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Faculty of Natural and Agricultural Sciences

The contribution of *Plasmodium falciparum* genetic diversity to differentiate drug response in gametocytes

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Summary

Late-stage gametocytes of *P. falciparum* parasites are responsible for the ongoing human-to-mosquito transmission of malaria disease. To block transmission, novel gametocytocidal compounds are required. *P. falciparum in vitro* assays that aim to study transmission-blocking compounds focuses mainly on lab adapted strains, which have been isolated years ago and do not reflect the current status quo of malaria transmission. It is thus important to determine *ex vivo* efficacy against diverse, contemporary clinical isolates as an early filter to provide confirmation of novel gametocytocidal activity in the field. This study hypothesizes that differences in drug responses in late-stage gametocytes of clinical isolates can be correlated with the extent of genetic diversity of clinical isolates.

Previously, potent phosphatidylinositol 4-kinase (PI4K) inhibitors indicated differential drug response, which was observed in transmissible stages of *ex vivo* southern African *P. falciparum* clinical isolates. These differential drug responses were not only limited to kinase inhibitors, but also seen with endoperoxide and ATP4 inhibitors that were used in the current study. In asexual parasites, drug resistance markers are used to ensure that there is no cross resistance inherent in newly developed drugs. The clinical isolates were characterised phenotypically based on asexual drug resistance markers; however, these drug resistant profiles were not able to clarify the differential drug response observed in late-stage gametocytes. Consequently, other genotyping techniques were used to characterise the clinical isolates. Microsatellite (MS) and single nucleotide polymorphism (SNP) markers were used to determine whether allelic variation and therefore genetic complexity can explain differential drug response in isolates.

The genetic fingerprint created by MS and SNP genotyping revealed allelic complexity within clinical isolates. Isolates from the high transmission areas were characterised by a high multiplicity of infection (MOI) and isolates exhibited a large number of unique alleles. Equally, clinical isolates with lower MOI were less genetically diverse, although they were from an area of high malaria transmission. This study also showed that parasites from neighbouring countries shared clones, which might suggest there were similar parasites circulating in those areas. This study also shows that there is a relationship between gametocyte production and isolates that are genetically diverse. By contrast, isolates with less genetic diversity produced less gametocytes. Furthermore, the study presents SNP barcoding as a more sensitive and

robust genotyping technique, as it was able to correlate differential drug response to genetic complexity of clinical isolates.

This master's dissertation shows the relevance of using SNP genotyping to clarify differential drug responses in late-stage gametocytes of clinical isolates. This work also provides contemporary clinical isolates that can potentially be used as early filter in the hit prioritisation to lead selection for testing novel drugs with transmission-blocking activity. Testing the gametocytocidal compounds on the contemporary clinical isolate will inform whether there is differential drug activity in different gametocyte populations *in vivo*. Possibly, drugs with very low susceptibility against clinical isolates will not progress through the drug testing cascade, in turn prioritizing compounds with activity to further development.

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List of abbreviations

ACT	Artemisinin-based combined therapy
APAD	3-Acetylpyridine adenine dinucleotide
ATP 4	P-type Na^+-transporting ATPase 4
CNV	Copy number variance
CQ	Chloroquine
CSP	Circumsporozoite surface protein
DBS	Dried blood spot
DBSs	Dried blood spots
FDA	Food and drug administration
G6PD	Glucose-6-phosphate deficiency
IRS	Indoor residual spray
ITN	Insecticide treated bed nets
LD	Linkage disequilibrium
MB	Methylene blue
MCA	Multiple correspondence analysis
MOI	Multiplicity of infection
MS	Microsatellite
MSP-1	Merozoite surface protein-1
MSP-2	Merozoite surface protein-2
MSs	Microsatellites
NAG	N-Acetyl glucosamine
NBT	Nitro-blue tetrazolium
PCA	Principe component analysis
PCR	Polymerase chain reaction
PfCRT	*P. falciparum* chloroquine resistance transporter
PfDHFR	*P. falciparum* dihydrofolate reductase
PfDHPS	*P. falciparum* dihydropteroate synthase
PfMDR1	*P. falciparum* multidrug resistance protein 1
PI4K	Phosphatidylinositol 4-kinase
pLDH	Parasite lactate dehydrogenase
PQ	Primaquine
SNP	Single nucleotide polymorphism
SNPs	Single nucleotide polymorphisms

TCP	Target candidate profile
TPP	Target product profile
WHO	World health organization

Chapter 1: Introduction

1.1. Global burden of malaria

Malaria is a parasitic disease that is caused by a protozoan from the genus *Plasmodium*. The disease remains a global health concern despite the availability of tools to help treat and eliminate it [1]. There are five species of *Plasmodia* that affect humans, namely, *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae and Plasmodium knowlesi* [2]. Together, these species accounted for an estimated 228 million cases of malaria across the globe in 2018, and a 23 million reduction in case numbers from 2010 [3]. However, in 2018, there was still an alarming 405 000 cases of death associated with the disease [3]. Of the five species, *P. falciparum* is the most prevalent malaria parasite as it was responsible for 99.7 % of malaria cases in the world health organization (WHO) African region in 2018 [3]. Alarmingly, the mortality rates (70 % of death cases) are highest amongst children under the age of 5 years in southern African countries [3].

Despite the high number of malaria incidence rates and malaria-associated mortality rates, 17 countries have managed to eliminate malaria between 2000 and 2015 [4]. There are still approximately 91 malaria endemic countries, most of which are in sub-Saharan Africa, Asia and South American countries (Figure 1).

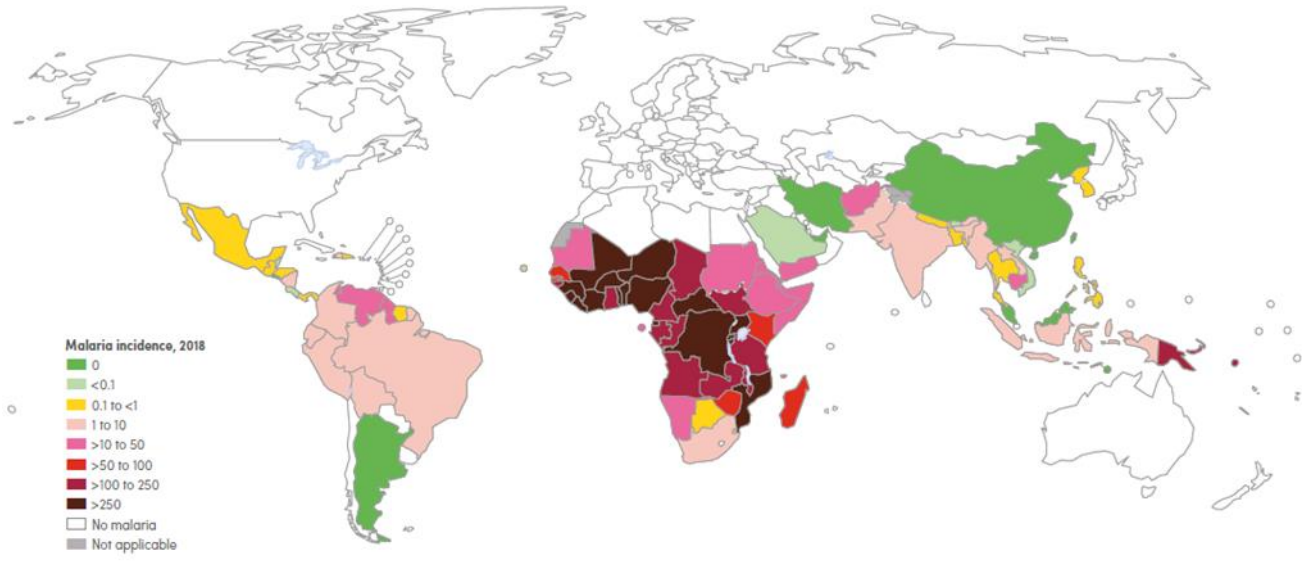


Figure 1: Map of malaria case incidence by country as reported in 2018.
Map shows malaria incidence rate by country as estimated by World Health Organization (WHO). Cases are estimated per 1000 population at risk per country. Countries arranged based on colour showing zero cases (green) to more than an estimated 250000 cases (dark brown) [3].

Malaria transmission is dependent on climatic conditions such as rainfall, temperature, and humidity. These conditions may affect the number and survival of mosquitoes. Approximately

14 million people from countries with low transmission in east and southern Africa are at high risk of malaria. The elimination-8 (E8) initiative includes frontline countries in southern Africa (Botswana, Eswatini, Namibia and South Africa) coordinating malaria elimination efforts, yet these countries reported an increase in cases between 2015 and 2018, setting back progress to reduce mortality rates by 40 % by 2020 and requiring adjusted timelines to achieve elimination [3]. There are multiple reasons for the setback, including imported cases from neighboring countries and inadequate coverage of vector control strategies [5].

1.2. *P. falciparum* parasite development

The protozoan *P. falciparum* parasite has a complex life cycle: asexual multiplication occurs in the mammalian host as a haploid organism during the liver stages and the asexual, intraerythrocytic developmental stages with obligate sexual reproduction occurring in the female *Anopheles* mosquito, where the parasite is diploid [6]. A high parasite load during the asexual cycle results in the clinical manifestations with symptoms such as high fever, anemia and chills [7].

1.2.1. Asexual developmental cycle

An infection is initiated when an infected female *Anopheles* mosquito transmits mature sporozoites into an uninfected human host during a blood meal. The sporozoites migrate through the circulatory system to the liver, where they infect the hepatocytes and mature into schizonts during exo-erythrocytic schizogony. Liver schizonts rupture and release merozoites. The 48 h asexual developmental cycle is initiated when the mature merozoites are released into the bloodstream, where they invade erythrocytes (Figure 2) [6]. Between 0 to 12 h after erythrocytic invasion, the merozoites develop into ring-stage parasites. The ring-stage parasites mature to form metabolically active trophozoite stages about 12 h post-invasion [8]. After subsequent cellular growth, the trophozoites undergo nuclear division to form schizonts that consists of approximately 8-32 individual merozoites [6]. Upon the rupture of schizonts, merozoites are released into the bloodstream to invade healthy erythrocytes (Figure 2) [8].

1.2.2 Sexual development and gametocytogenesis

Approximately 10 % of asexual parasites undergo a series of changes until they progress into both sexually mature male and female stage gametocytes in a process known as gametocytogenesis. These mature gametocytes can then be transmitted back to the mosquito and thereby complete the transmission cycle [9].

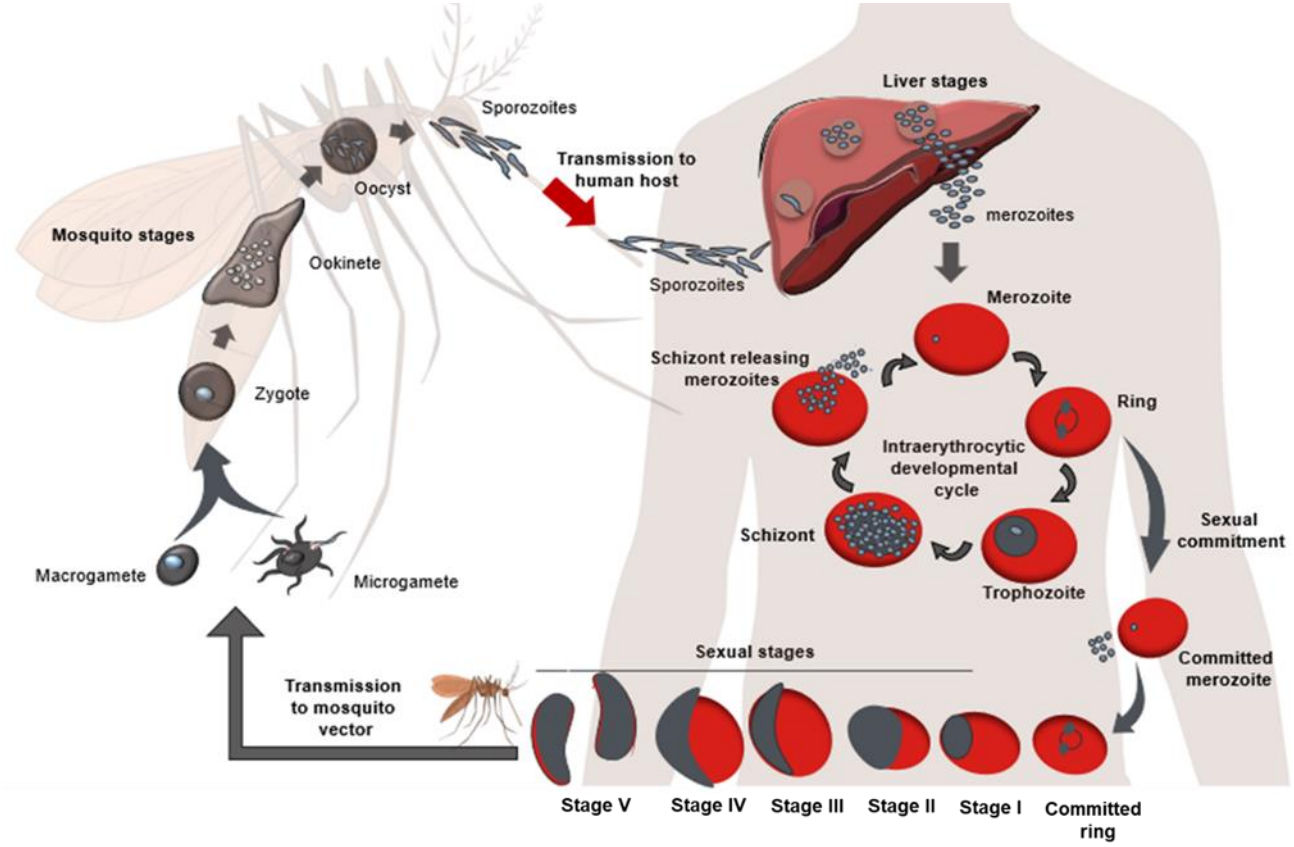


Figure 2: *P. falciparum* life cycle in the human host and mosquito vector .
An infected *Anopheles* mosquito infects the human tissue with sporozoites. After reaching the periphery circulation, sporozoites migrate to the hepatocytes within the liver, where they mature into merozoites. Upon maturation, merozoites are released into the bloodstream to invade healthy erythrocytes. The intraerythrocytic cycle starts with a ring stage that develops into a metabolically active trophozoite stage. Subsequently, trophozoites mature into schizonts that release merozoites, which further reinvade new erythrocytes. Through the process of gametocytogenesis, a proportion of the intraerythrocytic stages are committed into sexual gametocytes that migrate to the dermal tissue for subsequent uptake by the mosquito vector during another blood meal. *P. falciparum* gametocytes are classified into five developmental stages with distinct morphological traits. Developmental stages in the mosquito vector are initiated after the ingested gametes undergo fertilization. After subsequent sporogonic development in the mosquito midgut, infectious sporozoites form and reach the salivary glands for further transmission [10]. Images were generated from Servier Medical Art (URL link to the license: https://creativecommons.org/licenses/by/3.0/) and adapted based on colour and size.

Following erythrocytic invasion, sexually committed merozoites mature into sexually committed ring stages and subsequently, *P. falciparum* gametocytes uniquely develop for 10-12 days as marked by transition through five distinct morphological stages (stage I-V, Figure 2) [11-13]. Gametocytogenesis occurs in tissues including the bone marrow and only mature stage V gametocytes can be detected in peripheral circulation for the mosquito vector to ingest during a blood meal [14, 15]. Once in the mosquito vector midgut, a drop in temperature and the presence of xanthurenic acid, as well as an increase in the pH of the midgut environment enable the ingested stage V gametocytes to differentiate into male and female gametes [16]. After fertilization of the gametes, a diploid zygote forms and matures into motile ookinetes, which penetrate the midgut wall and develop into oocysts and release infectious sporozoites

after 11-16 days [17]. The sporozoites migrate to the salivary glands for further transmission into a human host [10].

To achieve malaria elimination and eradication, it is imperative to target the asexual disease-causing stages of the parasite, as well as the sexual stages of the parasite that are transmissible to the mosquito vector to prevent the spread of the disease.

1.3. Malaria control, elimination and eradication

Efforts to fight the battle against malaria include integrated vector control strategies, vaccine development, treatment and prophylactic chemotherapy. Challenges such as the emergence of resistance to antimalarials that act against the asexual stages of the parasite and a lack of approved drugs that block malaria transmission, hamper the progress towards malaria elimination and eradication [18]. Malaria transmission can be prevented in two ways (Figure 2): 1) by preventing mosquito-to-human transmission of sporozoites and 2) by preventing human-to-mosquito transmission by targeting mature stage V gametocytes.

1.3.1 Preventing mosquito-to-human transmission

Malaria transmission from the mosquito vector to the human host can be targeted by either vector control, or by preventing the parasites (sporozoites) from establishing the life cycle in the human host through either vaccines or chemotherapy.

1.3.1.1 Vector control

Vector control involves measures that aim to limit the mosquitoes' ability to transmit the disease [19]. Indoor residual spraying (IRS) involves the annual application of an insecticide on surfaces where malaria vectors rest before or after a blood meal [20]. However, the use of IRS as a vector control strategy has declined as it is not a cost-effective method [20]. Alternatively, human contact with infected mosquitoes can be reduced by using insecticide-treated bed nets (ITNs) [21]. Moreover, ITNs function as a physical barrier to mosquitoes and the net has a lethal effect due to the insecticide applied on the net. Both the IRS and ITN contain insecticides such as dichloro-diphenyl-trichloroethane (DDT) and pyrethroids, but the use of the latter is threatened by resistance development and DDT is associated with toxicity risks [22, 23]. Together, these strategies have effectively reduced malaria incidence rates by 37 % and the overall global malaria mortality rates by 60 % between the years 2000 and 2015 [24]. Novel exploratory interventions are currently being investigated. These include killing the vectors through the use of endectocides, which are drugs that kill mosquitoes that feed on individuals

who have taken the drugs [25]. The use of chemosensory is another form of vector control which targets the mosquitoes' olfaction through the use of odorant receptors as a way to control mosquito populations [19]. Vector control can also be achieved through the sterile insect technique which controls the mosquito population by releasing many sterile males to decrease the female mosquito's reproductive potential [26, 27].

1.3.1.2 Vaccine development

Despite extensive research on vaccine development against malaria, there is still no licensed vaccine that has been successfully developed to prevent *P. falciparum* parasite infection [28]. Several studies have explored parasite proteins that are targeted by immunity for their potential to be used in vaccine programs. Multiple antigens from various stages of the parasite life cycle have to be targeted for vaccine development [29]. Many of the challenges in vaccine development are due to lack of understanding of the interaction between *P. falciparum* parasites and the human immune system [30]. Malaria vaccine development is focused on 1) the pre-erythrocytic stage with the aim of disrupting the exo-erythrocytic cycle of the parasite, 2) preventing the erythrocytic stages through parasitaemia reduction and 3) blocking transmission by interrupting parasite development in the mosquito midgut [31].

The leading pre-erythrocytic stage vaccine, RTS,S developed in partnership by PATH Malaria Vaccine Initiative and GlaxoSmithKline, has shown to be effective in children younger than five years, as it has reduced severe malaria cases by 36 % in Africa [32, 33]. Unfortunately, RTS,S vaccine failed to provide long term protection as its efficacy reduced to 26 % when children only received three vaccine doses [32]. Potentially, RTS,S could be used to interrupt malaria transmission when used in combination with chemoprevention [34]. There are at present several clinical trials on erythrocytic vaccines such as the apical membrane antigen 1 (AMA1), glutamate rich protein (GLURP), merozoite surface protein-1 (MSP-1) MSP-2 and MSP-3. Yet none of the vaccines have shown efficacy in phase II and III trials due to their polymorphic nature [35-37]. As a result, novel antigens for the development of effective erythrocytic vaccines is a needed. Another approach to vaccine development includes vaccines which are focused on the sexual stages to block malaria transmission [32]. Using this approach, a conjugate vaccine that targets the female gametocyte markers such as Pfs25, Pfs48/45 and Pfs230 is already being tested clinically [38, 39]. Unfortunately, transmission-blocking vaccines are rendered ineffective in high *P. falciparum* transmission settings [30].

1.3.1.3 Chemoprevention

Malaria prevention can also be achieved through antimalarial chemoprophylaxis, which aims to prevent malaria from developing by preventing the establishment of asexual blood stage development [40]. The combination of atovaquone with either proguanil, doxycycline or mefloquine may be taken as a preventative measure against malaria infections [41]. Prophylaxis through administration of sulphadoxine-pyrimethamine to pregnant women living in areas that have a high malaria transmission can be made after the first trimester. By contrast, for infants living in Africa, a small dosage of the preventive treatment of sulphadoxine-pyrimethamine is used [42]. A preventive strategy recommended by WHO in sub-Saharan Africa is seasonal malaria chemoprevention that involves the administration of a combination of antimalarial agents to children under five years [43]. In high transmission areas, malaria clinical manifestations can be reduced by using post-treatment prophylaxis, which is the treatment of previously infected individuals to protect against future infections [44].

1.3.1.4 Chemotherapy

Several antimalarials have been used to treat patients symptomatic of the disease and include the quinoline antimalarials like chloroquine, as well as the antifolates (sulphadoxine-pyrimethamine) [45]. Unfortunately, all these first generation antimalarials have faced resistance development in the parasite and the majority are no longer used clinically [46]. The current mainstay of uncomplicated malaria treatment is with artemisinin-based combination therapies (ACTs), which are combinations of two drugs (an artemisinin derivative and a partner drug) at a fixed dosage. The fast acting artemisinin derivatives are combined with slow acting drugs of a different mode of action to provide adequate cure rate and to hamper the development of resistance [47]. ACTs have significantly reduced the number of malaria related mortalities and they are responsible for an 18 % decline in the incidence of malaria cases from 2010 to 2016 [48]. Unfortunately, the efficacy of ACTs is threatened by the emergence of resistance, which is characterized by slow parasite clearance and a high rate of recrudescence as reported in western Cambodia in 2008 [48].

1.3.2 Preventing transmission from human host to mosquito vector

Transmission from an infected human host to a susceptible mosquito is mediated through the uptake of the mature, circulating stage V gametocytes and transmission-blocking compounds are required to prevent this transmission. However, compounds active against the asexual forms of the parasite are typically inactive or show at least a 10-fold reduction in efficacy against stage V gametocytes, due to the differential biology underlying asexual parasites and stage V

gametocytes [49]. Due to the inability of antimalarials to target gametocytes, the parasite may still be transmitted during the recovery phase of the disease, despite the successful treatment of asexual parasites [50].

Primaquine (PQ), an 8-aminoquinoline, is a drug licenced for clinical use and it is able to target mature gametocytes [51]. PQ can block malaria transmission in areas that either face artemisinin resistance or in areas that are approaching elimination. A single 0.25 mg/kg dose of PQ has shown to reduce the likelihood of transmission of drug resistant parasites [52]. This single dose that was recommended by WHO is aimed to reduce transmission in low endemic areas, but the efficacy in higher transmission settings is not established. Moreover, 8-aminoquinoline antimalarials are poorly tolerated and their metabolites cause haemolysis in individuals with glucose-6-phosphate deficiency (G6PD) [53].

Artesunate and artemether have been reported to be effective in reducing gametocyte carriage, and some reduction of transmission to mosquitoes was seen for artesunate. Yet these derivatives are unable to kill the mature stages of the *P. falciparum* parasites, which questions their clinical use [54-56]. Methylene blue (MB) is another drug that can target sexual parasites by interfering with gametocyte development [57]. Like PQ, MB has toxicity issues and it is associated with haemolysis in G6PD deficient patients. However, it exerts hemolytic properties at a higher dosage than PQ [1]. Also, MB has limitations in clinical cure of malaria as it temporarily turns urine to a green colour and cause the sclera to become blue [58].

Therefore, the currently used compounds either only target the early stages of gametocyte development and not the late stages, which will be transmitted to the mosquito vector, or there are toxicity concerns with the antimalarials that target the late-stage gametocytes. Thus, it is important to find novel compounds that prevent the transmission of gametocytes from the human host to the mosquito vector.

1.4 Drug discovery: Finding novel antimalarials

Malaria treatment relies primarily on drugs that target the disease-causing asexual blood stages of *Plasmodium* parasites [59]. However, resistance to current antimalarials threatens the progress towards elimination and eradication of the disease. As a result, the need to find new compounds that target both the asexual and sexual stages of the parasite is a priority. Target candidate profiles (TCP) and target product profiles (TPP) are clearly established for new antimalaria candidates (Table 1) [60]. TCP refers to a single molecule that is in formal

regulatory preclinical safety assessment or studies pertaining to human volunteering [44]. On the other hand, TPP defines a final product that constitutes of two or more active candidates, which is a guideline for drug discovery and development [44]. An ideal antimalarial drug should be able to kill multiple stages of the parasite, thereby fitting into several (if not all) TCPs. For instance, a drug useful to malaria elimination strategies should target both the sexual and asexual stages of the *P. falciparum* parasite, characterised with TCP-1 and TCP-5 activity. In this way, individuals will be cured from the disease and the population will be protected by preventing further transmission of malaria [60]. For TCP-5, it is important for the candidate to have activity against all stages of gametocytes, as well as against the oocyst and sporozoite formation in the mosquito vector [60].

Table 1: An overview of newly defined target product profiles (TTPs) and target candidate profiles (TCPs).
Table obtained from [60].

Profile	Intended use
TPP-1	Uses a combination of TCP-1 and TCP-5 for the treatment of acute uncomplicated malaria in children or adults
TPP-2	Uses a combination of molecules with activity against hepatic schizonts and TCP-1 activity as chemoprotection for individuals migrating into endemicity areas
TCP-1	Molecules that clear asexual stages
TCP-2	Profile retired
TCP-3	Molecules with activity against *P.vivax* hypnozoites
TCP-4	Molecules with activity against hepatic schizonts
TCP-5	Molecules that block transmission by targeting gametocytes

Phenotypic screening has resulted in thousands of compounds with activity against whole cell asexual stages of *P. falciparum* parasites and some of them are already in the clinical development [60]. However, as mentioned, these are largely effective only against asexual parasites and novel, alternative strategies have been proposed to discover transmission-blocking antimalarials.

1.4.1 Proposed transmission-blocking screening cascade

To discover and validate transmission-blocking compounds, a three tiered testing cascade has been suggested (Figure 3) and each tier highlights the biological activity of the compounds for hit prioritization [61]. The tiered cascade is imperative, since testing for transmission-blocking

compounds is a lot more complex and complicated. Several cell types (gametocytes, gametes and oocysts) have to be produced and tested, which is time and economically constraining.

Tier 1 involves hit identification and validation by screening larger libraries of compounds that are either active against asexual parasites or with known activity at a single concentration (1 μM) against late-stage gametocytes (Figure 3). These hits should then be validated on unrelated platforms to remove false positives and decrease the possibility that a compound interferes with a specific read out. Following full dose-response investigation, the hits should be prioritized based on the cost of production as well as selectivity and toxicity profiles. The validated hits then enter tier 2 [61].

By contrast, tier 2 which is termed as hit prioritization/lead selection, involves using assays that provide different metabolic endpoints to filter hits for the consequent tier [61]. In this tier, the activity of compounds is tested against gametocytes from laboratory strains (Figure 3). Ideally, gametocytes from known *P. falciparum* resistant strains with diverse genetic backgrounds and resistance phenotypes should be evaluated [62]. Additionally, the gametocytocidal activity of hits should also be tested against sexual stages from primary clinical isolates that are from different geographical settings [61]. Lastly, male and female gamete assays should be used to determine the sex-specific activity of the potential compound.

The third tier involves the validation of transmission-blocking compounds. In this tier, compounds selected from the previous tier are exposed to gametocytes, which are used to infect the *Anopheles* mosquito vector (Figure 3). In these assays, an endpoint is the number of oocysts that are present in the mosquito midgut. Although technically challenging, this tier provides the highest biological content and it is the most critical filter before the compounds proceed to an *in vivo* setting [61].

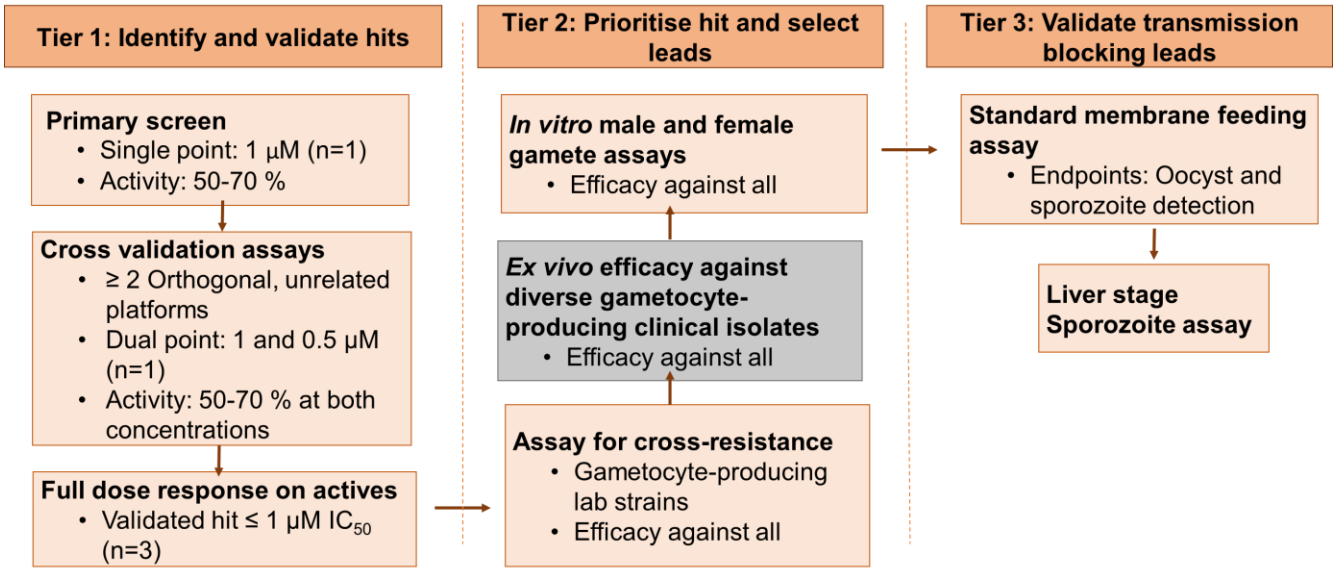


Figure 3: Three tier testing cascade for the malaria transmission-blocking compounds
Compounds progress through tier 1: Hit ID and validation, to tier 2 where the hits are prioritised, and leads are selected. Testing compounds against clinical isolates that produce gametocytes provides a realistic representation of gametocytocidal activity (grey). In tier 3, the transmission-blocking compounds are validated through different assays. The primary cascade is indicated in orange. Progression towards tier 3 includes more involved assay with a decreased throughput. (Figure adapted from [61]).

From the above cascade, the evaluation of a compound's action against clinical isolates is imperative. The main advantage of including clinical isolates is that they can be used as an early filter of *in vivo* efficacy and potentially minimize further downstream attrition rates [61]. Testing of gametocytocidal compounds against clinical isolates will determine whether there is differential drug activity in different gametocyte populations *ex vivo*. Ideally, drugs with very low activity against clinical isolates will not progress through the drug testing cascade, in turn prioritizing active compounds for further development.

1.4.2 Differential drug response in *ex vivo P. falciparum* clinical isolates

Previous work identified novel transmission-blocking hits from screens of kinase libraries and these hits were profiled against *ex vivo* clinical isolates with the aim of progressing them for effective transmission-blocking candidates [63]. Differential drug responses were observed for several of these kinase inhibitors, with the majority of the compounds retaining comparable activity on the clinical isolates compared to the lab strains tested. However, one of the compounds (MMV642944) displayed pronounced variability against the different compound pressure (Figure 4). This implies that the compound may therefore not be broadly effective against different clinical isolates and should therefore not be prioritized for development as a transmission-blocking drug.

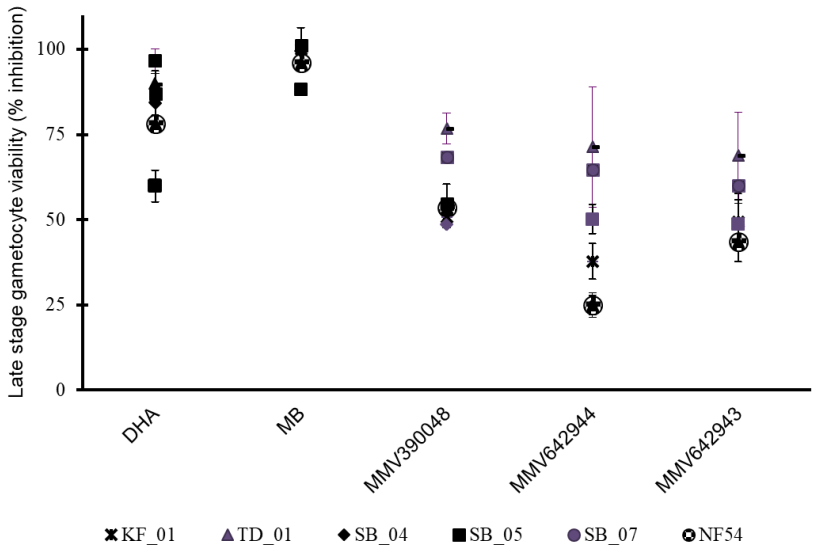


Figure 4: Late-stage gametocytocidal activity of lead compounds against *ex vivo P. falciparum* clinical isolates.
The inhibition (%) of PI4K inhibitors on late-stage gametocyte viability in clinical isolates using the parasite lactate dehydrogenase (pLDH) assay showed differential responses in various isolates [63].

This data also implies that different isolates have varied responses to transmission-blocking candidates. This raised the question as to what the underlying reason for this differential sensitivity is. As an initial investigation in the reported study [63], the isolates were evaluated for known antimalarial drug resistance markers (albeit for known TCP-1 asexual drugs).

Molecular markers associated with antimalarial drug resistance can either be mutations in genes or changes in the copy number of genes encoding the drug target in the parasite. It can also be mutations that alter the influx and efflux of drugs [64]. As such, chloroquine (CQ) resistance was indicated by mutations in *P. falciparum* CQ resistance transporter *(Pfcrt)* [65] and overexpression of multidrug resistance transporter, *Pfmdr*-1 [50, 66]. Antifolate resistance is typified by mutations in dihydropteroate synthase gene (*pfdhps*–sulphadoxine resistance) [67] and/or dihydrofolate reductase (*Pfdhfr*–pyrimethamine resistance) [24, 68].

Table 2: Genotypic characterisation of *ex vivo* clinical isolates from South African hospitals.
Table from [63]**.**

Clinical Isolate	Drug resistance markers	Drug resistance phenotype
KF14_01	*Pfdhfr, pfdhps, pfcrt*	Pyrimethamine, sulphadoxine, CQ
SB14_04	*Pfdhfr, pfdhps, pfmdr1*	Pyrimethamine, sulphadoxine
SB14_07	*Pfdhfr, pfdhps, pfcrt*	Pyrimethamine, sulphadoxine CQ
SB14_05	*Pfdhfr, pfdhps, pfcrt*	Pyrimethamine, sulphadoxine CQ
TD14_01	*Pfdhfr, pfcrt*	Pyrimethamine, CQ

Correlating the drug resistance profile of the clinical isolates from above to the differential transmission-blocking activity was not informative, with isolates such as KF14_01, SB14_07 and SB14_05, that display the same drug resistance profiles, having very different drug responses, specifically to candidate MMV642944 (Table 2). The standard drug resistance marker testing is therefore not applicable to effectively predict gametocyte drug responses. Consequently, a broader understanding of the genetic diversity associated with the clinical isolates is needed to describe a causal relationship between drug response and genetic diversity.

1.4.3 Genetic diversity and differential drug responses

The association of genetic diversity and differential response to drug action is well established, particularly in humans where e.g. genotype differences in CYP proteins result in different metabolic responses to drugs [69]. These extensive pharmacogenomics strategies are now widely employed during drug discovery.

Presently, population genomic strategies are used to identify genetic loci under selection in the malaria parasite, as well as finding polymorphisms that are associated with clinical phenotypes such as drug resistance [70]. The advantage of identifying resistance loci is that drug resistance involves significant contributions from individual genes and the causative alleles are often found at high frequencies in treated populations, due to strong selection (Figure 5) [71]. Further, drug resistance loci are determined from parasite samples with natural infections that exhibit varying drug responses and detailed genotypes, usually consisting of single nucleotide polymorphisms (SNPs) [70]. Drug resistance in malaria parasites is believed to be due to the selection of parasites that harbour point mutations [7].

P. falciparum parasites can be classified into two broad classes of natural selection, namely balancing and directional selection (Figure 5 A). Balancing selection maintains genetic diversity for loci, which are under immune selection [46]. Under balancing selection, loci have high genetic diversity, however, there is a low divergence, which is referred to as the amount of allelic variation between different populations (figure 5 A) [70]. By contrast, directional selection involves selecting mutations that are predisposed to survive under drug pressure after drug treatment [46]. In directional selection, genes are likely to have low diversity with populations with high divergence (Figure 5 A). Genes under directional selection include those with known drug resistance and they are selected by selecting alleles that confer resistance (Figure 5 B). This phenomenon is defined as the selection sweep. Due to this sweep, neighbouring alleles

remain maintained along with the allele that confers resistance, resulting in a large area on the genome with reduced genetic diversity. Consequently, identifying genome regions with reduced diversity in drug-resistant parasites reveals candidate drug resistant genes [70].

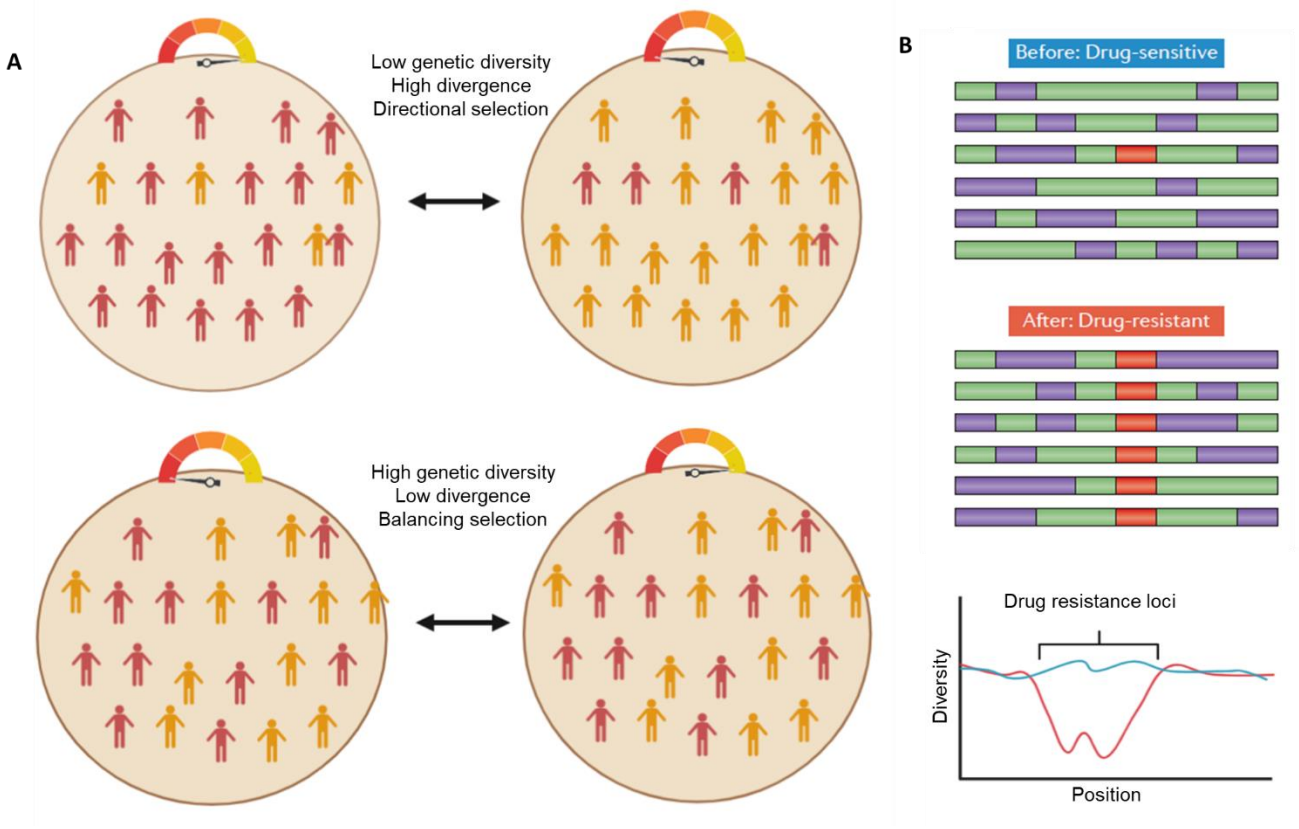


Figure 5: Genetic diversity and divergence in relation to drug resistance.
A) Within a given population (), numerous individuals each contain alleles (shown by red or yellow colour) across their genomes. Genetic diversity refers to the allelic variation among the individuals within a population, and divergence is the amount of allelic variation between different populations. Under balancing selection, high genetic diversity and low divergence is expected at a locus under selection. Under directional selection, known drug resistance genes have low diversity and a high divergence. **B)** Selective sweep is caused by the selection of an allele () that confers survival under drug pressure. This sweep is a consequence of selection for drug-resistant parasites and it causes a reduction in diversity (red line on the graph) compared to average diversity values for the genomic region (blue line on the graph). The figure was recreated using Biorender (http//app.biorender.com/) and adapted from [70].

In *P. falciparum*, pharmacogenomics is mostly limited to the evaluation of drug resistance marks as described above, but the association between drug resistance and genotyping is clearly established (Figure 5). To extend this to not only the analyses of drug resistance phenotypes but also the differential drug sensitivity, which is the focus of this study and the novelty is inherent in the characterization of transmission-blocking antimalarials.

1.5 Genetic diversity in *P. falciparum* parasites

The *P. falciparum* genome is 24 Mb in size, and it is distributed among 14 linear chromosomes, with two extra chromosomal DNA circles that constitutes the apicoplast and mitochondrial genomes [7]. The *P. falciparum* genome evolves in response to natural selection pressures of the human immune system, environmental factors, and the mosquito vector. Moreover, *P. falciparum* chromosomes have variable lengths, especially at subtelomeric regions. Due to recombination events between different parasite clones during meiosis in the mosquito, most clinical isolates exhibit extensive size polymorphism [72].

P. falciparum parasites from different transmission settings vary significantly in genotype and phenotype, with a correlation between malaria transmission intensity, infection complexity and the genetic structure of parasites (Figure 6) [73, 74]. Sub-Saharan Africa is defined as a high transmission malaria area with multiclonal *P. falciparum* infections and a high genetic diversity amongst the parasite populations [75]. The high incidence of multiple genotypes in areas of high transmission is due to the increased outbred population structure of *P. falciparum* parasites and extensive outcrossing. In these geographical areas of high malaria transmission, there is high multiplicity of infection (MOI), which is defined as the number of genetically distinct parasites that co-infect a single host. The high MOI is said to be from multiple bites from distinct *P. falciparum*-infected mosquitoes [70].

By contrast, patients infected with malaria in low transmission areas such as South American countries have infections caused by a single clone of *P. falciparum* parasites with limited genetic diversity [75]. The clonal population structure is due to high levels of linkage disequilibrium with limited recombination [76, 77]. As a result, there is a lowered prevalence of infections that are genetically identical [73, 76-78]. Individuals in these areas are said to have a low MOI and there is usually a less effective population size of people [73].

Figure 6: Genetic diversity of *P. falciparum* parasites from different trasnmission areas
In areas of high transmission, **A**) individuals within that population are expected to be infected with multiple parasite types, signifying high multiplicity of infection (MOI) with high parasite genetic diversity. Different colours show individuals harbouring multiple types of infections. **B**) In areas of low transmission, there is low genetic diversity of parasites, as individuals are expected to be infected with a single parasite type (homogenous colour represents single parasite type infections, black colour represents uninfected individuals). Figure adapted from [70].

Not only is the *P. falciparum* genome complex, also parasites from different transmission settings show the complexity in the genetics of the parasites. To study and understand different parasites, various strategies can genotype parasites to determine the extent of genetic diversity in parasites from different settings.

1.5.1 Genotyping strategies in *P. falciparum* parasites

Genotyping tools allow determination of genetic variation across the genome by typically assessing thousands of markers. Genetic diversity across the *P. falciparum* genome is manifested in the form of single nucleotide polymorphisms (SNPs), microsatellites (MSs), copy number variants (CNV) and insertions or deletions (indels) [7, 79]. The most applied genotyping techniques for *P. falciparum* include the antigenic size polymorphism, MSs and SNPs (Figure 7), which are discussed in this study.

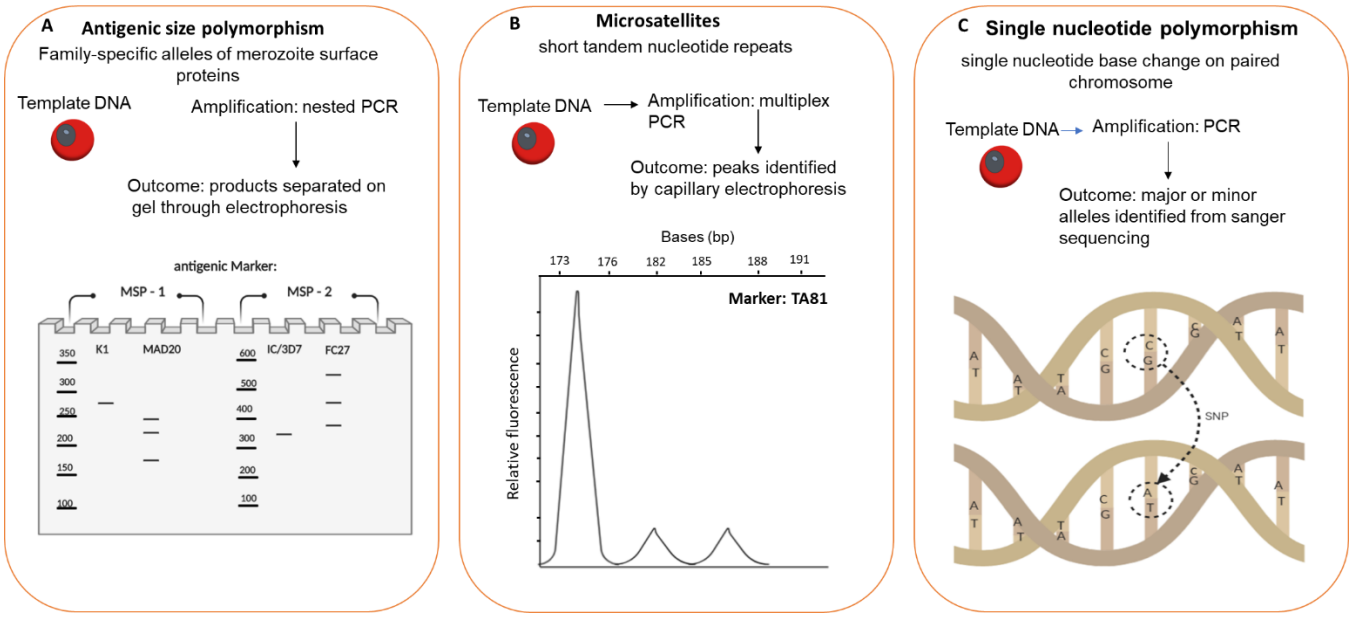


Figure 7: Genotyping techniques that dermine the genetic diversity of *P. falciparum* parasites
The genotyping techniques use template DNA from patient samples and polymorphic specific oligonucleotides to amplify the region of interest. **A)** In antigenic size polymorphism, family specific alleles of merozoite surface proteins 1 and -2 (MSP-1 and MSP-2) are detected within the patient samples. **B)** Microsatellites (MSs) are short tandem repeats which can be identified as peaks by using capillary electrophoresis. **C)** Single nucleotide polymorphism genotyping identifies major or minor allele changes on paired chromosomes. Figure created based on [76, 77, 80, 81].

1.5.1.1 Size polymorphic antigenic markers

Size polymorphism has been used to genotype of *Plasmodium* parasites and is mostly based on characterising size differences in surface proteins, some of which are associated with antigenic makers: merozoite surface proteins 1 (MSP-1) and 2 (MSP-2) (Figure 7 A), the glutamine rich protein (glurp) expressed throughout the asexual life cycle and the circumsporozoite surface protein (CSP), associated with sporozoites [82, 83].

This genotyping strategy amplifies the polymorphic regions of the parasite genome to identify haplotypes. The antigenic markers are amplified by PCR and the size of the amplicon can either be determined by gel or capillary electrophoresis (Figure 7 A) [80]. The strategy is useful for polygenomic infections, where more than one parasite genome contributes to the infection, which allows the estimation of the MOI level across patients [84]. The mean number of genetically distinct parasite lineages infecting a person can be estimated using this genotyping method to distinguish between high and low transmission levels. However, since size polymorphisms are dependent on characterising a limited set of genes, the level of detail obtained is obviously constrained [83]. Further drawbacks associated with this technique include that the interpretation of agarose gels shows significantly variable results when repeatedly performed on the same individual [85]. Genotyping accuracy can be improved with increasing the numbers of molecular markers assessed. Polymorphic markers with larger

repeats such as the CSP and glurp protein have been used, however they are less diverse compared to MSP-1 and MSP-2 [76, 85]. Other challenges are that loci are under strong immune selection will affect the distribution of alleles within a population. Further, there are non-random combinations of MSP-1 alleles in individuals with multiple infections, leading to underestimation of genetic diversity.

1.5.1.2 Polymorphic microsatellite (MS) genotyping

Multilocus genotyping in *P. falciparum* is used to investigate parasite genetics, population structure and dynamics of clinical isolates. Microsatellites (MSs) are defined as short tandem nucleotide repeats, generally limited to between 1 and 6 nucleotides each, found in specific genomic localisations across the genome. In the A+T-rich genome of *P. falciparum* [86], MSs are typically characterised as $(TA)_n$ and $(TAA)_n$ motifs, with the variation in the numbers of tandem repeats among strains [87]. These remain important to understand the genetic diversity, relatedness of parasite populations and understanding transmission dynamics.

With MS genotyping, genotypes are obtained accurately from several MS regions (over many loci), providing higher resolution data than the size polymorphisms described above. MSs can be detected from limited amounts of genomic DNA and the MS loci are amplified using multiplex PCR (Figure 7 B) [88]. The sizes of MSs can be highly reproducibly measured with capillary electrophoresis, which has a high resolution and precision to detect one nucleotide difference between MSs, that allows the diversity of loci to be fully detected [85]. MSs therefore describes allelic diversity at large number of loci which span across the genome and can type either individuals or populations [89]. Unlike antigenic markers, MSs are not under immune selection and the size of alleles have predictable lengths that are easily used to compare between various samples [85].

MS genotyping is limited due to variable mutation rates and the number of copies of the repeat, thus, the higher the repeat count, the higher the mutation rate, which may result in the detection of false positive alleles [90]. High mutation rates are due to the interplay between strand slippage events and mismatch repair, which counteracts DNA slippage during replication. As a result, the mutation rates allow the MSs to be used to understand the emergence of recent and local population genetic patterns [91].

P. falciparum parasites are characterised using 10 commonly used MSs, which are from seven chromosomes (Figure 8) [76, 80]. The different loci were selected at random with respect to

gene function [89]. Some of the loci are situated in genes with full sequences available, with majority of the loci uncharacterised [76]. This makes it challenging to reveal homology with other organisms, further making it difficult to determine the functions of the sequences [92]. Nonetheless, loci are situated in coding regions and are attributed to continuous reading frames with conserved sequences that flank the repeat regions [92].

In the present study, a panel of 26 MS loci were used to measure genetic diversity in clinical isolates. The 26 MS loci includes a panel of 16 newly identified loci that flank the 10 (PolyA, Ta81, TA87, TA1, TA109, TA40, ARA2, PfG377, PfPK2 and TA60) MS loci previously described (Figure 8) [93]. The 16 were selected and identified MS loci in the up- and down-stream flanking regions of the 10 MS loci, to allow the evaluation of short-range haplotypes. The 26 loci were carefully evaluated for their utility as genotyping markers and they were found to be neutral and polymorphic [93]. In previous studies, the panel was used to determine 1) the genetic diversity of southern African parasites to infer local and cross border infections, 2) to use genetic diversity as a surveillance tool to understand the dynamics of malaria parasites and 3) to evaluate temporal spatial clustering of parasites from Zambia [88, 94, 95].

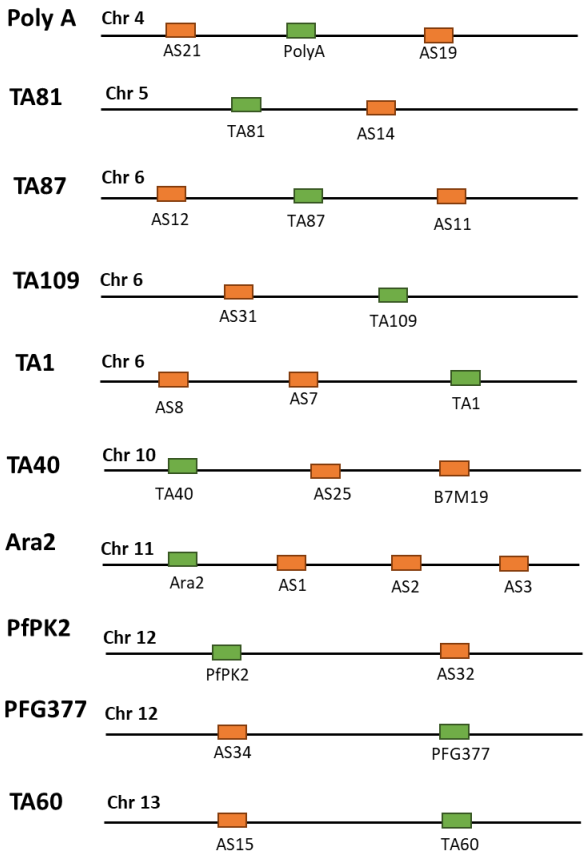


Figure 8: Chromosomal location and distribution of a panel of 26 microsatellite loci
Ten commonly studied microsatellite (MS) markers indicated in bold were used guide and select 16 new tandem repeats in the up- and down-stream flanking regions of the 10 MS markers. Newly selected MS loci (▭) and the original 10 MS loci (▭) are located on their respective chromosomes indicated by a single line, which is not drawn to scale. Figure generated using Biorender (https://app.biorender.com/) and adapted from [93]

1.5.1.3 High-throughput single nucleotide polymorphism genotyping

SNPs are defined as DNA sequence variations, which occur when either one of the four nucleotide bases [adenine (A), cytosine (C), guanine (G) or thymine (T)] in the genome differs between parasites or paired chromosomes in an individual (Figure 7 C). A SNP molecular barcode is comprised of different combinations of SNPs that together express a unique pattern of variation of sequences, and these patterns are then used to describe and distinguish parasite clones from each other. The technique can be easily applied to a variety of lab adapted parasites and field isolates with over 99 % success [79]. This genotyping technique is sensitive and requires small amounts of gDNA for amplification of genome fragments containing the SNP sites [81].

SNP genotyping has be applied to various *P. falciparum* lab strains including field isolates [96]. SNP-based barcodes are able to distinguish malaria parasites that originate from different geographic regions, hence they are used to identify sources of infections in pre-elimination settings [97, 98]. SNP barcodes have also been used in *P. vivax*, where a panel of 42-SNP barcode was developed using clinical samples from parasite populations originating from Africa, Asia and South America [98]. Of interest to this current study, a panel of 24 *P. falciparum* SNPs were selected from over 112000 SNPs from 18 parasite genomes (Figure 8) [79]. The selected SNPs segregate independently and they are broadly distributed across the genome, exhibiting a 35 % minor allele frequency, which are defined as the frequency at which the second most common allele occurs within a population [79]. The panel has been previously used 1) to study the vector and human interactions by tracking parasite genotypes over time with the aim of understanding parasite populations, 2) to investigate the impact of various intervention strategies in high transmission areas such as Zambia and Zimbabwe, and 3) to investigate clonal outbreaks of *P. falciparum* in Panama [79, 86, 99].

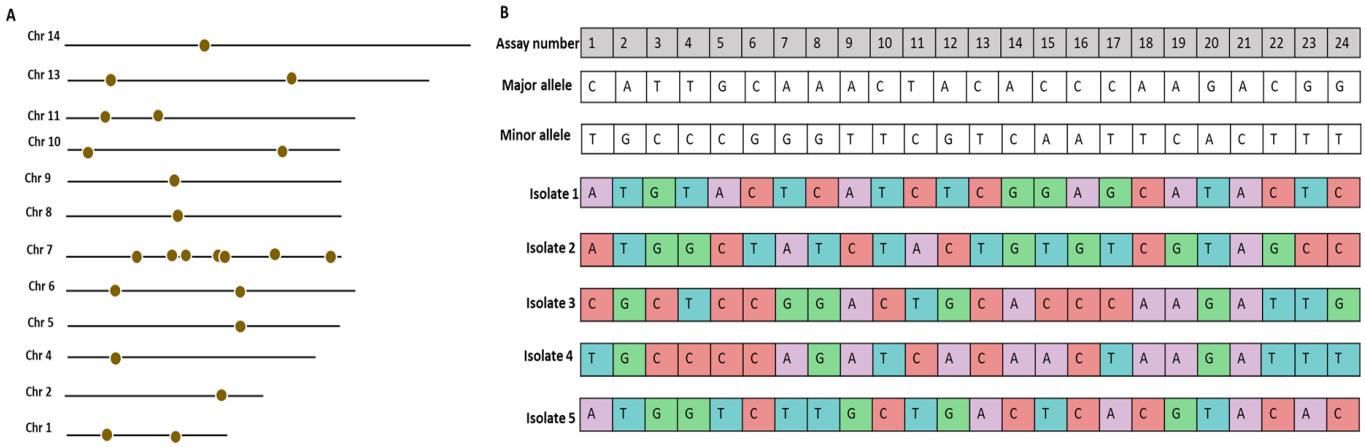


Figure 8: Distribution of the SNP assays and the resultant molecular barcode
A) The distribution of *P. falciparum* SNPs (●) across the 14 chromosomes of *P. falciparum* genome. **B**) An example of a molecular barcode indicating the major and minor alleles in field isolates. The assay number corresponds to the distribution of alleles on the chromosomes. Figure adapted from [79].

SNP-based barcodes have been described as more informative compared to the size polymorphism genotyping, as they have the capacity to differentiate between strains due to the variable number of alleles which exist among the 24 markers [100]. Compared to MSs, SNPs are more amenable [101]. A drawback of DNA based barcodes (24 *P. falciparum* and 42 *P. vivax* SNPs) is that they are located in the nuclear genomes, which makes them easily subject to recombination, thus exhibiting high mutation rates. As a result, the correct genotyping of the parasites could be impaired [102].

Genotyping parasites further aids the biological discovery by using genetic diversity to identify loci under selection or loci which are associated with clinical phenotypes, leading to the development of tools to monitor and evaluate interventions. For the first time, this study aims to address differential drug response observed in gametocyte producing southern African *ex vivo P. falciparum* isolates. The study interrogates the contribution of the extent of genetic diversity by using MS analysis and SNP genotyping to clarify the differential drug response observed in clinical isolates.

1.6 Aim and Objectives

1.6.1 Hypothesis

Genetic diversity of *P*. *falciparum* clinical isolates can describe differential drug response in sexual stages of the parasite**.**

1.6.2 Aim

Determine the allelic variation in *P. falciparum* clinical isolates to elucidate the contribution of genetic diversity to differential drug responses of late-stage gametocytes

1.6.3 Objectives

- Determine the inhibitory effect of antiplasmodial compounds using the pLDH assay for late-stage gametocytes from clinical isolates
- Determine extent of genetic diversity in clinical isolates through microsatellite analysis
- Characterize the SNP molecular barcode of *P. falciparum* clinical isolates
- Correlate extent of genetic diversity of *P. falciparum* clinical isolates to gametocyte differential drug responses

Chapter 2: Materials and Methods

Ethical clearance

P. falciparum clinical isolates (KF14_01, SB14_07, SB14_04, SB14_05 and TD14_01) were obtained from infected patients following informed consent from Steve Biko Hospital, Tshwane District Hospital and Kalafong under ethical clearance (University of Pretoria 417/2013). *Ex vivo* culture adaptation of clinical isolates was performed [63]. NF54 and 3D7 lab adapted strains were obtained from MR4. All experiments were performed at the Malaria Parasite Molecular Laboratory (M^2PL), under the supervision of Professor L. Birkholtz and Dr J. Niemand in an access controlled P2 facility. All *in vitro* experiments involving human blood donors and human malaria parasites holds ethics approval from the University of Pretoria Research Ethics Committee, Health Sciences Faculty (506/2018).

2.1 *In vitro* cultivation of the asexual stages of *P. falciparum* parasites

In vitro P. falciparum parasites (NF54, KF14_01, SB14_07, SB14_07, SB14_05 and TD14_01) were cultured in human erythrocytes at a haematocrit of 5 %. The parasites were maintained in complete media [RPMI-1640 (Sigma-Aldrich, USA) supplemented with 23.81 mM sodium bicarbonate (Sigma-Aldrich, Germany) to maintain a pH of 7.4; filter sterilized 0.5 % (w/v) Albumax II (Thermo Fisher Scientific, Germany); 80 mg/mL gentamycin (Aspen, Germany), 25 mM HEPES (Sigma-Aldrich, Germany); 20 mM glucose (Merck, South Africa) and 0.2 mM hypoxanthine Sigma-Aldrich, Germany)]. The asexual stages of the parasites were maintained at a 5 % haematocrit with a 3-5 % parasitaemia. Before incubation (37 ºC, shaking at 60 rpm), the parasites were placed in culture flasks and gassed with 90 % N_2, 5 % O_2 and 5 % CO_2 gas mixture for 30 s to create hypoxic conditions [103]. Culture media was replaced daily to provide nutrients and remove the lactic acid produced by the parasites. Parasite morphology and proliferation was monitored by Giemsa-stained slides visualized with light microscopy using a 100 x oil immersion lens at a 1000 x magnification.

2.2 Gametocyte induction and *in vitro* maintenance of sexual stages of *P. falciparum* parasites

Ring-stage intraerythrocytic *P. falciparum* parasites (5 % haematocrit, 5 % parasitaemia) were synchronized using 5 % (w/v) D-sorbitol for 15 min at 37 °C incubation, to obtain iso-osmotic lysis [104]. The synchronized ring-stage asexual parasites were used to initiate gametocytogenesis, with the haematocrit and parasitaemia adjusted to 6 % and 0.5 % respectively. The parasites were transferred to a glucose-deprived media to produce nutrient-

starved and stressed parasite cultures [105]. The parasites were maintained under hypoxic gas conditions (5 % CO_2, 5 % O_2 and 90 % N_2, 37 °C) in a stationary incubator for 11 days [105]. After 72 h, the volume of culture medium was increased to 1.5 times the initial volume to lower the haematocrit, thus, inducing gametocyte production. The gametocytes were cultured for 11 days in glucose deprived media [RPM-1640 (Sigma-Aldrich, USA), supplemented with 23.81 mM sodium bicarbonate (Sigma-Aldrich, Germany); filter sterilized 0.5 % (w/v) Albumax II (Thermo Fisher Scientific, Germany); 80 mg/mL gentamycin (Aspen, Germany), 25 mM HEPES (Sigma-Aldrich, Germany); and 0.2 mM hypoxanthine (Sigma-Aldrich, Germany)] and monitored daily under a microscope with Giemsa stained blood smears [105]. Gradual elimination of asexual parasites was achieved by supplementing the glucose deprived media with 50 mM N-acetyl-glucosamine (NAG) for 6 to 9 days [105, 106]. NAG treatment inhibits erythrocyte reinvasion by merozoites but does not interfere with gametocyte development [107, 108]. On day 11, gametocyte cultures were mostly stage IV and V. Gametocyte commitment was calculated as a ratio between a population of Stage II gametocytes on day 5 and intraerythrocytic ring-stage parasites on day 1. The gametocyte commitment was expressed as % conversion rate. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA) using an unpaired student's t-test.

2.3 Inhibition of intraerythrocytic *P. falciparum* viability by antiplasmodial compounds

Following cultivation of the late-stage *P. falciparum* gametocytes, the inhibitory effects of antiplasmodial compounds were evaluated using the pLDH assay. Test compounds used in this study include MMV00019, compound A, compound B at 1 μM concentration. MMV00019 (MMV'019), which is also known as OZ439 is an endoperoxide inhibitor. Compound A and compound B are both P-type Na^+-transporting ATPase 4 (ATP4) inhibitors, all compounds have shown gametocytocidal activity against late-stage gametocytes [109, 110]. MB at 5 μM was used as a positive control for gametocyte inhibition, while complete media was used as a positive control for gametocyte viability. The drugs were placed in 96-well plates in a final volume of 100 μL/well, followed by the addition of synchronized stage IV and V gametocyte parasites at 1 % haematocrit and 2 % gametocytaemia. The plates containing the parasites under drug pressure were incubated in a stationary incubator at 37°C for 72 h under drug pressure. Subsequently, 75 % of the spent drug medium was replaced by drug-free complete medium, followed by a further 72 h incubation [105, 111]. Gametocyte viability was determined spectrophotometrically by measuring the activity of pLDH [112]. In an infected erythrocyte, the *Plasmodium* lactate dehydrogenase can oxidize lactate to pyruvate while reducing the cofactor

3- acetylpyridine-adenine-dinucleotide ($APAD^+$) to APADH. Subsequently, APADH reduces a yellow tetrazolium dye, nitro-blue tetrazolium (NBT) to a blue diformazan compound. Malstat reagent at a volume of 100 μL [0.21 % v/v Triton X-100; 222 mM L-(+)- lactic acid, 54.5 mM Tris, 0.166 mM APAD (Sigma Aldrich); pH 9] was added to a 96-well plate. Subsequently, 20 μL of parasite suspension was transferred to the Malstat plate, followed by addition of 1.96 mM NBT and 0.0239 mM phenazine ethosulfate (PES). Plates were incubated in the dark for 40 min, after which absorbance was measured with a Multiskan-Ascent 354 multiplate scanner (Thermo Labsystems, Finland) at a wavelength of 620 nm. All experiments were unpaired and performed in technical triplicates with three biological repeats for each strain. The data were represented as percentage inhibition of the late-stage gametocytes for each strain after background subtraction. All graphs were made using GraphPad Prism 6.0 software and the unpaired student t-test was used to determine the significance of the % inhibition of each isolate by each test compound, compared to NF54.

2.4 Determining genetic diversity of *ex vivo P. falciparum* clinical isolates.

2.4.1 Multilocus genotyping of clinical isolates using MS analysis of 26 loci

All experimental procedures for the MS genotyping of clinical isolates were performed by PhD candidate Hazel B Gwarinda at the laboratory of Professor Bryan Greenhouse, University of California, San Francisco. The panel of MSs which was used for genotyping of *ex vivo* clinical isolates consist of 26 markers across the *P. falciparum* genome [76, 85]. The genomic DNA was extracted using the chelex method as described on section 2.4.2.1. Briefly, two rounds of PCR were applied to amplify the 26 MS loci for all five clinical isolates. Firstly, a multiplex PCR was used for amplification using two different PCR conditions described elsewhere [94]. A volume of 1 μL of the amplified product was subsequently used as template for the individual PCR for each marker. PCR products were diluted and sized by denaturing capillary electrophoresis, which was performed on an ABI 3730XL analyzer with GeneScan™ 400HD ROX™ size standard (Thermo Fisher Scientific). To automate the identification of true alleles and differentiate real peaks from artifacts, the electrograms obtained were analyzed using microSPAT software [88]. Data were obtained in a form of allele sizes with varying base pairs (bp) per loci for each clinical isolate. Loci were arranged in increasing number of varying alleles detected in all clinical isolates. To determine the number of clones which are present in each isolate, MOI was calculated as the second highest allele number detected for each isolate [88]. The box and whisker diagram were created in the RStudio software program v3.5.3 (2019-03-11) using the ggplot2 package (v3.2.1). Multiple correspondence analysis plot was created by

assigning each allele size per loci as a unique categorical value. The plot was also created using the RStudio software program (v3.5.3) and a Factoextra (v 1.0.5) package.

2.4.2 Single nucleotide polymorphism (SNP) genotyping of *ex vivo P. falciparum* clinical isolates

2.4.2.1 Genomic DNA isolation from *P. falciparum* parasites

Two different approaches were used to isolate gDNA from intraerythrocytic *P. falciparum* parasites. In the first approach, the chelex method was used to isolate gDNA from dried blood spots (DBSs) from cultured *P. falciparum* parasites [(clinical isolates, NF54, 3D7) at (5 % haematocrit, 10 % parasitaemia, 2 μL)] and were spotted on filter paper (Whatman®) [113]. A uniform section from each dried blood spot (DBS) were exercised using a hole punch. Punched DBSs were incubated in 0.5 % (w/v) saponin in 1 X PBS [137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4 and 8 mM Na_2HPO_4, pH 7] solution overnight at 4° C. Saponin is used to lyse erythrocytes for hemoglobin removal, while leaving the parasites plasma membrane and parasitophorous vacuole membrane intact [114]. After aspirating the saponin/PBS solution, the DBSs were washed using 1 X PBS with a brief vortex, followed by a 30 min incubation at 4 °C. The DBSs/1 X PBS were centrifuged using a MiniSpin® plus centrifuge (Eppendorf, Hamburg, Germany) at 1000 x g for 2 min and aspirated. Chelex solution at 10 % (w/v) dissolved in $dddH_20$ was transferred to the DBSs and incubated at 95 °C for 10 min. Chelex (Bio-Rad, Hercules, USA) is an ion chelator, which binds and removes ions such as magnesium ion. Removal of ions inactivate DNases that might damage the DNA. Applying heat to the solution disrupts the cell membrane and denatures proteins including heat liable enzymes [115]. Every 2 min for the 10 min incubation period, the DNA was extracted through brief, vigorous vortexing. Lastly, the samples were centrifuged at 14000 x g for 5 min. The gDNA containing supernatant from all samples were stored at -20 °C.

Alternatively, a commercial kit (Qiagen, Germany) was used to extract gDNA from trophozoite stages of the parasites. The proprietary components of the kit led to higher purity of DNA samples and increased yield compared to the chelex method. The kit uses the principle of chaotropic salt binding, where DNA binds to the silica membrane under high salt conditions [116]. The hydrate shell of nucleic acids is reversibly removed by chaotropic salt, followed by ethanolic wash steps which are vital for the removal of contaminants. DNA is released from the membrane using an elution buffer. Two volumes of genomic Lysis buffer were added to the *P. falciparum* intraerythrocytic parasites (5 % haematocrit, 10 % parasitaemia, 200 μL), vortexed

for 4 s and incubated at room temperature for 5 min. The lysate was then transferred to a Zymo-Spin IIN Column attached to a collection tube, and centrifuged using a MiniSpin® plus centrifuge (Eppendorf, Hamburg, Germany) at 14000 x g for 1 min. Wash buffer (500 µL) was used to wash the column to remove contaminants through centrifugation at 14000 x g for 1 min. The contents were transferred to a clean Eppendorf tube, followed by an addition of DNA elution buffer (70 µL) which was preheated to 37 °C. The column containing the preheated elution buffer was incubated for a further 5 min at room temperature before eluting the purified gDNA at 14000 x g for 30 s. The concentration and purity of the isolated gDNA was determined spectrophotometrically by measuring absorbance at 230, 260 and 280 nm determining the $A_{260/280}$ and $A_{260/230}$ ratios using the Nanodrop® ND-1000, v3.8.1 (Thermoscientific, USA) [117, 118].

2.4.2.2 PCR amplification of SNP containing genome fragments

SNP genotyping was performed using previously published oligonucleotides that were designed to amplify the genome region of more than 100 bp containing the SNP (Table 3). The name of each SNP consists of the chromosome in which it is found and its position on the chromosome as annotated in PlasmoDB v3. Oligonucleotides (Inqaba Biotech) were dissolved in 1 X TE buffer (0.1 mM Tris, 0.1 mM EDTA, pH 7) to a final concentration of 100 µM. The concentrations of oligonucleotides were determined spectrophotometrically using the NanoDrop ND-1000 (Agilent Technologies, Santa Clara, USA) spectrophotometer by substitution into Beer-Lambert equation ($c = \varepsilon \times A_{260\ nm} \times$ dilution factor) using the extinction coefficient for single stranded DNA as given by the manufacturer for each oligonucleotide. All PCR amplifications were carried out in the 2720 Thermal Cycler (Applied Biosystems, Foster City, USA). Amplification was achieved with 40 cycles of denaturation at 94 °C (2 min), annealing at 53 °C or 54 °C (30 s, Table 3) and extension at 72 °C (2 min); ending in a final extension at 72 °C (5 min). For all PCR reactions (25 µL), 12.5 µL of 2 X KAPA Taq ReadyMix [(0.5 U KAPA Taq DNA polymerase, KAPA Taq Buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and stabilizers), Kapa Biosystems, USA)], 10 pmol of each oligonucleotide and 60-120 ng template gDNA were added to the reaction mix.

Five to eight PCR reactions (25 µL) of each fragment were pooled prior to purification, with a fraction (10 µL) of each loaded with 2 µL of 6 X TriTrack DNA Loading Dye [0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 0.15 % orange G, 60 % glycerol, 60 mM EDTA and 10 mM Tris-HCl (pH 7.6)] (Thermofisher, Lithuania). GeneRuler Ultra Low Range DNA ladder 10-300 bp (Thermofisher, Lithuania) was included as a molecular marker for size

comparison. The PCR products were separated on a 5 % (w/v) analytical grade agarose gel (Promega, USA), which was prepared in TAE buffer (0.04 M Tris acetate, 1 mM EDTA) at 80 V/cm, with TAE as a running buffer. Following electrophoretic analysis, gels were stained in a TAE solution containing 5 μg/mL Ethidium bromide (EtBr) for 30 min. To visuals gels, a Gel Doc™ XR+ gel visualization system (Bio-Rad, Hercules, USA) was used and the gel images were analyzed using Image Lab™ Software, Version 3.0 (Bio-Rad, Hercules, USA).

Table 3: Oligonucleotides used in the amplification of SNP containing genome fragments.
(Table adapted from [79, 99])

Identity PlasmoDB v3	Forward oligonucleotide	Reverse oligonucleotide	Fragment size (bp)	Ta (° C)	Oligonucleotide used in Sanger sequencing
Pf_01_000130573	AGCAATCGAACCCTTTGATCTA	AAATGAGAAGAGAGAAATATGTGTTGATAA	139	53	Forward
Pf_02_000842803	ATGGAAATACACAATTCAATGGC	GAAATGCAGTGGTACTTGTTGCTA	163	53	Forward
Pf_04_000282592	TGTTATGTAAATTATTGTAATTATATGACTGG	AATATGAACGGTCAGGTAGAAA	112	53	Forward
Pf_05_000931601	GGGAAGAAATGGAAGATCAGGA	TAAATTGTCATCATCTTCATAATGTGGG	209	54	Reverse
Pf_06_000145472	TTTGTTCACAATATATGTTCATGTGTCC	AGGTAGAATATCAAATACAAGGGATGTTA	113	54	Forward
Pf_06_000937750	CTAGCTCAGCTTCCAATTTGTCA	ACAAGATGTCCACATCCATATACT	160	54	Forward
Pf_07_000490877	GAAACATATTAAAACGTAAACCAGGAG	ATAGATGAGTTAGCAACGAAACC	118	53	Reverse
Pf_07_000545046	GGTTTATAACTATTCTTTAAATATGGATGTATGAC	GAAATGCTTGGTATTGCTAGATTAGAT	242	53	Forward
Pf_07_000657939	ATATACGTCCACTTATGATAATGACCC	CTTTCGTTTATATTGCAACATTTCTTCATACT	115	53	Reverse
Pf_07_000671839	ACCATTCATAGTTTAGTAGAATCAACC	AGTCATGTGATATATGATTTTGT	118	53	Forward
Pf_07_000683772	ATCGTACCACCATTAACATTTTGGAT	GGTGAATACAATGAAAAAAATTATAAAGATTTATCATTCG	155	53	Reverse
Pf_07_000792356	AATGGGACTCTTCTGTTTCGG	AACTAAGTCCAATAGTTTGTATATTCCCT	128	53	Forward
Pf_08_000613716	ATGATAATAAGACACTTCAAATCTTTTCCAG	GGTATGTAATTTGGGAAAGAATTAACAATACT	181	53	Reverse
Pf_09_000634010	ACAGCAAAAAATAATGTTAATTTTATTTCATCATCCGCA	ATTGATTTAGTTGAAATACCATGTGTACCTGTTGTG	152	53	Forward
Pf_10_001403751	AGAACCCGTGGTACCTACAT	GGACCATCTACTACTGGTGTTTCC	147	53	Reverse
Pf_11_000406215	ACACAGGATGATGAATATAATAACTTAAATAAAAATATGG	GGACATTCTTTCAATGCTTCCG	248	53	Forward
Pf_14_000755729	GAATATGTCGTAACAAATCATCAATTAAGTC	TGAGGAATAGGTTCATATGCTGATGAAGA	125	53	Reverse
Pf_10_00082376	ACAACATACAGCTACAAATATATGTATCC	ATGTAATATAGAACGGTTACCAACGA	233	54	Reverse
Pf_01_000539044	AACCAAGAAGTCCAAGAATAATAGTTAC	ATAATTTCATGTGTGGCTGCT	196	53	Reverse
Pf_07_000277104	ACTCAGCATTATATCTTTATTAATATAATGTTCATCC	ATGATTGCATGTACTTATCAAATTATTTACAAATAG	174	54	Forward
Pf_07_0001415182	GAGGTACTGATTATTGGAATGATTTGAGC	AATAACTTTCCACCACTCATCACG	181	53	Forward
Pf_13_001429265	TTATGATAAGCATGAAGATTTCAATAATG	CATTGATAATAAGAACCTGAAGAACCAA	156	53	Forward

2.4.2.3 PCR purification and quantification of genome fragments containing the SNPs

The amplified genome fragments were purified using the NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel, Germany), which is based on the binding of DNA to silica membranes in the presence of chaotropic salts. Briefly, the PCR reaction mixture was loaded onto a silica membrane with two volumes of Buffer NTI (2.8 M potassium acetate, pH 5.1) per 1 volume of sample. The samples were then loaded onto a NucleoSpin® Gel and PCR Clean-Up column which was attached to a 2 mL collection tube. The column-assembly was centrifuged at 11 000 x g for 30 s (MiniSpin® plus centrifuge, Eppendorf, Hamburg, Germany) and the flow through was discarded. Subsequently, the silica membrane contained within the column was washed twice with 700 µL of Buffer NT3 (100 mM Tris-H_3PO_4, 15 % ethanol, 1.15 M KCl, pH 6.3) by centrifugation at 11000 x g for 30 s. After discarding the flow through, the silica membrane was dried by centrifuging the column for 1 min at 11000 x g and incubating the column at 70 °C for 5 min to allow the evaporation of residual ethanol. The DNA was eluted in 30 µL of a preheated (37 °C) low ionic alkaline Buffer NE (5 mM Tris-HCl, pH 8.5) through centrifugation at 11000 x g for 1 min. The concentrations of the purified PCR products were determined as described previously (section 2.4.2.1).

2.4.2.4 Sanger sequencing of purified PCR products and ethanol precipitation

The nucleotide sequences of the amplified genome fragments were determined using the Sanger dideoxy method. Fluorescently labelled di-deoxynucleotides (ddNTPs) incorporated with dNTPs results in the termination of elongating strand of DNA. A BigDye v3.1 sequencing kit (Applied Biosystems, USA) was used for the sequencing reactions. The sequencing reactions (20 µL) contained 4 µL of 5 X BigDye buffer (400 mM Tris-HCL, 10 mM $MgCl_2$, pH 9), 2 µL of a 0.5 X BigDye reaction mix (proprietary mix), 40-100 ng template per kb gene region and 5 pmol of either the forward or reverse oligonucleotides in individual reactions to sequence the pure and amplified genome fragments containing the SNPs (Table 3). The sequencing reactions included an initial denaturation step of 96 °C (1 min), followed by 25 cycles of denaturation at 60 °C (10 s), annealing temperature set at 53 °C or 54 °C (5 s) (Table 3) and extension at 60 °C (4 min). The sequencing PCR was performed in the 2720 Thermal Cycler (Applied Biosystems, Foster City, USA).

Ethanol precipitation was used to purify the amplified labelled DNA from excess, unincorporated fluorescent ddNTPs and salt residues. Briefly, the sequencing reactions were combined with 25 X absolute ethanol and 1/10 v of 3 M sodium acetate (pH 5.2) and incubated on ice for 15 min. The mixture was centrifuged at 13000 x g at room temperature using a

MiniSpin® plus centrifuge (Eppendorf, Hamburg, Germany) for 30 min. Following the removal of the supernatant, the pellet was washed with 250 μL of freshly prepared 70 % (v/v) of EtOH and centrifuged at 13000 x g for 10 min at room temperature. The supernatant was removed and residual EtOH was evaporated at 50 °C for 5 min to obtain precipitated PCR products. 3D7 *P. falciparum* DNA, sequencing analysis was performed at the AGCT Sequencing facility (University of Pretoria) using an ABI PRISM® Genetic Analyzer (Applied Biosystems, Foster City, USA). Genome fragments from NF54 and *ex vivo P. falciparum* clinical isolates DNA were exported to Macrogen Europe Sequencing Facility (Amsterdam, Netherlands). Sequence base-calling were performed using CLC Main Workbench version 8.0.1 (CLC Bio, QIAGEN®, Denmark).

3.1 Ex vivo gametocyte production from *P. falciparum* clinical isolates

3.1.1 Origin and sampling of *ex vivo P. falciparum* isolates

Ex vivo P. falciparum clinical isolates used in this study were obtained directly from patients from local hospitals in South Africa and adapted to culture conditions. Although patients were at the local hospitals, travel histories of the patients revealed that they are from areas of higher transmission areas such as Mozambique and Malawi (Figure 9).

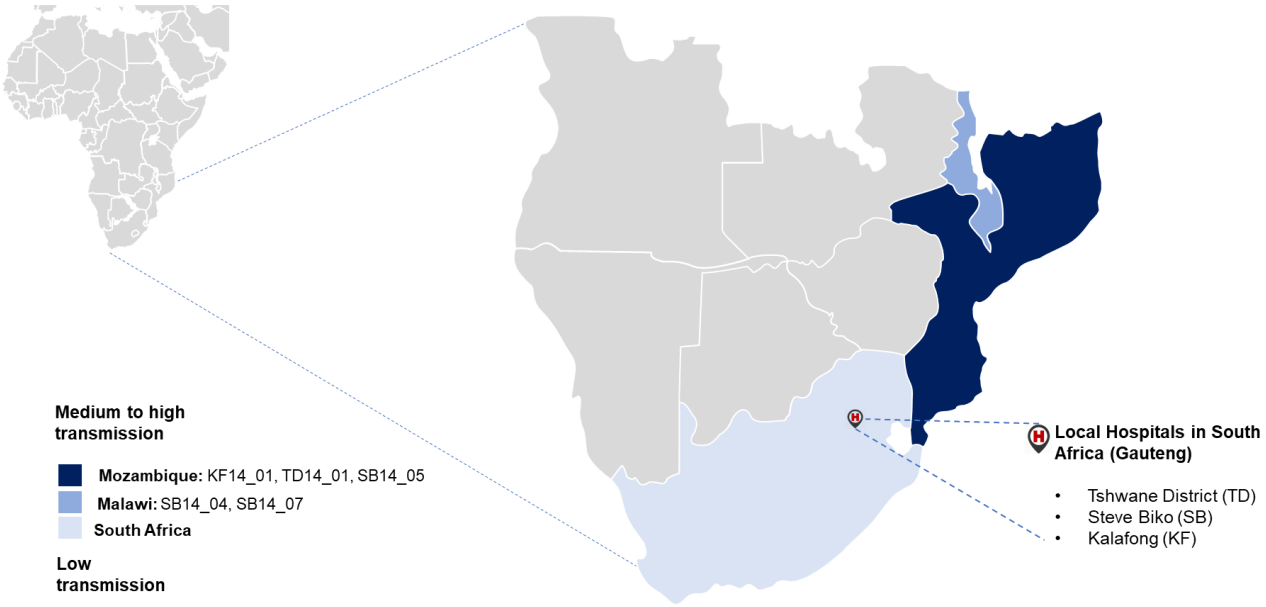


Figure 9: Location of local hospitals and the origin of each *ex vivo P. faliparum* clinical isolate on the African map.
Clinical isolates originate from Mozambique (■) and Malawi (■) based on patient travel history obtained from local hospitals. Blood samples were obtained from patients in local southern African hospitals (■). Clinical isolates are abbreviated based on the hospital from which they are obtained, namely Kalafong, Tshwane District and Steve Biko and abbreviated as KF, TD and SB respectively. [Map obtained from Servier Medical Art (URL:https://smart.servier.com/), and adapted based on colour].

3.1.2 Characterization of gametocytes of *ex vivo* clinical isolates and NF54 *P. falciparum* parasites

Gametocytes were produced from 5 individual cultures from clinical isolates (KF14_01, TD14_01, SB14_04, SB14_05 and SB14_07, Figure 10) and lab adapted NF54 *P. falciparum* parasites. Gametocytogenesis was initiated from asexual parasites synchronised to 95 % ring-stage asexual parasites from *P. falciparum* clinical isolates. Gametocytes were induced by a combination of nutrient starvation and lowered haematocrit. *P. falciparum* gametocytes undergo a long maturation period of >10 days, during which they develop from stage I to stage

V gametocytes [119]. Developmental stages of gametocytes were evaluated morphologically through Giemsa-stained microscopy (Figure 10).

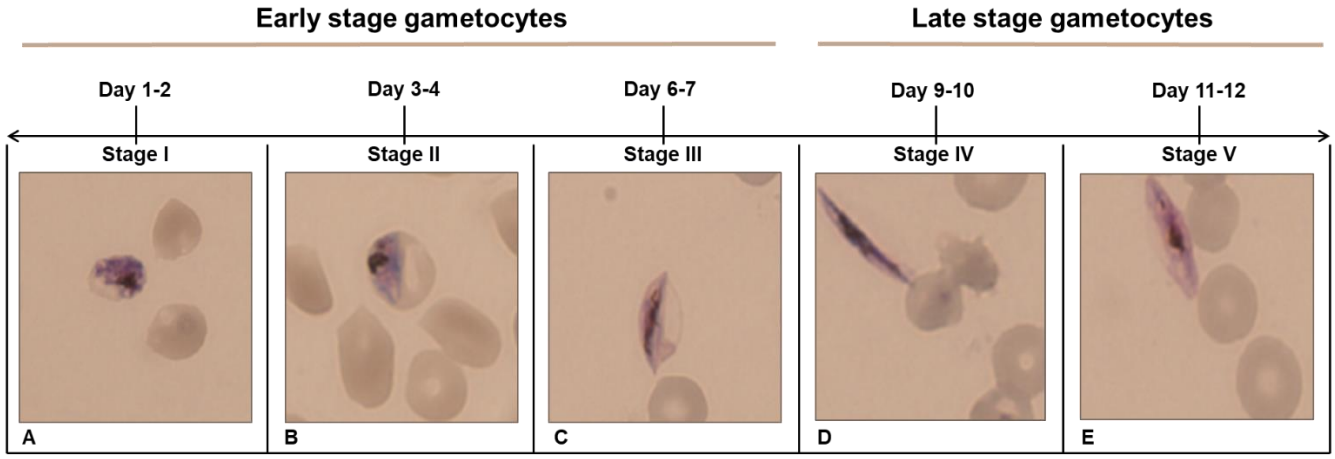


Figure 10: Five distinct morphological stages of NF54 *P. falciparum* gametocytes.
The sexual stages of the parasites are classified into five distinct morphologies which were observed from day 1-11 after gametocyte initiation (A-E). (**A**) Stage I (Day 1-2) gametocytes that resemble trophozoites. (**B**) Stage II (Day 3-4) gametocytes that have a D shape and an elongated stage III (Day 6-7) gametocyte (**C**). Stage IV (Day 9-10) gametocytes are further elongated with pointed ends (**D**). Stage V (Day 11) gametocytes have round ends with a pigmented center (**E**). Morphologies were visualized with Giemsa-stained thin smear under a light microscope at 1000 x magnification.

Stage I gametocytes were characterized by a round shape which is indistinguishable from the trophozoites of the asexual stages (Figure 10) [11]. The parasites developed into stage II gametocytes that have a D-shape, due to the formation of subpellicular microtubules. Subsequently, the parasite elongated into stage III gametocytes (Figure 10) [120]. It is during this stage where sexual dimorphism begins to be apparent. In stage IV gametocytes, osmiophilic bodies begin to form and the gametocytes were characterized by pointed ends. Stage V gametocytes were identified by round ends [9, 120].

3.1.3 Gametocyte production by *ex vivo P. falciparum* clinical isolates

To determine the gametocyte production in the different *ex vivo* clinical isolates, gametocyte development was monitored for 11 days after gametocytogenesis initiation. Parameters included in the evaluation was conversion factor (the ratio of the population of stage II gametocytes that appeared on day five to ring-stage parasites counted on day one from a total of 500 erythrocytes) and gametocytaemia for late-stage gametocytes (approximately 90 % of stage IV and V) (Figure 11).

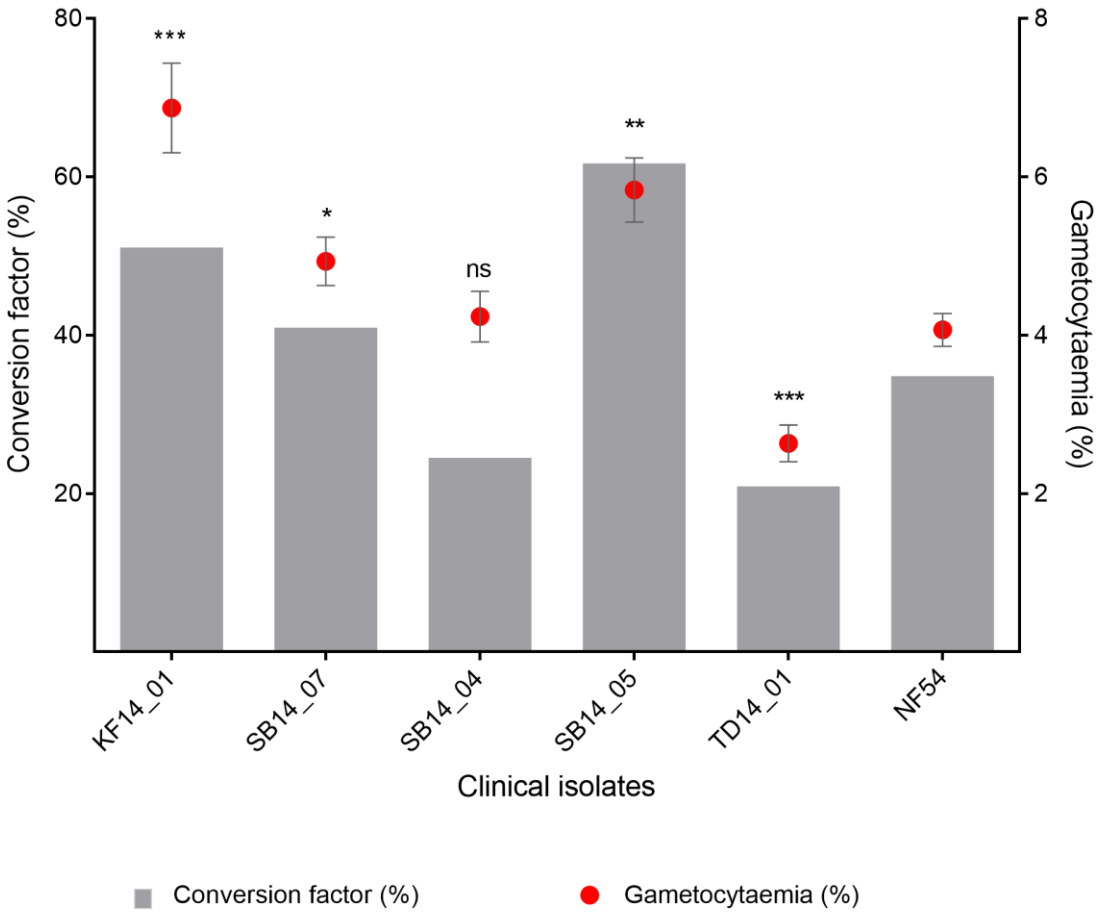


Figure 11: Gametocyte commitment relative to gametoycte production in *ex vivo P. falciparum* clinical isolates.
Conversion factor is calculated as the ratio of stage II gametocytes on day five to ring stage intraerythrocytic parasites on day one, expressed as percentage to represent gametocyte commitment (▮). Gametocytaemia (%) represents late-stage gametocytes (Stage IV and V) on day 11 (●). NF54 lab strain included as a positive control for viable gametocytes. For conversion factor, data are representative of one biological (n=1) repeat. For gametocytaemia, data are representative of three biological repeats (n=3, SD, unpaired t-test compared to NF54 ***P<0.001, **P<0.01, *P<0.05, ns= no significance). Gametocyte development was monitored with Giemsa-stained thin smears under a light microscope at 1000 x magnification.

Gametocytogenesis was induced from the *ex vivo* clinical isolates with a 100 % success rate in gametocyte production. Compared to *P. falciparum* NF54 (38 % conversion factor), KF14_01, SB14_07 and SB14_05 had higher conversion rates at 50 %, 40 % and 60 % respectively (Figure 11). By contrast, SB14_04 and TD14_01 poorly converted to sexual gametocytes at 23 and 20 % respectively (Figure 11).

KF14_01, SB14_07, SB14_05 and TD14_01 all produced statistically significant higher gametocytaemia relative to NF54 (P<0.05, n=3, unpaired student t-test) (Figure 11). Interestingly, although SB14_05 had the highest conversion rate, it did not produce the highest number of gametocytes as expected (Figure 11). This suggests that a portion of the committed ring stages counted on day 1 could have died, thus reducing the number of gametocytes

counted on day 11. Similarly, for SB4_04, the low conversion rate (22 %) was not proportional to the final relatively high gametocytaemia obtained (Figure 11). Together these findings support the notion that gametocyte production is strain dependent [121, 122]. However, this data is from a single biological repeat, with more repeats required to make conclusive observations.

3.1.4 Inhibitory effects of antiplasmodial compounds on the viability of late-stage gametocytes from *ex vivo P. falciparum* clinical isolates

It was previously reported that the inhibition of viability of late-stage gametocytes from *ex vivo P. falciparum* clinical isolates with selected PI4K inhibitors resulted in differential drug responses [63]. To explore this observation further, we evaluated the performance of additional inhibitors besides the kinase inhibitors for their efficacy against clinical isolates. Two ATP4 inhibitors (compounds A and B) [110]) and an endoperoxide (MMV00019 also known as OZ439, [109]) were used as representatives of different chemical classes with known *in vitro* gametocidal activity [109, 110] (Figure 12).

Varying drug response among clinical isolates was observed, indicating that clinical isolates not only exhibited differential drug response against PI4K inhibitors as previously published, but also against ATP4 inhibitors as well as endoperoxide inhibitor. Overall, compound A resulted in the highest degree of variability on the different clinical isolates, due to the different drug responses obtained. Comparatively, compound B was able to kill gametocytes from the majority of clinical isolates, more so than compared to the control NF54 lab isolate (Figure 12). Compound A significantly inhibited SB14_07 compared to NF54 (P<0.01, n=3, unpaired student t-test). In addition, KF14_01 and SB14_05 late-stage gametocytes showed statistically significant higher viability of inhibition relative to NF54 inhibited (P<0.05, n=3, unpaired student t-test) (Figure 12). The least variance was seen for the endoperoxide MMV'019, which was similarly active against all the clinical isolates. Notably, all the test compounds performed poorly against SB14_07 clinical isolate, whereas little differences were observed between the other clinical isolates and NF54 (Figure 12). Taken together, this data does indicate that differential drug response was observed between different clinical isolates, for inhibitors from different chemical classes.

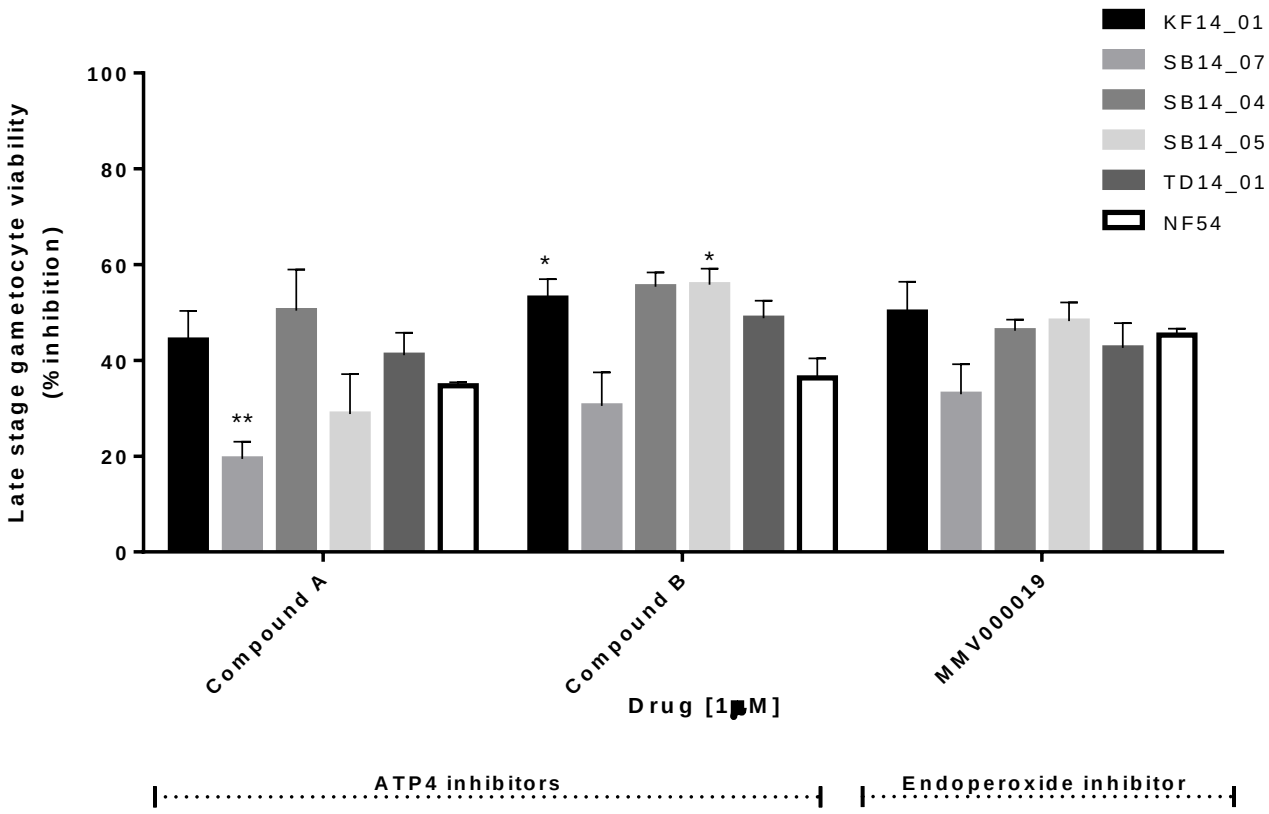


Figure 12: Late-stage gametocytocidal activity of selected compounds (1 µM) on the viability of *ex vivo P. falciparum* clinical isolates.
The late-stage gametocytes were exposed to 1 µM of each test compound (endoperoxide inhibitor: MMV'019 (OZ439), ATP4 inhibitors: compounds A and B) for 72 h at 37 °C. After a further 72 h incubation (37 °C) without compound pressure, the effect of gametocyte viability was determined using the pLDH assay. The data is representative of three biological repeats (n=3, SEM, unpaired t-test compared to NF54 ***P*<0.01, **P*<0.05, where not shown, viability of inhibition was not significant compared to NF54) performed in triplicates are shown. Structures were constructed using ChemSpider (www.chemspider.com/StructureSearch.aspx).

3.2 Genetic diversity in *ex vivo P. falciparum* clinical isolates

One postulation for the observed differential drug responses between clinical isolates of *P. falciparum* is that it could be linked to genetic diversity between these isolates. Both MS analysis and SNP genotyping were therefore used as complementary techniques to determine the genetic diversity associated with the five clinical isolates from above. The sections below will describe the data from these two approaches, after which the genotypic information will be compared to the drug response phenotypes.

3.2.1 Microsatellite characterization of *P. falciparum* clinical isolates

P. falciparum clinical isolates were genotyped using MS markers from 26 loci across the *P. falciparum* genome [85, 88]. Allelic peaks were successfully obtained for 24 of the 26 loci for all isolates. The data was first evaluated pertaining to each specific loci and the number and

identity of alleles detected. The more alleles detected within a locus, the higher the contribution to genetic diversity. The most variable loci are therefore the most informative to describe differences between and within isolates. The number of alleles also signifies the number of clones detected within each isolate.

Each MS loci showed different alleles detected for each clinical isolate at varying sizes (bp) (Figure 13). Consequently, the MS loci were distributed from the most conserved to more diverse based on the number of unique alleles detected. There were 23 variable loci, with only one conserved locus, namely AS1, which was indicated by the presence of a single private allele obtained across all clinical isolates (Figure 13). Consequently, AS1 was removed from downstream analysis as this locus had no variance and did not contribute towards genetic variability between isolates. Also removed from downstream analysis was one allele (165 bp) at locus AS12 that was present in all isolates. Loci AS34, AS7 and AS21 were attributed by two unique alleles, while AS12 and AS8 contained three unique alleles (Figure 13). There was a total of seven loci, namely: B7M19, AS19, AS2, AS32, Ara2, AS11 and PFG377, that were characterized by four unique alleles detected (Figure 13). Loci TA60, TA109 and AS3 all contained five unique alleles detected, while loci AS31, AS15 and TA87 all contained six unique alleles. Both AS14 and TA81 had seven unique alleles, while TA40, PolyA and AS25 were among the most variable loci with eight, nine and 10 unique alleles detected, respectively.

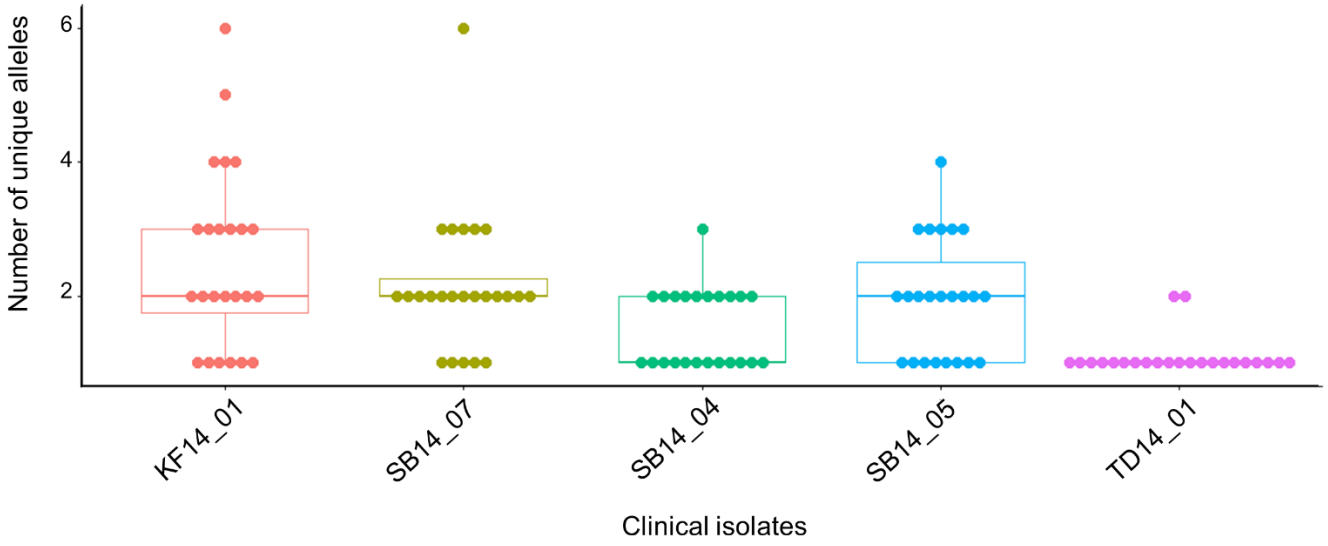
Locus	Allele size (bp)	Clinical isolates				
		KF14_01	SB14_07	SB14_04	SB14_05	TD14_01
AS1	173	■	■	■	■	■
	184	■	■	■	■	
AS34	187					■
AS7	171	■	■	■	■	
	174			■	■	■
AS21	156	■	■			
	160	■	■			
AS12	162	■	■	■	■	
	165	■	■	■	■	■
	168	■				
AS8	197		■	■	■	
	200	■	■		■	■
	204		■			
B7M19	138				■	
	146		■			
	155		■	■	■	■
	163	■				
AS19	166	■			■	
	179	■	■	■		
	182	■	■	■		
	185					■
AS2	186				■	
	193	■	■			
	195	■	■	■		■
	199		■		■	
AS32	227				■	
	230				■	
	242	■	■	■		■
	257	■			■	
Ara2	136	■	■	■	■	
	139		■	■	■	
	142	■			■	■
	145	■				
AS11	156	■	■	■		
	162	■	■		■	
	165	■				■
	171	■				
PFG377	97	■				
	100	■	■	■		
	104	■	■			■
	107				■	
TA60	205	■		■		
	208	■	■	■		
	211			■		
	217				■	■
	221		■			
TA109	152	■	■	■		
	155	■			■	
	165		■			■
	168	■			■	
	174		■			
AS3	164	■	■	■		
	167	■	■			
	170			■		■
	173		■		■	
	179	■				

Locus	Allele size (bp)	Clinical isolates				
		KF14_01	SB14_07	SB14_04	SB14_05	TD14_01
AS31	190				■	
	196	■		■		
	200	■	■	■	■	
	203	■	■			■
	206	■				
	209		■			
AS15	122	■			■	
	125		■	■		
	128		■			
	134			■	■	
	137	■				
	143					■
TA87	88					■
	91		■			
	97	■				
	100			■		
	110			■	■	■
	113	■				
AS14	197	■				
	206	■		■		■
	209				■	
	212	■	■	■	■	
	215	■	■		■	
	221	■			■	
	230	■				
TA81	121	■				
	125		■			
	128	■		■	■	
	131				■	
	134	■		■	■	
	137					■
	140	■	■			
TA40	232	■				
	235	■				
	243	■				
	247	■				
	249	■				
	253		■		■	
	267		■	■	■	
	270					■
PolyA	110	■				
	141				■	
	145				■	
	151		■			
	157				■	
	167			■		
	170					■
	176		■			
	179					■
AS25	82		■			
	91		■			
	101		■	■		
	104				■	
	110		■			
	113				■	
	120	■	■			■
	123	■				
	129				■	
	138		■			

Figure 13: Microsatellite (MS) markers display allelic variation in *ex vivo P. falciparum* clinical isolates.

Genetic variation was determined using 24 MSs, which are arranged from the lowest to the highest number of variable loci. Values indicate the sizes (bp) of different alleles detected within each locus. Detected alleles per loci also indicate the total number of unique clones present in isolates. Colour (■) signifies presence of allele detected within isolate. MS loci with no shaded blocks indicates absence of allele in clinical isolates.

The MS data was subsequently used to describe genetic diversity within an isolate and between isolates. The presence of more than one allele detected per loci reflected multiple clones, which have infected an individual (defining the MOI) [123]. To determine the variability within individual isolates, the MOI was therefore determined for each isolate and to minimize overestimation, MOI was set as the second highest number of alleles detected per locus for each isolate as previously described (Figure 14) [88, 124].



MOI	5	3	2	3	2
Loci contributing to MOI	TA40	AS31, TA109, AS8, AS3, AS2	AS3, AS7, AS12, AS14, AS15, AS19, AS31, Ara2, TA87, TA81	PolyA, TA81, Ara2, AS25, AS32	TA87, PolyA

Figure 14: Multiplicity of infection (MOI) as contributed by alleles detected on various MS loci. The loci are colour coded to correspond to the associated clinical isolate [KF14_01 (●), SB14_07 (●), SB14_04 (●), SB14_05 (●), and TD14_01 (●)]. The number of unique alleles for each of the 24 MS loci are indicated with a single dot. Inset below shows the MOI and identify the loci that had the second highest number of alleles per clinical isolate. Box plots indicate 75% distribution of data in box and hinges, and 25% in whiskers.

The MOI ranges from one to six different clones found within the various clinical isolates. KF14_01 is the most complex isolate with the highest MOI of five (Figure 14), indicating a total of six unique alleles. However, these alleles were associated with a single locus (TA40), which is one of the most variable loci detected as indicated above (Figure 14). SB14_07 and SB14_05 were the second most complex isolates, each with three clones, but variation was ascribed to five different loci for each isolate (Figure 14). SB14_04 and TD14_01 were the least complex isolates with at least two different clones present in the isolates (Figure 14). There was a total of 10 loci that contributed towards MOI for SB14_04 and two loci that could possibly account

for the clones present in TD14_01 (Figure 14). This further indicated that although both isolates have at least two clones, the clones within SB14_04 were more diverse from one another than the clones in TD14_01.

This data indicate that the isolates displayed a different distribution of unique alleles and that the loci that contribute towards MOI are completely different. Interestingly, the only exception is with locus TA87, which is shared between SB14_04 and TD14_01, indicating that it could be the locus that contributes to the two similar clones that are present in the isolates (Figure 14). The geographic association between the isolates were considered to determine if these isolates are from closely associated transmission areas. SB14_04 and TD14_01 originate from neighboring countries, Malawi and Mozambique, and the possibility that these isolates share clones that are distributed within this geographical region cannot be excluded.

To further delineate a plausible relationship between the different clinical isolates, allele variation across the 24 MS loci were compared using multiple correspondence analysis (MCA) (Figure 15) [125]. The alleles detected within each isolate were converted to categorical values to group the clinical isolates based on their similarities and differences. Only loci which contributed towards the genetic variance between isolates were used to differentiate between isolates.

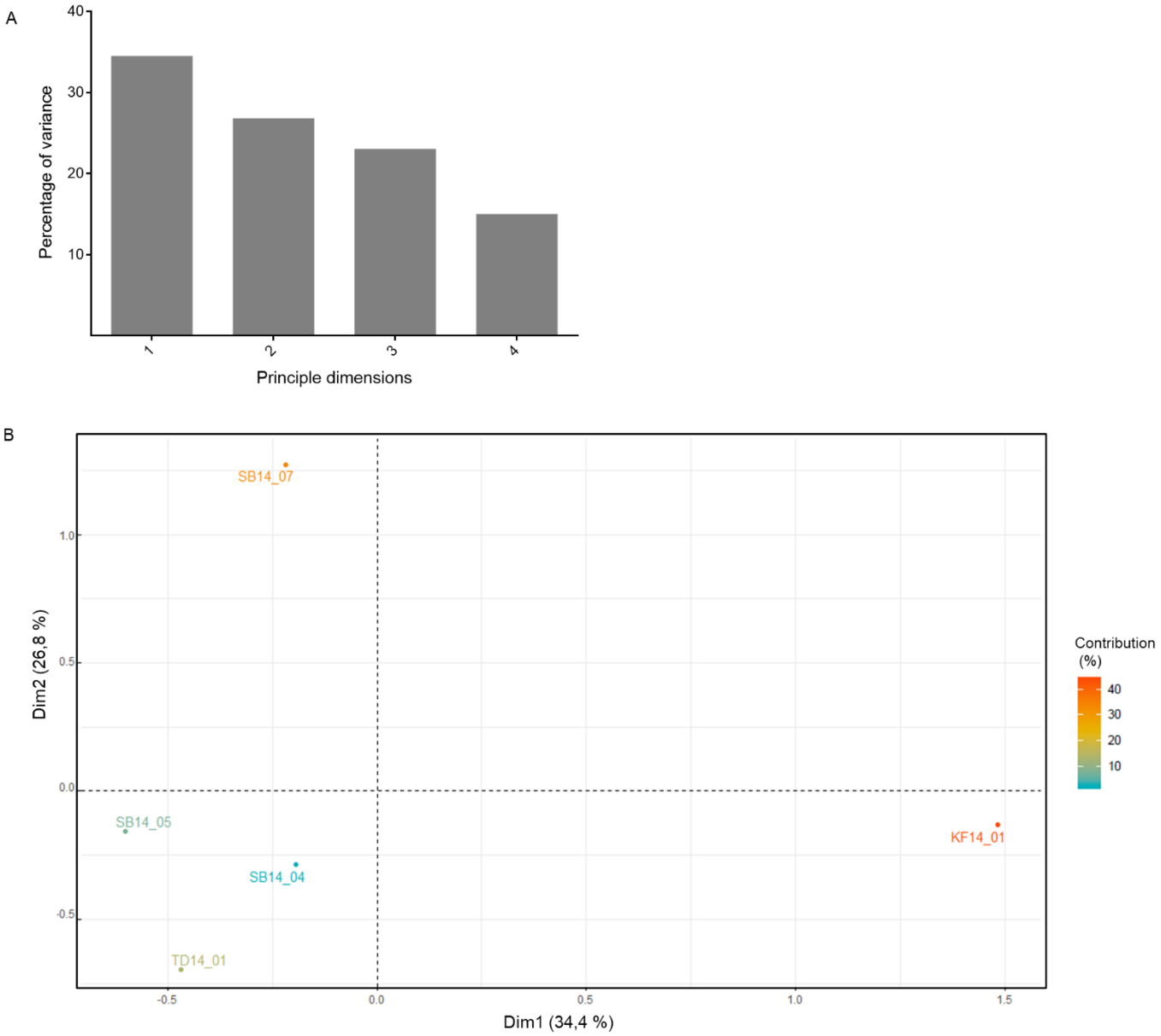


Figure 15: Multiple correspondence analysis (MCA) showing the relatedness between different *ex vivo P. falciparum* clinical isolates.
A) The variances explained by dimensions compared the *ex vivo P. falciparum* clinical isolates based on the allelic variation from the 23 MS loci. **B**) The score plot of the first two principal components is represented. The contribution associated with the principal components differentiates between clinical isolates according to the highest variance.

Based on the allelic profiles of the clinical isolates which were converted to categorical values, there were four principal components that were derived using MCA (Figure 15 A). The first two highest principal dimensions of 34 % and 27 % were used to explain the total variance of 61 % in the data (Fig 15 B). These two dimensions were then used describe the variance in the first and second component.

The MCA demonstrated clear distinction between the clinical isolates into either closely related or unrelated groups (Figure 15 B). Clinical isolates that group together have a similar genetic profile, while those that are far from each other are dissimilar. Clinical isolates SB14_05, SB14_04 and TD14_01 are genetically more related to one another as they cluster together and are close to the center of the MCA plot (Figure 15 B). These isolates contribute between 5-15 % to the genetic variance. Comparatively, KF14_01 and SB 14_07 are neither related to other isolates nor to each other (Figure 15 B). These isolates are far from the center of the MCA plot, showing that they contribute the most genetic variance towards the first and second dimensions. The data demonstrates that KF14_01 and SB14_07 contain more unique alleles in comparison to other isolates (Figure 15 B). Together these results confirm KF14_01 as the most genetically complex isolate, followed by SB14_07 (Figure 15 B).

Interestingly, SB14_05 and TD14_01 are closely related and originate from Mozambique. However, both are only distantly related to KF14_01, which comes from the same geographic location. Similarly, SB14_04 and SB14_07 from Malawi are distantly related. This data further supports that there is a degree of similarity between isolates that originate from neighboring countries. However a larger sample of isolates are needed to make conclusive statements.

3.2.2 SNP characterization of *ex vivo P. falciparum* clinical isolates

3.2.2.1 PCR amplification of SNP containing genome fragments

To SNP characterize the clinical isolates and lab strains, a panel of 22 SNP markers were used [79]. All 22 genome fragments containing the SNP loci were successfully amplified to obtain sufficient amounts of purified template for subsequent downstream experiments (Figure 16).

After each of the five clinical isolates were evaluated, expected fragment sizes were obtained for each of the 22 SNP loci, as indicated by single bands after agarose electrophoresis (Figure 16). After successful amplification, PCR products were purified to the desired concentration of more than 50 ng/μL for subsequent DNA Sanger sequencing.

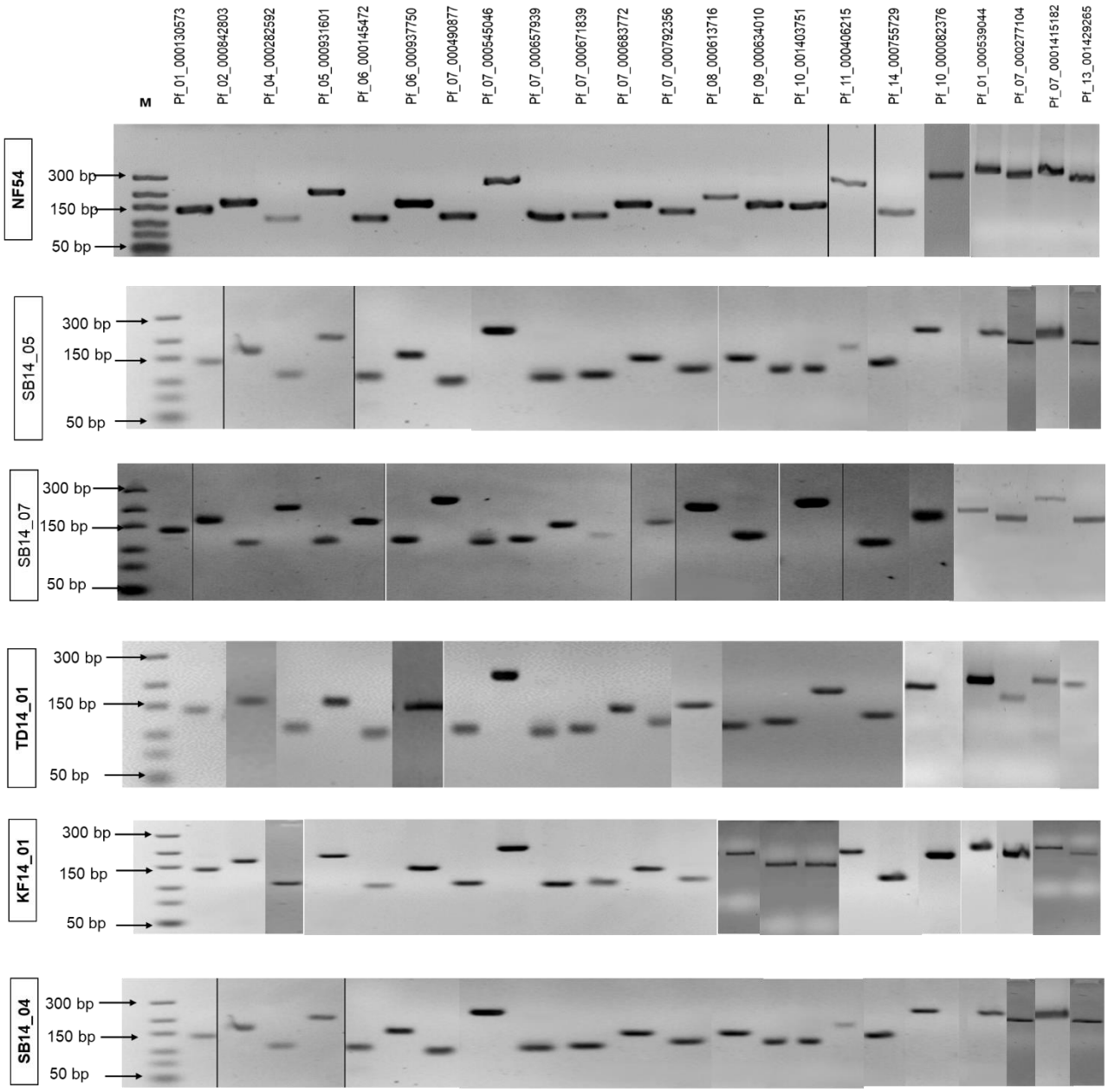


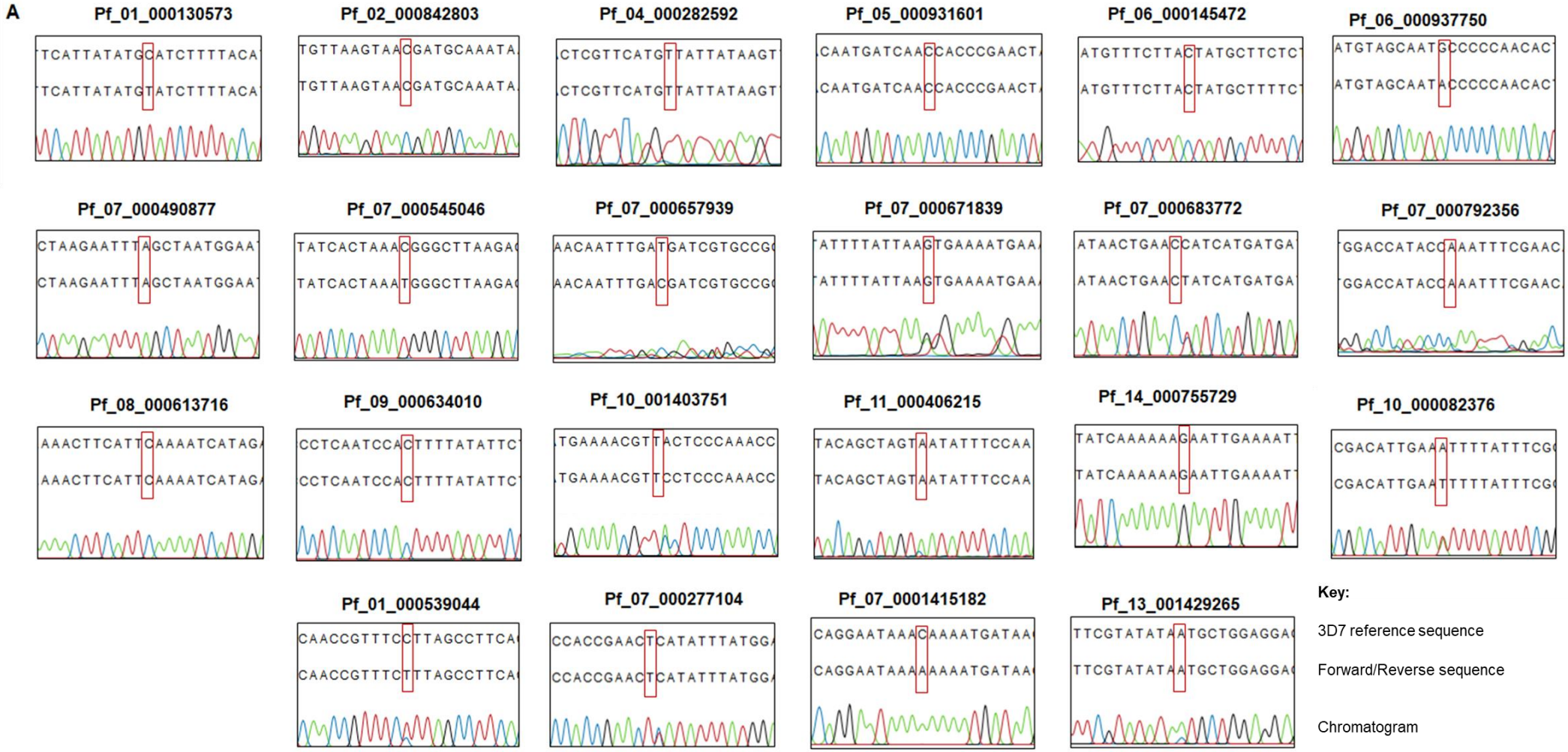
Figure 16: Amplification of NF54 and clinical isolates *P. falciparum* gDNA using a panel of 22 SNP markers.

The figure is a composite from different gels, sized based on the same molecular marker used for each gel. Unmodified, individual gels are found in appendix. Clinical isolates: **SB14_05, SB14_07, TD14_01, KF14_01**, **SB14_04** and lab strain **NF54** product sizes are indicated. **M**: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker, only 50-300 bp are indicated, full ladder available in appendix. The SNP ID above each lane show the products, which were separated on a 5 % agarose/TAE gel at a current of 80 V/cm and visualised with 5 µg/mL EtBr. Clinical isolates products were aligned to SNP IDs labelled for NF54.

3.2.2.2 SNP analysis from sequenced genome fragments

The SNP sites were identified by aligning the sequenced fragment to the original *P. falciparum* 3D7 genome sequence as obtained from PlasmoDB v3. Where variations were identified at the SNP site in sequenced fragments compared to the 3D7 sequence, the minor or major allele were recorded as previously published [79].

Nucleotide identity were determined by the program CLC MainWork bench v8.01, yet, for some fragments there were two nucleotides of equal or different peaks visible at a single locus (Figure 17 A-F). The isolates will be re-sequenced to determine whether these anomalies are not due to artifacts. The ambiguities in the chromatograms may indicate that there are different SNPs present in the same sample due to multiple clones within the isolate [126]. Interestingly, KF14_01 did not contain any multiple clones (Figure 17 A). For isolate SB14_07, two loci contained heterozygous SNPs, namely Pf_02_000842803 and PF_07_000792356 (Figure 17 B). SB14_04 had only one heterozygous SNP on locus Pf_01_000130573 (Figure 17 C). Interestingly, SB14_05 had the most loci with heterozygous mutations, namely, Pf_07_0000490877, Pf_07_00054046 and PF_08_000613716 (Figure 17 D). Loci Pf_08_000613716 within isolate TD14_01 contained heterozygous SNP (Figure 17 E). For further analysis, the nucleotide called by the program used was to create a SNP barcode for each isolate. In future studies, these isolates would have to be cloned to investigate the SNP barcode per clone.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Pf_07_000657939
Pf_07_000671839
Pf_07_000683772
Pf_07_000792356
Pf_08_000613716
Pf_09_000634010
Pf_10_001403751
Pf_11_000406215
Pf_14_000755729
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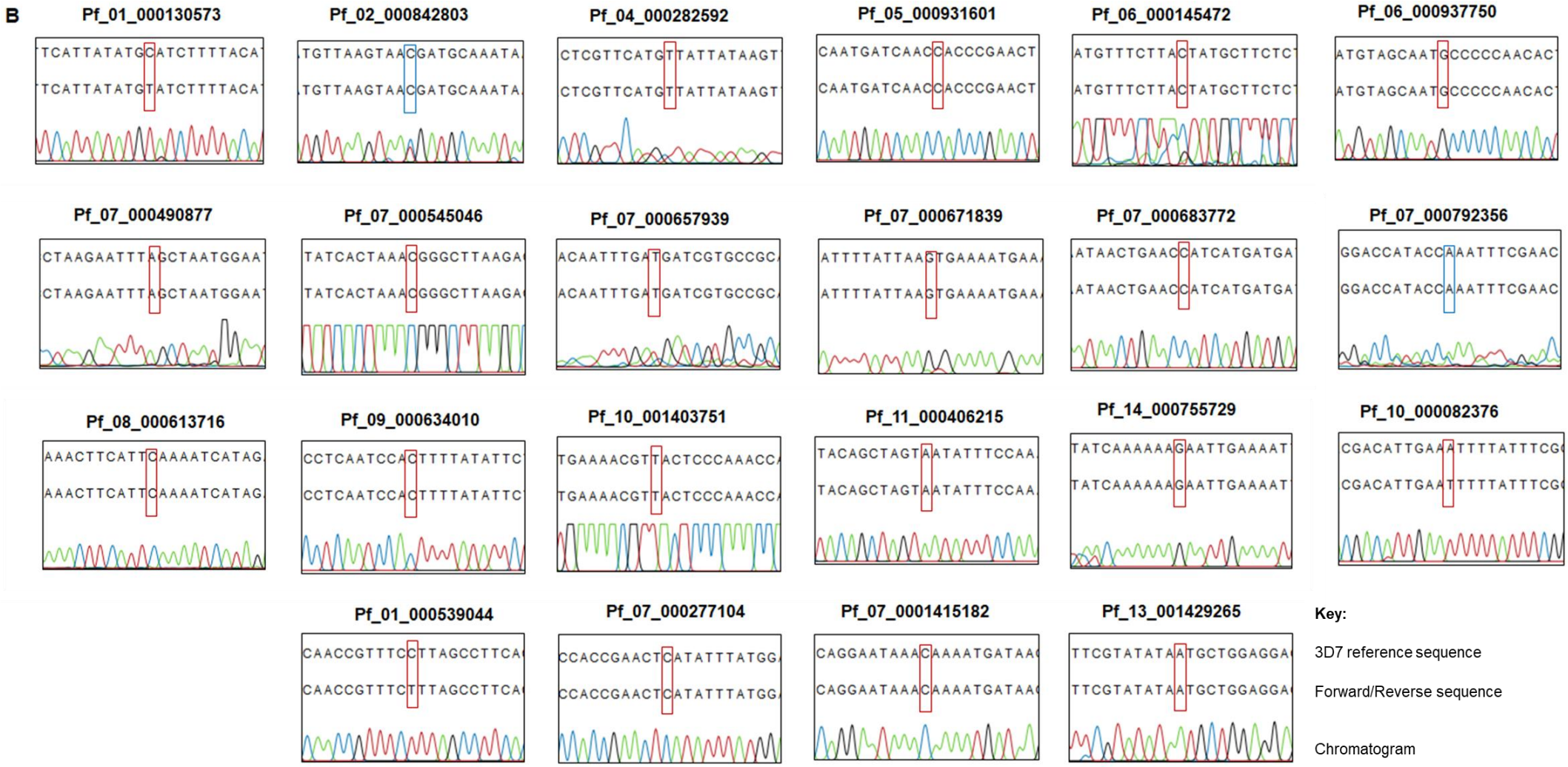
Key:
3D7 reference sequence
Forward/Reverse sequence
Chromatogram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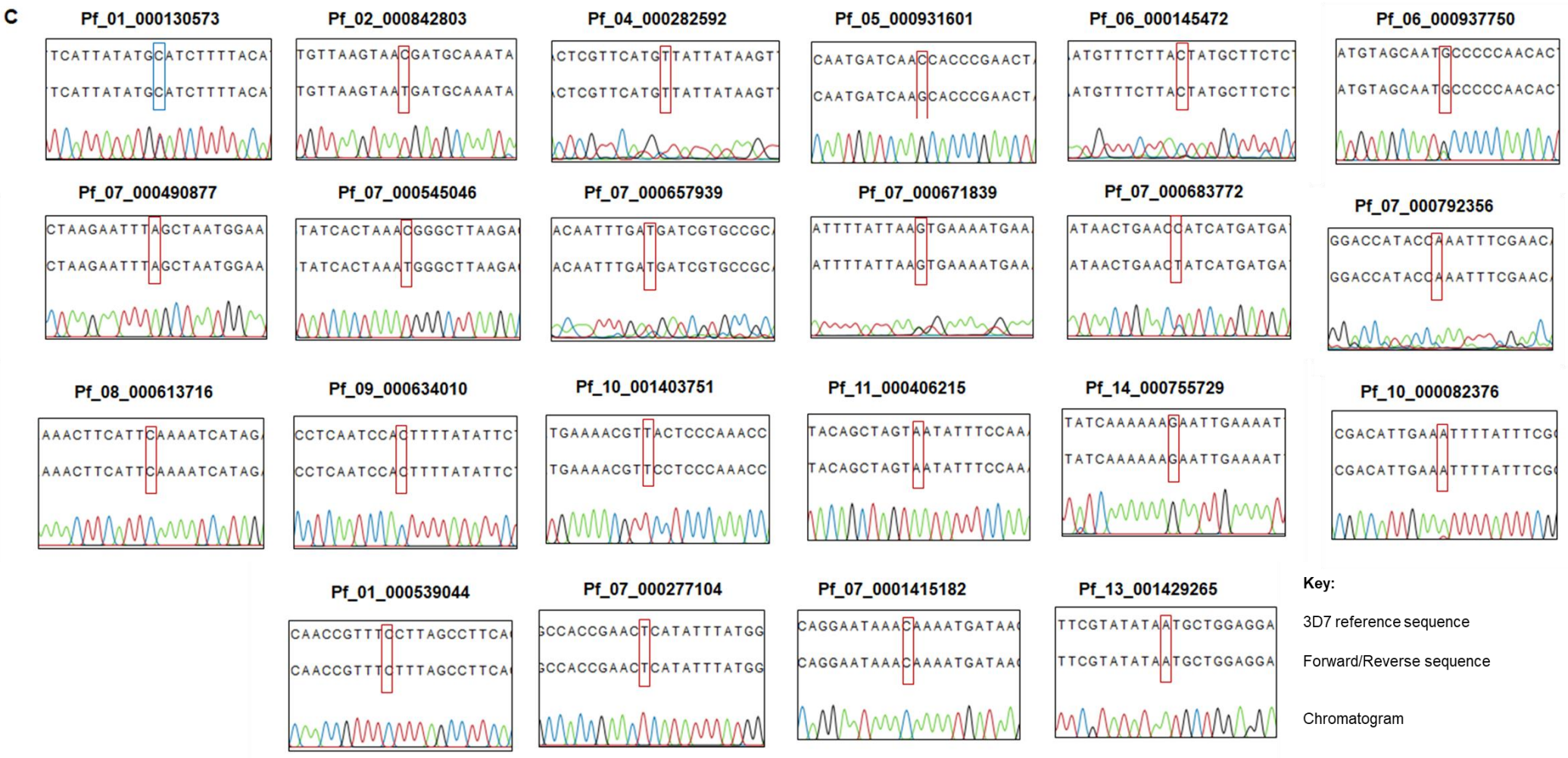
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Pf_11_000406215
Pf_14_000755729
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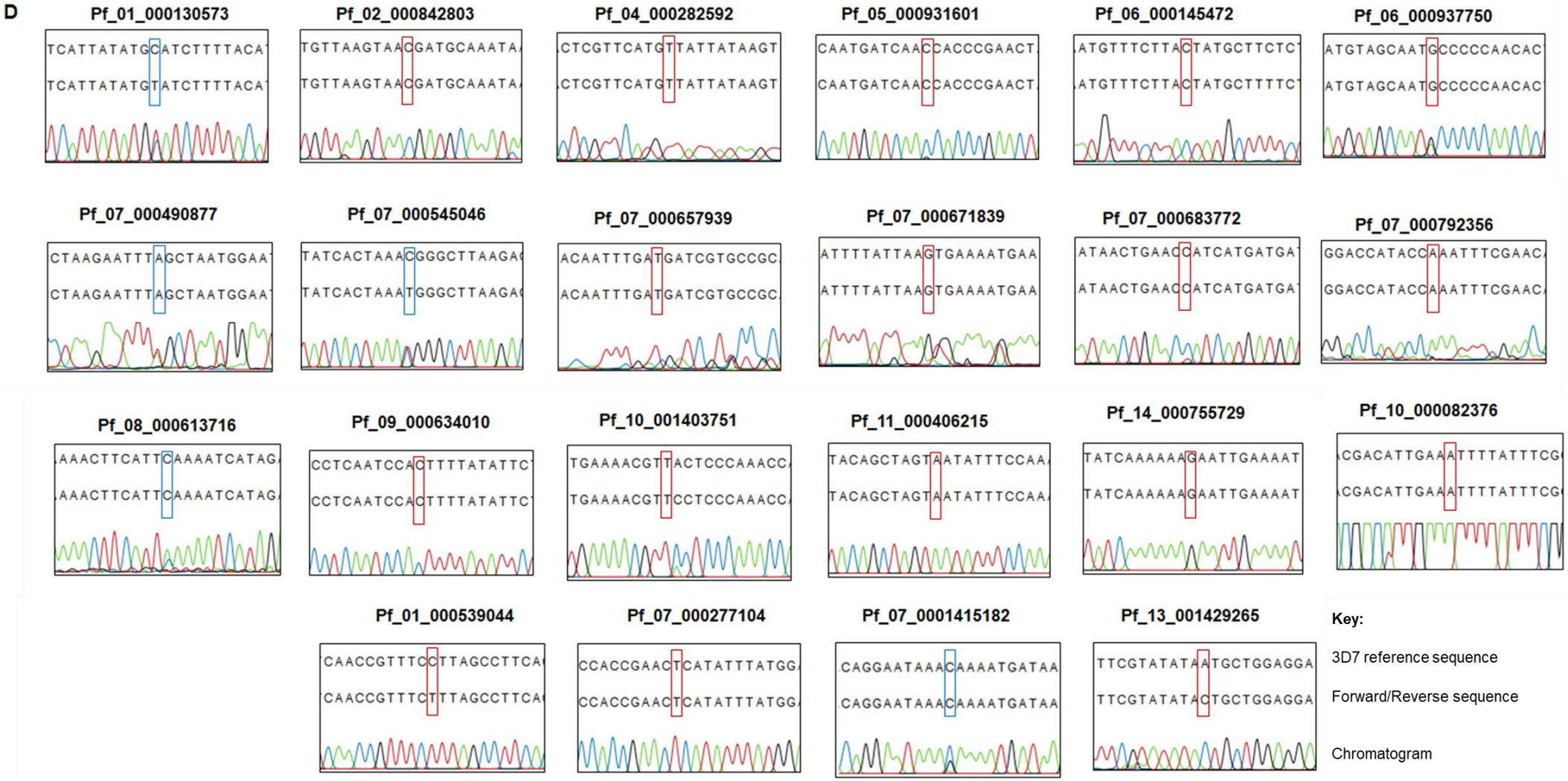
Forward/Reverse sequence
Chromatogram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Pf_07_000657939
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Pf_07_000683772
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Forward/Reverse sequence
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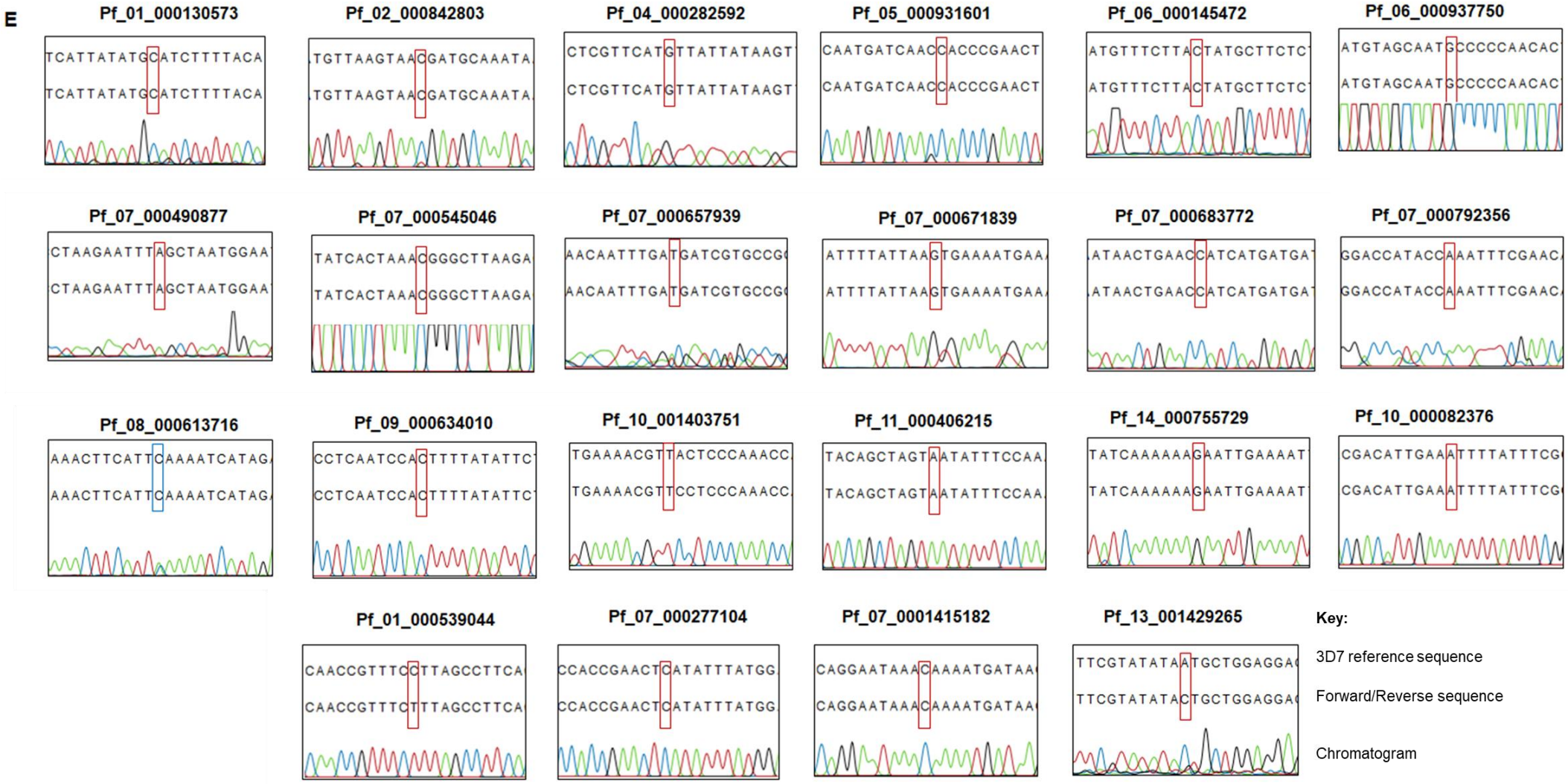
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Pf_07_000545046
Pf_07_000657939
Pf_07_000671839
Pf_07_000683772
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Pf_10_001403751
Pf_11_000406215
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Pf_10_000082376
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Key:
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Forward/Reverse sequence
Chromatogram

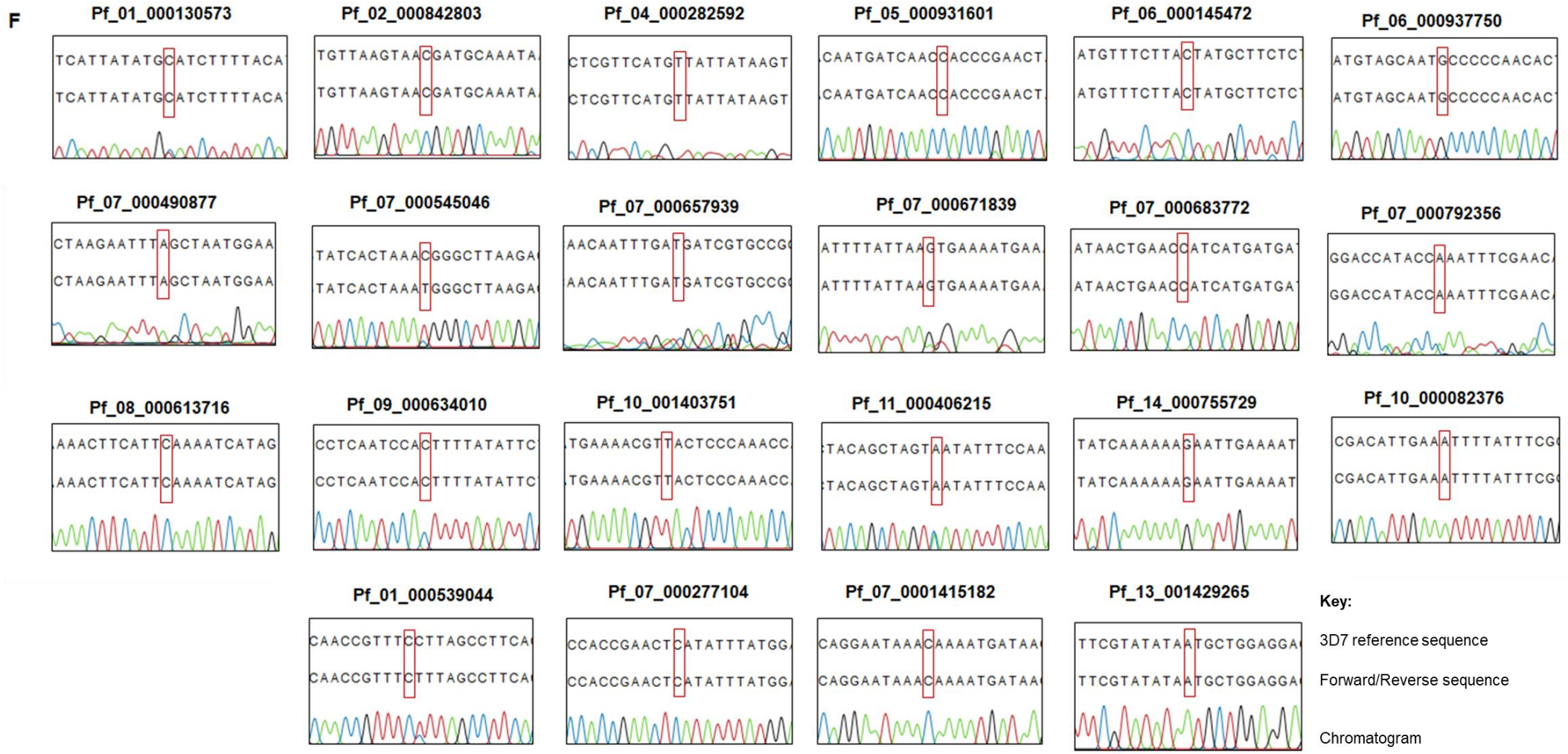

Figure 17: Chromatograms of *P. falciparum* isolates and lab strains sequenced regions using genome fragments from 22 polymorphic loci. Sequenced genome fragments of *P. falciparum* **A**) KF14_01, **B**) SB14_07, **C**) SB14_04, **D**) SB14_05, **E**) TD14_01 and **F**) NF54 strain containing SNP sites, which are represented with chromatogram peaks mapped to 3D7 genome sequence. Full fragments not shown, only 10 nucleotides downstream and upstream of SNP site are illustrated. SNP loci are represented with either the major or minor allele ( ). Multiple clones present in samples indicated by ambiguities due to heterozygous SNP sites ( ).

3.2.2.3 A SNP molecular barcode for lab strains and *ex vivo P. falciparum* clinical isolates

The SNP data after sequencing was used to generate a SNP molecular barcode, where the SNP identities per loci was evaluated across the different isolates and compared to NF54 lab adapted strain (Figure 18). *P. falciparum* 3D7 was used a control for correct barcoding as it was used in the original publication for SNP characterization [79].

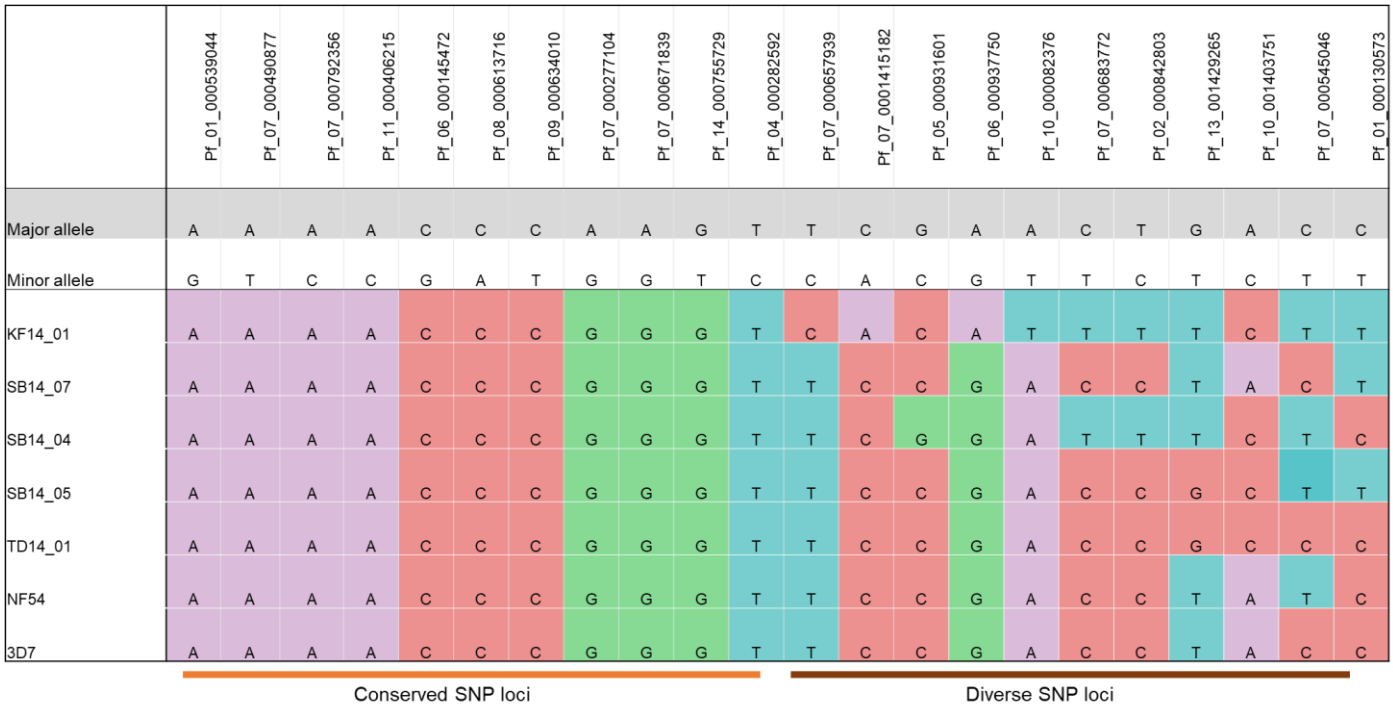

Figure 18: Molecular SNP barcode characterisation of lab strains and *ex vivo P. falciparum* clinical isolates.
SNPs across the *P. falciparum* genome were characterised for lab strains and *ex vivo* clinical isolates. Reference major alleles are indicated with a grey shaded colour as published [79]. Major and minor alleles of the SNPs are indicated as guanine (G), adenine (A), cytosine (C) and thymine (T). Barcode demonstrates conserved (—) and diverse (—) SNP loci.

The molecular SNP barcode reveals genetic diversity based on allelic variation in the clinical isolates. Isolate KF14_01 was the most variant as it had four loci (Pf_07_000657939, Pf_07_0001415182, Pf_06_00097750 and Pf_10_000082376) with completely different alleles compared to the other isolates (Figure 18). Out of the four loci, one SNP locus (Pf_06_00097750) contained a major allele (Figure 18). KF14_01 had a total of 11 minor alleles across all SNP loci. The second most variant isolate was SB14_04, which had one locus (Pf_05_000931601) with one unique major allele (Figure 18). SB14_04 isolate had seven minor alleles across all loci. Similarly, SB14_07 and NF54 also had seven minor alleles. SB14_05 had the eight minor alleles, making it the second highest isolate with minor alleles. TD14_01 and 3D7 both had the least number (six) of minor alleles across all isolates (Figure

18). Interestingly, NF54, and its daughter clone 3D7 differ from each other at SNP Pf_07_000545046.

All isolates had 11 SNP loci that were conserved and there were two loci (Pf_07_000277104 and Pf_07_000671839) that had minor alleles for all isolates. These isolates were subsequently removed from downstream analysis. Equally, there were 11 SNP loci that were diverse amongst all isolates. These loci contributed to genetic variance between isolates (Figure 18).

3.2.2.4 Genetic relatedness between *ex vivo P. falciparum* clinical isolates based on SNPs

Principal component analysis (PCA) was used to determine the relationship between different clinical isolates. PCA is defined as linear combinations of variables that account for the variance in sample data [127]. To demonstrate which of the loci contributed to the variance between isolates, a score plot showing various SNP loci was created. Eigenvectors are comprised of coefficients corresponding to the variables. The eigenvectors also inform the relative weight of each variable in the component. To further determine the relationship between different clinical isolates, allele variation across the 11 diverse SNP loci were used to draw the PCA.

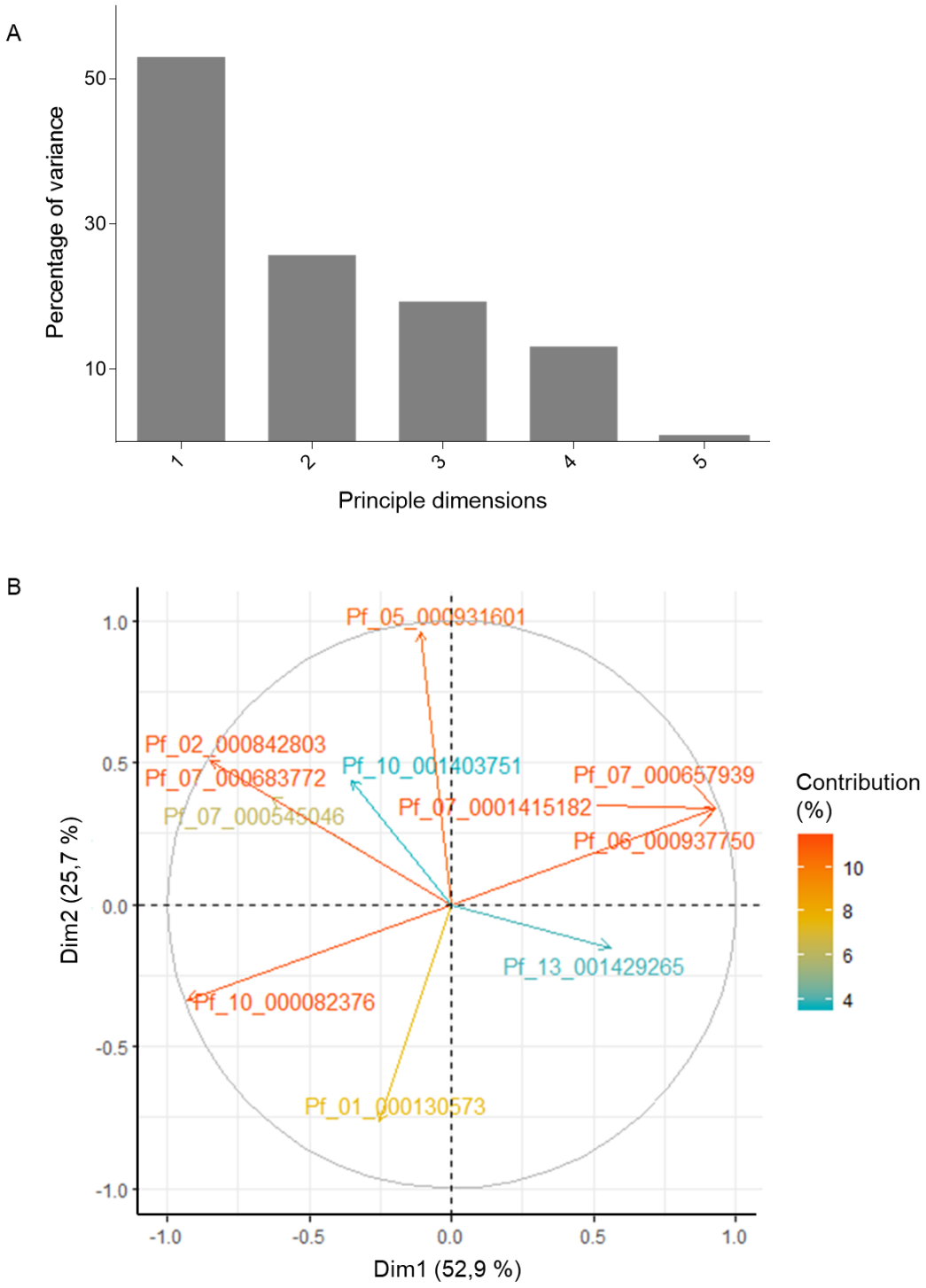

Figure 19: The score plot to determine the contribution of each SNP loci to the genetic variance.

A) The variances explained by dimensions. Loading variables were 11 diverse SNP loci that contribute towards genetic variability in clinical isolates. **B**) The score plot of the first two principal components are represented. The length of the eigenvectors and the direction in which they point, indicate their correlation and how influential the variables are, with a positive direction indicating positive correlation.

Based on the allelic profiles of the clinical isolates from the 11 diverse SNP loci, there were five principal components that were derived to explain the dimensions that contribute to the variance (Figure 19 A). The two principal dimensions of 52,9 % and 25,7 % which describe the

variance in the first and second components respectively, were used to explain the total variance of 78,6 % in the data (Figure 19).

The score plot revealed loci which contributes towards the variance in between clinical isolates (Figure 19 B). The PCA further differentiates between SNP loci based on the weight in genetic diversity as demonstrated by the length and weight of the eigenvectors pointing to each SNP loci (Figure 19 B). SNP loci with an orange colour correlate to a high eigenvalue which measures the variance in all the variables which are accounted for by the SNP loci. Conversely, the SNP loci indicated by a cyan colour correlate to the least eigenvalue, showing that the SNP loci contributes to the least amount of variance (Figure 19 B). SNP loci Pf_05_000931601, Pf_02_000842803, Pf_07_000683772, Pf_07_000657939 and Pf_06_000937750 positively contribute up to 10 % to the variance (Figure 19 B). Conversely, SNP loci Pf_10_00082376 negatively contribute up to 10 % to the genetic variance. The next most influential loci to contribute to the variance was Pf_01_000130573, which accounted for approximately 8 % to the variance. SNP loci Pf_07_000545046 contributed between 6-7 % to the genetic variation. The loci which contributed the least to the variance were Pf_10_001403751 and Pf_13_001429265 which contributed between 4 and 5 % to the genetic variance (Figure 19 B).

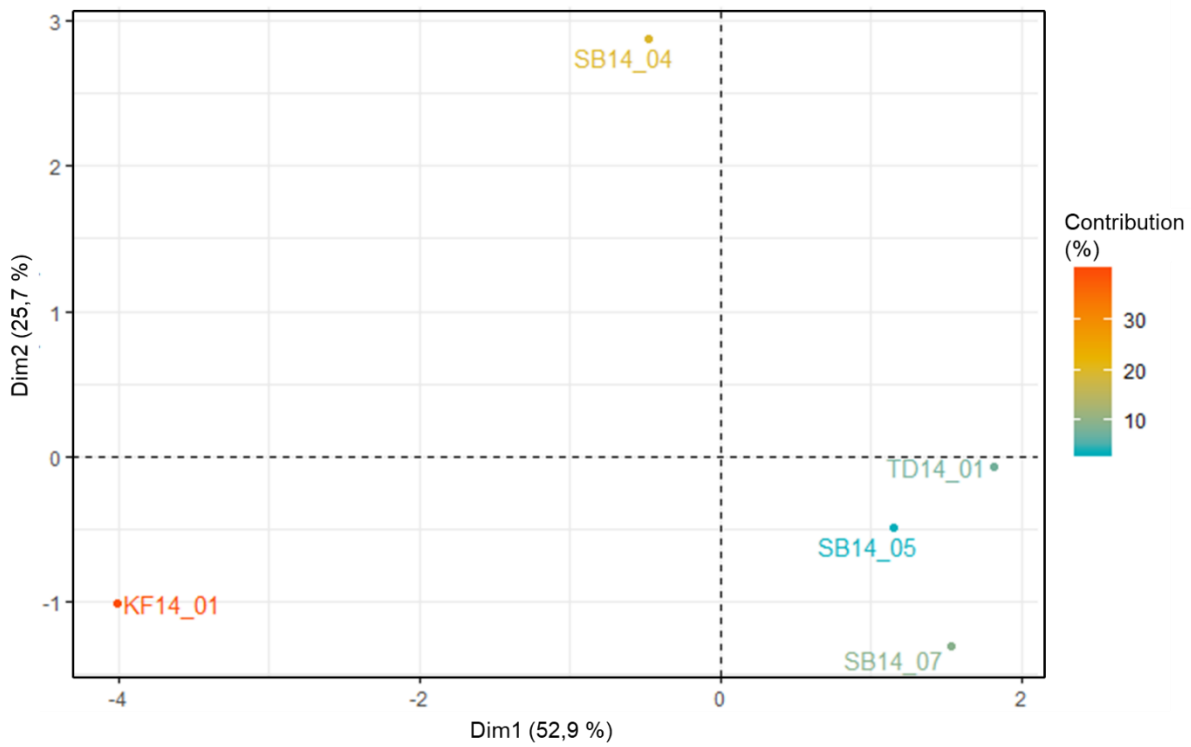

Figure 20: Principal component analysis show relationship betwen *ex vivo P. falciparum* clinical isolates.
The isolates are clustered according to the allelic variation from 11 single nucleotide polymorphism (SNP) loci. The contribution associated with the principal components differentiates between clinical isolates according to the highest variance.

The PCA demonstrated a clear distinction between the clinical isolates based on their allelic variation contributed from 11 SNP loci (Figure 20). The PCA revealed genetic similarity between isolates TD14_01, SB14_05 and SB14_07, as they cluster together (Figure 20). These isolates contribute up to approximately 10 % genetic variance to the first and second dimensions (Figure 20). In contrast, KF14_01 and SB14_04 were dissimilar to the other isolates as they do not cluster together nor to the other isolates. These isolates are far from the center of the MCA plot, showing that they contribute the most genetic variance towards the first and second dimensions. This difference shows the high genetic variance between KF14_01 and SB14_04 based on SNP loci, further demonstrating that both are the most genetically variable isolates (Figure 20).

3.3 Contribution of genetic diversity to differential drug response in *ex vivo P. falciparum* clinical isolates

This study hypothesised that the differential drug response observed in late-stage gametocytes of *ex vivo P. falciparum* clinical isolates could be ascribed to underlying genetic diversity between isolates [63]. To determine if there is an association between the genetic diversity of the clinical isolates with the differential drug response, clinical isolates were rank ordered based on the allelic diversity obtained when MS and SNP genotyping were used, respectively (Figure 21). Genetic diversity determined by MS and SNP genotyping techniques identified KF_01 as the most genetically variable and complex isolate. According to MSs, the second most variable isolate was SB14_07 (Figure 21: top panel). In contrast, SB14_04 was the second most variable isolate based on SNP barcode (Figure 21: top panel). Both techniques identified SB14_05 to be the third most variant isolate and TD14_01 to be the least variable isolate, thus the least genetically diverse with the lowest complexity (Figure 21: top panel).

Subsequently, the complexity of genetic diversity based on the rank order was correlated to drug response in late-stage gametocytes and the association evaluated based on linear regression (Figure 21 A and B). The differential drug response data for both the kinase inhibitors [63] and compounds used in section 3.1.4 (Figure 12) against late-stage gametocytes of *P. falciparum* clinical isolates was used. Overall, MS genotyping was poorly correlated to differential drug action, with very low strength of associations and linear correlations r^2 ranging from 0.0016 to at best 0.289. Comparatively, SNP genotyping seemed to be more informative with relatively strong positive associations obtained. This is particularly true for the ATP4 inhibitors (compounds A and B) as well as the endoperoxide inhibitor (Figure 21 A). For these inhibitors, genetic diversity in the isolates was positively (r^2 of >0.2) correlated to drug efficacy,

with these compounds able to effectively target even the most diverse clinical isolates. By contrast, genetic diversity and drug efficacy was negatively correlated for the kinase inhibitors (Figure 21 B). This class of inhibitors seem to not be effective on isolates with higher genetic complexity but can effectively target more clonal parasites.

Taken together, these findings indicate that differential drug action can be described by genetic complexity of malaria parasite isolates. Moreover, this is different for different chemical classes used. Lastly, these findings suggest that SNP barcoding is a generally more informative tool compared to MS to correlate genetic diversity to differential drug response of late-stage gametocyte viability.

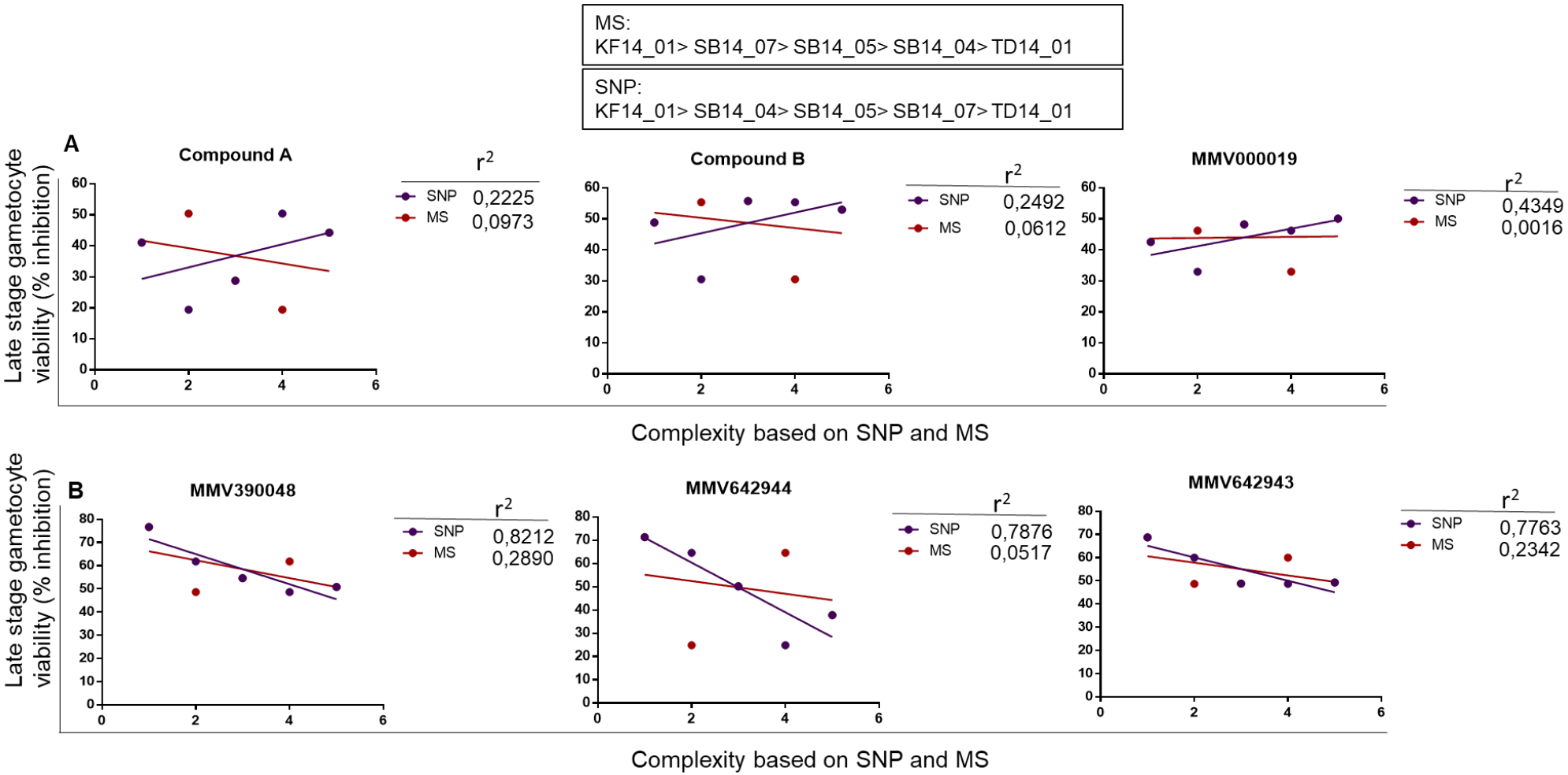

Figure 21: Correlation between genetic diversity in *ex vivo P. falciparum* clinical isolates and differential drug response in late-stage gametoytes. Top panel shows the rank order of genetic diversity in clinical isolates based on microsatellite (MS) and single nucleotide (SNP) genotyping. The rank order defines the complexity of isolates based on SNP and MS. Drugs from various chemical classes, **A**) diverse set comprised of endoperoxide inhibitor and ATP4 inhibitors (Compounds A and B). **B**) Kinase inhibitors were tested on late-stage gametocytes of *P. falciparum* clinical isolates. Graphs (**A** and **B**) shows the correlations between the % inhibition of late-stage gametocyte viability of clinical isolates and genetic complexity of isolates based on MS and SNP genotyping techniques. R^2 value for linear regression measures the strength of association and it is indicated for each SNP and MS linear correlation under each drug.

Chapter 4: Discussion

Blocking malaria transmission is key to achieving elimination and eradication of the disease. It is therefore important to prioritize the discovery and development of molecules that target the transmissible stages of the parasite [128]. Efforts to measure the activity of antimalarials on the intraerythrocytic sexual stage gametocytes, with the aim of identifying transmission-blocking compounds, have been reliant on the use of lab adapted parasite strains of *P. falciparum* that have been isolated many years ago [129]. Although reference strains such as 3D7 and NF54 are useful to standardize and validate high throughput assays, clinical isolates offer a more realistic representation of drug efficacy because they have been exposed to years of multiple drug pressure and natural transmission. Drug efficacy evaluated against gametocyte producing clinical isolates is important as antimalarial drugs have different effects on isolates compared to culture adapted reference strains [129]. Therefore, the aim of the work presented here was to determine if *P. falciparum* genetic diversity differentiates drug response in gametocytes of southern African *ex vivo P. falciparum* clinical isolates. Ultimately, this work hoped to provide contemporary clinical isolates that can be used as an early filter when measuring the activity of gametocytocidal drugs.

The findings presented in this study confirmed that the differential drug response in late-stage gametocytes of *P. falciparum* clinical isolates is not only limited to the kinase inhibitors as preciously published. This study provided evidence that the differential drug response initially observed can be achieved by using compounds that have other drug targets, namely endoperoxide and ATP4 inhibitors. The differential drug response is either attributed to the extent of genetic diversity of clinical isolates or it could possibly be due to the nature of the drug and their respective targets.

Interestingly, using SNP and MS genotyping techniques, both revealed KF14_01 and TD14_01 to be the most and least genetically complex isolates, respectively. According to the SNP barcode, KF14_01 was defined as the most complex isolated due to the highest number of unique alleles. Similarly, according to MSs, KF14_01 was the most complex isolate as it had the highest number of clones detected. The multiple clones detected in clinical isolates could result from independent bites of infected mosquitoes, a phenomenon defined as superinfection [130]. As a result, multiple strains can be transmitted simultaneously due to the high rate of genetic recombination of genetically distinct malaria parasites during the sexual stages in the mosquito host [124]. The clones could also result from a single mosquito bite, which transmitted

genetically diverse sporozoites into the human host [80]. Previous studies have shown that a high MOI is correlated to high malaria transmission levels [124, 131, 132] and, it is positively correlated with parasite density [132]. As the transmission intensity increases, so does the genetic diversity of the parasite [124]. Our findings also show that KF14_01 produced the highest number of gametocytes compared to other isolates. The high parasite density causes the frequent genetic recombination [131]. Our findings supported this notion as KF14_01 originates from Mozambique, a high malaria transmission area.

By contrast, TD14_01 produced the least number of gametocytes and this isolate was characterized by a low MOI and the least number of unique alleles based on MS analysis and SNP barcode, respectively. It was expected that the isolate would have similar characteristics as it came from the same transmission setting as KF14_01. However, it has been reported that within a country, there may be different malaria transmission areas [132, 133]. As a result, the least diverse TD14_01 clinical isolate with the least genetic diversity can originate from areas with high malaria transmission, possibly in pockets of low transmission within Mozambique [3, 134]. Our study shows that the relationship between MOI and transmission intensity is not always linear, due to transmission not being homogenous. Hence transmission occurs in hot spots depending on microscale differences in mosquito biting rates. Furthermore, our study showed that isolates originating from neighboring countries, namely Malawi and Mozambique, might share clones that are distributed within these countries. Another interesting discovery presented by the study is that clinical isolates SB14_04 and SB14_07 both from Malawi are distantly related. This finding coincides with a previous study, where isolates from Malawi were distantly related and shared no identical barcodes, due to the country being a high transmission area [135]. Furthermore, none of the African clinical isolates shared identical barcodes as expected, since Malawi and Mozambique are countries of high malaria transmission.

Furthermore, not only did the study show allelic variability within *ex vivo* isolates, diverse loci were revealed, with TA40 and PfG377 MS loci which were among the most variable loci that contributed to the genetic diversity in clinical isolates. This contrasts with another study, which looked at diversity of *P. falciparum* parasites using MS on a population level; showed that PfG377 revealed a low number of alleles per sample [136]. This MS marker is a gametocyte specific antigen, which is expressed in osmophilic bodies of gametocytes, further highlighting the relevance of including this marker in our study. It is also suggested that including gametocyte specific transcripts such as the polymorphic PfG377 genotyping marker during drug treatment on transmission might allow the study of the parasite clones, which are actually

transmitted [137]. In another study, it was speculated that the increase in PfG377 MS diversity at genomic level was a reflection of the parasite population adaptive and survival mechanisms due to reduced transmission [138, 139].

It is evident that both SNP and MS genotyping techniques were able to differentiate between different isolates from various backgrounds. For the first time, this study showed that both techniques can be used to correlate genetic diversity to differential drug response. Although MS genotyping and consequent analysis has been used extensively to distinguish between isolates from varying transmission intensities on a population level, this study showed that the technique is also sensitive to distinguish between different clinical isolates on a smaller scale. This technique has also been previously used to genotype both laboratory clones and field isolates from different transmission settings, especially in high transmission areas where there is a high incidence of polyclonal infections [85]. The study presented here, showed that SNP barcoding was more informative compared to MS as it showed positive correlation between drug inhibition and genetic diversity. For the kinase inhibitors, there was a clear correlation where the compounds were more active on genetically less diverse clinical isolates. In contrast, the opposite holds true for ATP4 and endoperoxide inhibitors, where the inhibitors were more active on genetically diverse clinical isolates. These findings show that the complex mixture of genetic types is due to haploid parasites that infect a single individual. Thus, it is of importance to detect all clones during drug trials as one of them might be drug resistant [124].

Ultimately, we hope that these isolates will serve as a benchmark to help guide the malaria drug discovery in assessing the potential of newly discovered transmission-blocking antimalarials. Further, they might provide insights into which chemical drug classes might provide transmission-blocking capabilities. However, a wide range of compounds with different chemical classes and targets will have to be included and evaluated in the study to make informed decisions about whether the differential drug response in gametocytes is due to the nature of the compounds. Testing of gametocytocidal compounds on clinical isolates has the capacity to inform whether there is differential drug activity in different gametocyte populations *in vivo*. Possibly, drugs with very low susceptibility against clinical isolates will not progress through the drug testing cascade, in turn prioritizing compounds with activity to further development. Limitation of the study includes a very low number of sample size. Most MS analysis are effective on large population sets. Also, most arguments in the study remain inclusive because one isolate is not representative of the entire transmission setting. It was also a challenge to directly correlate differential drug response to genetic diversity since the

MS markers and SNPs used are not directly correlated to known drug resistance markers, rather, they are standard genetic markers used to evaluate genetic diversity in a population. Future prospects include isolating individual clones from the isolates and assessing drug efficacy on those. Identifying informative MS and SNP markers that are based on gametocyte drug response is also an attractive future aspect that will enable researchers to make direct inferences between diversity and drug response

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Chapter 5: Conclusion

Targeting late-stage gametocytes, the transmissible stages of the *P. falciparum* parasite remain very complex due to their poorly understood biology. The late-stage gametocytes of *ex vivo P. falciparum* clinical isolates have previously been used to test the efficacy of PI4K inhibitors. A differential drug response was observed. This study further confirmed differential drug responses in clinical isolates using different classes of antiplasmodial compounds, namely ATP4 and endoperoxide inhibitors. It was thus hypothesized that the observed differential drug response is underpinned by underlying genetic diversity of *ex vivo* clinical isolates.

This work demonstrated that MS and SNP genotyping techniques both effectively differentiated between *ex vivo P. falciparum* clinical isolates to elucidate the genetic diversity and complexity of the isolates. The variability of the *P. falciparum* parasite genome aids the parasite to overcome therapeutic interventions. As a result, elucidating the underlying genetic diversity in *ex vivo* clinical isolates and how it correlates to differential drug response could aid in understanding why most drugs fail in clinical settings. Our study proved SNP genotyping to be more robust as it displayed positive correlation between drug response and extent of genetic diversity in isolates. Although MS markers were able to reveal genetic diversity and differentiate between different clinical isolates, it was not able to positively correlate genetic diversity to differential drug response.

Our findings do support the hypothesis that differential drug response can be directly correlated to *P. falciparum* genetic diversity given the markers used. The extent of genetic diversity revealed between isolates show that there is an interplay of genetic variation, which somewhat plays a role in the drug response observed. This could mean that the choice of genetic markers that were chosen were not informative enough to clarify the differential drug response. In addition, genotyping, and the use of *ex vivo* clinical isolates to identify novel transmission-blocking drugs is an important step towards translation of *in vitro* efficacy to public health applications. This work further presents clinical isolates which can be used to investigate drug susceptibility in gametocytes.

6 References

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7 Appendices

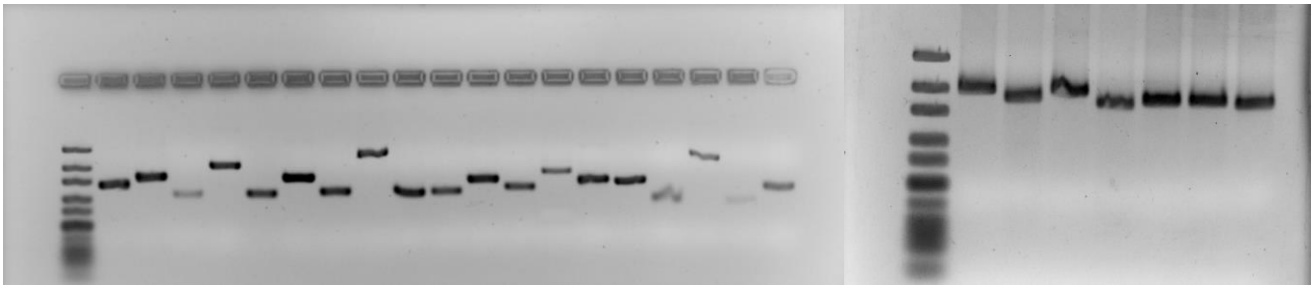

Figure 22: Amplification of NF54 gDNA using a panel of 22 SNP markers.
Unmodified gels that were used to make NF54 figure. M: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker. The products that were separated on 5 % agarose/TAE gel at a current of 80 V/cm and visualised with a 5 μg/mL EtBr.

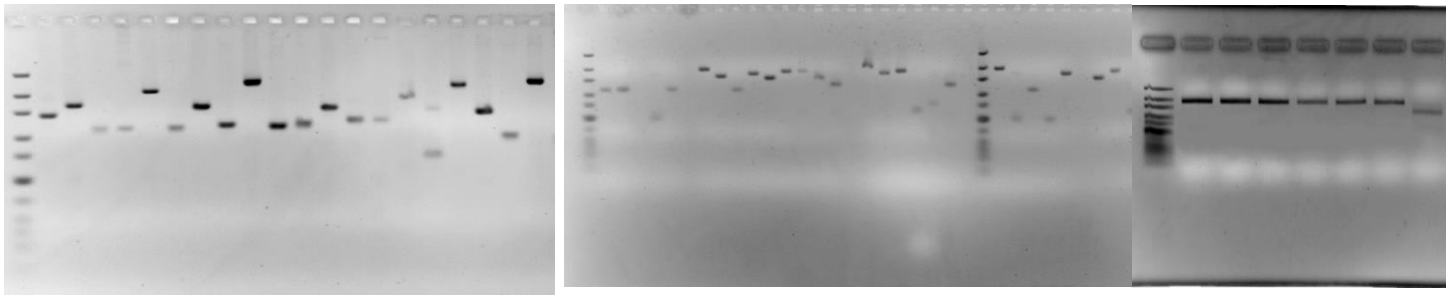

Figure 23: Aplification of KF14_01 gDNA using a panel of 22 SNP markers.
Unmodified gels that were used to make KF14_01 figure. **M**: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker. The products that were separated on 5 % agarose/TAE gel at a current of 80 V/cm and visualised with a 5 μg/mL EtBr.

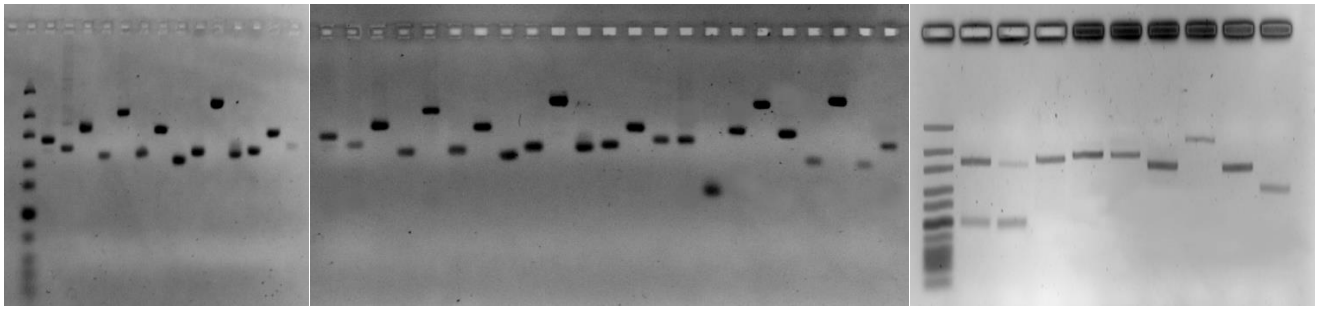

Figure 24: Amplification of SB14_07 gDNA using a panel of 22 SNP markers.
Unmodified gels that were used to make SB14_07 figure. **M**: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker. The products that were separated on 5 % agarose/TAE gel at a current of 80 V/cm and visualised with a 5 μg/mL EtBr.

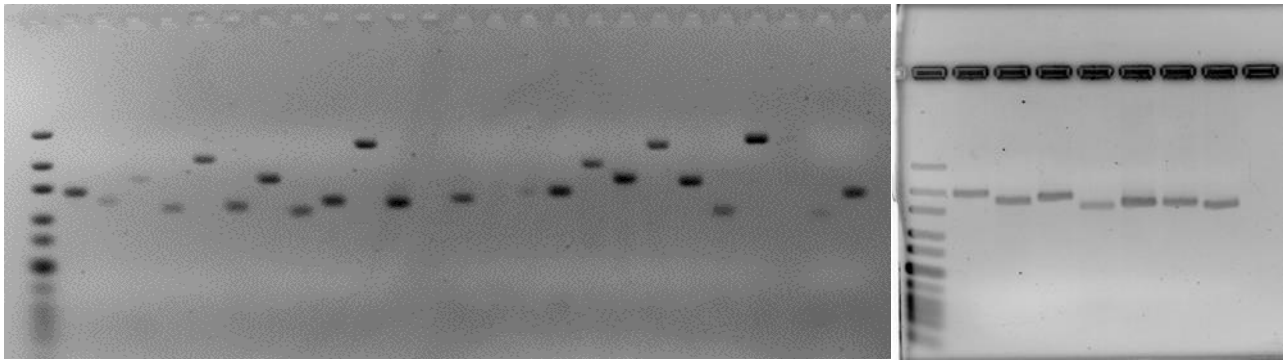

Figure 25: Amplification of SB14_04 gDNA using a panel of 22 SNP markers.
Unmodified gels that were used to make SB14_04 figure. **M**: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker. The products that were separated on 5 % agarose/TAE gel at a current of 80 V/cm and visualised with a 5 μg/mL EtBr.

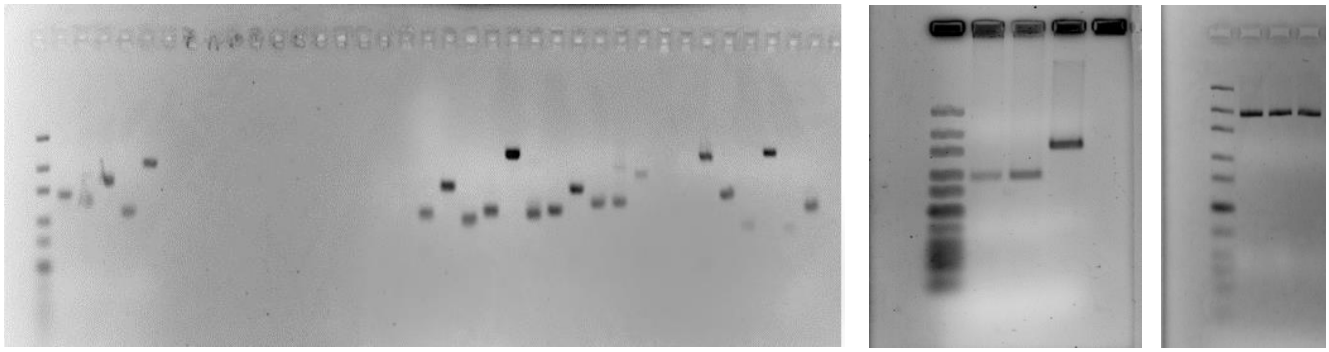

Figure 26: Amplification of SB14_05 gDNA using a panel of 22 SNP markers.
Unmodified gels that were used to make SB14_05 figure. **M**: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker. The products that were separated on 5 % agarose/TAE gel at a current of 80 V/cm and visualised with a 5 μg/mL EtBr.

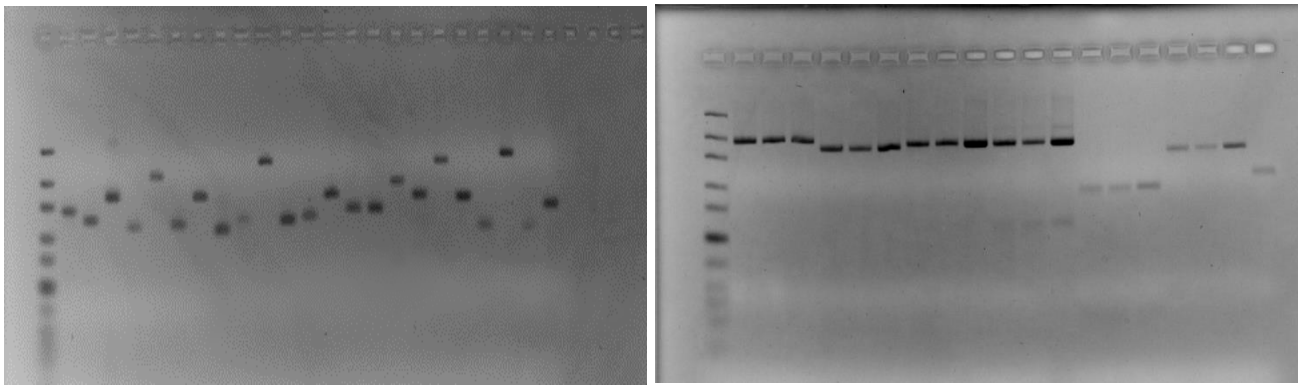

Figure 27: Amplification of TD14_01 gDNA using a panel of 22 SNP markers.
Unmodified gels that were used to make TD14_01 figure. **M**: GeneRuler Ultra Low Range DNA ladder 10-300 bp used as a molecular marker. The products that were separated on 5 % agarose/TAE gel at a current of 80 V/cm and visualised with a 5 μg/mL EtBr.