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ALLOZYME DIVERGENCE SUPPORTING THE TAXONOMIC SEPARATION OF *OCTOPUS MIMUS* AND *OCTOPUS MAYA* FROM *OCTOPUS VULGARIS* (CEPHALOPODA: OCTOPODA)

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ABSTRACT

Taxonomic status and phylogeny of shallow-water octopuses remain to be resolved. Recently the octopod inhabiting Peru and North Chilean waters has been identified on morphological basis as *Octopus mimus* instead of *O. vulgaris*. The former shows faint paired ocelli whereas the latter is a non-ocellated species. Another ocellated octopus is *O. maya*, which is restricted to the Gulf of Mexico. Thirty allozyme loci were studied in *O. mimus* collected from Peru, *O. vulgaris* from Spain and *O. maya* from the Gulf of Mexico, and in two other Spanish octopods (*Eledone cirrhosa* and *E. moschata*) which were used as outgroups. Genetic identity values (Nei, 1972) between *O. vulgaris* and *O. mimus*-*O. maya* (mean $I = 0.18$) were typical of confamilial genera, whereas genetic identity between the two latter species ($I = 0.44$) was typical of congeneric species. Moreover, neighbor-joining and parsimony trees showed that *O. mimus* and *O. maya* constitute a monophyletic clade. These genetic results suggest that *O. mimus* and *O. maya* should be considered as belonging to a different confamilial genus than *O. vulgaris*, and support the hypothesis of both taxa as Panamanian geminate species resulting from an allopatric speciation event associated with the uplift of Central America.

The systematics of Octopodinae is still unresolved (Voss et al., 1998; Toll, 1998; Söller et at., 2000). One of the key problems that needs to be addressed is whether *Octopus vulgaris* Cuvier, 1797 is a true cosmopolitan or simply a literature cosmopolitan species (Mangold, 1998). Its distribution area covers the western and eastern basins of the Mediterranean Sea and the Adriatic Sea. In the eastern Atlantic it is found from the South of England to the Gibraltar Strait, on the West and South coast of Africa, and the Azores, Canary, Cape Verde and St. Helena Islands. This species has also been cited in numerous localities from the West Atlantic Ocean. However, there is some recent evidence which indicates that these animals do not belong to the same species, suggesting that *O. vulgaris* is not a cosmopolitan species but rather one member of a species complex (see Mangold, 1998; Söller et at., 2000). One piece of evidence is the finding that the shallow-water commercial species inhabiting the Peru and North Chilean coast has clearly been identified on morphological and molecular (mtDNA sequences) basis as *Octopus mimus* Gould, 1852 instead of *O. vulgaris*, as was long time considered (Cortez, 1995; Guerra et al., 1999; Söller et at., 2000). *O. mimus* shows faint paired ocelli between the eyes and the bases of arms II and III, whereas *O. vulgaris* is not an ocellated species. Pairwise genetic distance estimated as percentage of substitutions between the Chilean *O. mimus* and the Mediterranean *O. vulgaris* was 12.7. At present, the limits of the distribution of *O. mimus* are unknown, although they have been cited from North of Peru to San Vicente Bay (Chile) (Fig. 1). Another ocellated species included within the *Octopus* genus is the shallow-water octopus *O. maya* Voss and Solís, 1966. This species is distributed from the north-eastern Yucatan Peninsula, around the Bay of Campeche, to near Vera Cruz (Mexico), where it supports a local commercial fishery (Roper et al., 1984) (Fig. 1). Present diagnosis and information on its biology and natural history can be found in Voss and Toll (1998) and references therein.

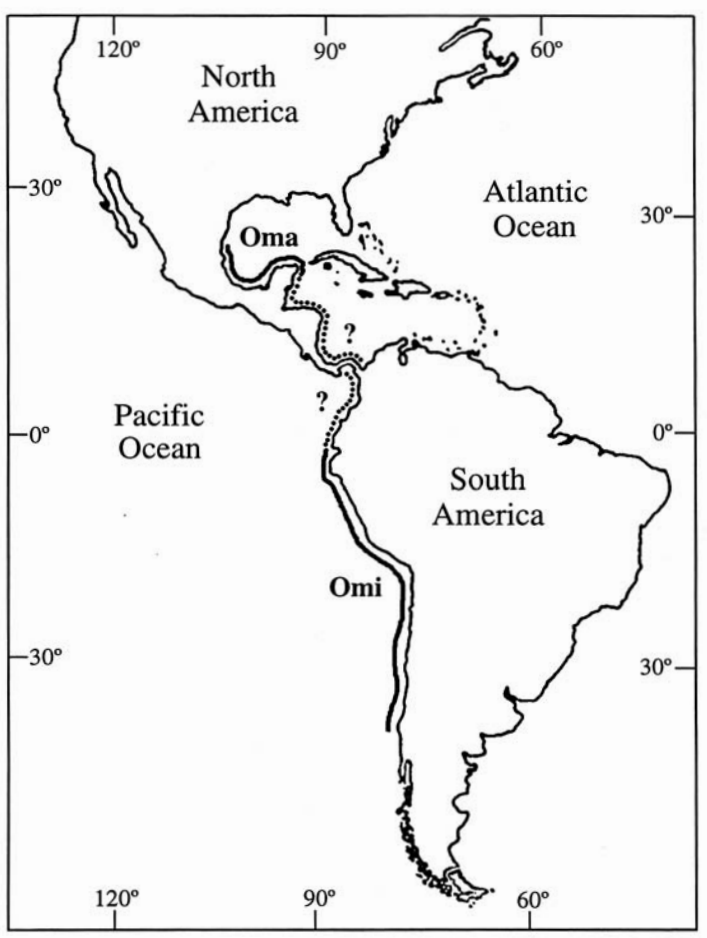

Figure 1. Map of America showing the known geographical distribution of *Octopus mimus* (Omi) and *O. maya* (Oma) (continuous line). ? indicates areas where the distribution of each *Octopus* species is unknown. Species codes are as Table 1.

One of the most important problems in constructing a consistent taxonomic and phylogenetic basis for the Octopodinae seems to be the identification, selection and standardisation of suitable morphological characters, mostly due to the body plasticity of these animals and almost non-existence of hard specific structures. Allozyme electrophoresis has several advantages over morphological criteria because allozymes are primary products of the genome (Ayala, 1983). Allozyme polymorphisms have proved to be effective for clarifying the taxonomic status of several cephalopod species, including cryptic species, from different genera, e.g., *Eledone*, *Sepia* and *Loligo* (see Levy et al., 1988; Brierley et al., 1996; Sanjuan et al., 1996 and references therein). The aims of the present study are to use allozyme electrophoresis to distinguish *O. mimus* and *O. vulgaris*, and to evaluate the systematic relationships between these two octopuses and *O. maya* using two species of *Eledone* as outgroup.

Materials and Methods

Sampling.—Samples of five octopod species were collected between October 1995 and December 1996 in the East Pacific: *Octopus mimus* Gould, 1852 from Peru (n = 12); the West Atlantic: *O. maya* Voss and SolEis, 1966 from the Gulf of Mexico (n = 31); and the Mediterranean Sea: *O. vulgaris* Cuvier, 1797 (n = 27), *Eledone cirrhosa* (Lamarck, 1798) (n = 6) and *E. moschata* (Lamarck, 1798) (n = 8), from the Northeast of Spain. Mediterranean specimens were taken from commercial catches on the day of capture and Pacific and Atlantic samples were bought in Spain after one month kept frozen on board (−20°C). They were immediately transported in dry ice to the laboratory where they were stored at −72°C until required.

Electrophoresis.—Horizontal starch gel electrophoresis based on the method of Murphy et al. (1996) was carried out. Samples of mantle muscle were prepared for electrophoresis using the methods previously described for *Sepia* spp. (Pérez-Losada et al., 1996). Twenty-six enzymes yielding 30 putative enzyme coding loci, displayed adequate activity and resolution for consistent interpretation and routine examination (Table 1). Detailed electrophoretic conditions and histochemical staining recipes for most of the enzymes are described in Pérez-Losada et al. (1996, 1999). For the enzyme PEPS the electrode buffer was Tris-Citrate pH 8.0 (gel buffer dilution 1:9), and the histochemical staining recipe was as in Murphy et al. (1996). Arabic numerical suffixes for multiple loci (*1* and *2*) and alleles (*100**, *110**, ...) are presented in order of decreasing and increasing anodal mobility, respectively. Cross-comparisons were made among species and gels to ensure scoring accuracy.

Data Analysis.—Genotype frequencies at polymorphic loci were tested for conformance to Hardy-Weinberg equilibrium expectations by exact tests. Estimates of genetic variability were calculated for each species (Nei, 1987). Genetic identity (*I*, Nei, 1972) between *Octopus* species and their bootstrap confidence estimates were also calculated. The 95% bootstrap confidence limit on *I* was constructed by the percentile method (Felsenstein, 1988). A dendrogram was constructed using the unweighted pair-group method using arithmetic averages (UPGMA, Sneath and Sokal, 1973). *Eledone cirrhosa* and *E. moschata* were used as outgroups for the phylogenetic study because Eledoninae constitute, presumably, a monophyletic group with Octopodinae (Voss, 1998). The chord genetic distances of Cavalli-Sforza and Edwards (1967) among samples were used to obtain a neighbor-joining tree (NJ, Saitou and Nei, 1987). Moreover, a cladistic analysis using the locus as the character and their alleles as unordered states were also carried out. Polymorphic loci in terminal taxa were treated as 'polymorphism' (see Swofford, 1993). To assess the confidence of the obtained phylogenetic hypotheses, 1000 bootstrap replicates of each data matrix were generated. The results were summarized using the 50% majority-rule consensus method. Four goodness-of-fit statistics, consistency index (CI), rescaled CI (RC), retention index (RI) and homoplasy index (HI) were also estimated according to Swofford (1993).

Genetic data were analysed using GENEPOP 3.1b computer program (Raymond and Rousset, 1995). Nei's (1972) genetic identities and their bootstrap confidence estimates were carried out with Dbot program (Zaykin, Tatarenkov and Pudovkin, pers. com.). PHYLIP 3.5c (Felsenstein, 1993) and PAUP 3.1 (Swofford, 1993) computer programs were used for the phylogenetic analyses.

Results

Allele frequencies at 30 enzyme loci are shown in Table 1. Within *Octopus* species, 21 loci were diagnostic between *O. vulgaris* and *O. mimus*, 25 between *O. vulgaris* and *O. maya*, and 16 between *O. mimus* and *O. maya*. No significant deviation from Hardy-Weinberg proportions was found at any polymorphic locus (data not shown). Estimates of genetic variability (bottom of Table 1) showed low values for all species (e.g., $H_e <$ 0.07), although due to the number of sampled individuals for some species (e.g., *Eledone* spp.), some of these estimates should be interpreted with caution.

Table 1. Allele frequencies at 30 enzyme loci and indices of genetic variability for *Octopus vulgaris* (Ovu, sample size: n = 27), *O. mimus* (Omi, n = 12), *O. maya* (Oma, n = 31), *Eledone cirrhosa* (Eci, n = 6) and *E. moschata* (Emo, n = 8).

	Species				
Locus	Ovu	Omi	Oma	Eci	Emo
AAT-1*					
100*	1	0	0	0	0
110*	0	1	1	0	0
120*	0	0	0	1	0
125*	0	0	0	0	1
AK*					
75*	0	1	1	0	0
100*	1	0	0	0	0
220*	0	0	0	1	1
ALPDH*					
100*	1	0	0	1	0
120*	0	1	0	0	1
170*	0	0	1	0	0
ARK-1*					
80*	0	0	0	1	0
90*	0	0	0	0	1
100*	1	1	1	0	0
ARK-2*					
100*	1	1	1	0	0
160*	0	0	0	1	1
DDH*					
80*	0	0	1	0	1
100*	1	0	0	0	0
110*	0	1	0	0	0
125*	0	0	0	1	0
EST*					
90*	0	1	1	0	0
100*	1	0	0	0	0
160*	0	0	0	1	0
180*	0	0	0	0	1
ESTD*					
90*	0.111	0	0	0	0
100*	0.889	1	0	0	0
110*	0	0	0	1	0
120*	0	0	1	0	0
200*	0	0	0	0	1
G3PDH*					
90*	0	0	0	1	1
100*	1	0	0	0	0
110*	0	1	1	0	0
G6PDH*					
50*	0	0	0	1	0
65*	0	0	0	0	0.813
75*	0	0	0	0	0.188

Table 1. Continued.

	Species				
Locus	Ovu	Omi	Oma	Eci	Emo
G6PDH*					
90*	0	0.125	0	0	0
100*	1	0.875	1	0	0
GAPDH*					
100*	1	0	0	0	1
190*	0	0.458	0	0	0
200*	0	0	0.935	0	0
210*	0	0.542	0	1	0
220*	0	0	0.065	0	0
IDDH*					
100*	1	1	0	0	0
120*	0	0	1	0	0
150*	0	0	0	1	0
160*	0	0	0	0	1
IDHP-1*					
80*	0	0	1	0	0
90*	0	0	0	1	1
100*	1	1	0	0	0
IDHP-2*					
70*	0	1	1	0	0
80*	0.037	0	0	0	0
100*	0.963	0	0	0	0
200*	0	0	0	1	0
250*	0	0	0	0	1
LAP*					
85*	0	0	0	1	1
100*	1	0	0	0	0
125*	0	0.750	0	0	0
130*	0	0.250	0	0	0
150*	0	0	1	0	0
MDH-1*					
70*	0	0	0	1	0
75*	0	0	0	0	1
85*	0	0	1	0	0
90*	0	1	0	0	0
100*	1	0	0	0	0
MDH-2*					
30*	0	0	0	1	0
60*	0	1	0	0	0
70*	0	0	1	0	0
75*	0	0	0	0	1
100*	1	0	0	0	0
MEP*					
60*	0	0	0	1	0
75*	0	0	0	0	1
100*	1	0	0	0	0

Table 1. Continued.

	Species				
Locus	Ovu	Omi	Oma	Eci	Emo
MEP*					
150*	0	1	1	0	0
MPI*					
100*	1	0	0	1	1
110*	0	1	1	0	0
OPDH-2*					
20*	0	1	0	0	0
100*	1	0	0	0	0
150*	0	0	1	0	0
700*	0	0	0	0	1
900*	0	0	0	1	0
PEPA*					
55*	0	0	1	0	0
75*	0	1	0	1	1
80*	0.370	0	0	0	0
100*	0.630	0	0	0	0
PEPB*					
95*	0	0.042	0	0	0
100*	1	0.958	0	0	0
110*	0	0	1	0	0
115*	0	0	0	0	1
120*	0	0	0	1	0
PEPD*					
80*	0.056	0	0.919	0	0
100*	0.944	0.375	0.081	0	0
110*	0	0.542	0	0	0
120*	0	0.083	0	0	0
150*	0	0	0	0	0.938
160*	0	0	0	0.833	0
170*	0	0	0	0	0.063
180*	0	0	0	0.167	0
PEPS-1*					
75*	0	0	0	1	1
95*	0	1	0	0	0
100*	0.963	0	0	0	0
110*	0.037	0	0	0	0
140*	0	0	1	0	0
PEPS-2*					
10*	0	0.083	0	0	0
20*	0	0	0	1	0
40*	0	0.917	1	0	0
90*	0	0	0	0	1
100*	1	0	0	0	0

Table 1. Continued.

	Species				
Locus	Ovu	Omi	Oma	Eci	Emo
PGDH*					
100*	0.870	0	0	0	1
160*	0.130	1	1	1	0
PGM*					
100*	1	0	0	0	1
200*	0	0.958	0.984	0	0
240*	0	0.042	0.016	0	0
600*	0	0	0	1	0
PK-1*					
100*	1	0	0	0	0
125*	0	1	0	1	1
175*	0	0	1	0	0
PK-2*					
100*	1	0	0	0	0
125*	0	1	0	1	1
175*	0	0	1	0	0
SOD*					
75*	0	0	0	1	0
100*	1	0	0	0	0
110*	0	1	0	0	0
150*	0	0	1	0	0
200*	0	0	0	0	1
He	0.039	0.068	0.010	0.010	0.015
(SE)	(0.018)	(0.029)	(0.006)	(0.010)	(0.011)
Ho	0.037	0.064	0.009	0.011	0.017
(SE)	(0.016)	(0.028)	(0.005)	(0.011)	(0.013)
Na	1.20	1.27	1.10	1.03	1.07
(SE)	(0.07)	(0.10)	(0.06)	(0.03)	(0.05)
P95	13.3	16.7	6.7	3.3	6.7

He: unbiased estimate of mean expected heterozygosity; Ho: mean observed heterozygosity; Na: mean number of alleles; P95: percentage of polymorphic loci with the 95% criterion.

Genetic identities (*I*, Nei, 1972) between the so-called *Octopus* species and their bootstrap estimates are shown in Table 2. Similar *I* values were obtained when unbiased genetic identities (Nei, 1978) were calculated (data not shown). *I* values were less similar between *O. vulgaris* (Ovu) and *O. mimus* (Omi) or *O. maya* (Oma) ($I < 0.260$) than between the latter two species ($I = 0.442$). Non-overlap of the bootstrap confidence intervals (i.e., significant differences) was observed between Ovu-Oma and Omi-Oma species pairs, but not between Ovu-Omi and Omi-Oma. The UPGMA dendrogram of Nei's *I* (Fig. 2) summarizes these results, showing the clear genetic differences between Ovu and the species pair Omi-Oma ($I < 0.20$).

The NJ tree based on Cavalli-Sforza and Edwards's (1967) chord genetic distance joined *Octopus mimus* and *O. maya* in a monophyletic group, clearly separated from *O. vulgaris*

Table 2. Genetic identities (*I*, Nei 1972) with their standard errors (SE), 95% confidence interval (95% CI) and bootstrap estimates (after 1000 runs) between all *Octopus* pairs. Species codes are as Table 1.

Species pairs	*I*	SE	95% CI	Bootstrap	
				5%	95%
Ovu-Omi	0.253	0.079	0.098–0.408	0.143	0.385
Ovu-Oma	0.111	0.057	0.000–0.223	0.022	0.214
Omi-Oma	0.442	0.093	0.261–0.624	0.304	0.609

(Fig. 3A). The frequency with which a particular clade occurred in the tree when majority-rule consensus method was applied to the data set generated by bootstrapping is shown at each node. All the nodes of the tree were strongly supported by bootstrapping (>90%). The maximum parsimony analysis (Swofford, 1993) of allele frequencies produced only one most parsimonious tree of 19 steps (excluding uninformative loci) with the same topology as NJ tree (Fig. 3B), although only the (Omi, Oma) clade was strongly supported by bootstrapping (94%). The CI, RC, RI and HI indices were 0.895, 0.671, 0.750 and 0.105, respectively.

Discussion

Estimates of genetic variability in the present study, the first reported for the genus *Octopus*, fall below the average for invertebrate species and within the range for most cephalopods, confirming the low genetic variability of the class (see Sanjuan et al., 1996). Although this low variation can generate difficulties with the use of electrophoretic data in intraspecific studies, the use of such data for taxonomic and phylogenetic purposes is made more straightforward since unique diagnostic alleles between species can be identified easily and unambiguously. The relatively small sample sizes of some of the species investigated here are likewise not considered to be problematic. Nei's (1972) genetic identity and NJ trees based on Cavalli-Sforza and Edwards's (1967) chord genetic distance presumably will function well when the average heterozygosities of the species under investigation are low, allele frequencies are near zero or one, and there are clear patterns of fixed or nearly fixed alleles unique to certain taxa, as observed here (see Archie et al., 1989). Phylogenetic relationships derived using parsimony are not affected by unique and fixed alleles (states) because they are based on shared alleles. However the

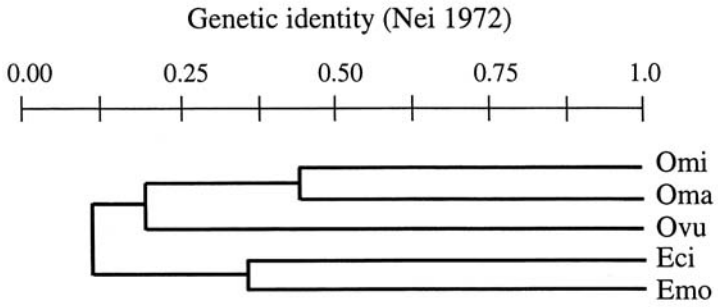

Figure 2. UPGMA dendrogram of Nei's (1972) genetic identity between all *Octopus* and *Eledone* pairs. Species codes are as Table 1

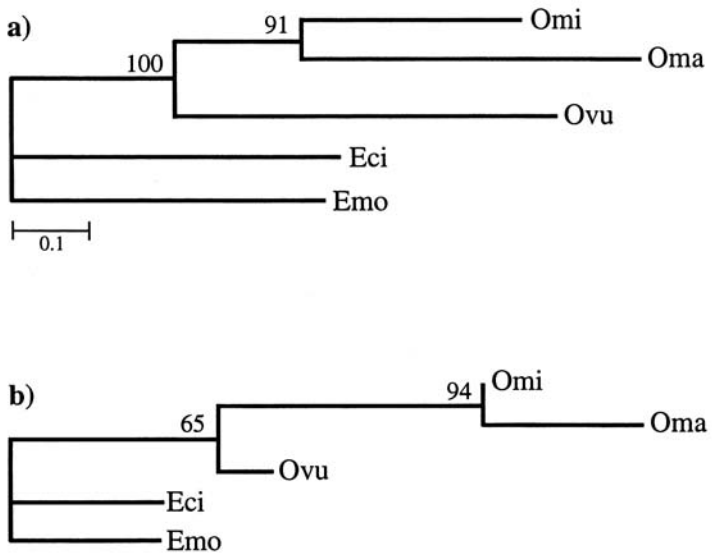

Figure 3. NJ tree based on Cavalli-Sforza and Edwards's (1967) chord genetic distance (a) and most parsimonious tree (b) for five octopus species using *Eledone cirrhosa* and *E. moschata* as outgroups. Numbers at each node are the bootstrap values after 1000 runs. The branch lengths are shown proportional to the amount of evolutionary change along the branches. Species codes are as Table 1

number of possible shared states for each character may be vulnerable to sampling error, thus alleles with a frequency lower than 0.05 were not considered (as suggested by Buth, 1984). Moreover, analysis of relatively high numbers of loci is of far greater importance to taxonomic studies than is the screening of large numbers of individuals per locus (Nei and Roychoudhury, 1974). Therefore, our screening of 30 loci can be considered enough to ensure reasonable accuracy in estimated trees (Nei et al., 1983).

It has been suggested that when conventional morphological studies leave taxonomic status in doubt, an estimate of genetic divergence from allozyme polymorphisms could provide an objective and useful criterion (Thorpe, 1983). Most genetic evidence collected to date from cephalopod species suggests that measures used to distinguish taxonomic hierarchies in the majority of other animal taxa are also appropriate for this mollusc class (Sanjuan et al., 1996). The UPGMA tree (Fig. 2) shows that the mean *I* value between *O. vulgaris* and the pair *O. mimus*-*O. maya* is much lower ($I = 0.18$) than the reported critical value ($I = 0.35$) for distinguishing between species and genera (77% of *I* values between confamilial genera fall below 0.35; Thorpe, 1983), whereas the genetic identity between *O. mimus* and *O. maya* ($I = 0.44$) is a typically expected value between congeneric species (76% of *I* values between congeneric species exceed 0.4, Thorpe, 1983). Moreover, it is in the range of the other octopus species considered here, *Eledone cirrhosa* and *E. moschata* ($I = 0.34$). Consequently, these genetic results suggest that *O. mimus* and *O. maya* should be removed from the genus *Octopus* to a distinct genus within the subfamily Octopodinae. The phylogenetic analysis of the octopuses using neighbor-joining and maximum parsimony procedures with the two *Eledone* species as outgroups (Fig. 3) did not confirm the present *Octopus* classification, as Söller et at. (2000) have suggested recently. In both trees *O. mimus* and *O. maya* constituted a clearly separated

and more recent lineage than the *O. vulgaris* lineage. However, the assignation of these species to one of the extant genera or the proposition of a new genus for both taxa is far from the scope of this study. A more extensive allozyme screening including octopuses from other genera will be necessary to establish the new taxonomic names of the so-called taxa *O. mimus* and *O. maya*. In addition, the generic revision of the Octopodinae has not been done yet and little is known about the octopuses from the Central and East Pacific, which will pose a problem to future studies on Octopodinae systematics.

Considering the present geographical distribution of *O. mimus* and *O. maya* (Fig. 1), the phylogenetic results reported here suggest the possibility of an evolutionary scenario where both species were derived from a common ancestor which inhabited the Central Pacific and Atlantic waters. Little is known about the phylogeography of the octopuses from this area. However Voight (1988) in her study about Trans-Panamanian octopuses suggests that their evolutionary history could be associated to the previous connection between the Pacific and the Atlantic Oceans through what is now Central America, as it has been proposed for other species on the basis of faunal affinities between the two regions (Ekman, 1953). These faunal affinities consist mainly of geminate species (i.e., morphologically similar species with distributions on either side of the isthmus). Close phylogenetic relationships between these species are postulated due to their similarity and the geological history of the area, which indicates that both oceans have been isolated for 3.0–3.5 MYBP (Jones and Hasson, 1985). Voight (1988) compared shallow-water octopods from the eastern tropical Pacific (including the Gulf of California) to those of the western tropical Atlantic on basis of their phenetic similarity. She found a high proportion of geminate species (6 out of 16) among which the pair *O. maya*-*O. bimaculatus* Verrill, 1883 was included. *O. mimus* was not analysed in this study because it was out of the geographical range considered; however, this species shares several morphological similarities with *O. maya* which would indicate they are geminate species. They have similar size, sexual dimorphism, paired ocelli between the eyes and the base of arms II and III, enlarged sucker distributions, minute ligula, small calamus and skin with patch and groove trellis arrangement (Guerra, pers. observ.). According to Voight (1988), these shared characters would be evidence of a recent common ancestor. Nevertheless, because this and the other species comparisons are based upon overall similarity, these species pairs represent hypotheses that remain to be tested by phylogenetic analysis (Voight, 1988). In this sense, the phylogenetic results presented here seem to uphold the hypothesis of *O. mimus* and *O. maya* as geminate species.

Several estimates have been proposed to calculate divergence times from allozyme data. To obtain a rough idea about evolutionary time (t) the formula $t = 5 \times 10^6 D$ was used (Nei, 1987), where D is the genetic distance of Nei (1972). For *O. mimus* and *O. maya* t is 4.0 mybp, a time longer than that considered for the complete closure of the Isthmus of Panama. However genetic divergence before final closure may have been facilitated by changing oceanographic conditions (Knowlton et al., 1993). By 5.0 myrbp, substantial divergence at even subgeneric level has been described for other shallow-water molluscs associated to the gradual closure of the Panamanian seaway (Jackson et al., 1993). Thus, the divergence time and the phylogenetic result reported here suggest for *O. mimus* and *O. maya* the development of reproductive isolation under the classical allopatric model of division into two large populations without secondary contact.

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