Genetic Indicators of Drug Resistance in the Highly Repetitive Genome of Trichomonas vaginalis

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Abstract

Trichomonas vaginalis, the most common nonviral sexually transmitted parasite, causes ~283 million trichomoniasis infections annually and is associated with pregnancy complications and increased risk of HIV-1 acquisition. The antimicrobial drug metronidazole is used for treatment, but in a fraction of clinical cases, the parasites can become resistant to this drug. We undertook sequencing of multiple clinical isolates and lab derived lines to identify genetic markers and mechanisms of metronidazole resistance. Reduced representation genome sequencing of ~100 T. vaginalis clinical isolates identified 3,923 SNP markers and presence of a bipartite population structure. Linkage disequilibrium was found to decay rapidly, suggesting genome-wide recombination and the feasibility of genetic association studies in the parasite. We identified 72 SNPs associated with metronidazole resistance, and a comparison of SNPs within several lab-derived resistant lines revealed an overlap with the clinically resistant isolates. We identified SNPs in genes for which no function has yet been assigned, as well as in functionally-characterized genes relevant to drug resistance (e.g., pyruvate:ferredoxin oxidoreductase). Transcription profiles of resistant strains showed common changes in genes involved in drug activation (e.g., flavin reductase), accumulation (e.g., multidrug resistance pump), and detoxification (e.g., nitroreductase). Finally, we identified convergent genetic changes in lab-derived resistant lines of Tritrichomonas foetus, a distantly related species that causes venereal disease in cattle. Shared genetic changes within and between T. vaginalis and Tr. foetus parasites suggest conservation of the pathways through which adaptation has occurred. These findings extend our knowledge of drug resistance in the parasite, providing a panel of markers that can be used as a diagnostic tool.

Key words: Trichomonas vaginalis, sexually transmitted infection, comparative genomics, genetic association study, antimicrobial drug resistance.

Introduction

Trichomonads are haploid unicellular microaerophilic members of eukaryotic phylum Parabasalia that infect a variety of vertebrates including humans, wildlife, farm animals, and pets (Maritz et al. 2014). Among the several important species in this group is Trichomonas vaginalis, which causes trichomoniasis, the most common nonviral sexually transmitted disease in humans, with 3.7 million cases reported each year in the US (Center for Disease Control and Prevention 2013). T. vaginalis can cause long-term symptomatic infections of vulvar and urethral regions of the genital tract (Petrin et al. 1998). Importantly, T. vaginalis infection is associated with increased risk of HIV infection (McClelland et al. 2007), development of prostate cancer (Sutcliffe et al. 2012; Twu et al. 2014), and complications during pregnancy, such as premature and low-weight births (Cotch et al. 1997). Another trichomonad, Tritrichomonas foetus, is an economically important cattle parasite, and while only distantly related to T. vaginalis, it nevertheless has an analogous site of infection and disease etiology (Yule et al. 1989; Foote 1996; Frey and Muller 2012).
A highly fragmented assembly of the ~160 Mb *T. vaginalis* strain G3 genome generated by Sanger sequencing was published in 2007 (Carlton et al. 2007). It consists of ~17,000 scaffolds containing ~60,000 predicted protein coding genes, many of them members of high copy number gene families, and ~40,000 transposable element (TE) genes distributed among ~59 TE families, members of which are highly similar (average ~97.5% identity) within a family. Identification of meiosis-specific genes in the genome suggested that the parasite might have a cryptic sexual cycle or may have recently lost the ability to undergo genetic exchange (Carlton et al. 2007; Malik et al. 2008). Genetic markers mined from the genome have been used in population studies of the species (Conrad et al. 2011; Cornelius et al. 2012), revealing significant *T. vaginalis* genetic diversity that is structured but unrelated to geographical origin (Conrad et al. 2012). Recently, a genome assembly for *Tr. foetus* strain K has been published, that reveals its genome, too, is also highly repetitive (Benchimol et al. 2017).

Metronidazole (Mz) and tinidazole are the only two United States Food and Drug Administration approved drugs effective against *T. vaginalis* (de Brum Vieira et al. 2017). However, clinical failure of Mz treatment has been reported since 1959 (Watt and Jennison 1960) and ranges from ~4% in the US (Kirkcaldy et al. 2012) to 17% in Papua New Guinea (Upcroft et al. 2009). Mz is a produg, which must be reduced at its nitro group in order to form the nitroradical anions that are toxic to the parasite. The electrons required for Mz activation are generated by several mechanisms, including the reduction of pyruvate by pyruvate:ferredoxin oxidoreductase (PFO) (Yarlett et al. 1986a,b; Brown et al. 1999; Kulda 1999), an enzyme found in membrane-bound organelles called hydrogenosomes that produce ATP and hydrogen, as well as several pathways hypothesized to exist in the cytosol (Leitsch et al. 2009). *Tr. foetus* is here again similar to *T. vaginalis* in displaying both Mz resistance and related hydrogenosomal metabolic pathways (Kulda 1999 and Kulda et al 1984).

Aerobic and anaerobic pathways of Mz resistance have been proposed. Clinical (aerobic) resistance is typically observed only in the presence of oxygen, which decreases Mz toxicity by re-oxidizing nitroradical anions before they can damage the parasite, reverting Mz to an inactive form. Clinically resistant *T. vaginalis* strains have impaired oxygen scavenging activity and elevated levels of intracellular oxygen. Oxygen stress response genes are thought to be involved in aerobic Mz resistance, with *NADH-dependent oxidase and flavin reductase* (FR) in particular being implicated as important enzymes involved in oxygen removal (Leitsch et al. 2012, 2014). In contrast, anaerobic resistance has typically been observed only *in vitro* (Upcroft and Upcroft 2001a,b), with the exception of strain B7268 where anaerobic resistance developed in a patient (Voolmann and Boreham 1993). Anaerobically resistant strains are marked by the absence of certain hydrogenosome metabolic enzymes that would normally activate Mz, including *PFO* and *ferredoxin* (Fd) (Yarlett, et al. 1986a; Kulda et al. 1993; Land et al. 2004), as well as *cytosolic thioredoxin reductase* (*TrxR*) (Leitsch et al. 2009). Aerobic and anaerobic resistance pathways are proposed to be fundamentally different, although FR, whose primary role is to reduce molecular oxygen to hydrogen peroxide, appears to be involved in both processes (Leitsch et al. 2014). To date, studies of the mechanism of Mz resistance have focused wholly on candidate genes known to be involved in oxygen scavenging or hydrogenosome metabolism, e.g., *PFO*, Fd, FR, and *TrxR* (Kulda 1999; Upcroft and Upcroft 2001b; Pal et al. 2009; Leitsch et al. 2010, 2014). However, alterations in these genes do not explain all cases of Mz resistance, nor have other genes been identified as possible candidates. The aim of our study was to address these issues by large-scale genetic comparison of Mz sensitive and resistant strains.

High-throughput methods for large-scale genetic comparison and analysis of mutational phenotypes are particularly difficult to implement in trichomonads. The highly repetitive nature of the *T. vaginalis* and *Tr. foetus* genomes makes them refractory to conventional short-read genome sequencing. We therefore employed double digest restriction-site associated DNA sequencing (ddRAD-Seq) to genotype 102 *T. vaginalis* isolates from eight countries, the largest sampling of *T. vaginalis* genomes reported to date. We identified single nucleotide polymorphisms (SNPs) in the sequences in order to explore population structure and genome-wide linkage disequilibrium (LD) in the isolates, and provide a basis for a genome-wide association study of Mz genetic indicators. In addition, we compared laboratory-derived Mz resistant isolates with their isogenic parents to identify SNPs that could indicate Mz resistance pathways common to resistant lines. Because there is no efficient high-throughput transfection system for large-scale assay of mutational phenotypes in *T. vaginalis*, we conducted whole transcriptome analysis of gene regulation in resistant and sensitive isolates, as a proxy for direct functional testing. Finally, to explore the universality of the genetic bases of Mz resistance among trichomonads, we analyzed genetic changes between a parental strain and *in vitro*-derived Mz resistant lines of *Tr. foetus*.

**Results**

We used ddRAD-Seq to partially sequence genomes of 102 *T. vaginalis* clinical isolates from eight countries (supplementary fig. S1 in supplementary file S1, Supplementary Material online). Sequence reads were filtered, aligned, and single nucleotide polymorphisms (SNPs) were identified (supplementary file S2, Supplementary Material online; see “Materials and Methods” for filtering criteria). Our final set of 3,923 high quality SNPs comprised 1,463 nonsynonymous mutations, 879 silent (synonymous) mutations, 10 nonsense mutations, and 1,571 intergenic mutations.
Population Structure and Fast LD Decay Suggest Recombination in \textit{T. vaginalis} Genomes

Population structure and linkage disequilibrium (LD) properties play a key role in determining success of mapping genotypes to phenotypes (Mu et al. 2005). The existence of genetic subpopulations and the occurrence of LD decay over short distances are strong indicators of genetic recombination and sexual reproduction in a population (Tibayrenc and Ayala 2002; Halkette et al. 2005), with implications for correlating genotypes to drug resistance. To characterize population structure, we performed principal components analysis (PCA) of SNPs from 102 \textit{T. vaginalis} isolates (fig. 1). The first two principal components accounted for the highest variation within the sampled isolates and explained 10.1 and 6.1\% of the variation, respectively. Similar to published findings, these two PCA axes split the global sample into two subpopulations (Conrad et al. 2012; Bradic et al. 2014), a structure congruent with sexual reproduction. Population structure did not correspond to the geographical origin of the isolates.

In asexual populations, LD is maintained over long genomic distances or even over the entire genome, while genetic exchange in sexually reproducing populations constrains LD to shorter distances. We plotted LD decay along the 898 genomic contigs containing 2,837 SNPs in one population, and along the 872 contigs containing 2,694 SNPs in the other population, and found decay occurring within 1\,kb in both populations (fig. 2), although the rate differed between the two. These results, together with the presence of population structure, provide strong evidence that genetic exchange occurs, or occurred sometime in the recent evolutionary past, of both \textit{T. vaginalis} populations. The difference in LD decay rates indicates potential differences in recombination, population size, or phenotypic traits (e.g., transmission, drug resistance, virulence) between the two populations.

Association Study of \textit{T. vaginalis} Isolates Identifies Candidate Biomarkers of Mz Resistance

Our findings suggested (1) that population structure should be accounted for in \textit{T. vaginalis} association studies in order to avoid spurious genotype-to-phenotype association; and that (2) fast LD decay allows for mapping of phenotypic traits in candidate regions. We first tested for association between \textit{T. vaginalis} population structure and resistance to metronidazole (Mz) as measured using a standard minimum lethal concentration (MLC) protocol under aerobic conditions (supplementary file S2, Supplementary Material online). Of the 95 isolates for which MLC could be determined, 63 were classified as “sensitive” and 32 as “resistant” (MLC $\geq 100$ $\mu$g/mL), of which 23 were also “highly resistant” (MLC $\geq 400$ $\mu$g/mL). Neither of the two subpopulations detected by PCA of ddRAD SNPs was significantly associated with Mz resistance (Pearson’s chi-squared test for MLC $\geq 100$ $\mu$g/mL: $\chi^2 = 0.2139$, df = NA, $P$-value = 0.8136; for MLC $\geq 400$ $\mu$g/mL: $\chi^2 = 0.2291$, df = NA, $P$-value = 0.7271; fig. 1).
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We next used Discriminant Analysis of Principal Components (DAPC) (Jombart et al. 2010) to identify SNPs that distinguish Mz resistant from Mz sensitive isolates, and quantified the contribution of each allele to this distinction (fig. 3). Our analysis showed partial overlap of alleles between sensitive and resistant groups (fig. 3A). We identified the SNPs that contribute the most to distinguishing sensitive from resistant phenotypes (fig. 3B). Among these, we identified a set of 72 SNPs associated with moderate or high Mz resistance (MLC \( \geq 100 \mu g/ml \) or \( \geq 400 \mu g/ml \), supplementary file S3, Supplementary Material online). Along with multiple silent (synonymous) and intergenic SNPs, we identified 16 nonsynonymous SNPs associated with moderate resistance in 13 genes (e.g., TVAG_197160 *CAMK* family protein kinase, TVAG_158720 Bspa-like protein), and two nonsynonymous SNPs in two conserved hypothetical genes (TVAG_124910, TVAG_493120) associated with both moderate (MLC \( \geq 100 \mu g/ml \)) and high (MLC \( \geq 400 \mu g/ml \)) resistance. A summary of these genes and their SNPs is shown in supplementary file S3, Supplementary Material online.

**SNP Changes in Lab-derived Mz Resistant *T. vaginalis* Lines Suggest Similar In Vivo and In Vitro Mechanisms of Drug Resistance.**

While the 72 SNPs we found associated with moderate and high resistance in disparate clinical isolates represent a potentially important set of genetic markers of the resistance phenotype, such association does not demonstrate causation. To identify SNPs that may be more strongly inferred to be consequences of adaptation to drug treatment, we exploited existing pairs of *T. vaginalis* lines, each consisting of a highly drug-resistant line derived *in vitro* from a naturally drug-sensitive or moderately resistant parental line by increasing drug selection pressure over time (supplementary file S1 and fig. S2, Supplementary Material online). We analyzed SNPs observed in ddRAD-Seq data from three such pairs of parental and derived lines: (1) Mz-sensitive B7708 and highly resistant B7708-M; (2) Mz-sensitive F1623 and highly resistant F1623-M (Brown et al. 1999); and (3) moderately resistant B7268 and highly resistant B7268-M (Wright et al. 2010). SNPs were identified by comparing the parental and derived line to the reference genome of the Mz-sensitive lab strain G3. For each pair, only bases that were the same in the parental line and the G3 reference but different in the derived strain were considered as SNPs (see “Materials and Methods”). We found 39 nonsynonymous SNPs that were common to all three derived, highly resistant lines, in 36 genes (fig. 4, supplementary file S5, Supplementary Material online). Changes common to two out of three derived lines included seven nonsynonymous mutations in seven genes in B7708-M and F1623-M; 137 nonsynonymous (including one nonsense) SNP in 141 genes in B7708-M and B7268-M; and 170 nonsynonymous and two nonsense SNPs in 155 genes in F1623-M and B7268-M. We also identified multiple synonymous and intergenic SNPs in common between the pairs (data not shown).

Of particular note are five genes (TVAG_124910, TVAG_197160, TVAG_226650, TVAG_393400, and TVAG_465880) containing nonsynonymous SNPs that were (1) present in at least two lab-derived resistant strains, and (2) associated with Mz resistance in our association study of \( \sim 100 \) clinical isolates described above. TVAG_197160 encodes a predicted *CAMK* kinase family protein...
above, while the rest encode conserved hypothetical proteins, one of which (TVAG_465880) contains multiple SNPs shared between the derived and clinical isolates. These five genes represent novel candidates for roles in both clinical and in vitro-derived Mz resistance. A summary of the genes and their SNPs described in this section is shown in supplementary file S4, Supplementary Material online.

Gene Expression Changes Are Associated with Resistance and SNPs in Clinical and Lab Derived Isolates

We employed whole transcriptome analysis to survey changes in gene expression associated with Mz resistance. RNA-Seq libraries in triplicate were sequenced from nine Mz-sensitive isolates (reference strain G3 and GOR69, NYCA04, NYCB20, NYCD15, NYCE32, NYCF20, NYCG31, and SD2), and three resistant isolates (moderately resistant clinical isolate B7268, its highly resistant in vitro derivative B7268-M, and highly resistant clinical isolate NYCC37). Of the 57,796 predicted protein-coding genes in the T. vaginalis G3 genome (excluding TE genes), a total of 28,919 (≈50%) showed evidence of expression (supplementary file S6, Supplementary Material online), a similar number as reported in a previous transcriptome study of T. vaginalis strain TO16 (Gould et al. 2013). A wide range of gene expression was seen between isolates (PC1, 22.38% and PC2, 14.35% variation explained), with the drug resistant pair B7268 and B7268-M clustering together and separately from the other isolates (fig. 5A). It is worth mentioning that a large proportion of gene expression diversity was apparent due to variation in paralogous genes (data not shown).

Fig. 4.—Common genetic changes in laboratory-derived T. vaginalis lines. Venn diagram circle sizes are based on numbers of genes that have nonsynonymous SNPs. A nonsynonymous SNP was defined as a position where the nucleotide in the ancestral strain was the same as in the reference G3 strain, but different in the derived (more resistant) strain.

Fig. 5.—Differential gene expression in laboratory-derived and clinically resistant T. vaginalis versus sensitive lines. (A) Principal components analysis of 12 isolates and their replicates based on normalized gene read counts. Replicates are indicated as shapes and different isolates as colors.
To identify genes whose expression is most strongly associated with Mz resistance, we compared each of the three resistant strains with the group of nine sensitive strains. Mz resistant strains B7268 and B7268M shared the highest number of pairwise down- (1208) and up-regulated genes (568) (supplementary file S6, Supplementary Material online), as expected given their common ancestry. All three resistant strains shared 28 upregulated and 162 downregulated genes (FDR = 0.05; fig. 5B and C and supplementary file S6, Supplementary Material online). Comparing these genes to results from our two SNP studies described above, we identified conserved hypothetical gene TVAG_465880 that was upregulated in at least two Mz-resistant lines (B7268M and B7268) and was also among the five novel candidate genes flagged in both of the studies. We also identified nine genes with a nonsynonymous SNP that were either up- or down-regulated in the experimentally derived B7268M isolate compared to its B7268 parent. For example, dynein heavy chain protein (TVAG_188120) had a nonsynonymous SNP and expression of the gene was downregulated in B7268M, strongly suggesting its involvement in drug resistance. Another example is a hypothetical gene (TVAG_258020) with one nonsynonymous SNP and downregulation of the gene in both B7268M and clinical strain NYCC37. A summary of the genes and their SNPs described in this section is shown in supplementary file S4, Supplementary Material online.

**Mz Resistance Is Associated with Regulation of Genes Involved in Drug Activation, Accumulation, and Detoxification**

We first asked if Mz resistance is more likely to occur by drug inactivation or cell repair processes by testing if the number of downregulated genes (fig. 5A) is significantly higher than the number of upregulated genes (fig. 5C). We established that the number of downregulated genes was significantly higher in all the comparisons, suggesting that Mz resistance predominantly occurs through processes that restrict drug activation. Next, we tested for association between the set of 162 downregulated and 28 upregulated genes identified as shared between the three Mz resistant strains and the Mz resistance phenotype by cluster analysis (fig. 6). Not only did the gene expression data cluster by phenotype, but the association was significant (chi-square test, \( P = 2.76 \times 10^{-8} \)).

Within our Gene Ontology (GO) enrichment analysis of the 162 downregulated genes, oxidoreductase, catalytic, and acid phosphatase activities were identified as the most enriched molecular processes (P-value < 0.05). These processes are involved in electron transfer and thus likely highly relevant to the activation of Mz (supplementary file S6 and table S6, Supplementary Material online). For example, *T. vaginalis* oxygen tolerance pathways are important mediators of Mz reduction (Yarlett et al. 1986b; Ellis et al. 1992; Rasoloson et al. 2001). We observed changes in multiple genes associated with oxygen detoxification (supplementary file S6, Supplementary Material online), including in the *flavin reductase* gene *FRT* (TVAG_517010), identified as downregulated in our three Mz resistant strains. It has been suggested that nitrosoimidazol, the reduced form of Mz, can form covalent bonds with cellular proteins such as cytosolic malate dehydrogenase (MDH) and *thioredoxin reductase* (*TrxR*) (Leitsch et al. 2009). We observed reduced expression of two variants of *MDH* (TVAG_354940 in all three resistant isolates, TVAG_412220 in B7268 and B7268M) suggesting their potential role in resistance. As a probable consequence of reduced *MDH* expression, we also detected an increase in *lactate dehydrogenase* (TVAG_381310) activity in B7268 and B7268M, which was reported previously (Leitsch et al. 2009). The *superoxide dismutase* (SOD) gene family is also involved in scavenging oxygen reactive species as part of an oxygen defense mechanism in *T. vaginalis* (Leitsch et al. 2009). We identified two downregulated paralogs of *SOD* (TVAG_039980, TVAG_079600) in B7268 and NYCC37 while comparing resistant and sensitive strains. Several other oxidoreductase genes that have not been previously reported were downregulated in the resistant strains (TVAG_414030, TVAG_044820, and TVAG_411220). Other proteins that might contribute to removal of reactive oxygen and help in establishing resistance are the *thioredoxin* genes (*Trx*). Paralogs of these genes were upregulated either in B7268 and B7268M (TVAG_277410) or in all three resistant strains (TVAG_253800). Finally, among the eleven Mz activating *nitroreductase* (*ntr*) genes, we observed lower expression of *ntr6* (TVAG_354010) in Mz resistant strains B7268 and B7268M, and downregulation of *ntr7* (TVAG_474290) (Pal et al. 2009; Paulish-Miller et al. 2014), and two other genes from the *ntr* family (TVAG_303860, TVAG_473580) in NYCC37. The *ntr* gene (TVAG_499730) whose protein product is found in the hydrogenosome (Beltran et al. 2013) and is involved in oxygen detoxification, was downregulated in all three resistant strains. The hydrogenosome is a key site of accumulation and activation of Mz. Of 359 *T. vaginalis* proteins predicted to be localized to the organelle (Garg et al. 2015) our data revealed down-regulation of several of them in the resistant strains, such as *iron-sulfur flavoproteins* (TVAG_04030, TVAG_154730, and TVAG_370510) (Smutna et al. 2014), *iron hydrogenase* (TVAG_182620), *alcohol dehydrogenase* (TVAG_328940), *malic enzymes* (TVAG_412220, TVAG_340290, TVAG_238830, and TVAG_416100), and...
ruberythrin (TVAG_064490; supplementary file S6, Supplementary Material online). In addition, of the three hydrogenosome-localized nitroimidazole reductase (Nim) paralogs that code for a protein associated with Mz detoxification in the reference strain G3, (TVAG_356810) was upregulated in resistant strains B7268 and B7268M, confirming previous functional studies that showed overexpression of this gene in Eschericia coli inactivates Mz (Pal et al. 2009). However, Nim1 (TVAG_005890) was downregulated in all three resistant strains, making its role less unclear.

Of the 28 upregulated genes found in all three resistant strains compared to the sensitive strains, 14 were enriched for antiporter and drug transmembrane transporter GO functional processes ($P < 0.05$; supplementary files S4 and S6, Supplementary Material online). Multidrug resistance pump (MDR) (TVAG_291970, TVAG_210540) and metal ABC transporter (TVAG_254060) genes showed overexpression in all three resistance strains, suggesting that T. vaginalis may also use drug efflux to develop resistance. A summary of the genes and their expression differences described in this section is shown in supplementary files S4 and S6, Supplementary Material online.

Similar Genetic Changes Associated with Mz Resistance Occur in Two Distantly Related Trichomonad Species

To investigate whether there are evolutionarily common mechanisms of Mz resistance in trichomonads, we undertook whole genome shotgun sequencing and SNP detection of three strains of Tritrichomonas foetus, a trichomonad distantly related to T. vaginalis that also causes a venereal disease in cattle. We used Mz-sensitive and Mz-resistant lines
Table 1
Characteristics of Whole Genome Shotgun Sequencing Datasets of Three *Tr. foetus* Strains, KV1, KV1_M100, and KV1_1MR100.

|                      | Tr. foetus KV1 | Tr. foetus KV1_M100 | Tr. foetus KV1_1MR100 |
|----------------------|---------------|---------------------|-----------------------|
| Library insert size  | 350 bp        | 350 bp              | 350 bp                |
| Read length          | 100 nc        | 100 nc              | 100 nc                |
| Total no. reads      | 67,088,722    | 75,792,262          | 70,337,248            |
| Coverage             | 44.27×        | 46.23×              | 49.48×                |
| Avg. GC%             | 30            | 30                  | 30                    |
| Assembly             | Velvet        | Not done            | Not done              |
| No. contigs          | 194,695       | –                   | –                     |
| Contig N50           | 2,054 bp      | –                   | –                     |
| No. orthologs with *T. vaginalis* | 28,362       | –                   | –                     |

KV1_M100 (exhibiting *in vitro* aerobic resistance) and KV1_1MR100 (exhibiting *in vitro* aerobic and anaerobic Mz resistance) derived by Kulda and colleagues from KV1 (Kulda et al. 1984) (supplementary file S1 and fig. S3, Supplementary Material online). Details of the sequencing are shown in table 1. Sequenced reads of the KV1 genome were assembled into 194,695 contigs (N50 = 2,054 bp) with a size range of 61–19,994 bp. A BLASTX search of the KV1 assembly against the *T. vaginalis* G3 reference assembly returned 28,632 orthologs of *T. vaginalis* genes (data not shown). We identified 183 SNPs in the orthologs, 13 of which (in 13 orthologs) were present in both Mz-resistant *Tr. foetus* lines (supplementary file S7, Supplementary Material online). Of these 13 orthologs two include genes encoding an *actin-like protein* (TVAG_371880) and *TPR domain-containing protein* (TVAG_371930), both upregulated in lab derived B7268M resistant strain compared to the sensitive strains.

A few *Tr. foetus* hypothetical proteins with SNP changes in either of the two *Tr. foetus* strains are also notable for their orthologs in *T. vaginalis* exhibiting reduced transcription (TVAG_151770, TVAG_451120, and TVAG_400730). Moreover, two of 13 orthologs in the two species (1) *CAMK family protein kinase* (TVAG_139460), and (2) *GPE3-like adhesion* molecule (TVAG_295740), had SNP mutations and were downregulated in *T. vaginalis* (supplementary files S4 and 7, Supplementary Material online). It is also worth noticing that we identified SNP changes in other orthologs that might be important for Mz resistance including *tvntr3* (TVAG_35682) and *pyruvate:ferredoxin oxidoreductase* genes (TVAG_198110, TVAG_242960) that did not show changes in our *T. vaginalis* transcriptome dataset (supplementary file S4, Supplementary Material online).

**Discussion**

This study of genetic variation and gene expression related to drug resistance in multiple *T. vaginalis* isolates is the most comprehensive in this species to date. We successfully used ddRAD-Seq as a method to genotype unique regions of the highly repetitive *T. vaginalis* genome, demonstrating that it can be applied to protists whose genomes are refractory to cost-effective whole genome sequencing. Our dataset of 3,923 ddRAD-Seq SNP markers also represents a high confidence set of genetic variants, and greatly expands upon the limited number of genetic markers available for this parasite. Using these markers, we explored the genetic diversity of *T. vaginalis* and present a picture of the parasite’s population structure and linkage disequilibrium (LD) at higher resolution than previous studies (Conrad et al. 2012; Bradic et al. 2014). Our study also offers a panel of biomarkers that can be used to advance personalized treatment and prevention of trichomoniasis.

Genetic variation and exchange through recombination is important to consider in parasites as it influences the spread of drug resistance and virulence genes among them. Our study confirms the existence of two genetically distinct *T. vaginalis* populations (Conrad et al. 2012; Cornelius et al. 2012; Bradic et al. 2014; Paulish-Miller et al. 2014), congruent with sexually reproducing organisms (Tibayrenc and Ayala 2002). Genome-wide LD, reported from analysis of a small number of genes in previous studies (Conrad et al. 2012; Cornelius et al. 2012), was found to decay rapidly within 1–5 kb in both populations, providing evidence for recent genetic exchange. Differences in LD decay between the two populations suggest that different degrees of inbreeding and rates of transmission might be responsible for maintenance of the two-type population structure. Based on previous work (Conrad et al. 2012; Paulish-Miller et al. 2014), we also expected to observe a difference in average drug susceptibility between the two *T. vaginalis* populations. Higher mean values of Mz resistance were found in one population compared to the other (98.5 mg/ml vs. 126.2 mg/ml), although the difference was not statistically significant.

A primary goal of generating the SNP markers in this study was to undertake a genome-wide association analysis to identify novel genetic indicators of Mz resistance. From analysis of ~100 global *T. vaginalis* Mz resistant and sensitive strains, we identified 72 SNPs associated with moderate or high Mz resistance - a panel of biomarkers that can be used to advance personalized treatment and prevention of trichomoniasis.
Currently only a single “reflex test” that identifies a mutation in the \( T. \) \( \text{vaginalis} \) ntr6 gene by real-time PCR (http://www.prnewswire.com/news-releases/medical-diagnostic-laboratories-llc-announces-a-complimentary-reflex-test-to-determine-metronidazole-resistance-in-trichomonas-vaginalis-145736975.html) is available for clinical testing of resistance. Of our 72 biomarkers, several (e.g., those found in TVAG_465880 [conserved hypothetical] and TVAG_308310 [bromodomain containing protein]) were also recurrently identified in other analyses, such as a comparison of three laboratory-derived Mz resistant lines with their isogenic parental strains. While we were unable to undertake a direct functional analysis of these new putative resistance mutations and genes because of the limitations of \( T. \) \( \text{vaginalis} \) transfection, whole transcriptome analysis of three resistant and nine sensitive isolates showed several of the mutations occurred in genes that also showed changes in expression. We also noticed that diversity in gene expression between isolates is primarily due to variation in expression of paralogous genes, as has been noted in previous studies (Pal et al. 2009; Horvathova et al. 2012). For this reason, we suggest that multiple isolates should be used as a control panel in expression studies of \( T. \) \( \text{vaginalis} \).

Drug resistance in eukaryotic microbes is a complex process and usually involves either drug activation/inactivation, or active drug efflux/reduced drug uptake (Penuliar et al. 2015). A major finding of this study was the many changes found in genes coding for proteins involved in drug reduction, efflux, and inactivation (fig. 7). First, we confirmed that down-regulation of proteins involved in Mz activation such as nitro-reductase (ntr) and flavin reductase 1 (FR1) are a primary response of \( T. \) \( \text{vaginalis} \) against Mz. We observed that previously identified mutations in the ntr6 gene (Paulish-Miller et al. 2014) are associated with down-regulation in the resistant lab pair B7268 and B7268M, and that down-regulation of other ntr genes also appears to play an important role (supplementary file S4, Supplementary Material online). Our observation of FR1 reduced expression in resistant isolates is in agreement with Leitsch et al., who first showed reduced activity of this gene in six resistant \( T. \) \( \text{vaginalis} \) strains, and importantly, restoration of Mz sensitivity upon transfection of resistant B7268 with a plasmid bearing a functional FR1 gene (Leitsch et al. 2014). In addition, iron metabolism in \( T. \) \( \text{vaginalis} \) is highly dependent on FR1, and thus resistant parasites must “optimize” their iron-dependent pathways in the absence of this protein (Leitsch et al. 2009). It is possible that the reduced expression of many cytosolic and hydrogenosomal metabolic pathways that we observed, as well as reduced expression of iron-SOD and iron-sulfur flavoproteins and genes coding for proteases, was as a consequence of that optimization. The effect that iron-induced changes have on the transcriptome and proteome of \( T. \) \( \text{vaginalis} \) hydrogenosomes and proteolytic activity has also been described by others (Horvathova et al. 2012; Beltran et al. 2013; Arroyo et al. 2015). As such, these changes in iron-dependent processes...
processes represent consequences rather than causes of Mz resistance, and thus may play an important role in parasite fitness.

*T. vaginalis* contains a large 911 BspA gene family, members of which have been localized to the cell surface and may be involved in pathogenicity (Noel et al. 2010). Interestingly, we detected 14 BspA-like genes as down-regulated in our three resistant strains compared to the sensitive strains (supplementary file S6, Supplementary Material online). This is in contrast to a recent finding by Ansell and colleagues (Ansell et al. 2017) who showed up-regulation of ~10% of VSP (variant surface protein) genes in drug resistant *Giardia* strains. Because of the possible modulation of various pathways by iron in *T. vaginalis*, we consider any association between BspA-like protein regulation and drug resistance to be most likely an epiphenomenon.

We also confirmed a possible role for certain NimA genes in Mz detoxification. Transfection of the nim1 gene in *E. coli* made the bacterium 20× more resistant to Mz through detoxification of the drug to nontoxic amine (Pal et al. 2009). While the nim2 paralog in our studies was upregulated in our two anaerobically resistant lab strains suggesting its importance in anerobic resistance to Mz, nim1 was downregulated in all three resistant strains indicating that its function must be furthered studied. Finally, our study identified 14 possible membrane transporter genes that were upregulated in resistant strains, indicating that efflux pumps may play a significant role in the phenotype. Previous work surveyed a single-domain P-glycoprotein-like gene (Pgp) (Johnson et al. 1994), however there was no strong evidence that this protein might be involved in Mz resistance.

While our study is the first to provide a genome-wide scan of genetic variation and transcriptomes in tens of *T. vaginalis* strains in an attempt to identify novel genes involved in the Mz resistance phenotype, it has limitations. For example, the *PFO-ferredoxin* pathway in hydrogenosomes and *TxnR* pathway in the cytosol have been described as potent activators of Mz and linked to anaerobic resistance (Cerkasovova et al. 1984; Leitsch et al. 2009; Rasoloson et al. 2001, 2002). However, our *TxnR* gene expression results are the opposite to the expression pattern shown in other studies (Leitsch et al. 2009), possibly due to experiments being performed in different strains, or under anaerobic conditions, or through the analysis of gene expression at the RNA level rather than at the protein level. In addition, our use of the “reduced representation” sequencing method means that partial SNP coverage of the highly repetitive, ~160 Mb genome was obtained, because re-sequencing such a repetitive genome using short read technology would have been too challenging to analyze.

An important question arising from this work is what our analyses reveal about the evolution of Mz resistance in *T. vaginalis* or other trichomonads. We observed mutations in genes shared between several genetically distinct laboratory-derived *T. vaginalis* lines and >100 *T. vaginalis* clinical isolates, as well as with the distantly related *Tr. foetus* that causes venereal disease in cattle, suggesting strong natural selection on the same sets of genes and the presence of shared pathways of resistance. We propose that one of the first means for developing Mz resistance in trichomonads is the development of tolerance to high oxygen levels, which provides a protective mechanism against the drug (e.g., FR1, ntr).

*T. vaginalis* already harbors mechanisms to cope with a highly fluctuating oxygen environment (Rasoloson et al. 2001), which may make it “pre-adapted” to Mz treatment. This hypothesis is further supported by the extremely rapid development of aerobic resistance under lab conditions (~<50 in vivo passages), and the emergence of clinical Mz resistance within only 2 years of introduction of the drug (Robinson 1962). Other genes may play an important role in developing subsequently higher levels of resistance. For example, several genes in highly resistant isolates show the same mutation recurrently appearing accompanied by transcriptional change; e.g., dynein heavy chain protein (TVAG_188120), and numerous other hypothetical proteins whose function has yet to be determined (supplementary file S4, Supplementary Material online). Many genes identified in our study have also been associated with Mz resistance in other microaerophilic parasites such as *Giardia* and *Entamoeba* (Pal et al. 2009; Penuliar et al. 2015). For example, a recent transcriptomic profile of resistance in *Giardia* suggests also modification of nitroreductase and oxidoreductase gene families (Ansell et al. 2017). However, in these other species, it has been proposed that laboratory-evolved resistant strains of those parasites accumulate multiple changes not only beneficial for resistance but also those related to decreased fitness. As a consequence, clinical resistance in those parasites has been purported to be rare. It is tempting to speculate in the case of *T. vaginalis* that due to its “Swiss army knife” of a genome, which shows highly duplicated gene families that may bestow the parasite with extraordinary versatility (Barratt et al. 2016), it is able to establish drug resistant infections because of being able to easily compensate for fitness costs. While the details of these mechanisms and their fitness costs should be further explored, we provide here a first insight into adaptation of the unicellular eukaryote *T. vaginalis*.

**Materials and Methods**

Parasite Strains, Culturing and DNA Extraction

We used a set of 102 *T. vaginalis* isolates originating from eight countries: Australia (13), Italy (2), Mexico (8), Papua New Guinea (18), South Africa (9), Mozambique (1), United States (50), and the United Kingdom (1) (supplementary file S2, Supplementary Material online). Most of the strains were isolated from women undergoing routine examination in sexual health clinics and then adapted to growth in culture. Additional isolates included the reference lab strain G3, and
three in vitro-derived Mz resistant lines and their isogenic parents, which were made Mz resistant using the method of Kulda et al. [21] (see Fig. S2 in Supplementary file S1, Supplementary Material online): Mz-sensitive parental strains F1623 and B7708 and their Mz-resistant derivatives F1623-M and B7708-M (MLC ≥ 400 μg/ml, anaerobic) (Brown et al. 1999), and moderately resistant parental strain B7268 (MLC ≥ 200 μg/ml) (Voolmann and Boreham 1993) and its derivative B7268-M (MLC ≥ 400 μg/ml, aerobic and anaerobic) (Wright et al. 2010). All three derived lines are stably resistant in the absence of drug pressure. We also obtained three Trichomonas foetus lines derived using a similar selection method [47]: Tr. foetus KV1 is the Mz-sensitive parental strain to two resistant lines: KV1_M100 (exhibits both in vivo and in vitro aerobic resistance) and KV1_1MR100 (exhibits in vitro anaerobic resistance only), respectively (see supplementary fig. S3 in supplementary file S1, Supplementary Material online). T. vaginalis and Tr. foetus isolates were cultured in modified Diamond’s media as described (Conrad et al. 2011), pelleted by centrifugation, and parasite DNA extracted using a standard phenol–chloroform procedure (Conrad et al. 2012).

Metronidazole Susceptibility Phenotyping

T. vaginalis susceptibility to Mz was determined using a standard minimum lethal concentration (MLC) protocol under aerobic conditions (Rojas et al. 2004). Each isolate was grown in culture media in 96 well plates in the presence of serial dilutions of Mz ranging from 0.2 to 400 μg/ml for 48 h. The lowest concentration at which no motile parasites were observed by microscopy was recorded as the MLC. Each assay was repeated two times. Control strains [CDC 252, resistant to >400 μg/ml Mz, and Mz sensitive CDC 520 (Crowell et al. 2003)] were obtained from the Centers for Disease Control and Prevention (CDC) and used in each assay. Low-level resistance was defined as aerobic MLC 50–100 μg/ml, moderate-level resistance as 100–400 μg/ml, and high-level resistance as 400 μg/ml or greater, as per previous studies (Lossick et al. 1986; Kirkcaldy et al. 2012).

In Silico Restriction Enzyme Optimization for ddRAD Sequencing

We used ddRAD-Seq to sample unique regions of the highly repetitive T. vaginalis genome. We first evaluated in silico digests of the reference assembly of strain G3 using several common choices of restriction enzymes implemented in GMBX_digest_v1.0.pl script (https://github.com/Genomics CoreLeuven/GBSX) and the SimRAD package in R (Center for Disease Control and Prevention 2013), starting with digestion with six-base cutting site enzymes (EcoRI, PstI, SdaI, SgrAI, and SbfI) to determine the best enzyme for cutting within unique regions of the genome [defined as regions free of highly repetitive TEs or genes in multicopy families, based on the current genome annotation (www.Trichdb.org)]. EcoRI was found to cut most frequently in the unique regions of the genome (fig. S4A in supplementary file S1, Supplementary Material online). Subsequent testing of EcoRI in combination with other restriction enzymes, both experimentally (data not shown) and in silico, revealed that a double digest of EcoRI and NlaIII produced the optimal 650–850 bp fragment size distribution for Illumina library preparation and sequencing (fig. S4B and C in supplementary file S1, Supplementary Material online).

ddRAD Library Preparation and Illumina Sequencing

ddRAD sequencing libraries were prepared using EcoRI and NlaIII digested DNA followed by the protocol described in (Peterson et al. 2012). Sequencing adapters containing a combinatorial in-line barcode and standard Illumina multiplexing read index were used to individually barcode samples such that the identity of each isolate was kept intact. Libraries were sequenced on an Illumina HiSeq 2000 following standard methods for cluster generation and sequencing, and paired end (PE) reads of 100 bp were generated. Samples were demultiplexed and individual FASTQ files obtained using Google Documents spreadsheets and database tools in Python (Peterson et al. 2012).

SNP Discovery and Filtering

To estimate the SNP false discovery rate and optimize SNP filtering, the Perl script IRMS.pl (Farrer et al. 2013) was used to introduce 99 random SNPs into the reference G3 genome in silico. SNPs were then detected by aligning ddRAD sequencing G3 data to the G3 reference with introduced SNPs using BWA version 5.09 to align reads (Li and Durbin 2009) and GATK to call variants (DePristo et al. 2011). Multiple filtering steps included removing SNPs based on quality (QUAL > 30), approximate read depth (DP < 5), minimal genotype quality (GQ < 25), quality by depth (QD < 5) and minor allele frequency (MAF) < 0.05. We recovered 82% of introduced SNPs upon alignment with the ddRAD re-sequenced G3 data, thus the same criteria were applied to our actual ddRAD dataset (fig. S5 in supplementary file S1, Supplementary Material online).

Illumina PE reads generated for 102 isolates were aligned to the G3 reference genome in the TrichDB database (Aurrecoechea et al. 2009) using BWA. BAM alignment files of all sequenced isolates were merged and locally realigned to the reference assembly using GATK RealignerTargetCreator in order to minimize the number of mismatching bases across all the reads (DePristo et al. 2011). Genotypes were called from merged BAM files using GATK. In addition to the above-mentioned filtering criteria, SNPs detected from multiple samples were further filtered by removing those with >40% missingness, and those found within TEs. These filtering steps produced a final set of 3,923 high quality filtered SNPs from
the 191,671 total SNPs identified from 102 *T. vaginalis* libraries. Direct SNP comparisons between parental and drug resistant derived pairs were made for each SNP such that those SNPs found in ancestral pair must match with reference G3 strain (sensitive), and must differ from derived stains with at least three reads for each SNP.

**Sanger Sequencing for SNP Validation**

Using the reference G3 assembly in the TrichDB database (Aurrecoechea, et al. 2009), primers were designed to 150–300 bp regions flanking six of the 72 SNPs associated with MLC ≥ 100 µg/ml and used to amplify DNA from 20 to 24 *T. vaginalis* isolates (supplementary table S1 in supplementary file S1, Supplementary Material online). PCRs were performed in 25 µl reactions containing ~20 ng genomic DNA, 0.2 µM of each primer, 200 µM of each dNTP, 1.5 mM MgCl₂, 1 U of Taq DNA polymerase (Sigma) and 1 x PCR buffer. Standard PCR amplification programs were used with an initial denaturation for 5 min and 30 cycles, and PCR products visualized on 1.5% agarose gels. Amplicons were cleaned with QIAquick PCR purification kit (Qiagen) and sequenced using standard Sanger sequencing chemistry. The identity of ~73% (range ~63% to 88%) of the SNPs identified through ddRAD sequencing were confirmed by Sanger sequencing.

**Population Structure**

Population structure was assessed from the 3,923 high-quality SNPs representing 100 isolates using Discriminant Analysis of Principal Component (DAPC) implemented in the R adegenet package, using “average” in the snpzip function (Jombart 2008; Jombart et al. 2010). snpzip uses DAPC to calculate the contribution (loadings) of each SNP to each population group or phenotype. Correction of population structure was performed by regressing along the first PCA axis. DAPC with the snpzip function and centroid clustering was also used to identify SNPs that distinguish Mz-resistant and Mz-sensitive isolates. SNP effects from the final data set was predicted using SnpEff (Cingolani et al. 2012), and functional categories were determined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000; Kanehisa et al. 2014) metabolic enrichment tools in TrichDB (Aurrecoechea et al. 2009).

**Linkage Disequilibrium**

After filtering out rare variants (MAF < 0.05), which could give spurious LD, we recovered 3,894 SNPs from the 3,923 SNPs used to determine population structure. Filtering this set within each population gave 2,837 SNPs covering 898 genomic contigs in one population, and 2,694 SNPs covering 872 contigs in the other. Linkage disequilibrium decay from SNPs was determined using the function $r^2$ from the tootset PLINK (http://pngu.mgh.harvard.edu/purcell/plink/), with pairwise $r^2$ only considered within each contig. The decay of LD over distance was estimated using the Hill and Weir formula (1988), and a nonlinear model was used in order to fit the data to the decay function.

**RNA-seq of *T. vaginalis* Mz Resistant and Sensitive Parasites**

Three Mz-resistant *T. vaginalis* isolates (B7268, B7268-M, and NYCC37), and nine Mz-sensitive isolates (G3, GOR69, NYCA04, NYCB20, NYCD15, NYCE32, NYCF20, NYCG31, and SD2) were grown overnight in 14 ml sealed tubes and total RNA was isolated using the Qiagen® RNeasy Mini Kit, including a column DNase treatment using the Qiagen® RNase-Free DNase kit. Polyadenylated RNA was purified from 5 µg of total RNA using the Dynabeads® mRNA DIRECT™ Purification Kit. All experiments were carried out in triplicate. First strand synthesis was carried out by mixing the entire fraction of isolated polyA+ RNA (8 µl) with 0.5 µl Random Primers (3 µg/µl, Invitrogen), 10 mM DTT and 0.25 µl ANTI-RNase (15–30 U/µl, Ambion) in 1 x First-Strand Synthesis Buffer (5 x, Invitrogen), with incubation at 65 °C for 3 min to remove RNA secondary structures, then placed on ice. A total of 0.5 µl SuperScript® III enzyme (200 U/µl, Invitrogen) and dNTPs to a final concentration of 0.125 mM were added to the mixture and reverse transcription was carried out using the following incubations: 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min. The resulting cDNA/RNA hybrid was purified from the mix using Agencourt RNAClean XP Beads according to manufacturer’s instructions. Second-strand synthesis was carried out by mixing the purified cDNA/RNA hybrid with 1 µl dUTP mix (10 mM, Roche), 0.5 µl Ribonuclease H (2 U/µl, Invitrogen). About 1 µl DNA polymerase I (5–10 U/µl, Invitrogen) in 1 x NEB buffer 2 with 2.5 mM DTT. This mixture was incubated at 16 °C for 2.5 h. The resulting double stranded cDNA was purified using Agencourt AMPure XP beads according to manufactures instructions. The cDNA was end repaired by mixing 5 µl T4 DNA Polymerase (3 U/µl, NEB), 2 µl Klenow DNA Polymerase (3–9 U/µl, Invitrogen), 5 µl T4 Polynucleotide Kinase (10 U/µl, NEB) in 1 x T4 DNA Ligase buffer with 10 mM ATP (NEB) with 0.4 mM dNTPs. The mixture was incubated at room temperature for 30 min. The end-repaired cDNA was purified using Agencourt AMPure XP beads according to manufactures instructions. The purified end-repaired cDNA was then taken through A-tailing, adapter ligation and PCR enrichment using the Illumina TruSeq Stranded mRNA Sample Preparation Kit with different barcodes for each sample. The libraries were pooled and sequenced on the Illumina HiSeq 2500 with 101 cycles, paired-end reads, and multiplexing.

**Transcriptome Analyses**

Approximately 10–20 million reads were generated per RNA-seq library. The RNA-seq data sets were aligned to the G3 reference genome sequence using BWA version 5.09 (Li and
### Data Access
This project has been deposited at the Sequence Read Archive under the accession SRP057357 and SRP057311.

### Supplementary Material
Supplementary data are available at Genome Biology and Evolution online.

### Authors’ Contributions
MB designed the study, performed experiments, analyzed the data, and drafted the paper. SWV made the RNA-seq libraries. GT performed Sanger sequencing validation experiments. PS helped with ddRAD sequencing experimental design. WS provided lab lines and training in drug resistance assays. PH, TC, CL, and PT performed NGS sequencing and raw data processing of three whole genomes. SS undertook data checking, proofreading, and writing the manuscript. JC oversaw all aspects of the research and experimental design, as well as manuscript writing.

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