Molecular phylogeny of Mexican species of freshwater prawn genus *Macrobrachium* (Decapoda: Palaemonidae: Palaemoninae)

Filogenia molecular de las especies mexicanas de camarones dulceacuícolas del género *Macrobrachium* (Decapoda: Palaemonidae: Palaemoninae)

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ABSTRACT

Molecular phylogenetic analyses of 16 species of the freshwater prawn genus *Macrobrachium* from Mexico are presented. The phylogenetic reconstructions are based on partial sequences from16S rDNA mitochondrial gene. The results show a clear separation of the species with extended larval development (ED) from those with abbreviated larval development (AD). Within the ED clade, the species of the Olfersii Group clustered together, which is in agreement with their morphological similarity, whereas the position of the three pairs of geminate species within this clade suggests different origins. Five Mexican species are grouped into a single clade suggesting a common origin in a species with few larval stages and relatively large eggs. The estimated time of evolutionary divergence suggests that the ED and AD clades diverged in the Middle Eocene; the three geminate pairs differentiated at different times, and the species of the AD group originated during the Early Pliocene.

Key words: *Macrobrachium*, mitochondrial r16S, phylogenetic reconstruction.

RESUMEN

Se presenta una filogenia molecular para las 16 especies mexicanas de camarones dulceacuícolas del género *Macrobrachium.* La reconstrucción filogenética está basada en secuencias parciales del gen mitocondrial 16S ADNr. Los resultados muestran una clara separación entre las especies con desarrollo larval extendido (ED) y las de desarrollo larval abreviado (AD). Dentro del clado ED se agrupan las especies del Grupo Olfersii, taxa morfológicamente similares, aunque la posición de los tres pares de especies geminadas dentro de este clado sugiere diferentes orígenes para las mismas. Las cinco especies mexicanas con AD se agruparon en un solo clado, sugiriendo un origen común de las especies con pocos estadios larvales y ovas relativamente grandes. El tiempo de divergencia evolutiva estimado sugiere que los clados ED y AD se separaron en el Eoceno medio que los tres pares de especies geminadas presentaron diferentes tiempos de divergencia evolutiva y que las especies del clado AD se originaron durante el Plioceno temprano.

Palabras clave: *Macrobrachium*, r16S mitocondrial, reconstrucción filogenética.

INTRODUCTION

The freshwater prawns of the genus *Macrobrachium* are successful decapods with a circumtropical distribution. Species occupy a wide variety of habitats, such as estuaries, coastal lagoons, lakes, rivers (from the coastal plain to an altitude of 1100 m), sinkholes and caves. Approximately 75% of the species have an extended larval development (ED) with 10-12 larval stages. At different stages these species require brackish water to complete their development. Adults of *Macrobrachium* species may be associated to estuaries as well or can be permanent freshwater inhabitants. The other 25% of species correspond to those with strictly freshwater species characteristics typical of abbreviated larval development (AD) with only 1-3 larval stages (Sollaud, 1923; Jalihal *et al*., 1993; Álvarez *et al*., 2002; Gonzalez, 2002). Females lay up to 70 large eggs, rich in vitelum. After 58 days on average, the egg hatches an advanced form that quickly reaches the juvenile stage (Sollaud, 1923; Boschi, 1961; Holthuis, 1952; Shokita, 1977; Gamba, 1980; Rodríguez, 1982; Pereira, 1985, 1986, 1989, 1993; Pereira & García, 1995; Magalhaes & Walker, 1988; Mashiko, 1992; Bueno & de Almeida, 1995; Álvarez *et al*., 2002; González, 2002). To date, more than 200 species are described and there are numerous yet undescribed cryptic species (Cai *et al*., 2004; Short, 2004). All the species within the genus show a highly conserved morphology. The species identity has been traditionally based on: the shape and dentition of the rostrum, and the shape, ornamentation and relative length of the articles of the second pair of pereiopods (Holthuis, 1950, 1952; Villalobos, 1967a, 1982; Jayachandran, 2001). Some species groups were proposed based on morphological similarities, mainly of the rostrum and the second pereiopod (Johnson, 1973). The phylogenetic affinities between *Macrobrachium* world species has not been resolved, even when in the last decade major contributions like Murphy and Austin (2002, 2003, 2004, 2005) who based on studies of mitochondrial DNA (mtDNA), 16S rDNA and cytochrome c oxidase I (COI) gene, have contributed to the classification of Australian species of *Macrobrachium*. Liu *et al.* (2007), also using mitochondrial DNA sequences, have contributed in molecular systematics of East Asia; Pileggi and Mantelatto (2010) analyzed the phylogeny of Indo-Pacific species and some American species of *Macrobrachium.* In Mexico, nineteen species of *Macrobrachium* have been recorded, three of them have been reported only once: *M. quelchi* De Man, 1900, in the Otolun River, east from Palenque, Chiapas (Rodríguez de la Cruz, 1965); *M. nattereri* Heller, 1862 in the Sierra River, Tabasco (Rodríguez de la Cruz, 1965) and *M. jelskii* Miers, 1877 nearby the Port of Veracruz, Veracruz (Wicksten, 2005). However, given the lack of other records in Mexico and the likelihood of misidentifications, these species are not included in this study. The remaining 16 species are widely distributed on both Pacific and Atlantic slopes or their records have been well documented, however their relations have not been previously determined.

Eleven of the Mexican *Macrobrachium* species have ED. Six of these species (three pairs) share similar morphology and they are considered to be geminate species or twin sibling species, where every geminated species inhabits an opposite side of the geographic barrier that separates them (Jordan, 1908). At the present study geminate species are separated by the continental mass, so one of the pair species is present along the Pacific slope of the Americas and the other one along the Atlantic slope. The three species that are found along the Pacific slope are: *M. tenellum* (Smith, 1871); *M. americanum* Bate, 1868; *M. occidentale* Holthuis, 1950, and their respective geminate pairs along the Atlantic slope are: *M. acanthurus* (Wiegmann, 1836); *M. carcinus* (Linnaeus, 1758) and *M. heterochirus* (Wiegmann, 1836). The remaining ED species have amphiamerican distributions, *M. hobbsi* Villalobos & Nates, 1990 and *M. olfersii (*Wiegmann, 1836) or are only distributed along the Pacific slope, such as *M. digueti (*Bouvier, 1895), *M. acanthochirus* Villalobos, 1967b, and *M. michoacanus* Villalobos & Nates, 1990.

In an early study of Mexican *Macrobrachium* species based on the similarity on the morphology of the second pereopod, Villalobos (1967a) proposed that the "Olfersii Group" included six species from Mexico, Central and South America: *M. digueti*, *M. acanthochirus*, *M. hancocki* Holthuis, 1950, *M. faustinum* de Saussure, 1857; *M. crenulatum* Holthuis, 1950, and *M. olfersii*, and suggested that they probably derived from a type species similar to *M. olfersii*. Villalobos (1967a) hypothesized that both, the Isthmus of Tehuantepec and the Isthmus of Panama played a significant role in the diversification of this species group. Recently, Hernández *et al*. (2007) based on a morphological study of the *Macrobrachium* species distributed along the Baja California Peninsula, suggested the synonymy of *M. acanthochirus* with *M. digueti*, a proposal that could modify the original Olfersii Group.

From the five Mexican species of *Macrobrachium* with AD, *M. villalobosi* Hobbs, 1973b and *M. acherontium* Holthuis, 1977 are stygobitic with adaptations to cave life. The other three *M. tuxtlaense* (Villalobos & Álvarez, 1999), *M. vicconi* (Román *et al*., 2000) and *M. totonacum (*Mejía *et al*., 2003) are epigean species, occurring in geographically isolated springs or small streams distant from the coast. None of the species with AD co-occur with species with ED. The populations of species with AD are small genetically structured, have no gene flow among them and are highly endogamic (low genetic variation), characteristics that favour genetic differentiation (Acuña, 2002). The first description of *Macrobrachium* species with AD in Mexico was that of *M. tuxtlaense* (Villalobos & Álvarez, 1999) followed by that of *M. vicconi* (Román *et al.,* 2000) and *M. totonacum* (Mejía *et al*., 2003). However, a careful examination of voucher specimens from the Colección Nacional de Crustáceos (CNCR) at Universidad Nacional Autónoma de México (UNAM), revealed that a number of popula-

tion samples dated from 1921, were collected from the states of Chiapas, Oaxaca, Veracruz and Tabasco and had small females carrying few large eggs, a characteristic typical of species with AD. Despite the difficulty in finding enough characters to describe new species within the *Macrobrachium* with AD, it is clear that many more species from southern Mexico will be recognized in the future.

The 16S rDNA mitochondrial gene is a structural, non-coding gene that has been widely used in phylogenetic and phylogeographic studies of crustaceans (Bucklin *et al*., 1995; Crandall & Fitzpatrick, 1996; Kitaura *et al*., 1998; Crandall *et al*., 1999; Daniels *et al.,* 2002; Murphy & Austin, 2002, 2003, 2004, 2005), basically because their maternal inheritance, its rapid substitution rates, and because permit to test if speciation patterns of endemic species result from multiple lineages or from a single event, and allow to elucidate cryptic species that are difficult to distinguish using more-traditional techniques (Schubart *et al*., 2000; Knowlton, 2000; Ellis *et al*., 2006) .

The objective of the present study was to infer the molecular phylogenetic relations of the Mexican representatives of *Macrobrachium* species based on partial sequences of the 16S rDNA mitochondrial gene and by using sequences available from GenBank and to test various hypotheses on the origin of geminate species, the conformation of Olfersii Group and the divergence between species with ED and AD.

MATERIALS AND METHODS

We used two specimens of each of the 16 species of *Macrobrachium* described for Mexico until 2007. We worked with biological material preserved in the CNCR of the Institute of Biology, UNAM; for seven of these species was possible to work with type series (Table 1). Several external groups were selected as out-groups for the analysis as follows: *Paratya australiensis* Kemp, 1917, from Australia (GenBank accession number AF374469) was selected because it has been used in several phylogenetic analyses of species of *Macrobrachium*, making it a useful reference for comparisons (Murphy & Austin, 2003; 2005), five species of the related genera *Palaemon* and *Palaemonetes* (subfamily Palaemoninae) were included in the analysis: *Palaemon northropi* Rankin, 1898, from Ubatumirin, Brasil (CNCR), (GenBank accession number JF491339); *Palaemon serenus* Heller, 1862, from Hopkins River, Victoria, Australia (GenBank accession number AF439518); *Palemonetes atrinubes* Bray, 1976, from Australia (GenBank accession number AF439520); *Palaemonetes australis* Dakin, 1915, from Australia (GenBank accession number AF439517) and *Palaemon intermedium* Stimpson, 1860 from Swan River, Western Australia, (GenBank accession number AF439516).

DNA Extraction, amplification and sequencing. DNA was extracted from abdominal muscle samples (0.5-1.0 g). The tissue was fragmented and digested for 24 h at 57 °C in a solution containing: 500 µl STE buffer, 10 mg/ml of proteinase K and 75 µl of 10% SDS. DNA extraction was carried out using a phenol-chloroform-isoamylic alcohol technique (Hillis *et al*., 1996). A fragment of the 16S rDNA gene was amplified by PCR using primers developed by Vázquez-Bader *et al*. (2004) for penaeid shrimp: 16ScF (5´ GAC CGT GCG AAG GTA GCA 3´), 16 ScR (5´ AAT TCA ACA TCG AGG TCG CA 3´). The amplification reaction was prepared in a final volume of 50 µl containing: 5 µl of 10x PCR buffer, 0.4 mM of each dNTP, 0.8 µM of each primer, 0.4 mM $MgCl_2$, 1 unit of Taq polimerase, 2 µl of DNA extract and bi-distilled water. The PCR amplification was done in a 9700 PE Applied Biosystems Cycler under the following temperature profile: initial denaturation 95 °C for 3 min, 30 cycles of 95 °C for 30 sec, annealing temperature of 50 °C for 30 sec, extension temperature of 72 °C for 30 sec, and additional extension of 72 °C for 3 min. PCR products were purified with a Qiagen QIA quick PCR Purification Kit with a final recovery volume of 50 µl. The purified PCR products were sequenced following a standard Perkin-Elmer protocol; the final sequence reaction was carried out using a final volume of 10 µl containing ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit, v. 3.0 polymerase (Applied Biosystems) and the corresponding oligonucleotides. The incorporated dideoxynucleotides were removed through Sephadex filtering (G-25 Sigma). The purified products of the sequence reaction were sequenced in both directions in an automatic ABI Prism 3100 (Applied Biosystems) sequencer.

Phylogenetic reconstruction. Electropherograms from each sample were manually aligned and edited using CHROMAS 2.01 (Pro Version, Technelysium Pty Ltd) and Sequencer (Gene Codes) to create a consensus sequence for each species. The initial sequence alignment was done with CLUSTAL X (Thompson *et al*., 1997) and multiple alignments with BIOEDIT (Hall, 1997-2001). Pairwise sequence comparisons provided an assessment of levels of saturation when plotting the number of transitions and transversions against the uncorrected proportional distances (p-distances) for each pair of unique sequences. Sequences were analyzed with DNAMAN 4.15 (bio soft 1994-2001, Lynnon Corporation, Quebec, Canada) to determine the genetic distance/identity matrix and the similarity percentages among sequences. Aligned sequences were imported into PAUP 4.0b (Swofford, 1998) to run Minimum Evolution (ME), Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses.

Inter and intra-specific genetic distances were calculated using the Kimura (1980) 2-parameter model with the pairwise deletion option in the MEGA 4 program (Tamura *et al*., 2007).

The best-fit model of evolution for ML was obtained with MODELTEST 3.7 (Posada & Crandall, 1998) using the Hierarchical Likelihood Ratio test (Huelsenbeck & Crandall, 1997). For the ML analysis (Huelsenbeck & Ronquist, 2001) heuristic searches were performed with 100 random replicates as sequence additions; the

Table 1. Mexican species of the genus *Macrobrachium* sequenced for a fragment of the 16S rDNA gene. Collecting site and Ocean slope are indicated as well as habitat, larval stage, and identification number at the CNCR and GenBank.

Species	Collection locality	Slope	Habitat	Larval development	Type Series Catalog number in CNCR	GenBank Access Number
M. acanthochirus Villalobos, 1966	Chamela, Jalisco	Pacific	Epigean	Extended	—	KF383299
M. acanthurus Wiegmann, 1836	Los Tuxtlas, Veracruz	Atlantic	Epigean	Extended	—	KF383300
M. acherontium Holthuis, 1977	Oaxaca	Atlantic	Caves	Abbreviated	8694	KF383301
M. americanum Bate, 1868	Chamela, Jalisco	Pacific	Epigean	Extended	—	KF383302
M. carcinus (Linnaeus, 1758)	Veracruz	Atlantic	Epigean	Extended	—	KF383303
M. digueti (Bouvier, 1895)	Baja California Sur	Pacific	Epigean	Extended	—	KF383304
M. heterochirus (Weigmann, 1836)	Los Tuxtlas, Veracruz	Atlantic	Epigean	Extended	13333	KF383305
M. hobbsi Villalobos & Nates, 1990	Pijijiapan, Chiapas	Amphiamerican	Epigean	Extended	2239a	KF383306
M. michoacanus Villalobos & Nates, 1990	Michoacán	Pacific	Epigean	Extended	3550	KF383307
M. occidentale Holthuis, 1950	Chamela, Jalisco	Pacific	Epigean	Extended	—	KF383308
M. olfersii (Wiegmann, 1836)	Chamela, Jalisco	Amphiamerican	Epigean	Extended	—	KF383309
M. tenellum (Smith, 1871)	Chamela, Jalisco	Pacific	Epigean	Extended	—	KF383310
M. totonacum Mejía, Álvarez & Hartnoll, 2003	River San Antonio, Oaxaca	Atlantic	Epigean	Abbreviated	19915	KF383311
M. tuxtlaense Villalobos & Álvarez, 1999	Lake Catemaco, Veracruz	Atlantic	Epigean	Abbreviated	13174	
	KF383312					
M. vicconi Román, Ortega & Mejía, 2000	Ocosingo, Chiapas	Atlantic	Epigean	Abbreviated	17034	KF383313
M. villalobosi Hobbs, 1973b	Cave of San Gabriel, Oaxaca	Atlantic	Caves	Abbreviated	19220	KF383314

confidence level was determined with 100 non-parametric bootstrap replicates and 10 sequence additions. Maximum Parsimony and ME analyses were carried out through heuristic searches with 2000 non-parametric bootstrap replicates. A Bayesian Analysis (BA) was performed with MrBAYES 3.0b4 (Huelsenbeck & Ronquist, 2001) by running a Markov chain Monte Carlo algorithm for 10 millions of generations, sampling 1 tree every 100 generations starting with a random tree. A burn-in value of 10,000 generations was applied before obtaining a 50% majority rule consensus tree was obtained from the remaining saved trees. An additional 50% majority consensus tree was computed using all the obtained reconstructions with PAUP 4.0b (Swofford, 1998), computing the bootstrap values for: ME, MP and ML analyses and Posterior Probabilities for the BA analysis.

The divergence times between the ED and AD clades and between the nodes that support the geminate species pairs were estimated under a molecular clock model with the ML reconstruction using the Langley-Fitch method with r8s (Sanderson, 2002a). The fossil species *Palaemon antonellae* (Garassino & Bravi, 2003) from the lower Cretaceous (99-112 million years ago, mya) was used for calibration at the well-supported node where *Macrobrachium* separates from *Palaemon* and *Palaemonetes*. Estimates were obtained using two values, 99 and 112 mya for this particular calibration point.

RESULTS

A total of 385 base pairs were aligned, excluding primers and ambiguous regions, of which 179 were variable and 133 parsimony-informative. The best-fit model selected with the Hierarchical Likelihood Ratio test was the Hasegawa-Kishino-Yano model (Hasegawa *et al.,* 1985), accounting for invariable positions and differential substitution rates under a gamma distribution; the specific parameters under this model (HKY+I+G) are as follows: nucleotide frequencies A = 0.318, C = 0.100, G = 0.185, T = 0.396; substitution model with a transition/transversion ratio = 3.625; proportion of invariable sites I = 0.368; variable sites followed a gamma distribution with shape parameter = 0.622.

The four methods of phylogenetic analysis resolved similar results, particularly the trees obtained with ME and MP. Topologies obtained with ML (two trees) and BA (two selected trees) were also similar, but with few variations in the position of the *M. acanthurus-M. tenellum* and *M. hobbsi-M. olfersii* geminate pairs. Based on the few observed changes, high congruency across the four different methodologies and their agreement to current taxonomic classification we will direct the remaining analyses and discussion to the tree obtained by MP (Fig. 1).

The Mexican species of *Macrobrachium* form a monophyletic group resolving two well defined and statistically supported clades: one including the 11 species with ED and a second clade with the five AD species (Fig. 1). The species in the two clades diverged on average 14%, corresponding to a maximum of 54 nucleotides (Table 2). The monophyly of the *Macrobrachium* species is well supported; with the species of *Palaemonetes* and *Palaemon* clearly forming a sister group (Fig. 1).

Within the AD clade, the average similarity among sequences was 96%, corresponding to a minimum of four and a maximum of 30 nucleotide substitutions (Table 2). From the five species in the clade, the most divergent is *M. vicconi* with a similarity of 93% (Table 2; Fig. 1); whereas *Macrobrachium villalobosi* is the only species in all four topologies that resolves on its own, not grouping in to any node. The genetic distances among AD species seems to be independent of being epigean or stygobitic. The epigean species, *M. tuxtlaense,* has a similar genetic distance (0.030) with the epigean species *M. totonacum* and with the stygobitic *M. villalobosi*. A similar result occurs between the stygobitic spe-

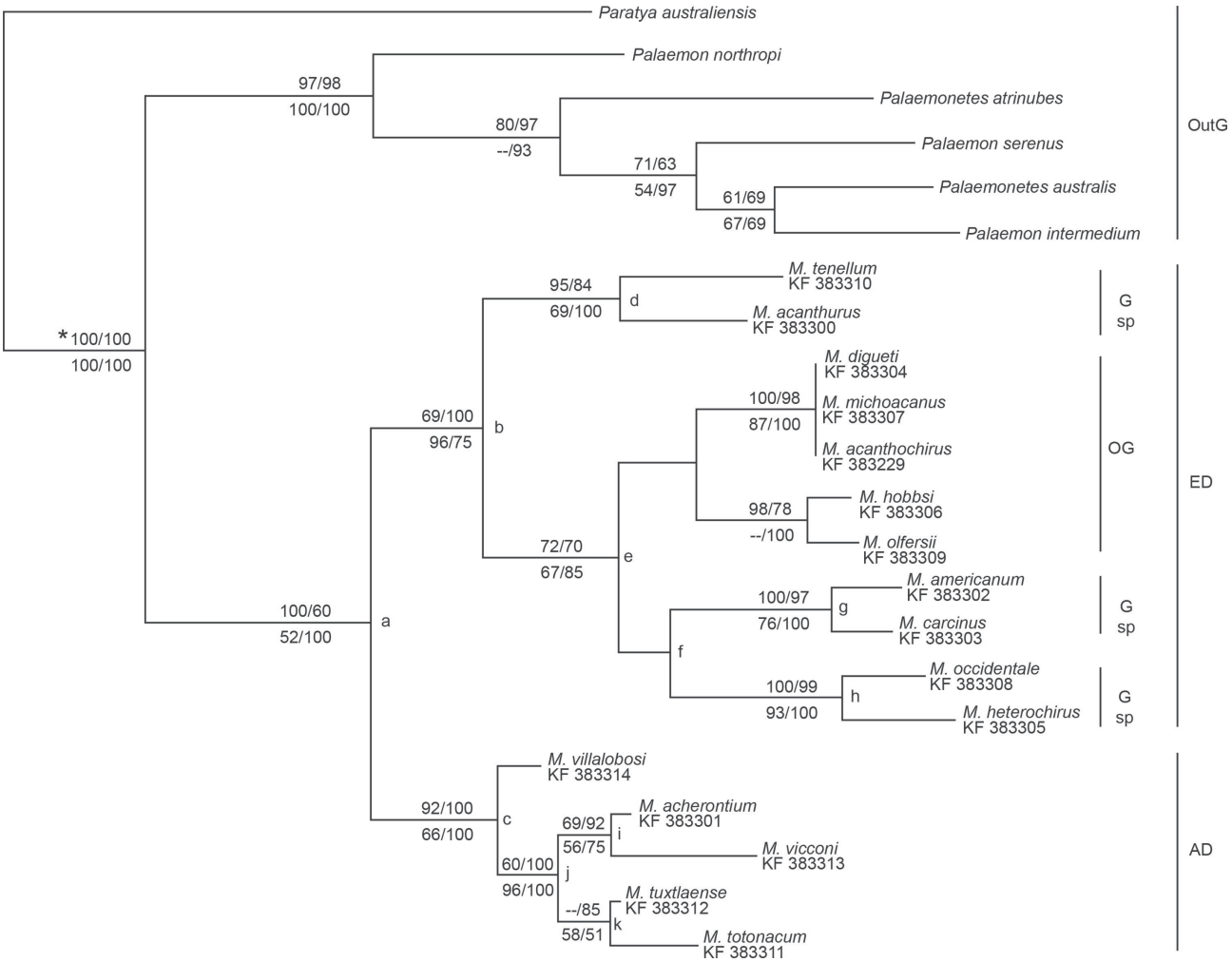

Figure 1. Parsimony tree for the 16 species of Mexican *Macrobrachium* generated from the analysis of 380 base pairs of the 16S rDNA gene. Bootstrap values of MP/ME are above the branch, and ML/Posterior Probabilities of BA are below the branch. Letters to the right of nodes denote nodes for which divergence estimates were obtained (Table 3). AD, species with abbreviated larval development; ED, species with extended larval development; OG, species in the Olfersii Group; G sp, geminate species and (*) calibration point.

Table 2. Genetic distances (above diagonal) and number of nucleotide substitutions (below diagonal) for the 16 species of *Macrobrachium* included in this study, based on a 380 bp fragment of the 16S rDNA gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *M. tenellum*		0.075	0.129	0.129	0.129	0.131	0.132	0.139	0.129	0.129	0.129	0.167	0.134	0.144	0.124	0.145
2. *M. acanthurus*	29		0.134	0.134	0.134	0.118	0.118	0.131	0.129	0.134	0.131	0.151	0.126	0.136	0.116	0.142
3. *M. digueti*	49	47		0.000	0.000	0.059	0.065	0.097	0.094	0.099	0.115	0.151	0.124	0.136	0.134	0.153
4. *M. michoacanus*	49	47	0		0.000	0.059	0.065	0.097	0.094	0.099	0.115	0.151	0.124	0.136	0.134	0.153
5. *M. acanthochirus*	49	47	0	0		0.059	0.065	0.097	0.094	0.099	0.115	0.151	0.124	0.136	0.134	0.153
6. *M. hobbsi*	50	45	23	23	23		0.013	0.105	0.102	0.088	0.102	0.134	0.110	0.125	0.110	0.147
7. *M. olfersi*	50	45	25	25	25	5		0.102	0.099	0.091	0.110	0.134	0.111	0.112	0.111	0.142
8. *M. americanum*	53	50	37	37	37	40	39		0.024	0.115	0.123	0.151	0.126	0.136	0.132	0.145
9. *M. carcinus*	49	49	36	36	36	39	38	9		0.113	0.115	0.147	0.124	0.133	0.129	0.142
10. *M. occidentale*	49	53	38	38	38	34	35	44	43		0.048	0.151	0.129	0.141	0.124	0.153
11. *M. heterochirus*	49	50	44	44	44	39	42	47	44	18		0.151	0.129	0.144	0.121	0.155
12. *M. acherontium*	64	58	58	58	58	53	52	58	56	58	58		0.020	0.037	0.037	0.064
13. *M. tuxtlaense*	53	48	47	47	47	42	43	48	47	50	49	8		0.030	0.030	0.070
14. *M. totonacum*	55	52	52	52	52	47	43	52	51	54	55	4	12		0.044	0.071
15. *M. villalobosi*	48	45	53	53	53	42	43	51	49	46	46	4	12	17		0.078
16. *M. vicconi*	55	54	59	59	59	56	54	56	55	59	60	25	27	27	30	

cies, *M. acherontium,* (genetic distance 0.037) and the stygobitic species *M. villalobosi* and the epigean *M. totonacum*. However, when comparing among the five AD species, *M. tuxtlaense* and *M. acherontium* are the closest genetic species (genetic distance 0.020). While *M. vicconi,* has a greater genetic distance with other AD species (Table 2).

The ED clade groups 11 species in two main subgroups, the first one containing the geminate pair *M. tenellum-M. acanthurus* which differ 8% from each other and 13% on average from the rest of the species in the subgroup (Table 2; Fig. 1). The second subgroup is divided into two resolved nodes, each with a pair of geminate species: *M. americanum-M. carcinus*, with 98% similarity and *M. occidentale-M. heterochirus*, with 95% similarity; and the Olfersii Group, which includes the species proposed by Villalobos (1967a): *M. digueti*, *M. olfersi* and *M. acanthochirus*, this analysis also includes *M. michoacanus* and *M. hobbsi*. *Macrobrachium olfersii* and *M. hobbsi* have 99% similarity, whereas *M. digueti*, *M. michoacanus* and *M. acanthochirus* have 100% similarity and consequently form an unresolved trichotomy (Fig. 1).

The divergence time analyses using the Langley-Fitch method indicate that the molecular change rates were not constant. Therefore, the age of the clades was then estimated through the penalized maximum likelihood test (Sanderson, 2002b), a semiparametric method that allows the molecular clock assumption and independent calibrations (Table 3). The estimated divergence time between the ED and AD clades ranged between 47.8 and 41.7 mya.

Table 3. Estimated divergence times at selected branch nodes for *Macrobrachium*, with particular calibrations for 99 and 112 mya, obtained through the penalized maximum likelihood test.

Node	99 mya	112 mya
a	41.69	47.82
b	29.55	33.07
c	8.71	9.02
d	16.04	16.11
e	12.50	14.81
f	17.80	20.35
g	1.92	2.03
h	4.32	4.98
i	5.48	5.96
j	4.99	6.14
k	4.07	4.52

Within the ED clade, the geminate pair *M. acanthurus-M. tenellum* shows an early separation from the rest of the subgroup, with a divergence of 16.1-16.0 mya from each other; the other two geminate pairs suggested a later formation, *M. heterochirus-M. occidentale* 4.9-4.3 mya and *M. americanum-M. carcinus* 2.0-1.9 mya. Within the AD clade, it is suggested that the five species included in this subgroup appeared 9.0-8.7 mya, with the more recent separation of *M. tuxtlaense* and *M. totonacum* occurring 4.5-4.0 mya.

DISCUSSION

The nucleotide frequencies obtained for the 16S rDNA gene fragment studied here are similar to those reported by Murphy and Austin (2005) for 30 species of *Macrobrachium* around the world.

The results of the four methods used to infer the phylogenetic relationships of the *Macrobrachium* species from Mexico were highly congruent. We selected the tree obtained with MP in order to further discuss the relationships of these species, basically because with this method we obtained one single tree and because it better reflects current taxonomic information (Villalobos, 1967b; Hernández *et al.,* 2007). The two most important features of the analyses are that the Mexican *Macrobrachium* species form a monophyletic group and that the species with ED are clearly separated from those with AD. Although the resolved monophyly of the group is not surprising when only Mexican species are included in the analysis, it important to highlight it, particularly *a posteriori* in testing if the hypothesis of the monophyly of the Mexican group is retained when including the characteristic separation of a single clade for species with DA and another with species with ED, and to probe it in the overall phylogeny of the genus.

Compiled information from several studies of crustaceans using r16S sequences revealed that the levels of variation estimated among con-generic species range from 2 to 17% (Suno-Ughi *et al.,* 1997; Ponniah & Hughes, 1998; Jarman *et al*., 2000; Tong *et al*. 2000; Murphy & Austin, 2002, 2003, 2004, 2005; Lefebure *et al*., 2006; Costa *et al*., 2007). The results presented herein show a range of variation from 0 to 16.7%, with the maximum genetic difference observed between *M. acherontium*, a cave adapted species with AD, and *M. tenellum*, an epigean, estuarine species with ED.

Species with abbreviated development. The marked ontogenetic and morphological divergence between *Macrobrachium* species with ED from those with AD has promoted the idea of considering them as two separate lineages that split very early in the history of the group during the Cretaceous, being all the species with AD closely related (Pereira & García, 1995) (the AD species are more closely related among them than to those ED species). However, the worldwide distribution of species with AD can also suggest independent origins in each geographic region (Murphy & Austin, 2005). In other words, the species with AD from South America, for example, are probably the result of different invasion events of the freshwater habitat relative to those species present in southern Mexico.

The phylogeny resolved in this study, also suggest the divergence between the species with ED from those with AD. The mean genetic difference between the two groups was 14% or 54 nucleotides. This difference could be used to erect two different genera, as Pereira and García (1995) proposed. However, AD is a widespread characteristic among the *Macrobrachium* species and it is also present in several other related palaemonid genera, so it may not be a very informative character as has been concluded in a similar study (Murphy & Austin, 2005). Furthermore, the emergence of AD in every lineage has not been analyzed within a phylogenetic context in order to determine if: a) it has arisen one or many times, b) it has evolved several times, with each event corresponding to a different geographic region, and c) it has a different origin relative to ED or if one gave rise to the other one.

In contrast to those ideas other authors proposed a more recent origin of this fauna placing major radiations in the Miocene (Murphy & Austin, 2005) and the origin of AD in post-Miocene or Pliocene times (Shokita, 1979a; b; Villalobos, 1982; Magalhaes & Walker, 1988; Mashiko, 1992).

The Olfersii Group. In our study, the species in the Olfersii Group clustered together in agreement to Villalobos (1967b). Low genetic differences (below 2%) were found among all of the species within this clade. For *M. digueti*, *M. michoacanus* and *M. acanthochirus* the resolved sequences were identical. Hernández *et al*. (2007) described in detail the misunderstanding on the morphology of *M. digueti* and *M. acanthochirus*, which prompted Villalobos (1967b) to describe *M. acanthochirus*. Furthermore, the morphological analyses of *M. digueti* and *M. acanthochirus* by Hernández *et al.* (2007) are supported by our molecular data, both in agreement to the synonymyzation of *M. acanthochirus*. Interestingly, in our analysis *M. michoacanus* also exhibited a 100% similarity to *M. digueti* and *M. acanthochirus*; however, as Hernández *et al*. (2007) concluded, the morphology of *M. michoacanus* clearly separates this species from *M. digueti*. We agree with his result after the examination of specimens of both species. This apparent contradiction deserves further studies in order to determine if in fact *M. michoacanus* represents a different species, or to elaborate more on the nature of this species complex. Synonymyzation of species based on evidence drawn from mitochondrial genes alone should be avoided as processes such as introgressive hybridization can have a confounding effect (Sites & Crandall, 1997; Harrison, 2004).

The remaining two species in the Olfersii Group, *M. hobbsi* and *M. olfersii*, with 99% genetic similarity can be distinguished by morphological analyses. However, both species exhibit a certain degree of variation and have an amphiamerican distribution (with co-occurrence in the same localities both on the Pacific and the atlantic coasts; Villalobos Hiriart & Nates Rodríguez, 1990; Hernández *et al*., 2007), two conditions that can create uncertainty in their identification.

Geminate species. The geographic separation of a species with widespread distribution and a continuous gene flow for an extended period of time due to the formation of geographical barriers (ei. oceans, deserts or ridges), lead to the conformation of two

big but isolated populations, with restricted gene flow and with adaptations to local environments that lead to their independent evolution and geographic speciation (Jordan, 1908; Knowlon *et al.,* 1993; Knowlton 2000). The formation of the Isthmus of Panama led to the isolation of many species populations, particularly aquatic, which currently inhabit both the Pacific and Atlantic oceanic slopes, but which are subjected to independent evolution, an example of them are shrimps of the genus *Alpheus,* for which seven species pairs have been reported, with one unit of each pair located on each of the oceanic slopes, maintaining considerable morphological similarity but completely genetic isolated, therefore they are recognized as sibling species or geographic. Molecular evidence indicated that these morphologically similar *Alpheus* species are genetically different and that divergence followed after the geographical barrier, in this case the Isthmus of Panama (Knowlon *et al.,* 1993; Knowlton, 2000).

In this study of *Macrobrachium*, the close relationship between the species in the geminate pairs is clearly represented in the phylogenetic analysis. Their positions resolved by MP, ME and ML analyses suggest that the splitting of the pairs occurred at different times, although previously all three pairs were believed to have derived as a result of the closing of the Isthmus of Panama (Villalobos, 1982; Camacho *et al*., 1997). In this study the divergence time estimates derived from calibrating the molecular clock with an unrelated event, which is the record of the fossil species *Palaemon antonellae* (Garassino & Bravi, 2003), can be used to test if the closing of the Isthmus was the single most important event in the formation of the geminate pairs or if there were other processes involved in their differentiation.

The divergence time estimate of 16.1-16.0 mya obtained for the *M. acanthurus-M. tenellum* pair clearly sets back their splitting well before the formation of the Isthmus of Panama. Assuming that the ancestor of both *M. acanthurus* and *M. tenellum* had a widespread distribution along the coast of the Chortis Block, which consisted of Guatemala, Honduras and Nicaragua, the northward movement of the southern Central America land mass and its final connection with the Chortis Block, could have separated the primitive species giving rise to the two species. This geological setting is depicted in Iturralde-Vinent and MacPhee (1999) for the Middle Miocene. Coates *et al*. (1992) have proposed that the Isthmus of Panama started forming 12.9-11.8 mya, at a time when these two species had already diverged. Interestingly, although the most genetically different of the geminate pairs, the morphological separation of specimens of the two species without knowing where they were collected can be very difficult since their rostrum and second pair of pereiopods are extremely similar and both species also exhibit a high degree of geographic variation (Camacho *et al.,* 1997).

According to our results, the pair composed by *M. heterochirus-M. occidentale* split 4.9-4.3 mya, an estimate that is clearly related to the formation of the Isthmus of Panama. Although the final closure of the Isthmus is situated at 3.5-3.1 mya, this was a gradual process that had probably started by the end of the Miocene (7.0-6.3 mya) when a rising of the coastal areas occurred as indicated by the fauna of foraminiferans (Coates *et al.,* 1992). Thus, the differentiation of *M. heterochirus* and *M. occidentale* could be attributed to the barrier that was effectively operating before the final closure of the Isthmus. Morphologically, the two species can be distinguished by the more robust second pereiopod of *M. occidentale*, since the relative proportions of the articles of the second pereiopod of both species are almost identical (Holthuis, 1952).

The third geminate pair, composed by *M. carcinus* and *M. americanum* diverged 2.0-1.9 mya. In this case the two species started diverging long after the Isthmus of Panama had closed, a scenario that suggests that other processes, such as the adaptation to different hydrological regimes along both continental slopes, might have influenced their differentiation. Among the three geminate pairs of *Macrobrachium*, this pair contains the two most morphologically similar species and their correct identification depends on knowing where they were collected.

The different divergence estimates obtained for the three geminate pairs in this study are consistent with those views that consider the emergence of the Isthmus of Panama as a gradual process that took place over at least 3.0 my (Coates *et al.,* 1992). Marko (2002) questioned the validity of using the time of the final closure of the Isthmus as the fixed time at which all geminate pairs originated. It seems unlikely that the differentiation of populations for all taxa occurred exactly when water exchange between the eastern Pacific and Caribbean ceased, and thus molecular clock calibrations based on this final event ignore the long previous process. Knowlton and Weigt (1998) using multiple geminate pairs of the genus *Alpheus* identified the staggered speciation pattern as we do in this study; however, studies with one or a few geminate pairs and no independent calibrations might be underestimating divergence times.

Therefore at least three important events have been involved in the differentiation of the Mexican species of *Macrobrachium*. First, there is an early separation of the species with ED from those with AD, probably due to a generalized invasion of the freshwater habitat in the Middle Eocene that originated the ancestral freshwater stock from which the five species with AD later derived. Second, within the clade with ED, the Olfersii Group is formed with highly adaptable forms, two species remain with amphiamerican distributions and a species complex emerges with moderate morphological variability and low genetic variation whose differentiation is not yet completely understood. Third, also within the ED clade, species with large distribution ranges on both continental slopes were impacted by the formation of Central

America and then by the closure of the Isthmus of Panama, giving rise to the geminate pairs.

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