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Karyological and electrophoretic differences between *Pomacea flagellata* and *P. patula catemacensis* (Caenogastropoda: Ampullariidae)

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Key words: Apple snails, endangered species, isoelectric focusing, karyotype, Mexico, Native-PAGE electrophoresis.

ABSTRACT: The widespread Mexican apple snail *Pomacea flagellata* (Say 1827) and the strictly endemic "tegogolo" *P. patula catemacensis* (Baker 1922) (restricted to Lake Catemaco), are the only known American Ampullariidae that have haploid complements n=13. *Pomacea patula catemacensis* has suffered a critical reduction in abundance due to immoderate fishing for human consumption. Chromosome slides were obtained from colchicine-injected *Pomacea* snails collected from nine locations along the coastal zone of the Gulf of Mexico, including Lake Catemaco, for use in principal component analysis (PCA). Total proteins in foot homogenates were analyzed through isoelectric focusing (IEF) and native-PAGE electrophoresis on polyacrylamide gels. The chromosome number 2n=26 was confirmed for snails from all locations, with a uniform 9 m + 4 sm formula. However, *P. patula catemacensis* showed significantly larger chromosomes (absolute size) than any population of *P. flagellata*. *Pomacea patula catemacensis* also differed from all populations of *P. flagellata* in a PCA with standardized data, i.e., independently of the absolute size difference between species. Proteins with an acid isoelectric point were dominant in the foot of both species. The electrophoresis analysis showed that *P. flagellata* has 17 protein bands, with an upper bound at IEF=7.6, while *P. patula catemacensis* has only 15 bands, with an upper bound at IEF=7 and a more evenly spaced band pattern. Molecular weights ranged from 40 to approximately 130 kDa in both species. Proteins with high values (>94 kDa) were the most abundant. *Pomacea patula catemacensis* showed a band of 93 kDa, which was absent from all specimens of *P. flagellata*. Samples of *P. flagellata* did not cluster according to any geographical pattern in the statistical analyses, nor did they show any taxonomically useful differences in their electrophoretic patterns that merit sub-specific discrimination.

Introduction

Most species of apple snails (Ampullariidae) have haploid complements of n=14 chromosomes (Choudhury and Pandit, 1997). Among the species living in the Americas, only the two species of *Pomacea* Perry 1810 thriving in Mexico, *Pomacea flagellata* (Say 1827) and *P. patula catemacensis* (Baker 1922), are known to be exceptions, with haploid complements of n=13 chromosomes (Diupotex, 1994; Diupotex-Chong *et al*., 2004).

Pomacea flagellata is a widespread species that is distributed from Mexico and Central America to northern Colombia (Pain, 1964), while *P. patula catemacensis*, also a member of the "*Pomacea flagellata* group", is confined to its type locality, Lake Catemaco, in southeastern state of Veracruz, Mexico (Naranjo-

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García, 2003; Naranjo-García and García-Cuba, 1986). The shared unusual number of chromosomes and the restricted geographical location of *P. patula catemacensis* within the natural realm of *P. flagellata* induced us to look further into the consistency of the differences between these two taxa.

The quantitative differences in chromosome morphology of various *Pomacea* populations along the coastal zone of the Gulf of Mexico were examined, and the possible specific discrimination among species, were analysed using electrophoretic techniques. Results confirmed that the two taxa, *P. flagellata* and *P. patula catemacensis*, which previously had been discriminated only by shell and anatomical features, are also clearly distinguished by karyological and electrophoretic characteristics.

Materials and Methods

Specimens were collected from nine locations along the coastal zone of the Gulf of Mexico: 1) state of Veracruz: Catemaco, Tlacotalpan, Alvarado, and three sampling points along the Misantla River (M1: near the river mouth, M2: mid course, and M3: near the headwaters), 2) state of Tabasco: near El Espino and San Miguel, and 3) state of Campeche: lake El Vapor adjacent to Laguna de Términos. The coastal zone of the Gulf of Mexico is characterized by low land and shallow clean water bodies of 2-5 ppm salinity, a mean water temperature of 26ºC, and a mean pH of 7.5 (Pérez-Rojas and Torres-Orozco Bermeo, 1992). The climate is humid and warm, and the prevailing vegetation is evergreen rainforest.

The specimens were kept alive in the laboratory in filtered and aerated water at room temperature, and were fed *Vallisneria* sp. Some specimens were sacrificed, dissected, and immediately frozen at -30ºC for a further study of protein patterns.

Five specimens from each sampling site were injected, 45 min before sacrifice, with 1 ml of colchicine 0.04% per 50 g of animal weight, for the chromosome study. Samples of fresh gonadic tissue were cut into 3 mm thick pieces, mashed and stained with 2% acid orcein, in agreement with Griffin *et al*. (1997), in order to determine the haploid complement. Gill tissue was also excised, cut into 3 mm thick slices, and put in Petri dishes in a hypotonic solution (0.075M KCl) for 2 h. The sediment was fixed in a 3:1 mixture of methanol and acetic acid after maceration and centrifugation at 1500 rpm for 10 min. The sequence of centrifugation and fixation was repeated for three periods of 5 min and the samples were stored for 24 h at 4ºC in the same fluid. The samples were treated according to the method of Kligerman and Bloom (1977), modified by Coullin and Pellestor (1997). Cell desquamation was performed in 60% glacial acetic acid. The sediment was resuspended and three drops of the liquid for each specimen were transferred to each of five slides at 50ºC. The slides were air-dried during 24 h before staining with Giemsa solution (Sigma) in a pH 6.4 phosphate buffer. The slides were washed in distilled water, clarified with xylene, and mounted with Canada balsam.

Metaphase plates were selected and photographed with a Zeiss phase contrast microscope. The plates were represented in ideograms according to the criteria established by Al-Aish (1969) and Levan *et al*. (1964). The chromosome sets used were those of adequate quality for statistical evaluation, with whole uniformly stained mitotic fields, non-superposed or shrunken chromosomes, and all chromosomes suitable for accurate measurement. Thus, the number of sets differs among populations in the analysis. Statistical comparisons were possible as all materials were processed under the same methodological protocol, by the same person (the senior author) and in a short time period, minimizing the effects of a differential contraction of the chromosomes, staining differences, and variations due to conservation. Homology of the chromosomes according to their order from the longest to the shortest in each ideogram was assumed.

The descriptive parameter calculations have been described by Diupotex-Chong *et al*. (2004). The absolute length of the chromosomes was compared by one-way ANOVAs and Tukey's HSD multicomparison test. Karyotype asymmetry was assessed through the indices defined by Romero-Zarco (1986): A1 estimates the intrachromosomal asymmetry as

$$A1 = 1 - \frac{\sum_{i=1}^{n} \frac{b_i}{B_i}}{n}$$

where n is the number of homologous chromosome pairs, bi is the average length for short arms, and Bi is the average length for long arms in every chromosome pair (i). A2 depicts the interchromosomal asymmetry as the ratio of the standard deviation to the mean length of the chromosomes.

A principal component analysis (PCA) was applied to a matrix of mean relative lengths of the short (bi) and long (Bi) arms. In this analysis, 13 representative metaphases of *P. patula catemacensis* from Lake

Catemaco and 52 representative metaphases of *P. flagellata* from Tlacotalpan (5), Alvarado (5), Misantla-1 (4), Misantla-2 (5), Misantla-3 (4), San Miguel (6), El Espino (13) and El Vapor (10) were included.

Foot flesh samples of specimens from each sampling location were macerated in an Ultraturax homogenizer with a Tris-HCl damper (0.05 M, pH 8) for a minimum of 7 min in short intervals of less than 1 min each at low temperature (4ºC) in order to avoid denaturalization of proteins. The extracts were centrifuged at 30,000 g and 4ºC for 30 min in a Bechman J2-21 centrifuge. The supernatant was dialyzed with a Tris-HCl damper (0.05 M, pH 8) for 9 h in a continuous dialysis system (Arreguín and Taboada, 1968). The protein contents of the extracts were quantified and concentrated to a single dilution in order to be able to make comparisons. Quantification was completed by the method described by Smith *et al*. (1985) which uses bicinchoninic acid (BCA; Protein Assay Reagent) and a spectrophotometer at 562 nm.

Electrophoretic studies were performed on samples from the six locations in the state of Veracruz: Catemaco, Tlacotalpan, Alvarado, and three locations along the Misantla River (M1, M2, and M3).

Isoelectric focusing (IEF) was carried out with a Pharmacia's Phast System equipment on 35 x 43 x 50 mm microplates, for a gradient of pH 3-9 calibrated with a Pharmacia electrophoresis calibration kit, running 1 µg of protein in each lane. The band position was read with a laser LKB 2202 Ultro Scan densitometer.

Native-PAGE electrophoresis on homogeneous 20% polyacrylamide gels was used to analyze the composition of native proteins and estimate their molecular weight on 45 x 43 x 50 mm microplates at 500 volts, 10 mA rising voltage and 15ºC. Reference samples of known molecular weight from 14.4 to 94 kDa (Pharmacia electrophoresis calibration kit) were used, and 1 µg of protein was run in each lane. Fixing, staining and lightening techniques were performed according to Neuhoff *et al*. (1985). The position of the bands was read with a laser LKB 2202 Ultro Scan densitometer.

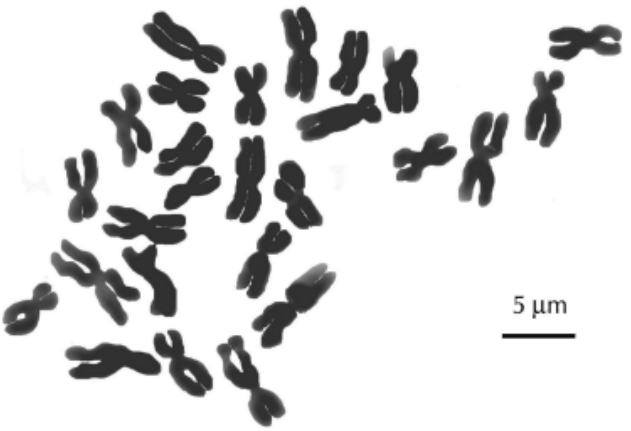

FIGURE 1. A representative metaphase plate of *Pomacea flagellata* (Say, 1823).

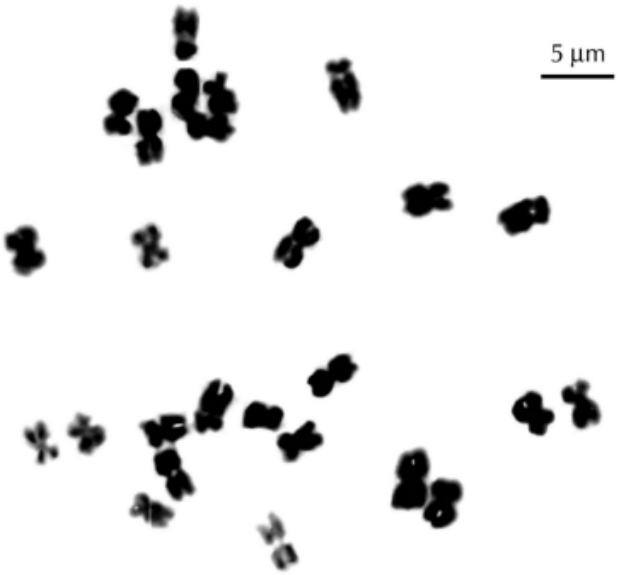

FIGURE 2. A representative metaphase plate of *Pomacea patula catemacensis* (Baker, 1930).

Five collections of shells were deposited as voucher specimens in the Museo de La Plata, Argentina, under the following collection numbers: *Pomacea patula catemacensis* (Baker 1922), Lake Catemaco, Veracruz, MLP 11670; *Pomacea flagellata* (Say 1823), San Miguel, Tabasco, MLP 11929; Tlacotalpan, Veracruz, MLP 11925; Alvarado, Veracruz, MLP 11926; and El Vapor, Campeche, MLP 11924.

Results

Chromosome numbers 2n=26 were confirmed for the apple snails of all locations, and haploid complement n=13 was confirmed for gonadic tissues. Both *Pomacea patula catemacensis* and *P. flagellata* showed a fundamental number of FN=52. Neither negative heteropicnosis nor chromosomal heteromorphism were observed, and there was no visual evidence of sexual chromosomes.

The chromosomes of *P. patula catemacensis* (Fig. 1; absolute mean length = 4.94 µm ± 0.84 SD) were significantly longer than those of *P. flagellata* (Fig. 2; absolute mean length = 4.03 µm ± 0.58 SD): F=7.19,

d.f.=8, p<0.0001 (data were transformed to ln(x) to improve homocedasticity before comparison). The mean chromosome length was not significantly different among populations of *P. flagellata* (Table 1).

The chromosome formula was 9 m + 4 sm for all the studied populations (Table 2). Figure 3 shows the respective ideograms.

Karyotype asymmetry of the two species corresponded to category A2 (Stebbins, 1971) for two reasons: a) the ratio of the largest to the smallest chromosome in the karyotype was <2 (1.76 for *P. patula catemacensis* and from 1.19 (Misantla-1) to 1.53 (Tlacotalpan) for *P. flagellata*), and b) less than 50% of the chromosomes showed a mean arm proportion >2:1.

TABLE 1.

Chromosomal absolute lengths (μm) for nine Mexican populations of *Pomacea* (two species) from the coastal zone of the Gulf of Mexico. All localities refer to *Pomacea flagellata* (Say 1823) except Lake Catemaco which is *Pomacea patula catemacensis* (Baker 1922). Results of a Tukey's HSD multicomparison test are included (critical values: Tukey $_{0.05}$=5.27; Tukey $_{0.01}$=6.67).

	Mean	Standard	Tukey's test values							
	length	deviation	EV	A	EE	M-1	M-2	SM	M-3	T
Lake Catemaco (LC)	4.938	0.839	9.221**	8.571**	8.039**	7.980**	7.389**	7.330**	6.739**	6.561*
Tlacotalpan (T)	4.054	0.521	2.660	2.010	1.478	1.419	0.828	0.768	0.177	0.000
Misantla 3 (M-3)	4.046	0.572	2.483	1.832	1.300	1.241	0.650	0.591	0.000	
San Miguel (SM)	3.962	0.474	1.892	1.241	0.709	0.650	0.059	0.000		
Misantla 2 (M-2)	3.946	0.371	1.832	1.182	0.650	0.591	0.000			
Misantla 1 (M-1)	3.877	0.224	1.241	0.591	0.059	0.000				
El Espino (EE)	3.800	0.277	1.182	0.532	0.000					
Alvarado (A)	3.800	0.498	0.650	0.000						
El Vapor (EV)	3.700	0.381	0.000							

TABLE 2.

Chromosomal measurements for nine Mexican populations of *Pomacea* (two species) from the coastal zone of the Gulf of Mexico. All localities refer to *Pomacea flagellata* (Say 1823) except Lake Catemaco which is *Pomacea patula catemacensis* (Baker 1922). Data on each cell is mean absolute length in μm, mean arm ratio in brackets, and chromosome classification following Levan *et al.* (1964): m, metacentric and sm, submetacentric chromosomes.

Chromosome pair	Catemaco	El Espino	San Miguel	Misantla 1	Misantla 2	Misantla 3	Tlacotalpan	El Vapor	Alvarado
1	6.7 (1.23) m	4.5 (1.05) m	5.1 (1.04) m	4.3 (1.26) m	4.5 (1.05) m	5.4 (1.00) m	5.2 (1.08) m	4.5 (1.05) m	4.8 (1.09) m
2	6.0 (1.40) m	4.1 (1.05) m	4.8 (1.09) m	4.1 (1.41) m	4.5 (1.05) m	5.0 (1.00) m	5.0 (1.00) m	4.3 (1.15) m	4.6 (1.09) m
3	5.6 (1.33) m	4.0 (1.35) m	4.0 (1.11) m	3.9 (1.60) m	4.5 (1.50) m	4.5 (1.05) m	4.2 (1.21) m	3.9 (1.05) m	4.5 (1.14) m
4	5.4 (1.45) m	3.9 (1.29) m	3.9 (1.29) m	3.9 (1.29) m	4.2 (1.33) m	4.1 (1.16) m	4.1 (1.56) m	3.8 (1.38) m	3.9 (1.60) m
5	5.1 (1.32) m	3.8 (1.38) m	3.8 (1.24) m	3.7 (1.18) m	4.0 (1.67) m	3.9 (1.29) m	4.1 (1.28) m	3.7 (1.06) m	3.9 (1.44) m
6	4.8 (1.40) m	3.7 (1.06) m	3.8 (1.06) m	3.7 (1.06) m	3.9 (1.29) m	3.9 (1.05) m	4.0 (1.67) m	3.5 (1.33) m	3.9 (1.29) m
7	4.5 (1.25) m	3.6 (1.40) m	3.7 (1.31) m	3.7 (1.06) m	3.8 (1.38) m	3.7 (1.47) m	4.0 (1.35) m	3.5 (1.19) m	3.9 (1.05) m
8	4.1 (1.16) m	3.5 (1.50) m	3.5 (1.06) m	3.6 (1.12) m	3.8 (1.01) m	3.7 (1.31) m	3.9 (1.60) m	3.4 (1.43) m	3.7 (1.31) m
9	3.8 (1.11) m	3.5 (1.06) m	3.5 (1.06) m	3.6 (1.12) m	3.7 (1.06) m	3.6 (1.57) m	3.8 (1.71) m	3.3 (1.54) m	3.6 (1.40) m
10	5.2 (1.89) sm	3.8 (1.92) sm	4.0 (1.86) sm	4.2 (1.80) sm	3.8 (2.45) sm	3.8 (1.92) sm	3.8 (1.92) sm	3.9 (1.79) sm	3.9 (2.25) sm
11	4.5 (2.00) sm	3.7 (1.85) sm	3.9 (1.79) sm	4.0 (1.86) sm	3.6 (2.00) sm	3.7 (1.85) sm	3.7 (1.85) sm	3.7 (1.85) sm	3.7 (1.85) sm
12	4.5 (1.81) sm	3.7 (1.85) sm	3.9 (1.79) sm	3.9 (2.25) sm	3.6 (2.00) sm	3.7 (1.85) sm	3.5 (2.18) sm	3.3 (2.30) sm	3.7 (1.85) sm
13	4.0 (1.67) sm	3.6 (2.27) sm	3.6 (2.27) sm	3.8 (1.71) sm	3.4 (2.40) sm	3.6 (2.27) sm	3.4 (2.09) sm	3.3 (1.75) sm	3.6 (2.27) sm

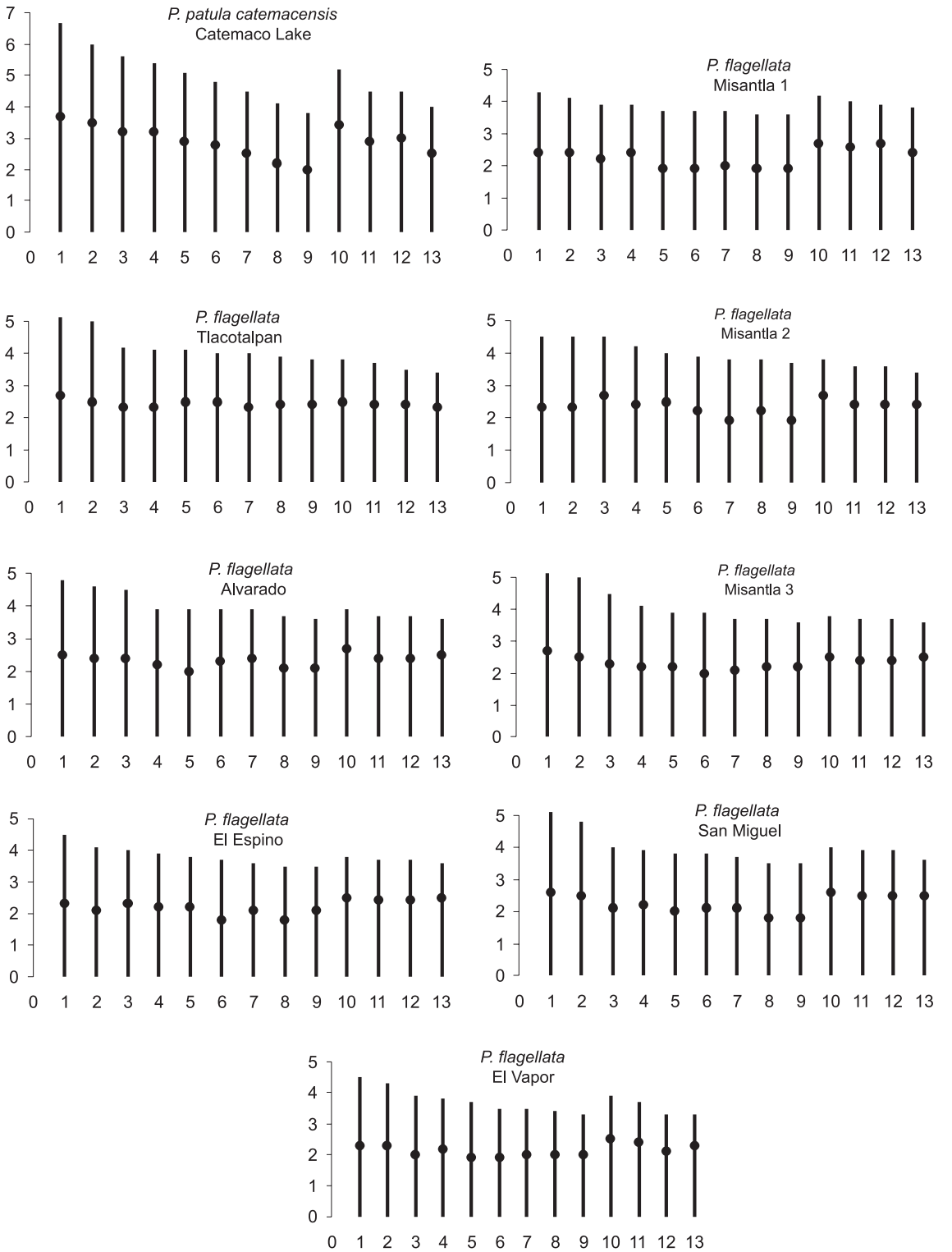

FIGURE 3. Ideograms of the haploid complement of nine *Pomacea* populations (two species) from the coastal zone of the Gulf of Mexico. Ordinate values are absolute lengths of the chromosomes (in μm).

Only 1 of the 13 chromosomes (7.69%) in most populations, except in Tlacotalpan and Alvarado (two chromosomes with mean arm proportion >2:1, i.e. 15.38%), and Misantla-2 (four of these chromosomes, i.e. 30.77%).

Figure 4 shows a scatter plot of the intra- (A1) and inter-chromosomic (A2) asymmetry indices for the nine studied populations. A1 was 0.29 and A2 was 0.17 for *P. patula catemacensis*, the latter value being the highest among the studied populations. A1 ranged from 0.23 to 0.33 and A2 ranged from 0.06 to 0.14 for *P. flagellata.* There was no significant correlation between A1 and A2 (r=0.207, p>0.1) or any clear pattern derived from the geographical origin of the specimens. It should be noted that the variability recorded for the three samples of *P. flagellata* from the Misantla River (M1, M2 and M3: Fig. 4, circle) enfolds the variation of most populations of this species, except for those from San Miguel (SM) and Tlacotalpan (T) that scored the lowest and highest values of A1, respectively.

Before we performed a principal component analysis, we standardized the lengths of the chromosome arms as relative lengths of the total extent of the chromosome complement to avoid the effect of the absolute size difference between species. Even so, *P. patula catemacensis* differed from all populations of *P.*

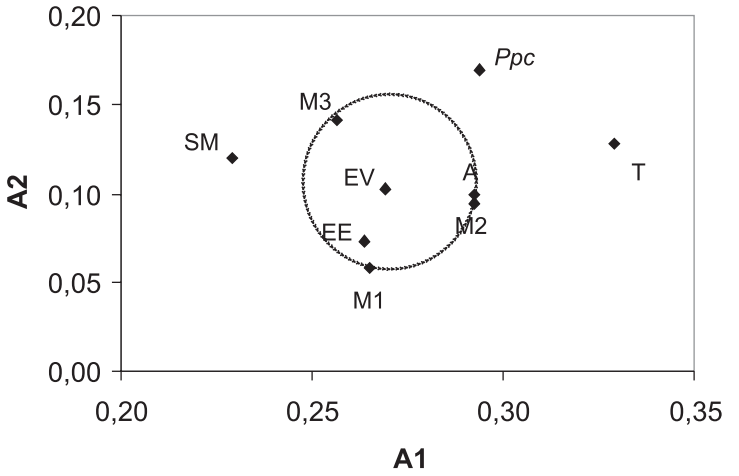

FIGURE 4. Scatter plot of the karyotype asymmetry indices (Romero-Zarco, 1986) for nine *Pomacea* populations (two species) from the coastal zone of the Gulf of Mexico. A1: intrachromosomal asymmetry index; A2: interchromosomal asymmetry index. *Ppc* identifies *Pomacea patula catemacensis* (Baker 1922) from Lake Catemaco. The remaining references are for *Pomacea flagellata* (Say 1827) from: Alvarado (A), El Espino (EE), El Vapor (EV), three localities along the Misantla River (M1: river mouth, M2: mid course, M3: headwaters), San Miguel (SM) and Tlacotalpan (T).

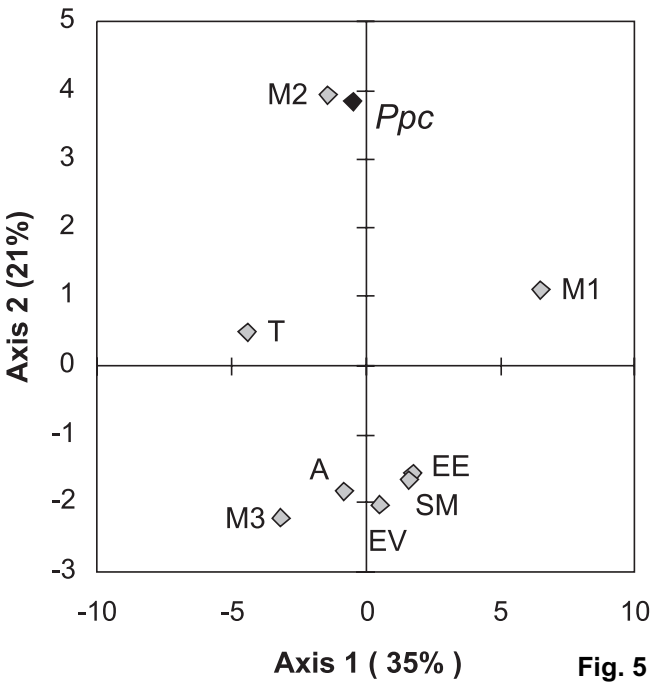

Fig. 5

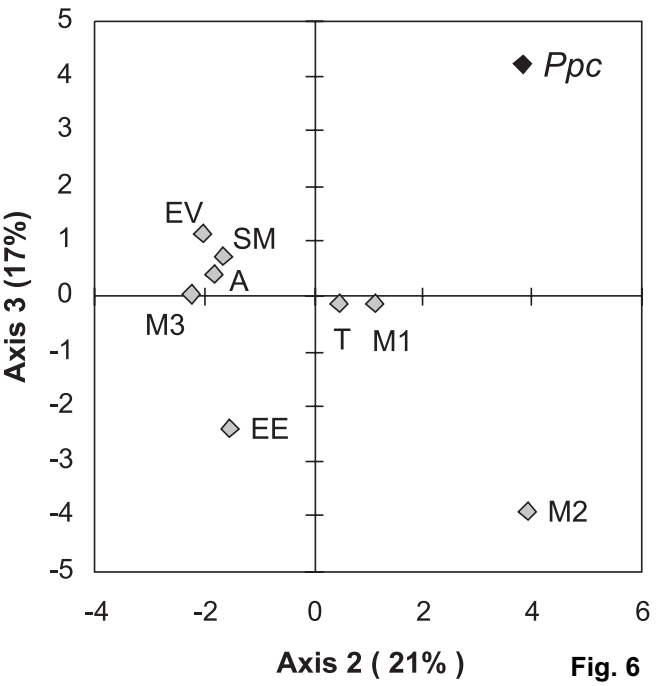

Fig. 6

FIGURES 5-6. Plots of the Principal Component Analysis on the standardized lengths of the chromosome arms for nine *Pomacea* populations from the coastal zone of the Gulf of Mexico. Black square: *Pomacea patula catemacensis* (Baker 1922), grey squares: *Pomacea flagellata* (Say 1827). Other references as in figure 2.

flagellata. Most populations of *P. flagellata* scored low on axis 2; only the snails from the middle course of the Misantla River (M2) are located near *P. patula catemacensis* at high values on axis 2. This is positively correlated with the length of the long arms in chromosomes 3 and 4 (B_3, B_4) and the length of the short arm in chromosome 7 (b_7), and negatively correlated with B_5, B_7 and B_{13} (Fig. 5); on axis 3, *P. patula catemacensis* and *P. flagellata* from Misantla-2 were the populations that received the highest and the lowest scores respectively (Fig. 5). Axis 3 is positively correlated to B_1 and B_2 and negatively correlated with B_{13}. The three samples from the Misantla River were located far from each other on these charts, which represents a remarkably high variability for co-specific populations from a single basin, and exceeds the observed variation among populations from different localities.

Out of the five tested protein concentrations (1, 1.5, 2, 3 and 5 mg/ml) with the isoelectric focusing tests, the best-defined bands were obtained with the 3 mg/ml concentration. Proteins with acid isoelectric points were dominant in the two electrophoretic patterns, and the most intense bands were located around the 5.8, 5, 4 and 3.5 isoelectric points. One of the bands below the lowest IEF marker (3.5) was also quite intense (Fig. 7). The main differences between the two specific patterns were that *P. flagellata* showed 17 bands and the highest upper bound at isoelectric point pI 7.6, which was relatively far from the next five bands and concentrated between isoelectric points 6.6 and 6.15. The IEF on *P. patula catemacensis* showed only 15 bands, with the upper bound at isoelectric point pI 7 and a more evenly spaced band pattern.

The molecular weights of the proteins on the polyacrylamide gels are shown in figure 8. Most of the identified proteins had a molecular weight of 40 to >94 kDa (probably up to 130 kDa), and the most abundant proteins were those with the highest values. *Pomacea flagellata* showed 12 bands, while *P. patula catemacensis* showed 13 bands. Both patterns were very similar, except for a band of 93 kDa in the *P. patula catemacensis* specimens from Lake Catemaco, which was absent in all samples of *P. flagellata.*

Discussion

Apple snails have variable morphologies (Pain, 1964; Rangel-Ruiz, 1988; Estebenet *et al*., 2006), genetics and molecular characteristics (Keawjam and Upatham, 1990; Thaewnon-Ngiw *et al*., 2003).

The most common ampullariid snail in Mexico is *Pomacea flagellata,* which has countless local forms that have received many specific names. Pain (1964: 224) said that this species is "variable to an extent which fills the investigator with dismay".

Pomacea flagellata has a distribution range that spans from the state of Veracruz and throughout the

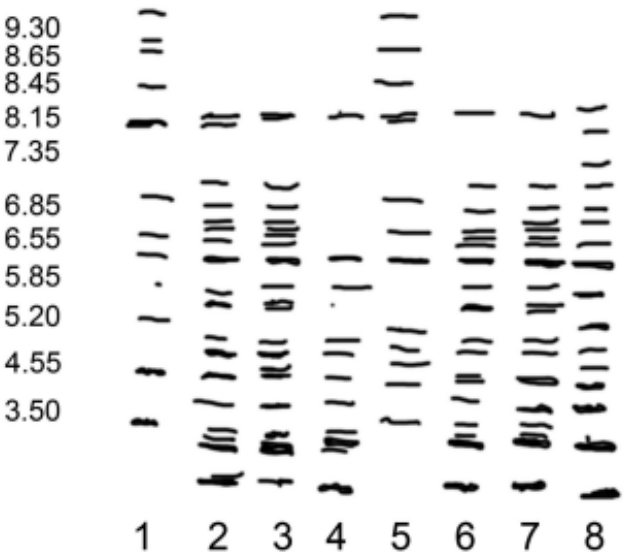

FIGURE 7. Isoelectric focusing (IEF) on a gradient of pH 3-9 for some *Pomacea* populations from the coastal zone of the Gulf of Mexico. All localities refer to *Pomacea flagellata* (Say 1823) except Lake Catemaco which is *Pomacea patula catemacensis* (Baker 1922). The order of lanes is as follows: (1) protein standards, (2) Misantla-3, (3) Misantla-1, (4) Misantla-2, (5) protein standards, (6) Alvarado, (7) Tlacotalpan, and lane 8 is Lake Catemaco.

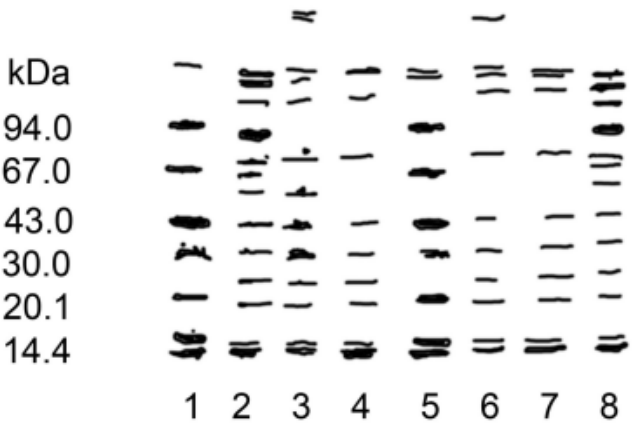

FIGURE 8. A representative microplate with results of native PAGE electrophoresis on homogeneous 20% polyacrylamide gels for some *Pomacea* populations from the coastal zone of the Gulf of Mexico. All localities refer to *Pomacea flagellata* (Say 1823) except Lake Catemaco which is *Pomacea patula catemacensis* (Baker 1922). The order of lanes is as follows: (1) molecular weight standards, (2) Misantla-3, (3) Misantla-1, (4) Misantla-2, (5) protein standards, (6) Alvarado, (7) Tlacotalpan, and (8) Lake Catemaco.

Mexican Gulf slope and Yucatan Peninsula, south to Panama and northern Colombia (Naranjo-García, 2003). In contrast, *P. patula catemacensis* is restricted to a single lake. Given that apple snail species commonly have wide distribution ranges, the strict endemism of *P. patula catemacensis* in Lake Catemaco is a remarkable exception.

Lake Catemaco is a relatively isolated 7250 hectare water body that lies in a volcanic crater at an altitude of 333 m, in the volcanic Los Tuxlas area, within the Papaloapan River basin. It is the most productive natural lake in Mexico, with approximately 1800 tons of fishing products harvested per year (Pérez-Rojas and Torres-Orozco Bermeo, 1992). *Pomacea patula catemacensis* is an important source of food for human consumption (26.6% of the local fishery production). However, overexploitation has resulted in a dramatic reduction in the abundance of this apple snail, from over 5000 tons between 1980 and 1989 to only 24 tons in 2001 (Carreón-Palau *et al*., 2003). At present, and according to the criteria of IUCN (2001), the endemic *P. patula catemacensis* may be considered "endangered" or "critically endangered", and measures to control fishing activities in the lake are the subject of legislative concern (Ochoa-Muñoz, 2006).

Pomacea patula catemacensis was originally described as *Ampullaria patula catemacensis* by Baker (1922), based only on shell characteristics. Its identity as a well defined taxon was reaffirmed recently by Carreón-Palau *et al*. (2003) on the basis of genital anatomy. The results presented in this study provide additional evidence that support these previous findings. Indeed, despite their morphological heterogeneity, all populations of *P. flagellata* have similar karyotypes and common patterns of protein bands (IEF and molecular weights) that differ from those of *Pomacea patula catemacensis*. The chromosomes of *P. patula catemacensis* were found to be significantly longer, and a multivariate analysis with standardized data clearly separated the two species in this study.

Landa and Nader (1991) reported for *Pomacea flagellata* a chromosome number of 2n=16, while Diupotex (1994) found a modal number of 2n=26 for the same species. This latter report is consistent with our findings for eight populations collected along the coastal zone of the Gulf of Mexico. Data published by Landa and Nader (1991) have not been confirmed.

After placing more than 30 species names within the synonymy of *Pomacea flagellata*, Pain (1964) recognized only four morphs as probable subspecies. However, these morphs do not seem to represent actual examples of independent geographic variation, and Rangel-Ruiz (1988) stated the inconvenience of retaining such trinomials. Our samples of *P. flagellata* did not cluster according to any geographical pattern in the statistical analyses. The chromosomes were found to vary among the three samples collected from the Misantla River (M1, M2, M3), and the karyotype and electrophoretic results did not lead to a subspecific discrimination.

Diupotex-Chong *et al*. (2004) stated that *P. patula catemacensis* has a more asymmetric karyotype than *P. flagellata.* The results obtained here verify this previous finding for the inter-chromosomal asymmetry index (A2), but not for the intra-chromosomal asymmetry index (A1), given that the *P. flagellata* samples from Alvarado and Misantla-2 are almost asymmetric and the snails from Tlacotalpan are much more asymmetric than *P. patula catemacensis.*

The presence of sexual chromosomes in the Gastropoda is rare. Only about 7% of the 230 species studied of Caenogastropoda were reported to have a chromosomal system of sex determination (Patterson, 1969; Thiriot-Quiévreux, 2003). Most previous authors found no evidence of sexual heterosomes in species of Ampullariidae (Choudhury and Pandit, 1997; Diupotex, 1994; Diupotex-Chong *et al*., 2004; Kawano *et al*., 1990; Mercado-Laczkó and Lopretto, 1998; Yaseen *et al*., 1991). Only von Brand *et al*. (1990) described the existence of male XY heterogamety in *Pomacea canaliculata* from Japan. Our extensive sampling along the Mexican coast does not support the hypothesis that sex is chromosomally determined in *P. flagellata* and *P. patula catemacensis.* Yusa and Suzuki (2003) suggested that a polyfactorial genetic determination of sex for *P. canaliculata* was the most likely mechanism to explain their findings. Also, more recent studies suggest the involvement of either a small number of sex-determining genes or a more complicated system such as sex-ratio or sex-determining polygenes that act non-additively (Yusa, 2005).

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