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Molecular Markers Associated with Resistance to *Schistosoma mansoni* Infection in the *Biomphalaria glabrata* Snails

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Abstract: Identification and characterization of molecular markers involved in parasite resistance in *Biomphalaria glabrata* snail is importance and constitutes a critical first step towards the understanding of, not just the interaction between snail and schistosome, but also more generally a variety of aspects of other host-parasite systems. Due to the high applicability of RAPD-PCR and the quality of the results obtained, we have used this technique to investigate the intra and interpopulational variability related to susceptibility and resistance to Egyptian strain of *S. mansoni* infection of *Biomphalaria glabrata* snail to detect genetic marker associated with resistant snails which may be responsible for schistosome resistance in *Biomphalaria glabrata* snails. The obtained results using primer OPA-06 in this field reported great genetic variations between the two strains susceptible and resistant in the genomic DNA of parent snails and these variations were heritable from parent to F_1 and F_2 progenies. The molecular biological techniques used in our studies are geared toward a better understanding of the molecular processes involved in influencing parasite development in the snail host and how this information eventually can apply in natural settings for an effective and potentially low-cost biological control strategy for human schistosomiasis.

Key words: Snails, *Biomphalaria*, RAPD-PCR, resistance, susceptible, Egypt

INTRODUCTION

Schistosomiasis is a parasitic disease, which is considered a worldwide problem in various parts of the world including Egypt. As estimated by WHO (1993) 600 million people are at risk of infection and > 200 million are currently infected (Mckerrow and Salter, 2002). The importance of the disease lies in the fact that it affects not only the overall health status and fitness of the infected people, but also the human productivity and national economy (El-Garem *et al*., 1994).

In order to bring this hazardous disease under an adequate control, the concept of snail control on genetic basis has gained a considerable interest, since snail control is one of the most rapid and effective means available for reducing transmission of schistosomiasis. *Biomphalaria glabrata* is the second important hermaphrodites species related to the transmission of schistosomiasis in Egypt (Yousif *et al*., 1996, 1998). One strategy is based on the premise that snails resistant to parasitic infection could be used as biological competition agents to replace existing susceptible snails in endemic areas (Hubendick, 1958). That approach, however, depends on a more thorough understanding of the genetics of both the parasite and snail in their complex interrelationship (Knight *et al*., 1999).

Richards *et al*. (1992), employing techniques of classical genetic, proposed that susceptibility of *B. glabrata* to infection by *S. mansoni* would present distinct patterns of inheritance dependable on the snail. In fact up to the moment, the patterns on inheritance, regarding susceptibility, were not well established (Spada *et al*., 2002).

Several techniques of molecular biology for studies of genetic variability in schistosomiasis vectors have been used (Knight *et al*., 1991, 2000; Abdel-Hamid *et al*., 1999; Vidigal *et al*., 2000, 2001; Jannotti Passos and Souza, 2000; Tuan and Bortolato, 2001; Souza and Jannotti Passos, 2001; Spada *et al*., 2002; Da Silva *et al*., 2004). An introduction of the random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique (Welsh and McClelland, 1990; Williamns *et al*., 1990) has amplified the possibilities of polymorphism analysis.

Vernon *et al*. (1995) showed the potential of RAPDs as molecular markers for analysis of fertilization in wild-type *B. glabrata*. In addition, promising results concerning resistance and susceptibility of *B. glabrata* to *S. mansoni* were reported by Larson *et al*. (1996), Lewis *et al*. (1997), Knight *et al*. (1999, 2000), Spada *et al*. (2002), Abdel-Hamid *et al*. (1999) and Da Silva *et al*. (2004).

As reported by Lockyer *et al*. (2000) that, there is not a single morphological or biochemical marker that can

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reliably distinguished a susceptible from non-susceptible snails. We have used RAPD technique for analysis of the genomic DNA, in laboratory *B. glabrata* strains, in an attempt to associate possible polymorphic markers related to susceptibility/resistance to infection by single Egyptian strain of *S. mansoni.*

MATERIALS AND METHODS

The present study was conducted during 2005 in our laboratory in the Medicinal Chemistry Department-National Research Center-Cairo-Egypt.

Snail stocks: *S. mansoni*-resistant and susceptible *Biomphalaria glabrata* snails (NEg strain) in this study come from Egyptian stock maintained at Medicinal Chemistry Department, National Research Center (NRC), Egypt. These two strains were originally selected by self and cross fertilization of mollusks from parental down to F_2 generations in a well-prepared snail room, belongs to our laboratory, under suitable environmental conditions, in glass aquaria containing Snail-conditioned Water (SCW) in a density of 10 snails L^{-1}. The snails were fed according to (Madsen, 1992).

Snail infection: Each mollusk was challenged with 10-12 miracidia (Egyptian strain) obtained from Schistosome Biological Supply Project (SBSP), (Theodur Bilharz Research Institute, Egypt) according to Zanotti-Magalhaes *et al.* (1997), Langand and Morand (1998). Adult snails that did not develop any sporocysts after 12 weeks of observation were scored as nonsusceptible as suggested by Knight *et al.* (1999).

Snail rearing: Afterwards, the respective pairs (cross-fertilization) and single (self-fertilization) snails were separately put into glasses in order to obtain F_1 descendants. In this experiment, the egg-layings from the resistant snails, as well as from the susceptible ones, were collected for five weeks. The egg-layings from F_1 generation were transferred to an aquarium until the mollusks could reach 5 mm in diameter. F_2 generation was obtained by means of self and cross-breeding between F_1 individuals according to same procedures previously described.

Segregation of nonsusceptible/susceptible phenotypes in the adult (i.e., those after the onset of egg laying) progeny snails (F_1 and F_2) was determined by exposing snails to miracidia.

DNA extraction: A fragment from the cephalopodal region of the individual snails (resistant and susceptible strains) was collected for DNA extraction of the successive generations (F_0, F_1 and F_2), by the method of Winnepenninckx *et al.* (1993). DNA concentration and purity were determined according to the Southern (1979) and Brown (1987).

DNA amplification by RAPD-PCR: The protocol used was described by Simpson *et al.* (1993).

Polyacrylamide gel electrophoresis and gel staining: DNA amplification products were submitted to 6% polyacrylamide gel electrophoresis and silver stained according to Santos *et al.* (1993).

Phonetic analysis of bands: To calculate percentage band differences between susceptible and resistant strains, the bands observed in a given lane were compared with those in other lanes of the same gels, as described by Vidigal *et al.* (1994).

Nei's estimate of similarity, based on the probability that an-amplified fragment from one isolate will also be found in another (Nei and Li, 1979).

Polymorphism analysis: Genetic variation of the susceptible and resistant strains evaluated by analyzing the electrophoretic band patterns obtained on the gels and by determining the similarity coefficient as described by Dice (1945).

RESULTS

Resistant and susceptible snail populations were initially confirmed by means of a phenotypic marker (infection experiment).

Five specimens from resistance stock (F_0) and 5 from susceptible ones, as well as 8 individuals from F_1 generation (4-self and 4-cross fertilized snails) and four from F_2 generation were aleatory selected and were submitted to RAPD-PCR analysis.

Of 20 random primers that were screened in RAPD analysis for their ability to produce sufficient amplification products, eight gave amplification products. One out of the eight primers (OPA-06,) was selected on the basis of the number and frequency of polymorphism produced among the different snail strains.

As shown in Fig. 1, a clear difference is found between the two snail lines (susceptible and resistant) of *B. glabrata* where, in resistant genotype snails (lanes 1-5), OPA-06 produced a major two strain-specific markers, approximately 400 and 800 bp. In contrast, DNA amplification with primer OPA-6 generated one strain-specific marker discernable approximately 1100 bp for all

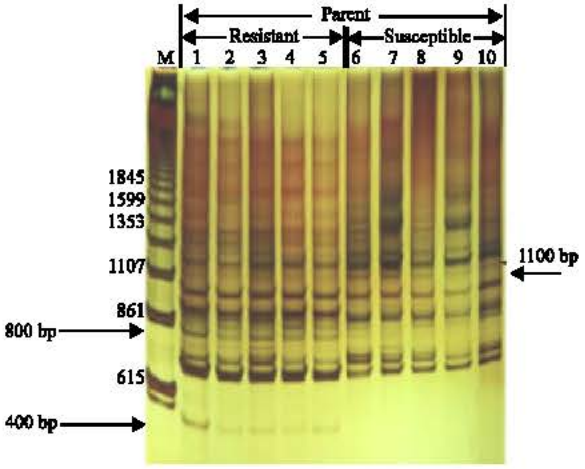

Fig. 1: Primer OPA-06 RAPD products from genomic DNA of five individual parental self-resistant *Biomphalaria glabrata* snails (lanes 1-5) and five individual parental susceptible *B. glabrata* snails (lanes 6-10). Lane (M) contains size marker (123 bp Ladder). Arrow indicates the position of 400 and 800 bp bands in the resistant parent snails only, and 1.1-kb marker amplified only in the susceptible parent snails. Samples were analyzed by 6% Polyacrylamide Gel Electrophoresis (PAGE) and visualized by silver staining

susceptible genotype individual snails (lanes 6-10). These results revealed that this major OPA-06 marker represents invariable structural genotype differences between the two strains of *B. glabrata* parental snails.

With the exception of the intensity of bands as shown in Fig. 1, the amplified fragments within the same isolates either resistant or susceptible were identical, indicating a high degree of genetic similarity. The only reproducible differences were noticed between the two different isolates of resistant and susceptible strains.

However, the average percentage of shared bands between each pair within the specimens of the same strain (resistant or susceptible) was 100% while between all possibly shared pairs from the two different strains was 96%.

Genetic heritability of resistant markers in individual F_1 resistance progeny derived by cross-fertilization *B. glabrata*: Due to the RAPD-PCR amplification using the random primer OPA-6 was stable, reproducible and shown to discriminate between the two snail lines of *B. glabrata*. Therefore we focused our efforts on this primer and examined the inheritance pattern of markers

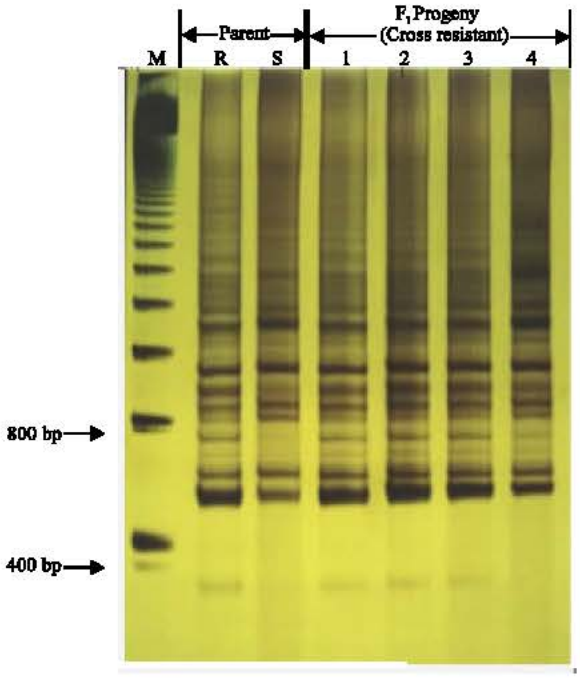

Fig. 2: Comparison between OPA-06 RAPD-PCR products of genomic DNA from four individual F_1 cross-resistant *B. glabrata* snails (lanes 1-4), parental self-resistant snail (lane R) and parental self-susceptible (lane S). Lane (M) contains size marker (123 bp DNA ladder). Arrow shows the position of 400 and 800 bp markers bands in the resistant snails either self-parent or cross-F_1. Samples were analyzed by 6% PAGE and visualized by silver staining

identified by this primer from parental resistant snails to F_1 resistant progeny snails derived by cross-fertilization between these two parental snails. Template snail DNA used in the assay was prepared from parent snails (resistant and susceptible) and cross-F_1 resistant progeny snails (Fig. 2).

As shown in Fig. 2, by using primer OPA-06 the 400 and 800 bp, resistant-line-specific fragments were consistently inherited in a dominant fashion from self-resistant individual of parental snail (lane R) to all resistant-cross individuals of F_1 progeny snails (lanes 1-4). On the other hand, these specific fragments were absent in parent susceptible strain (lane-S).

From Table 1, it is also noticed that, the self-fertilization is genetically very close to that of cross-fertilization, since similarity coefficient between them was 1.0 and the average percentage of shared bands was 100%.

Table 1: Nei's similarity coefficient among three successive progeny of *B. glabrata* snails with two different strains using primer OPA-06

			Parent		F1		F2
			Resistant	Susceptible	Resistant		Resistant
			Self	Self	Self	Cross	Self
Parent	Resistant	Self	1.00	0.96	1.00	0.98	1.00
	Susceptible	Self	0.96	1.00	0.96	0.95	0.95
F1	Resistant	Self	1.00	0.96	1.00	0.99	1.00
		Cross	0.98	0.95	0.99	1.00	0.98
F2	Resistant	Self	1.00	0.95	1.00	0.98	1.00

Similarity coefficient = $2a/F_A + F_B$, Where a: Number of shared bands. FA + FB: Number of fragments in A + number of fragments in B

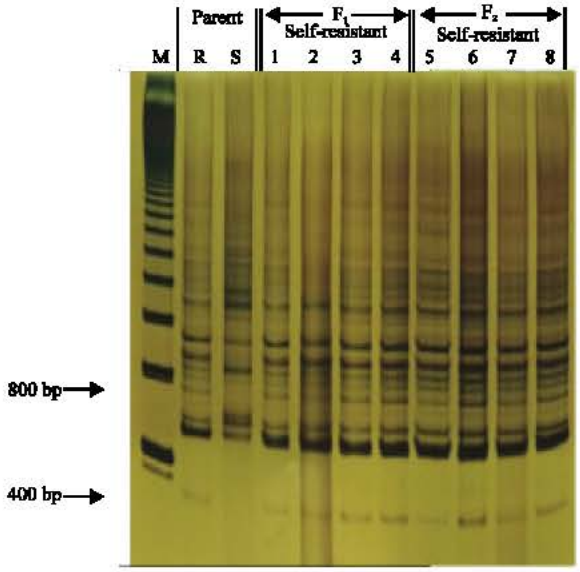

Fig. 3: Heritance pattern of OPA-6 RAPD-PCR products from parent-resistant individual *B. glabrata* snail (lane R) to F_1 self-resistant four individual *B. glabrata* snails (lanes 1-4) to F_2 self-resistant four individuals of *B. glabrata* snails (lanes 5-8). Lane (S) represents one parental susceptible *B. glabrata* snail and lane (M) contains size marker (123 bp ladder). Arrow indicates heritance of 400 and 800 bp markers in resistant snails. Samples were analyzed by 6% PAGE and visualized by silver staining

The inheritance patterns of resistant markers identified by OPA-06 primer from parent to F_1 to F_2 progeny snails by self-fertilization: To determine whether the segregation pattern of 400 and 800 bp specific-markers in individuals of parent progeny snails would correspond to results obtained with self-resistant F_1 and F_2 progeny snails, we examined DNA from 10 individuals, two from parent snails (resistant and susceptible), four from self-F_1 resistant progeny individual snails and four from self-F_2 resistant individual progeny with primer OPA-06 (Fig. 3).

Comparison of the inheritance pattern of 400 and 800 bp bands among four individuals of self-resistant F_1 and four individuals F_2 progeny snails showed that the non-susceptible parent snail markers (lane R) were consistently inherited in a dominant fashion from it to all their offspring F_1 (lanes 1-4) and F_2 individual (lanes 5-8).

Also, Fig. 3 shows that some bands within the eight amplified products of DNA extracted from resistant individuals F_1 and F_2 progeny snails (lanes 1-4) and (lanes 5-8), respectively were more intense than others. The fragments amplified from these individuals compared with their resistant parent (lane R) showed a high degree of similarity and identity since the number and percentage of shared bands between each pair is about 100%. On the other hand, the percentage of shared bands between each pair from these different isolates and susceptible strain (lane S) is 96%.

In conclusion, the amplified fragments of self-resistant genotype snails within F_1 and F_2 progeny snails presented very similar profiles that complicated their identification at molecular level as well as between them and their parents snails suggesting a very clear genetic similarity, since similarity coefficient among them as shown in Table 1 is 1.0. On the other hands, significant genetic differentiation was demonstrated between parental susceptible genotype snails (lane-S), F_1 and F_2 offspring resistant individuals (lanes 1-4) and (lanes 5-8) respectively with similarity coefficient among them 0.96.

DISCUSSION

Since, it was lately reported by Yousif *et al.* (1996, 1998) that, *B. glabrata* form a new threat for schistosomiasis transmission in Egypt invading the irrigation areas and drainage systems and also water courses in reclaimed areas leading to increase schistosomiasis transmission. So the present study focused on using *B. glabrata* snails as the specific intermediate hosts for Egyptian *Schistosoma* species which is responsible for wide spread schistosomiasis in Egypt.

In the present study, laboratory-reared snails were selected to investigate resistant or susceptible ones in the original stocks of Egyptian *B. glabrata* snails upon first exposure with their homologous single strain of *S. mansoni*.

Several techniques of molecular biology for studies of genetic variability in schistosomiasis vectors have been used (Abdel-Hamid *et al*., 1999; Vidigal *et al*., 2000, 2001; Calderia *et al*., 2000; Jannotti Passos and Souza, 2000; Knight *et al*., 2000; Souza and Jannotti Passos, 2001; Tuan and Bortolato, 2001; Spada *et al*., 2002; Florence *et al*., 2004).

The introduction of RAPD-PCR technique has amplified the possibilities of polymorphisms analysis, as it allowed the use of small arbitrary nucleotide segments without the need of a previous knowledge of genes and/or genomic sequences (Welsh and McClelland, 1990; Williams *et al*., 1990). Also, RAPD assay clearly has certain practical advantages for detecting DNA variation: It is technically less demanding, cheaper and quicker than other molecular techniques (Stothard and Rollinson, 1996).

Rollinson *et al*. (1998) reported that, snails resistant to infection occur naturally and there is a genetic basis for this resistance, in *B. glabrata* resistance to *S. mansoni* is known to be polygenic trait. So, we have initiated a preliminary search for snail genomic regions linked to, or involved in resistance by using a RAPD-PCR based approach in conjunction with progeny individual methods.

The present study, demonstrated the utility of RAPD-PCR method for the differentiation of snail lines of Egyptian *B. glabrata*. The snail lines studied were selected on the basis of well-characterized resistance/susceptibility phenotypes upon exposure to *S. mansoni*. Reproducible and inheritable stable polymorphic markers for *B. glabrata* were identified with one out of the twenty arbitrary primers tested. Several of the primers produced monomorphic bands among the stocks, or the polymorphism identified were not reproducible. In previous studies, genetic diversity among *B. glabrata* snails was evaluated either by allozyme or Restriction Fragment Length Polymorphism (RFLP) analysis (Mulvey and Vrijenhoek, 1981; Knight *et al*., 1991; Mulvey and Bandoni, 1994). Both methods suggested a significant degree of genetic variability within laboratory-maintained lines of *B. glabrata*.

In a study comparable to present study, Langand *et al*. (1993) demonstrated that the RAPD method was a useful tool for the evaluation of the degree of genetic diversity within the genus *Bulinus*. Also, RAPD analysis of Brazilian field isolates of *B. glabrata* showed that a high degree of genetic diversity exists among natural populations of these snails (Vigidal *et al*., 1994; Abdel-Hamid *et al*., 1999; Spada *et al*., 2002). Recently, Da-Selva *et al*. (2004) reported that, RAPD-PCR methodology represents an adequate approach for analysis of genetic polymorphism associated to resistance/susceptibility of *Biomphalaria* species.

Based on the fact that, the intriguing interaction between host and parasite allows a favorable balance for them to reproduce simultaneously, defining the genes that influence this interaction should lead to a better understanding of host-parasite relationship. One major finding of the present study was that although the snail stocks utilized (susceptible and resistant) were maintained and inbred in our laboratory, they constitute inter-strain genetic variation i.e. genetic heterogeneity has been reported within *B. glabrata* snail maintained under the laboratory conditions.

Larson *et al*. (1996) showed that the genetic differentiation among several laboratory-maintained pedigree snail lines of *B. glabrata* (with different susceptibility phenotypes to *S. mansoni*) exhibit an intra-strain genetic variation using RAPD analysis method.

Also, several attempts have been made to define markers for susceptibility /resistance in *B. glabrata* (Mulvey and Vrijenhoek, 1981; Mulvey and Woodruff, 1985; Sire *et al*., 1999; Knight *et al*., 1999). Most attempts involved linkage analysis by examining pigmentation and allozyme phenotypes in resistant and susceptible snails.

Our interest was to identify only stable invariant genetic markers in the laboratory Egyptian strain of *B. glabrata* stocks. So, with the primer (5`-GGTCCCTGAC-3`), which corresponds to primer OPA-06 employed by Larson *et al*. (1996), two polymorphic markers of 400 and 800 bp were found (Fig. 1), those bands were repeatedly found only in the resistant lineage, also, using the same primer under the same condition one strain-specific marker was discernable approximately 1.100 bp for all susceptible genotype individual snails.

In support of these results, Larson *et al*. (1996) obtained, with primer OPA-06 specific markers of 150, 400 and 800 bp, characteristic of resistant lineages of *B. glabrata* (Brazilian strains) (BS-90 lines), also they found that in susceptible M-line snail a major specific marker of approximately 1100 bp was amplified by OPA-06. The distinct origin of the resistant strain employed in the current work Egyptian *B. glabrata* strain (NEg-strain) (ES-R line), could justify the acquisition of distinct polymorphic bands from the amplification with a primer with equal sequence of nucleotides. In other words, the resistant strain in question presents specific markers but they are different among themselves.

The current study also showed that, the average percentage of shared bands between each pair from the same strain (either susceptible or resistant) was nearly 100% and between all possible different strains was 96% which indicate that, the RAPD-PCR profiles of the same strains of laboratory maintained *B. glabrata* were highly homogenous while profiles from two different strains showed a degree of genetic variability.

In a study comparable to the current work, Abdel-Hamid *et al.* (1999) demonstrated that there is a very limited difference in the amplified bands of resistant or susceptible *B. tenagophila* and the only reproducible differences were noticed between the two types of snails.

Also, the present findings coincide well with the results obtained by Larson *et al.* (1996) who demonstrated that, with primer OPA-06 a band of 1100 bp, characteristic of susceptible lineages (M-line). That lineage was obtained by Newton (1955). Although Spada *et al.* (2002) detected with the same primer (OPA-06) a polymorphic marker of 1100 bp only in the susceptible *B. glabrata* snails (BH strain) obtained by self-fertilization.

B. glabrata snails are hermaphrodite freshwater snails and can self-fertilize but prefer to produce by cross-fertilization when paired. These biological characteristics are evolutionarily very important, providing the organism with the ability to establish colonies from an individual organism while maintaining genetic variation through sexual reproduction (Paraense, 1955; Jaenike, 1978; Hamilton, 1980; Vidigal *et al.*, 1994, 1998).

In the present study using RAPD-PCR technique findings showed also that, F_1-crossed offspring patterns shared nearly the same number of bands within self-resistant lines of the same parental *B. glabrata* snails (Fig. 2). Moreover, using primer OPA-06 the 400 and 800 bp, resistant-line specific fragments for *B. glabrata* were consistently inherited in a dominant fashion from self-resistant individual of parental snail to all resistant-cross individuals of F_1 progeny and self-resistant individuals of F_1 and F_2 progeny of snails (Fig. 3).

In this respect, Larson *et al.* (1996) indicated that, the RAPD profile of *B. glabrata* resistant lines (BS-90, 10-R2 and LAC) gave reproducible markers with the arbitrary primer OPA-06. The results were in agreement with our results where these specific markers were inherited in a dominant or codominant fashion.

The present results also are in accordance with the findings of Knight *et al.* (1999) who analyzed through RAPD-PCR F_1 and F_2 generations of *B. glabrata* (Brazilian strains) and the F_1 versus parental backcross progeny. They identified molecular markers associated with resistant DNA genome of these snails and showed that 1200 bp amplified by primer OPM-04 and 1000 bp marker produced by primer OPZ-11 segregated and was inherited in the same dominant fashion.

The results represented in the present study also showed the stability of markers in susceptible phenotype within each individual of parental *B. glabrata* with the specific strain-markers (1100 pb) depending on the utilized primer OPA-06 for *B. glabrata* snails. Only some-minor differences were occasionally observed and concerned with intensity. Previous work indicates similar results. According to Lewis *et al.* (1997), certain strain specific markers were inherited in a co-dominant fashion in the majority of F_2 *B. glabrata* snail progeny under their utilized primer. Also they added that, in DNA from F_2 progeny resulting from cross-fertilization between parasite-resistant and susceptible *B. glabrata* snails, the molecular markers show linkage to either the resistant or susceptible phenotype. By using both Restriction Fragment Length Polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) approaches revealed that more promising results have been found with RAPD approach, where 1300 bp marker appears in nearly all resistant progeny and 1000 bp marker appears in all susceptible ones. Based on all the previous recorded information, as well as the present observations it is evident to conclude that, the resistant character is heritable like susceptible character.

In the present study, descendants from snails maintained in the laboratory were used (*B. glabrata*). Thus, brave as resistant and susceptible strains were studied and we could expect that the variability was low, since laboratory strains are submitted to much less intense selective pressures than field isolated one, i.e. natural conditions. This fact may explain the high coefficient of similarity observed for the strains studied here.

Present results demonstrate experimentally that the distribution patterns of schistosome larvae among the snail host population may differ depending upon the host-parasite combination considered, a parameter that can have significant consequences on the transmission dynamics of the parasite and on the distribution of the genetic diversity of adult schistosomes among the definitive host population. Other molecular techniques such as analysis of repetitive polymorphic DNA element (Minchella *et al.*, 1995) or random amplified polymorphic DNA markers (Barral *et al.*, 1996; Dabo *et al.*, 1997) are now useful tools for investigating the distribution patterns of trematode larvae among naturally infected snail hosts.

In summary, the present study shows that the RAPD-PCR method can be used quickly and efficiently to discern genetic differences within laboratory-maintained Egyptian stocks of *B. glabrata* snails. Results showing the stability

of markers in individual samples should allow us to evaluate the inheritability of RAPD markers by individual segregation analysis of DNA from resistant and susceptible progeny snails.

Finally, the identification and characterization of genomic regions associated with schistosome resistance in the present study may provide insight into the mechanism of snail resistance and the role of the snail immune system, not just in *B. glabrata* but also in other snail intermediate hosts of schistosomes. In the long-term, understanding the genetic basis of schistosome resistance in snails may provide novel opportunities to develop alternative control strategies for schistosomiasis.

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