# Lack of divergence between 16S mtDNA sequences of the swimming crabs *Callinectes bocourti* and *C. maracaiboensis* (Brachyura: Portunidae) from Venezuela*

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**Abstract**—Lake Maracaibo, a large Venezuelan estuarine lagoon, is reportedly inhabited by three species of the genus *Callinectes* Stimpson, 1860 that are important to local fisheries: *C. sapidus* Rathbun, 1896, C. *bocourti* A. Milne Edwards, 1879, and *C. maracaiboensis* Taissoun, 1969. *Callinectes maracaiboensis*, originally described from Lake Maracaibo and assumed endemic to those waters, has recently been reported from other Caribbean localities and Brazil. However, because characters separating it from the morphologically similar *C. bocourti* are noted to be vague, we have compared these species and several congeners by molecular methods. Among our specimens from Lake Maracaibo and other parts of the Venezuelan coast, those assignable to *C. bocourti* and *C. maracaiboensis* on the basis of putatively diagnostic characters in coloration and structural characteristics do not differ in their 16S mtDNA sequences. These molecular results and our re-examination of supposed morphological differences between these species suggest that *C. maracaiboensis* is a junior synonym of *C. bocourti*, which varies markedly in minor features of coloration and structural characteristics. Genetic relationships of this species to other species of swimming crabs of the genus *Callinectes* are also explored and presented as phylogenetic trees.

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Swimming crabs of the genus *Callinectes* Stimpson, 1860 are widely distributed throughout the American tropics and subtropics, where many species are exploited commercially (Norse, 1977; Williams, 1984). Members of this genus play key trophic roles in coastal habitats that range from sandy-mud bottoms to seagrass meadows (Arnold, 1984; Orth and van Montfrans, 1987; Wilson et al., 1987; Lin, 1991). Of the nine species of *Callinectes* from the tropical western Atlantic, seven have been reported from western Venezuela (Rodríguez, 1980; Williams, 1984; Carmona-Suárez and Conde, 1996). However, knowledge of these Venezuelan populations remains inadequate, despite the importance of several species in both large-scale commercial harvests and artisanal fisheries of coastal villages in the area (Oesterling and Petrocci, 1995; Ferrer-Montaño, 1997; Conde and Rodríguez, 1999). Persistent difficulty in identifying mixed samples of these species hampers understanding of their distribution, abundance, and population dynamics, which are essential to fishery-management decisions. In particular, consistent morphological distinction of the commonly encountered *C. bocourti* from *C. maracaiboensis* cannot be achieved with confidence.

Initially described as endemic from Lake Maracaibo, *C. maracaiboensis* has been reported from other Venezuelan waters, as well as from sites in Jamaica, Curaçao, Colombia, and Brazil (Norse, 1977; Carmona-Suárez and Conde, 1996; Sankarankutty et al., 1999). Although detailed descriptions have been provided by Taissoun (1969, 1972) and Williams (1974) to diagnose *C. maracaiboensis*, the same authors have repeatedly emphasized its resemblance to *C. bocourti*. Morphological distinction of the two species is based upon postulated defining characters of the frontal teeth, direction and form of

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the anterolateral spines, convexity of the carapace, surface granulation patterns, and varied morphometric measurements (Taissoun 1969, 1972). However, neither these characters nor additional ones proposed by other authors (Williams, 1974; Rodríguez, 1980) have proven to be of consistent use in separating the species. Carapacial color and granulation vary widely, and the outer frontal teeth of many specimens cannot be classified as a definitive triangular (*C. maracaiboensis*) or obtuse (*C. bocourti*) shape. These concerns have led some authors to report only possible co-occurrence of *C. maracaiboensis* in their samples of *C. bocourti*, as in records from Puerto Rico (Buchanan and Stoner, 1988).

Recently, molecular markers have proven particularly valuable in decapod crustacean systematics. In addition to reconstructing phylogenetic relationships, molecular sequences are of value in recognizing questionable species separations (e.g. Geller et al., 1997; Sarver et al., 1998; Schneider-Broussard et al., 1998; Schubart et al., 1998; Schubart et al., in press). In our study, we used sequences of a mitochondrial gene (16S rRNA) to examine genetic differentiation between the morphologically similar species *C. bocourti* and *C. maracaiboensis* and to compare these sequences to those of other swimming crabs of the genus *Callinectes* in a phylogenetic analysis.

## Materials and methods

Swimming crabs were caught by hand in shallows of the Golfete de Cuare (68°37′W, 10°06′N, State of Falcón, Venezuela) in November–December 1998 and March 1999, and in the shallows of El Moján, Lago de Maracaibo (71°39′W, 10°58′N, State of Zulia, Venezuela) in October 1999. Crabs were transported to the Instituto Venezolano de Investigaciones Científicas, Caracas, in liquid nitrogen and then stored in a –70°C freezer. Two walking legs and one swimming leg were separated from each specimen, preserved individually in 85% ethanol, and shipped to the University of Louisiana, Lafayette (U.S.A.), for molecular analyses.

Specimens were assigned to *Callinectes bocourti* or *C. maracaiboensis* according to morphological characters provided by Taissoun (1969, 1972), Williams (1974, 1978), Fischer (1978), and Rodríguez (1980). Eight specimens of *C. bocourti* and six of *C. maracaiboensis* from Venezuela were used for the molecular analysis. In addition, a short fragment (~300 base pairs) of 16S mtDNA was obtained with an internal primer for one formalin-preserved specimen of *C. bocourti* from Colombia. These voucher specimens, lacking limbs (which had been removed for analysis), were cataloged in collections of the Laboratorio de Ecología y Genética de Poblaciones, Instituto Venezolano de Investigaciones Científicas (IVIC), and in the University of Louisiana Zoological Collection (Table 1). For outgroup comparisons, specimens of *C. sapidus*, *C. ornatus* Ordway, 1863, *C. danae* Smith, 1869, and *Portunus ordwayi* (Stimpson, 1860) were sequenced (Table 1).

Genomic DNA was isolated from the muscle tissue of one walking leg with a phenol-chloroform extraction (Kocher et al., 1989). Selective amplification of a 585-basepair (bp) product (547 bp excluding primer regions) from the mitochondrial 16S rRNA gene was carried out by a polymerase-chain-reaction (PCR) (35–40 cycles: 1 min at 94°C, 1 min at 55°C, 2 min at 72°C (denaturing, annealing, and extension temperatures, respectively) with primers 16Sar (5′-CGCCTGTTTATCAAAAACAT-3′), 16Sbr (5′-CCGGTCTGAACTCAGATCACGT-3′), 1472 (5′-AGATAGAAACCAACCTGG-3′), and 16L15 (5′-GACGATAAGACCCTATAAAGCTT-3′) (for references to primers see Schubart et al., 2000). 16L15 is an internal primer and, in combination with 16Sbr, was used for partial amplification of the formalin-preserved specimen. PCR products were purified with Microcon-100® filters (Millipore Corp., Bedford, MA) and sequenced with the ABI BigDye® Terminator Mix (PE Biosystems, Wellesley, MA) in an ABI Prism 310 Genetic Analyzer® (Applied Biosystems, Foster City, CA). Sequences were manually aligned with the multisequence-editing program ESEE (Cabot and Beckenbach, 1989). New sequences were accessioned to the European Molecular Biology Laboratory (EMBL) genomic library (see Table 1). Sequences available online (by electronic database accession numbers that follow) were obtained for "*Callinectes sapidus*" (U75267), *C. ornatus* (U75268), *C. similis* (U75269), and *C. sapidus* (AJ130813).

Sequence divergence was analyzed by using Kimura 2-parameter distances, UPGMA cluster analysis, and neighbor-joining (NJ) distance analysis (Saitou and Nei, 1987) with the program MEGA (Kumar et al. 1993). Statistical significance of groups within inferred trees was evaluated by the interior branch method (Rzhetsky and Nei, 1992). As a second phylogenetic method, a maximum parsimony (MP) analysis was carried out with a heuristic search and random sequence addition (tree-bisection and reconnection as branch-swapping option) and by omission of gaps with the program PAUP (Swofford, 1993). Significance levels were evaluated with the same software and the bootstrap method with 2000 replicates.

## Results

Sequencing of 15 specimens of *Callinectes bocourti* and *C. maracaiboensis* from Golfete de Cuare and El Moján (both Venezuela) and the Río Sinú estuary (Colombia) revealed the existence of seven different haplotypes. Two haplotypes (ht-1 and ht-5) clearly predominated (together 64.3%) and were found in both species (ht-1 in two *Callinectes bocourti* and three *C. maracaiboensis* from Golfete de Cuare, ht-5 in three *C. bocourti* and one *C. maracaiboensis* from El Moján). The other five haplotypes (ht-2, ht-3, ht-4, ht-6, ht-7) each differed from ht-1 or ht-5 in not more than two positions and were found in only single individuals (Fig. 1, Table 2). Consequently there is not a single diagnostic molecular character in our sequenced unit of 16S mtDNA that would discriminate between the two species of swimming crabs. On the other hand, haplotype distributions differed between the two sampled populations; ht-1 is found in only the Golfete de Cuare (~550 km east of Lake Maracaibo) and ht-5 is restricted to El Moján (within Lake Maracaibo). Comparison of the

**Table 1**

Swimming crabs used in sequencing of 16S mtDNA and subsequent phylogenetic analyses. IVIC-LEGP = Laboratorio de Ecología y Genética de Poblaciones, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela; ULLZ = University of Louisiana Zoological Collections, Lafayette, U.S.A.; specimens bearing numbers for both collections are archived in ULLZ; EMBL = European Molecular Biology Laboratory.

| Species | Locality of collection | Catalog number | EMBL accession no. |
|---|---|---|---|
| *Callinectes maracaiboensis* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-40 = ULLZ 4181 | AJ298171 |
| *Callinectes maracaiboensis* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-70 | AJ298182 |
| *Callinectes maracaiboensis* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-71 | AJ298172 |
| *Callinectes maracaiboensis* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-72 | AJ298173 |
| *Callinectes maracaiboensis* | Venezuela: Zulia: El Moján | IVIC-LEGP-MZ-1 | AJ298177 |
| *Callinectes maracaiboensis* | Venezuela: Zulia: El Moján | IVIC-LEGP-MZ-6 | AJ298183 |
| *Callinectes bocourti* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEPG-C-30 = ULLZ 4180 | AJ298170 |
| *Callinectes bocourti* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-109 | AJ298174 |
| *Callinectes bocourti* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-112 | AJ298175 |
| *Callinectes bocourti* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-113 | AJ298176 |
| *Callinectes bocourti* | Venezuela: Zulia: El Moján | IVIC-LEGP-MZ-2 | AJ298181 |
| *Callinectes bocourti* | Venezuela: Zulia: El Moján | IVIC-LEGP-MZ-3 | AJ298178 |
| *Callinectes bocourti* | Venezuela: Zulia: El Moján | IVIC-LEGP-MZ-4 | AJ298179 |
| *Callinectes bocourti* | Venezuela: Zulia: El Moján | IVIC-LEGP-MZ-5 | AJ298180 |
| *Callinectes bocourti* | Colombia: Río Sinú | ULLZ 4186 | AJ298169 |
| *Callinectes sapidus* | Florida: Fort Pierce | ULLZ 3766 | AJ298189 |
| *Callinectes sapidus* | Venezuela: Zulia: El Moján | ULLZ 4188 | AJ298190 |
| *Callinectes ornatus* | Venezuela: Falcón: La Vela de Coro | IVIC-LEGP-LV-CO-1 | AJ298187 |
| *Callinectes ornatus* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-CO-3 | AJ298188 |
| *Callinectes ornatus* | Brazil: Ensenada de Ubatuba | ULLZ 4178 | AJ298186 |
| *Callinectes danae* | Brazil: Ensenada de Ubatuba | ULLZ 4179 | AJ298185 |
| *Callinectes danae* | Venezuela: Falcón: Golfete de Cuare | IVIC-LEGP-C-1 | AJ298184 |
| *Portunus ordwayi* | Venezuela: Falcón: La Vela de Coro | IVIC-LEGP-LV-9 | AJ298191 |

**Table 2**

Percent (uncorrected) genetic divergence and number of differences between 547 base pairs of 16S mtDNA among 7 haplotypes (ht) corresponding to 14 specimens of *Callinectes bocourti* and *C. maracaiboensis* from Venezuela (s: transition, v: transversion, i: indel). Catalog numbers shown below correspond to IVIC-LEGP specimens listed in Table 1.

| | ht-1 | ht-2 | ht-3 | ht-4 | ht-5 | ht-6 | ht-7 |
|---|---|---|---|---|---|---|---|
| | C-30 | C-70 | C-112 | C-113 | MZ-1 | MZ-2 | MZ-6 |
| | C-40 | | | | MZ-3 | | |
| | C-71 | | | | MZ-4 | | |
| | C-72 | | | | MZ-5 | | |
| | C-109 | | | | | | |
| ht-1 | — | 0.18 | 0.18 | 0.18 | 0.18 | 0.55 | 0.18 |
| ht-2 | 1v | — | 0.37 | 0.37 | 0.37 | 0.73 | 0.37 |
| ht-3 | 1s | 1s, 1v | — | 0.37 | 0.37 | 0.73 | 0.37 |
| ht-4 | 1s | 1s, 1v | 2s | — | 0.37 | 0.73 | 0.37 |
| ht-5 | 1s | 1s, 1v | 2s | 2s | — | 0.37 | 0.37 |
| ht-6 | 2s, 1i | 2s, 1i, 1v | 3s, 1i | 3s, 1i | 1s, 1i | — | 0.73 |
| ht-7 | 1s | 1s,1v | 2s | 2s | 2s | 3s, 1i | — |

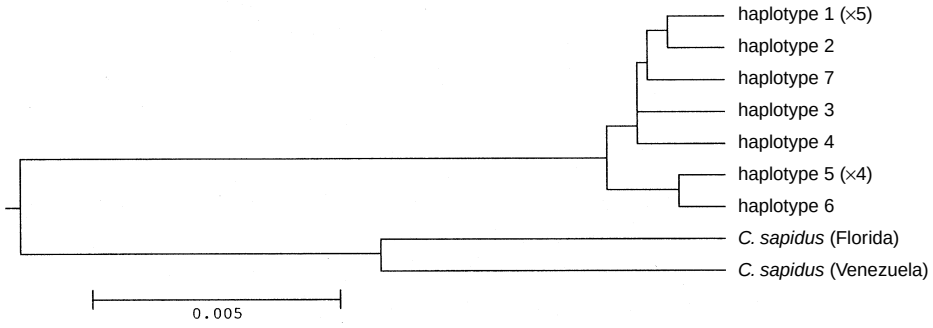


**Figure 1**

UPGMA (unweighted pair-group method with arithmetic averaging) analysis of Kimura 2-parameter genetic distances based on 547 base pairs of 16S mtDNA among seven haplotyopes of *Callinectes maracaiboensis* and *C. bocourti* (see Table 2 for species designation) corresponding to 14 specimens from Golfete de Cuare (haplotypes 1–4) and El Moján (haplotypes 5–7) (both Venezuela) and two populations of *C. sapidus*.

294 base pairs obtained for one *Callinectes bocourti* from Colombia did not reveal any difference from ht-1, ht-3, or ht-5, suggesting that genetic homogeneity in these species can be expected in other Caribbean localities.

Comparing sequences from *C. danae*, *C. ornatus*, *C. sapidus*, and *Portunus ordwayi* with those of *C. bocourti* and *C. maracaiboensis* allowed us to postulate phylogenetic relationships among these selected portunid taxa (Fig. 2). Marked genetic differences (Table 3) characterized all intra- and interspecific separations within the genus *Callinectes*, except in pairings of *C. bocourti* and *C. maracaiboensis*.

## Discussion

Comparison of 15 sequences of the 16S mtDNA of *Callinectes maracaiboensis* and *C. bocourti* from within and outside Lake Maracaibo revealed no consistent differences between these two species of swimming crabs. These results support observations from previous studies in which morphological characters have proven unreliable in separating these two species. Rather than constituting a separate species, *C. maracaiboensis* would appear from these findings to represent only a phenotypic extreme of *C. bocourti*, a species of known morphological plasticity (Williams, 1974).

We are highly confident that our sequenced samples of *C. maracaiboensis* represent the population originally assigned to that species. In addition to assuring that our samples were topotypic, we confirmed individual specimen identities by direct comparisons with deposited paratypes of Taissoun (IVIC-LEGP 425). However, we cannot make similar claims for the analyzed materials of *C. bocourti*. Although published records (Williams, 1984) indicate that *C. bocourti* is widely distributed (from Jamaica and Belize to Brazil, occasionally northward to Florida, Mississippi, and North Carolina), the type locality is southern Belize. To date, topotypic materials of *C. bocourti* have not been available to us for use in either our sequencing or morphological comparisons with Venezuelan material; such comparisons should, however, ultimately be undertaken to firmly anchor the synonymy we have proposed. Among specimens that we would presently assign to *C. bocourti* on the basis of structural features, one from the Río Sinú of Colombia shared complete identity in almost 300 base pairs of mtDNA with the most common haplotypes from Venezuela. This finding at the very least suggests a large overall range for materials that we refer to as *C. bocourti* and a relative homogeneity among forms that we have assigned to this name on the basis of structural characteristics, even over large geographic distances.

The reported phylogenetic relationships and genetic distances (Fig. 1) reflect a low level of genetic divergence among the seven haplotypes of *C. maracaiboensis* and *C. bocourti*, especially when compared with sequences of two populations of *C. sapidus*. The tree resulting from the phylogenetic analysis (Fig. 2) also suggests that *C. maracaiboensis* and *C. bocourti* are closer to *C. sapidus* than to *C. ornatus*, *C. similis*, and *C. danae*, which is in accord with preliminary findings of Norse and Fox-Norse (1979). However, our results must also be regarded as preliminary because other species of *Callinectes* should be sequenced before we can claim full understanding of sister-species relationships. Published studies to date that have attempted to separate questionable brachyuran species on the basis of 16S mtDNA have consistently reported at least a few nucleotide differences that are diagnostic for the species in question (Geller et al., 1997; Schneider-Broussard et al., 1998; Schubart et al. 1998; Schubart et al., 2000). Only the Mediterranean species *Brachynotus sexdentatus* and *B. gemmellari* may constitute an exception to this pattern because they are identical in the sequenced fragment of 16S mtDNA (Schubart et al., in press).

Comparison of our sequences with those from a previous study (Geller et al., 1997) revealed important, apparently intraspecific differences between specimens of *C. ornatus* as well as between specimens of *C. sapidus*. Genetic

**Table 3**

Percent genetic divergence (uncorrected; excluding indels) between 553 base pairs of 16S mtDNA among species of *Callinectes* and the outgroup *Portunus ordwayi*, as used for the phylogeny in Figure 2 (1=*C. similis* (U75269); 2="*C. sapidus*" (U75267); 3=*C. sapidus* (FL); 4=*C. sapidus* (Venezuela); 5=*C. bocourti* (ht-1); 6=*C. bocourti* (ht-5); 7=*C. danae* (Venezuela); 8=*C. danae* (Brazil); 9=*C. ornatus* (U75268); 10=*C. ornatus* (Venezuela CO-1); 11=*C. ornatus* (Venezuela CO-3); 12=*C. ornatus* (Brazil); 13=*P. ordwayi*).

| | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.18 | 5.61 | 5.79 | 6.33 | 6.15 | 0.54 | 0.54 | 2.53 | 3.07 | 3.07 | 2.89 | 13.02 |
| 2 | | 5.06 | 5.24 | 5.79 | 5.61 | 0.54 | 0.54 | 2.53 | 3.07 | 3.07 | 2.89 | 12.12 |
| 3 | | | 1.27 | 2.53 | 2.35 | 5.79 | 5.79 | 5.97 | 6.87 | 6.87 | 6.69 | 13.92 |
| 4 | | | | 2.71 | 2.53 | 6.15 | 6.15 | 6.15 | 7.05 | 7.05 | 7.05 | 13.92 |
| 5 | | | | | 0.18 | 6.51 | 6.51 | 6.33 | 6.51 | 6.51 | 6.33 | 14.10 |
| 6 | | | | | | 6.51 | 6.51 | 6.15 | 6.33 | 6.33 | 6.33 | 14.29 |
| 7 | | | | | | | 0.00 | 2.71 | 3.62 | 3.62 | 3.44 | 13.92 |
| 8 | | | | | | | | 2.71 | 3.62 | 3.62 | 3.44 | 13.92 |
| 9 | | | | | | | | | 1.63 | 1.63 | 1.45 | 12.66 |
| 10 | | | | | | | | | | 0.36 | 0.18 | 12.84 |
| 11 | | | | | | | | | | | 0.18 | 13.02 |
| 12 | | | | | | | | | | | | 13.38 |

differences between Geller et al.'s (1997) specimen from North Carolina (U75268) and three specimens of *C. ornatus* from South America can be attributed to geographical distance. In the case of *C. sapidus*, however, differences clearly exceed a level that could be interpreted as ancestral polymorphism or biogeographical variation. Although our new sequence of *C. sapidus* from Florida perfectly matches one previously registered for a specimen from Louisiana (AJ130813), and both of these show limited divergence from a Venezuelan specimen, another sequence previously reported for *C. sapidus* (U75267) by Geller et al. (1997) differs markedly from the aforementioned set. The phylogeny of all 16S sequences presently available for *Callinectes* (Fig. 2) shows that the sequence reported for "*C. sapidus*" by Geller et al. (1997) instead pairs closely with *C. similis*, a grouping supported by high bootstrap values. Because no morphological voucher specimens appear to exist for this sequenced specimen,[1] we must conclude that the reported sequence was based upon a misidentified specimen of *C. similis*.

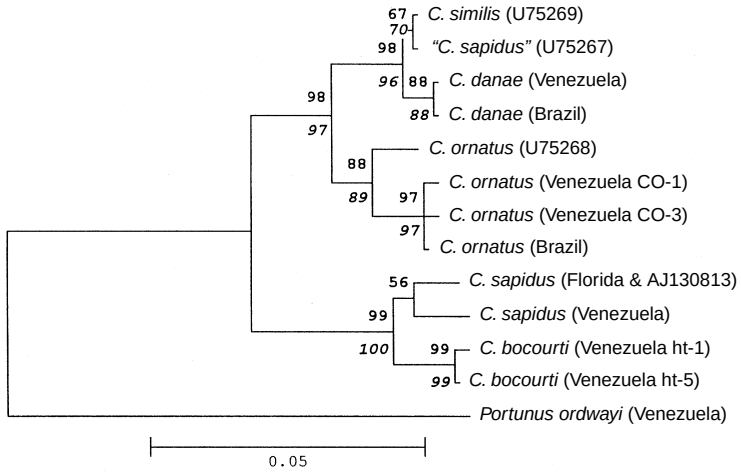


**Figure 2**

Phylogenetic relationships of five species of *Callinectes* based on 553 base pairs of 16S mtDNA. Tree topology is based on neighbor-joining (NJ) analysis with Kimura 2-parameter genetic distances. Sequences accessed online for *C. similis*, putative "*C. sapidus*," and *C. ornatus*, and *C. sapidus*, are indicated solely by electronic database accession numbers. Analyses for Venezuelan *C. ornatus* include specimens from adjacent localities (see Table 1); those for *C. bocourti* include haplotypes (ht) 1 and 5 (see Table 2 and Fig. 1). Confidence values ≥50 were obtained with the interior branch method for NJ distance analysis (upper values) and by maximum parsimony analysis after 2000 bootstrap replicates (lower values, in Italics).

Differences in color of some of the Brazilian specimens (see Sankarankutty et al., 1999) could indicate that two or more species might be involved in the *C. bocourti* complex, regardless of the synonymy that we have herein proposed. However, color differences should be judged with precaution, given the known high variability of this feature in *C. bocourti* (see Williams, 1984). Our recent color photography of specimens from Colombia, Venezuela, and

[1] Geller, J. 1999. Personal commun. Moss Landing Marine Laboratories, Moss Landing, CA 95039.

Florida has revealed marked variations; dorsal surfaces of the carapace of some specimens were predominantly olive green, those in other specimens were largely rust brown or rust patterned on green, and in yet others a rust pattern on a background of almost totally blue.

As a result of our molecular analyses, especially in the absence of consistent characters to distinguish *C. maracaiboensis* on the basis of color or structural characteristics, we must conclude that Lake Maracaibo and adjacent Venezuelan waters are inhabited by populations assignable to *C. sapidus* and by only one other species of the genus, *C. bocourti*. It appears that these are in turn the target species of a largely unregulated crab fishery which represented more than U.S. $6 million in exports to the United States in 1992 (Oesterling and Petrocci, 1995), even though it has declined since 1989 in probable response to overharvesting. As maximum sustainable yield has likely been achieved, these fisheries, which have been proposed for Miller's phase II management (resource-mapping, gear related regulations), should in the near future proceed to phase III (basic biological studies, long-term management) (Miller, 1999). This phase can be undertaken only with a clear understanding of the genetic entities upon which the fishery is based.

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