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Genetics

The identification of markers segregating with resistance to *Schistosoma mansoni* infection in the snail *Biomphalaria glabrata*

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ABSTRACT **Both snail and parasite genes determine the susceptibility of the snail *Biomphalaria glabrata* to infection with the trematode *Schistosoma mansoni*. To identify molecular markers associated with resistance to the parasite in the snail host, we performed genetic crosses between parasite-resistant and -susceptible isogenic snails. Because resistance to infection in adult snails is controlled by a single locus, DNA samples from individual F_2 and F_1 backcross progeny, segregating for either the resistant or susceptible phenotypes, were pooled (bulked segregant). Genotypes for both parents were determined with 205 arbitrary decamer primers by random amplified polymorphic DNA–PCR. Of the 205 primers, 144 were informative, and the relative allele frequencies between the pools for these primers were determined. Two primers, OPM-04 and OPZ-11, produced fragments in the resistant parent of one cross that were inherited in a dominant fashion in the resistant F_2 and backcross-bulked segregant progeny. Subsequent typing of DNA samples of individual progeny snails showed that the 1.2-kb marker amplified by primer OPM-04 and the 1.0-kb marker produced by primer OPZ-11 segregated in the same dominant fashion with the resistant phenotype. Sequence analysis of the 1.2-kb marker showed that it corresponds to a repetitive sequence in the snail genome with no homology to existing DNA sequences in the public databases. Analysis of the 1.0-kb marker showed that it also corresponds to a repetitive sequence in the *B. glabrata* genome that contains an imperfect ORF, with homology to retrovirus-related group-specific antigens (gag) polyprotein.**

It is estimated that 600 million people in the tropics are at risk for schistosomiasis (1). Agricultural and irrigation projects designed to improve the quality of life for the human population often expand the aquatic habitat of the schistosome's intermediate snail hosts, causing the spread of the disease into new areas. A wide range of strategies has been adopted to interrupt transmission of the parasite. One of the more promising strategies involves the combined use of molluscicides to reduce the snail population and mass chemotherapy of the human population, although there is growing evidence for drug resistance among schistosomes and concern that they may become resistant to the most effective drug currently available, praziquantel (2). Several vaccine candidate antigens also have been identified, but their development as effective agents against this disease has not been realized (3). As with many other parasitic diseases, alternative strategies to control them are being sought. For schistosomiasis, 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 (4). That approach, however, depends on a more thorough understanding of the genetics of both the parasite and snail in their complex interrelationship.

The survival of *Schistosoma mansoni* in the snail *Biomphalaria glabrata* is greatly influenced by both snail and parasite genes. Studies by Richards (5, 6) with various stocks of *B. glabrata* exposed to a single strain of *S. mansoni* demonstrated four separate susceptibility phenotypes based on age-related susceptibility and nonsusceptibility patterns. A fifth category, describing a delayed susceptibility phenotype, has also been described (7). Genetic crosses between snails from different phenotypic categories show that adult resistance is a Mendelian trait governed by a single locus, with resistance dominant (8).

In earlier studies, Richards (9) examined several visible morphological markers, such as pigmentation, pearl formation, and antler tentacles, with the hope of demonstrating linkage between some of these genetic characters with resistance in the snails. Similarly, linkage for nonsusceptibility and isoenzymes were sought by Mulvey and Vrijenhoek (10). Although these studies showed no linkage of markers and resistance, they did establish the pedigree snail lines and the crossing/mating compatibility systems that formed the basis of the present study.

By restriction fragment length polymorphism analysis of rDNA, we previously demonstrated the occurrence of inter- and intrastrain genetic variation in *B. glabrata* (11). We also studied with random amplified polymorphic DNA–PCR (RAPD–PCR) the stable inheritance of polymorphic RAPD markers in individual progeny derived from a single cross between resistant and susceptible parent snails (12). To identify molecular markers associated with adult resistance, we first performed crosses between laboratory-maintained resistant (BS-90) and susceptible (M line) snails. DNA from individual F_2 and F_1 backcross progeny that segregated for either the resistant or susceptible phenotypes were pooled (bulked segregant). This method of bulked segregant analysis was chosen to reduce the sample size while allowing for the rapid screening of polymorphic markers. Markers detected when the DNA fingerprint profiles from the two pools are compared have been shown to be genetically linked to the loci that determine the phenotypic trait used to construct the pools (13). We used bulked segregant analysis and RAPD–PCR in the reported study and describe markers in *B. glabrata* that segregate with resistance to *S. mansoni* infection and show the heritability of these two markers in resistant offspring.

MATERIALS AND METHODS

Snails. The BS-90 snail line is a pigmented laboratory-maintained line derived from Brazilian field isolates (14). It is resistant at any age to *S. mansoni* infection and was made

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Abbreviations: RAPD, random amplified polymorphic DNA; gag, group-specific antigens; NMRI, Naval Medical Research Institute.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF078108 and AF078109).

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available to us by E. S. Loker (University of New Mexico, Albuquerque, NM). The black-eye 10-R2 snail is another parasite-resistant strain of *B. glabrata* that was established by Richards (15). Snails of the M line and Naval Medical Research Institute (NMRI) stock are albino and were selected for high susceptibility to infection with the PR1 and NMRI strains of *S. mansoni* (16, 17). All stocks were reared in isolation, as described by Larson *et al.* (12).

Snail Crosses. Two independent crosses between BS-90 and M line snail parents (with the BS-90 snails serving as males) were conducted as described by Richards (15). Successful crosses were determined by the inheritance of pigmentation (dominant Mendelian trait) in offspring from the albino M line parent. Progeny snails were considered adults at the onset of egg laying (average size 7 mm). Adult progeny snails (F_1 and F_2) were each exposed to 100 miracidia and examined at weekly intervals for 2 months for signs of infection. Adult snails that did not develop any sporocysts after 3 months of observation were scored as nonsusceptible.

F_1 backcross progeny were derived from a cross between an adult F_1 hybrid and the M line parent. Segregation of the nonsusceptible/susceptible phenotypes in the adult backcross progeny was determined by exposing snails to miracidia as described above.

DNA Extraction and RAPD Analysis. DNA was extracted from individual frozen snails as described (18). The genotypes of the resistant and susceptible parent snails were determined with 205 arbitrary 10-mer primers (Operon Technologies, Alameda, CA) by RAPD–PCR as described (12), with the exception that agarose gel electrophoresis [1.2% TBE (90 mM Tris/90 mM boric acid/2.5 mM EDTA, pH 8.0)] was used to resolve amplified fragments. Primers that produced reproducible DNA fingerprint profiles from the parent snails were selected to screen progeny snail DNA. Bulk segregants were prepared by combining 5 μg of DNA from 10 individual progeny snails that displayed either the nonsusceptible or susceptible phenotype.

DNA Cloning and Sequence Analysis. Amplified markers were gel purified by Gene Clean (Bio 101) and cloned into the vector PCR 2.1 with the TA cloning kit (Invitrogen). Recombinant plasmid DNA was isolated with the Wizard miniprep DNA purification system (Promega) and sequenced by the dideoxy-sequencing method with the T7 Sequenase kit (Amersham) with universal primers M13 and synthetic commercial primers (Genosys, The Woodlands, TX) to complete the sequencing of both strands of the template.

Cluster Analysis. The statistical analysis to identify RAPD–PCR resistance markers proceeded by applying a blind protocol. For each individual, the absence or presence of each RAPD–PCR band was scored as 0 or 1, respectively. A total of 30 RAPD–PCR bands were scored for 56 individuals, including the parent snails and progeny, and a "binary" distance matrix was calculated for the group by treating the data for each individual as a vector. The binary matrix is the proportion of non-zeros that two individuals do not have in common (the number of occurrences of a 0 and a 1 or a 1 and a 0 divided by the number of times at least one individual has a 1). The resulting matrix was used for a hierarchical cluster analysis. The resulting cluster tree was plotted as an unrooted tree. Only after the cluster analysis was completed was the resistance information provided. Subsequently, a χ^2 calculation was done for each band to identify bands and primers that clearly identify the resistant/susceptible phenotype. Statistical analysis was done with programs written in S-PLUS 4.0 (MathSoft, Seattle, WA).

Southern Hybridization. Southern blots of digested genomic DNA (5 μg per lane) onto ZetaProbe GT membranes were obtained by the alkaline blotting method according to the manufacturer's instructions (Bio-Rad). Hybridizations with ^{32}P-labeled probes were conducted overnight at 65°C in 0.5 M Na_2HPO_4, pH 7.2. Membranes were washed at 65°C: twice for 30 min each in 40 mM Na_2HPO_4, pH 7.2, and 5% SDS and twice for 30 min each in 40 mM Na_2HPO_4, pH 7.2, and 1% SDS. Blots were set up for autoradiography for 2–4 days at −70°C.

RESULTS

Snail Crosses. *B. glabrata* snails used in these experiments came from two independent crosses between resistant BS-90 and susceptible M line parents. As shown in Table 1, all adult F_1 progeny from both crosses were refractory to infection. After 64 adult F_2 progeny from cross 1 and 73 adult F_2 progeny from cross 2 were exposed, an approximate 3:1 segregation ratio occurred in the nonsusceptible vs. susceptible phenotypes from both crosses, confirming the Mendelian dominant, single-gene nature of this trait.

Results of parasite exposure of adult F_1 backcross progeny are shown in Table 1. Of 48 snails exposed, 23 were resistant and 25 were susceptible, or an approximate 1:1 segregation pattern in the nonsusceptible/susceptible phenotypes in these snails.

Identification of Markers in Bulk Segregants of Adult Resistant F_2 Progeny. Genotypes of the original parental snails were determined by RAPD analysis of 205 arbitrary primers. Those 144 primers that consistently revealed invariant polymorphic markers between the parent snails were selected to investigate the segregation of polymorphic bands in either the bulked segregant nonsusceptible or susceptible progeny (F_2 and backcross F_1).

Of the 144 informative arbitrary primers tested in either nonsusceptible or susceptible F_2 progeny, two primers (OPM-04 and OPZ-11) that revealed, respectively, the presence of a 1.2- and 1.0-kb band unique to the nonsusceptible parent (Fig. 1*A* and *B*, lane B) amplified the same sized bands in nonsusceptible progeny. Comparison of the inheritance pattern of the 1.2-kb band among four groups of nonsusceptible and one group of susceptible progeny showed that the nonsusceptible parent snail marker was consistently inherited in a dominant fashion in the nonsusceptible progeny (Fig. 1*A*, lanes 1–4) but not in the susceptible progeny (Fig. 1*A*, lane 5). Similarly, analysis of the inheritance of the 1.0-kb marker among four nonsusceptible and one susceptible bulk segregants of progeny snails showed that this marker was also inherited in the nonsusceptible (Fig. 1*B*, lanes 1–4) but not susceptible groups (Fig. 1*B*, lane 5).

Segregation of both markers was also examined in adult exposed backcross F_1 progeny. As shown in Fig. 1*C*, RAPD–PCR amplification of nonsusceptible (lane 1) and susceptible (lane 2) backcross F_1 progeny with primer OPM-04 revealed that the 1.2-kb marker was inherited in the nonsusceptible, but not susceptible, backcross F_1 progeny. A similar finding also resulted when amplification of F_1 backcross progeny bulk segregants was done with primer OPZ-11 (Fig. 1*D*).

Heritability of Markers in Individual F_2 Nonsusceptible and Susceptible Progeny. To determine whether the segregation pattern of the 1.2- and 1.0-kb markers in individual progeny snails would correspond to results obtained with bulked samples, we examined DNA from 10 individual F_2 nonsusceptible and 10 individual susceptible progeny with primers OPM-04 and OPZ-11 (Fig. 2). The 1.2-kb marker was uniformly inherited in a dominant fashion in all individual nonsusceptible (Fig. 2*A*), but not susceptible, progeny (Fig. 2*B*). We also detected the presence

Table 1. Resistant and susceptible progeny from independent crosses and backcrosses

	Snails exposed	Resistant	Susceptible
Cross 1			
F_1	37	37	0
F_2	64	50	14
Cross 2			
F_1	74	74	0
F_2	137	105	32
Backcross 1, F_1	28	15	13
Backcross 2, F_1	20	8	12

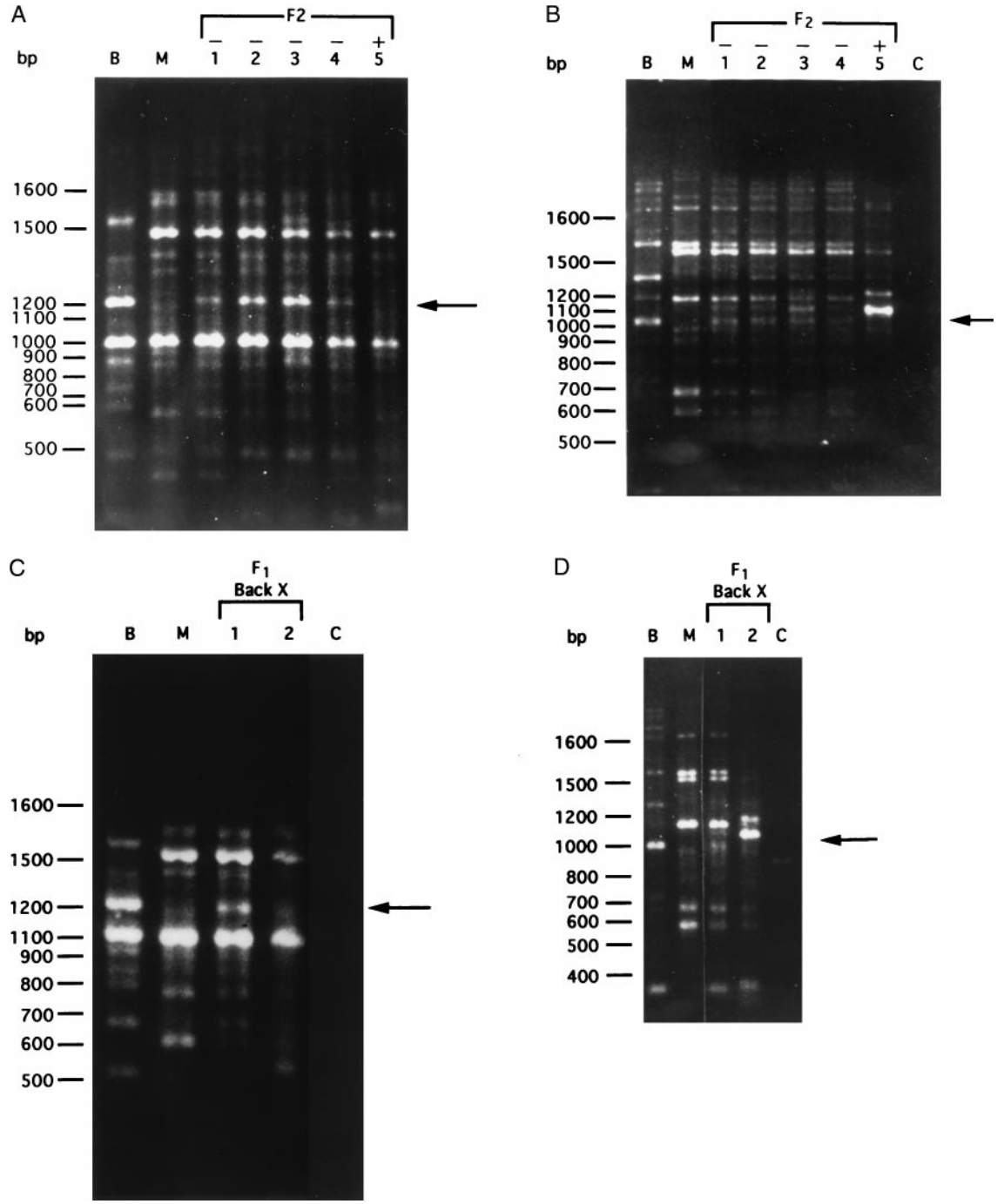

FIG. 1. (*A*) Primer OPM-04 RAPD products from genomic DNA of a resistant BS-90 parent (B), a susceptible M line parent (M), and four groups of resistant (1–4) and susceptible (5) F_2 progeny. Arrow indicates the position of a 1.2-kb band in the resistant parent and offspring DNA. (*B*) Primer 0PZ-11 RAPD bands amplified from genomic DNA of resistant BS-90 (B), susceptible M line parents (M), and four groups of resistant (1–4) and susceptible (5) F_2 progeny. Lane C shows amplification in the absence of template DNA. Arrow indicates position of 1.0-kb marker in resistant parent and progeny DNA. (*C*) Primer 0PM-04 amplification of resistant parents (B), susceptible parents (M), and F_1 backcross resistant (1) and susceptible (2) progeny. Lane C represents amplification in the absence of DNA. Note the position of a 1.2-kb band (arrow) amplified only in resistant parent and progeny. (*D*) Primer OPZ-11 amplification of resistant (B), susceptible (M) parents, and resistant (1) and susceptible (2) F_1 backcross progeny. Lane C shows RAPD–PCR without DNA template. Arrow shows position of 1.0-kb marker amplified only in the resistant parent and progeny.

of a 1.1-kb parasite-specific band (lane S) in some susceptible progeny snails (lanes 4–6).

RAPD–PCR of individual nonsusceptible and susceptible snails with primer OPZ-11 showed that the 1.0-kb marker was also present in nonsusceptible (Fig. 2*C*), but not susceptible (Fig. 2*D*), F_2 progeny. As with the case of primer OPM-04, a parasite-related band (≈1.1 kb) was amplified from *S. mansoni* DNA (Fig. 2*D*, lane S) in the majority of the susceptible progeny tested. In a few cases (30%), however, we failed to detect the 1.1-kb parasite band in those snails originally scored as susceptible, but we detected the 1.0-kb marker instead. To date, genotypes of 54 individual resistant F_2 progeny examined with primers OPM-04 and OPZ-11 have shown the presence of the 1.2- and 1.0-kb bands in 90% and 100% resistant offspring, respectively. Both markers were absent in all susceptible F_2 progeny and in another susceptible strain of *B. glabrata* (NMRI, data not shown). In addition, the 1.2- and 1.0-kb markers were detected in another isogenic parasite-resistant strain of *B. glabrata* (10-R2, Fig. 3). The relat-

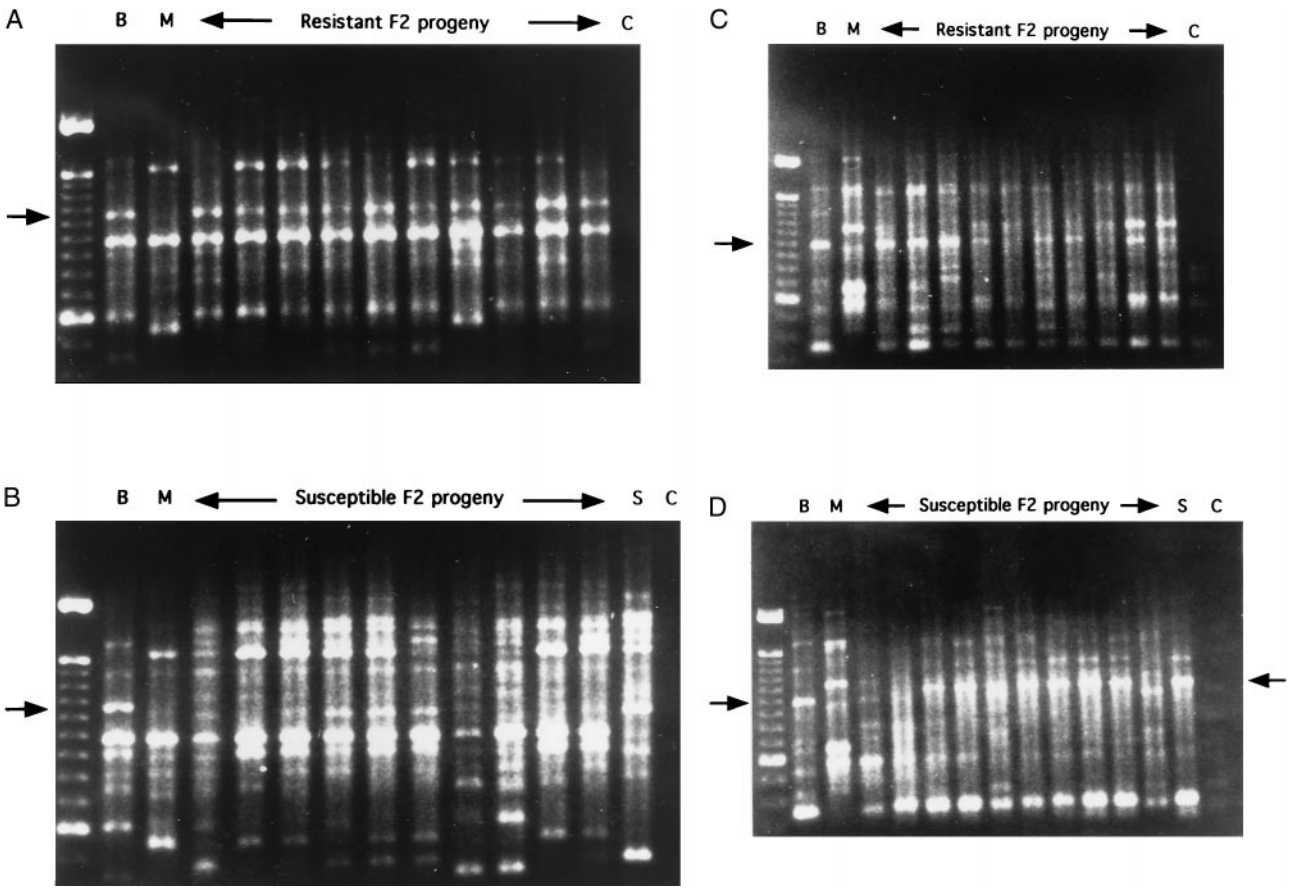

FIG. 2. (*A*) OPM-04 RAPD–PCR amplification of genomic DNA from resistant BS-90 (B), susceptible (M) parent snails, and 10 individual resistant progeny. Lane C represents amplification in the absence of template DNA. (*B*) OPM-04 amplification of DNA from resistant (B) and susceptible (M) parents and 10 individual susceptible progeny. Lane S represents amplification of *S. mansoni* DNA, and lane C shows amplification without DNA. (*C*) OPZ-11 amplification of DNA from resistant (B) and susceptible (M) parents and 10 individual resistant progeny. Lane C shows amplification in the absence of template DNA. (*D*) OPZ-11 amplification of resistant (B) and susceptible (M) parent snail DNA and DNA from 10 individual susceptible progeny. Lane S represents amplification of parasite DNA and lane C shows amplification without DNA.

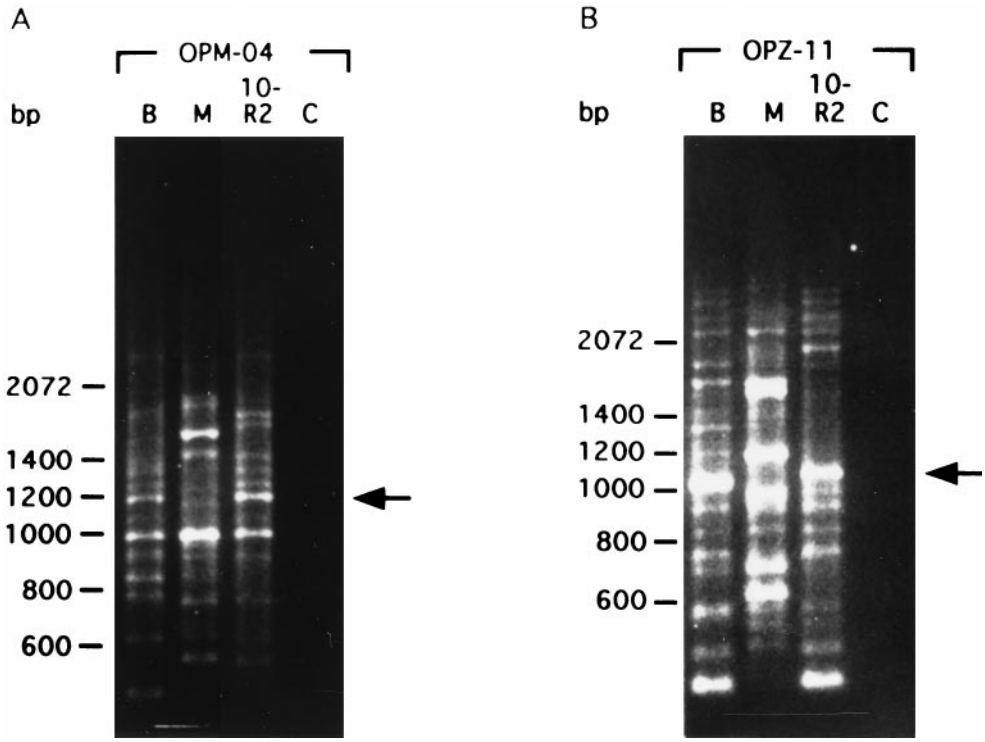

FIG. 3. Amplification of DNA from *B. glabrata* resistant strain BS-90 (B), susceptible strain M line (M), and resistant strain 10-R2 using primers OPM-04 (Fig. 3*A*) and OPZ-11 (Fig. 3*B*). Lane C in both cases represents the amplification in the absence of DNA template.

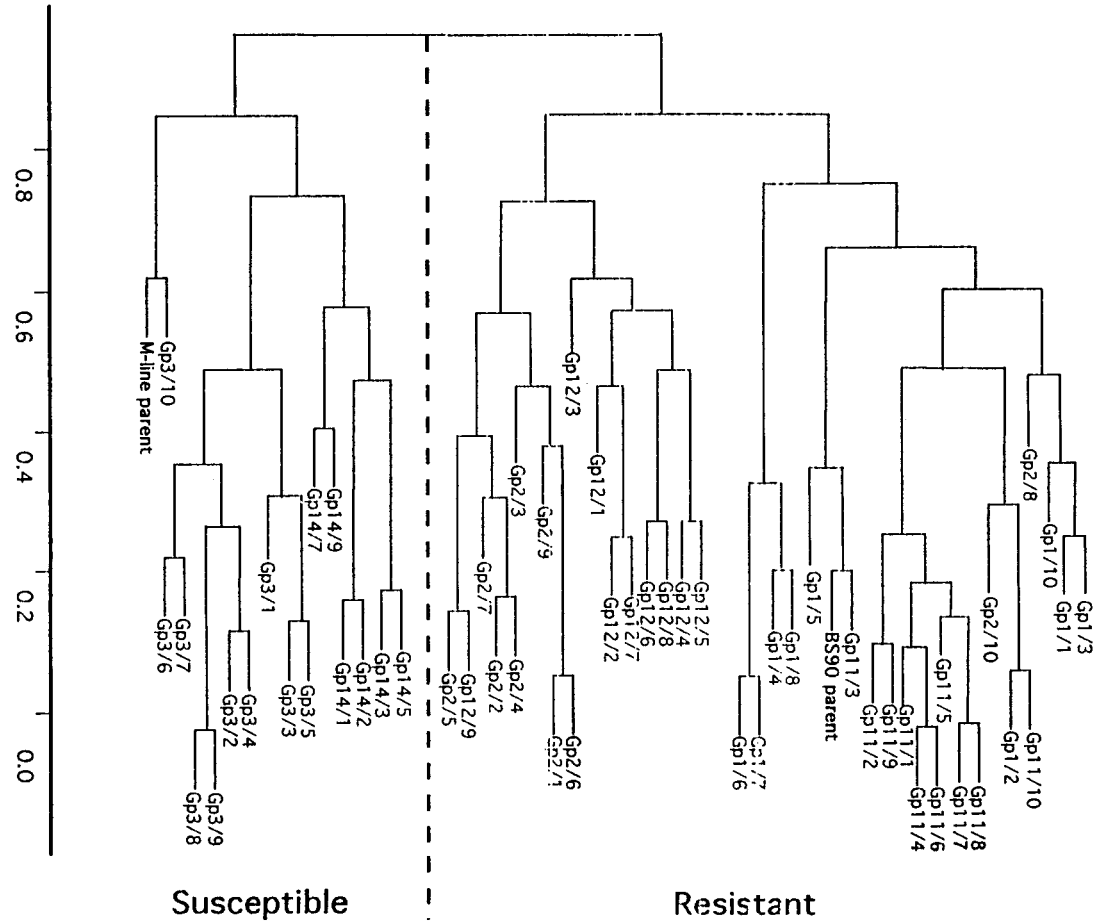

FIG. 4. Cluster analysis of the RAPD data was performed as described in *Materials and Methods*. A total of 30 bands from the 2 RAPD primers (OPM-04 and 0PZ-11) were scored for 56 individuals, including the parent snails and offspring in a blind fashion. The hierarchical cluster analysis was performed with a binary distance matrix calculated from the data with a cluster analysis program that works with S-PLUS 4.0. Those individuals to the right are all resistant to the disease, and conversely, those to the left of the dashed line are susceptible.

edness of the 10-R2 strain bands to the BS-90 resistant snail markers has been verified by PCR analysis. In separate experiments, the 1.2- and 1.0-kb markers were detected in F_2 resistant progeny from a second independent cross between BS-90 and M line snails (cross 2, data not shown).

Cluster Analysis of the RAPD Bands. We performed a hierarchical cluster analysis of the data from the RAPD gels of individual F_2 progeny (Fig. 4). Without knowing the susceptibility phenotype of the snails beforehand, a total of 30 reproducible, prominent bands from the parent snails (identified by the OPM-04 and OPZ-11 primers) were scored for 54 randomly chosen, individual progeny. This blind cluster analysis segregated the F_2 progeny into two major groups. When identifying the phenotypes after cluster analysis was performed, we found that individuals to the right of the dotted line were all nonsusceptible to infection, whereas those to the left were susceptible. Also, the major group displaying the nonsusceptible phenotype further segregated into two approximately equal groups (as determined by a cutoff of 0.8). To assess statistical independence, we performed a χ^2 analysis with the two primer sets. With a significance level of 0.05, the Bonferonni procedure (19) controls the multiple comparison error rate by comparing against $0.05/30 = 0.00167$. The inheritance of the 1.2- and 1.0-kb bands in the individual F_2 progeny snails showed significant 1-df χ^2 values of 29.4 ($P = 5.8 \times 10^{-8}$) and 43.0 ($P = 5.5 \times 10^{-11}$), respectively.

Table 2. Molecular analysis of RAPD markers

Marker, kb	Length, bp	%AT	Copy no.	Homology
1.2	1210	61	28	Novel
1.0	1022	62	40	gag polyprotein

Generation of Sequence-Amplified Characterized Regions. Both the 1.2- and 1.0-kb markers were gel purified, cloned, and sequenced. Results of the molecular characterization of these markers are summarized in Table 2. The use of the cloned markers as probes for Southern blot analysis of snail DNA indicated that both markers represented repetitive elements in the snail genome. A BLAST search of public databases (March 26, 1998) showed no homology of the 1.2-kb marker with existing DNA sequences. However, an imperfect ORF detected in the 1.0-kb marker showed a relationship (45% identity) with a retrovirus-related group-specific antigens (gag) polyprotein (GenBank accession no. P04023). The nucleotide sequences of the 1.0- and 1.2-kb markers have been deposited in GenBank with accession numbers AF078108 and AF078109, respectively.

DISCUSSION

In this study, we combined bulked segregant analysis and RAPD–PCR methodologies to identify two markers that segregate with adult nonsusceptibility in *B. glabrata*, which provided an opportunity to define the target gene(s) responsible for conferring nonsusceptibility to *S. mansoni* in this snail. We envisioned applying the molecular approach here to target the adult nonsusceptibility locus for two reasons: (*i*) previous work by Richards clearly showed the single-gene nature of this character and (*ii*) segregation of the pigment trait, a single, dominant character, allowed us to easily identify hybrid progeny.

The use of DNA-based markers to analyze genotypic differences that reveal whether polymorphic loci are linked to a particular response to an invading pathogen has been widely applied in both plant and animal genetics. For insect vectors of parasites, most notably *Anopheles gambiae* and *Aedes aegypti*, which are responsible for the transmission of malaria and filarial pathogens, respectively, genetic linkage maps based on restriction fragment length polymorphism and microsatellite markers to identify parasite susceptible/resistant genes are being developed (20, 21). It is hoped that genetic transformation of *A. gambiae* with parasite-resistant genes may provide refractory strains that can be used to control the transmission of malaria (22).

In the case of human schistosomiasis, to our knowledge linkage studies with molecular markers has been applied only to the vertebrate host of the infection. In a recent analysis of Brazilian families, a locus on human chromosome 5q31-33 was identified that influences the intensity of infection to *S. mansoni* (23).

Although mechanisms involved in the genetic control of parasite survival in *Biomphalaria* sp. are not completely understood, ample evidence exists of an immunological type of response to infection from studying the cellular and humoral components of the snail's internal defense system. After miracidial penetration of a resistant snail, primary sporocysts can be encapsulated within several hours by host hemocytes and destroyed. This cellular response also involves the interaction of humoral components such as lectins. A cloned fibrinogen-related lectin that is induced in *B. glabrata* in response to echinostome infection was recently described (24).

Age also has an effect on the susceptibility of the snail, and in some cases snails that are susceptible as juveniles become resistant as adults (8). From earlier work, adult resistance is known to be a single-gene trait, but juvenile snail resistance to *S. mansoni* infection is believed to be polygenic, and genes involved may be expressed throughout the life of the snail, masking the presence of the adult nonsusceptibility gene (25). Results from the cluster analysis of the RAPD data showed that the adult resistant progeny segregates into two groups. It is possible that this separation represents, in one group, progeny snails that inherited both combinations of juvenile and adult resistant genes and, in the other, those that inherited only the adult resistant trait.

Because the markers we describe here represent repetitive sequences in the snail genome, it might preclude their use in chromosome-walking experiments to identify more associated sequences. We have, however, identified nonrepetitive regions within the sequences that are being used as probes for screening a resistant snail cosmid library. Sequence-tagged sites generated from positive clones will be used to identify more target sequences associated with the markers identified in this study.

Results showing the presence of the 1.0-kb marker and absence of the parasite specific 1.1-kb band in some susceptible progeny snails indicate that a misscoring of the phenotype might have occurred in these progeny snails. The misscoring of snails as susceptible or nonsusceptible based on microscopic examination for the presence or absence of sporocyts is always possible because, for example, the presence of a granulomatous body in the snail's headfoot could be mistaken for a sporocyst (C. S. Richards, personal communication).

Because there are no genetic maps for *Biomphalaria* sp. snails, it is hoped that RAPD and restriction fragment length polymorphism markers identified from an ongoing search of polymorphic markers among *B. glabrata* expressed sequence tags (18) will form the basis of generating a coherent map of this snail's genome. Genotypes of *B. glabrata* snails collected from different geographical regions have been shown by RAPD–PCR to be highly variable (26). Similarly, variations in the susceptibility of field-collected snails when experimentally exposed to a single strain of parasite have been reported (14). Whether differences in susceptibility patterns reflect either genotypic heterogeneity of *B. glabrata* snails or the reported genetic variation in the parasite population (27, 28) remains unknown. In this study we have shown that the two markers identified are present in resistant strains, BS-90 and 10-R2, and absent in the susceptible stocks, M line and NMRI. These snails represent the best characterized laboratory-maintained resistant and susceptible stocks of *B. glabrata*. The impact of genetic variation among parasite and snail populations on the epidemiology of human schistosomiasis is unknown. We may be able to assess, from the relative frequencies of the markers developed from this study and from other epidemiological information, whether the presence or absence of these markers among field-collected snails has any significance on the dynamics of *S. mansoni* transmission.

In summary, we have identified two markers (1.2 and 1.0 kb) that segregate with adult nonsusceptibility in bulked segregant F_2 and F_1 backcross progeny snails. The molecular characterization and further mapping of regions associated with the 1.2- and 1.0-kb sequences may lead to a better understanding of the molecular interactions between the snail and parasite.

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