimal trees only, collapse zero branch lengths, random stepwise addition of taxa with 100 replications, tree bisection and reconnection for branch swapping, and MULPARS option on. Character states were unordered, and all characters were equally weighted. The neighbour-joining searches assumed the generalized nucleotide substitution model of Hasegawa *et al.* (1985), HKY85, for calculating distances (Swofford *et al.* 1996). To calculate phylogenies under the maximum-likelihood optimality criterion, we used an iterative strategy (Swofford *et al.* 1996). Initially, we used a maximum-parsimony tree as a starting tree for the analysis. We then performed a heuristic search, assuming the HKY85 substitution model, with a gamma correction for among-site rate variation of 0.50, and simultaneously estimating model parameters. The likelihood of the maximum-parsimony tree was significantly improved by the addition of these parameters to the model at each step (Huelsenbeck and Crandall 1997). We used the resulting phylogeny as the starting point for another search and repeated this process until the analysis converged on a single phylogeny. The following model of evolution for the maximum-likelihood algorithm was therefore used

for the 16S data set: maximum-likelihood estimates of the base-pair frequencies under the HKY85 model, transition to transversion ratio of 3.175, and gamma distribution shape factor of 0.379. The model of evolution for the maximum-likelihood algorithm used for the COI data set was: maximum-likelihood estimates of the base-pair frequencies under the HKY85 model, transition to transversion ratio of 7.639, and gamma distribution shape factor of 0.512. Models used for both data sets had four categories of substitution rate.

The next step in analysis involved combining the alignments from 16S and COI into a single data file and subjecting this combined data set to maximum-parsimony, neighbour-joining, and maximum-likelihood analyses. Maximum-parsimony and neighbour-joining analyses used the same parameters employed in the analyses of the individual data sets. The model of evolution for the maximum-likelihood algorithm used for the combined data set was: maximum-likelihood estimates of the base-pair frequencies under the HKY85 model, transition to transversion ratio of 4.097, and gamma distribution shape factor of 0.517. The model had four categories of substitution rate.

To evaluate the degree of phylogenetic signal in both data sets, 16S and COI, we performed two tests for phylogenetic structure. First, we performed the cladistic permutation tail probability (PTP) test (Archie 1989; Faith and Cranston 1991) as implemented in PAUP\*. This test uses the original data set to produce 100 randomly generated data sets, randomizing only the data among the ingroup taxa. Parsimony analyses using heuristic searches were then performed on each of the random data sets. The observed minimum length of the trees resulting from analyses of the random data sets was then compared to tree length of the minimal-length trees observed from a heuristic search performed on the original data set. The null hypothesis of no difference between tree lengths of original and random data sets can be rejected at the 0.05 level if 5% or fewer of the random data sets have a tree length equal to or less than that of the original most-parsimonious tree(s) ( $PTP \leq 0.05$ ).

A second test for phylogenetic signal in both 16S and COI data sets employed an estimation of skewness of tree-length distributions from maximum-parsimony analyses (Huelsenbeck 1991; Hillis and Huelsenbeck 1992), again as implemented in PAUP\*. The shape of a tree-length distribution provides a good indication of the presence of phylogenetic signal in a data set (Huelsenbeck 1991). Distributions of tree lengths with a strong left skew indicate that relatively few solutions exist near the optimal solution compared to elsewhere in the distribution. This pattern, in turn, is an indication of correlation among characters beyond that expected at random and therefore of significant phylogenetic signal in the data set (Hillis and Huelsenbeck 1992). For our analyses, we evaluated 10,000 random trees each for the 16S and COI data sets.

Finally, we calculated bootstrap values (Felsenstein 1985) to examine the degree of support for the nodes of trees resulting from maximum parsimony and neighbour joining for the separate and combined data sets. For each data set we performed 1000 bootstrap iterations using heuristic searches and the same parameters as those used in the original analyses.

## Results

### Nucleotide Composition of COI and 16S Alignments

The alignment of COI sequences for all 22 taxa was 643 bp in length. Of these, 341 characters were constant, 64 were variable but uninformative, and 238 were parsimony-informative. All sequence differences among taxa consisted of single-base-pair substitutions except for two 3-bp deletions found in *P. gracilis*. Sequences have been deposited with GenBank (Table 2).

The COI region for these 22 taxa was 57% A + T rich, which is similar to the 59.5% A/T bias reported among species of *Jasus* (Ovenden *et al.* 1997) and slightly less than that reported for insects (range of 68–75%, Lunt *et al.* 1996). Nucleotide content averaged 24.8% A, 32.2% T, 22.6% C, and 20.4% G, with very little deviation among taxa. The transition/transversion ratio estimated from the maximum parsimony analysis was 6.97. This bias favouring transitions is similar to that reported by other studies of substitutions in mtDNA (Moritz *et al.* 1987).

The alignment of 16S sequences for all 22 taxa was 487 bp in length. Twenty-seven characters were excluded because of ambiguities in the alignment of these regions. Of the remaining 460 characters, 269 were constant, 69 were variable but uninformative, and 122 were parsimony-informative. Sequence differences among taxa consisted of single-base-pair substitutions and 29 indel mutations. Of these 29 gaps, two regions had a 2-bp indel and one region had a 4-bp indel. The remainder of these mutations consisted of single-base-pair indels. Gaps were treated as missing data in the analyses. Sequences have been deposited with GenBank (Table 2).

The 16S region for these 22 taxa was 64.2% A + T rich, which is similar to the 62% A/T bias reported for species of *Jasus* (Ovenden *et al.* 1997) but less than that reported for insects (range of 77–82%, Simon *et al.* 1994). Nucleotide content averaged 31.1% A, 33.1% T, 13.6% C, and 22.2% G. The transition/transversion ratio estimated from the maximum-parsimony analysis was 2.99.

The pairwise sequence divergences based on Kimura's (1980) two-parameter model, which corrects for the 2:1 transition bias in mtDNA nucleotide substitutions, were consistently lower in the 16S than in the COI region (Table 3). This result suggests that the 16S gene is more conserved than the COI gene in *Panulirus*, a finding similar to that reported for species of *Jasus* (Ovenden *et al.* 1997) and several insect genera (Simon *et al.* 1994).

A total of 1104 characters was included for analysis in the combined COI/16S data set. Of these, 610 characters were constant, 134 were variable but uninformative, and 360 were parsimony-informative.

#### Phylogenetic Relationships

A significant level of phylogenetic signal was detected in both 16S and COI data sets. The randomization technique of Faith and Cranston (1991), the PTP test, confirmed that both 16S and COI original data sets were phylogenetically informative (PTP = 0.01 for 16S and PTP = 0.001 for COI). Critical values of  $g1$  (test statistic for skewness of tree-length distributions) were less (i.e. more negative) than tabled values (Hillis and Huelsenbeck 1992) for both 16S and COI data sets, indicating that each was significantly more structured than were random data. The value of  $g1$  for the 16S data set was  $-0.47$ , which is less than the tabled

value of  $g1 = -0.20$  (no. of taxa = 15, no. of characters = 100,  $P = 0.01$ ). The value of  $g1$  for the COI data set was  $-0.45$ , which is less than the tabled value of  $g1 = -0.16$  (no. of taxa = 15, no. of characters = 250,  $P = 0.01$ ). Thus parsimony trees generated by phylogenetic analyses of both 16S and COI data sets should show more structure than expected at random.

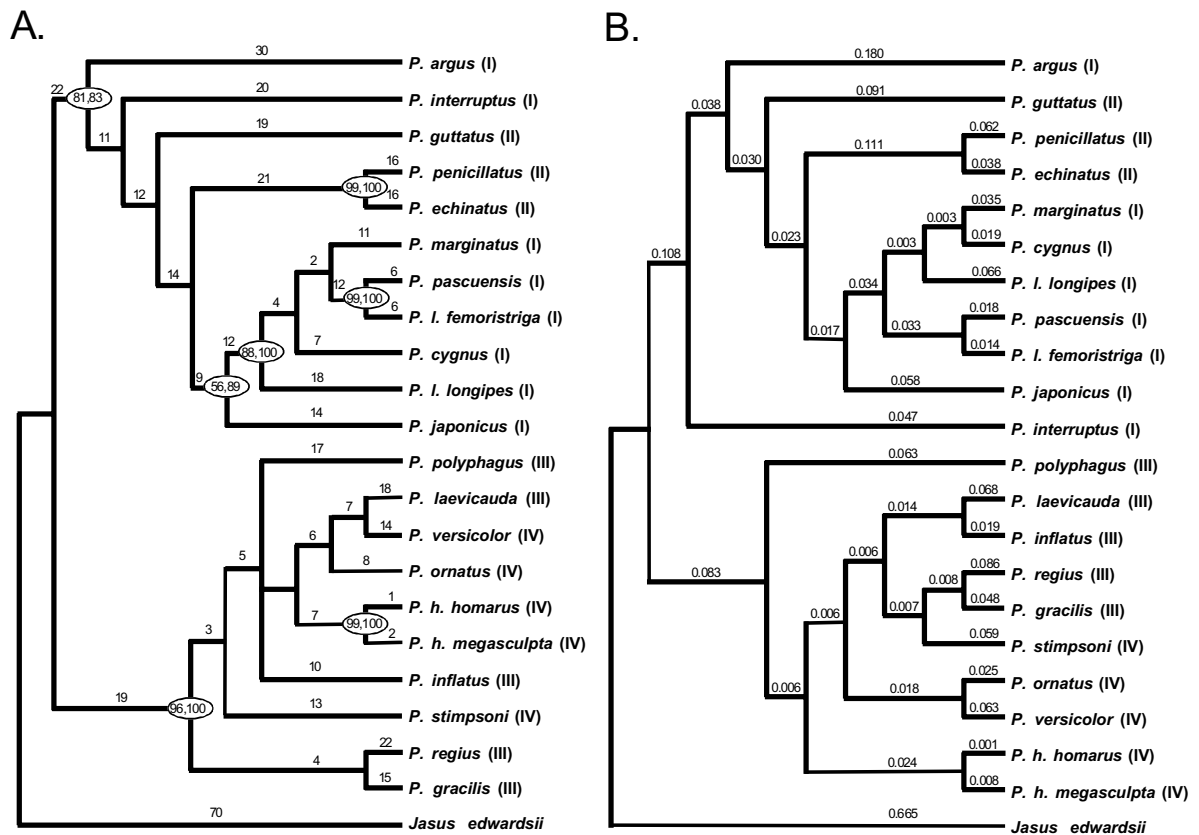
Maximum-parsimony, neighbour-joining, and maximum-likelihood analyses of the 16S data set all yielded trees with the same overall topology. Two well-supported major lineages of *Panulirus* species were recovered by all three methods of phylogenetic analysis. The first major lineage included all species of the morphologically based Groups I and II; the second lineage contained all species included in Groups III and IV (George and Main 1967).

Maximum-parsimony analysis of the 16S data set recovered two minimal-length trees (Fig. 1A). Neighbour joining recovered a topology similar to the maximum-parsimony trees with some minor differences in relationships among species within the Group III/IV lineage. The maximum-likelihood tree (Fig. 1B) also recovered the same two major lineages among species of *Panulirus*, but its topology differed from those of maximum parsimony and neighbour joining in its placement of *P. interruptus* as the sister to the remaining Group I/II species and *P. polyphagus* as the sister to the remaining Group III/IV species. In contrast, the maximum-parsimony and neighbour-joining trees placed *P. interruptus* as the second lineage to branch off (after *P. argus*) in Group I/II, and *P. polyphagus* was nested within the Group III/IV lineage. Placements of these two species were not strongly supported by bootstrap analyses, however (Fig. 1A). Trees resulting from the different analysis methods also differed in relationships among Group III/IV species.

Maximum-parsimony analysis of the COI data set yielded three minimal-length trees, again showing two major lineages of species of *Panulirus*, a Group I/II lineage and a Group III/IV lineage (Fig. 2A). Neighbour joining recovered a similar overall topology but differed with respect to some of the sister relationships recovered among species within each of the two major lineages. Bootstrap support for the Group I/II lineage was lower than that from the 16S data set (Fig. 2A). Bootstrap support for the Group III/IV lineage was strong for both 16S and the COI data sets. The maximum-likelihood analysis also recovered the same two major lineages of *Panulirus* as were recovered by maximum-parsimony and neighbour-joining analyses (Fig. 2B). The maximum-likelihood tree differed from the parsimony and neighbour-joining trees in its placement of *P. argus* as the sole sister taxon to the remaining Group I/II species. In contrast, the maximum-parsimony trees placed *P. guttatus*, *P. echinatus* and *P. penicillatus* along with *P. argus* as a sister clade to the remaining species in Group I/II. The neighbour-joining tree placed *P. guttatus* as the

**Table 3. Interspecific sequence divergence estimates for pairs of *Panulirus* species for 16S (above diagonal) and COI (below diagonal)**  
 Sequence divergence estimates are based on Kimura's (1980) two-parameter model. See Table 2 for species abbreviations

	arg	cyg	ech	gra	gut	hoh	hom	inf	int	jap	lae	lof	lol	mar	orn	pas	pen	pol	reg	sti	ver	jas
arg	---	0.157	0.176	0.190	0.160	0.175	0.179	0.166	0.142	0.139	0.168	0.187	0.164	0.157	0.181	0.185	0.194	0.173	0.189	0.175	0.175	0.286
cyg	0.211	---	0.145	0.172	0.122	0.170	0.174	0.167	0.126	0.079	0.178	0.062	0.068	0.047	0.193	0.065	0.144	0.176	0.205	0.173	0.194	0.253
ech	0.248	0.238	---	0.203	0.149	0.184	0.189	0.199	0.148	0.127	0.187	0.145	0.158	0.133	0.190	0.140	0.076	0.200	0.224	0.194	0.185	0.240
gra	0.253	0.265	0.272	---	0.177	0.071	0.080	0.065	0.156	0.169	0.080	0.203	0.201	0.194	0.079	0.205	0.212	0.100	0.089	0.081	0.105	0.257
gut	0.218	0.209	0.239	0.255	---	0.165	0.166	0.171	0.118	0.121	0.178	0.134	0.150	0.131	0.168	0.134	0.153	0.172	0.200	0.169	0.183	0.256
hoh	0.262	0.242	0.268	0.126	0.252	---	0.007	0.051	0.145	0.146	0.075	0.188	0.183	0.170	0.051	0.196	0.206	0.071	0.097	0.074	0.076	0.234
hom	0.269	0.221	0.280	0.152	0.216	0.140	---	0.053	0.149	0.145	0.073	0.190	0.186	0.175	0.054	0.197	0.211	0.072	0.103	0.075	0.080	0.237
inf	0.237	0.243	0.230	0.172	0.242	0.156	0.148	---	0.153	0.160	0.067	0.180	0.155	0.177	0.067	0.185	0.189	0.080	0.087	0.068	0.092	0.261
int	0.307	0.289	0.318	0.329	0.271	0.288	0.297	0.332	---	0.121	0.155	0.134	0.127	0.131	0.170	0.130	0.159	0.154	0.181	0.163	0.170	0.256
jap	0.248	0.210	0.261	0.269	0.233	0.241	0.251	0.280	0.280	---	0.165	0.106	0.109	0.087	0.181	0.109	0.138	0.170	0.199	0.161	0.164	0.260
lae	0.263	0.253	0.301	0.159	0.234	0.170	0.162	0.154	0.310	0.263	---	0.196	0.203	0.185	0.080	0.198	0.209	0.111	0.093	0.086	0.078	0.257
lof	0.218	0.136	0.237	0.282	0.212	0.275	0.231	0.272	0.297	0.232	0.243	---	0.078	0.065	0.200	0.028	0.150	0.207	0.238	0.200	0.207	0.280
lol	0.209	0.135	0.236	0.272	0.198	0.270	0.215	0.266	0.315	0.221	0.233	0.060	---	0.076	0.207	0.081	0.151	0.195	0.232	0.183	0.208	0.290
mar	0.223	0.139	0.261	0.264	0.218	0.248	0.220	0.256	0.287	0.211	0.253	0.140	0.124	---	0.197	0.067	0.149	0.192	0.228	0.179	0.197	0.268
orn	0.257	0.242	0.264	0.147	0.247	0.127	0.145	0.154	0.318	0.253	0.188	0.254	0.236	0.247	---	0.199	0.215	0.079	0.099	0.071	0.069	0.238
pas	0.236	0.175	0.222	0.304	0.234	0.268	0.256	0.268	0.297	0.238	0.290	0.198	0.171	0.169	0.276	---	0.148	0.206	0.236	0.202	0.202	0.268
pen	0.226	0.219	0.131	0.251	0.227	0.270	0.259	0.243	0.292	0.260	0.288	0.234	0.207	0.230	0.250	0.212	---	0.201	0.225	0.191	0.196	0.267
pol	0.242	0.245	0.288	0.175	0.234	0.173	0.170	0.175	0.296	0.255	0.173	0.258	0.239	0.228	0.179	0.247	0.273	---	0.118	0.090	0.103	0.253
reg	0.266	0.258	0.291	0.162	0.277	0.186	0.173	0.181	0.322	0.263	0.170	0.266	0.259	0.250	0.185	0.280	0.262	0.185	---	0.089	0.132	0.264
sti	0.233	0.242	0.275	0.164	0.249	0.183	0.173	0.173	0.301	0.259	0.184	0.258	0.237	0.251	0.173	0.246	0.252	0.164	0.186	---	0.089	0.233
ver	0.246	0.244	0.260	0.174	0.276	0.196	0.171	0.159	0.305	0.261	0.181	0.254	0.258	0.253	0.178	0.284	0.246	0.179	0.189	0.147	---	0.257
jas	0.287	0.255	0.251	0.289	0.293	0.264	0.256	0.258	0.323	0.279	0.265	0.286	0.279	0.264	0.281	0.268	0.257	0.250	0.256	0.263	0.240	---



**Fig. 1.** (A) Strict consensus of two minimal-length trees based on maximum-parsimony analysis of the 16S data set for the 22 taxa examined (tree length = 533; consistency index = 0.552; homoplasy index = 0.448; retention index = 0.644). Numbers on the branches are branch lengths measured as number of nucleotide substitutions. Bootstrap support (>50%) for 1000 iterations for maximum parsimony and neighbour joining, respectively, is given in circles at the nodes. (B) Optimal tree topology from maximum-likelihood analysis with branch lengths indicated (-Ln = 2978). (I) = Group I; (II) = Group II; (III) = Group III; (IV) = Group IV, all based on the morphological groups of George and Main (1967).

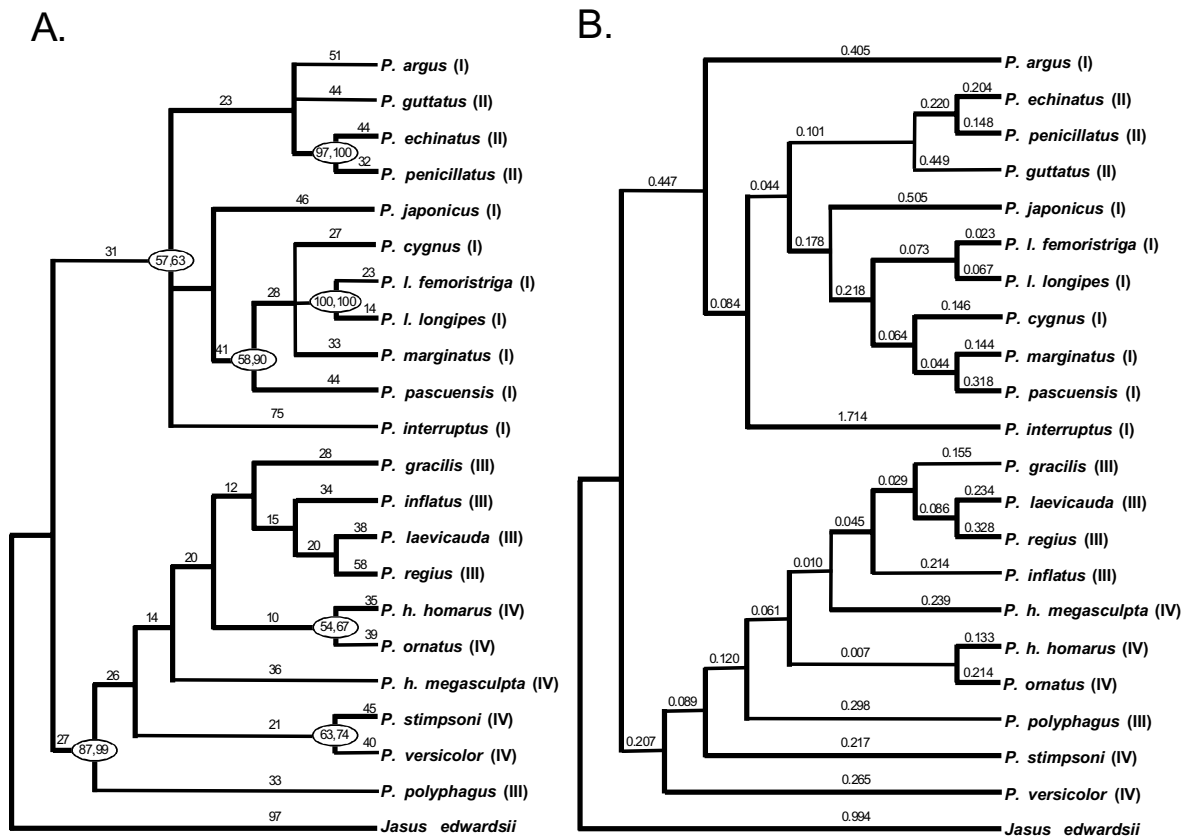
sister to *P. argus* and with this clade as sister to all the remaining Group I/II species except *P. interruptus* and *P. japonicus*, the first clade coming off of the Group I/II lineage. A *P. echinatus*, *P. penicillatus* lineage was nested within the Group I/II clade in the neighbour-joining tree. Bootstrap support was weak ( $\leq 38\%$ ), however, for all of these arrangements in either the maximum-parsimony trees or the neighbour-joining tree. All three topologies differed slightly in their relationships among species within the Group III/IV lineage, but these differences were also not strongly supported by bootstrap analyses.

The combined COI/16S data set yielded seven minimal-length trees from maximum-parsimony analysis (Fig. 3A). The same two major lineages (Group I/II and Group III/IV) were again recovered and strongly supported by bootstrap analyses for both maximum-parsimony and neighbour-joining analyses (Fig. 3A). Neighbour-joining analysis produced a topology nearly identical to the maximum-likelihood tree (Fig. 3B). The major difference between the maximum-likelihood tree and the maximum-parsimony tree

topology was in the placement of *P. argus*. Similarly to the separate analyses of each data set, the maximum-likelihood analysis of the combined data placed *P. argus* as sister to the remaining Group I/II species. In contrast, maximum-parsimony analysis placed *P. interruptus* as sister to the remaining Group I/II species and *P. argus* as sister to a *P. echinatus*/*P. penicillatus* clade within the Group I/II lineage. Bootstrap support for these arrangements in the maximum-parsimony trees was weak, however ( $< 50\%$  for each node; Fig. 3A). Again, maximum-parsimony, neighbour-joining, and maximum-likelihood trees differed slightly in relationships among species within the Group III/IV lineage.

## Discussion

All methods of phylogenetic reconstruction used on the two gene regions either separately or combined consistently recovered two major evolutionary lineages among species of *Panulirus* that correspond to the morphologically based Group I/II and Group III/IV, but the molecular phylogeny

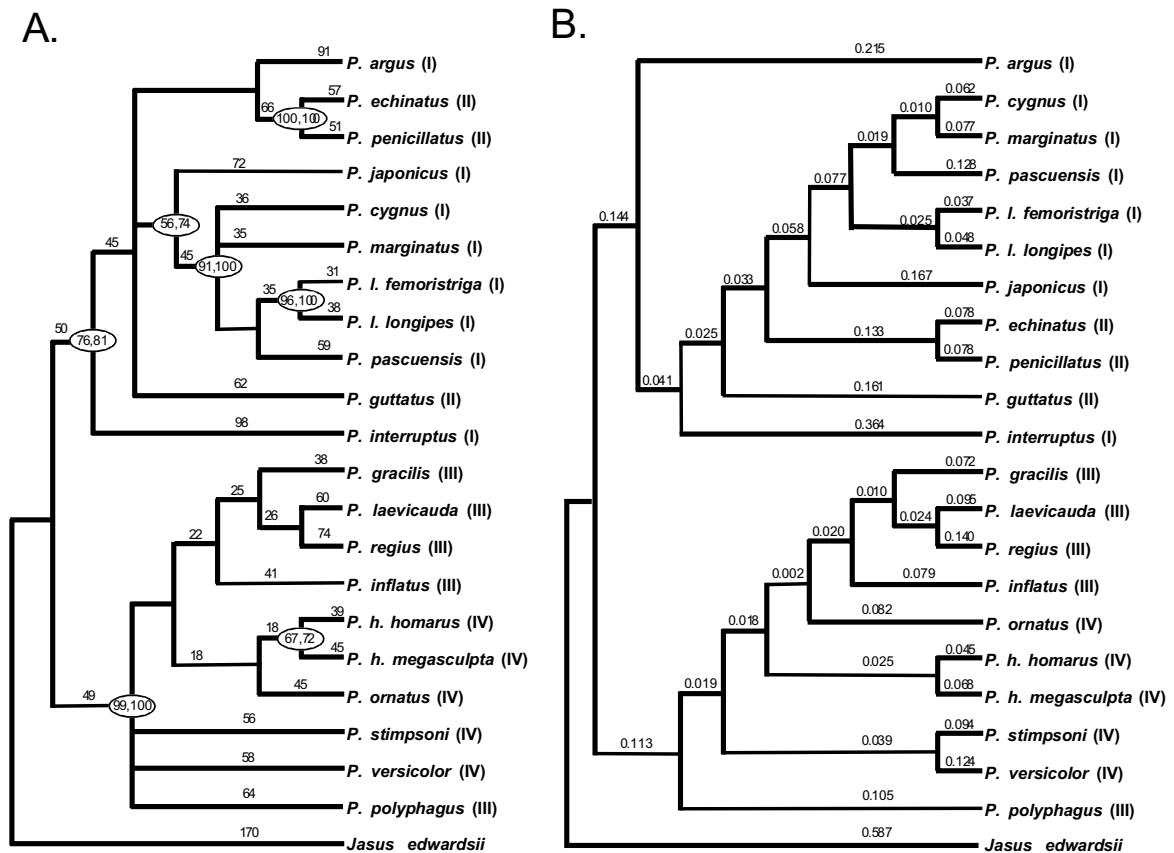


**Fig. 2.** (A) Strict consensus of three minimal-length trees based on maximum-parsimony analysis of the COI data set for the 22 taxa examined (tree length = 1394; consistency index = 0.367; homoplasy index = 0.633; retention index = 0.420). Numbers on the branches are branch lengths measured as number of nucleotide substitutions. Bootstrap support (>50%) for 1000 iterations for maximum parsimony and neighbour joining, respectively, is given in circles at the nodes. (B) Optimal tree topology from maximum-likelihood analysis with branch lengths indicated (-Ln = 6032). (I) = Group I; (II) = Group II; (III) = Group III; (IV) = Group IV, all based on the morphological groups of George and Main (1967).

does not support the monophyly of any of the individual Groups I–IV (George and Main 1967). The degree of genetic divergence between species suggests that species in Group I/II may have radiated earlier than species in Group III/IV. The level of sequence divergence was consistently higher among species in Groups I and II (16S: 2.8–19.4%; COI: 12.4–31.8%) than that among species in Groups III and IV (16S: 5.3–13.2%, COI: 12.6–19.6%). However, the higher level of sequence divergence among species in Groups I and II may also be due to a higher rate of molecular evolution at these mitochondrial genes for species in this lineage. The observed pattern of sequence divergence is consistent with previous hypotheses suggesting that species in Groups I and II represent an early radiation of *Panulirus*. These hypotheses were based on the retention of presumably more primitive or ancestral morphological traits in adult (George and Main 1967), phyllosoma, and puerulus forms (McWilliam 1995) for species in Groups I and II.

The bootstrap support for a major division between *Panulirus* species of Group I/II and Group III/IV is reasonably strong, especially for the analyses of the 16S and

combined data sets (Fig. 1A and 3A), but within each of the two major lineages, support for relationships among species is much weaker. Within the Group I/II lineage, support is strong for a monophyletic lineage containing Group I species *P. cygnus*, *P. marginatus*, *P. longipes* and *P. pascuensis*. This group was consistently recovered by all three methods of phylogenetic analysis for each data set separately and for the combined data set. In addition, all analyses placed *P. japonicus* as sister to this lineage, although bootstrap support for this node is weak (e.g. Fig. 3A). However the remaining Group I species, *P. argus* and *P. interruptus*, are well outside of this Group I species clade and are clearly distantly related. Little support exists for Group II of George and Main (1967). Group II species *P. echinatus* and *P. penicillatus* are well-supported sister taxa, but they clearly do not share a recent common ancestor with *P. guttatus*, the third species in Group II. In addition, maximum-parsimony and maximum-likelihood trees differ considerably in the placement of these two taxa within the Group I/II lineage (Fig. 3A and 3B). McWilliam (1995) also argued that *P. guttatus* did not fit with other Group II



**Fig. 3.** (A) Strict consensus of seven minimal-length trees based on maximum-parsimony analysis of the combined 16S and COI data sets for the 22 taxa examined (tree length = 1962; consistency index = 0.411; homoplasy index = 0.589; retention index = 0.473). Numbers on the branches are branch lengths measured as number of nucleotide substitutions. Bootstrap support (>50%) for 1000 iterations for maximum parsimony and neighbour joining, respectively, is given in circles at the nodes. (B) Optimal tree topology from maximum-likelihood analysis with branch lengths indicated ( $-Ln = 9208$ ). (I) = Group I; (II) = Group II; (III) = Group III; (IV) = Group IV, all based on the morphological groups of George and Main (1967).

species on the basis of its possession of more derived phyllosoma characters.

Within the Group III/IV major lineage of *Panulirus*, there is little support for any groupings among species; bootstrap values are low for most nodes within this lineage (Fig. 3A). Trees resulting from the different analysis methods also produced different relationships among species in Group III/IV (e.g. Fig. 3A and 3B), indicating poor resolution within this lineage. The molecular phylogeny therefore provides no support for a separation of Group III from Group IV whether these groupings are based on adult (George and Main 1967), phyllosoma, or puerulus traits (McWilliam 1995).

Because most of the relationships within each of the two major lineages are only weakly supported, we can only begin to make tentative predictions concerning the pattern of speciation of *Panulirus*. Our findings based on mtDNA sequence divergences seem to agree with allozyme-based genetic distances (Chan and Chu 1996) in suggesting that species in Groups I and II radiated much earlier than the

Pleistocene, the time previously proposed by George and Main (1967), Pollock (1992, 1993), and Sekiguchi (1995). Indeed, George (1997) has recently revised his hypotheses regarding the timing of the initial radiation of *Panulirus* in light of the recent genetic evidence and suggests a late Miocene radiation of species in Group I/II.

George and Main (1967) proposed an evolutionary hypothesis for speciation in *Panulirus* that in many ways is consistent with the pattern recovered by our molecular phylogeny. They proposed that the ancestral species of *Panulirus* was able to inhabit the sublittoral of an extensive geographic area in the equatorial region with free access to all oceans because of its wide environmental tolerance in both the larval and adult condition. They further suggested that, among modern species, those that fit this description most closely are *P. longipes* and *P. argus*. George and Main (1967) chose *P. longipes* to represent the ancestral species because 'it is better known to us'. Our maximum-likelihood phylogeny of the combined data suggests a radiation of Group I/II species from an 'argus-like' ancestor from which

*P. argus* and *P. interruptus* diverged (Fig. 3B). Indeed, all methods of phylogenetic analysis of all three data sets (16S, COI, and combined) show an early split between a *P. argus* lineage and a *P. interruptus* lineage, followed by the radiation of the Indo-West Pacific species in Group I: *P. cygnus*, *P. marginatus*, *P. longipes*, *P. pascuensis*, and *P. japonicus*. Interoceanic connections and westward equatorial currents present in the late Miocene would support this pattern of evolutionary diversification between Group I taxa in different oceans (Pollock 1992; George 1997).

A more recent radiation of species in Groups III and IV has been hypothesized on the basis of their smaller genetic distances from allozyme studies (Chan and Chu 1996; Macaranas *et al.* personal communication, cited by George 1997). This pattern is also supported by our mtDNA analyses, which show lower levels of sequence divergence and shorter branch lengths among species in Groups III and IV than among species in Groups I and II (Table 3, Fig. 3A). George and Main (1967) hypothesized that an Indo-West Pacific ancestor gave rise to the more recent radiation of species in Group III/IV, probably speciating in response to temporary isolation within restricted parts of the vast region of the Indo-West Pacific. Species in Group III/IV with restricted distributions such as *P. regius*, *P. polyphagus*, *P. stimpsoni*, and two subspecies of *P. homarus*, *P. h. megasculpta* and *P. h. rubellus* are good examples (George 1997). Our molecular phylogeny provides some support for George and Main's (1967) hypothesis of allopatric speciation within the Group III/IV lineage. For example, *P. polyphagus* is the first species to split off in the Group III/IV clade in maximum-likelihood trees for both the 16S and combined data sets and in the maximum-parsimony trees for the COI data set, suggesting an early divergence of this species.

The overall pattern of speciation recovered by our mtDNA phylogenies supports earlier hypotheses, based on morphological characters (George and Main 1967; McWilliam 1995), suggesting the early divergence of two major lineages in *Panulirus*. Further phylogenetic analyses are required to test relationships among species within these two major lineages. For example, the taxonomic status of the subspecies of *P. homarus* and *P. longipes* warrants further investigation. In both instances, subspecies are sister taxa within trees generated from the combined analyses, but levels of sequence divergence at the two genes suggest genetic differentiation has occurred between subspecies (Table 3). Future studies should address questions of genetic differentiation both within and between species of *Panulirus*.

In summary, our molecular phylogeny has begun to clarify the relationships within the genus *Panulirus*. Our phylogenetic results represent a first step toward understanding the pattern of speciation in *Panulirus* solely

on the basis of molecular characters. We hope that future researchers will add to our molecular data set by including sequences of additional genes from both the nuclear and mitochondrial genomes for species and subspecies of *Panulirus*. In addition, we encourage others to use our phylogenetic framework as a starting point in attempts to uncover the origin and likely direction of change in key morphological, ecological, and behavioural traits possessed by members of this fascinating and highly divergent genus.

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