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Genetic crosses and linkage mapping in schistosome parasites

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Abstract

Linkage mapping—utilizing experimental genetic crosses to examine cosegregation of phenotypic traits with genetic markers—is now 100 years old. Schistosome parasites are exquisitely well suited to linkage mapping approaches because genetic crosses can be conducted in the laboratory, thousands of progeny are produced, and elegant experimental work over the last 75 years has revealed heritable genetic variation in multiple biomedically important traits such as drug resistance, host specificity and virulence. Application of this approach is timely because the improved genome assembly for *Schistosoma mansoni* and developing molecular toolkit for schistosomes increase our ability to link phenotype with genotype. We describe current progress and potential future directions of linkage mapping in schistosomes.

Keywords

Schistosoma; Linkage; heritability; phenotype; fine mapping; functional analysis

Why linkage mapping?

Schistosomes, and in particular *Schistosoma mansoni*, are unique among human helminths because the complete lifecycle can be maintained in the laboratory using aquatic snail intermediate and rodent definitive hosts. As a consequence, these parasites have been intensively studied over the past 75 years, and there is a rich experimental literature revealing multiple phenotypic traits that show heritable genetic variation among different schistosome lines. These include traits associated with parasite transmission and virulence (cercarial production, pathogenicity to snail and vertebrate host) [1–3], host specificity and co-evolution with snails [4–7], and drug resistance to praziquantel [8–10], oxamniquine and hycanthone [11–13]. An understanding of the genetic and biochemical basis of drug resistance has obvious benefits. This would allow us to determine the mechanism of drug action and to develop improved drugs targeting the same biochemical pathway. We can also exploit genetic markers for tracking resistance spread within populations — now a widely

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used approach to managing resistance in other parasitic diseases [14, 15]. Similarly, traits such as host specificity are fundamental to multiple parasite species, but the genetic and biochemical basis of specificity is poorly understood at the molecular level. Manipulation of interactions between mosquito vectors and the pathogens they transmit is an active field of research [16]. We anticipate that similar approaches may be possible for controlling schistosome transmission, but we first need to understand snail-schistosome interactions at a molecular level.

Linkage (see Glossary) mapping utilizes experimental genetic crosses, followed by genotypic and phenotypic characterization of progeny to identify genome regions where genetic markers co-segregate with phenotypes of interest [17, 18]. This approach to genetic mapping is not new: 100 years ago, Morgan and Sturtevant first developed linkage maps to establish the linear order and relative distance of genes determining visible mutants such as eye color and wing shape on the *Drosophila* genome [19, 20]. Since then, with the advent of dense linkage maps utilizing restriction fragment length polymorphism (RFLP) or simple sequence repeats (SSR), and development of a robust mathematical framework [21], linkage mapping has been widely used for localizing phenotypic traits in organisms ranging from yeast to humans. Among parasitic organisms, linkage mapping has proved to be remarkably successful for parasitic protozoa: characterization of just three controlled *Plasmodium falciparum* genetic crosses has resulted in the identification of major genes underlying resistance to chloroquine, quinine, sulfadoxine, as well as growth rate, red cell specificity, and male gametocytogenesis [22, 23]. Similarly, *Toxoplasma* and trypanosome genetic crosses lead to identification of genes underlying pathogenesis and host specificity [24–27]. This impressive body of work with parasitic protozoa originally inspired us to apply linkage mapping approaches to *S. mansoni*. Two developments now make this approach especially powerful for schistosomes. First, we have genome sequence for *S. mansoni* [28], *S. haematobium* [29] and *S. japonicum* [30]; the *S. mansoni* genome in particular has been refined and assembled [31]. Second, we have a developing molecular tool kit for genetic manipulation of schistosomes with efficient RNAi and transfection approaches [32–34], as well as cell biology tools [35–39]. Hence, we are poised to map genes determining interesting phenotypes to genome regions (quantitative trait loci or QTLs), and to identify causative genes and mutations through functional genomics experiments.

In this review, we describe prospects and progress with linkage mapping in schistosomes. We outline details of the schistosome lifecycle that make this organism uniquely suited to linkage mapping (see Box 1), highlight heritable traits of interest, and describe experimental approaches and study designs for QTL mapping with this organism. To illustrate the utility of this approach we provide published case examples. We also show how linkage mapping can be used to inform genome sequencing and assembly efforts which are critical for linkage analysis. Finally, as linkage mapping is a relatively new approach for schistosome biologists, we summarize exciting future prospects and new directions.

Heritable traits in *S. mansoni*

Host specificity – Schistosome provides a classical example of host parasite co-evolution. As schistosomes castrate their host snails, snail populations are under strong selection to

evolve barriers to infection, while schistosomes are selected to overcome snail host defenses. Linkage analysis should allow us to identify the parasite genes that underlie host specificity as well as the snail genes underlying resistance. *S. mansoni* shows dramatic differences in host specificity both at the intraspecific level to different snail genotypes or populations of *Biomphalaria* spp., and at the interspecific level to different species of *Biomphalaria* [40]. Similarly, *S. haematobium* shows well documented variation in specificity to different *Bulinus* species and populations [4, 41]. Four lines of evidence demonstrate that this variation in host specificity is heritable — i.e. has a genetic basis. (1) Reciprocal infection experiments between parasites and hosts from different geographical regions tend to show higher infection levels between sympatric host parasite combinations demonstrating adaptation of parasites to local hosts [2, 3, 42] but with counter examples [43]. (2) Infection experiments using different laboratory populations of *S. mansoni* isolated against panels of inbred snails show distinctive and highly repeatable infection patterns [5, 6] (Fig. 1A–B). (3) Host specificity can be selected in the laboratory, and response to selection is rapid, demonstrating the genetic variation for host specificity occurs within populations [40]. (4) Genetic crosses show that compatibility with the snail host – along with host finding behavior, another component of host specificity (Fig. 1C) – has a simple recessive inheritance [7]. Genetic crosses between parasites with distinctive patterns of host specificity provide a straight forward approach to locate genes underlying host specificity in *S. mansoni*. Furthermore, the *Biomphalaria* genome has now been sequenced [44] and assembled with the aid of linkage maps [45], and several snail genome regions underlying host resistance have been identified [46, 47]. We are therefore positioned to identify both co-evolving parasite and snail genes using linkage mapping to provide an understanding of host-parasite co-evolution at the molecular level. We note that in malaria parasites (*P. falciparum*), genetic crosses have identified the *Plasmodium* genes that allow parasites to infect owl monkey red blood cells [48], and the genetic basis of mosquito specificity [49, 50]. However, we have no comparable molecular knowledge of host specificity in helminth parasites.

Virulence and transmission – Parasite virulence can be quantified by measuring the impact of schistosome infection on snail mortality and lifetime reproductive success, or by measuring pathogenicity in the rodent host, while transmission potential can be quantified by measuring cercarial production [1, 3, 51]. There is extensive evidence that both virulence and transmission potential are heritable traits. Davies *et al.* [52] generated 5 inbred parasite lines from the PR1 laboratory line (originally isolated from Puerto Rico) showing distinctive patterns of virulence to the snail and rodent host. Intriguingly, they found that low cercarial production was associated with high virulence to snails [52]. This counters theoretical work suggesting that virulence is expected to be a byproduct of increased transmission stage production [53, 54]. They further showed that high virulence to the snail host was associated with lower virulence to the rodent host suggesting a mechanism for maintenance of these traits. Cercarial production responded rapidly to selection in the laboratory, strongly suggesting that this is controlled by genetic factors. For example, five-fold variation in cercarial production was selected in just three generations (Fig. 1D) [1]. Genetic crosses and linkage analysis would provide a straightforward approach to determining the genetic and

molecular basis of both virulence and transmission potential underlying these important parasite traits.

Schistosomes also vary in the time of day at which cercariae are shed [55, 56]. *S. mansoni* infected snails typically shed cercariae in late morning but some parasite populations (Guadalupe, South America and Oman) showing late afternoon or nocturnal shedding, and most likely represent *S. mansoni* adaptation to rodent hosts (Fig. 1E). Genetic crosses between schistosome lines showing distinctive shedding profiles demonstrate that the timing of cercarial release from snails is under genetic control, and that the genetic basis for this differs among populations studied. Similarly, *S. japonicum* show contrasting patterns of cercarial release, with nocturnal shedding in marshy areas where rodents are the primary host [57]. Cercarial shedding profiles are easily quantified by moving single infected snails to new beakers each hour and counting cercariae released. Genetic crosses combined with genotyping or sequencing of progeny and linkage analysis would provide a powerful approach to determining the genetic and molecular basis underlying this fascinating transmission-related trait.

Drug resistance - Widespread deployment of a praziquantel monotherapy for treating schistosome infections raises the spectre of resistance emergence. There is clear evidence that parasites tolerant to praziquantel treatment occur in natural populations [10, 58] and that PZQ-resistant parasites are readily selected in the laboratory [8, 59–61]. In both Egypt and Kenya infections have been found that were not cured after multiple rounds of treatment [10, 58]. Subsequent laboratory studies demonstrated that parasite isolates from these patients showed increased resistance to treatment suggesting that treatment failures are due to parasite factors and that this trait is heritable [10, 58]. In the laboratory, several groups have selected parasites with reduced response to PZQ treatment by treatment of adult worms in the rodent host [8, 59, 61] or of larval parasites in the intermediate snail host [60]. These experiments, and genetic crosses between PZQ-sensitive and resistant parasites [61], clearly demonstrate that PZQ-resistance is a heritable genetic trait. Resistance to two further drugs, oxamniquine and hycanthone, has also been found in natural parasite populations and an elegant series of papers by Cioli and colleagues helped to decipher fundamental aspects of the genetics and biochemistry of resistance to these compounds prior to the sequencing of the *S. mansoni* genome [11] [62]. We use this case example to illustrate the power of linkage mapping approaches. Identification of resistance genes and mutations provide tools for understanding mechanism of drug action, development of modified drugs with improved properties and development of genetic markers for monitoring resistance spread. Linkage approaches have substantial advantages over candidate gene approaches, as we do not need any prior knowledge of the mode of action.

Linkage mapping as a genome assembly tool

Good genome assemblies are essential for linkage mapping, as the aim is to localize genetic determinants of traits to genome regions. However, linkage analyses also provide a powerful approach for guiding genome assemblies and assessing assembly quality, because scaffolds can be placed on chromosomes simply by examining their segregation patterns relative to other genetic markers used in the linkage map. As such, linkage can be used to confirm

order of scaffolds inferred by sequence alignment methods, and provide unambiguous assignment of problem scaffolds to chromosomal positions. We note that successful completion of both the *P. falciparum* and the *Trypanosoma brucei* genome sequences was greatly aided by linkage maps for these species [26, 63]. The initial *S. mansoni* genome assembly (v.4) [28] contained 19,022 scaffolds with 50% assembled into scaffolds of 824.5 kb. Assembly was problematic because ~40% of the genome consists of repetitive regions and transposable elements. To help resolve this problem, we conducted a three generation genetic cross, and genotyped parents, F1s and multiple F2s, using 243 microsatellite markers [64]. We placed these markers in the 203 longest scaffolds in the draft genome to maximize the amount of the genome assembled. Using this strategy we were able to assign 70% of the genome to chromosomes. We also placed 2 microsatellites on 37 of the longest scaffolds. In 16/37 cases, markers on these scaffolds mapped to different chromosomes indicating that they were misassembled. This information was key to the v.5 *S. mansoni* assembly which contained just 885 scaffolds with 50% of scaffolds 2Mb [31] (Fig. 2). Linkage based scaffold placement is considerably more powerful with SNP-based exome or genome resequencing of genetic crosses. Such dense linkage data provides an independent genetic quality check for assembling genomes that complements long read sequence data and optical mapping approaches [65]. This approach has recently been used to assemble a notoriously difficult helminth genome (*Haemonchus contortus*) [66].

Classical linkage mapping in schistosomes

We summarize the identification of the oxamniquine resistance (OXA-R) locus, because this work illustrates the power of linkage mapping approaches [13] (Fig. 3). OXA-R is a dramatic phenotype: resistant worms show ~500-fold reduction in sensitivity to drug exposure. Cioli and colleagues pioneered work on OXA-R using elegant genetic approaches in the 1980–90s prior to the genomic era. Through genetic crosses they demonstrated that oxamniquine resistance was recessive [11], that resistance to oxamniquine and to a closely related drug (hycanthone) involved the same gene [67], while through biochemical analyses they showed that the resistant parasites lacked a critical protein (~30 kDa) with properties of a sulfotransferase [62]. To determine the genome location of the gene involved we staged a genetic cross between an OXA-R parasite (SmHR) and an OXA-sensitive parasite (SmLE), by infecting a hamster with male OXA-R cercariae from a single miracidia snail infection and female OXA-S cercariae from a second single miracidia infection. We then mated F1 progeny to isolate multiple F2 progeny. We measured resistance phenotypes in parents, F1s and F2 progeny, by placing groups of adult worms of a single genotype in wells of a 24 well plate, exposing them to OXA for 45 minutes and plotting survival curves. We can do this with great accuracy, because clonal reproduction within the snail host allows us to recover thousands of single genotype cercariae, which can then be used to infect rodents to obtain adult worms.

Genetic markers for linkage mapping

Dense genotyping of F2 progeny is not a requirement for linkage analysis. In our initial work we were able to localize OXA-R to the end of chr. 6 using just 62 microsatellite markers spaced at ~20 CentiMorgan (cM) intervals across the genome (Fig. 3). However,

whole genome sequence (WGS) data from the two parents involved in the cross can be extremely useful for narrowing down the causative gene(s) underlying the trait of interest. For example, we can prioritize genes showing non-synonymous differences between the parental parasites, or major structural differences (deletions or copy number changes). We use a simple strategy to maximize our power to fine map causative genes, while minimizing genotyping costs. This involves WGS of parental parasites, followed by microsatellite genotyping, or exome sequencing of progeny parasites [68, 69]. The *S. mansoni* exome is ~15 Mb while the genome is 363 Mb [31]. Generating exome data from hundreds of progeny is currently cheaper than WGS and exomes contain ample marker information for linkage analysis. Furthermore, exome sequence can be unambiguously aligned, and is easier to store and manipulate than WGS data.

The power of pooling

Classical linkage mapping requires phenotyping and genotyping of multiple individual parasites and is laborious, time consuming, and expensive. This constrains the numbers of individual progeny analyzed which limits both the statistical power of mapping experiments and the precision with which traits can be mapped. In work on plants, Michelmore *et al.* [70] circumvented this problem by pooling progeny with similar phenotypes. They then quantitatively genotyped genetic markers in the pooled progeny and identified genome regions underlying the traits of interest by searching for regions enriched for alleles from one of the parents. This approach, named bulk segregant analysis (BSA) (Fig. 4), has been revived for trait mapping in malaria parasites where it was rechristened “linkage group selection” [71] and yeast where it was named “extreme QTL” [72]. These modern iterations of bulk segregant analysis utilize selection based approaches (e.g. with drugs) to enrich large progeny pools for different phenotypes, and then compare pools of individuals with extreme phenotypes using microarray or next deep sequencing approaches, that allow for accurate measurement of allele frequencies from read depth.

We examined the utility of BSA methods for mapping the oxamniquine resistance locus in *S. mansoni*. In these experiments we compared pools of F2 adult worms that were either treated with oxamniquine or with drug diluent only. Despite using quite small pools of adult worms (16 adult worms remained in one pool following OXA treatment) we found a QTL with a peak just 200kb (<1 cM) from the known mutation underlying OXA-R [68] (Fig. 4).

BSA methods have pros and cons relative to classical QTL approaches. In addition, to reducing costs and increasing statistical power, BSA approaches are the only viable approach for mapping of some schistosome traits. For example, host specificity is difficult to map using classical approaches, because miracidium larvae that are incompatible with their snail hosts do not establish infections so cannot be genotyped. However, host specificity genes can be mapped using BSA by selecting pools of progeny through compatible or incompatible snails. We are currently using this approach to determine genes underlying compatibility between SmLE and the snail BgBS90, using crosses between SmLE (compatible) and SmBRE (incompatible) (Fig 1a). On the negative side, epistatic interactions between genes cannot be deduced from BSA, because we cannot determine which genotypes at different loci are found together within individual parasites. Using

genotypes and phenotypes from individual progeny and classical linkage approaches however, there is a robust statistical framework for quantifying epistatic interactions [17].

Prospects for interspecific crosses

Crosses between closely related species provide a powerful means to understand traits that underlie species differences [73, 74]. Closely related schistosome species differ in intermediate host snail use [41], in development patterns and adult maturation site within the vertebrate hosts. While reproductive barriers prevent interspecific mating in most organisms, hybridization between schistosomes occurs in nature [75–78]. Furthermore, crosses between species can be staged in the laboratory: an impressive body of work during the 1980 and 1990s demonstrated intercrosses between species within the *S. haematobium* species group (*S. mattheei*, *S. intercalatum*, *S. guineensis*, and several species that predominantly infect livestock *S. bovis*, *S. curassoni*, *S. leiperi* and *S. margrebowiei*) could be maintained until at least the F4 generation and remained viable and fertile [79, 80]. Interspecific crosses remain an exciting but as yet untapped opportunity for genetic analysis of schistosome traits.

One obvious obstacle to conducting crosses is that schistosome species differ in host snail use [41]. Fortunately, *Bulinus wrighti* is susceptible to most members of the *S. haematobium* species group [81]. This snail can be used to transport different species from the field and for maintaining lifecycle and hybrid progeny in the laboratory.

Fine mapping

QTLs identified using linkage analysis are typically large and span multiple genes. For example, in our genetic crosses to identify OXA-R we were able to narrow down the genome region involved to 17 genes [13]. Typically QTL regions are much larger encompassing hundreds of genes [17]. There are several approaches to narrow down the genes involved. For traits important in adult worms (e.g. drug resistance) we expect the genes involved to be expressed in adults, while for traits involved in compatibility with the snail intermediate we would expect genes to be expressed during the miracidia or sporocyst stage. Similarly, candidate genes with amino acid variants distinguishing the two parents, or with segregating indels or copy number variants, can be prioritized as candidates. We were able to use both polymorphism and expression data to prioritize genes within the OXA-R QTL (Fig 3). In this example, we were also able to use two additional cues: previous work determined that the protein required to activate OXA was between 25–35 kDa, and showed the hallmarks of a sulfotransferase [62]. Prioritization of candidate loci is most powerful when multiple lines of evidence are used. For example, searching for amino acid variants within genes may be an unreliable criteria for prioritizing genes if non-coding variants (e.g. promotor variants) underlie the trait of interest.

Population genomic data from natural schistosome populations also provides a powerful resource for prioritizing candidate loci from laboratory linkage mapping studies. For example, scans for signatures of selection can highlight genome regions showing extreme levels of positive selection or population differentiation [82] that might be expected in candidate drug resistance or host specificity loci. While obtaining adult schistosomes is

problematic, we can obtain genome or exome sequence from miracidium larvae from eggs recovered from feces or urine samples [69] and large collections of miracidia from multiple geographical locations are available [83].

Functional validation

Having identified candidate genes for the trait of interest, functional validation is required to validate candidates. Fortunately, the *S. mansoni* molecular toolkit is rapidly increasing in sophistication. RNAi is well established for specific knockdown of gene expression [34, 84] and can be used for knockdown of thousands of genes [38]. In addition, methods for stable transfection and integration of genes have been developed. The current state-of-the-art approach uses modified viral vectors (pseudotyped murine leukemia virus), into which genes of interest and appropriate promoter sequences are inserted [32, 33]. The viral vector DNA is introduced into parasite cells by electroporation of either eggs, schistosomules or adult worms. Levels of integrated genes can then be assessed in the adult worms by quantitative PCR, and integration sites can be determined by sequencing of the 363 Mb genome [33]. Drug selection provides a powerful approach to enrich for transfectants. Incorporation of bacterial resistance genes (against neomycin) into transfection plasmids have been employed for schistosome transfection, and can enrich for transfected parasites [85]. Newer methods such as CRISPR-Cas9 gene editing and knockouts are currently in development for *S. mansoni* and likely to be available soon. Further work is required to develop tools for manipulation of the intramolluscan stages of the parasite lifecycle; this will be critical for understanding genes involved in snail-parasite interactions.

We used two approaches for functional validation of candidate genes determining OXAR. First, we showed that OXA-S parasites became OXA-R when Smp-089320 was knocked down using RNAi (Fig. 3). We also used a simple biochemical assay: homogenates of resistant worms do not bind labelled OXA. However, addition of recombinant protein for Smp-089320 rescued OXA binding, demonstrating that this sulfotransferase enzyme (SmsULT-OR) is key to OXA activity [13]. This biochemical binding assay also revealed the precise mutation responsible. Recombinant proteins with the deletion at amino acid 142, failed to rescue OXA-binding in resistant worm homogenates, demonstrating that this mutation was responsible for the OXA-R phenotype segregating in our cross [13].

Cell and developmental biology methods available for schistosomes are also increasing in sophistication. These include whole worm approaches for examining gene expression localization and co-expression, and single cell methods for determining cell types and development of parasite stages [38]. We expect that these approaches may be extremely useful for understanding the cellular basis of phenotypes likely to have a developmental basis. These include variation in numbers of cercariae produced or the timing of cercarial production.

Concluding Remarks

Multiple schistosome traits are amenable to linkage mapping: we highlight several traits of particular interest (see Outstanding Questions). This review focuses on schistosomes, but the

approaches outlined are also applicable to multiple helminth species. Genetic crosses can be staged for other trematode species, such as liver fluke (*Fasciola hepatica*), using ruminant vertebrate hosts [86]. A genomic sequence is now available [87] and a trait of great economic importance (triclabendazole resistance) is currently the target of genetic mapping efforts. Filarial nematodes are also an attractive parasite for linkage analysis because the complete lifecycle of *Brugia malayi* can be maintained using jird (*Meriones* spp.) vertebrate hosts and mosquito vectors [88]. In particular, bulk segregant approaches provide an attractive route for linkage mapping of helminth species without convenient rodent hosts [89].

Finally, we highlight the potential utility of systems biology using multiple layers for molecular data (e.g. transcriptomics, proteomics, metabolomics) as a target for linkage analysis and an aid to understanding the link between genotype and phenotype. Systems biology datasets are increasingly easy to generate in large numbers of individuals and are particularly well suited to analysis in a linkage mapping framework (see [90, 91] for examples from the malaria literature). Levels of each transcript, protein or metabolites can be mapped to genomic locations to detect expression (eQTLs), protein (pQTL) or metabolite (mQTL) QTLs, while single cell approaches promise to refine these data to the level of different parasite tissues. The interplay between these multiple layers of data will provide a powerful approach to understand the intermediate steps linking genotype and phenotype for important schistosome traits.

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Glossary

Miracidia

short-lived, swimming larval schistosomes that actively locate and infect snail intermediate hosts (aquatic snails)

Sporocyst

Intramolluscan larval stages: a globular primary sporocyst develops from each infecting miracidium. This divides clonally to produce multiple daughter sporocysts, which each produce 100–1000s of cercariae

Cercariae

short-lived, free-living, motile larval schistosomes that are released from the aquatic snail host. These locate and penetrate vertebrate hosts

Genotype

Genetic description of organism: genetic markers commonly used to construct genotypes are microsatellite loci, or single nucleotide polymorphisms (SNP) from exome or whole genome sequence

Exome sequencing

sequencing of protein coding genome regions only, following enrichment using oligo based exome capture

Phenotype

Measurable characteristics of organisms: these include morphological (e.g. size, shape, color), behavioral (e.g. virulence, response to host cues) and biochemical (e.g. drug resistance, gene expression) traits

Segregation

the manner in which genetic markers or phenotypes are inherited between generations

Linkage

When a genetic marker segregates with a phenotype in a genetic cross in violation of Mendel's law of independent assortment. This indicates that they are inherited together on the same chromosome. Two genetic markers that co-segregate are also inferred to be located close together on the genome: this is used for making genetic (linkage) maps

CentiMorgan (cM)

A measure of the proximity of two markers on a chromosome based on recombination. When two markers show discordant segregation at 1/100 progeny, these two markers are separated by 1 cM

Quantitative trait locus (QTL)

A genome region, often spanning multiple genes, containing genetic markers that showing statistical linkage to a phenotype

LOD

a statistical measure (Log10 of the Odds) to determine the likelihood of linkage between marker and phenotype (or between two markers)

Monogenic vs polygenic

traits determined by a single locus are monogenic, while traits determined by two or more loci are polygenic

Recessive vs Dominant

describes the inheritance pattern of phenotypic traits. Dominant traits are expressed in heterozygote or homozygote genotypes, while recessive traits must be homozygous for the trait to be expressed

Bulk Segregant Analysis (BSA)

Linkage mapping by comparing populations of progeny with similar phenotypes. Quantitative genotyping is used to measure allele frequency in progeny pools. Differences between pools in allele frequency are used to identify QTL regions

Extreme QTL / Linkage group selection

Synonymous terms for BSA when selection (e.g. using drug treatment) is used to enrich progeny pools for particular phenotypes

Systems biology

Quantitative analysis of complex interactions within biological systems. Such analyses may utilize multiple layers of molecular data from the transcriptome, proteome, metabolome and epigenome

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Highlights

- Schistosomes are experimentally tractable organisms and show heritable variation in a wide variety of biomedically important and biologically interesting phenotypes
- The schistosome lifecycle can be maintained in the laboratory and a good genome sequence and genome assembly is now available, so we can stage genetic crosses and use linkage analysis to locate genes and mutations underlying key parasite traits.
- Early successes include the identification of the gene and mutations underlying oxamniquine resistance and the mode of action of oxamniquine: we are now using this information for rational redesign of OXA derivatives.
- Linkage information also provides a powerful approach for improving genome assemblies and provides a valuable and underutilized complement to bioinformatics methods.
- Bulk segregant approaches using pooled progeny, and next generation sequencing for allele frequency estimation now allow powerful alternative approaches for gene location, and are uniquely suited to understanding host specificity.

Box 1.**The Schistosome lifecycle through the eyes of a geneticist**

Schistosomes have a complex life cycle involving both a snail intermediate and a vertebrate definitive host, but from a genetics standpoint these trematodes are standard diploids, with two sexes, just like *Drosophila* or mice. We can therefore apply experimental and statistical frameworks developed for these model organisms to schistosomes. However, several aspects of the schistosome lifecycle are worth noting, as they appear custom designed for experimental genetic crosses and linkage analysis (see Figure I).

- Schistosomes show a catholic host range, naturally infecting both humans and rodents (A). This makes schistosomes one of very few major human parasites that can be maintained across the complete lifecycle in the laboratory. All stages of the *S. mansoni* life cycle can be maintained in the laboratory using *Biomphalaria* spp. snails and mouse or hamster vertebrate hosts. In this respect, schistosomes have many advantages over malaria parasites (*P. falciparum*) in which only blood stage culture is well established, and genetic crosses require splenectomised chimpanzees or humanized mice transfused with human red blood cells [92], to complete the liver stage of the lifecycle.
- *S. mansoni* females (A) produce hundreds of eggs per day (B), so the number of progeny that can be analyzed is extremely large. As a consequence we can use statistically powerful study designs for QTL mapping of phenotypic traits [93].
- Clonal reproduction of parasites within the snail (C) allows replicate measurement of phenotypes in thousands of genetically identical cercariae (D) or adult worms (A). This natural facet of the trematode lifecycle allows us to obtain extraordinarily accurate measures of phenotypic variation. In contrast, researchers working on more standard genetic model systems (e.g. mice) are forced to use recombinant inbred lines to achieve the same end result.
- Sex is genetically determined, so PCR based sexing of cercariae (D) is possible [94, 95], which simplifies staging of genetic crosses. The recent development of markers for sexing *S. haematobium* now greatly simplifies linkage analyses in this species [96].
- Cercariae (D) can be induced to form schistosomula *in vitro* and then be cryopreserved in liquid nitrogen [97]. Later, the schistosomula can be revived and used to infect mice or hamsters. This allows genetic crosses can be repeated if further progeny are required for reexamination of new phenotypes or detection of additional recombination events.

Figure 1. Heritable phenotypes in schistosomes.

(A and B) Host specificity of *S. mansoni* to the snail intermediate host (redrawn from [6]). (A) Infection rates of a Brazilian snail (BgBRE) challenged with miracidia from two different *S. mansoni* lab populations (SmBRE and SmGUA), with doses ranging from 1–50 miracidia/snail. The sympatric Brazilian *S. mansoni* (SmBRE) infection rate reaches 100% with 10 miracidia, but parasites from Guadalupe (SmGUA) are unable to infect BgBRE. Similarly, (B) shows infection rates of another Brazilian snail (BgBS90) challenged with miracidia from two different *S. mansoni* lab populations (SmBRE and SmLE). Only SmLE, from Puerto Rico) is able to infect BgBS90. Note that infection rates of SmLE plateau at ~50% indicating that this snail population is polymorphic for susceptibility to SmLE. (C) Larval response to snail odors (data from [7]). The behavior of miracidia to host snail

species varies between *S. mansoni* populations. Egyptian *S. mansoni* (SmEG) respond strongly (showing rapid change in swimming direction) only to their sympatric host snail (*B. alexandrina*), while Brazilian *S. mansoni* are indiscriminate and respond to *Biomphalaria* spp. from both the New and Old World and even to unrelated species (*Lymnaea*). Genetic crosses [7] suggest recessive inheritance of snail specific behavior in SmEG. (D) Cercarial shedding intensity. *S. mansoni* (SmPR1) show rapid change in numbers of cercariae shed in response to laboratory selection for high or low shedding (redrawn from [1]). Surprisingly, high levels of virulence to the snails is associated with low rather than high cercarial shedding. The rapid response to selection of both cercarial shedding intensity and virulence strongly suggest that these traits are heritable. (E) Cercarial shedding time in omani *S. mansoni* (redrawn from [55]). A parasite population primarily recovered from rodents (grey shading) shows a nocturnal shedding of cercaria larvae from the snail (*B. pfeifferi*), while populations infecting humans (no shading) show a normal diurnal shedding of cercaria larvae. Genetic crosses indicate that this trait is heritable [56] in other population studied.

Figure 2. Genetic crosses and linkage information aid genome assembly.

A *S. mansoni* genetic map [64] generated using a genetic cross allows unordered scaffolds assembled from short-read genome sequence to be anchored and ordered to chromosomes (only chr. 6 is shown here). Neighboring scaffolds can then be stitched together using additional sequence data (adapted from [31]).

Figure 3. Classical linkage mapping of oxamniquine resistance.
(A) Linkage analysis. Measurement of OXA resistance and genotyping of individual worms from a two generation cross (insert) resulted in unambiguous mapping (LOD = 31, dotted line shows genome wide threshold for significance) of a single genome region underlying OXA resistance (red line). When the marker showing the highest LOD is used as a cofactor in the analysis (blue line) no additional peaks are observed, suggesting that OXA-R is a monogenic trait. (B). This genome region contains 17 genes (Red and blue genes are transcribed in opposite directions) (C). We narrowed down this list to one gene (Smp_089320, red outline; Y: Yes, N: No) by searching for genes that show amino acid differences between the parents, are expressed in adult worms, and encode for proteins of a size consistent with previous biochemical work [62]. (D). Functional analysis: RNAi

knockdown (left panel: control RNAi treatment; right panel: RNAi targeted against Smp_089320) of OXA-sensitive parasite demonstrate involvement of Smp_089320, a sulfotransferase. Figure adapted from [13].

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Figure 4. Schistosome linkage analysis using Bulk Segregant analysis (BSA).

(A). BSA using pooled F2 progeny – in this case one pool is selected while a second pool is left unselected. Pools of surviving parasites are then quantitatively genotyped across the genome. This can be done by genome or exome sequencing of pools, and estimation allele frequencies from read depth of variable sites. (B). Validation of BSA methods for detecting OXA resistance. Plot reveals genome regions that show significant ($-\log_{10}$ p-value) differences in alleles frequencies between pools of parasites that were either treated with OXA or treated with drug diluent only [68]. The peak observed in chr. 6 is within 1 cM of the locus mapped using classical QTL methods [13]. Figure redrawn from [68] using version 7 of the *S. mansoni* genome. Alternating gray and white shading marks chromosome boundaries, while unassembled genome regions are shown in black.

Figure I.
(in Box 1). Key lifecycle features relevant to linkage mapping.