GENETIC VARIATION AT THE LEUCINE AMINOPEPTIDASE (LAP) LOCUS IN *CRASSOSTREA VIRGINICA* FROM EASTERN VENEZUELA

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ABSTRACT In this work we examine by means of starch gel electrophoresis the genetic variability at the Leucine Aminopeptidase (*LAP**) locus in samples of *Crassostrea virginica* taken from two locations differing in salinities (Güiria and Guariquén; Eastern Venezuela). The enzyme system is determined by two loci: *LAP-I* and *LAP-II* (*LAP-II* is monomorphic). *LAP-I* showed 8 alleles in Guariquén and 6 in Güiria. The distribution of genotype frequencies in *LAP-I* from both locations was significantly different from the Hardy-Weinberg expectation. For the Guariquén population, the observed heterozygosity was 0.45, the expected one was 0.72 and the heterozygote deficiency was 0.371. For the Güiria population, the observed heterozygosity was 0.54, the expected one was 0.76 and the heterozygote deficiency was 0.442. The heterozygote deficiency is discussed in terms of inbreeding, mixture of genetically different populations, null alleles and selection. We relate the differences in genetic structure between both populations of *Crassostrea virginica* with the environmental conditions in both locations.

RESUMEN La variabilidad genética del locus Leucil Aminopeptidasa (*LAP**) de *Crassostrea virginica* de las localidades de Güiria y Guariquén fue examinada mediante electroforesis en gel de almidón. El sistema enzimático está determinado por dos loci, *LAP-I, LAP-II,* siendo este último monomórfico. *LAP-I* presentó 8 alelos en Guariquén y 6 alelos en Güiria. La distribución de las frecuencias genotípicas en el locus *LAP-I* de ambas localidades fue significativamente diferente de lo esperado según el principio de equilibrio de Hardy-Weinberg. Para la población de Guariquén la heterocigosidad observada fue 0,45; la esperada fue de 0,2 y la deficiencia de heterocigotos fue -0,29. En la población de Güiria, la heterocigosidad observada fue 0,54 y la esperada de 0,76; la deficiencia de heterocigotos fue de -0,29. La deficiencia de heterocigotos del locus *LAP-I* se discute en términos de intracruzamiento, efecto Wahlund, alelos silentes y selección. La diferencia de la estructura genetica de las poblaciones de Crassostrea virginica se relaciona con la variación de las condiciones ambientales en ambas localidades.

INTRODUCTION

The effect of environmental salinity in determining the patterns of leucine aminopeptidase (LAP EC.3.4.1.1) variation in natural populations of *Mytilus edulis* has been successfully demonstrated (KOEHN & IMMERMAN, 1981; KOEHN & HILBISH, 1987). These studies, as well as others carried out on *M. galloprovincialis* and *Perna canaliculus* (GARDNER & KATHIRAVETPILLAI, 1997), *Mercenaria mercenaria* (KOEHN *et al*., 1980), *Geukensia demissa* (GARTHWAITE, 1986; 1989) *Crassostrea virginica* (BUROKER, 1983; ROSE, 1984) *C. rhizophorae* (NIRCHIO *et al*., 1991) and C. *angulata* (MICHININA & REBORDINOS, 1997) provide a large body of evidences that strongly supports the hypothesis that LAP variation would generally be of selective importance in marine bivalves.

In *Crassostrea virginica*, the enzyme is determined by two polymorphic loci: *LAP-I* and *LAP-II* (BUROKER *et al*., 1979 a, b; BUROKER, 1983; ROSE 1984; FOLTZ, 1986 a, b; SARVER *et al*., 1992). However, in *C. virginica* from Venezuela, a study by GUTIÉRREZ e*t al.* (1989) indicates that only *LAP-I* is polymorphic, with two codominant alleles.

This report presents data on allelic frequencies at the LAP-I locus coupled with assays of protein concentrations and LAP activity in the oyster *Crassostrea virginica* sampled at two localities, (Guariquén and Güiria) in the gulf of Paria, in Eastern Venezuela (Fig. 1), which differs greatly in salinity. Oysters from Guariquén (southeast area of the gulf) were previously studied by GUTIÉRREZ *et al.* (1989). The area features a great quantity of canals that irrigate the mangrove formation, a muddy bottom and very turbid waters. At the time of sampling (December

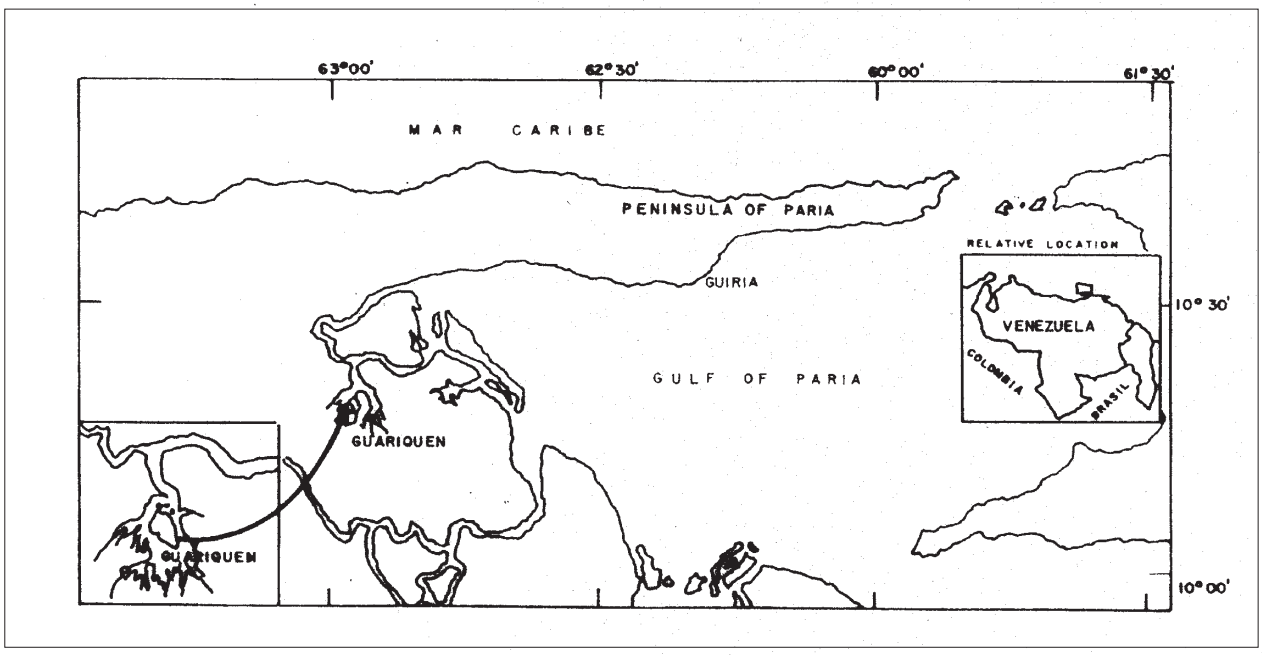

Figure. 1 Map of the gulf of Paria

1995) temperature was 28.95 ºC and salinity was 13.83 ‰ (the annual fluctuation range is 12-22 ‰ [JIMÉNEZ *et al.*, 1992]).The other sample was taken from Güiria, located on the northern area of the gulf. The area is an open bay that features shallow waters, less turbid than those at Guariquén. At the time of sampling (April 1996) temperature was 25.33 ºC and salinity was 24.66‰ (data collected during two years in Punta de Piedras, very close to Güiria, indicate an annual salinity fluctuation range between 17 and 33 ‰ [ALTUVE, 1997]).

MATERIALS AND METHODS

Specimens of *C. virginica*, 10-14 cm in length, were collected in both locations and transported to the laboratory where the oysters were separated from their valves and blotted with absorbent paper. Portions of 100 ± 5 mg of digestive glands and adductor muscles were dissected from each oyster and homogenized separately, in 2 ml of bidistilled water and centrifuged at 12,500 r.p.m. during 20 mins at 4 ºC. The supernatant was used as enzyme source for analyses. Genes that codify for LAP are expressed in both tissues. Electrophoresis on the digestive gland yielded a better visualization of enzyme bands, but muscle was selected for enzyme activity determination to assure that any variation in enzyme activity could only be attributed to osmoregulation requirements and not to digestive processes.

Electrophoresis was carried out in horizontal starch gel (12.5%, SIGMA CHEMICAL CO.). The gel buffer employed was Tris-citrate 0.008 M. The buffer used in the tray was Borate-NaOH 0.3 M, pH 8.60 (NIRCHIO *et al.*, 1991). Samples were electrophoresed at 200 V and 4 ºC (in refrigerator) for 2 hours, until the marker (bromophenol blue) ran 8 cm. Two types of gel were electrophoresed simultaneously under the same conditions of time and voltage, but under different gel buffer pH (7.00 and pH 8.00). Electromorphs were visualized pouring the gel slice in 50 ml Tris-HCl, 0.05 M buffer, adjusted at either pH 7.00 or 8.00 and containing 10 mg of L-leucyl-b-naphthylamide and 20 mg of Fast Garnet. Samples with all the previously identified electromorphs were used as pattern of comparison for each run.

The most frequent allele was identified with the superscript "^{100}" and the others were named according to their relative mobility to it. Allele frequencies were tabulated within the standard error (LEVINGTON & KOEHN, 1976). Conformance to H-W expectation was

assessed with Log-likelihood G-test (SOKAL & ROHLF, 1981). Heterozygote deficiency was measured using the F_{IS} estimator of WEIR & COCKERHAM (1984). The Genes in Populations (version 2.0) software was used for these computations (PERKINS & PAUL, 1995).

Chi-square contingency test was employed to assess for significant differences in allele frequencies at the LAP locus between locations. The proportion of contribution of each allele to the total Chi-square value was also calculated (NIRCHIO *et al.,* 1991).Enzyme activity (EA) of LAP was determined using the methodology described by KOEHN & IMMERMANN (1981). Since specific activity can be confounded by two fairly independent variables: enzyme activity, a function of both the catalytic properties of the enzyme and its concentration, and the total protein baseline with which this activity is compared (KOEHN, 1978), EA was expressed as unit of activity per extract volume (U/ml). Total protein (TP) was determined by the FOLIN method with a standard Bovine Serum Albumin curve. All the determinations were carried out in duplicate.

Analysis of variance (SOKAL & ROHLF, 1981) was employed to establish differences in EA and TP between genotypes and localities, taking into account homozygotes and heterozygotes only. Testing for differences in EA and TP among all genotypes and localities will be examined in another contribution.

RESULTS AND DISCUSSION

Two zones of activity were detected in the gel: *LAP-I* and *LAP-II*. *LAP-I* turned out to be polymorphic, while *LAP-II* was monomorphic. A band of *LAP-I* at pH 7.00 overlaps the only *LAP-II* electromorph. Activity of *LAP-II* products was inhibited at pH 8.00; only the electromorphs of the *LAP-I* locus were visible, so the assignment of genotype was performed without confusions.

Eight electromorphs were recorded at Guariquén (*LAP-I* ^{92}, *LAP-I* ^{94}, *LAP-I* ^{96}, *LAP-I* ^{98}, *LAP-I* ^{100}, *LAP-I* ^{102}, *LAP-I* ^{104}, *LAP-I* ^{106}) and six at Güiria (*LAP-I* ^{94}, *LAP-I* ^{96}, *LAP-I* ^{98}, *LAP-I* ^{100}, *LAP-I* ^{102}, *LAP-I* ^{104}). Both *LAP-I* ^{92} and *LAP-I* ^{106} at Guariquén are rare and were not recorded at Güiria population, hence the polymorphism consists mainly of six alleles.

At Güiria the observed heterozygosity was 0.452 and the expected one was 0.718; the heterozygote deficiency was of 0.371, and 18 genotypes out of possible 21 were observed. At Guariquén, the observed heterozygosity was 0.432 and the expected one was 0.775; the heterozygote deficiency was 0.442 and 17 genotypes were observed. The distribution of the genotype frequencies for the *LAP-I* locus in both locations, was significantly distinct ($P<0.001$) from the H-W expectation (Table 1).

It has been previously indicated that *LAP* in *Crassostrea virginica* from other latitudes is genetically determined by two polymorphic loci, *LAP-I* and *LAP-II*, with the presence of up to 9 alleles for *LAP-I* and 23 alleles for *LAP-II* (BUROKER *et al*., 1979 a, b; BUROKER, 1983; ROSE 1984; FOLTZ *et al*., 1986 a, b; SARVER *et al*., 1992). Our results differ from the aforementioned results due to the fact that LAP-*II* was monomorphic as in a previous study by GUTIÉRREZ *et al.* (1989) on oysters also from Guariquén, but, conversely, the eight different LAP alleles observed by us represent a much higher level of polymorphism than reported in GUTIÉRREZ and coworkers´ study in which they found solely two alleles for *LAP-I*. Although we employed the same buffers utilized by GUTIÉRREZ *et al.* (1989) to resolve the *LAP* loci, differences in the length run of the samples (8 cm) during the electrophoresis explain the discrepancy

Table 1.- Allele frequencies, expected heterozygosity (He), observed heterozygosity (Ho), Allele number in the sample (N), Hardy-Weinberg test (G), heterozygote deficiency index (F_{IS}), independence test between allele frequencies vs locations with all the alleles (c_1^2) and without alleles 96 and 104 (c_2^2) and percentage of contribution to the c^2 value for the *LAP I* locus in the localities of Güiria and Guariquén.

Allele	Guariquen	Guiria	Contingency test	Contribution
94	0.056 ± 0.018	0.067 ± 0.017		1.41%
96	0.198 ± 0.031	0.101 ± 0.021		41.43%
98	0.185 ± 0.031	0.255 ± 0.030		13.75%
100	0.358 ± 0.037	0.438 ± 0.034		9.98%
102	0.093 ± 0.023	0.091 ± 0.020		0.01%
104	0.111 ± 0.025	0.048 ± 0.015		33.41%
N	162	212	$c1^2 = 14.31$;(P< 0.025)	
			$c2^2 = 3.60$; (0.10< P 0.50)	
Fis	0.442	0.371		
Ho	0.432	0.452		
He	0.775	0.718		
G_{test}	66.969**	65.915**		

** = P< 0.001

between the results obtained by those authors, who ran the gels only 3 cm, and those described by us. An insufficient separation in the gel would lead to misscore different electromorphs as products of the same allele.

Contingency test revealed a significant dependence between locations and allele frequencies (Table 1). Analysis of the relative importance of specific alleles on account of the statistical differences between sampling sites, reveals that the *LAP-I*^{*96} and *LAP-I**^{104} alleles, contribute 41.43% and 33.41%, respectively, of the difference between locations. The remaining alleles contribute on the whole 25.16% of the difference. When alleles *LAP-I*^{*96} and *LAP-I**^{104} were excluded, Chi-square value was not significant (Table 1).

The influence of salinity on the determination of the population genetic structure for the locus LAP of *Mytilus edulis* is well known thanks to the studies of KOEHN and co-workers [see KOEHN & HILBISH (1987) for references]. A similar influence has been corroborated in several populations of *Crassostrea virginica* by BUROKER (1983) who pointed out that in oysters living in an environment with high salinity, the most frequent allele, *LAP-II*^{94}, seems to be favored, whereas it is disfavored in an environment with excessive freshwater discharge (while the *LAP-II*^{92} allele seems to be favored in such an environment). Differences in some LAP alleles has also been established between *C. rhizophorae* populations from locations that differ in salinity and temperature; where salinity was greater, the frequency of the *LAP-3*^{98} allele was higher, whereas for the *LAP-3*^{100} allele, the situation was the opposite (NIRCHIO *et al.*, 1991).

If significant differences of allele frequency in a population indicate selective pressures operating on their genetic structure, the *LAP-I*^{*96}, *LAP-I**^{104}alleles would be at a selective advantage in lower salinity (Guariquén). Genetic differences being the result of selection seems to be the best explanation. Nevertheless no corroborating evidence, such as consistent differences in allele frequencies when multiple contrasting salinity environments are sampled with replication, or reciprocal transplant experiments showing greater mortality of particular genotypes when moved to a different salinity regime, is available.

C. virginica features external fertilization and a planktonic stage, which lasts from two to three weeks, providing ample opportunity for larval dispersal (BUROKER, 1983). The current patterns within the Gulf, are strongly influenced by the Guayana Current, which, entering the Gulf from the South, between Trinidad and Venezuela, moves water masses in a general North direction (GADE, 1961; OKUDA, 1974; BONILLA, 1977; BONILLA & LIN, 1979; MASCIANGOLI, 1982). This pattern suggests a unidirectional gene flow from Guariquén to Güiria, the opposite not being likely. This gene flow from Guariquén to Güiria is not enough to prevent the genetic differentiation of the two populations at the *LAP-I** locus.

The F_{IS} estimator revealed a heterozygote deficiency in both populations (Table 1). Significant heterozygote deficiencies for the LAP locus in diverse species of marine mollusks have been pointed out as well (KOEHN *et al.*, 1973; TRACEY *et al.*, 1975; BUROKER *et al.*, 1975; LASSEN & TURANO, 1978; BUROKER *et al.*, 1979a,b; BUROKER, 1983; FOLTZ, 1986 a; GOSLING & MCGRATH, 1990; NIRCHIO *et al.*, 1991

One of the most extended theories to explain the heterozygote deficiencies is the presence of null alleles. These alleles do not manifest their activity on the gel and lead to misinterpret the heterozygote combinations as homozygotes (MICHININA & REBORDINOS, 1997). FOLTZ (1986 a) has obtained experimental evidences in favor of null alleles in *C. virginica*.

It is possible to estimate the frequency of null alleles by means of the relationship v =(1- t)/(1+ t), being "t" the quotient between the observed heterozygote frequency and the expected one (BOYER, 1974). Thus, for *C. virginica* the frequency of a null allele would be of 0.17 at Guariquén and 0.23 at Güiria. Since no individual with zero activity was observed in the gel, it is possible to assume that null alleles, if present, would be lethal in homozyigosis and so, their persistence in the population would depend on the balance between selection and mutation. Although the possibility of null alleles as an explanation of the observed heterozygote deficiencies could not be discarded, we consider that this is not likely since mutation frequencies of 2.89 x 10^2 (Guariquén) and 5.29 x 10^2 (Güiria), too high to be reasonable, would be required.

An alternative explanation to account for the

observed deficiencies is the occurrence of selective forces on the heterozygotes if there are functional differences between genotypes and the heterozygote represents some disadvantage.

EA was different in both locations (416.57±17.98 UEA at Guariquén; 349.11 ± 18.41 UEA at Güiria) ($P<0.025$) but no difference was demonstrated between homozygous-heterozygous genotypes (396.8 ± 20.68 in Homozygotes; 369.49 ± 15.38 in Heterozygotes) ($p>0.05$) (Table 2). There were significant differences in protein concentration ($P<0.05$) between the locations of Guariquén (279.09 ± 5.91 mg/ml) and Güiria (254.83 ± 6.04 mg/ml). Differences in protein concentration were also evident ($P<0.05$) between homozygous (276.31 ± 6.79 mg/ml) and heterozygous (257.62 ± 5.03 μg/μl) genotypes (Table 3).

Oysters from Güiria (the most saline location) showed a reduction of 8.69% in protein concentration as compared to those from Guariquén. This could be attributed to greater energy expenses in terms of aminoacid requirements for the adaptation of *C. virginica* to the most saline environment. BISHOP *et al.* (1981), have indicated that in *Mytilus edulis* during the process of acclimatization to hyperosmotic water, simultaneously to accumulation of Free amino acids (FAA), a reduction between 10% and 15% in the levels of proteins is produced. In the same manner, NIRCHIO & PÉREZ (1997) found that the FAA increment in *Crassostrea rhizophorae* exposed to hypersaline conditions was accompanied by a decrease of approximately 20% in protein concentration.

NIRCHIO & PÉREZ (1997) studied the variations of the EA of LAP, concentration of proteins and concentration of FAA in the muscular tissue of *C. rhizophorae* exposed to salinities of 5 ‰ (minimal tolerable), 38 ‰ (habitual) and 60 ‰ (maximum tolerable). In the groups maintained in salinities of 60 ‰ and 5 ‰, the enzymatic activity of LAP incremented 55.7% and 17.45% respectively in relation with the control group. An increase in the salinity was accompanied by the accumulation of FAA (as Ninhidrin Positive Substances), while the exposure to low salinity determined their decrease. The high mean value of LAP activity in 60 ‰ salinity coincided with a decrease in the protein concentration of about 20% and with an increment in SPN concentration.

Fig. 2 shows that in both locations protein concentration was lower in the heterozygous individuals. This difference could be interpreted as an

Table 2. Analysis of variance for Enzyme Activity in oyster (*C. Virginica*), between locations and Heterozygous-Homozygous genotypes, with degrees of freedom (df), sum of squares (SS), mean squares (MS), and F-value (F).

Sourface of variation	SS	df	MS	F	Sig.level
Between locations	112,752.97	1	112,752.97	6.868	0.0101
Between genotypes	17,655.07	1	17,655.07	1.075	0.3021
Locations x genotypes	3,578.11	1	3,578.11	0.218	0.6465
Error (residual)	1,756,633.00	107	16,417.13		
Total	1,931,017.70	110			

Table 3. Analysis of variance for total protein in oyster (*C. Virginica*), between locations and Heterozygous-Homozygous genotypes, with degrees of freedom (df), sum of squares (SS),

Sourface of variation	SS	df	MS	F	Sig.level
Between locations	14,579.01	1	14,579.01	8.233	0.005
Between genotypes	8,664.22	1	8,664.22	4.893	0.0291
Locations x genotypes	400.92	1	400.92	0.226	0.6402
Error (residual)	189,474.29	107	1,573.60		
Total	216,771.89	110			

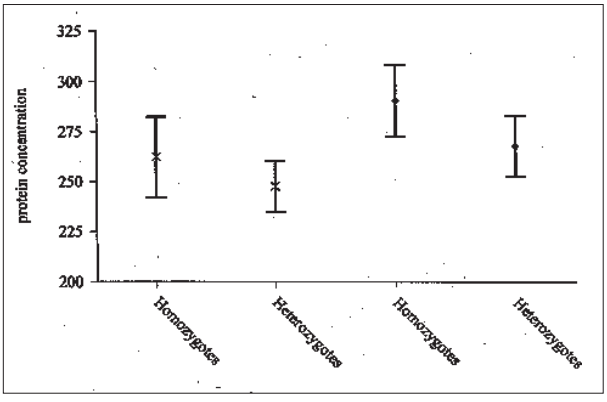

Figure 2. Mean protein concentration (mg/ml) in homozygous-heterozygous oysters for LAP*-I in Guiria (*) and Guariquen ( ). Bar represents 95% confidence limits for the mean.

indicator of greater energy demand per part of the individuals in order to confront the environmental salinity conditions. Thus, it is to be expected that under relatively elevated salinity conditions like in Güiria, an additional demand of free intracellular aminoacid would be required to maintain the cell volume within physiologically appropriate limits. The additional catabolism of proteins would add an energy expense that, if it could not be supported by many heterozygotes would lead to their selective mortality, explaining their frequency reduction, and, as a consequence a greater heterozygote deficiency.

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