CHAPTER 4

EXPERIMENTAL CROSSES OF *L. TROPICA*

4.1. Introduction

Protozoan parasites of the genus *Leishmania* are thought to be facultative sexual organisms, capable of - at least occasionally - generating "hybrid" strains, bearing genetic markers of different parental lines of the same species (Akopyants, Kimblin et al. 2009, Sadlova, Yeo et al. 2011, Inbar, Akopyants et al. 2013, Calvo-Alvarez, Alvarez-Velilla et al. 2014, Rogers, Downing et al. 2014) or even different species (Romano, Inbar et al. 2014). Despite the fact that the frequency of these hybridization events in the wild is currently disputed, laboratory experiments have demonstrated that several species, including *L. major*, *L. infantum*, and *L. donovani*, can undergo genetic exchange during their extracellular growth and development inside the sand fly vector, resulting in hybrids bearing a full complement of genetic markers from each parent. These unicellular organisms therefore retain the molecular machinery necessary for carrying out cellular fusion and segregation of the parental genetic material to the progeny, and possibly also perform homologous recombination.

The first experimental cross in *Leishmania* was carried out in *Phlebotomus duboscqi* sand flies between *L. major* Friedlin and LV39 strains (Akopyants, Kimblin et al. 2009). Using clonal lines of each strain in which two different drug resistance

markers were introduced, hybrids that were resistant to both hygromycin B and nourseothricin were recovered from sand fly midguts. Attempts to recover double-drug resistant hybrid lines from *in vitro* co-culture or from *in vivo* co-infections of mice were unsuccessful. Out of 18 hybrid lines recovered, 7 showed 3n DNA content by propidium iodide staining, while the rest showed 2n DNA content. Intermediate levels of DNA content were not observed. SNP-CAPS analysis confirmed triploid and diploid patterns in the sequencing traces of these hybrids. At all markers where the parental lines were homozygous, the parental lines were heterozygous for each of the parental alleles. The hybrids were fully viable, and were capable of reinfecting mice and undergoing metacyclogenesis in the sand fly. Markers on the kDNA maxicircle appeared to follow uniparental inheritance rules.

Several other crosses have been performed in *L. major* with strains originating from different geographic regions. Inbar and colleagues (Inbar, Akopyants et al. 2013) recovered 9 additional double drug resistant lines from the same lines that were used in the first experimental cross, Friedlin SAT, resistant to nourseothricin, and LV39 HYG, resistant to hygromycin B. Pairing *L. majo*r Friedlin SAT with a different drug resistant strain, Sd HYG, gave yield to 15 additional hybrids. Lastly, crosses between the LV39 HYG strain and two other strains, Sd BSD and Ryan SAT, gave yield to 4 and 5 more hybrids, respectively. In addition to these crosses, which were performed in *P. duboscqi*, crosses were also performed in *Lutzomyia longipalpis*, a non-natural but permissive vector of *L. major*, between strains Friedlin SAT and LV39 HYG. A total of 61 hybrids were generated from this last cross.

These experiments suggest that *Leishmania* lacks distinct "mating types", and that there are no barriers to hybridization between strains originating from different geographic regions. Hybrids were recovered at different stages of metacyclogenesis, with no strict association between timing of hybrid recovery and developmental stages present in the sand fly midgut. The developmental stage most closely associated with hybrid recovery appeared to be the nectomonad stage, although more mature forms, such as haptomonads and metacyclics, may retain mating competency. Early replicating procyclic forms are unlikely to be mating competent, as *in vitro* promastigotes do not advance past these stages, and no hybrids have ever been recovered from selection of *in vitro* promastigote cultures. Mating competent developmental forms must therefore arise from late promastigote forms that only appear *in vivo* in the sand fly.

At the time this dissertation was written, a cross between *L. infantum* and *L. major* had also been successfully performed (Romano, Inbar et al. 2014). *L. major* Friedlin strain resistant to nourseothricin was crossed with *L. infantum* strain LLM-320 resistant to hygromycin B in *L. longipalpis* sand flies, and 11 double drug resistant hybrids were recovered. The hybrids lines differed in their phenotypes in mouse models of visceral and cutaneous disease, suggesting differential inheritance of genes involved in tissue tropism and pathogenesis.

The process through which parasite lines can undergo hybridization and the characteristics of this genetic exchange remain largely undescribed. In the first cross in *L. major*, the majority of hybrids were diploid, although triploid hybrids were also observed. In subsequent crosses, again triploid and even tetraploid hybrids were

seen. A tetraploid *L. major* hybrid line reverted to diploid and lost resistance to the two antibiotics following passage through the mouse, while all other lines were stably diploid or triploid both *in vitro* and *in vivo*. While kDNA markers were inherited uniparentally in all progeny clones, most nuclear markers followed biparental inheritance rules and were for the most part heterozygous for each parental allele. In a few cases, however, loss of heterozygosity and reversion to one of the two parental alleles was seen for a number of loci (< 3% of all genotypes markers). Whether this is due to aneuploidy, a common phenomenon in *Leishmania* (see Chapter 3), or to a more specific gene conversion mechanism remains unknown.

In the inter-specific cross between *L. major* and *L. infantum*, again diploid and triploid hybrid clones were recovered, in addition to a single tetraploid hybrid clone. The tetraploidy remained present following repeated *in vitro* passage and recovery of tissue amastigotes from infected mice, suggesting that polyploidy is not intrinsically unstable. Triploid - or near-triploid - hybrids showed intermediate tissue tropism phenotypes in a dose-dependent manner depending on the parental origin of the supernumerary chromosomes. As discussed in Chapter 3, gene dosage seems to have a major effect on transcription and may subsequently affect infection phenotypes, as seen for these inter-specific cross progeny in a mouse model of visceral and cutaneous disease. Interestingly, some of the *L. major* x *L. infantum* hybrid lines established better infections in a vector species, *P. duboscqi*, which is normally refractory to *L. infantum*, with the 3n hybrids showing a dosage-dependent

trend in their ability to infect this vector species based on how many of the supernumerary chromosomes came from the *L. major* parental line.

Following passage through the mouse, one of the inter-specific hybrids reverted to the *L. major* allelic variant at one of the heterozygous nuclear markers on chromosome 29, resulting in a homozygous genotype. The same nuclear marker was also stably homozygous in one other hybrid following maintenance of the line under *in vitro* and *in vivo* conditions, despite being heterozygous at all other nuclear markers, indicating the possible presence of a mechanism such as gene conversion reverting some heterozygous loci to one or the other of the parental alleles, as documented in previous crosses of *L. major*. Again, kDNA maxicircle inheritance appeared to be strictly uniparental. It is important to note that in *T. brucei*, a related kinetoplastid parasite, hybrids recovered from earlier time points showed that progeny inherited both parental copies of kDNA maxicircles, and later lost one of the two allelic variants due to unbalanced segregation in subsequent mitotic divisions (Gibson and Garside 1990).

These experiments confirm that although rare, genetic exchange does occur in *Leishmania* at low frequencies, with approximately 10^{-4} to 10^{-5} or less meiotic events per mitotic division, after correcting for recovery of only double drug resistant hybrids. Considerable work has been done on studying cellular processes underlying reproduction in the related kinetoplastid species *T. brucei*, a pathogenic organism that shares many life cycle similarities with *Leishmania*. Genomic hybrids were recovered from tse-tse fly salivary glands (Jenni, Marti et al. 1986), suggesting that hybridization happens in these stages, as later confirmed using fluorescently

tagged parasites (Gibson, Peacock et al. 2008). Many features of parasite development, such as epithelial attachment through the flagellum, are present in both *Leishmania* and *T. brucei*. A similar approach screening for double drug resistant hybrids in *T. cruzi*, responsible for American trypanosomiasis, recovered double drug resistant hybrids in mammalian host stages (Gaunt, Yeo et al. 2003), suggesting there may be important differences between trypanosome species.

As mentioned already, *T. brucei* hybrids have biparental inheritance of kDNA maxicircles and minicircles, but one of the two parental copies is subsequently lost, while the mature hybrid parasite retains minicircle kDNA from both parents. Similarly, nuclear DNA is also inherited biparentally in a Mendelian fashion, and homologous non-sister chromosomes undergo meiotic recombination. Unlike in *Giardia*, a distantly related binucleated excavate that performs genetic exchange without meiosis (Poxleitner, Carpenter et al. 2008), expression of meiosis-specific genes that are widely conserved in eukaryotes and that act at different stages during homologous recombination seems to be closely linked to a meiotic process in *T. brucei* salivary gland stages (Peacock, Ferris et al. 2011). Tracking the expression of fluorescently tagged proteins that function during prophase of meiosis I (SPO11, MND1, HOP1, and DMC1, a homologue of RAD51) in *T. brucei* epimastigotes, a distinct meiotic-competent stage was identified in which replication of the flagellum and kinetoplast precedes cellular division into daughter cells containing 2n nuclear DNA. These cells then undergo meiosis II without further DNA replication to produce haploid gametes, although the exact process – which may or may not involve nuclear fission without cell division - remains uncharacterized. Haploid

promastigote-like cells are the final product of meiosis II, making *T. brucei* an essentially meiotic organism (Peacock, Bailey et al. 2014).

There is no reason to believe that similar processes may not also underlie genetic exchanges in *Leishmania*. In addition to the crosses mentioned above, fluorescently tagged clonal lines have been used to generate crosses in *L. infantum* and in *L. donovani*, although the number of hybrid lines recovered was much smaller in these experiments (Sadlova, Yeo et al. 2011, Calvo-Alvarez, Alvarez-Velilla et al. 2014). Natural hybrids between different species of *Leishmania*, including *L. tropica*, have also been reported from many different countries (Chapter 2).

In conclusion, *Leishmania* species appear able to interbreed if presented the opportunity, both within their species and across species designations. *L. tropica* isolates often bear within their genome signatures of past occurrences of this genetic exchange (Chapter 2 and 3). Cell fusion between promastigote-like stages was documented in *L. tropica* (ISER/MA/89/LEM/1685) originating from a sand fly captured in Morocco (Lanotte and Rioux 1990) (video recording available online at: http://goo.gl/X6xs1L). It is therefore expected that *L. tropica* lines should be able to give rise to hybrid lines in a laboratory setting. This chapter describes experiments performed to generate experimental hybrids.

4.2. Methods

4.2.1. Laboratory infections of *L. longipalpis* and *P. arabicus* sand flies

Sixteen parasite lines were selected at the time of this study, fourteen of which were confirmed to be *L. tropica* by MLST (See Chapter 2). These isolates were selected from the collections deposited at NIH in the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases. Isolates whose sequences fully aligned with over 90% agreement to *L. tropica* sequences after a BLAST search in GeneBank were prioritized for sand fly feeding assays.

Batches of approximately 50 individuals of 3-5 days old female *L. longipalpis* and *P. arabicus* sand flies from laboratory colonies that were initiated from specimens collected in Jacobina, Brazil (LLJB strain) and in North Israel (PAIS strain), respectively, were used in the feeding assays. The colonies were permanently housed at the Walter Reed Army Institute of Research, in Rockville, Maryland. Sand flies were placed in feeding cups, starved overnight, and fed through a chick skin membrane with heparinized mouse blood kept at 37 °C in glass feeders, containing 4 x 10^6 parasites per mL of log-phase growth promastigotes that had been previously harvested from *in vitro* culture and washed in PBS (See Section 2.2.1 for detailed description of promastigote *in vitro* culture). Laboratory feeding of promastigotes has been previously shown to be the best model to study sexual processes, even if amastigotes are normally taken up by sand fly bites *in vivo* (Inbar, Akopyants et al. 2013).

Sand fly midguts were dissected at two time points: when the blood meal was still present within the midgut, at day 2 post-infection (n = 5 sand flies); and after the blood meal had passed, at day 8 post-infection (n = 10 sand flies). Individual midguts were dissected with sterile needles, and homogenized with a pestel in 50 µL of complete M199 medium (see Chapter 2) in a 1.5 mL eppendorf tube. The proportion of procyclic, nectomonad, haptomonad, and metacyclic forms was determined by counting the number of each form present in the midgut homogenate using a haemocytometer and a light microscope. Some *ad hoc* exploratory feedings were also performed with dissections every 2-3 days for 14 days, to further assess fluctuations of parasite development in the laboratory colonies available to us for each species of sand fly. Promastigote stages were identified based on morphological criteria previously described in the literature (Saraiva, Pimenta et al. 1995).

4.2.2. Generation of drug resistant parasite lines

Plasmid constructs bearing markers conferring resistance to nourseothricin (NTC/SAT), hygromycin B (HYG), and neomycin/G418 (NEO) were kindly provided by Dr Alain Debrabant, Division of Emerging and Transfusion Transmitted Diseases, U.S. Food and Drug Administration. Each construct integrated a cassette, containing a drug resistance marker and a fluorescence marker, into one of approximately 20 different tandem copies of the small subunit RNA domains on chromosome 27. Previous studies have shown that disruption of this redundant locus does not result

in impaired parasite growth *in vitro* or in *in vivo*. Plasmid DNA was transformed into *E. coli* and grown at 37 °C under standard procedures. Plasmid DNA was then extracted and purified using the Qiagen MiniPrep Kit following manufacturer's specifications.

Plasmid DNA was cut with SwaI restriction enzyme at 30 °C for 15 minutes, precipitated in 100% ethanol, and resuspended in 20 µL of TE buffer. Approximately 2 x 10^8 promastigotes were washed in cold PBS and resuspended in electroporation buffer (21mM Hepes, pH = 7.0, 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM glucose). Parasites were then electroporated on a BioRad BTX ECM 630, with settings at 450 V, resistance at 15 Ω, and capacitance at 500 µF. The transfected parasites were incubated at 26 °C overnight prior to drug selection with the chosen antibiotic (25 µg/mL hygromycin, 100 µg/mL nourseothricin, 50 µg/mL G418). After 1-2 weeks of drug selection, cultures with drug resistant parasites were diluted and plated on cM199 agar plates. Single parasite colonies were picked with a pipette tip, and individual clones were then grown briefly in culture for genotyping. Clones positive for integration were stored in freezing solution under liquid nitrogen conditions. Successful genomic integration of the resistance cassette was verified by PCR using previously designed primers that span the 5' and 3' SwaI restriction sites (Figure 4.1), and by expression of the expected fluorescent protein in drug-resistant transgenic parasites as verified by fluorescent microscopy and flow cytometry.

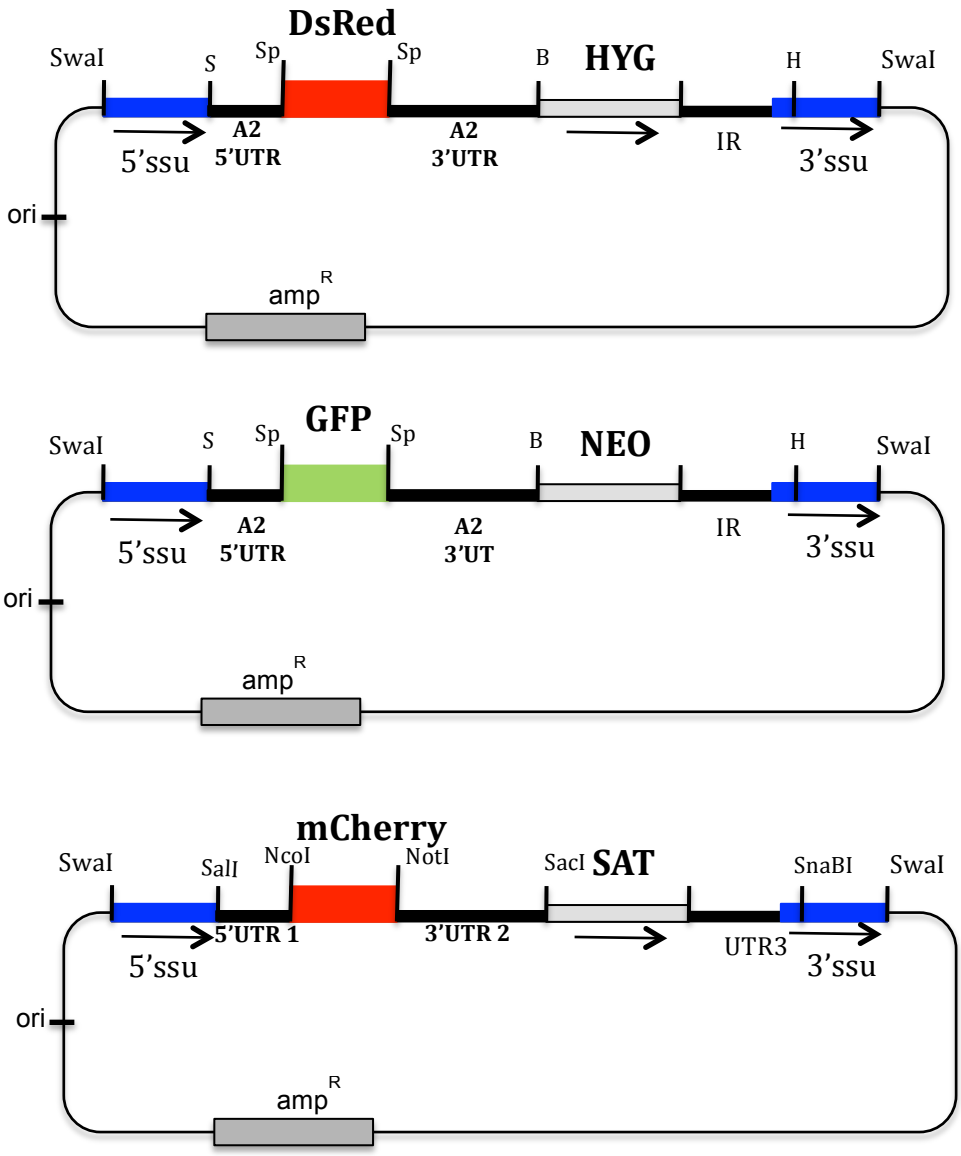

Figure 4.1. Targeting vectors used in this study for integration of different drug resistance markers in the *L. tropica* genome. From top to bottom, the plasmids are pA2-RFP-HYG, pLEXSY-cherry-SAT2, and pA2-GFP-NEO. In blue are the homologous arms for integration into the small subunit RNA locus. The HYG marker codes for resistance to hygromycin B (Hyg B), SAT codes for

resistance to nourseothricin (NTC), NEO codes for resistance to neomycin and geneticin (G418).

4.2.3. Screening for double drug resistant hybrids

A number of different crosses were attempted using drug resistant lines that were generated as described in Section 4.2.2. In each attempted cross, we seeded heparinized mouse blood with a mixture of 4 x 10^6 parasites of two different lines of drug-resistant *L. tropica*, each line being resistant to a different antibiotic. Cross-resistance to both antibiotics by a single drug resistance marker was excluded by *in vitro* susceptibility assays for all drug resistance markers used (data not shown). *L. longipalpis* (LLJB strain) and *P. arabicus* (PAIS strain) sand flies were fed on this mixture of parasites as described in Section 4.2.1. Blood-fed sand flies were separated from unfed females the day following the infectious feed. At different time points after the infectious feed, ranging from 7 to 13 days post-infection, a variable number of blood-fed sand flies were anesthetized with CO_2, and their midgut were dissected, homogenized in 100 µL of complete M199 medium, and placed in a single well of a 96-well flat bottom plate. Plates were incubated at 26 °C overnight, and the appropriate combination of antibiotics for selection of double-drug resistant parasites was added the following day (100ug/mL NTC, 50 µg/mL G418, 25 µg/mL Hyg B). Double-drug resistant parasites were cloned by limiting dilution, or by plating the diluted culture on cM199 agar plates, as described in Section 4.2.2. The

presence of both drug resistance markers in the putative hybrid lines was confirmed by PCR. A summary of the screening procedures is shown in Figure 4.2.

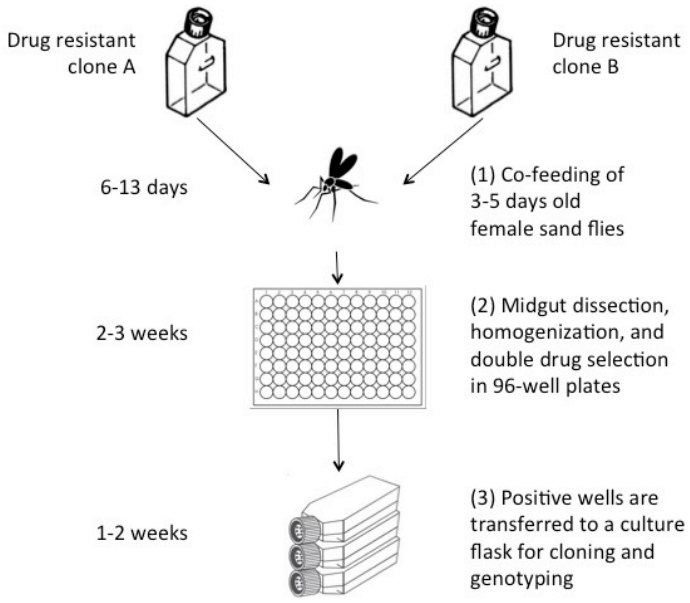

Figure 4.2. Schematic representation of the screening procedures for recovery of double-drug resistant hybrids in sand fly co-infections. Clones A and B have been engineered to be resistant to two different antibiotics. The two lines are mixed in heparinized mouse blood and co-fed to female sand flies (1). At different points during metacyclogenesis, individual sand fly midguts are dissected and placed in a 96 well plate following homogenization, and both antibiotics are added to the culture medium (2). Following 2-3 weeks or longer of double drug selection, any positive wells are transferred to a larger

culture flask for cloning and genotyping by PCR to verify the presence of both drug resistance markers (3).

4.3. Results

4.3.1. Laboratory infections of *L. longipalpis* and *P. arabicus* sand flies

All 14 isolates confirmed to be *L. tropica* by MLST were able to infect the non-natural vector *L. longipalpis* LLJB strain, a well-established permissive laboratory model to study parasite-vector interactions (Figure 4.3). Five parasite isolates (Melloy, Ackerman, 188, E50, Azad) had a lower parasite load present in the midgut on day 8 post-infection compared to the parasite load on day 2, when the blood meal was still present. All other parasite isolates had a higher parasite load on day 8 post-infection, suggesting successful attachment to the inner surface of the midgut during bloodmeal discharge and subsequent parasite replication. Examination of the stomadeal valve at day 13 post-infection showed promastigote secretory gel plug formation, suggesting that *L. tropica* can complete its life cycle within this insect vector and may be infectious to mammalian hosts.

Four isolates (Rupert, Kubba, E50, L747) were also tested in the natural vector *P. arabicus* PAIS strain. All isolates successfully developed within the sand fly midgut, showing an increase in parasite load and progression from procyclic to nectomonad, haptomonad, and metacyclic forms on day 8 (Figure 4.4). Comparison with *L. longipalpis* infections shows similar parasite loads and comparable

proportions of each developmental stage, except for one isolate, E50, which seems to infect *L. longipalpis* LLJB sand flies with lower effectiveness than *P. arabicus* PAIS. The isolate L747 showed appreciable infection at day 8 post-infection, but due to the limited size of the laboratory colony and the small proportion of sand flies that completed blood feeding in our feeding assays no sand flies could be dissected at day 2 (data not shown).

Two exploratory feedings were also performed in *P. sergenti* PSSS strain, another natural vector of *L. tropica* throughout most of its range in the Middle East. Due to the instability of the laboratory sand fly colony and the low infection levels in the small number of sand flies that successfully blood fed in laboratory conditions (data not shown), this vector species was abandoned in subsequent experiments.

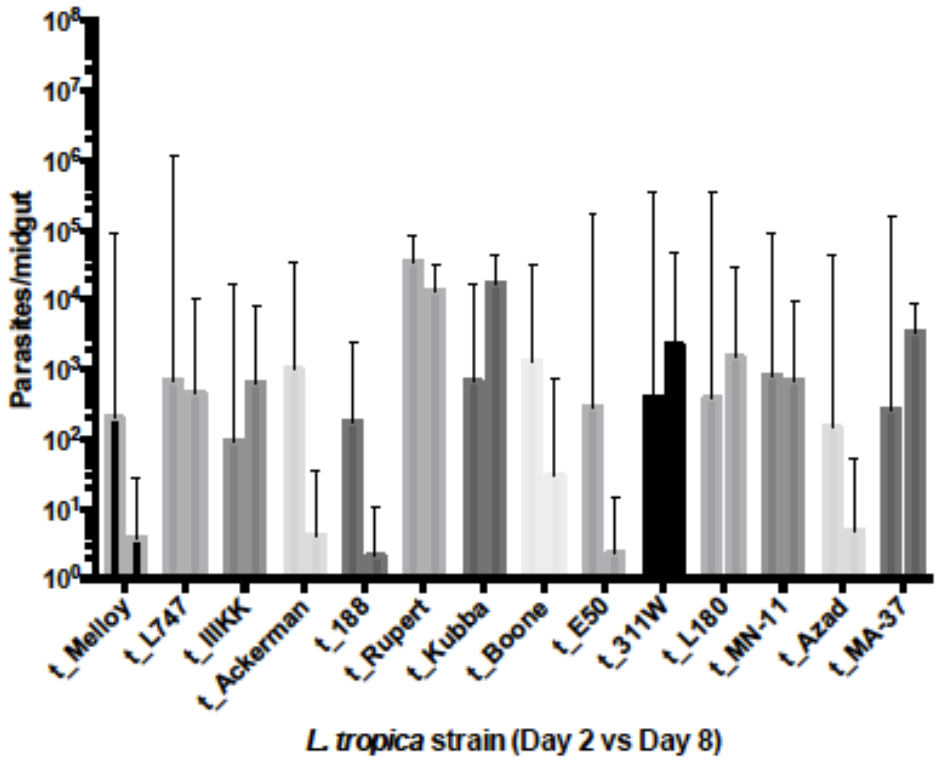

Figure 4.3. Infection loads of 14 *L. tropica* isolates in *L. longipalpis* LLJB sand flies, at day 2 and day 8 post-infection. Each pair of bars represents the geometric mean number of parasites (all developmental stages) in 5 infected midguts at day 2 (left bar) and in 10 infected midguts at day 8 (right bar). The 95% confidence interval around the geometric mean is shown with error bars.

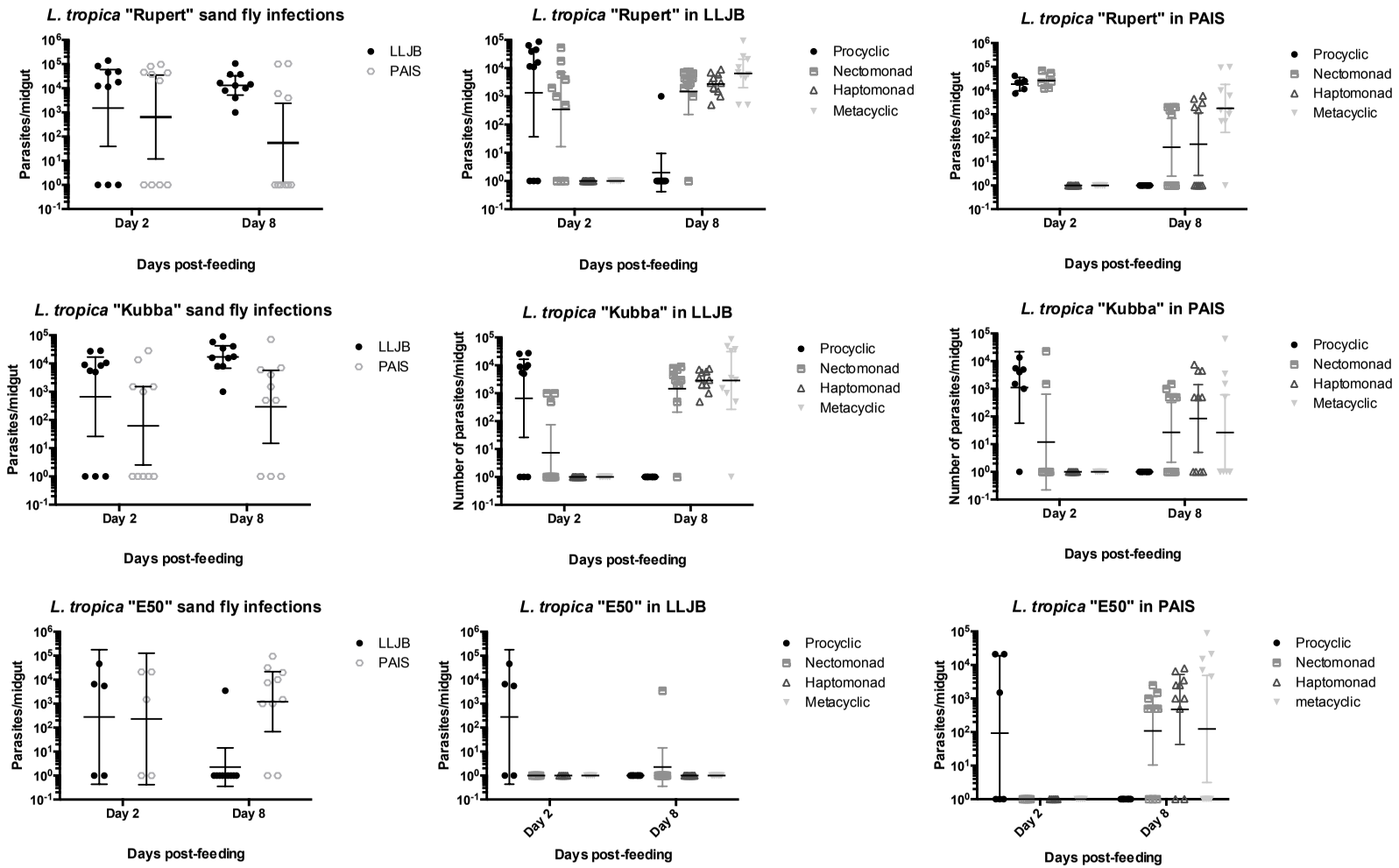
L. tropica "Rupert" sand fly infections
Parasites/midgut
Day 2
Day 8
Days post-feeding
LLJB
PAIS
L. tropica "Rupert" in LLJB
Procyclic
Nectomonad
Haptomonad
Metacyclic
L. tropica "Rupert" in PAIS
L. tropica "Kubba" sand fly infections
L. tropica "Kubba" in LLJB
Number of parasites/midgut
L. tropica "Kubba" in PAIS
L. tropica "E50" sand fly infections
L. tropica "E50" in LLJB
L. tropica "E50" in PAIS
metacyclic

Figure 4.4. Infection loads and developmental stages of *L. tropica* isolates Rupert, Kubba, and E50 in *L. longipalpis* LLJB and *P. arabicus* PAIS sand flies. The geometric mean and 5% confidence interval of each experiment is shown. The number of infected midguts dissected at day 2 and day 8 were 5 and 10, respectively.

4.3.2. Generation of drug resistant parasite lines

Five strains were selected based on country of origin, robust growth phenotypes throughout *in vitro* culture and *in vivo* sand fly feedings, and variation in genotype (Chapter 2). One of three possible constructs containing a fluorescent marker and a drug resistant marker was integrated into the genome of isolates Kubba, Rupert, MN-11, MA-37, and L747, in one of approximately 20 small subunit RNA domains on chromosome 27 (Table 4.1). Constructs stably integrated in the genome as determined by PCR (data not shown). Fitness of the transgenic line was confirmed by passage through the sand fly (Figure 4.5). Drug resistance and fluorescence was confirmed during repeated *in vitro* culture and stable inheritance of the integrated construct was confirmed by flow cytometry (Figures 4.6, Kubba SAT and MN-11 NEO shown as examples).

Line	Origin	Fluorescent marker	Drug resistant marker	Plasmid
Kubba SAT	Syria	mCherry	Nourseothricin	pLEXSY-cherry-SAT2
Rupert HYG	Afghanistan	dsRed	Hygromycin B	pA2-RFP-HYG
Rupert NEO	Afghanistan	GFP	Neomicin/G418	pA2-GFP-NEO
MN-11 HYG	Jordan	dsRed	Hygromycin B	pA2-RFP-HYG
MN-11 NEO	Jordan	GFP	Neomicin/G418	pA2-GFP-NEO
MA-37 NEO	Jordan	GFP	Neomicin/G418	pA2-GFP-NEO
L747 HYG	Israel	dsRed	Hygromycin B	pA2-RFP-HYG

Table 4.1. Transgenic drug resistant lines generated for crossing experiments with drug resistance markers NEO, HYG, and SAT.

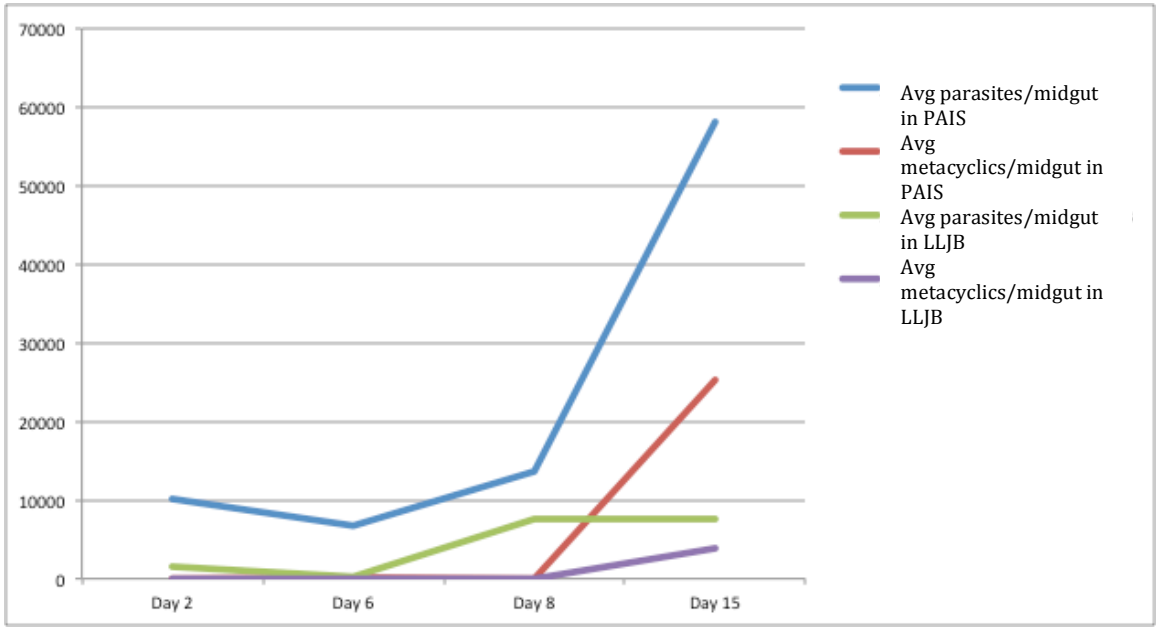

Figure 4.5. Growth phenotype of the transgenic line Kubba SAT in *P. arabicus* PAIS and *L. longipalpis* LLJB sand flies. For both PAIS and LLJB, the first line represents the average number of parasites per midgut at days 2, 6, 8, and 15 post-infection. The second line represents the average number of infectious

metacyclic promastigotes per midgut, and is therefore a fraction of the total number of parasites per midgut. Notice the increase in metacyclic forms at days 8 and 15 post-infection, meaning successful establishment of infection and progression to infectious mature stages.

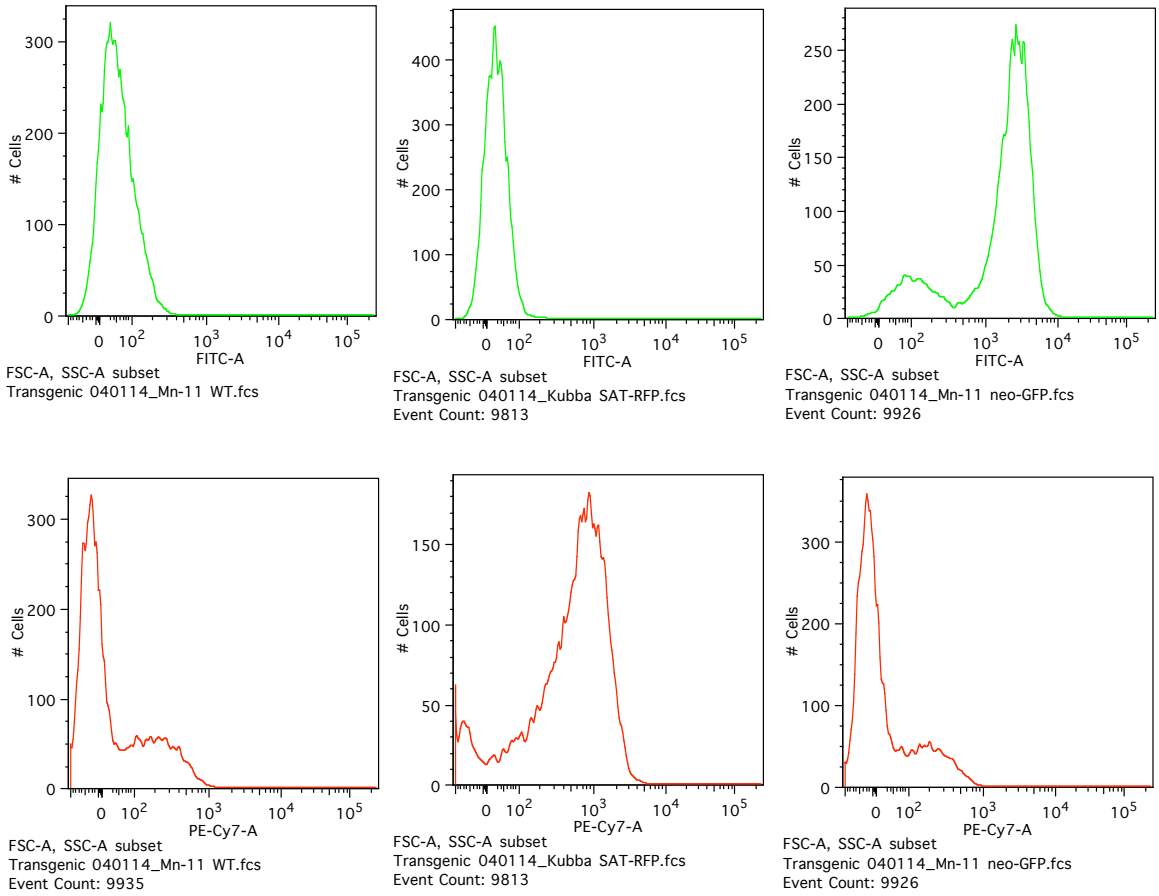

Figure 4.6. Confirmation of expression of the fluorescent markers in the transgenic drug-resistant lines Kubba SAT and MN-11 NEO. From left to right, each panel shows fluorescence in channels FITC-A (in green, top row) and PE-Cy7-A (in red, bottom row) for lines MN-11 wild type, Kubba SAT (expressing

mCherry fluorescent protein), and MN-11 NEO (expressing GFP). Kubba wild type had a similar fluorescence profile as MN-11 wild type (negative in both FITC-A and PE-Cy7-A, not shown).

4.3.3. Screening for double drug resistant hybrids

A total of 7 crosses were attempted using different combinations of drug resistant lines in both *P. arabicus* PAIS and *L. longipalpis* LLJB sand flies (Table 4.2). Recurrent health problems with the laboratory colony of *P. arabicus* PAIS limited the number of females available for feeding assays. A total of 931 sand flies were dissected, and 178 wells (~19%) were lost to fungal or bacterial contamination despite our attempts at maintaining sterile conditions. Sand flies cannot be kept under aseptic laboratory conditions and harbour many bacteria within their gut. A double-drug resistant recovery rate of 2.26 % was seen in the MN-11 HYG x Kubba SAT cross (2.23 % in LLJB, 2.13 % in PAIS). Parasites from 6 wells were positive for double drug resistance (resistant to both NTC and Hyg B) in this cross, however they failed to grow after transfer to a new culture flask with fresh antibiotics, and were therefore lost. A double-drug resistant recovery rate of 31.5 % was seen in the MA-37 NEO x L747 HYG cross in LLJB sand flies, with 3 double-drug resistant parasites recovered from 15 clean wells at day 7 post-infection, 27 recovered from 82 clean wells at day 8, and 15 out of 46 clean wells at day 11. A total of 5 positive wells showed strong double drug resistance even following passage of the parasites into a new culture flask with the same concentration of fresh antibiotics (50 µg/mL

G418, 25 μg/mL Hyg B). These represent 5 independent possible mating events (Table 4.2). Two clonal lines were obtained from each of these double drug resistant parasite cultures. Each pair of clonal lines can be considered "sibling" hybrid lines, which may or may not have originated from the same mating event. The drug resistance profile of each clone was ascertained (Table 4.3) and the Inheritance of each drug resistance marker, NEO and HYG, in each of the hybrid lines was confirmed by PCR (Figure 4.7). The uncloned, double drug resistant parasite population from each positive well was also preserved as a frozen stock for subsequent analyses.

Cross	Sand fly	No. dissected	No. contaminated	No. double-drug resistant	No. hybrids recovered
MN-11 NEO x Kubba SAT	PAIS	46	26	0	0
MN-11 NEO x Kubba SAT	LLJB	138	2	0	0
MN-11 HYG x Kubba SAT	PAIS	48	7	1	0
MN-11 HYG x Kubba SAT	LLJB	282	58	5	0
Rupert HYG x Kubba SAT	PAIS	50	18	0	0
Rupert HYG x Kubba SAT	LLJB	217	42	0	0
MA-37 NEO x L747 HYG	LLJB	150	7	45	5

Table 4.2. Number of sand fly dissections, wells lost to bacterial or fungal contamination, and positive wells with double-drug resistant parasites for each of the attempted crosses. Variation in the number of sand flies dissected is due to fluctuations in colony size and in the number of sand flies that successfully fed in each experiment.

	Clone	Positive well	NEO	HYG
1	H1a	H1	+	+
2	H1b	H1	+	+
3	H2a	H2	+	+
4	H2b	H2	+	+
5	H3a	H3	+	+
6	H3b	H3	+	+
7	H4a	H4	+	+
8	H4b	H4	+	+
9	H5a	H5	+	+
10	H5b	H5	+	+
11	L747 HYG	NA	-	+
12	MA-37 NEO	NA	+	-

Table 4.3. Summary of the 10 hybrid lines originated from the MA-37 NEO x L747 HYG cross, and PCR positivity for presence of each drug resistance marker. Hybrid lines that were cloned from the same positive well may or may have arisen from independent mating events.

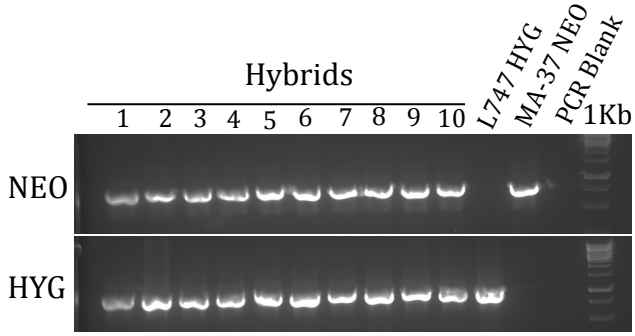

Figure 4.7. Confirmation by PCR of inheritance of the drug resistance markers NEO and HYG in the 10 putative hybrid lines generated in the MA-37 NEO x L747 HYG cross. Hybrid lines are numbered as in Table 4.3.

4.4. Discussion

The experiments summarized in this chapter demonstrate that *L. tropica*, like other *Leishmania* species, is able to perform genetic exchange in the sand fly stages. A total of nearly 1000 sand flies were dissected, and only about 50 wells showed strong growth in the presence of both antibiotics. Low double-drug resistant recovery rates were seen in the MN-11 HYG x Kubba SAT crosses (~2%), but since these subsequently failed to grow *in vitro* under drug selection, they cannot be considered real hybrids. Possible rationales for this phenomenon include loss of either drug resistance marker, poor initial exposure to drug in culture due to high parasite density, or generation of hybrids with reduced fitness. An additional area that needs further investigation is the possibility that some hybrids may be arising after midgut dissection in extremely low numbers, although extensive previous experimentation has effectively ruled out this possibility as exceedingly rare (Akopyants, Kimblin et al. 2009).

A relatively high double-drug resistant recovery rate was seen in the L747 HYG x MA-37 NEO crosses (~30%), with a true hybrid recovery rate of 3.5%. No double drug resistance was observed in any of the other cross combinations. These differences may indicate that there are mating incompatibilities between strains of different genetic backgrounds. As described in Chapters 2 and 3, some isolates appear to be homozygous at the majority of typed marker (Chapter 2) and have long stretches of homozygosity throughout their genomes (Chapter 3), while others harbour considerable heterozygosity. An intriguing interpretation of these findings

is that as heterozygosity arises through outcrossing events between homozygous strains, the mating potential of these outbred hybrids is reduced, both in crosses with either homozygous parent, or with other heterozygous hybrids. In yeast interspecific crosses, F1 heterozygous hybrids produce only 1% viable gametes, while parental lines produce 90-100% viable gametes (Greig, Louis et al. 2002, Xu and He 2011). Different mechanisms could be mediating this phenomenon, from deleterious epistatic interactions to dominant genic incompatibility. A similar process may reduce the mating potential of heterozygous hybrids in *L. tropica*: isolates Rupert and Kubba appear to be heterozygous throughout the majority of their genomes both by MLST and WGS, and crosses involving either as one of the parental lines yielded no or very few positive wells (6 out of a total of 781 dissected midguts). Post-zygotic barriers to hybrid fertility might reduce interbreeding between the hybrid and parental populations and favour speciation in *Leishmania* as in yeast (Greig 2007, Schumer, Cui et al. 2015). Interestingly, in *T. brucei*, F1 hybrid progeny show a range of mating compatibilities that do not readily fit a simple two mating type system (Peacock, Ferris et al. 2014).

The vector species used for the crosses may also play a role in hybridization. In previous crosses in *L. major*, the same *L. longipalpis* LLJB strain used in this study gave much higher hybrid recovery rates than the natural vector, and at earlier time points of parasite development within the sand fly. Reports of natural interspecific hybrids are quite common in South America, where the *Lutzomyia* genus is endemic. Parasite development within *L. longipalpis* has been shown to occur faster

compared to Old World vector species, with nectomonads being the dominant life cycle stage by day 3 post-infection (Walters, Irons et al. 1993).

The 5 positive wells found in this study were recovered at days 7, 8, and 11 post-infection, confirming an association between the nectomonad and later stages and the presence of hybrids. The infection assays I performed in *L. longipalpis* show a large number of mature forms at day 8, with a large number of elongated nectomonad forms and fully mature metacyclics being present. The relatively smaller parasite load seen in *P. arabicus* may reflect intrinsic physiological differences in the amount of blood consumed per blood meal by the sand fly, rather than parasite fitness within the midgut environment. It is important to note that the minimum infectious dose necessary to ensure transmission of the parasite to the host is very small, and previous crosses have recovered hybrids from midguts with very low parasite infections, making a heavy parasite load in the midgut a condition which may not necessarily improve parasite survival (Kimblin, Peters et al. 2008, Stamper, Patrick et al. 2011).

As reviewed in Chapter 2, the heterozygosity observed in natural populations of *L. tropica* is larger than that seen in other species. This and additional lines of evidence suggest that *L. tropica* is capable of Mendelian genetic exchange. I have here reported the first successful cross between two isolates of *L. tropica*, L747 from Israel and MA-37 from Jordan, with different genetic backgrounds as elucidated by MLST and WGS, and demonstrated biparental inheritance of the two drug resistance markers used for selection of double drug resistant hybrids. In the next chapter, I introduce the results of WGS data analyses from these 10 hybrid clones to elucidate

genome-wide patterns of inheritance and genetic exchange that arise through hybridization.