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RESEARCH ARTICLE

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Population genetics of the swimming crab *Callinectes bellicosus* (Brachyura: Portunidae) from the eastern Pacific Ocean

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Abstract The population genetics and historical demography of the swimming crab *Callinectes bellicosus* from the eastern Pacific were assessed using mitochondrial DNA (mtDNA) sequences from portions of the cytochrome *c* oxidase subunit I (*COI*) and cytochrome *b* (*Cyt*b) genes. Analysis of molecular variance of sequence data from crabs collected from nine localities, ranging from the upper to lower Gulf of California and the outer coast of the Baja California peninsula, revealed an absence of population structure, suggesting a high level of gene flow over a wide geographic area. Maximum-likelihood estimates of long-term effective population size obtained with the program FLUCTUATE, in addition to highly significant values obtained from neutrality tests (Tajima's *D*, Fu and Li's *D*, and Fu's F_S) and mismatch analysis, are consistent with a population expansion dating to the Pleistocene epoch. Phylogenetic analysis of *C. bellicosus* sequences using both neighbor-joining and Bayesian methods revealed a widely distributed subclade (clade II) cryptically embedded at low frequency in the main (clade I) population. Although sequence divergence between the two clades was low (1.1% *COI*; 0.6% *Cyt*b), statistical support for the split was high. The Kimura-2-parameter genetic distance between *C. bellicosus* and the sympatric and morphologically similar *C. arcuatus* was high ($d=0.17$) and similar to the genetic distance between *C. bellicosus* and the allopatric *C. sapidus* from the western Atlantic ($d=0.18$), suggesting an ancient (Miocene) divergence of *C. bellicosus* and *C. arcuatus*.

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Introduction

The ecology, life history and population biology of eastern Pacific swimming crabs (Brachyura: Portunidae) belonging to the genus *Callinectes* are poorly understood. This lack of knowledge has important practical implications because within the last 20 years *C. bellicosus* (Stimpson) has become the focus of an important commercial fishery in Mexico, especially in the eastern Gulf of California and at Bahía Magdalena, a hypersaline estuary on the outer coast of Baja California Sur (Sánchez-Ortiz and Gómez-Gutiérrez 1992; Molina 1999; Bourillón-Moreno 2002). A second, slightly smaller species, *C. arcuatus* Ordway, occurs sympatrically with *C. bellicosus* throughout most of the gulf and on the outer Pacific coast of the Baja California peninsula in sandy and soft-bottom habitats. Of the two species, *C. arcuatus* shows the widest distribution, being found as far south as Peru (Williams 1974).

The southern limit of the distribution *C. bellicosus* is most likely the lower Gulf near Mazatlán, Sinaloa (Brusca 1980; Paul 1982a). A third eastern Pacific species, *C. toxotes* Ordway, reaches the northern limit of its distribution at the extreme southern end of the gulf near Mazatlán, and then occurs sympatrically with *C. arcuatus* southward to northwestern South America (Paul 1982a).

The few studies that have been conducted on eastern Pacific *Callinectes* spp. indicate that they share similar life-history characteristics with their western Atlantic congener, *C. sapidus* Rathbun. As in *C. sapidus*, ovigerous females of eastern Pacific *Callinectes* spp. migrate to the mouth of estuaries and bays to spawn (Norse and Estevez 1977; Paul 1982a, 1982b; Sánchez-Ortiz and Gómez-Gutiérrez 1992). Recently hatched larvae (zoeae) are then carried by currents to the open ocean, where larval development occurs over a period of about 50–70 days (Paul 1982b). The protracted pelagic larval stage suggests that dispersal potential is high (Scheltema 1986; but also see Knowlton and Keller 1986). Dispersal capabilities of benthic adults are unknown. After pelagic development, the zoeae molt into benthic megalopae that begin recruiting to inshore habitats.

The high potential for larval dispersal predicts that little genetic differentiation would be found over the geographic range of *Callinectes* spp. in the eastern Pacific. There is increasing evidence, however, that a variety of physical oceanographic factors, including temperature gradients, oceanic currents and wind patterns, can restrict larval dispersal, leading to population structure in marine species with pelagic larvae (e.g. Reeb and Avise 1990; McCartney et al. 2000). Both oceanic currents and geological features have been implicated in restricting the distribution of marine organisms in the Gulf of California. The midriff islands that form a boundary between the relatively shallow waters of the northern gulf and the deeper waters of the central and southern gulf (Fig. 1) have been suggested to represent a barrier affecting the distribution of brachyurans in the gulf (Correa-Sandoval and Carvacho-Bravo 1992). The island barrier, together with localized oceanic currents, is thought to restrict dispersal of pelagic larvae of penaeid shrimp in the gulf and contribute to population structure, as has been detected with allozyme and DNA markers (Aubert and Lightner 2000; de la Rosa-Vélez et al. 2000). The possible existence of genetically distinct populations, or stocks, of *C. bellicosus* would have important implications for management of the developing fishery.

Mitochondrial DNA sequence data have been used increasingly for inferring phylogenetic relationships and species identity in decapod crustaceans (e.g. Harrison and Crespi 1999; Schubart et al. 2001; Stillman and Reeb 2001). Molecular methods also can provide indirect estimates of larval dispersal and gene flow in marine organisms with pelagic larvae in which direct estimates are impractical (Hamm and Burton 2000). Furthermore, statistical tests designed for assessing whether nucleotide

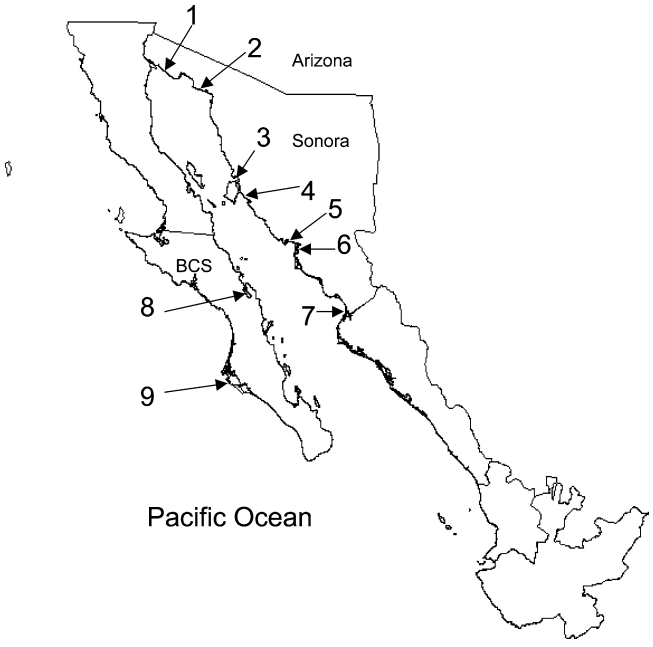

Fig. 1 Map showing collecting localities in the Gulf of California and on the outer coast of the Baja California peninsula, with locality abbreviations in parentheses [*1* El Golfo de Santa Clara (St); *2* Bahía San Jorge near Puerto Peñasco (Sj); *3* Canal del Infiernillo near Punta Chueca (In); *4* Bahía Kino (Bk); *5* Guaymas (Gy); *6* Guásimas (Gi); *7* Agiabampo (Ag); *8* Bahía Concepción (Bc); *9* Bahía Magdalena near San Carlos (Bm); *BCS* Baja California Sur]

polymorphisms deviate from expectations under neutral theory also can reveal details on population demographics. Ramos-Onsins and Rozas (2002) recently have compared these statistical tests, which are based on either: (1) the frequency of segregating sites [e.g. Tajima's (1989) *D* and Fu and Li's (1993) *D**], (2) the distribution of haplotypes [Fu's (1997) F_S], or (3) the distribution of pairwise sequence differences (mismatch distribution; Harpending 1994), and showed that Fu's F_S was the most powerful test for detecting population growth when sample size was large.

In the present study we analyzed sequence data from cytochrome *c* oxidase subunit I (*COI*) and cytochrome *b* (*Cyt*b) gene segments to assess whether structure was present in populations of *C. bellicosus* from the Gulf of California and the outer Pacific coast, and to characterize the demographic history of the species utilizing the representative statistical tests described above, together with maximum-likelihood estimates of population growth (Kuhner et al. 1998). We chose to present results on the two genes individually in order to compare our divergences between *Callinectes* subclades (see below) with those reported for other marine invertebrates that had been based on analysis of single genes, mainly *COI*. However, the results were similar, and the same conclusions were indicated, when we conducted the analyses with a combined data set.

Materials and methods

Animals

Individuals of *Callinectes bellicosus* were collected from eight localities within the Gulf of California, spanning a distance of about 600 km, and from Bahía Magdalena on the western coast of Baja California Sur (Fig. 1). Fresh whole crabs or individual claws (chelipeds) were either packed in ice or preserved in ethanol and transported to Guaymas. Samples transported on ice were either used immediately or frozen at −18°C. Individuals of the sympatric species *C. arcuatus* were collected at Bahía Kino and Guaymas and used as outgroups, along with several individuals of the western Atlantic species *C. sapidus* from Beaufort, North Carolina. *C. arcuatus* is very similar morphologically to *C. bellicosus*, but the two species can be separated by several characters, including the length of the lateral spines and the spines between the eyes on the carapace, and coloration. Also, the inner face of the chelae of *C. bellicosus* lacks a ridge of shallow denticles that characterize *C. arcuatus* (Brusca 1980).

DNA extraction, gene amplification, sequencing and alignment

Total genomic DNA was extracted from muscle tissue obtained from the cheliped using either the DNAzol (Molecular Research Center, Cincinnati, Ohio) or the DNeasy (QIAGEN, Valencia, Calif.) protocol with proteinase K digestion. The polymerase chain reaction (PCR) was used to amplify a 709-bp segment of the *COI* gene using the primers LCO1490f (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198r (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). A 425-bp segment of the *Cyt*b gene was amplified with the primer set 144F (5′-TGAGSNCARATGTCNTWYTG-3′) and 270R (5′-AANAGGAARTAYCAYTCNGGYTG-3′) (Merritt et al. 1998). PCR was performed on either a Perkin-Elmer Thermal Cycler 480 or an Eppendorf Mastercycler in a reaction mixture containing 1 µl template DNA, 5 µl of 10× PCR buffer (0.1 M Tris-HCl, 0.5 M KCl, 0.015 M $MgCl_2$, pH 8.3), 5 µl of 2.5 mM dNTP, 2 µl of each 10 µM primer, 5 µl of 50 mM $MgCl_2$ and 1.5–2.5 U *Taq* DNA polymerase (Takara Shuzo, Shiga, Japan or Fisher Scientific, Fair Lawn, N.J.) and brought up to 50 µl with water. After an initial denaturation at 94°C for 3 min, PCR reaction conditions for *COI* were 30 cycles of 94°C for 1 min of denaturation, 45°C for 1 min of annealing, and 72°C for 1 min of extension, followed by a final extension of 10 min. For *Cyt*b, conditions were 94°C for 4 min and then 40 cycles of 94°C for 1 min of denaturation, 48°C for 1 min of annealing, and 72°C for 2 min of extension, followed by a final extension of 6 min. Verification of successful amplification was assessed by agarose gel electrophoresis.

PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN). Forward and reverse sequencing reactions were performed on an Applied Biosystems (Foster City, Calif.) ABI 3700 DNA sequencer using the PCR primers. Alignments were performed in ClustalX 1.81 (Thompson et al. 1997). Forward and reverse sequences (95–100% overlap for *COI*; 93% overlap for *Cyt*b) were in complete agreement. The first base in *COI* (657 bp) and *Cyt*b (382 bp) corresponds to position 4,073 and 14,694, respectively, in the complete mitochondrial genome of the anomuran decapod *Pagurus longicarpus* (GenBank Accession no. NC_003058: Hickerson and Cunningham 2000). Representative sequences for both genes have been deposited in GenBank under accession numbers AY465907–AY465910 (*C. bellicosus*), AY465911–AY465914 (*C. arcuatus*) and AY465915–AY465916 (*C. sapidus*).

Data analyses

Aligned DNA sequences were imported into MEGA version 2.1 (Kumar et al. 2001) for analysis of base composition and determination of genetic distances using Kimura's (1980) 2-parameter (K2P) method. Neighbor-joining (NJ) analysis (Saitou and Nei 1987) implemented in MEGA and based on the matrix of K2P distances was used to examine relationships between *Callinectes* spp. Relative support for tree topology was obtained by bootstrapping (Felsenstein 1985) using 1,000 iterations of the data matrix. All codon positions and types of substitutions were weighted equally in all analyses.

The two distinct clades of *C. bellicosus* and *C. arcuatus* identified in the NJ trees for *COI* (see "Results") were also confirmed by Bayesian methods implemented in MrBayes version 2.01 (Huelsenbeck and Ronquist 2001). Clade support was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm. Parameters in MrBayes were set to one-million generations and 4,000 trees sampled (sampled every 250th generation). Log-likelihood values from four simultaneous MCMC chains stabilized at about 80,000 generations, resulting in the first 320 trees being discarded from the analysis (burnin = 320). The 50% majority rule consensus tree was based on the general time-reversible (GTR) model of DNA substitution using the default random tree option to begin the analysis and selecting site-specific rate variation by codon.

Analysis of molecular variance (AMOVA) was used to test for population structure in *C. bellicosus* using ARLEQUIN version 2.000 (Schneider et al. 2000). The calculation of significance (5% level) of pairwise comparisons (hierarchical analysis) of the F_{ST} analogue Φ_{ST} among collection localities was based on 1,000 permutations of the data matrix.

Tests for population size changes

Neutrality tests [Tajima's D, Fu and Li's D^* and Fu's F_S] were performed in DnaSP version 3.51 (Rozas and Rozas 1999). The approximate 95% confidence intervals for each test statistic were obtained using 10,000 coalescent simulations implemented in DnaSP. Mismatch analysis of the number of pairwise differences between haplotypes was performed in ARLEQUIN.

To estimate long-term effective population size (N_e) of *C. bellicosus*, and to evaluate whether N_e has remained stable or changed over time, aligned *COI* and *Cyt*b sequences were analyzed with the computer program FLUCTUATE version 1.4 (Kuhner et al. 1998) (available at http://evolution.genetics.washington.edu/lamarc.html). Simultaneous maximum-likelihood estimates of the mutation parameter (θ) and the exponential population growth parameter (g) were estimated, where $\theta = 2N_{ef}\mu$ and N_{ef} is the female effective population size; μ is the neutral mutation rate per site per generation. We assumed an equal sex ratio such that $N_e = 2N_{ef}$.

Search strategies in FLUCTUATE were initially set at ten short chains of 1,000 steps each, followed by two long chains of 20,000 steps each, sampling every 20th step. Increasing the steps to 5,000 and 50,000, respectively, resulted in increased stability in values of θ and g in four successive iterations. The values reported for θ and g were based on a final extended run of ten short chains of 100,000 steps each and two long chains of 200,000 steps each (Garrigan et al. 2002). As all nucleotide substitutions were transitions, except for a single transversion in *COI*, the Ti/Tv ratio was set at 10. The initial estimates of θ were based on the number of segregating sites (Watterson 1975); the initial estimate of g was arbitrarily set at 10 and then adjusted in subsequent iterations to reflect the value of the previous iteration. The random tree default setting was selected for the starting genealogy.

An estimate of N_e can be obtained if μ is known. Molecular clock calibrations based on geminate species separated by the Isthmus of Panama estimate the average pairwise sequence divergence rate at about 2.3% per million years for the *COI* gene in crustaceans and other arthropods (Knowlton et al. 1993; Brower 1994; Daniels et al. 2002). Unless stated otherwise, we used this rate here (i.e. the estimated single-lineage value for μ was 1.15×10^{-8}), but it must be emphasized that a calibrated molecular clock for mtDNA in brachyuran crabs has yet to be described (Chu et al. 1999), and substantial errors may be associated with the estimates. Marko (2002), for example, has pointed out that speciation could have begun in geminate pairs before habitat separation, resulting in an overestimation of the actual divergence rate. Studies on grapsid crabs suggest divergence rates as low as 1.32% per million years for *COI* (Schubart et al. 1998). A 1-year generation time, based on our unpublished field observations, was assumed in all analyses of *C. bellicosus*. This estimate also agrees with time to reach maturity in females of *C. arcuatus* (Paul 1982b; Dittel and Epifanio 1984).

Results

Sequence analysis

Mean nucleotide composition of the *COI* and *Cyt*b gene fragments in *Callinectes* spp. is given in Table 1. For each gene, nucleotide composition was similar in the three species, with a strong bias against G, especially at the third codon position. Percentages of third position G for *COI* and *Cyt*b, respectively, were 4.6% and 4.5% (*C. bellicosus*), 8.5% and 6.8% (*C. arcuatus*) and 1.8% and 0.8% (*C. sapidus*). As expected for protein-coding genes, no insertions or deletions were found, and no stop codons were detected in either of the translated gene products. A search of crustacean *COI* and *Cyt*b sequences in GenBank revealed high amino acid sequence homology with *Callinectes*. Together these results indicate that our sequences represent mtDNA, and are not nuclear pseudogenes, which are known for a variety of organisms, including crustaceans (Zhang and Hewitt 1996; Williams and Knowlton 2001).

Variable positions in *COI* and *Cyt*b in *C. bellicosus* are shown in Table 2. The *COI* gene fragment contained 26 variable sites, all at the third position. Except for a single transversion (C to G) mutation at position 561, all substitutions were transitions. Twenty-three *COI* haplotypes were found. There were no non-synonymous substitutions. The last four haplotypes (haplotypes 20–23) possessed five mutations (positions 48, 264, 273, 414 and 540) not shared with the other 19 haplotypes (Table 2). These four unique haplotypes characterized a subclade (clade II) of *C. bellicosus* (see following subsection).

The *Cyt*b gene fragment in *C. bellicosus* contained 21 variable sites, four at the first codon position and the remainder at the third position. All of the variable sites were transitions. Twenty-one haplotypes were found (Table 2). A single non-synonymous substitution (A to G transition at site 281) was seen in one individual from Guaymas, resulting in the replacement of Met94 with Val94. For *Cyt*b, the three haplotypes representing clade II (19–21) differed from the common (clade I) haplotypes only at a single site (position 376).

Analysis of *COI* sequences in *C. arcuatus* ($N = 7$; 14 variable sites; 7 haplotypes) and *C. sapidus* ($N = 2$; 3 variable sites) revealed no replacement substitutions. For *Cyt*b sequences in *C. arcuatus* ($N = 8$; 13 variable sites; 8

Table 1 *Callinectes*spp. Mean nucleotide composition (%) of the *COI* (657 bp) and *Cyt*b (382 bp) gene fragments (*N* number of organisms sequenced)

Species	Gene	(*N*)	T	C	A	G
C. bellicosus	*COI*	(67)	34.1	22.2	26.2	17.5
	*Cyt*b	(74)	39.0	21.7	26.2	13.1
C. arcuatus	*COI*	(7)	34.2	21.9	25.0	18.9
	*Cyt*b	(8)	36.4	24.2	25.6	13.8
C. sapidus	*COI*	(2)	34.9	22.1	26.4	16.6
	*Cyt*b	(1)	41.1	19.6	27.0	12.3

Table 2 *Callinectes bellicosus*. Variable positions in the *COI* and *Cyt*b gene fragments. *Outlined boxes* show clade II haplotypes, with mutations not shared with clade I in *bold print*. *Dots* indicate base identity with haplotype 1

COI Haplotype	Position
	` 1122333344444555555556666`
	`40067125711799024566680234`
	`82843541514758150214723438`
1	`ACATAGGGCGCTAAAACTCGTATGCA`
2	`..................G.......`
3	`.......A..................`
4	`...............G..........`
5	`.........A................`
6	`.................C........`
7	`...........C..............`
8	`.............G...........G`
9	`.............G............`
10	`.T...A....................`
11	`.....A....................`
12	`....................C..A..`
13	`............G..........A..`
14	`.......................A..`
15	`.....................G....`
16	`...................A.G....`
17	`..G.....T..............A..`
18	`......................C...`
19	`.........................G`
20	`G..CG....AT.....T.........`
21	`G..CG.A..AT.....T.........`
22	`G..CG....AT...G.T.........`
23	`G..CG.A..AT.....T.......T.`

*Cyt*b Haplotype	Position
	`        1111222333333`
	` 11267792569068256667`
	`439373647169381821276`
1	`TGTCAGACCATTTGATACCTG`
2	`................G....`
3	`...................C.`
4	`..................T..`
5	`........T............`
6	`..............G......`
7	`.............A.......`
8	`C....................`
9	`.................T...`
10	`......G..............`
11	`.....A...............`
12	`...T...T.............`
13	`...T......C..........`
14	`...T.................`
15	`.A.............C.....`
16	`.........G..C........`
17	`..C.G................`
18	`.A.........C.....T...`
19	`....................A`
20	`.A..................A`
21	`.A...A..............A`

haplotypes), there were no replacement substitutions either. In the one complete *Cyt*b sequence obtained in *C. sapidus*, a single amino acid replacement was seen (Asn17 to Gly17). This substitution was confirmed in two additional shorter (351 bp) *Cyt*b sequences that were omitted from the summary analysis for *C. sapidus* (Table 1).

No apparent geographical relationship among haplotypes was seen for either *COI* or *Cyt*b (Table 3). For both genes, haplotype 1 predominated at all localities and rare haplotypes of *COI* (haplotype 9) and *Cyt*b (haplotype 15) were each found at three widely separated localities.

Sequence divergence and phylogenetic analyses

Average pairwise distances between clades I and II in *C. bellicosus* were 1.1% (*COI*) and 0.6% (*Cyt*b). Relative rate tests (Tajima 1993), using *C. arcuatus* as an outgroup, were not significant, indicating a similar rate of sequence evolution within the two clades. K2P distances for both *COI* and *Cyt*b between the sympatric *C. bellicosus* and *C. arcuatus* were similar (16.4% and 17.6%) and approximately the same as the divergences between *C. bellicosus* and the allopatric *C. sapidus* from the western Atlantic (16.2% and 18.4%).

Neighbor-joining analysis of *Callinectes COI* sequences based on K2P distances revealed that the separation of *C. bellicosus* into clades I and II was reasonably well-supported (Fig. 2). In addition, the well-resolved *C. arcuatus* lineage, composed of seven individuals collected from a single locality (Guaymas), also showed two subclades with bootstrap support values similar to those of *C. bellicosus* (Fig. 2). Examination of the 14 variable positions in the seven *C. arcuatus* haplotypes (not shown) revealed four unshared transitions (positions 180, 459, 547 and 580) in haplotypes 6 and 7, representing a divergence of 1.1%, a situation reminiscent of the clade II separation in *C. bellicosus*, where five unshared mutations separated subclades. Bayesian analysis yielded a topology similar to the NJ tree, with high support for clade II in *C. bellicosus* (97%); in *C. arcuatus*, the two clade II individuals were paraphyletic, but all of the clade I individuals formed a well-supported (83%) clade.

As expected based on only a single fixed difference, NJ analysis of *Cyt*b sequences (not shown) was not as informative in separating clades I and II in *C. bellicosus*, but clustering of all clade II individuals except two (Bm4 and Bk18) was seen, but with very low (33%) bootstrap support. Interestingly, one sample omitted from the *COI* analysis because it was a partial sequence (Gi1) also clustered with clade II in the *Cyt*b NJ tree. Because of the low bootstrap support, and the fact that only a single fixed *Cyt*b difference separated the subclades, the partial *COI* sequence for Gi1 [composed of sites 297–657 which included 19 of the 24 variable sites (Table 2)] was re-examined and found to possess the diagnostic C to T transitions at sites 414 and 540, thus confirming that sample Gi1 belonged to clade II.

The overall frequency of clade II individuals of *C. bellicosus*, including partial sequences omitted from analysis, was 10%. The ratios of clade II to the combined clade I + II individuals by locality were: Golfo de Santa Clara (1:10), Puerto Peñasco (1:9), Canal del Infiernillo (0:10), Bahía Kino (1:10), Guaymas (0:9), Guásimas (2:10), Agiabampo (0:6), Bahía Concepción (1:6) and Bahía Magdalena (2:10). The relatively consistent appearance of clade II crabs was noteworthy, especially considering the low sample sizes, and indicated that the subclade was widely distributed geographically.

Table 3 *Callinectes bellicosus*. Summary of number and distribution of *COI* and *Cyt*b haplotypes among the nine eastern Pacific localities

Population	Clade I haplotypes																		Clade II haplotypes			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
COI																							
Golfo de Santa Clara	3					1	1		1	1	1										1		
Puerto Peñasco	5														1	1				①			
Canal del Infiernillo	8											1					1						
Bahía Kino	3						1						1							1			
Guaymas	2	1			1			1						①									
Guasimas	5		1	1	1															1			
Agiabampo	4								①		①												
Bahía Concepción	3								1					①								①	
Bahía Magdalena	3																	①	①	①			①
Total	36	1	1	1	2	1	2	1	3	1	2	1	1	2	1	1	1	1	1	4	1	1	1

Population	Clade I haplotypes																		Clade II haplotypes			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21		
*Cyt*b																							
Golfo de Santa Clara	4									1	1						1	1		1			
Puerto Peñasco	3							1	2					1						①			
Canal del Infiernillo	5						1					1			1	1							
Bahía Kino	6						1			1									1				
Guaymas	7					1								1									
Guásimas	5				1								1		1					2			
Agiabampo	3		1	1											1								
Bahía Concepción	4	1																		1			
Bahía Magdalena	6																			1	1		
Total	43	1	1	1	1	1	2	1	2	2	1	1	1	2	3	1	1	1	1	6	1		

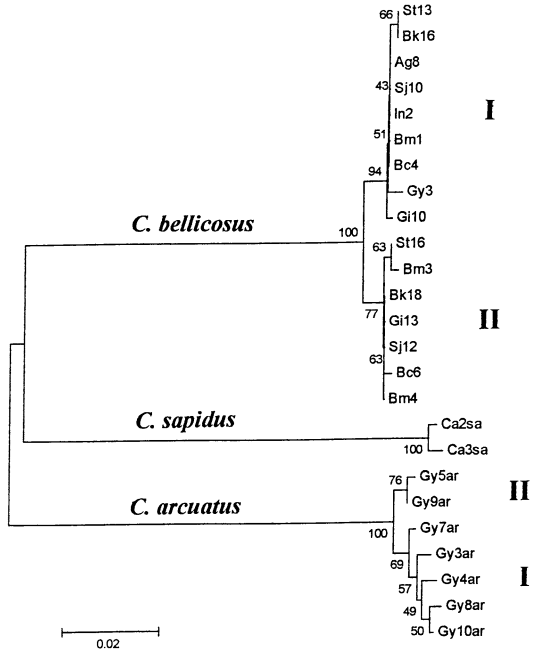

Fig. 2 *Callinectes* spp. Neighbor-joining tree based on *COI* sequence data in *C. bellicosus*, *C. arcuatus* (from Guaymas, Sonora) and *C. sapidus* (from Beaufort, North Carolina). Only a partial and representative data set for clade I in *C. bellicosus* is shown (see legend to Fig. 1 for locality abbreviations). Resolution of two subclades (I and II) is seen for both *C. bellicosus* and *C. arcuatus*. Bootstrap support values are shown on branches. Scale indicates sequence divergence

Population analyses

AMOVA results for *COI* and *Cyt*b from *C. bellicosus* revealed that 100% of the genetic variation occurred within populations (Table 4), indicating a lack of genetic structure across localities both within and outside the Gulf of California. None of the global Φ_{ST} values were significant. AMOVAs in Table 4 were conducted with the entire data set for each gene under the assumption that there is presently no reproductive isolation between subclades. Repeating the analyses after removing clade II individuals gave the same results. For pairwise comparisons among the nine localities, *COI* and *Cyt*b sequence data were combined (1,039 bp; $N = 63$), as were clades I and II. None of the pairwise Φ_{ST} values were significant, indicating a panmictic population.

Results of neutrality tests for the separate *COI* and *Cyt*b data sets in *C. bellicosus*, and the effect of excluding clade II, are given in Table 5. For all test statistics, significant negative values were obtained for both genes. Significant negative values were also obtained after exclusion of clade II. Given that all of the mutations were silent, with the exception of a single nonsynonymous substitution in *Cyt*b, we interpret these results as evidence of a population expansion in which genetic drift has not yet had a chance to eliminate the excess singleton mutations from the population.

Further support for a population expansion was obtained from analysis of sequence data with the computer program FLUCTUATE. We ran separate analyses on clade I and on the combined clades I and II as before. The maximum-likelihood estimates of the mutation parameter (θ) and the exponential population growth parameter (g) are given in Table 6. For all analyses, the likelihood surface showed well-defined peaks and the resulting standard deviations of the estimates were relatively low. For a stable population, which is not increasing in size, $g = 0$. All analyses indicated that g was positive and significantly different from zero. When clade I was analyzed separately, values of g showed a significant increase over the combined analysis, and, for each gene, the values were similar and not significantly different. Values for θ were significantly higher for *Cyt*b than for *COI*, and, for each gene, a significant increase in θ occurred when clade I was analyzed separately.

The mismatch distributions for *COI* and *Cyt*b pairwise differences (clade I only) were both unimodal (not shown) as expected for an expanding population (*COI*: mean = 1.01, variance = 0.97; *Cyt*b: mean = 0.95, variance = 1.04). Values for the raggedness statistic (*rg*), which measures the smoothness of the mismatch distribution (Harpending 1994), were low for both genes (0.0521 and 0.0453 for *COI* and *Cyt*b, respectively) and not statistically significant ($P = 0.693$ and 0.904, respectively). Because the mismatch distributions conformed to the sudden-expansion model, the time since the population expansion (τ), in units of $1/2u$ generations (Rogers and Harpending 1992) could be estimated, where u is the mutation rate for the entire gene segment (i.e. μ times the number of nucleotides). The value for τ (with 95% CI) for the *COI* data set was 1.01 (0.49, 1.68), resulting in an estimated time since the expansion began of 67,000 (33,000–111,000) years ago.

Discussion

Population characteristics

Analysis of the *COI* and *Cyt*b sequence data from *Callinectes bellicosus* indicated a lack of population

Table 4 *Callinectes bellicosus*. AMOVA results with clades I and II combined

Gene	Source of variation	*df*	Sum of squares	Variance components	Percent variation	Φ_{ST} (*P*-value)
COI	Among	8	7.214	−0.02893	−2.66	−0.027 (0.767)
	Within	58	64.727	1.11598	102.66	
*Cyt*b	Among	8	4.250	−0.01112	−1.82	−0.018 (0.797)
	Within	65	40.453	0.62235	101.82	

Table 5 *Callinectes bellicosus*. Results of neutrality tests on the *COI* and *Cyt*b data sets from *C. bellicosus* with 95% confidence limits shown *in brackets* (*N* number of organisms sequenced)

Gene	(*N*)	Tajima's *D*	Fu and Li's *D*	Fu's F_S
COI				
Clade I	(60)	−2.26 [−1.64, 1.87]	−2.99 [−2.57, 1.66]	−20.91 [−6.57, 6.07]
Clade I+II	(67)	−1.91 [−1.66, 1.88]	−2.47 [−2.47, 1.50]	−16.64 [−7.90, 6.65]
*Cyt*b				
Clade I	(66)	−2.37 [−1.67, 1.87]	−3.90 [−2.30, 1.31]	−18.87 [−7.25, 6.24]
Clade I+II	(74)	−2.17 [−1.65, 1.89]	−3.45 [−2.25, 1.34]	−20.70 [−7.39, 6.68]

structure over a wide geographic area in the eastern Pacific, suggesting a high level of gene flow consistent with a high potential for larval dispersal. Previous population genetic studies of *Callinectes* based on allozymes in *C. sapidus* from the western Atlantic and the Gulf of Mexico have suggested both a high level of gene flow (Cole and Morgan 1978; McMillen-Jackson et al. 1994) as well as population structure (Burton and Feldman 1982; Kordos and Burton 1993). Even in the presence of high gene flow over a broad area (New York to Texas), McMillen-Jackson et al. (1994) noted spatial and temporal genetic patchiness at several allozyme loci, and clinal variation at one of these loci (*Est-2*) in the Atlantic, probably maintained by selection and local larval retention. In the Gulf of Mexico, Kordos and Burton (1993) documented substantial temporal and spatial heterogeneity in allele frequencies in *C. sapidus* in Texas and, in addition, showed that larvae and adults from the same area differed in allele frequencies. Whether allozyme analysis would reveal similar heterogeneity in the apparently panmictic population of *C. bellicosus* in the eastern Pacific remains to be determined.

A major finding from our mtDNA analyses was evidence for a population expansion in *C. bellicosus*. This conclusion was supported by the significant values obtained from several statistical tests (Tajima's *D*, Fu and Li's *D**, Fu's F_S and the mismatch distribution) and by maximum-likelihood estimates of θ and the exponential population growth parameter *g* (Kuhner et al. 1998). Although the values obtained for effective population size and growth rate per generation (Table 6) depend on the accuracy of the molecular clock, and thus can be considered only rough estimates, the conclusion that *g* is positive and significantly different from zero would remain the same. Evidence for population expansions based on mismatch analysis of mtDNA data also have been reported recently for *C. sapidus* (McMillen-Jackson and Bert 2004) and the brown shrimp (*Farfantepenaeus aztecus*) (McMillen-Jackson and Bert 2003) in the Atlantic and Gulf of Mexico. The time of the most recent expansion of the brown shrimp population was dated to the late Pleistocene, about 74,000 years ago, similar to the estimated time of the population expansion of *C. bellicosus* in the Gulf of California (67,000 years ago).

Callinectes subclades in the eastern Pacific

We found that two separate lineages of *C. bellicosus* coexisted at most of our eastern Pacific sampling localities. Although this finding was unexpected, other mtDNA studies on marine invertebrates with high potential for larval dispersal have yielded similar results, with divergences between subclades similar to those reported here (ca. 1–3% based on either *COI* or mitochondrial control region sequences) (Chu et al. 1999; Gopurenko et al. 1999; Luttikhuizen et al. 2003; McMillen-Jackson and Bert 2003). The explanation for the presence of closely related but separate lineages is often attributed to a Pleistocene vicariant event associated with sea level changes (Haq et al. 1987) that isolated subpopulations and restricted gene flow among them. As a result, the fragmented subpopulations began to diverge genetically, and are then thought to have been reconnected secondarily as sea levels rose at the end of the Pleistocene. Indeed, Palumbi (1994) has pointed out that Pleistocene sea level changes probably isolated many populations of different near-shore species that appear panmictic today.

In some cases populations of marine invertebrates that are thought to have been isolated during the Pleistocene continue to be isolated by contemporary barriers (e.g. Reeb and Avise 1990), and some have diverged to the point where they may now be separate species (e.g.

Table 6 *Callinectes bellicosus*. Effective population sizes (N_{ef} and N_e) and exponential growth rate (*g*). Maximum-likelihood estimates of θ and *g* (±1.96 standard deviations) were obtained by separate analysis of aligned gene sequences, with clade II both included and excluded, using the computer program FLUCTUATE. The neutral mutation rate per site per generation (μ) was assumed to be 1.15×10^{-8} for each gene. An equal sex ratio was assumed (i.e. $N_e = 2N_{ef}$)

Gene	Number of sequences	θ	N_{ef}	N_e	*g* ($1/\mu$ generations)	Growth rate per generation
COI						
Clade I	60	0.0886 ± 0.0141	3.85×10^{6}	7.70×10^{6}	5,726 ± 328	6.585×10^{-5}
Clade I+II	67	0.0348 ± 0.0063	1.51×10^{6}	3.02×10^{6}	1,638 ± 272	1.884×10^{-5}
*Cyt*b						
Clade I	66	0.4123 ± 0.0886	1.79×10^{7}	3.58×10^{7}	5,205 ± 269	5.986×10^{-5}
Clade I+II	74	0.2636 ± 0.0378	1.15×10^{7}	2.30×10^{7}	2,320 ± 124	2.669×10^{-5}

Gopurenko et al. 1999). Whether the sympatric subclades noted here for *C. bellicosus* resulted from a Pleistocene vicariant separation, or whether they are a result of some other factor such as a population bottleneck or incipient speciation in sympatry is unclear. Because of this uncertainty, and because the possibility existed that gene flow could have been restricted between subclades potentially allowing for reproductive isolation mechanisms to arise (Palumbi 1994), we took a conservative approach and both included and excluded clade II from our analyses; we found that our main conclusions of a lack of population structure and of a population expansion in *C. bellicosus* remained the same.

Morphological examination of a complete voucher specimen of a clade II individual (Bk18) revealed no external differences compared to those from clade I, which is not surprising given that even in *C. arcuatus*, which has been separated from *C. bellicosus* probably since the Miocene, morphological differences are subtle. Our results from the small sample ($N=7$) of the sympatric *C. arcuatus* from Guaymas also suggest that two *COI* lineages are present in this species, but more extensive sampling will be required to determine if both lineages coexist throughout its broad range in the eastern Pacific.

Comments on phylogenetic relationships in the genus *Callinectes*

Our mtDNA analyses suggest that *C. bellicosus* and *C. arcuatus* are as widely diverged from each other as each is from the allopatric *C. sapidus* from the western Atlantic. Determining relationships among species of *Callinectes* will require a comprehensive phylogenetic analysis, but, at present, the limited evidence suggests that *C. bellicosus* and *C. arcuatus* are not sister species. Schubart et al. (2001) conducted a molecular phylogenetic analysis on several species of western Atlantic *Callinectes* using 16S rRNA and showed that *C. sapidus* was more closely related to *C. bocourti* (= *C. maracaiboensis*) than to *C. danae*, *C. similis*, or *C. ornatus*. *C. danae* and *C. arcuatus* are almost indistinguishable morphologically, and are considered each others closest relative (Williams 1974; Norse and Estevez 1977). The large divergence we noted between *C. bellicosus* and *C. arcuatus* is consistent with the findings of Schubart et al. (2001), but the question as to which species is the closest relative of *C. bellicosus* is still unanswered. It would not appear to be the third eastern Pacific species, *C. toxotes*, because *C. toxotes* is closely related to *C. bocourti* (Norse and Estevez 1977). It is possible then that *C. bellicosus* is most closely affiliated with the western Atlantic clade identified by Schubart et al. (2001) as comprising *C. danae*, *C. similis* and *C. ornatus*.

The large genetic distances between *C. bellicosus* and *C. arcuatus* suggest that the two species diverged roughly 7–14 million years ago during the Miocene epoch, assuming a 1.3–2.3% sequence divergence rate per million years. The estimated divergence predates the formation of the Isthmus of Panama during the Pliocene, approximately 3.5 million years ago (Coates et al. 1992), and places it during the early stages, or possibly predating, the formation of the Gulf of California during the late Miocene (Holt et al. 2000; Riddle et al. 2000). Thus, *C. bellicosus* and *C. arcuatus* may have already speciated by the time they began to invade the newly forming Gulf of California.

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