Morphological and Polymerase Chain Reaction-Restriction Fragment Lenght Polymorphism Characterization of *Biomphalaria kuhniana* and *Biomphalaria amazonica* from Colombia

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In Colombia, five Biomphalaria *planorbid species are known:* B. kuhniana, B. straminea, B. peregrina, B. canonica *and* B. oligoza (*var.* B. philippiana). *Among them,* B. straminea *is intermediate host of* Schistosoma mansoni *and* B. peregrina *has been found to be experimentally susceptible to this parasite.* B. straminea *is commonly confused with* B. kuhniana *and they have been clustered together with* B. intermedia *in the complex named* B. straminea. *The difficulties involved in the specific identification, based on morphological data, have motivated the use of new techniques as auxiliary tools in cases of inconclusive morphological identification of such planorbid. In the present study, five* Biomphalaria *populations from the Colombian Amazon region and from Interandian Valleys were morphologically identified and characterized by polymerase chain reaction-restriction fragment lenght polymorphism directed at the internal transcribed spacer region of the rRNA gene, followed by digestion of the generated fragment with restriction enzymes* (Dde*I,* Alu*I,* Rsa*I,* Mva*I and* Hae*III). Known profiles of the Brazilian species* B. straminea, B. peregrina, B. kuhniana, B. intermedia *and* B. amazonica, *besides* B. kuhniana *from Colombia, were used for comparison. The five populations under study were morphologically and molecularly identified as* B. kuhniana *and* B. amazonica.

Key words: *Biomphalaria kuhniana* - *Biomphalaria amazonica* - snails - polymerase chain reaction - restriction fragment length polymorphism - Colombia

The Colombian snail fauna of the genus *Biomphalaria* is represented thus far by five species: *B. kuhniana* (DeJong et al. 2001), *B. peregrina* (Malek 1985), *B. straminea* (Barbosa 1968), *B. canonica* and *B. oligoza* var. *B. philippiana* (Uribe 1950). Among them, *B. straminea* is intermediate host of *Schistosoma mansoni*, being one of the main species responsible for schistosomiasis transmission in many localities of the Northeastern region of Brazil. This species was described by Dunker (1848) and according with Paraense (1963) "The type locality was vaguely mentioned as South America, where several species answering to that description are known to occur" and "Martens (1873) restricted the type locality of *P. stramineus* to Venezuela (Lagunilla and Caracas), and also referred it to the State of Ceará, Brazil". Being experimentally susceptible to *S. mansoni*, the species *B. peregrina* is regarded as a potential host of the trematode (Paraense & Corrêa 1973). Concerning the epidemiological importance of *B. straminea* for schistosomiasis, this species is commonly confused with *B. kuhniana* and *B. intermedia*. For this reason, these three species were clustered into a group named *B. straminea* complex (Paraense 1988). The difficulties involved in specific identification based on morphological characters have motivated the use of more modern techniques such as molecular biology.

The type locality of *B. kuhniana* (Clessin, 1883) is Paramaribo, Surinam but it is also found in Cayenne, French Guyana (Floch & Fauran 1954a,b, Floch & Lajudie 1945), Venezuela (Baker 1930), Tucuruí, Pará, Brazil (Paraense 1988), and Panama (Paraense - pers. commun. 1998). Regarding susceptibility, Floch and Fauran (1954b) showed that *B. kuhniana* is resistant to *S. mansoni* infection.

The type locality of *B. amazonica* Paraense, 1966, is Manaus, Amazonas, Brazil. To date, its distribution is restricted to the Brazilian states of Acre, Amazonas, Rondônia (Paraense 1983), Mato Grosso (Paraense 1983), Mato Grosso do Sul (Dorval & Silva 1990) and Bolivia (Pontier et al. 2002). Experiments on susceptibility showed that such species is a potential host of *S. mansoni* (Corrêa & Paraense 1971, Paraense & Corrêa 1985). Vidigal et al. (2000a) characterized this snail by polymerase chain reaction-restriction fragment lenght polymorphism (PCR-RFLP) with the enzyme *Dde*I and detected three species-specific profiles in specimens from the same or different localities.

The PCR-RFLP technique has been successfully used in studies on *Biomphalaria* (Vidigal et al. 1998, 2000a, Caldeira et al. 1998, 2000, Spatz et al. 1999), *Oncomelania* (Hope & McManus 1994) and *Bulinus* species (Stothard et al. 1996, Stothard & Rollinson 1997). This methodology was also used for molecular identification of Mammalia,

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Coleoptera and Platyhelminthes (Clark et al. 2001, Dynes et al. 2001, Verkaar et al. 2002) and for the distinction of cryptic species within the *Trypanosoma brucei* group (Agbo et al. 2001). The technique is based on the amplification of a particular genomic DNA region, followed by digestion of the generated fragment with restriction enzymes. Considering the simplicity and easy execution of the technique, in the current study we used PCR-RFLP and morphological identification in order to characterize *Biomphalaria* snails from the Colombian Amazon region and the Valles Interandinos.

MATERIALS AND METHODS

Snail populations - This study was carried out using snail populations from five localities in Colombia (Fig. 1), together with specimens identified as *B. peregrina, B. straminea, B. intermedia, B. amazonica* and *B. kuhniana* (Table) used for comparison. All the snails obtained from the field were examined for the presence of *S. mansoni* cercariae.

Morphological identification - Ten specimens of each population from Colombia were killed, their feet removed and conserved in ethanol. The remaining material was fixed and dissected for morphology of the shell and reproductive organs as described by Deslandes (1951) and Paraense (1975, 1976, 1988).

DNA extraction - Total DNA was extracted from the feet of the snails by phenol-chloroform extraction and ethanol precipitation (Vidigal et al. 1994).

PCR-RFLP analysis - The entire ITS region (which includes the 5.8S rDNA gene together with the flanking ITS1 and ITS2 spacers) was amplified using the primers ETTS2 (5-TAACAAGGTTTCCGTAGGTGAA-3) and ETTS1 (5-TGCTTAAGTTCAGCGGGT-3) (Kane & Rollinson 1994). PCR amplification conditions were the same as used by Vidigal et al. (1998). Several enzymes employed in our previous studies with *Biomphalaria* snails (Vidigal et al. 1998, 2000a, Caldeira et al. 1998, 2000) were used here: *Dde*I, *Alu*I, *Rsa*I, *Mva*I and *Hae*III. Digestion and RFLP analysis were performed as described by Vidigal et al. (1998).

RESULTS

Morphological identification - The snails were morphologically identified as *B. amazonica* (Fig. 2) and *B. kuhniana* (Figs 3-4)**.** The vaginal corrugation, so characteristic of the *B. straminea* complex, show a difference among the *B. kuhniana* populations studied. Specifically, in the snails from Llanogrande (located at 2,100 m altitude), minimal swellings in the vaginal wall were found, in sharp contrast with the finding in snails from Acacias (530 m altitude).

All populations showed to be negative for *S. mansoni* cercariae. Some specimens of *B. kuhniana* from Acacias were infected with unidentified trematode cercariae.

Restriction profile analysis - DNA amplification with the ETTS1 and ETTS2 primers generated a fragment of approximately 1,300 bp for all specimens. Fig. 5 shows the profiles obtained with the enzyme *Dde*I for Brazilian populations of *B. peregrina* (lanes 1, 2), *B. intermedia* (lanes 3, 4), *B. straminea* (lanes 5, 6), *B. kuhniana* (lanes 7, 8), for *B. kuhniana* populations from Venezuela (lanes 9, 10) compared with populations from the Interandean Valleys (lanes 11 to 22). The four populations from the Interandean Valleys showed species-specific profiles for *B. kuhniana*. The enzymes *Alu*I, *Rsa*I, *Mva*I and *Hae*III generated spe-

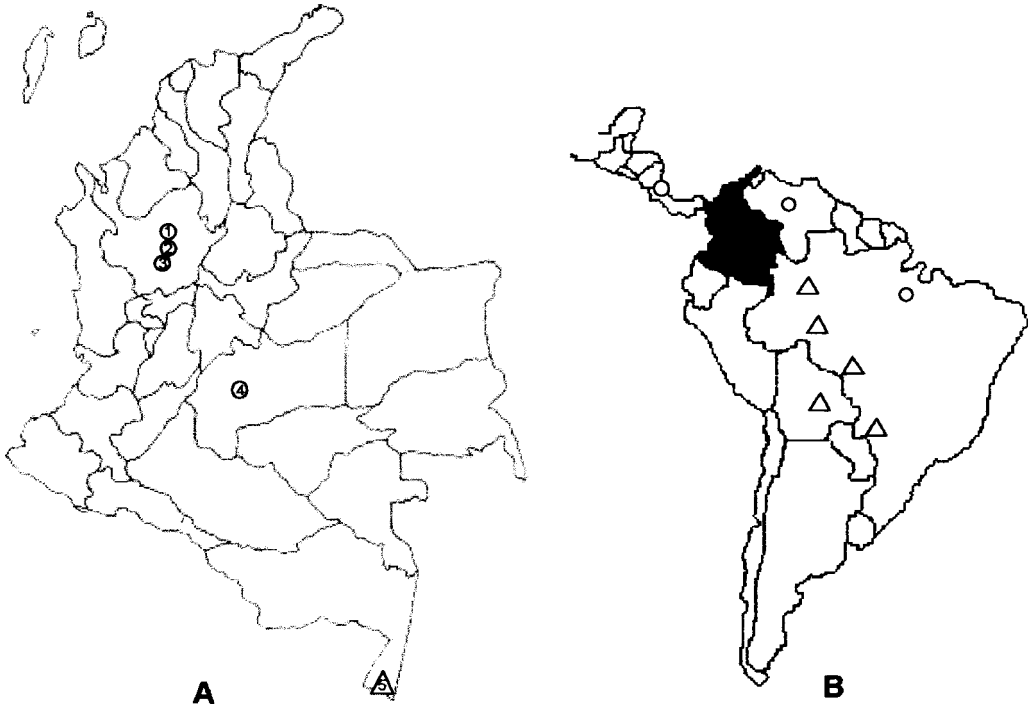

Fig. 1: geographic distribution of *Biomphalaria kuhniana* (○) and *B. amazonica* (△). A: in Colombia; municipality: 1 Segovia; 2 Porce; 3 Llanogrande; 4 Acacías; 5 Leticia; B: in Central and South America

TABLE

Origin of the *Biomphalaria* species used in the study

Species of *Biomphalaria*	Locality	State/Country	Geographic coordinates	Altitude
B. kuhniana	Tucuruí	Pará/Brazil	03S46/49W40	50
	Villa de Cura	Aragua/Venezuela	10N01/67W29	550
	Segovia	Antioquia/Colombia [a]	07N04/74W42	900
	Porce	Antioquia/Colombia	06N91/75W13	950
	Acacías	Meta/Colombia	04N35/71W48	523
	Llanogrande	Antioquia/Colombia	06N06/75W27	2100
B. amazonica	Benjamin Constant	Amazonas/Brazil	04S22/70W01	100
	Manaus	Amazonas/Brazil	03S07/60W01	50
	Barão Melgaço	Mato Grosso/Brazil [a]	16S11/55W41	200
	Leticia	Amazonas/Colombia [a]	04S13/69W56	82
B. straminea	Picos	Piauí/Brazil [a]	07S04/41W28	50
	Florianópolis	Santa Catarina/Brazil	27S35/48W32	100
B. intermedia	Pindorama	São Paulo/Brazil	21S11/48W54	150
	Itapagipe	Minas Gerais/Brazil	19S54/49W22	400
B. peregrina	Alfenas	Minas Gerais/Brazil	21S25/45W56	900
	Taim	Rio Grande do Sul/Brazil	32S30/52W30	50

a: laboratory populations

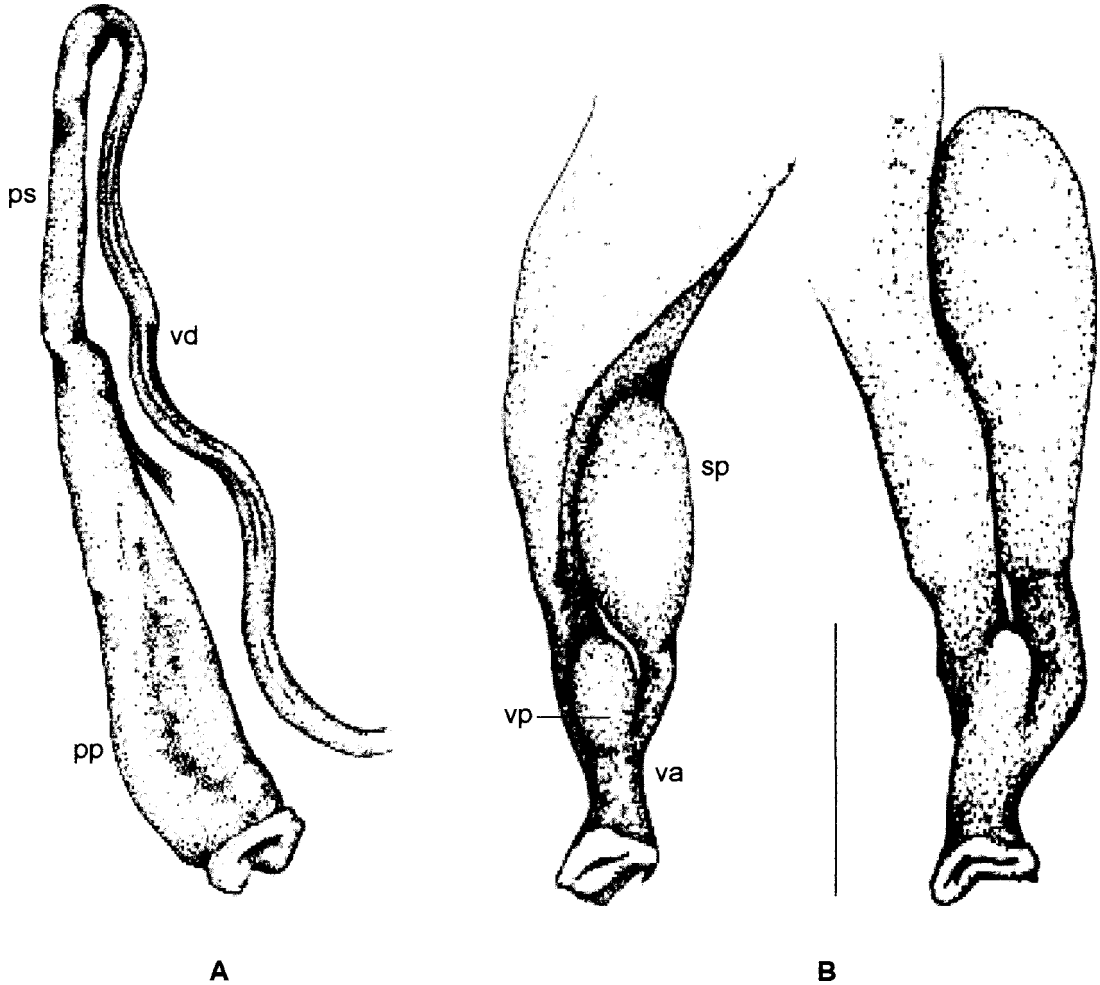

Fig. 2: *Biomphalaria amazonica* from Leticia, Colombia. A: penial complex, pp: prepuce, ps: penis sheath, vd: vas deferens; B: vaginal complex, sp: spermatheca, va: vagina, vp: vaginal pouch. Bar = 1 mm

cies-specific profiles for the four *B. kuhniana* populations from the InterAndean Valleys (data not shown).

Fig. 6 shows the profiles obtained with the enzyme *Dde*I for Brazilian populations of *B. amazonica* (lanes 1 to 11; 16-17) compared with the samples from Colombian Amazonia (lanes 12 to 15). The profiles of the latter proved similar to each other and also to one of the three profiles of the Brazilian *B. amazonica*.

DISCUSSION

We report here for the first time the presence of *B. amazonica* in Colombia. The genitalia characters of this species correspond to those described by Paraense (1966). It does not present phenotypic plasticity but has well-defined morphological characters. On the other hand, the molecular profile of these snails sometimes shows three variants in the same locality (Vidigal et al. 2000a). Such intraspecific variation was further studied by Vidigal et al. (2000b), when the ITS2 region of Brazilian *Biomphalaria* snails was sequenced. Through PCR-RFLP analysis, using the same snail samples, these investigators could observe, in all trees, a polymorphism concerning the position of the three individuals (two from Amazonas and one from Mato Grosso, Brazil). Dejong et al. (2001) observed an intraspecific variation in two *B. amazonica* Brazilian snail populations (the same under study by Vidigal et al. 2000a, b) and in another from Bolivia, using combined data of the sequenced regions ITS1, ITS2 of rRNA gene and partial subunit 16S mitochondrial of rRNA gene of 23 *Biomphalaria* species (16 Neotropicals and 7 Africans). However, the Colombian specimens showed only one of the three profiles, reported by Vidigal (2000a), which mirrors less polymorphism when compared with Brazilian specimens. The phylogenetic relationship of *B. amazonica*, obtained through sequencing analyses by Vidigal et al. (2000b) and DeJong et al. (2001), with species of the same genus, from Neotropical and African regions, showed that *B. amazonica* possesses high affinity with the species of the complex *B. straminea*.

Among the populations of *B. kuhniana* under study presented variability of the vaginal corrugation, with more marked differences among the populations from Llanogrande, which showed very slight swellings, and from Acacias, which exhibited conspicuous corrugation. Interestingly, this kind of morphological variability was not detected at the intrapopulational level in contrast with the finding reported in *B. kuhniana* from Tucuruí, Brazil (Paraense 1988).

Owing to the morphological similarity and short genetic distance between *B. straminea* and *B. kuhniana* (Paraense 1988, Caldeira et al. 1998), these species are commonly confused by health technicians who are not specialized in Malacology. Indeed, snails from Venezuela identified as *B. straminea* were actually *B. kuhniana* (Caldeira et al. 2000). Such misclassification may also have occurred in Colombia since some populations of *B.*

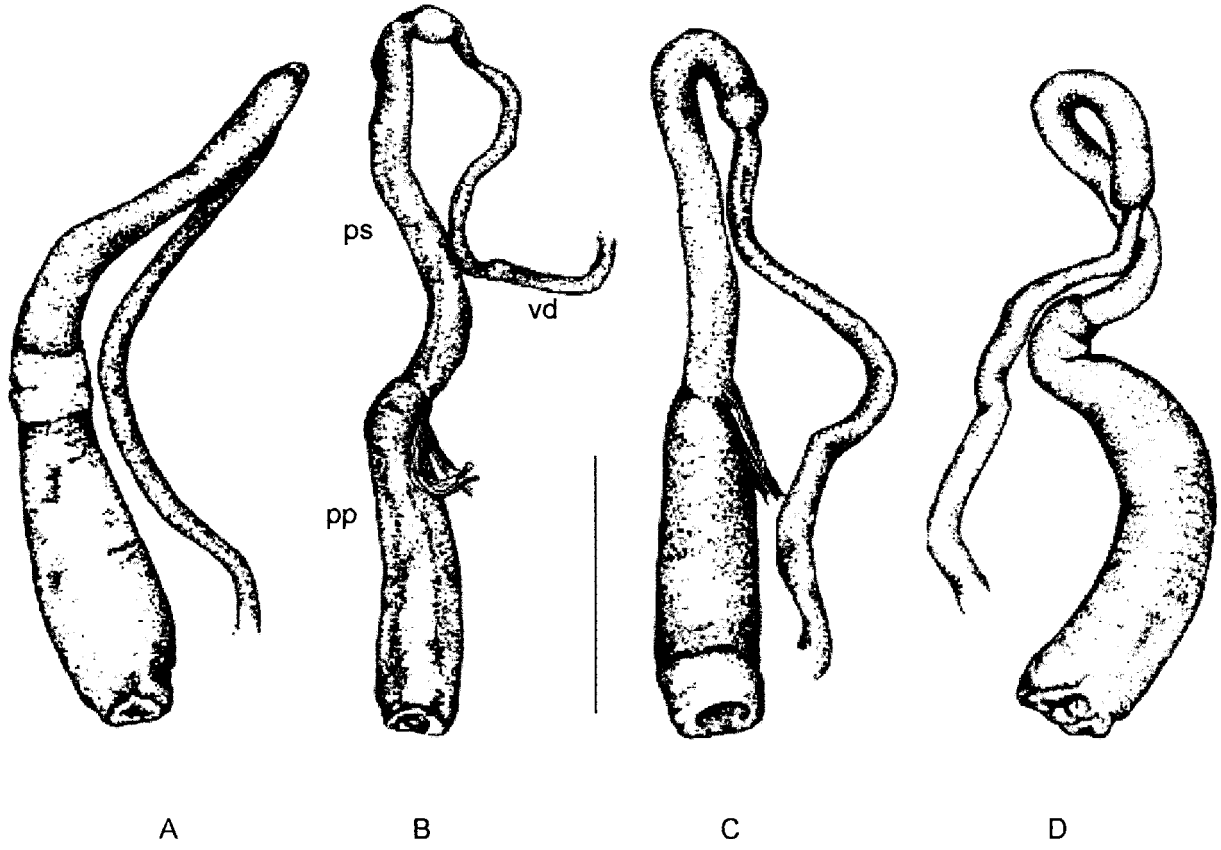

Fig. 3: penial complex of *Biomphalaria kuhniana* in different localities from Colombia: A: Llanogrande; B: Porce; C: Segovia; D: Acacías, pp: prepuce, ps: penis sheath, vd: vas deferens. Bar = 1 mm

kuhniana, previously identified as *B. straminea* (Velásquez & Vélez 1999), were molecularly characterized as *B. kuhniana* in the present study.

Caldeira et al. (1998), by using PCR-RFLP analysis of the ITS region of rRNA of *B. straminea*, *B. intermedia*, *B. kuhniana* and *B. peregrina* observed the cluster of three groups, which comprise: I) *B. straminea* and *B. kuhniana*; II) *B. intermedia* and III) *B. peregrina*. Groups I and II are more closely related while the third one showed to be a distant group. It was very clear the close relationship between *B. straminea* and *B. kuhniana*, and despite the morphological similarity of *B. straminea* and *B. intermedia* with *B. peregrina*, it could not be included in the complex, which is supported by the morphological information reported by Paraense (1988). Following this, Vidigal et al. (2000b), through the sequencing of ITS2 of rRNA of the ten Brazilian *Biomphalaria* species, confirmed that *B. kuhniana* (from Venezuela and Brazil) is more closely related with *B. straminea* (from Brazil) than with *B. intermedia* (from Brazil). Afterwards, Dejong et al. (2001)

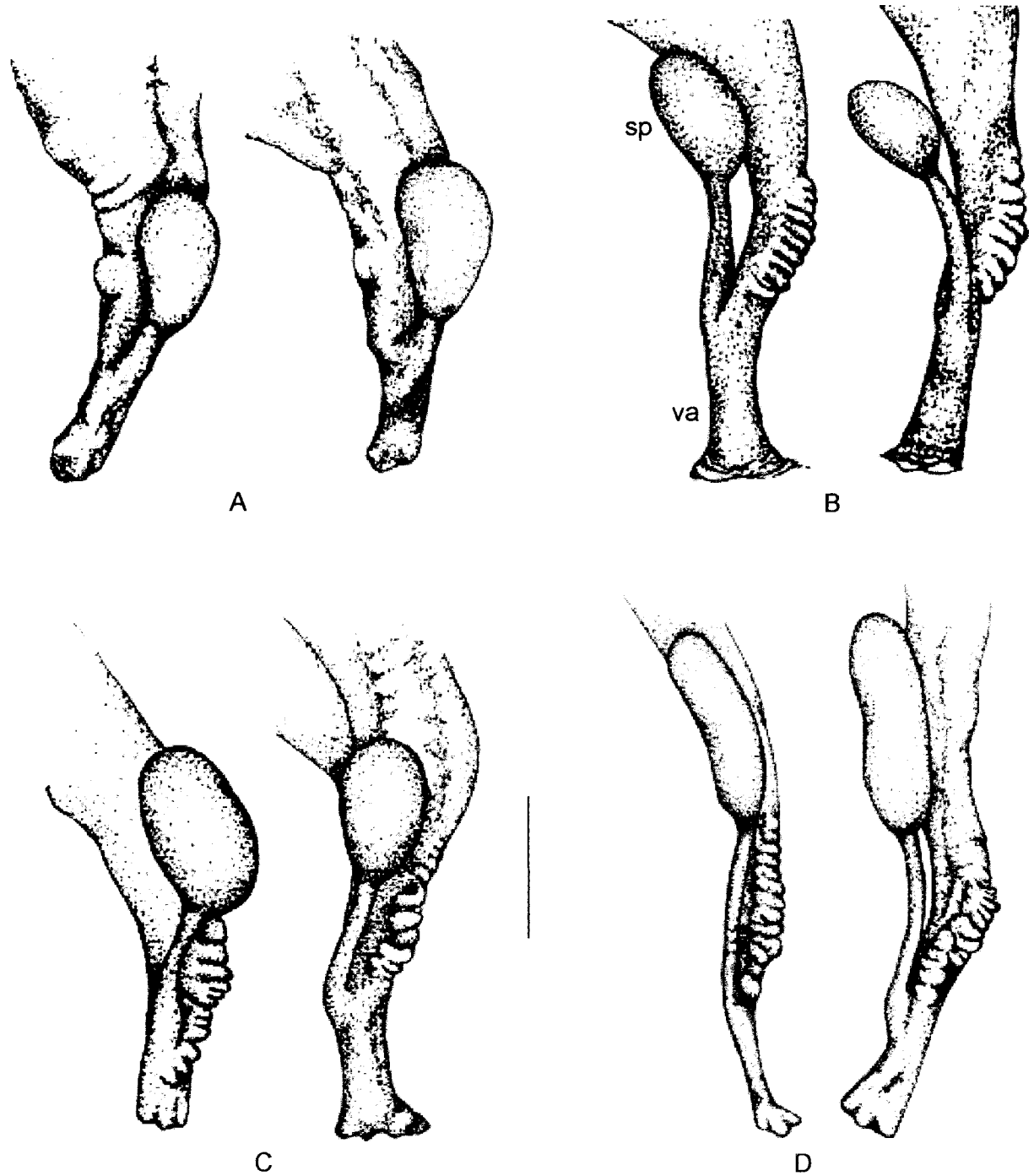

Fig. 4: diversity of vaginal corrugation in *Biomphalaria kuhniana* from Colombia: A: Llanogrande; B: Porce; C: Segovia; D: Acacías; sp: spermateca, va: vagina. Bar = 1 mm

observed that, among the 23 *Biomphalaria* species studied, the most closely related group was that formed by *B. straminea* (Brazil), *B. kuhniana* (Dominica, Colombia and Venezuela) and *B. intermedia* (Paraguay). However, these authors remark that when the region 16S was separately analyzed, two groups were then formed: (1) *B. straminea* (Pará, Brazil) and *B. kuhniana*; (2) *B. straminea* (São Paulo, Brazil) and *B. intermedia*. Only after the analysis of the three DNA regions was it possible to observe the cluster of a single group. These authors speculate about a possible hybridization among these species, which could explain the close relationship among them. It is also impor-

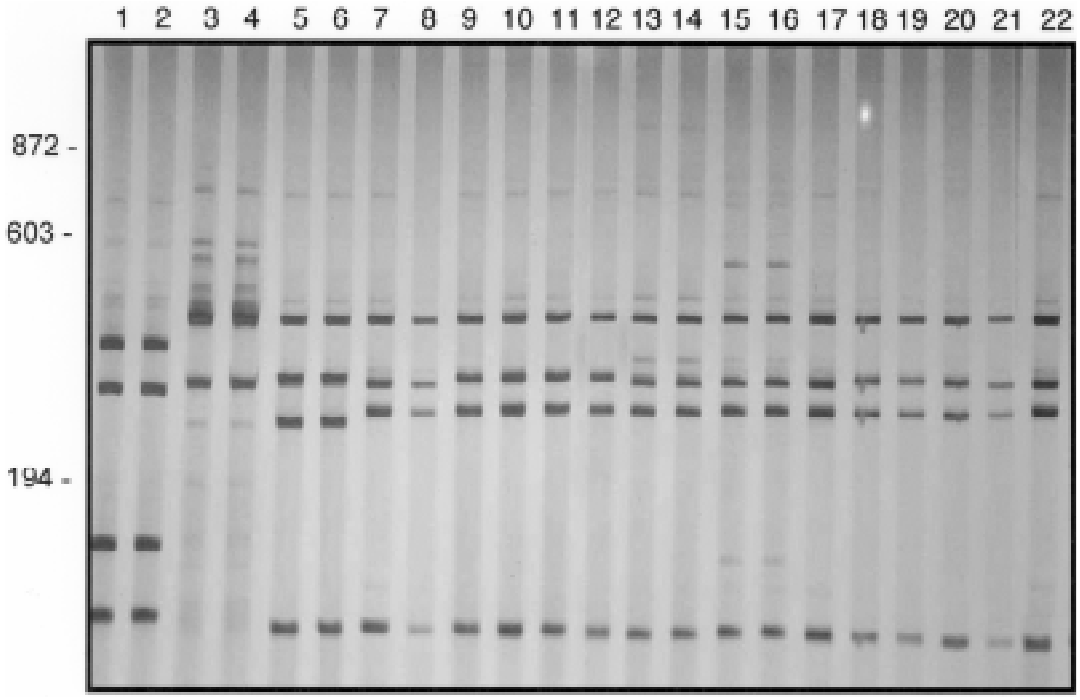

Fig. 5: silver-stained 6% polyacrylamide gels showing restriction fragment length polymorphism profiles obtained after digestion of the rDNA internal transcribed spacer with *Dde*I enzyme. *Biomphalaria peregrina* - Lanes: 1 - Alfenas (Minas Gerais, Brazil); 2 - Taim (Rio Grande do Sul, Brazil); *B. intermedia*: 3 - Pindorama (São Paulo, Brazil); 4 - Itapagipe (Minas Gerais, Brazil); *B. straminea*: 5 - Picos (Piauí, Brazil); 6 - Florianópolis (Santa Catarina, Brazil); *B. kuhniana*: 7, 8 - Tucuruí (Pará, Brazil); 9, 10 - Villa de Cura (Aragua, Venezuela); 11-13 - Segovia (Antioquia, Colombia); 14-16 - Porce (Antioquia, Colombia); 17-19 - Acacías (Meta, Colombia); 20-22 - Llanogrande (Antioquia, Colombia).

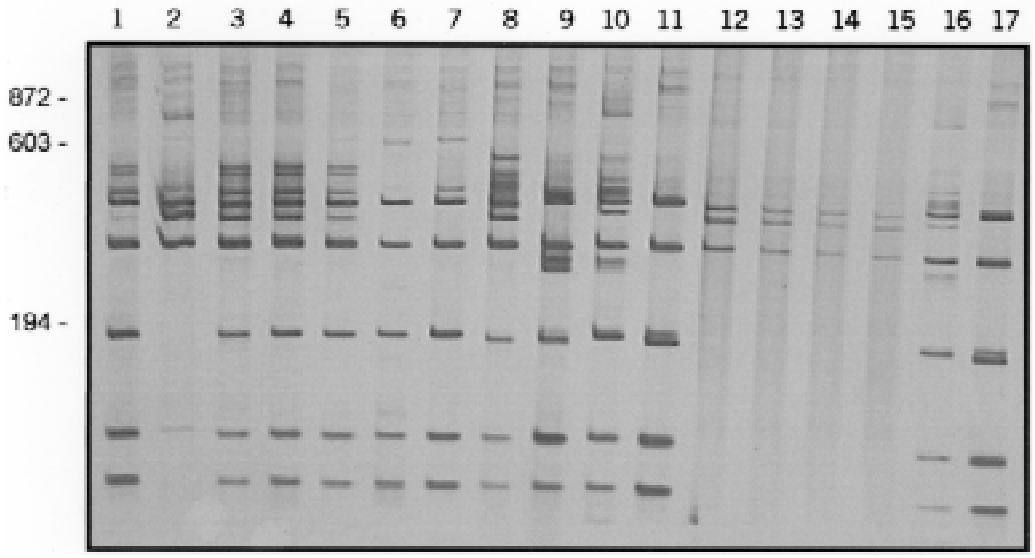

Fig. 6: silver-stained 6% polyacrylamide gels showing the restriction fragment length polymorphism profiles obtained by digesting the rDNA internal transcribed spacer with *Dde*I enzyme. *Biomphalaria amazonica* - Lanes: 1-5 - Benjamim Constant (Amazonas, Brazil); 6-8 - Barão de Melgaço (Mato Grosso, Brazil); 9-11 - Manaus (Amazonas, Brazil); 12-15 - Colombian Amazon (Amazonas, Colombia); 16-17 - Manaus (Amazonas, Brazil).

tant to remark that for Venezuela, country considered the type-locality of *B. straminea*, such species appear not to exist anymore, as recently reported by Caldeira et al. (2000), perhaps suggesting a misclassi-fication of the species, or, yet, a progressive substitution of *B. straminea* for *B. kuhniana*.

Therefore, the correct identification of these snails is of great importance since it allows the detection of species in areas of schistosomiasis transmission, as well as in areas free of the disease, which might become schistosomiasis foci, owing to the presence of natural or experimentally susceptible species. Thus, the methodology using PCR-RFLP proved to be effective for the characterization of Colombian *Biomphalaria* snails since it was able to confirm the classical morphologic identification.

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