Identification of *Biomphalaria havanensis* and *Biomphalaria obstructa* Populations from Cuba Using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism of the Ribosomal RNA Intergenic Spacer

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In Cuba, several Biomphalaria *species have been reported such as* B. orbignyi, B. schrammi, B. helophila, B. havanensis *and* B. peregrina; *only the latter three are considered as potential hosts of* Schistosoma mansoni. *The specific identification of* Biomphalaria *species is based on anatomical and morphological characters of genital organs and shells. The correct identification of these snails is complicated by the high variation in these characters, similarity among species and in some cases by the small size of the snails. In this paper, we reported the classical morphological identification, the use of PCR and RFLP analysis of the internal transcribed spacer region of the ribosomal RNA genes for molecular identification of seven snail populations from different localities in Cuba. Using morphological and molecular analysis, we showed that among the studied Cuban* Biomphalaria *populations only* B. havanensis *and* B. obstructa *species were found.*

Key words: *Biomphalaria havanensis* - *Biomphalaria obstructa* - polymerase chain reaction - internal transcribed spacer - ribosomal DNA - snails - Cuba

Schistosomiasis mansoni is endemic in several countries of the Americas and Africa (WHO 1993). Although the disease has not been reported in Cuba yet, one can mention some *Biomphalaria* species such as: *B. orbignyi* Paraense, 1975b, *B. schrammi* (Cross, 1864), *B. helophila* (Orbigny, 1835), *B. havanensis* (Pfeiffer, 1839), *B. peregrina* (Orbigny, 1835). The latter three species are considered as potential hosts of *Schistosoma mansoni* (Richards 1963, Paraense & Corrêa 1973, Michelson 1976, Yong et al. 1984, 1989, Yong & Perera 1989, Paraense 1996). *B. obstructa* was mentioned in Cuba by PAHO (1968), Malek (1985) and Yong et al. (1989), however, in such papers it is not possible to identify the authors who found, for the first time, *B. obstructa* in this country. This species was described by Morelet (1849) based on specimens from Isla del Carmen, State of Campeche, Mexico.

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The correct identification of *Biomphalaria* snails is difficult because of the high variation in the anatomical and morphological characters and similarity among species (Paraense 1975a, Yong et al. 1991, 1995). The taxonomy of Cuban *Biomphalaria* species remains confused and the correct identification of *B. havanensis* is always in discussion. This species has been considered as very similar to *B. obstructa, B. orbignyi* (Yong et al. 1991, 1995) and *Biomphalaria* sp., recently mentioned in Cuba by Durand et al. (1998). Paraense and Deslandes (1957) reported that *B. havanensis* (identified as *Taphius maya*) is indistinguishable from *B. peregrina* by shell features, however they can be differentiated by the radular characteristic (Paraense 1966). *B. peregrina* and *B. orbignyi* are morphologically recognized as similar species also (Paraense 1975b, Yong & Perera 1989, Yong et al. 1991).

Yong et al. (1991) and Durand et al. (1998) used multilocus enzyme electrophoresis to identify Cuban snails such as *B. havanensis*, *B. orbignyi*, *B. helophila* and *Biomphalaria* sp.

The polymerase chain reaction and restriction fragment length polymorphism ribosomal DNA internal trancribed spacer (rDNA-ITS) have been used in studies on identification of *Biomphalaria*

species from South America (Vidigal et al. 1998, 2000, Caldeira et al. 1998, 2000, Spatz et al. 1999, 2000).

In the current study, we analyzed snail populations from seven different localities, in Cuba, using morphological and PCR-RFLP techniques and we also propose the use of this molecular method as an auxiliary tool to identify the Cuban *Biomphalaria* snails.

MATERIALS AND METHODS

Snails - Seven snails of Cuban populations from Canasi, Zanja Ferrer, Arroyo Arenas, Hanabanilla, Santa Rita, Guatao and Hanabanilla Vaqueria, maintained for six months, approximately, in the Department of Malacology of Pedro Kouri Institute, Havana, Cuba, were used.

Morphological identification and DNA extraction of the snails - Ten specimens of each population were killed and fixed (Paraense 1976). Before fixation, the foot of each specimen was removed for subsequent DNA extraction (Vidigal et al. 2000).

Following fixation, the specimens were identified according to Paraense and Deslandes (1958), and Paraense (1975a, 1990, 1996).

The PCR amplification and RFLP profile analysis - The entire ITS region (ITS1 + 5.8S + ITS2) of rDNA was amplified using the same primers and conditions used by Vidigal et al. (1998). The most effective enzymes used in our previous studies with *Biomphalaria* snails (Vidigal et al. 1998, 2000, Caldeira et al. 1998, 2000, Spatz et al. 1999, 2000) were used in this study: *Alu*I, (New England Biolabs, USA) *Mnl*I, *Dde*I and *Hae*III, (Promega Co, USA). Digestion and RFLP analysis were performed as described by Vidigal et al. (1998).

RESULTS

Morphological identification - In six localities (Canasi, Arroyo Arenas, Hanabanilla, Santa Rita, Guatao, and Hanabanilla Vaqueria), the specimens were morphologically identified as *B. havanensis*. The specimens from Zanja Ferrer were identified as *B. obstructa*. In addition, snails previously characterized as *B. obstructa* from Isla del Carmen, Mexico (type locality) and two localities from Dominican Republic (Villa Vasquez and Santo Domingo), maintained in the Departments of Malacology of the Oswaldo Cruz Institute, Rio de Janeiro, and Pedro Kouri Institute, Havana, Cuba, respectively, were included in this study for morphological and molecular comparisons with snails from Cuba.

Comparison of the Biomphalaria rDNA-ITS RFLP profiles - PCR amplification of the ITS region of the snails resulted in a product of approximately 1.3k bp (data not shown). The *Dde*I (Fig. 1) produced a simple profile of three or five fragments for each specimen in which two fragments are present only in snails morphologically identified as *B. obstructa* (lanes 1, 6 to 9, and 30 to 36). Similar profiles obtained among the specimens from Zanja Ferrer, Cuba (lanes 6 to 9), Isla del

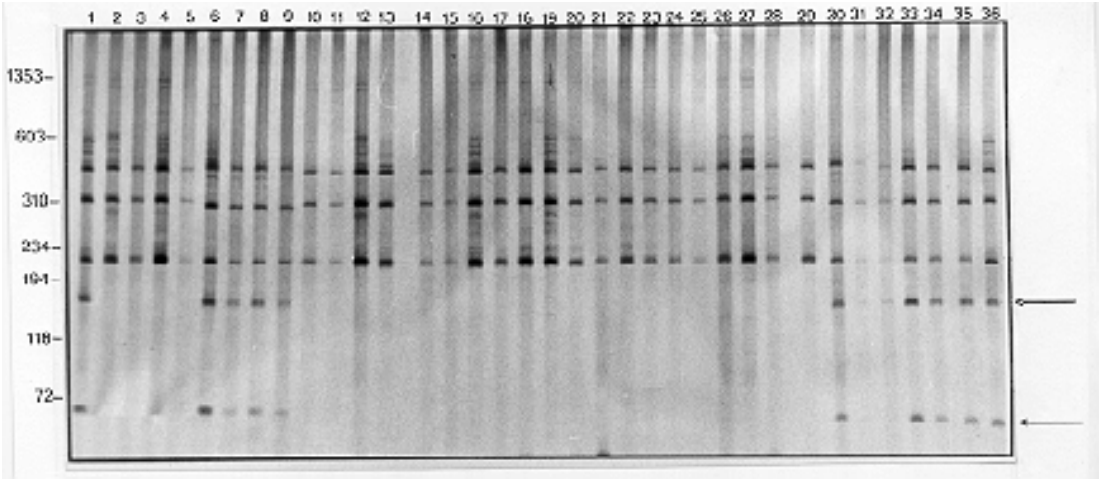

Fig.1: silver stained polyacrylamide gel 6% showing the PCR and RFLF profiles obtained following the digestion of the rDNA internal transcribed spacer region with *Dde*I. Lane 1: *Biomphalaria obstructa* from Isla del Carmen, México; lanes 2-5: *B. havanensis* from, Canasi, Cuba; lanes 6-9: *B. obstructa* from Zanja Ferrer, Cuba; lanes 10-13: *B. havanensis* from Arroyo Arenas, Cuba; lanes 14-17: *B. havanensis* from Hanabanilla, Cuba; lanes 18-21: *B. havanensis* from Santa Rita, Cuba; lanes 22-25: *B. havanensis* from Guatao, Cuba; lanes 26-29: *B. havanensis* from Hanabanilla Vaqueria, Cuba; lanes 30-32: *B. obstructa* from Villa Varquez, Cuba; lanes 33-36: *B. obstructa* from Dominican Republic. Molecular size markers are shown on the left of each gel. The arrows indicate species specific fragments for *B. obstructa*.

Carmen, Mexico (lane 1) and Dominican Republic (lanes 30 to 36) suggest that snails from Zanja Ferrer belong to *B. obstructa* species. The other Cuban snail populations from Canasi, Arroyo Arenas, Hanabanilla, Santa Rita, Guatao, Hanabanilla Vaqueria, identified by morphological methods as *B. havanensis*, were molecularly characterized by the presence of three fragments (Fig. 1, lanes 2 to 5, and 10 to 29).

To confirm the molecular identification obtained with *Dde*I enzyme, three other enzymes were tested using two specimens of each population. Figs 2A, B, C show, the rDNA-ITS RFLP profiles obtained with *Alu*I, *Mnl*I and *Hae*III enzymes, respectively. The *Alu*I profiles were very similar for *B. havanensis* and *B. obstructa*, both with only one distinct fragment characterizing each species (Fig. 2A, lanes 1 to 5, *B. obstructa*; lanes 6 to 11, *B. havanensis*). Two polymorphic profiles were obtained using the *Mnl*I enzyme. However, both global profiles were very clear allowing to separate these two species (Fig. 2B). *B. havanensis* was characterized by the presence of two fragments (lanes 6 to 11) that permitted a clear distinction of such species from *B. obstructa* (lanes 1 to 5). The *Hae*III showed RFLP profiles (Fig. 2C) which included two invariable profiles with species-specific products for the two species. *B. obstructa* snails (lanes 1 to 5) showed distinct profiles from *B. havanensis* which exhibited one clear profile with five fragments (6 to 11). Comparing the products obtained with all enzymes (*Dde*I, *Alu*I, *Mnl*I and *Hae* III) for snails from Zanja Ferrer (Cuba) with the ones obtained for *B. obstructa*, from Isla del Carmen and Dominican Republic, it was possible to conclude that these populations belong to the same species.

DISCUSSION

The molecular techniques based on PCR-RFLP analysis of the rDNA ITS region have been extensively used for many analyses of schistosomiasis intermediate host of the genera *Bulinus*, *Oncomelania* and *Biomphalaria* (Hope & McManus 1994, Vidigal et al. 1998, 2000, Caldeira et al. 1998, 2000, Jones et al. 1999, Spatz et al. 1999, 2000). In Cuba, malacological surveys have been considered very important to better know the geographical distribution and also to help the health institution programs, in order to prevent the introduction of schistosomiasis in the country. Yong et al. (1997) discussed about the type locality of *B. havanensis* suggesting that specimens described by Paraense and Deslandes (1958), as *B. havanensis* (collected in the Country Club of Havana), may in fact belong to another species. The first authors suggest that the Country Club of Havana species does not correspond to the type locality of *B. havanensis*. In previous studies, *B. havanensis* has been mentioned as very similar to *B. obstructa*, *B. orbignyi* (Yong & Perera 1989, Yong et al. 1995) and *B. peregrina* (Yong et al. 1989) based on their shell

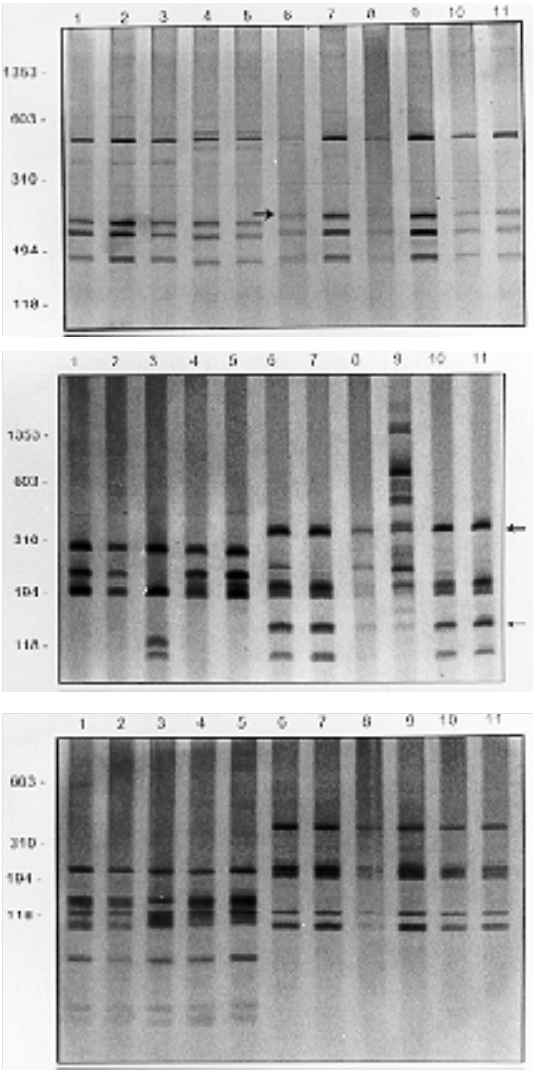

Fig. 2: silver stained polyacrylamide gels 6% or 8% showing the PCR and RFLP profiles obtained following the digestion of the rDNA internal transcribed spacer region with *Alu*I (A), *Mnl*I (B) and *Hae*III (C). In each gel the snails specimens are: lanes 1-2: *Biomphalaria obstructa* from Isla del Carmen, México; lane 3: *B. obstructa* from Santo Domingo, Dominican Republic; lane 4: *B. obstructa* from Zanja Ferrer, Cuba; lane 5: *B. obstructa* from Villa Vasquez, Dominican Republic; lane 6: *B. havanensis* from Canasi, Cuba; lane 7: *B. havanensis* from Arroyo Arenas, Cuba; lane 8: *B. havanensis* from Hanabanilla, Cuba; lane 9: *B. havanensis* from Santa Rita, Cuba; lane 10: *B. havanensis* from Guatao, Cuba; lane 11: *B. havanensis* from Hanabanilla Vaqueria, Cuba. Molecular size markers are shown on the left of each gel. The arrows in A and B indicate species specific fragments for *B. havanensis.*

characteristics. The identification of these species is very important because *B. obstructa* and *B. orbignyi* have been considered refractory to infection by *S. mansoni* (Richards 1963, Paraense 1975b, Sullivan & Hu 1996), whereas *B. havanensis* is considered as a potential intermediate host (Richard 1963, Michelson 1976). The latter species is considered widely spread in Cuba (Yong et. al. 1995) and has also been found in Antillean region, Mexico, Central America and probably in some localities of northern South America (Malek 1985). *B. orbignyi* had been previously found only in Argentina (Paraense 1975b) and, recently, in Cuba (Yong & Perera 1989). This species is morphologically very similar to *B. peregrina*, which is one of the most widespread planorbid species in the Neotropical region and a potential intermediate host of *S. mansoni* (Paraense 1966, 1975a, Paraense & Corrêa 1973). Recently, Spatz et al. (2000), using PCR-RFLP, showed that it is possible to separate *B. havanensis* from *B. peregrina* and *B. orbignyi*.

B. obstructa occurs in regions of the gulf coastal of the United States, Caribbean and Mexico (Malek 1985). However, since 1995, Yong et al. considered *B. obstructa* as "*nomina spurea*" [sic]. According to Yong (1998) the topotypic *B. obstructa*, reviewed by Paraense (1990), would correspond perfectly to the topotypic *B. havanensis* and would be a synonym of the latter.

In spite of the important questions on *B. obstructa* (Yong 1998) and the type locality of *B. havanensis* (Yong et al. 1997), in the present work, the Cuban snail populations were identified in accordance with the classical morphological systematics adopted by Paraense, which has produced the most complete description of the majority of the Neotropical *Biomphalaria* species (Paraense 1975a,b, 1990, 1996, Paraense et al. 1992).

As a result of our current analysis, two clear profiles were obtained with *Dde*I enzyme, identifying two species as shown by morphological criteria. The profiles among specimens from different areas were highly reproducible.

The products obtained with *Alu*I, *Mnl*I and *Hae*III confirmed morphological data and reinforced the molecular results provided by *Dde*I enzyme. The observation of two profiles for all analyzed snails demonstrated that among those populations only *B. havanensis* (Canasi, Arroyo Arenas, Hanabanilla, Santa Rita, Guatao, and Hanabanilla Vaqueria) and *B. obstructa* (Zanja Ferrer) species were found. Therefore, it is important to mention that (a) Zanja Ferrer and Calle Pila have recently been reported as type localities of *B. havanensis* (Yong et al. 1997), (b) Yong (1998) and Durand et al. (1998) reported the presence of *Biomphalaria* sp. in Canasi, Arroyo Arenas, Santa Rita, Guatao, and Hanabanilla, and (c) *B. peregrina* has also been reported in Hanabanilla (Yong et al. 1989).

We showed that PCR-RFLP of the *Biomphalaria* rDNA ITS region, using *Dde*I, *Hae*III, *Mnl*I or *Alu*I enzymes allowed the differentiation of two Cuban *Biomphalaria* species. Such molecular data were supported by classical systematics of Paraense and Deslandes (1958) and Paraense (1990).

As already pointed out, PCR-RFLP methodology has the advantage of producing simple profiles for each species and also shows to be helpful in Cuban snail systematics.

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