Molecular Diagnosis of Malaria Infection and Evolutionary Genetics of Drug Resistant Genes in *Plasmodium falciparum* in Cameroon

A thesis submitted to Goa University for the award of the Degree of

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In

BIOTECHNOLOGY

By

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Lovely dedicated to my:

Mum, husband and son

CONTENTS

LIST OF TABLES

LIST OF FIGURES

STATEMENT

As required under the Goa University Ordinance OB-9.9(ii), I state that the present thesis entitled "**Molecular Diagnosis of Malaria Infection and Evolutionary Genetics of Drug Resistant Genes in *Plasmodium falciparum* in Cameroon**" is my original contribution and the same has not been submitted to any University/Institute on any previous occasion for any degree. To best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestion have been availed of.

Place: Goa University **Mrs. Ngassa Mbenda Huguette Gaelle**

Date:

CERTIFICATE

This is to certify that the thesis entitled "**Molecular Diagnosis of Malaria Infection and Evolutionary Genetics of Drug Resistant Genes in *Plasmodium falciparum* in Cameroon**" submitted by **Mrs. Ngassa Mbenda Huguette Gaelle**, for the award of the Degree of Doctor of Philosophy in Biotechnology is based on original studies carried out by her under my supervision.

The thesis or any part thereof has not been submitted for any other degree or diploma in any university or institution.

Dr. Aparup Das

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Ngassa Mbenda H. G.

ABBREVIATIONS

µl	: Micro liter
ACTs	: Artemisinin-combination based therapies
AQ	: Amodiaquine
BLAST	: Basic local alignment search tool
CQ	: Chloroquine
CQR	: Chloroquine resistant/resistance
CQS	: Chloroquine sensitive
CSP	: Circumsporozoite protein
DARC	: *Duffy* antigens receptor for chemokines
dN	: Number of non-synonymous mutations
dS	: Number of synonymous mutations
DV	: Digestive vacuole
FLD	: Fu and Li's D
FLF	: Fu and Li's F
FWH	: Fay and Wu H
LD	: Linkage disequilibrium
MEGA	: Molecular evolutionary genetics analysis
MK	: Mac-Donald and Kreitman
ml	: Milli liter
MSP	: Merozoite surface polymorphic
^{o}C	: Degree Celsius
PCR	: Polymerase chain reaction
RBCs	: Red blood cells
SP	: Sulfadoxine-Pyrimethamine
TBE buffer	: Tris-Boric-EDTA buffer
TD	: Tajima's D
TE buffer	: Tris-EDTA buffer

INTRODUCTION

INTRODUCTION

Malaria is a highly infectious vector-borne disease of the tropical and sub-tropical countries of the globe. Almost all the African countries are endemic to malaria, contributing about 90% of the total worldwide malaria death incidences (WHO, 2013). Apart from global effort to control malaria, this disease remains as a frontline infectious disease in Africa and in other malaria endemic regions of the world. Relief and management of malaria majorly rely on treatment of infected patients by chemotherapy. Since as many as five different species of the genus *Plasmodium* (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*) are known to infect humans (Singh et al., 2004; Tyagi et al., 2013) either singly or as mixed parasitic infections, proper diagnosis of specific malaria parasitic infection holds the key for effective treatment and management of malaria. Although microscopy is traditionally considered as gold standard for malaria diagnosis, in recent years, molecular diagnostic approaches by Polymerase Chain Reaction (PCR) assays have evolved as the most sensitive method to accurately diagnose single as well as mixed malaria parasite infections (Snounou et al., 1993; Johnston et al., 2006; Gupta et al., 2010). Several field epidemiological studies in different malaria endemic countries of the globe (including some African countries) (Gupta et al., 2010; Ryan et al., 2006; Culleton et al., 2009; Menard et al., 2010; Dhorda et

al., 2011; Mendes et al., 2011) have adapted this highly sensitive malaria diagnostic approach.

In recent times, malaria diagnosis by PCR assays have not only confirmed high incidences of malaria due to *P. falciparum* infections in Africa, but instances of *P. vivax* infections in *Duffy*-negative individuals have surfaced in many African countries, *e.g.,* Madagascar (Menard et al., 2010), Mauritania (Wurtz et al., 2011), Angola (Mendes et al., 2011), Equatorial Guinea (Mendes et al., 2011), Ethiopia (Woldearegai et al., 2013), Republic of Congo (Culleton et al., 2009) and Kenya (Ryan et al., 2006). Historically, *P. vivax* is known to be majorly prevalent in Asian and Latin American countries but largely absent in west and central African countries. This situation is explained by the fact that (i) the human *Duffy* Antigen Receptor for Chemokines (DARC) is used by *P. vivax* merozoites to invade the human red blood cells (RBCs) (Miller et al., 1976), (ii) a mutation (T-33C) located in the promoter region of the human *Duffy* gene (otherwise known as *Duffy*-negative individuals) blocks the RBCs invasion of *P. vivax* and (iii) the T-33C (causing *Duffy*-negativity) is nearly fixed in Africans (Mendis et al., 2001; Gething et al., 2012). Since the *Duffy*-negative Africans are naturally protected from *P. vivax* infection, it has been proposed that the T-33C mutation has been selected in humans (Hamblin and Di-Rienzo, 2000). Considering the recent hypothesis on the African origin of *P. vivax* (Liu et al., 2014) it is quite possible that the fixation of this T-33C mutation might have been propelled by long exposure to *P. vivax* infection in Africans.

To this respect, Cameroon, a West-Central African country is inhabited mostly by *Duffy*-negative humans (95-99%) (Culleton et al., 2008), with high prevalence of *P. falciparum* malaria (up to 100%) (WHO, 2012). Incidences of *P. vivax* infection have earlier been reported in Cameroon but all such patients were non-native Cameroonians (Guerra et al., 2010). However, a recent study has demonstrated that native Cameroonians (*Duffy*-negative and *Duffy*-positive individuals) can also be infected by *P. vivax* (Fru-Cho et al., 2014). Moreover, mixed parasitic malaria infection with this species has also been reported in native Cameroonians (Fru-Cho et al., 2014). Considering Cameroon harbors not only rich biological and vector species diversity (Hervy et al., 1998; Ndoye and Kaimowitz, 2000; Fontenille and Simard, 2004; Ayala et al., 2009) but also borders with malaria endemic countries (Congo and Equatorial Guinea) reporting *P. vivax* infection in *Duffy*-negative individuals. It is therefore important to survey the extent of malaria due to infection of different species of malaria parasites in native Cameroonians.

The emergence and spread of multidrug resistance in *P. falciparum* remains a foremost obstacle and a pending problem for a successful chemotherapeutic control of the disease. This malaria parasite (considered as the most dangerous one) has developed resistance to almost all the antimalarial drugs available with the most common ones being resistance to chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) broadly widespread in sub-Saharan Africa, South Asia and South America (Wang et al., 1997; WHO, 2001). Of particular

concern is the reduced responsiveness of *P. falciparum* to quinolone antimalarial drugs. Quinoline derivatives have been used in malaria chemotherapy for more than a century and they are still indispensable, serving as partner drugs to artemisinin derivatives.

Evolution and spread of chloroquine-resistant-malaria parasite *P. falciparum* have posed great challenge in malaria control efforts all over the malaria endemic countries in the world. Although CQ has been banned from national malaria control programs of many endemic countries; due to high efficacy, easy avaibility and affordability, CQ still continues to be non-officially used in many countries including Africa (Sayang et al., 2009; Mbacham et al., 2010; Ndo et al., 2011; Menard et al., 2012). Further, in endemic countries like India and Southeast Asia where malaria parasites *P. vivax* and *P. falciparum* co-occur, and due to susceptibility of the former parasite to CQ, this drug is still used to treat uncomplicated *P. vivax* malaria. Thus continuous drug pressure exerted by CQ usage possibly had helped in the emergence of new varieties of CQ resistant (CQR) *P. falciparum* isolates in these epidemiological setting and thereafter have spread to other malaria endemic countries previously dominated by CQ sensitive (CQS) isolates. In parallel, increasing failure of amodiaquine (AQ) has been denoted in areas of intense transmission (Happi et al., 2006; Holmgren et al., 2006; Borrmann et al., 2008) and increasing selection of *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) in asexual and sexual parasites following treatment of infections with artemether-lumefantrine

(AL) has been depicted (Happi et al., 2009). Moreover, artemisinin resistance has already been reported in some parts of Southeast Asia (Phyo et al., 2012). Hence, there is a growing spectre of reduced responses to artemisinin-based combination therapies (ACTs) and a chance that artemisinin resistance could spread too in Africa. Two factors appear to be frequently linked with altered drug susceptibility: the gene encoding the *P. falciparum* chloroquine resistance transporter (*pfcrt*) known as the principal determinant of chloroquine resistance (CQR), and the *P. falciparum* multidrug resistance 1 gene (*pfmdr1*).

In regard to this, African countries are known to be mostly populated with CQR *P. falciparum* isolates imported from either Southeast Asia *via* India or from South America (Awasthi et al., 2012). Such historical migration events have been traced with population genetics analysis based on the distribution of CQR-*pfcrt* haplotypes, formed by differential arrangement of amino acid sequences from the 72^{nd} to 76^{th} position of the *pfcrt* polypeptide chain. It is well known that these haplotypes are genetically associated with CQR; while $C_{72}V_{73}M_{74}N_{75}K_{76}$ is considered to be a wild (sensitive) type, two CQR mother haplotypes ($C_{72}V_{73}I_{74}E_{75}T_{76}$ and $S_{72}V_{73}M_{74}N_{75}T_{76}$) have given rise to an array of other haplotypes following different mutational steps (Awasthi et al., 2012). The SVMNT haplotype bearing *P. falciparum* isolates are known to be generally fitter in the CQ environment compared to the CVIET bearing parasites. Further, the CQR (CVIET) types can be reversed to wild type (CQS) with the treatment with CQ reversal agent, verapamil, while those bearing SVMNT haplotypes are not

(Sa et al., 2009; Awasthi et al., 2011). Furthermore, the SVMNT haplotypes are differentiated into two different genetic types based on nucleotide sequence arrangements; S(agt)VMNT and S(tct)VMNT types, with different evolutionary histories. While the S(agt)VMNT type has been reported to be originated in the Papua New Guinea (PNG), the S(tct)VMNT has south American origin (Sa et al., 2009; Awasthi et al., 2011).

Malaria due to *P. falciparum* is highly endemic in Cameroon with several reports of CQR isolates in the field (Basco and Ringwald, 2001; Basco et al., 2002; Mbacham et al., 2010). Since genotyping the *pfcrt* gene of 72nd to 76th amino acid position of the *pfcrt* polypeptide chain is the best way to determine molecular and genetic epidemiology of CQR malaria, earlier studies have indicated that in Cameroon, the CQR malaria parasites are categorized mostly into the CVIET type (Menard et al., 2012).

In face of all these epidemiological scenarios, monitoring the level of *P. falciparum* resistance against anti-malarial drugs seems to be one of the keys to a successful malaria control in malaria endemic parts of the globe in general, and in Cameroon in particular. Even though controlled clinical trials are the best available tool for assessing the relevance of anti-malarial treatments, molecular monitoring offers some advantages, *e.g.* studies on Single Nucleotide Polymorphisms (SNPs) and duplication of genes involved in

resistance can be carried out with more ease and are less time-consuming. Furthermore, molecular monitoring may reveal trends, permitting anticipation in the changes of treatment policies. Latest advanced approaches such as evolution by natural selection offers innovative way not only to understand the genetic polymorphism of drug resistant genes, but also to understand the selective force which is controlling evolution of these genes based on their variation at the genetic level, in order to unravel the mechanism by which resistance occurs, how it spreads and how to prevent it.

Main Objectives of the study

The main objective of this project is to explore the application of one of the recent approaches for malaria diagnosis in Cameroon. This is not only with a view to survey the extent of malaria due to infection of different species of malaria parasites in native Cameroonians, but to explore too, other regions of the country for the possible detection of *P. vivax* malaria infection. Besides, considering high migration potentiality of malaria parasites, with recent entry of CQR *P. falciparum* to many African countries (Awasthi et al., 2012), to conduct a large scale molecular epidemiological study following high-sensitive DNA sequencing of the Cameroonian *pfcrt* and *pfmdr1* genes in order to describe distributional patterns of different CQR-*pfcrt* and/or haplotypes in Cameroon.

To resume, we specifically intend to:

- To perform molecular diagnosis of malaria infection in Cameroon.
- To understand the genetic diversity of two drug resistant genes (*pfcrt* and *pfmdr1*) in Cameroonian *P. falciparum.*
- To analyse the DNA sequences and infer evolutionary patterns of these two genes in Cameroonian *P. falciparum.*

CHAPTER 1:

REVIEW OF LITERATURE

1. REVIEW OF LITERATURE

1.1. Malaria

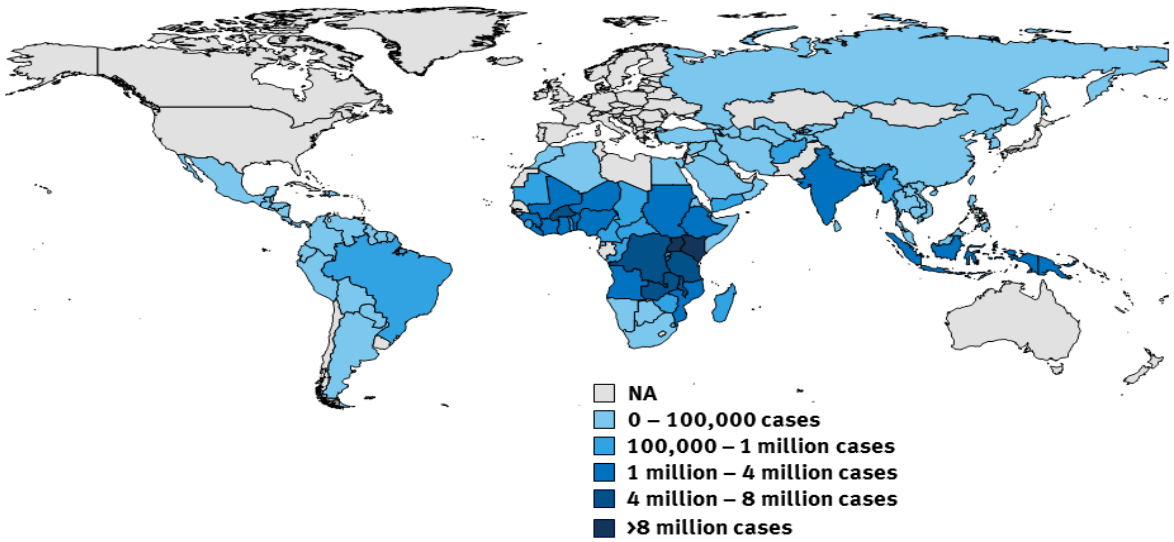
1.1.1. Definition and history

Malaria is an infectious disease which affects humans. It is transmitted by a protozoan parasite belonging to the genus *Plasmodium via* female *Anopheles* species mosquitoes (Cox, 2010). This disease constitutes one of the three deadliest infectious diseases of the recent years, competing with HIV and tuberculosis as a killer disease in tropical and subtropical regions (WHO, 2005). Malaria history is very ancient (2700 BC according to Chinese document) and for several years (around 2500 years) the exact cause of this disease was uncertain. With the discoveries of bacteria by Antoni Van Leeuwenhoek in 1676, the ability to cause infectious diseases attributed to microorganisms/the development of the germ theory of infection by Louis Pasteur and Robert Koch in 1878-1879, the search of the exact cause of malaria has intensified. The real explanation of malaria came with the discovery of the parasites in the blood of a patient by Charles Louis Alphonse Laveran in 1880 and furthermore, with the discovery by Ronald Ross in 1897 that mosquitoes can be vectors for avian malaria. Later on, Italian scientists Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava between 1898 and 1900 have found that mosquitoes can also be vectors for human malaria (Celli, 1933; Cox, 2010).

1.1.2. Brief worldwide epidemiology

Approximately 627000 deaths occur due to malaria infection every year in the globe with the majority of cases (90%) concentrated in sub-Saharan Africa (Figure 1.1 WHO, 2013). The principal targets of malaria are children less than five years old and pregnant women (WHO, 2013). Four species of *Plasmodium* were commonly known to infect humans: *P. falciparum* (, *P. vivax*, *P. malariae* and *P. ovale*. However, report on the existence of a fifth human malaria parasite (*P. knowlesi*) has recently been emerged in Southeast-Asia (Singh et al., 2004; Tyagi et al., 2013) with no report yet on its existence in Africa. Between those five species, the most distributed are *P. falciparum* and *P. vivax* with *P. falciparum* the most virulent.

Figure 1.1. Worldwide distribution of malaria



Source: Kaiser Family Foundation, www.GlobalHealthFacts.org, based on WHO, world malaria report 2012.

1.1.3. Transmission and biology of *Plasmodia*

The lifecycle of malaria parasites is composed of two stages involving two obligatory hosts: an asexual stage in humans and the sexual one in the female *Anopheles* mosquito (Su et al, 1999). During its life cycle, the parasite undergoes a period of haploidy (for the majority part of its life) and a brief period of diploidy (especially when it is following sexual reproduction in the mosquito). In this last period, there is occurrence of recombination coupled with a high crossover rate (Su et al, 1999) resulting in a mixture of different alleles. Through this phenomenon of recombination, the chance to get novel mutations in the parasite genomes is very high if the blood meal contains two or more genetically distinct clones (Barry, 2005). This makes the malaria diagnostic more complicated and the treatment less successful. Furthermore, in regions of high malaria endemicity like Africa and PNG where there are many multiple infections, it is common to see a phenomenon of out-crossing occur (Babiker et al, 1994; Paul et al, 1995). This leads to genetic diversity and provides opportunities for the propagation of advantageous alleles among parasites of a population (Barry, 2005).

1.1.3.1. *Plasmodium* life cycle in humans

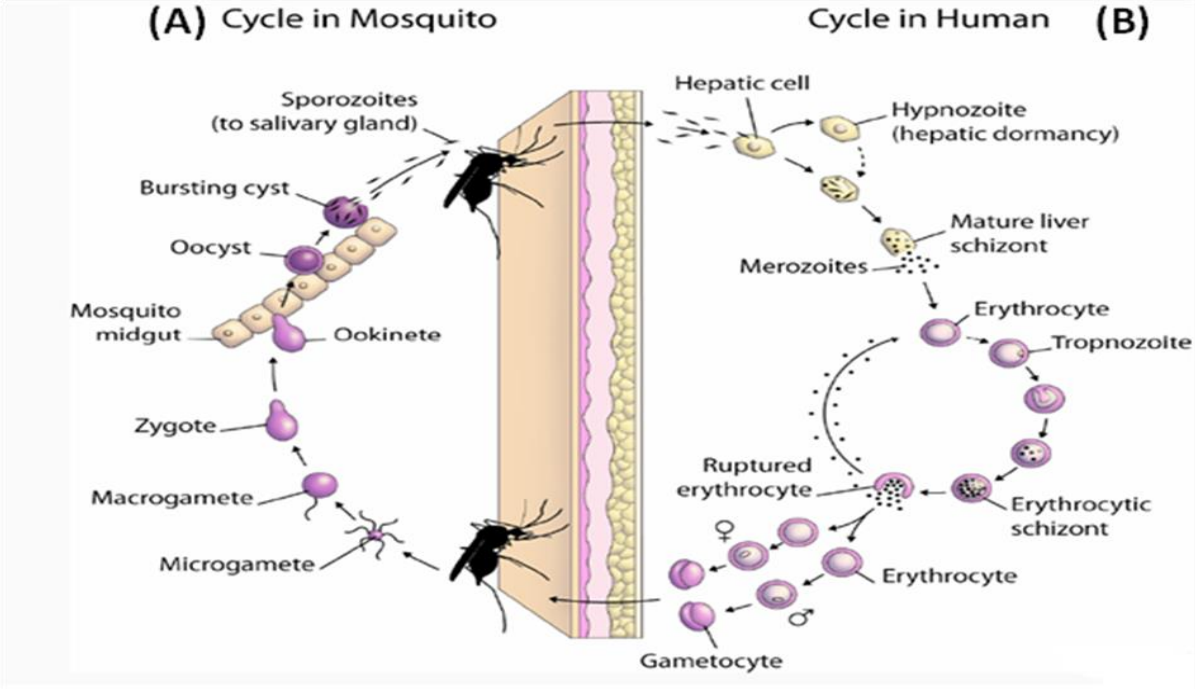
In the process of feeding of an infected mosquito on the human host, it injects few number of sporozoites contained in its salivary glands to the human blood circulation. The sporozoites then travel to the host liver and invade the hepatocytes. At this step, two malaria parasites (*P. vivax* and *P. ovale*) have the capacity to form dormant stages commonly known as hypnozoites in the liver. Because of this characteristic, the relapse is possible for several years after the initial infection. Approximately two weeks of maturation to exo-erythrocytic schizonts, the schizonts rupture and form the merozoites. The merozoites burst out of the hepatocytes and invade the red blood cells (erythrocytes). Afterward, the merozoites transform into many different forms, starting from rings to trophozoites and to erythrocytic schizonts. This process takes around two days (three days in the case of *P. malariae*). After the mature schizonts rupture, the merozoites released re-invade new erythrocytes. This process of replication of the parasite can continue asexually using that mechanism, but some can also form transmission stages called gametocytes [Barry, 2005; Figure 1.2 (B)].

1.1.3.2. *Plasmodium* life cycle in the *Anopheles*

The phase which involves the mosquito takes around two weeks time and starts with the ingestion of the gametocytes during the mosquito blood meal. These gametocytes are differentiated into gamete forms (male gamete and female gamete) that will further fuse in the mosquito midgut and form the zygotes. Zygotes develop into ookinetes which can pass through the gut wall to

develop into oocysts. The oocyts then explode and release the sporozoites that migrate to the salivary glands, ready to be transmitted to the human host during the blood meal and thus a new lifecycle will begin [(Barry, 2005; Figure 1.2 (A)].

Figure 1.2. Lifecycle of the malaria parasite



Source: www.malwest.gr

1.1.4. Different forms of malaria

There are mainly two different forms of malaria: uncomplicated malaria and severe malaria. The major complications of severe malaria are cerebral malaria and malarial anemia (Biemba et al., 2000; Idro et al., 2005).

1.1.5. General clinical presentation of malaria

Patients suffering from *P. falciparum* malaria infection generally present the signs of high fever (sometimes accompanied by chills), rigors, sweats, headaches, generalized weakness, backache, myalgias, vomiting and pallor. These symptoms are almost similar in the case of children too. However, for children who are partially immune (*e.g.* newly arrived immigrants or refugees from areas where malaria infection is highly endemic like Africa), there are some signs such as hepatomegaly, anemia, jaundice, anorexia, decreased activity or sometimes they are asymptomatic (Maroushek and Aguilar, 2002). Moreover, besides these malaria specific symptoms, there are others abnormalities common with this disease: elevated C-reactive protein levels, thrombocytopenia, elevated liver enzymes levels and elevated procalcitonin level. In the case of severe malaria, there are signs like hypoglycaemia, hyponatremia, hypoalbuminemia, hyperlactatemia, decreased plasma calcium, total cholesterol, magnesium levels, etc (English et al., 1996; Mfonkeu et al., 2010). Patients infected with *P. vivax* present signs of paroxysmal fever, chills, headaches, myalgias, anemia and hypersplenism (in case of chrocically infected children). *P. malariae* and *P. ovale* often cause fever but not a toxic appearance (in some individuals, *P. malariae* may coexist as a commensal organism causing infection but no clinical disease). Infection due to *P. falciparum* manifests days to weeks after initial exposure while *P. vivax* and *P. ovale* which have the hepatic hypnozoite stage (see above), may present much later (Stauffer and Fisher, 2003).

1.1.6. Diagnosis of malaria infection

There are two ways of malaria diagnosis: (i) the clinical diagnosis and (ii) laboratory diagnosis.

Clinical diagnosis: this method is based on the signs presented by the patient. In several malaria endemic areas where the resources and trained health personnel are scarce, this technique is the only realistic option even though reliable diagnosis cannot be made solely on the basis of signs and symptoms (because of the non-specific nature of clinical malaria and the fact that the patient can be asymptomatic). Moreover, substantial overlap exists between the malaria symptoms and signs and other frequent diseases; this can lead to an increase of the frequency of misdiagnosis and treatment (Redd et al., 1992). This method also presents advantages to be easy realizable, low of cost and speedy. However, since the symptoms presented by a malaria patient significantly overlap with other diseases, malaria treatment based only with this method is not advised. Therefore, laboratory tests are the most accurate way to diagnose malaria infection.

Laboratory diagnosis

Laboratory diagnosis is composed of several techniques as described below.

Microscopy: this technique is based on the use of thick and thin blood smears. Because the ingredients used are very cheap and easily affordable for the populations, it is considered as the golden technique for malaria diagnosis.

Thick blood smears is used to ascertain the presence or absence of the parasite, whereas thin blood smears is used for the speciation and quantification of the parasites (Ohrt et al., 2002; Coleman et al., 2002). The sensitivity and specificity of microscopy vary greatly and it is influenced by many factors such as laboratory skills (expertise of the personnel), timing and quality of smear collection, level of parasitemia (Ohrt et al., 2002; Coleman et al., 2002) etc. Because this technique is mostly personnel-dependent, it often leads to a misdiagnosis and therefore it is not 100% reliable.

Rapid antigen detection tests: most of these tests are basically used for the differentiation of *P. falciparum* from the non-*falciparum* infections through the detection of histidine rich proteins and/or parasite lactate dehydrogenase (Moody, 2002; Craig et al., 2002). These tests have revealed varied results in numerous trials, but a great number of them seem to be more efficient compared to the blood smears method when performed by an adequate (trained) personnel (Moody, 2002; Craig et al., 2002). These tests also offer advantage to be easy realizable and fast as neither light nor any source of energy is required. However, the tests present also some limits as they cannot quantify the parasitemia and they cannot differentiate non-*falciparum* species (Craig and Sharp, 1997).

Antibodies tests (serology technique): these tests are based on the detection of antibodies in the blood of the malarial patient in special circumstances. Firstly, antibody detection is used when the patient is a newly arrived

immigrant or refugee with a hyperactive malarial splenomegaly (term used to categorize people having a large spleen) who are suspected to have chronic malaria. In this condition, antibody IgM levels are strikingly elevated and once an antimalarial treatment is done, these levels decrease as well as the splenomegaly. Secondly, it is used when the patient is a returned traveler (who has a history of malaria diagnosis and treatment during its recent travels) and whom a serologic confirmation is desired. This technique has been proven to be useful. There is some evidence on travelers who have received a diagnosis of malaria whereas in the tropics they received an incorrect diagnostic. Unfortunately these tests are not useful for diagnosing an acute malaria infection (Schwartz et al., 2001).

Molecular techniques:

The molecular techniques are the most recent approaches taken to diagnose malaria infection. The methods aim to detect the parasite genetic material through the use of Polymerase Chain Reaction (PCR) technique. PCR tests have been demonstrated to be more sensitive and specific as compared by the traditional technique which is microscopy (Snounou et al., 1993; Gaye et al., 1999; Johnston et al., 2006; Gupta et al., 2010a) and the tests can detect parasite levels of 1 parasite/ml. Furthermore, PCR offer the advantage that the diagnostic might continue to be positive even for some days (approximately 11 days) after treatment due to the persistence of antigen in the serum. One very important use of this technology is in the detection of cases of mixed parasitic

infections. Besides, it is also important in the confirmation of the positive blood smears and particularly in the identification of malaria species when results of smears are not conclusive (Stauffer and Fisher, 2003). Beyond these PCR diagnostic assays, there are complementary techniques such as sequencing of the genes specific of each malaria parasite like the ribosomal RNA (particularly the 18S region as described by Snounou et al. in 1993), or involved in antimalarial drugs resistance of each specific species too, or polymorphic markers such as Merozoite Surface Polymorphic (MSP) and Circumsporozoite Protein (CSP) genes for the different malaria parasites species.

1.1.7. Overview on measures of prevention and important drugs for treatment of malaria

At present, there is no malaria vaccine widely efficient. Thus, different malaria control programmes rely on the measures of prevention: use of long lasting insecticides (LLINS), insecticides residuals doors (IRS), use of impregnated mosquito nets, use of long sleeves dresses and on the chemotherapy. Concerning the treatment of malaria, it is important to notice that it is selected on the basis of the infecting *Plasmodium* species, the severity of the infection, the drug susceptibility of the infecting parasites and the avaibility of medications and resources (Stauffer and Fisher, 2003). For the past 50 years, the most widely used drugs for prevention or treatment of malaria are quinine and its derivatives (quinidine, mefloquine, halofantrine, 8-aminoquinoline primaquine used for its gametocidal effect and its action on the liver stage of *P.*

vivax, the 4-aminoquinolines: CQ and its relative AQ) and antifolate combination drugs which embrace the diaminopyrimidines, such as pyrimethamine and trimethoprim; the biguanides, represented by proguanil (cycloguanil) and chlorproguanil; the sulfa drugs including the sulfonamides and the sulfones (Bloland, 2001). Since 2007 till date, the utilization of Artemisinin Combination Therapies (ACTs) has been implemented and adopted as a first line for the treatment of uncomplicated malaria due to infection of *P. falciparum* (Ashley et al., 2007).

1.1.8. Challenges for the malaria control programmes

Due to the absence of competent malaria vaccine, the malaria control programmes are relying mostly on chemotherapy. However, their efforts are repeatly jeopardized by the emergence and spread of drug resistance. Furthermore, a proper diagnosis of specific malaria parasitic infection holds the key for effective treatment and management of malaria.

1.1.8.1. Mixed parasitic infections

Mixed parasitic malaria infection can be defined as the coexistence of more than one malaria parasite species in the same individual. Microscopy has been demonstrated to be a golden malaria diagnostic technique (Chotivanich et al., 2007), especially in areas where there are less financial resources (*e.g.* some parts of the African continent) to afford more efficient techniques. It is easily practicable by the clinicians (Chotivanich et al., 2007). However, in regions of high endemicity (sub-Saharan Africa) where the rate of multiple infections is

high, this technique becomes less efficient to detect mixed parasitic malaria infection rendering the patient's treatment more complicated. The reduction of efficiency of microscopic detection of two or more than two parasites can be explained by the tendency of one parasite to dominate the other one (Sethabutr et al. 1992). In recent years, molecular diagnostic approaches by PCR assays have evolved as the most sensitive method to accurately diagnose single as well as mixed malaria parasite infections (Snounou et al., 1993; Johnston et al., 2006; Gupta et al., 2010). With the recent reports of the capacity of *P. vivax* (the most distributed malaria parasite outside of sub-Saharan Africa, Guerra et al., 2009) to infect *Duffy*-negative individuals (Carvalho et al., 2012) and in particular *Duffy*-negative Africans (Table 1.1 Mendes et al., 2011; Ryan et al., 2006; Culleton et al., 2009; Menard et al., 2010; Wurtz et al., 2011; Woldearegai et al., 2013; Fru-Cho et al., 2014), it becomes highly important more than before, to use and implement techniques enable to detect very efficiently not only cases of mixed parasitic malaria infection but also single species infections; particularly in areas such as sub-Saharan Africa where species like *P. vivax* is not yet very prevalent such as *P. falciparum* species. It appears therefore that the detection of mixed infections is not only important for the successful medical treatment of malaria, but also for the study of malaria epidemiology.

Table1.1. List of the African countries where *P. vivax* has been detected on *Duffy*-negative individuals

Country	Origin of individuals	Type of analysis for identification	*Duffy* gene characteristic if performed	Material used	References
Angola	Indigenes	PCR targeting the small-subunit rRNA + *pvcsp* analysis	*Duffy* negative individuals	Finger prick, Whole blood samples, Mosquitoes	Mendes et al., 2011
Equatorial Guinea	Foreigners (White people), Indigenes	PCR targeting the small-subunit rRNA + *pvcsp* gene	*Duffy* negative individuals *Duffy* positive (Only White people)	Whole blood samples, Mosquitoes	Mendes et al., 2011
Kenya	Indigenes	PCR targeting the small-subunit rRNA + *pvcsp* gene	*Duffy* negative individuals	Whole blood samples, Mosquitoes	Ryan et al., 2006
Mauritania	Indigenes	Real time PCR	*Duffy* negative individuals	Finger prick blood samples	Wurtz et al., 2011
Ethiopia	Indigenes	Microscopy, PCR targeting the small-subunit rRNA	*Duffy* negative individuals	Finger prick blood samples	Woldearegai et al., 2013
Congo	Indigenes	*pvcsp* , *pvmsp* genes analyses	*Duffy* negative individuals	Whole blood samples	Culleton et al., 2009
Madagascar	Indigenes	Microscopy, PCR targeting the small-subunit rRNA, PCR based on Cytochrome oxidase I, *pvdbp gene*	*Duffy* negative individuals	Whole blood samples	Menard et al., 2010
Cameroon	Indigenes	Microscopy, PCR targeting the small-subunit rRNA, *pvcsp* gene	*Duffy* negative and positive individuals	Finger prick blood samples	Fru-Cho et al., 2014

***pvcsp*:** *P. vivax* circumsporozoite protein **pvdbp:** *P. vivax Duffy* binding protein

***pvmsp*:** *P. vivax* merozoite surface polymorphic **rRNA:** ribosomal RNA

1.1.8.2. Emergence and spread of drugs and insecticides resistance

Antimalarial drugs resistance as well as insecticides-resistance of malaria vectors has emerged as one of the biggest challenges faced by the various malaria control programmes today including those implemented in Cameroon. Drug resistance has been involved in the continue spread of malaria infection to new areas and in the re-emergence of this disease in areas where it had already been eradicated (Foster, 1991; Ridley, 1997). Drug resistance has also significantly contributed to the occurrence and severity of epidemics in some parts of the world; due to the population movement, resistant strains of parasites have been introduced to areas previously free of drug resistance (Foster, 1991; Ridley, 1997). *P. falciparum* (the most dangerous malaria parasite) has developed resistance to almost all the antimalarial drugs available starting with the cheap and widely available first-line drug CQ up to the latest which is artemisinin. Arylaminoalcohol drugs such as mefloquine and halofantrine have been introduced as second-line drugs in areas where there was a high level of CQR. Unfortunately, resistance to these drugs has also emerged and in certain areas such as parts of South-East Asia (with reference to the borders of Thailand), their use as monotherapy has been compromised due to the severity of the resistance (Price et al., 1995). The recently introduced endoperoxide drugs (artemisinin and its derivatives) have demonstrated a great efficacy against parasite lines resistant to the other antimalarials. But nonetheless, resistance to these novel drugs has also been detected in some areas such as South-East Asia (Phyo et al., 2012) and *in vitro* studies have

indicated that there is a reciprocal relationship between CQR and resistance to the arylaminoalcohols and endoperoxides (Duraisingh and Cowman, 2005). The genetic basis of the resistance to these antimalarial drugs has also been determined in recent studies and many genes have been identified as key determinants of antimalarial resistance. However, the mechanism of resistance of this parasite has been well explored only for the two famous antimalarial drugs: CQ and SP.

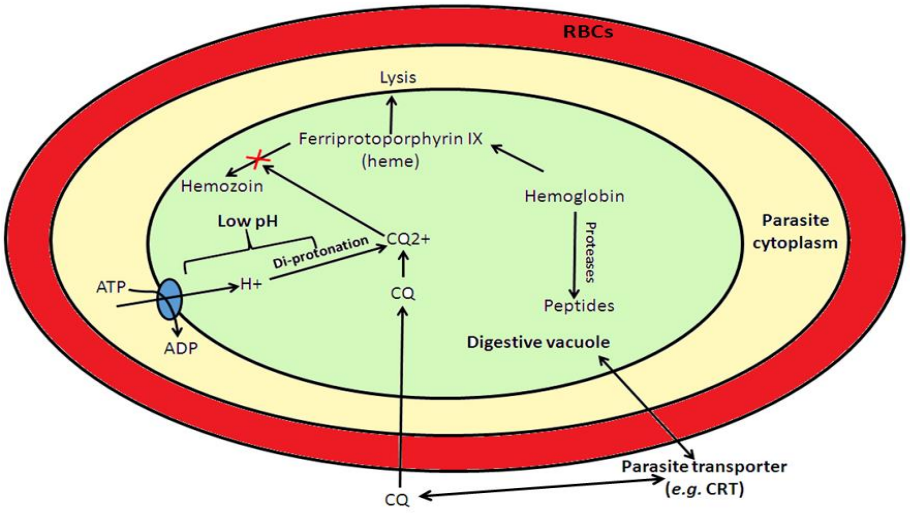
i. Chloroquine resistance (CQR) and the mechanisms

The discovery of the exceptional antimalarial properties (efficacy, safety, affordability) of CQ 65 years ago has made this drug a golden one for the treatment of malaria due to *P. falciparum* worldwide (Hay et al., 2004). However, after around one decade of use, CQR has emerged in few sites: Southeast Asia, South America, the Western Pacific region and from there has spread gradually all over the malaria-endemic areas including Africa which harbors the greatest number of malaria deaths (Trape et al., 2002; Wellems et al., 2009). Due to this scenario, ACTs has been adopted recently in all the malaria endemic parts of the world (Nosten and White, 2007; Wells et al., 2009). Unfortunately, none of the current first-line antimalarials could compete the encouraging properties once held by CQ. The mechanism of action of CQ takes place into a lysosome-like organelle, the digestive vacuole (DV) of the parasite (Figure 1.3). In fact, the infection of RBCs by the pathogen *Plasmodium* results in the ingestion of large amounts of host cell hemoglobin

into the DV. Therein, hemoglobin is proteolytically cleaved, releasing globin fractions that are further degraded into small peptides providing sources of amino acids for protein synthesis (Goldberg, 2005). During the degradation of hemoglobin, there is also the release of toxic Fe^{2+}-heme, which rapidly oxidizes to form Fe^{3+}-heme (also known as ferriprotoporphyrin IX or FPIX). This in turn liberates an excess of electrons that can trigger the production of bio-reactive oxygen species including hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2). Fe^{3+}-heme is largely insoluble and can disrupt membrane function. To avert this, parasites form iron-carboxylate coordinated FPIX dimers, also known as β-hematin, which bio-mineralize to form chemically inert crystals known as hemozoin (visible by microscopy as malaria pigment) (reviewed in [Egan, 2008; Roepe, 2009]). CQ (which is a weak base) can easily diffuse across membranes in its neutral form and therefore concentrates inside the acidic DV, according to the Henderson-Hasselbach equilibrium (Bray et al., 2006). Then, CQ will bind to FPIX and then prevent the heme detoxification leading to poisoning the parasite with its own waste products (Roepe, 2009). However, there is an alternative model which has been proposed, that CQ may act at least in part by binding to a transporter called *P. falciparum* chloroquine resistance transporter (*pfcrt*) and thus inhibits an endogenous function. Resistance to CQ was thought to show an amazing resemblance with multidrug resistance seen in mammalian cells due to the reduced accumulation of the drug and also due to the fact that resistance was reversed in the presence of verapamil (Krogstad et al., 1987; Martin et al., 1987). Despite the fact that CQ

itself is officially no longer recommended for the treatment of *P. falciparum* malaria, it maintains its efficacy against *P. vivax* in many geographical areas (Price et al., 2009). In Africa, self-medication is well practiced and Cameroon does not depart from the rule (approximately 60% of Cameroonian populations practice a self-medication), CQ continues to be non-officially used (Ongolo-Zogo and Bonomo, 2010). Furthermore, its structural analog AQ is used as a partner to artemisinin derivatives in most of the African countries including Cameroon. This increases the importance to study and understand deeply the mode of action of CQ as well as the mechanism of CQR. Since the genetic basis of CQR has been already found (Fidock et al., 2000), continuous studies into can provide insights of the parasite biology and aid future drug design. Moreover, observations of CQ-sensitive parasites capable to re-emerge after a strict removal of CQ (Kublin et al., 2003; Mwai et al., 2009) raise hopes that CQ (or derivatives) may again later rejoin the antimalarial armamentarium.

Figure 1.3. Mechanism of action of CQ



ii. Sulfadoxine-pyrimethamine resistance (SPR)

SP has been widely used as first-line therapy for uncomplicated *P. falciparum* malaria throughout sub-Saharan Africa. Because of its affordability, ease of administration and until recently, effectiveness, the drug is widely used to treat malaria. It is currently the only option for intermittent treatment of malaria during pregnancy (McCollum et al., 2006). Antifolate combination drugs, such as sulfadoxine plus pyrimethamine, generally act through sequential and synergistic blockade of two key enzymes involved in the process of folate synthesis. Pyrimethamine and related compounds inhibit the step mediated by dihydrofolate reductase (*dhfr*) preventing the biosynthesis of purines and pyrimidines and consequently halting the processes of DNA synthesis, cell division and reproduction. It acts primarily on the schizonts during the hepatic and erythrocytic phases (Kuznetsov et al., 1984). While sulphadoxine is focused on inhibiting the use of para-aminobenzoic acid during the synthesis of dihydropteroic acid, a step mediated by the enzyme dihydropteroate synthetase (*dhps*). Scientific studies have demonstrated that some specific mutations in the respective genes *dhfr* and *dhps* are responsible for the occurrence of resistances to SP. In the *dhfr* gene, a point mutation (causing a change of Serine to Asparagine: Ser→Asn) at the codon position 108th has been pinpointed to be involved in the resistance's mechanism (Basco et al., 1996; Reeder et al., 1996). Furthermore, high level of *in-vitro* resistance has shown that mutations at residue 51 resulting in Asn→Ile, 59 (Cys→Arg), and/or 164 (Ile→Leu) confer some level of resistance (Sirawaraporn et al., 1993; 1997a;

1997b). None of the mutations associated with *dhfr* appear to affect the function of the enzyme harmfully. As only a single- or double-step mutation is required to move from 'sensitivity' to 'resistance; the *dhfr* type of resistance occurs readily (White, 1992). Concerning sulfadoxine, sequence analysis of the coding region of the *dhps* has identified a number of point mutations correlated with *in-vitro* resistance to sulfa drugs. These mutations include: the amino acid change at the codon 437 (Alanine to Glycine, Ala→Gly); at the codon 581 (Alanine to Glycine, Ala→Gly); 436 (Serine to Phenylalanine, Ser→Phe), and 613 (Alanine to Serine, Ala→Ser). The mutations occurring at the last three codons positions of the list are generally additional to the first one for conferring high levels of resistance (Basco and Ringwald, 1998; Wang et al., 1995). Moreover, transfection studies in *P. falciparum* have proved that mutations occurring in the *dhps* gene confer effectively resistance to sulfonamides and sulfa drugs as well (Triglia et al., 1998; Kublin et al., 2002).

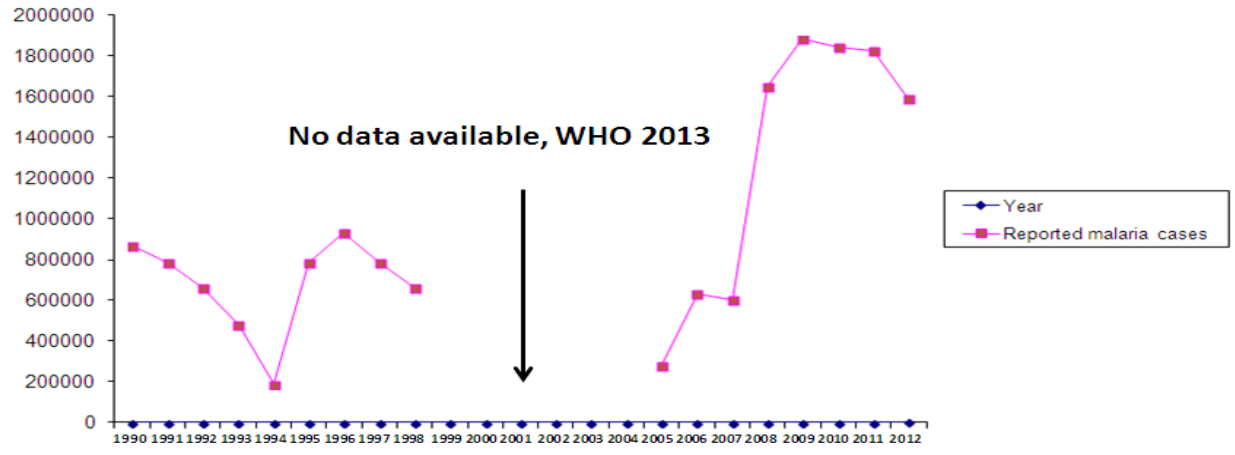
1.2. Malaria havoc in Africa with special reference to Cameroon

Almost the whole of Africa is highly endemic to malaria infection. The sub-Saharan Africa remains the most endemic part with the children below five years and pregnant women at the highest risk (WHO, 2013). This fact is corroborated by the prevalence of about 80% of 207 million clinical episodes of malaria in 2012 in African regions only with about 90% of 627 thousands malaria deaths reported in the whole world (WHO, 2013). Despite of the continuous efforts done by diverse malaria control programmes to trim down

the incidence of malaria, a very modest breakthrough has been made so far globally. Thus malaria remains as one of the principal human infectious diseases in Africa (Frech and Chen, 2011). Reflecting the situation of whole of Africa, in Cameroon [a country located at latitudes 2^{o}N and 13^{o}N, placed between West and Central Africa, often described as "Africa in miniature" because of the diverse natural environment spread throughout the country in the 10 regions (Wiysonge et al., 2005; Efon et al., 2013)], malaria is the primary cause of morbidity and mortality. It is also the major cause of illness and mortality in children under five years (WHO, 2013). Cameroon is one of the major contributors to the sub-Saharan African malaria incidences. This is justified by the fact that, out of 46 African regions as categorized by the WHO, Cameroon stands 11th in the list of the most malaria-endemic countries. Furthermore, among all central African countries recorded by WHO for malaria incidences, Cameroon occupies the 3rd position (WHO, 2010; WHO, 2012; WHO, 2013). Compared to the 1990's where the numbers of malaria cases were relatively less than one million, malaria incidence has drastically increased after 2004 to reach close to two millions in 2009, 2010 and 2011 (Figure 1.4). In fact, malaria is responsible for about 48% of hospital admissions, 30% of morbidity and 67% of childhood mortality per year in Cameroon (WHO, 2011; Antonio-Nkondjio et al., 2013). Recent records from the Cameroon Ministry of Health have indicated the fact that of the 20 million total populations, over 90% are at risk and about 41% get at least one episode of malaria each year (Antonio-Nkondjio et al., 2008; Minsante, 2008; Ndo et al., 2011). Moreover,

severe malaria cases (including cerebral and anemic malaria) which are known to be the two major contributors to overall malaria mortality are frequent in Cameroon (Forlack et al., 2005; Dongho et al., 2011). Rapid urbanization with increased population growth in-and-around Cameroonian cities accompanied by poor housing, lack of proper sanitation and drainage facilities frequently lead to flooding during rainy seasons, consequently increasing the mosquito breeding sites and helping in the spread of several vector borne diseases that include malaria, dengue or chikungunya (Nimpaye et al., 2001; Kamgang et al., 2010). Changing man-made eco-climatic conditions (see above) coupled with high diversity in malaria vectors species and insecticides-resistant malaria vectors, complex human histories and evolution and spread of drug resistant malaria parasites (see below) have put greater challenges on malaria management in Cameroon much steeper than before.

Figure 1.4. Malaria trend in Cameroon



Source: Ngassa et al., 2014

1.2.1. Complexity of malaria infection and drug-resistant *P. falciparum* in Cameroon

Four (*P. falciparum, P. vivax, P. malariae* and *P. ovale)* of the five commonly known species of *Plasmodium* capable to infect human are prevalent in Africa as well as in Cameroon (Table 1.2). However, malaria burden due to *P. malariae* and *P. ovale* infections are much lesser than *P. falciparum* infection alone and the latter species accounts for the majority (between 80 and 90% for *P. falciparum*; 8% for *P. ovale*; between 2 and 3% for *P. malariae* and between 1 and 10% for *P. vivax*) of malaria infection in Africa (http://www.malariasite.com/malaria/MalarialParasite.htm). Although reports on the existence of a fifth human malaria parasite (*P. knowlesi*) has recently been emerged in Southeast Asia including Andaman Islands of Indian (Singh et al., 2004; Tyagi et al., 2013), there is no report yet on its existence in Africa. Historically, Africans are known to be naturally resistant to *P. vivax* malaria infection (otherwise the most wide-spread species in South America and Asia) possibly due to the absence of *Duffy* antigen (Pogo and Chaudhuri, 2000; Chittoria et al., 2012). However, recent reports from some African countries (*e.g.* Angola, Equatorial Guinea, Congo, Mauritania, Madagascar, Kenya and Ethiopia) indicate prevalence of *P. vivax* in these countries with human infection by this malaria parasite (Ryan *et al.* 2006; Culleton et al., 2009; Menard et al., 2010; Mendes et al., 2011; Woldearegai et al., 2013; Wurtz et al., 2011). In this concern, it was revealed so far that humans in Cameroon found to be infected with *P. vivax* were in fact of non-African origin (Guerra et al.,

2010). However, a recent study demonstrated that Cameroonians can also be infected with *P. vivax* as others Africans (Fru-Cho et al., 2014). Since the majority of Africans infected with *P. vivax* have been reported to be *Duffy* negative, it seems probable that *P. vivax* can infect the *Duffy*-negative Africans by modifying the *Duffy* pathway or this species has found an alternative route to invade human immune system. Furthermore, in Africa, finding of several parasite clones in a single infected individual (multiclonal infections) indicates high rates of genetic recombination in parasites due to cross fertilization (Lekana-Douki et al., 2011). Probabilities of many different species of *Anopheles* biting a single person at a time cannot entirely be ruled out for the occurrence of such multiclonal infections (Read et al., 2011). Considering Africa as the homeland to *P. falciparum* (Das et al., 2007) and some indirect evidence on origin of *P. vivax* in Africa (due to fixation of *Duffy* negative allele in humans as a protective mechanism due to positive natural selection (Zimmerman et al., 1999; Carter and Mendis, 2002), mixed parasitic infection and malaria severity can be well justified. A very recent finding on the evidence on the African origin of *P. vivax* (Liu et al., 2014) has provided further impetus to understand malaria epidemiology of Africa in general. Since Cameroon possesses almost all the malaria epidemiological features that are broadly present in other African countries, Cameroon could serve as a model field for understanding malaria epidemiology in African perspectives.

Like the evolution and spread of insecticide-resistant mosquitoes in Cameroon, malaria parasites in Cameroon have long developed resistance to many

antimalarial drugs used in malaria control program. The most common cases of resistance for malaria treatment are CQ and SP (Ringwald et al., 1996; Basco et al., 2002a; Mbacham et al., 2009; Figure 1.5). Although some resistance to ACTs has been reported in South East Asia (Dondorp et al., 2009; Phyo et al., 2012), there is no published report yet in any African country including Cameroon. In fact, in Africa, CQR in malaria parasites was first reported in eastern part (Kenya) in 1978 (Kihamia and Gill, 1982; Trape, 2001); followed by a progressive dissemination in the whole of African continent. In Cameroon, CQR was noted for the first time in the South-western region, a hyperendemic area, specifically in Limbe in 1985 (Sansonetti et al., 1985) and then it has spread all over the 10 regions very rapidly (Soula et al., 2000; Basco et al., 2002a). It is now known from evolutionary genetic studies of the *pfcrt* gene that CQR-*P. falciparum* has not been originated in Africa but been imported mostly from Asia and South America through India (Fidock et al., 2000; Awasthi et al., 2011; 2012) and a particular *pfcrt*-haplotype (CVIET) is prevalent in almost all of the African countries. In Cameroon, molecular epidemiological studies suggest the association of K76T (Lysine amino acid replaced by threonine amino acid at the 76th position) mutation in the *pfcrt* gene with CQR (Basco and Ringwald, 1998; Menard et al., 2012). In the face of failure of CQ, Cameroonian Ministry of public health decided to opt for an alternative first-line drug (AQ) with SP as a second-line drug for the treatment of uncomplicated malaria (Basco et al., 2002a). Introduced in late 1970's, SPR has been detected around 1996 in the country. The occurrence of

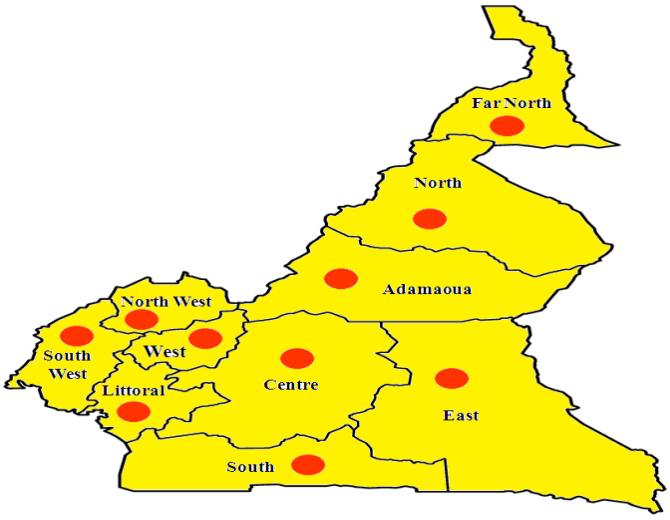
polymorphisms in the *dhfr* gene which has been attributed to consumption of SP as well as some antibiotics containing antifolates (*e.g.* co-trimoxazole), has also been indicated as responsible for SPR (Basco et al., 2002a). The efficiency of monotherapies for the treatment of uncomplicated malaria (CQ, mefloquine *etc.*) is challenged in Africa due to the ability of parasite to escape these drugs (Mbacham et al., 2009; Menemedengue et al., 2011).

Table 1.2. Distribution of principal human malaria *Plasmodium* species in Cameroon

Ecological facies	*P. falciparum* (%)	*P. malariae* (%)	*P. ovale* (%)
Adamaoua facies	100	0	0
Savannah-forest	93.6 - 98.7	0.0 - 6.4	0.0 - 1.3
Transition facies	89.8 – 100	4.3 - 8.4	0.0 - 1.8
Forest facies	62.0 - 96.3	0.6 - 3.0	1.1 - 35.0
Altitude facies	91.5 - 96.0	1.7 - 7.0	0.0 - 6.8
Coastal facies	97.7 – 100	0.0 - 0.7	0.0 - 2.3

Source: Ngassa et al., 2014

Figure 1.5. Distribution of CQ and SP resistance in Cameroon



Source: Ngassa et al., 2014

1.2.2. Complex human histories, migration and host susceptibility

Several studies on anthropology, archeology, paleontology and molecular genetics have pointed Africa to be the center of origin of humans (Foley, 1998; Raghavan et al., 2009). It has been pointed out that human migration in developing countries is generally influenced by urbanization, colonization, trade, agricultural labors and conflicts. All these elements interact with the transmission and epidemiology of some vector-borne diseases (*e.g.* malaria) (Sutherst, 2004). Defined as a social and political disease, malaria transmission and epidemiology can be affected by the migration of human populations as well as the vectors (Garfield, 1999). Accordingly, the sub-Saharan Africa is the most rapidly urbanizing continent with the highest rates of *P. falciparum* transmission (Trape et al., 1993; Granja et al., 1998; United Nations, 1999). The flux of Cameroonian populations both within and out of the country is intense, which can be explained by the presence of poverty, lack of employment, and economic crisis, to name a few (Fleisher, 2007). Traditionally, the principal income-generating activities in Cameroon are trade and agriculture. In the stream from Cameroon to Germany for example, Cameroonian migrants constitute the third largest group after Ghana and Nigeria (Fleisher, 2007). Also, Cameroon is one of the African countries having many refugees camps for displaced people coming from Democratic Republic of Congo, Chad, Central African Republic and Sudan and since 2001, the number of central African refugees inside the country is increasing (Overview of Displacement in Central African Republic, 2011) favoring the movement of

parasites among different countries. This also generates chances of evolution of new (virulent and drug-resistant) parasite clones by genetic recombination as well, in association with eco-climatic and environmental factors. These factors both in isolation or in combination possibly have contributed to the spread of drug resistance and increased incidences of malaria morbidity and mortality in Cameroon. This is especially true as it has been reported in some areas of the country (*e.g.* Northern Cameroon) the intense population migration was accompanied by an increase in human malaria cases (Antonio-Nkondjio et al., 2008). Moreover, it has been shown that international transit of infected people with malaria play a significant role in the global dispersal of the drug resistance against two most commonly used antimalarial drugs: CQ and SP (Lynch and Roper, 2011; Awasthi et al., 2012).

Considering malaria is associated with human since time immemorial, like evolution of resistance in malaria parasites (to antimalarials) and mosquito vectors (to insecticides), humans have also evolved different mechanism of resistance from malaria parasite infection. In this context, one of the main scientific evidence comes from the presence of human *Duffy*-negative individuals in Africa, where malaria due to *P. vivax* is very negligible. However, in regions where *P. vivax* malaria is highly endemic, humans are grossly *Duffy*-positive (*e.g.* India; Chittoria et al., 2012). This mechanism is explained by the fact that while the commonly found T nucleotide at the – 33rd position of the *Duffy* gene promoter region (Mendis et al., 2001) is responsible for the expression of this gene (*Duffy*-positive), the C-mutation at this position

dysfunctions the *Duffy* gene (*Duffy*-negative). Because of the C-mutation, the human DARC which is used by *P. vivax* merozoites to invade the human RBCs becomes silent causing blockage of the invasion. However, recent reports have emerged on the capability of *P. vivax* to infect *Duffy*-negative Africans (see above) and on the possible implication of gorillas and chimpanzees in the transmission of *P. vivax* malaria infection in West and Central Africa (Culleton and Ferreira, 2012). Cameroon not only belongs to the region where ~95% to 99% of the inhabitants are *Duffy*-negative (Culleton et al., 2008), it is also one of the African countries (including those which are borders like Democratic Republic of the Congo, Gabon and Equatorial Guinea) harboring a great number of gorillas and chimpanzees (Nkemnyi et al., 2011; Culleton and Ferreira, 2012). This situation points out the fact that the non-human primates might be the reservoir of *P. vivax* malaria (Culleton and Ferreira, 2012) and opportunistically infect humans.

1.3. Evolutionary genetics: application in malaria

Testing for processes rather than only looking for associations, opens new possibilities for those interested in public health issues. An integration of laboratory and field based studies, and closed coordination between evolutionary biologists and biomedical researchers will provide excellent opportunities to understand the phenotypic variations of malaria. It is well known that malaria infection engrosses the dynamics between three components in a given environment: the vector (*Anopheles*), the host (humans),

the parasite (*Plasmodium*). In order to evolve towards a world where individual-based-medicine (Evolutionary Medicine) is possible, population geneticists have found a way to integrate the concept of evolution into some diseases. This integration can also be possible with malaria by looking at the variability (at the genetic level) of genes involved in the functioning of malaria triangle (see above); *e.g.* genetic polymorphism of the genes involved in the resistance to antimalarial drugs, genetic polymorphism of the genes involved in the host susceptibility to malaria infection, genetic polymorphism of the genes involved in the resistance of malaria vectors to insecticides.

1.3.1. Basics of the evolution's law

The concept of evolution has started with Charles Darwin (1809-1882) when he was looking at the diversity of natural populations. In his book entitled: "On the origin of species by means of Natural Selection" written in 1859, he stated on his theory about Variation and Evolution, he said: << variation is a feature of natural populations and every population produces more progeny than its environment can manage. The consequence of this overproduction is that those individuals with the best fitness for the environment will produce more offspring that can more successfully compete in that environment. Thus the subsequent generation will have a higher representation of these offspring and the population will have evolved >>. The main key of evolution resides around the instinct of survival that each living organism has. This is because every living organism wants to reproduce in a given environment. These two factors:

survival and reproduction are controlled by an evolutionary force called “Natural Selection”. In an ideal population, there would be genetic equilibrium (meaning no change in gene frequencies) and no evolution would occur. Therefore, the conditions that would be needed in a population to have genetic equilibrium as proposed by Hardy and Weinberg are:

• Population must be "infinitely" large (large enough to eliminate chance or random gene frequency fluctuations).

• Population should be isolated from other such populations (no immigration or emigration; no gene flow).

• Mutation should not occur

• Mating should be random

• All genotypes are equally viable; natural selection should be absent

Any change in gene frequency from generation to generation can then be foreseeable and it is possible to look for the reasons or agents responsible for the change. As a result of the Hardy-Weinberg Equilibrium, biologists could search for the "agents" of evolution, or those factors that result in the change of gene frequency.

1.3.2. Concept of natural selection: effect in malaria parasites

As explained above, natural selection is the basic mechanism of evolution. This process has a great impact on malaria parasites genetic diversity. It is not a

secret that malaria is an infectious disease which displays great geographical diversity including not only ecological and epidemiological characteristics, but also wide-ranging polymorphisms in the genes encoding antigenic proteins. The expressed phenotypic variation arisen from that diversity is the raw material on which natural selection operates. Therefore, natural selection is brought up in the malaria literature whenever linkage is found between alleles and a phenotype, such as drug resistance, or a stimulated immune response is detected.

Evolution by natural selection generally occurs because of differences in reproduction of phenotypic variants in a given environment; this differential reproduction affects the frequencies of the associated genotypes in the next generation. There are three necessary conditions for evolution by natural selection to occur: (i) phenotypic variation; (ii) differences in the reproductive capacity of those phenotypic variants, given the environment; and (iii) genetic variation associated with the phenotypic variation. The processes driving natural selection (also called selective forces or selective pressure) are those capable of affecting the reproduction of the exposed phenotypes. In the context of malaria parasites, studies of natural selection have focused on antimalarial drugs and immune responses as selective pressures; however, natural selection will occur as the result of any process that differentially affects the reproduction of the parasite population, based on phenotypes linked to genetic variation. Consequently, genetic variants favored by a selective pressure will increase in frequency or will be maintained in the population (positive

selection), while those negatively selected will decrease or will be eliminated (negative selection) (Escalante et al., 2004). Since the parasite intra-host dynamics are indispensable in understanding the origin of phenotypes of public health relevance and natural selection being a part of this dynamic (Escalante et al., 2004), these processes (see above) represent what population geneticists try to capture wherever they suspect that natural selection is important in explaining the observed genetic diversity.

1.3.3. Genetic markers to infer natural selection: SNPs Approach

Genetic polymorphisms have been used as molecular markers to determine the evolution and the population genetics of the malaria parasites. Thus, various genetic markers have been found to be useful for inferring the effect of natural selection on a particular gene in a given population starting with the discovery of allozymes after several biochemical studies till the use of protein as well as DNA sequence variation since 1990s onwards. SNPs are simply defined as single base substitutions found at a single genomic locus (Xing et al., 2005). These are the point mutations in the nucleotide sequence that may or may not encode amino acid changes (called “non-synonymous mutation” in case of amino acid change and “synonymous mutation” in case of no amino acid change). SNPs represent the most widespread type of sequence variation in genomes (Brumfield et al., 2003). Around 90% of genetic variation in the human genome is in the form of SNPs (Collins et al., 1998) and they have only

emerged recently as valuable genetic markers for revealing the evolutionary history of populations.

How SNPs are ascertained?

SNPs can be ascertained by sequencing all individuals or a panel (subset) of individuals for an entire DNA region of interest, identifying variable sites, defining which of those sites will be considered SNPs and then screening only those sites in a sample of interest by using SNP-specific primers or probes.

Why to choose SNPs for the studies of population history?

There are several characteristics which make SNPs an ideal marker for population genetic studies. Looking into the mutation pattern, compared to markers such as microsatellites which are known to have high mutation rate per generation (of the order of 10^{-4}), SNPs have relatively low mutation rates (between 10^{-8} and 10^{-9}). Thus, the occurrence of multiple mutations at a single locus is improbable and consequently most of SNPs are bi-allelic. This characteristic is a quality that makes easy high-throughput genotyping and diminishes frequent substitutions at a single site that would stun the population history (Brumfield et al., 2003). Comparative studies on the use of genetic markers like allozymes, nuclear SNPs, mitochondrial (mt) SNPs and microsatellites have demonstrated that nuclear SNPs are measured on the same mutational scale as mtSNPs rendering intergenomic comparisons studies easier (Allendorf and Seeb, 2000). Moreover, there are many tests looking at the deviations from neutrality, the population size changes and the recombination

presence, which exist for SNPs data than microsatellites, and the fit of models to data is probably better in the case of SNPs.

In summary, by mapping SNPs all over the genome, it is possible to discover novel antigens that may be vaccine candidates, or other functionally important genes (under selection).

1.3.4. Methods and approaches to detect positive natural selection

There are diverse approaches (based on the polymorphisms) for inferring natural positive selection in malaria literature. The first group of tests of positive selection are based on polymorphisms within species: Tajima's D (TD) [a statistic which measures the difference between two estimators of the population mutation rate: θ_w and π (Tajima, 1989)]; Fu and Li's D (FLD) and F (FLF) [two tests similar to TD because they test for a skew in the allele frequency spectrum. These tests make the distinction between old and recent mutations as determined by where they occur on the branches of genealogies. The FLD and FLF statistics compare an estimate of the population mutation rate based on the number of derived variants seen only once in a sample (referred to as singletons) with θ_w or π, respectively (Fu and Li, 1993)]; Fay and Wu's H (FWH) test [a statistic that detects the presence of an excess of high frequency derived alleles in a sample, which is a hallmark of positive selection (Fay and Wu, 2000)]; Linkage disequilibrium (LD) test [LD is defined as a non-random association between particular haplotypes or alleles. Some of the most powerful tests for detecting recent positive selection are based on the levels of

LD. An allele highly prevalent in a given population is likely to be a target of positive selection. Strong positive selection is expected to accelerate the frequency of the advantageous allele, with the result that linked loci remain in remarkably strong LD with that allele (Slatkin, 2008). Therefore, the evidence of positive selection decreases the genetic variation, but leads to the increase in LD]; F_{ST} [this is a statistic test which quantifies levels of differentiation between subpopulations (Weir and Cockerhan, 1984). It is conceptually based on the estimation of heterozygosity [(HT-HS)/HT with HT representing an estimate of the total heterozygosity and HS a measure of the average heterozygosity across subpopulations].

The second group of tests is based on polymorphisms within species and the divergence between species. The neutral theory usually predicts a positive correlation between levels of polymorphism within species and divergence between species (Hudson et al., 1987). In this group, there are tests such as: Hudson–Kreitman–Aguade test (used to determine if levels of nucleotide variation within and between species at two or more loci conform to the expectation of neutral theory as explained by Hudson et al., 1987); McDonald Kreitman (MK) test [which compares the ratio of synonymous versus non-synonymous mutations within and between species (McDonald and Kreitman, 1991)].

The third group is composed of the tests based on polymorphisms between species. There are also called as “dN/dS tests”. The tests based on the

estimation of dN/dS ratio are mostly applied in protein coding regions and they have been proved to be the strongest ones for detecting signature of positive natural selection on these regions of the genomes. In the simplest forms, the ratio of non-synonymous (dN) to synonymous (dS) substitutions is compared in protein coding loci (Nei and Gojobori, 1986; Nielsen and Yang, 1998; Suzuki and Gojobori, 1999). The dN/dS ratio delivers information about the evolutionary forces operating on a particular gene. For example, under neutrality it is expected to have dN/dS=1. For genes that are subject to functional constraint such that non-synonymous amino acid substitutions are deleterious and removed from the population, dN/dS<1. For positively selected genes, dN/dS>1. Although the observation of dN/dS>1 provides strong evidence for positive selection, it is conservative if only a few sites have been targets of adaptive evolution. The basic dN/dS test has been extended to include models of codon and transition and/or transversion bias, to detect variation in dN/dS ratios among lineages and to identify specific sites that might be under selection.

1.3.5. Key determinants of *P. falciparum*-antimalarial drugs resistance

A feature of antimalarial resistance in *P. falciparum* is the inverse correlation observed between resistance to CQ and resistance to arylaminoalcohols and endoperoxides observed in several populations. Additionally cross-resistance has repeatedly been observed between the structurally unrelated

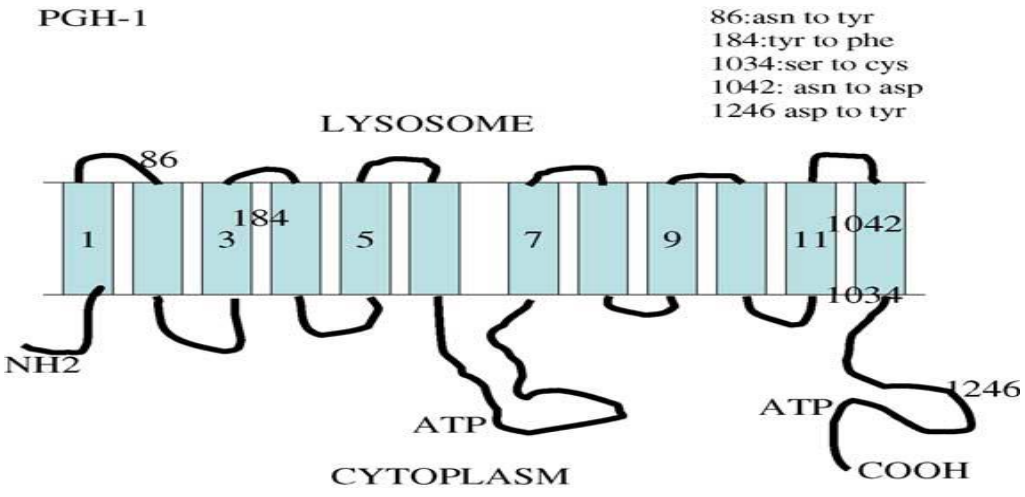
arylaminoalcohol and the endoperoxide drugs suggesting a true multidrug resistance-like mechanism.

i. The *pfmdr1* gene in *P. falciparum*

The story with the utilization of *pfmdr1* gene as a target of CQR starts with cancer cells, where drug resistance frequently arises from the presence of an over-expressed P-glycoprotein, an ABC transporter that is capable of actively expelling a wide range of structurally and functionally diverse chemotherapeutic agents in a verapamil-sensitive manner (Higgins, 2007). Inspired then by the apparent phenotypic similarities between multi-drug resistance in cancer cells and CQR in *P. falciparum*, it was then thought that a *P. falciparum* homologue of the mammalian P-glycoprotein, later termed as *pfmdr-1*or *Pgh-1*, might be the major candidate molecule for conferring resistance to CQ (Foote et al., 1989, 1990; Wilson et al., 1989; Sanchez et al., 2010). In fact, multidrug resistance arises when cells selected for resistance to one agent, become resistant to a wide range of structurally unrelated drugs (Juliano and Ling, 1976). The foremost protein mediating this process in several mammalian cell-lines is known to be the multidrug-resistance (*mdr*) transporter or P-glycoprotein. Thus, *pfmdr1*, a gene located on chromosome 5 of the *P. falciparum* genome and which codes for the plasmodial homologue of mammalian *mdr* genes in *P. falciparum*, was cloned and sequenced (Foote et al., 1989). It was found to be a member of the ABC transporter superfamily and has been named Pgh1 (P-glycoprotein homolog 1) (Cowman et al., 1991;

Higgins, 1992). Furthermore, presence of Pgh1 was noticed inside the parasite digestive vacuole during its asexual cycle where it is hypothesized that Pgh1 regulates intracellular drug concentrations (Cowman et al., 1991). During the development of mammalian multidrug resistance, both gene amplification and mutation of the *mdr* transporter genes has been shown to occur. Similarly with the *pfmdr1* gene there are two ways by which increased levels of drug-resistance can be conferred: *via* gene amplification and *via* mutation (Foote et al., 1990; Foote et al., 1989) but inheritance of point mutations compared to gene amplification is likely to be more efficient for transmitting a resistance genotype through a population (Foote et al., 1990). After sequencing *pfmdr1* gene of a series of laboratory strains from different geographical areas, polymorphism was observed at five loci at the codon level: 86th, 184th, 1034th, 1042nd and 1246th positions (Foote et al., 1990; Figure 1.6).

Figure 1.6. The Pgh1 protein of *P. falciparum* with the polymorphic amino acids indicated



Source: Duraisingh and Cowman, 2005

Several studies have been conducted in order to find out the role played by the *pfmdr1* gene in CQR as well as other antimalarial drugs resistance and the results are still controversial. Some studies (*in vitro* and *in vivo*) depict *pfmdr1* gene to be strongly correlated with the CQR phenotype. The point mutations in the gene mostly selected are those sitting at the amino acids positions 86 and 184. However, the 86th point mutation appears to be more selected for CQR, especially in African countries (Foote et al., 1990; Basco et al., 1995; Adagu et al., 1996; Duraisingh et al., 1997; Duraisingh and Cowman, 2005; Gupta et al., 2010b). Moreover, in some West-African countries *e.g.* the Gambia, this 86th mutation has shown to be selected not only for CQR but also for its structurally sister AQ (Duraisingh et al., 1997). This situation highlights the importance to pursue investigations on the genetic polymorphism of the *pfmdr1* gene since amodiaquine is highly used in Africa (the most malaria endemic part of the globe) as a partner for the ACTs and since it is well known that a self-medication is highly followed in the continent making the use of CQ in a non-official manner. Additionally, it seems that there is a kind of cross relationship between CQR, mefloquine-resistance and endoperoxide artemisinin derivatives-resistance in most populations including those in sub-Saharan Africa: *pfmdr1* deamplification and mutations (particularly the 86th mutation) are selected for in CQ-resistance parasites, concomitantly conferring on them a reduced sensitivity to arylaminoalcohols (mefloquine and halofantrine) and endoperoxide drugs (Duraisingh and Cowman, 2005). Others studies have also failed to find an association between the *pfmdr1* alleles and

antimalarial drugs resistance (*e.g.* (Basco and Ringwald, 1997; Pillai et al., 2001). Whatever the case might be, it is seems clear that *pfmdr1* gene plays a modulator role in the phenomenon of chloroquine-resistance but its genetic background differs geographically. In contrary, it seems to have a primary role in the development of mefloquine-resistance in regions like bordering areas of Thailand, Myanmar where this antimalarial drug is extensively used (Duraisingh and Cowman, 2005). It becomes very vital to understand the mechanism along with the parameters involved that lead to that variability of the utility of *pfmdr1* alleles as molecular markers of antimalarials resistance in different epidemiological settings. With this respect, approaches like evolutionary genetics are quite novel and so far non-explored yet in Cameroon.

ii. The *pfcrt* gene in *P. falciparum*

The PFCRT defined as *Plasmodium falciparum* chloroquine resistance transporter is a protein located at the membrane of the parasite's digestive vacuole (Fidock et al., 2000). The exact function of *pfcrt* still remains unknown; however, the transmembrane structure it possesses along with its cellular localization suggest that it might be involved in the transport of metabolites such as drugs and it has the ability to maintain the pH balance in the digestive vacuole of *P. falciparum* (Ecker et al. 2012; Awasthi and Das, 2013). Furthermore, it has been demonstrated that *pfcrt* plays a great role in the expulsion of amino acids from hemoglobin digestion from the DV and indirectly it might maintain the H^+ balance in the DV (Jiang et al., 2008). Also,

phylogenetic analyses have confirmed *pfcrt* to be a member of the drug/metabolite transporter superfamily supporting therefore its numerous roles in *P. falciparum* (Martin and Kirk, 2004). In fact, it is thought that this protein is responsible of the efflux of some drugs (in particular CQ) out of the DV, away from the molecular target which is the heme detoxification pathway (Ecker et al. 2012; Sanchez et al., 2010). Quinoline drugs other than CQ may have a complex mode of action and may target pathways in addition to heme detoxification (Ecker et al. 2012). The identification of *pfcrt* gene [localized on the 7th chromosome of *P. falciparum*; This gene spanning approximately 3.1 kb has 13 exons ranging in size of 45 to 269 bp (Awasthi and Das, 2013) and encodes for a 424 amino acids protein having 10 transmembrane domains (Fidock et al., 2000)] and its relevant role in the occurrence of CQR has been confirmed after several clinical and epidemiological studies (Wellems et al., 1991; Fidock et al., 2000). These studies followed genetic crosses between CQR and CQS strains. In particular, genetic loci on chromosome 5 (*pfmdr1* gene) and chromosome 13 (*pfnhe1* gene meaning sodium/hydrogen exchanger, N^+/H-antiporter in *P. falciparum*) have been proposed to be associated with higher IC50 values in the offspring derived from the genetic crosses (Wellems et al., 1991; Ferdig et al., 2004; Awasthi et al., 2013). Unfortunately, mutations in none of these genes have not been directly correlated with CQR (Cooper et al., 2002; Hayton and Su, 2004; 2008). Although mutations in the *pfmdr1* were somehow found to be associated with CQR (see above), but their exact contribution in modulating CQR remains controversial (Hayton and Su, 2004;

2008). Later on, after a genetic crossing between clones CQR Dd2 (from Indochina) and CQS HB3 (from Honduras), it has been established that a single locus was determinant for the CQ sensitivity (Wellems et al. 1991, Fidock et al. 2000). Further evidence establishing the *pfcrt* gene as the key determinant of CQR came from studies of culture-adapted field isolates (Wootton et al., 2002). Besides, *pfcrt* has been demonstrated to be a transporter for some quinoline derivatives, with a preference for 4-aminoquinolines (CQ, AQ) and arylaminoalcohols (quinine, quinidine, mefloquine and halofantrine) (Sanchez et al., 2010). Thus, mutations within the *pfcrt* gene have not only been linked with CQR, but also with responses to others antimalarial drugs such as quinine, quinidine (Cooper et al., 2002, 2007; Sanchez et al., 2010), AQ, halofantrine and possibly mefloquine (Sidhu et al., 2002; Lakshmanan et al., 2005; Sa et al., 2009; Sanchez et al., 2010). Shown to be a highly polymorphic protein (4 to 10 amino acid substitutions), different variants of *pfcrt* have independently arisen in numerous geographic foci, possibly due to the local drug pressures acting on the parasites (Wellems et al., 2009; Summers et al., 2012). Moreover, it was found that some CQR strains were also displaying resistance to the CQ analog, AQ, a drug of a great interest since it is a partner of one of the recently introduced combination therapy regimes, together with artemisinin or one of its analogs. Many African *P. falciparum* strains (including those from Cameroon) are still sensitive to, or only moderately resistant to AQ while being highly resistant to CQ (Sa et al., 2009; Sanchez et al., 2010). Among the mutations that *pfcrt* gene harbors, there is

one substitution at position 76 (lysine to threonine substitution) in the second exon of the gene which is crucial for *pfcrt*-mediated drug responses. Once *pfcrt* is mutated at that position, the transport of CQ is nullified (Lakshmanan et al., 2005). The high levels of resistance to AQ have been strongly correlated to the high prevalence of SVMNT polymorphism within *pfcrt* at codons 72nd to 76th as found in the south American *P. falciparum* strain "7G8" (Sa et al., 2009; Sanchez et al., 2010). The PFCRT protein thus seems to be a carrier for multiple antimalarial drugs and some researchers recently have hypothesized that it is possible that in the nearby future, this gene may evolve and acquire a multi-drug resistance capability and if so, this will render the entire famous class of antimalarial drugs inefficient. This is because mainly several of the antimalarial drugs that are currently used as first line treatment in combination with artemisinin reveal a response association with the *pfcrt* gene (Ecker et al., 2012), Moreover, it also seems that the 76th mutation historically described as the crucial key for CQR might not be the case or some additional/compensatory mutations have taken the trophy. Whatever the case might be, continuous investigation on the real function of the *pfcrt* gene (along with its mutations) is needed at every malaria endemic setting (including Cameroon).

iii. Interactions between the *pfcrt* and *pfmdr1* genes

Both the genes *pfcrt* and *pfmdr1* encode proteins of the parasite's digestive vacuolar membrane and their mutations are believed to confer resistance to

quinolines by preventing these drugs from accumulating in the digestive vacuole to the concentrations required to inhibit endogenous heme detoxification processes (Egan and Ncokazi, 2005). Interestingly, *pfcrt* and *pfmdr1* seem to facilitate drug movement in opposite directions (Sanchez et al., 2010). The *pfcrt*, when mutated, seems to function as a carrier (Summers and Martin, 2010) or as a voltage gated channel of drugs (Paguio et al., 2009), facilitating efflux of these drugs from the digestive vacuole. Whereas *pfmdr1* seems to act as a drug importer, with polymorphic amino acid substitutions reducing its ability to transport a given drug and therefore, to concentrate it in the DV (Sanchez et al., 2008a). LD has been observed in several studies between alleles of *pfmdr1* (particularly mutations at positions 86th and 184th) and the chromosome 7 locus including the major CQR gene *pfcrt* (Adagu and Warhurst, 2001; Gupta et al., 2010), despite their presence on different chromosomes, suggesting a strong interaction between these genes. This LD is probably due to high selection pressure by CQ. To briefly conclude, combination of *pfcrt* and *pfmdr1* polymorphisms together result in higher levels of CQR as mentioned by several researchers (see above).

How the *pfcrt* and *pfmdr1* genes interact together to bring a resistance phenotype is only partially understood. Understanding their rapport has been complicated by the fact that various *pfcrt* and *pfmdr1* alleles have emerged in *P. falciparum* strains in different geographic sites. However, these different polymorphic forms are the result of independent founding events and seem to reflect distinct solutions to regional histories of drug use and hence drug

selection (Sa et al., 2009; Wellems et al., 2009). For example, the South American *pfcrt* and *pfmdr1* alleles have arisen in an area where amodiaquine use has preceded that of CQ (Sa et al., 2009).

With a view to understand the complexities in malaria infection dynamics in Cameroon and to understand evolutionary genetic patterns of two drug resistance genes (which is not yet studied), the present work has been initiated.

CHAPTER 2:

MATERIALS AND METHODS

2. MATERIAL AND METHODS

The present study aims: to perform molecular diagnosis of malaria infection in Cameroon; to understand genetic diversity of two drug resistant genes (*pfcrt* and *pfmdr1*) in Cameroonian *P. falciparum*; and to analyse the DNA sequences and infer evolutionary patterns of these two genes in Cameroonian *P. falciparum.* In order to achieve these objectives, several materials and methods have been used a0nd performed. Furthermore, numerous statistical approaches have been conducted for the analysis of generated data.

2.1. Material

2.1.1. Ethical clearance

This study has been cleared by the Ethical Committee of Cameroon (*No003/CNE/SE/2012*) and written informed consents were obtained from all adult patients and the guardians of the minor patients.

2.1.2. Study areas and samples collection

Finger-prick blood samples were collected from 545 symptomatic patients attending six different district hospitals located (Table 2.1) in five distinct areas from the southern part of the country: Ebolowa, Douala, Bertoua, Yaounde and Kye-ossi in the border of Cameroon, Gabon and Equatorial Guinea on whatman paper as form of 4 or 5 spots (see Figures 2.1 and 2.2 below).

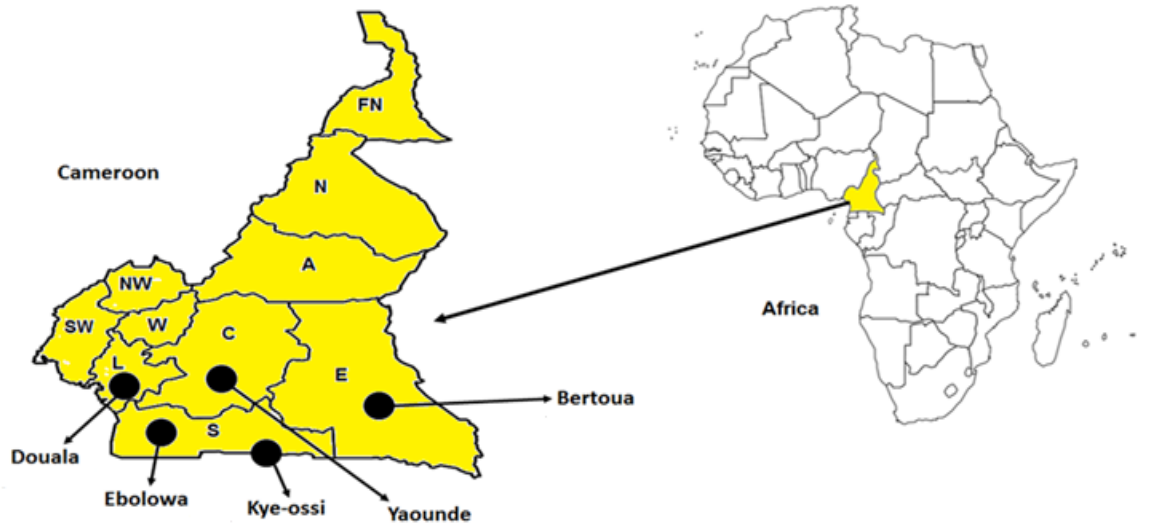
Table 2.1. Details of sampled areas

Regions	City	Geographic coordinates	District hospital	Nº of patients recruited
South	Ebolowa	2°55′N 11°9′E	EPC Enongal hospital	91
Littoral	Douala	3°48'N 10°08'E	Bossama hospital	111
			New-Bell hospital	60
East	Bertoua	4°35′N 13°41′E	Bertoua hospital	101
Centre	Yaounde	3°51'N 11°30'E	Cité-verte hospital	93
Border Cam-EG_Gab	Kye-ossi	2°15' N11°15'E	Kye-ossi hospital	89

°: Degree **Cam:** Cameroon **N:** North **EG:** Equatorial Guinea

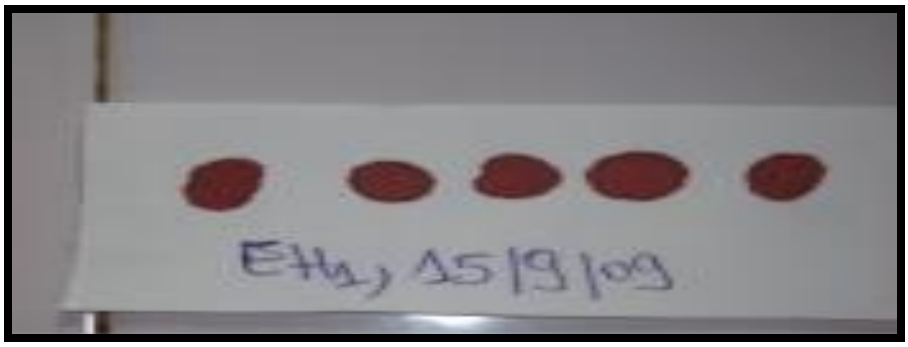
E: East **Gab:** Gabon **Nº:** Number

Figure 2.1. Maps of Africa and Cameroon with the different areas of samples collection



FN: Far North; **N:** North; **A:** Adamaoua; **C:** Centre; **NW:** North-West; **W:** West; **SW:** South-West; **L:** Littoral; **S:** South; **E:** East;

Figure 2.2. Photo picture of a finger-prick blood sample collected



2.2. Methods

2.2.1. Parasite genomic DNA extraction

Genomic parasite DNA was extracted from the blood spots using Qiagen kit with the following protocol:

1- Punched out circles (done with a single-hole paper punch) from a dried blood spot were placed into a 1.5 ml micro-centrifuge tube, then minimum 180 µl of ATL buffer was added and samples were incubated at room temperature overnight.

Note: It was made sure that the punches were fully covered with the buffer

2- Then, tubes were incubated at 85°C for 10 minutes on the next day and briefly centrifuged to remove drops from inside the lid.

3- 20 µl of proteinase K solution (with a concentration of approximately 20mg/ml) was added, the sample mixed by vortexing, briefly centrifuged and

tubes incubated at 56°C for one hour. After incubation, they were briefly centrifuged.

4- 200 µl of AL buffer were added to the samples, mixed thoroughly by vortexing, briefly centrifuged and then incubated at 70°C for 10 minutes. After incubation, they were briefly centrifuged.

5- 200 µl Ethanol (96-100%) was added to the samples, then mixed thoroughly by vortexing and briefly centrifuged.

6- The mixture was carefully applied from slips to QiaAmp spin column (which is present in a 2 ml collection tube) without wetting the rim. Then the cap was closed and samples centrifuged at 8000 rpm (6000 x g) for 1 min.

7- The QiaAmp S.C (spin column) was then placed in a new 2 ml collection tube and the tube containing the flow-through was discarded.

8- The QiaAmp spin column was opened carefully and 500 µl AW1 buffer (prepared already before by adding the indicated volume of 96-100% ethanol) added without wetting the rim. The cap was closed and then samples centrifuged at full speed (8000 rpm for 1 minute).

9- Again the QiaAmp S.C (spin column) was placed in a new 2 ml collection tube and the tube containing the flow-through discarded.

10- Then the QiaAmp spin column was carefully opened and 500 µl AW2 buffer (prepared already before by adding the indicated volume of 96-100% ethanol) added without wetting the rim. The cap was closed and samples centrifuged at

full speed (14000 rpm for 3 minutes). This step was repeated once again but for 1 minute only.

11- The QiaAmp spin column was placed in a clean 1.5 ml micro centrifuge tube and the tube containing the flow-through discarded.

12- The QiaAmp column was carefully opened and then 50 µl of AE buffer was firstly added. The columns were incubated at room temperature for 5 min and then centrifuged at 8000 rpm for 1 min.

13- Again 25-50 µl of AE buffer was added. The columns were incubated at room limp for 1 min and then centrifuged at 8000 rpm for 1 min.

14- After the DNA elution, the columns were discarded; the parasite DNA isolated was aliquoted and saved at - 20°C for further usage.

2.2.2. Molecular diagnosis of malaria infection

2.2.2.1. Detection and identification of *Plasmodium* species

i) PCR diagnostic assay

After extraction of the parasite genomic DNA, the presence as well as the type of malaria parasites was detected using PCR diagnostic assays (highly sensitive compared to the most common technique used: Microscopy) as described in several previous studies (Snounou et al., 1993; Johnston et al., 2006; Gupta et al., 2010a). For each isolate, a nested PCR was performed using published primers sets and programs with very less modification (details of the primers

sequences as well as the PCR programs and protocol are provided into Tables 2.2, 2.3, 2.4, 2.5 and 2.6 below) to amplify the 18S region of the ribosomal RNA for each species (*P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale*).

Table 2.2. Primers sequences used for the PCR assay diagnostic

Primers Name	**Sequences**	**Product Size**
1st Step primer forward	5'-CCTGTTGTTGCCTTAAACTTC-3'	1100 bp
1st Step primer reverse	5'-TTAAAATTGTTGCAGTTAAAACG-3'	
Fal 1 Forward	5'-TTAAACTGGTTTGGGAAAACCAAATATATT-3'	205 bp
Fal 1 Reverse	5'-ACACAATGAACTCAATCATGACTACCCGTC-3'	
Viv 1 Forward	5'-CGCTTCTAGCTTAATCCACATAACTGATAC-3'	120 bp
Viv 1 Reverse	5'-ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA-3'	
Mal 1 Forward	5'-ATAACATAGTTGTACGTTAAGAATAACCGC-3'	144 bp
Mal 1 Reverse	5'-AAAATTCCCATGCATAAAAAATTATACAAA-3'	
Ova 1 Forward	5'-ATCTCTTTTGCTATTTTTTAGTATTGGAGA-3'	800 bp
Ova 1 Reverse	5'-GGAAAAGGACACATTAATTGTATCCTAGTG-3'	

Fal 1: for the species *falciparum*

Viv1: for the species *vivax*

Mal 1: for the species *malariae*

Ova 1: for the species *ovale*

bp: bases pairs

Table 2.3. Step 1 (for *P. falciparum*, *P. vivax* and *P. malariae*)

Ingredients	1 tube	**Cycling conditions:**
Buffer	2.5 µl	Initial denaturation: 94°C for 5 minutes Start cycle: 30 cycles Denaturation: 94°C for 1 minute Annealing: 60°C for 2 minutes Extension: 72°C for 2 minutes End cycle Final extension: 72°C for 10 minutes Store: 4°C
dNTPs	0.5 µl	
25µM primer Forward	0.4 µl	
25µM primer Reverse	0.4 µl	
DNA template	1 µl	
Taq Polymerase	0.33 µl	
Water	19.87 µl	
Total	25 µl	

Table 2.4. Step 1 (*P. ovale*)

Ingredients	**1 tube**	**Cycling conditions:**
Buffer	2.5 µl	Initial denaturation: 94°C for 5 minutes Start cycle: 30 cycles Denaturation: 94°C for 30 seconds Annealing: 45°C for 30 seconds Extension: 72°C for 1 minute 30 seconds End cycle Final extension: 72°C for 10 minutes Store: 4°C
dNTPs	0.5 µl	
25µM primer Forward	0.4 µl	
25µM primer Reverse	0.4 µl	
DNA template	1 µl	
Taq Polymerase	0.33 µl	
Water	19.87 µl	
Total	25 µl	

Table 2.5. Step 2 (for *P. falciparum*, *P. vivax* and *P. malariae*)

Ingredients	1 tube	**Cycling conditions:**
Buffer	2.5 µl	Initial denaturation: 94°C for 5 min. Start cycle: 30 cycles Denaturation: 94°C for 1 minute Annealing: 55°C for 2 minutes Extension: 72°C for 2 minutes End cycle Final extension: 72°C for 10 minutes Store: 4°C
dNTPs	0.5 µl	
PCR product of the 1st step	3 µl	
25µM primer (Pf) Forward	0.4 µl	
25µM primer (Pf) Reverse	0.4 µl	
25µM primer (Pv) Forward	0.4 µl	
25µM primer (Pv) Reverse	0.4 µl	
25µM primer (Pm) Forward	0.4 µl	
25µM primer (Pm) Reverse	0.4 µl	
Taq Polymerase	0.33 µl	
Water	16.27 µl	
Total	25 µl	

Table 2.6. Step 2 (*P. ovale*)

Ingredients	1 tube	**Cycling conditions:**
Buffer	2.5 µl	Initial denaturation: 94°C for 5 minutes Start cycle: 45 cycles Denaturation: 94°C for 30 seconds Annealing: 45°C for 30 seconds Extension: 72°C for 1 minute 30 seconds End cycle Final extension: 72°C for 10 minutes Store: 4°C
dNTPs	0.5 µl	
PCR product of the 1st step	3 µl	
25µM primer (Po) Forward	0.4 µl	
25µM primer (Po) Reverse	0.4 µl	
Taq Polymerase	0.33 µl	
Water	17.87 µl	
Total	25 µl	

ii) Visualization of PCR products through agarose gel electrophoresis

After amplification by PCR, the different PCR products obtained were run on an agarose gel 2%). The gel was stained with ethidium bromide. The DNA isolation (from a separate blood spot) and PCR amplification processes were repeated two more times for reconfirmation in samples presenting infections of *P. vivax* following similar protocol as described above.

2.2.2.2. Validation of results of the PCR assays

i) Purification of the PCR products and sequencing of the 18S region of the rRNA gene

In order to sequence and determine sequence identity/variations in the respective 18S rRNA genes of the Cameroonians malaria parasites with the respective reference sequences, the respective PCR products were first purified with Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences) using the PCR thermal cycler (37^{o}C for 1 hour and then 85^{o}C for 15 minutes). The purified PCR products were then processed for DNA sequencing reactions with Big Dye Terminator as per standard protocol of the Applied Biosystems (ABI). The products were then run in the ABI 3730 XL DNA analyzer (in-house facilities of NIMR, New Delhi). The successful PCR products were purified with the use of Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences). In 10 μl of the PCR product, 1 microliter of the master mix (0.025 µl of exonuclease + 0.5 µl of shrimp alkaline phosphatase + 0.1 µl of buffer + 0.375 µl of water) was added. The mixture was then briefly

centrifuged and carried on a thermocycler with the following program: 37°C for 1 hour and 85°C for 15 min.

For sequencing reactions approximately 2 - 4 μl of the purified PCR product, 5 μl of big dye terminator (BDT) ready reaction mix, and 0.8 pmoles of primer were used. The cycle sequencing was performed in a thermal cycler as follows: initial denaturation at 95°C for five minutes, followed by 25 cycles of final denaturation at 95°C for 10 seconds, annealing at 50°C for five seconds and extension at 60°C for four minutes. The probes were then transferred to a 96-well plate and DNA sequencing was performed after the cleaning of the sequencing plates (as described below in the next paragraph) on a 3730XL DNA analyzer (Applied Biosystem), an in-house facility of NIMR**.** Each fragment was sequenced in both the forward and reverse directions (2X coverage), assembled and edited using the SeqMan computer program (DNASTAR, Madison, WI, USA). Homologous DNA fragments were then aligned using the MegAlign program of DNASTAR following the ClustalW algorithm.

ii) Cleaning of the 96 well sequencing plates

For sequencing of the probes in the automated DNA Sequencer, the cleaning of the sequencing reaction products was performed as follows:

Two types of master mix were prepared: master mix I (10µl nuclease-free water was mixed with 2µl of 125 mM EDTA per reaction) and master mix II (2 µl of 3M NaOAc (pH 4.6) was mixed with 50µl of ethanol per reaction). The final volume of 12µl of master mix I was added to each well of 96 well sequencing

plate containing already 10µl of the sequencing reaction mixture and the contents were mixed properly. 52 µl of master mix II was added to each well of sequencing plate. The plate was sealed with adhesive cover and the contents were mixed by inverting the plates 3 times. The sequencing plate was incubated at room temperature for 15 minutes before be centrifuged at a speed of 3000 x g for 30 minutes. The supernatant was decanted by inverting the plate on the paper towels and the plate in inverted condition was shortly centrifuged up to 180 x g to remove residual supernatant. Subsequently, 100µl of 70% ethanol was added, the plate was sealed and spinned at 3000 x g for 5 minutes at room temperature. The supernatant was decanted again on the paper towels and residuals were removed by inverting the plate. The step with ethanol addition and residual removal was repeated two more times in order to complete clean up procedure. At the end, 10µl of Hi-Di formamide was added and the plate was spinned, denatured, snap-chilled and proceeded for DNA sequencing from both the directions, *i.e.* forward and reverse (2X coverage) in an automated DNA analyzer, ABI 3730XL (Applied Biosystems), an in-house facility of NIMR.

iii) BlastN search and multiple DNA sequence alignments

For each DNA fragment, sequencing was performed from both the 3' and 5' directions (2X coverage). For each individual (parasites alike), separate contigs were formed with the two sequences from both the directions (2X coverage) using the SeqMan module of the DNASTAR (Maddison, USA) computer

program. The different sequences of *P. vivax* cases found (mono and mixed parasite infections) as well as the sequences of *P. falciparum* for the mixed parasite infections have been blasted in GenBank in order to find out if there are some similarities with the published sequences of those two species worldwide. Sequences having between 95%-99% similarity with the queries sequences have been then retrieved for further analysis (see below).

Multiple sequences of each homologous gene of the malaria parasites (18S rRNA gene in both, *P. falciparum* and *P. vivax*) were aligned using the MegAlign module of the DNAStar (Madison, USA) computer program to ascertain similarities and differences with the respective reference sequences. For example, the respective 18S rRNA gene sequences of *P. vivax* and *P. falciparum* were aligned with the reference sequences of *P. vivax* 18S rRNA gene of the SAL-1 strain (accession number U03079.1) and with the reference sequence of *P. falciparum* 18S rRNA gene of the 3D7 isolate (accession number AL844501), independently. All the references sequences (18S rRNA genes of *P. vivax* and *P. falciparum*) were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/) using the BLAST search.

iii) Phylogenetic analysis of the *P. vivax* sequences data

In order to infer genetic inter-relationships of the Cameroonian *P. vivax* with the global isolates at this species-specific 18S rRNA gene a neighbor-joining (NJ) phylogenetic tree , was constructed using MEGA software version 4.0 (Tamura et al., 2007).

2.2.2.3. PCR amplification, sequencing and multiple DNA sequences alignment of additional genes for *Plasmodium* species identification

In order to verify the PCR detection of *P. vivax* infection in Cameroonians based on the 18S rRNA gene, two other genes of *P. vivax*: *pvmdr1* and *pvcsp* were additionally PCR amplified. Whereas the *pvmdr1* gene is associated with resistance to multiple antimalarial drugs (*pvmdr1*), the *pvcsp* is a highly polymorphic gene, commonly used in the characterization of *P. vivax* populations for different strain variants (VK 210, VK 247 and *P. vivax*-like) circulating around the world (Qari et al., 1993; Imwong et al., 2005). The published primers (Lekweiry et al., 2012) of the *pvmdr1* gene amplify a 543 bp DNA fragment and the primers for the *pvcsp* gene amplify a 507 bp product. The details of the PCR conditions of both the genes are presented in Table 6. Similarly, in order to verify the presence of *P. falciparum* in the mixed infections (*P. vivax* + *P. falciparum*) as detected with PCR diagnostic assay with the 18S rRNA gene (Snounou et al., 1993; Johnston et al., 2006 see above), we PCR amplified a 510 bp DNA fragment of the *pfmdr1* gene by nested PCR using the published primers (Basco et al., 2002; Vathsala et al., 2004).

Multiple sequences of each homologous gene of the malaria parasites (*pvmdr1*, *pvcsp* and *pfmdr1*) were aligned using the MegAlign module of the DNAStar (Madison, USA) computer program to ascertain similarities and differences with the respective reference sequences. For example, the *pvmdr1* and *pfmdr1* genes were aligned with the respective reference strains of *P. vivax* (SAL-1 strain,

accession number XM_001613678) and *P. falciparum* (3D7 strain, accession number AL844504). Similarly, the *pvcsp* sequences generated in the present study were aligned with the reference sequence GU339059.1 (*pvcsp* SAL-1 strain). All the references sequences were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/) using the BLAST search.

2.2.2.4. Sequencing analysis of the promoter region of the *Duffy* gene

Since *P. vivax* is reported to be infecting *Duffy*-negative Africans (Ryan et al., 2006; Mendes et al., 2011; Wurtz et al., 2011; Woldearegai et al., 2013) with an interest to know if the *P. vivax*-infected Cameroonians are also *Duffy*-negative.

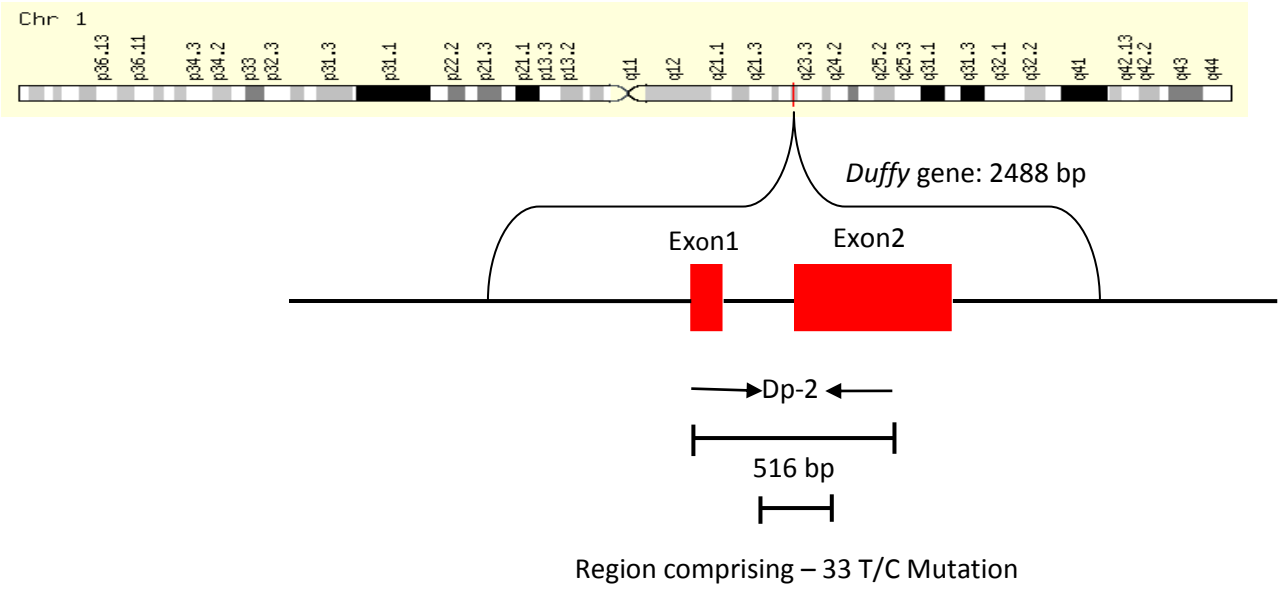
i) Human genomic DNA isolation

Human DNA has been isolated following the same process as for the isolation of genomic parasite DNA. Therefore, the isolated DNA from samples presenting malaria parasites (which also contain human genomic DNA) was used for PCR amplification of the promoter region of the human *Duffy* gene.

ii) Primers designing

We have used published primers pair sequences (Chittoria et al., 2012) to amplify a region of 516 bp of the promoter region (Figure 2.3) covering the mutation characteristic of the *Duffy* negativity of Africans (- 33 T/C mutation).

Figure 2.3. Part of the promoter region of *Duffy* gene sequenced



iii) PCR amplification of the promoter region of the *Duffy* gene

The promoter region has been PCR amplified following the program: initial denaturation at 95°C for 5 min; 35 cycles (denaturation at 95°C for 30 sec; annealing at 64°C for 30 sec; elongation at 72°C for 30 sec); final elongation: 72°C for 5 min. We have followed this approach because, it is well known that DNA sequencing of the promoter region of the human *Duffy* gene with respect to the -33rd nucleotide mutation can adeptly determine the *Duffy*-status of humans (Hamblin and Di-Rienzo, 2000; Mendes et al., 2011). This approach relies on the fact that finding the C homozygotes (C in both the sequences) at the -33rd position specifies complete *Duffy*-negative status (meaning no expression of the *Duffy* protein on the erythrocytes) (Tournamille et al., 1995). The details of the PCR protocols are provided in the Table 2.7.

Table 2.7. PCR cycling conditions used for the *pvmdr1*, *pfmdr1*, *pvcsp* and *Duffy* genes

	Reactions	Cycling conditions	Amplicons size (bp)
pvmdr1	1st PCR	Initial denaturation: 94°C for 5 minutes; Start cycle: 45 cycles (Denaturation: 94°C for 50 seconds; Annealing: 53°C for 1 minute; Extension: 72°C for 1 minute) End cycle; Final extension: 72°C for 10 minutes	543
	2nd PCR	Initial denaturation: 94°C for 5 minutes; Start cycle: 45 cycles (Denaturation: 94°C for 50 seconds; Annealing: gradient temperature from 55°C to 65°C for 1 minute; Extension: 72°C for 1 minute) End cycle; Final extension: 72°C for 10 minutes	
pfmdr1	1st PCR	Initial denaturation: 95°C for 5 minutes; Start cycle: 35 cycles (Denaturation: 95°C for 50 seconds; Annealing: 58.8°C for 1minute; Extension: 72°C for 1 minute) End cycle; Final extension: 72°C for 5 minutes	510
	2nd PCR	Initial denaturation: 95°C for 5 minutes; Start cycle: 35 cycles (Denaturation: 95°C for 50 seconds; Annealing: 58.8°C for 1minute; Extension: 72°C for 1 minute) End cycle; Final extension: 72°C for 5 minutes	
pvcsp		Initial denaturation: 95°C for 5 minutes; Start cycle: 35 cycles (Denaturation: 95°C for 1 minute; Annealing: 61.3°C for 1minute; Extension: 72°C for 1 minute) End cycle; Final extension: 72°C for 10 minutes	507
Duffy		Initial denaturation: 95°C for 5 minutes; Start cycle: 35 cycles (Denaturation: 95°C for 30 seconds; Annealing: 64°C for 30 seconds; Extension: 72°C for 30 seconds) End cycle; Final extension: 72°C for 5 minutes	516

iv) Purification of the PCR products, sequencing and multiple DNA sequences alignment

The PCR products were purified using the Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences) and then processed for DNA sequencing following standard protocols of Applied Biosystems after the cleaning of the sequencing plate (as described above, see 2.2.2.2). Each DNA fragment was sequenced from both the directions (2X coverage) and the products were run in an ABI 3730 XL DNA analyzer (in-house facilities of NIMR). Sequences were assembled and edited using the SeqMan and EditSeq computer programs (DNAStar, Madison), respectively. All the sequences were aligned using the MegAlign computer program (DNAStar, Madison) and scanned for Single Nucleotide Polymorphisms (SNPs) present in the -33rd position of the promoter region.

Multiple sequences of the homologous of the human *Duffy* gene and Cameroonians (*Duffy*) were aligned using the MegAlign module of the DNAStar (Madison, USA) computer program to determine similarities and differences with the reference sequence. The human *Duffy* gene sequences generated in the present study were aligned with the reference sequence (accession numbers NG_011626.1) retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/) using the BLAST search.

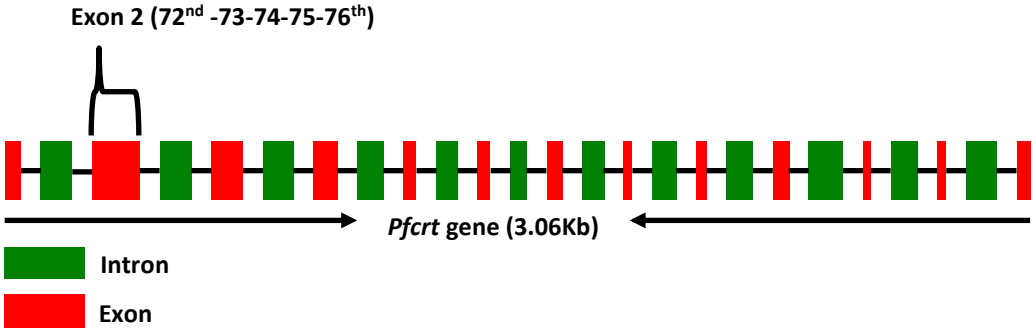
2.2.3. Genotypic characterization of the *P. falciparum* CQR genes in Cameroonian isolates: Molecular methods used and statistical analyses to infer evolutionary patterns

2.2.3.1. The *pfcrt* gene

i) Primer designing

The primers used were the published ones (Vathsala et al., 2004; Awasthi et al., 2011) for the amplification of 264 bp of the *pfcrt* gene covering the whole second exon (Figure 2.4.) and comprising the highly mutable region which is constituted of amino acids from 72nd to 76th position.

Figure 2.4. Schematic representation of the *pfcrt* gene and location of the exon 2



ii) PCR amplification of the second exon

For determination of the amino acid sequences of the *pfcrt* polypeptide chain, the entire second exon of the *pfcrt* gene was PCR amplified following standard protocols: initial denaturation at 95°C for 5 min; 35 cycles (denaturation at

95°C for 50 sec; annealing at 58.9°C for 1 min; elongation at 72°C for1 min); final elongation: 72°C for 5 min.

iii) Visualization of the PCR products and purification of the successful samples

The PCR products were then visualized on an Agarose gel prepared at 2% and stained with ethidium bromide. For the successful isolates, the PCR products were then purified using the Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences) in a thermal cycler at 37°C for 1 hour and 85°C for 15 min.

iv) DNA sequencing and multiple sequences alignment

Purified PCR products were processed for DNA sequencing following standard protocols of Applied Biosystems after the cleaning of the sequencing plate (as described above, see 2.2.2.2). For 10 µl DNA sequencing reaction, around 3 µl of the purified PCR product, 5 µl of the big dye terminator (BDT) ready master mix and 2 µl of the primer was used in a 96 wells plate and cycle sequencing was performed in a thermal cycler as follows: initial denaturation at 95°C for 5 min; 25 cycles (denaturation at 95°C for 10 sec; annealing at 50°C for 5 sec; elongation at 60°C for 4 min). Each DNA fragment was sequenced from both the directions, forward - reverse (2X coverage) and the products were run in an ABI 3730 XL DNA analyzer (in-house facilities of NIMR).

All the sequences were edited, aligned (following the ClustalW algorithm) using the SeqMan, EditSeq, MegAlign computer programs (DNAStar, Madison) for the further analysis of the sequences data.

2.2.3.2. The *pfmdr1* gene

i) Primer designing

The primers used were the published ones (Table 2.8. Basco and Ringwald, 1998; Das Sutar et al., 2011). For the two first populations, the first pair of primers (Mdr1forward/Mdr1 reverse) has successfully amplified the region of the *Pfmdr1* gene covering the 86th mutation strongly correlated with the phenomenon of CQR as reported in recent studies. For the populations from Bertoua, Yaounde, Kye-ossi and New-Bell (Douala) where parasitaemia was not that much high, a nested PCR was performed with the combination of the first pair and second pair (MdrG forward/MdrG reverse) of primers.

Table 2.8. Details of primers for PCR amplification of the *pfmdr1* gene

Primers Name	**Sequences**
Mdr1 forward	5'-ATGGGTAAAGAGCAGAAAGA-3'
Mdr1 reverse	5'-AACGCAAGTAATACATAAAGTCA-3'
MdrG forward	5'- AGAGAAAAAAGATGGTAACCTCAG-3'
MdrG reverse	5'- ACCACAAACATAAATTAACGG-3'

ii) PCR amplification of the *pfmdr1* fragments

For determination of the amino acid sequences of the *pfmdr1* polypeptide chain which has been targeted as correlated with the CQR phenomenon (covering the 86th mutation), a fragment of this gene was PCR amplified following standard protocols: initial denaturation at 95°C for 5 min; 35 cycles (denaturation at 95°C for 50 sec; annealing at 58.8°C for 1 min; elongation at 72°C for1 min); final elongation: 72°C for 5 min for some populations and individuals presenting a good parasitaemia. For the ones having less parasitaemia, a nested PCR (same protocol and same program for both of PCR reactions) was performed as well as with extra concentration of $MgCl_2$ added.

iii) Visualization of the PCR products and purification of the successful samples

The PCR products were then visualized on an Agarose gel prepared at 2% and stained with ethidium bromide. For the successful isolates, the PCR products were then purified using the Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences) in a thermal cycler at 37°C for 1 hour and 85°C for 15 min.

iv) Sequencing and multiple sequences alignment

Purified PCR products were processed for DNA sequencing following standard protocols of Applied Biosystems after the cleaning of the sequencing plate (as described above, see 2.2.2.2). Each DNA fragment was sequenced from both

the directions (2X coverage) and the products were run in an ABI 3730 XL DNA analyzer (in-house facilities of NIMR).

Cameroonian *pfmdr1* sequences generated were assembled and edited using the SeqMan and EditSeq computer programs (DNAStar, Madison), respectively. All the sequences were aligned using the MegAlign computer program (DNAStar, Madison) in order to further be scanned for Single Nucleotide Polymorphisms (SNPs) present in the total fragment of *pfmdr1* sequenced.

2.2.4. DNA sequences data analyses and evolutionary studies of the *pfcrt* and *pfmdr1* genes

Apart from diagnosing malaria infection in Cameroon on the basis of molecular approaches, this study also aimed to understand genetic diversity of two drug resistant genes (*pfcrt* and *pfmdr1*) in Cameroonian *P. falciparum* and to analyze the DNA sequences and infer evolutionary patterns of these two genes in Cameroonian *P. falciparum*. After alignment of the different Cameroonian *pfcrt* and *pfmdr1* sequences generated in the present study (see above), the sequences have been scanned for the presence of SNPs and the number of SNPs was determined. The *pfcrt* sequences were scanned for Single Nucleotide Polymorphisms (SNPs) not only present in the 72nd-76th amino acid positions to define different *pfcrt* haplotypes, but also for eventual detection of new mutations in the second exon. Similarly work has been done for the *pfmdr1* sequences with respect to the mutations sitting at the 86th and 184th positions.

2.2.4.1. Estimation of genetic diversity

The genetic diversities of the both genes have been estimated using the computer program MEGA version 5.0. Two factors of the diversity have been estimated. Firstly, the haplotype diversity (Hd) that measures the number and frequency of haplotypes in a given population and secondly the nucleotide diversity. The estimation of nucleotide diversity within each Cameroonian population has been done by calculating two measures of nucleotide diversity: the Watterson's θw (Watterson, 1975) based on the number of segregating sites in the sample and π (Tajima, 1983) based on the average number of pair-wise differences between sequences.

2.2.4.2. Tests of neutrality

The neutral model of molecular evolution is based on the fact that at the equilibrium, there should not be any evolutionary force acting in the population. Therefore, any deviation from the neutral model of molecular evolution signifies the reject of the neutral hypothesis. In order to find out if the observed patterns of nucleotide diversity in the Cameroonian *pfcrt* and *pfmdr1* genes are deviating from the neutral model of molecular evolution, many neutrality tests have been computed. They are: TD (Tajima, 1989), FLF (Fu and Li, 1993), FLD (Fu and Li, 1993), FWH (Fay and Wu, 2000) and Fu's Fs (Fu, 1997). For this, the software DNASp v.5.10.01 (Librado and Rozas, 2009) has been used. For all statistical analyses the P-value of ≤0.05 was considered as the level of significance.

i) Tajima's D test

The TD test compares two estimators of θ: the first Watterson's estimator (θw) based on the number of segregating sites (S) and the second based on the average number of pairwise differences (π). In fact, Tajima's D= π-θw. Under the null hypothesis, θw is expected to be equal to π, means Tajima's D value equal to zero. A positive Tajima's D value suggests a more number of pairwise differences between the sequences (θπ), indicating either the signature of balancing selection or a decrease in population size. In contrary to this, a negative Tajima's D suggests an excess of low and high frequency alleles in the region indicating directional selection (positive or negative selection) or population expansion.

ii) Fu and Li's F and D tests

FLF and FLD statistics measure the difference between number of derived singletons and the number of total mutations and average number of pairwise differences. For both tests, negative values indicate an excess of low-frequency polymorphisms, whereas positive values indicate an excess of intermediate polymorphisms (Fu, 1997; Fu and Li, 1993; Tajima, 1989).

iii) Fay and Wu's H test

FWH measures an excess of high frequency derived alleles. A positive value of FWH statistics indicates the presence of intermediate frequency variants that may depict the action of balancing selection. A negative value of FWH statistics indicates the presence of high frequency derived variants in the population that may be a signal of a recent selective sweep at the locus.

iv) Fu's Fs test (F_S)

This test has been used in order to determine if there is some demography force acting on the *pfcrt* and *pfmdr1* genes. This is because F_S statistic is strongly affected by demography. It compares the observed number of alleles in each sample to the number expected under the assumption of an infinite sites model of mutation. Fs is the test for determination of excess of rare alleles. A negative value of F_S suggests excess of alleles as expected under population expansion and genetic hitchhiking. A positive value indicates a deficiency of alleles as expected under recent population bottleneck

2.2.4.3. Inference of positive natural selection: synonymous versus non synonymous mutations based tests (dN/dS)

Since the regions of interest of the project are all coding regions, to infer the positive natural selection, Synonymous (dS) versus non synonymous (dN) mutations based approaches have been taken. Under the null hypothesis, dS is expected to be equal to dN. An excess of nonsynonymous substitutions (dN>dS

or dN/dS ratio also called "ω" greater than 1) can be interpreted as positive selection, indicative that replacement substitutions increase fitness. Whereas a rarity of replacement changes (dN<dS or dN/dS ratio lesser than 1) indicates that negative selection is working to remove such substitutions from the gene pool.

i) Codon based Z-test

For each Cameroonian population the values of dN and dS from averaging over all sequences have been calculated using MEGA version 4.0 (Tamura et al., 2007) with modified Nei-Gojobori method. A codon based Z-test of positive selection for analysis averaging over all sequence pairs was performed to get the level of significance of selection. Thus, the null hypothesis of no selection (H0: dN=dS) versus the positive selection hypothesis (H1: dN>dS) was tested. To be considered as being under positive selection, *pfcrt* and *pfmdr1* genes had to exhibit a dN/dS ratio larger than 1 and a P-value for the Z-test below the significance level 0.05.

ii) Mac-Donald and Kreitman test

MK test (McDonald-Kreitman, 1991) was performed in coding sequences of both the genes (*pfcrt* and *pfmdr1*) using DnaSP 5.10.01 (Librado and Rozas, 2009). For this test, the homologous sequences from *P. reichenowi* as an out-group data were retrieved from Genedb database and used. Under the null hypothesis, all non-synonymous mutations (replacement, dN) are expected to be neutral and the ratio of the non-synonymous to synonymous variations

within species (Pn/Ps) is expected to be equal to the ratio of non-synonymous to synonymous variation between species (Dn/Ds) (Egea et al., 2008). However, if non-synonymous polymorphisms are under some selective pressure, then the ratio will not be equal (Wayne and Simonsen, 1998). The MK test creates 2x2 contingency table that contains number of synonymous and non-synonymous (replacement) sites, polymorphic within species and between species. The test of significance was evaluated by Fisher's Exact test. Also, the directionality of the MK test was checked by neutrality index (NI) (Rand and Kann, 1996). The NI value >1 is consistent with negative selection, while NI value <1 is consistent with positive selection (Egea et al., 2008).

ii) Ka/Ks ratio test

The Ka/Ks ratio was estimated using DnaSP version 5.0. For each Cameroonian population, the consensus alignment of the respective *pfcrt* and *pfmdr1* genes has been aligned with the homologous sequences of these genes in *P. reichenowi*. This statistic provides an indication of selection for amino acid changes during evolution. This ratio is based on the rate of the nonsynonymous (amino acid changing) nucleotide substitutions compared with the rate of synonymous (non-amino acid changing) nucleotide substitutions between two taxa (Altheide et al., 2006). It is also another denotation of ω=dN/dS. The interpretation for positive and negative selection is same as mentioned at the beginning of the statistical analysis section.

iv) Phylogeny based test of positive selection

Apart from the above methods (see i and ii) for detecting deviation from the neutral expectation, others likelihood methods that allow ω to vary among the branches in a phylogeny, as well as between codons, have been conducted. *P. reichenowi* sequence was utilized as the closely related species of *P. falciparum* for the application of these methods. The phylogenetic tree was constructed using Mr. Bayes and coding sequences of the *pfcrt* and *pfmdr1* genes of all the individuals of six different populations and *P. reichenowi* were used for detection of natural selection using 'codeml' computer program embedded in the PAML4.4e software (Yang et al., 2000; Yang, 2007). PAML software v 4.4e was used to generate the "ω" value among different coding regions. In case of positive selection, the "ω" value would be greater than 1 (ω > 1), whereas under purifying selection, the "ω" value would be less than 1 (ω < 1). Furthermore, in order to determine the exact amino acid sites under selection, we used the NEB (Naive Empirical Bayes) and BEB (Bayes Empirical Bayes) methods incorporated in the PAML program (Yang et al., 2005). According to the "codeml" program, the site model pairs that appear to be particularly useful for real data analysis, are M1a vs M2a, and M7 vs M8 (Yang, 2007).

With a view to give robustness to the results on the identification of amino acids sites evolving under selective pressure as given by PAML test, Fast Unbiased Bayesian Approximation test has also been conducted (FUBAR; Murrell et al., 2013). This test was executed by the software HyPhy (Pond et al.,

2005a) and available in the web-based interface Datamonkey (http://www.datamonkey.org/; Pond et al., 2005b). As recombination can break the linkage and could be falsely interpreted as adaptive selection significant recombination events were tested by genetic algorithm for recombination detection (GARD; Pond et al., 2006) implemented in Datamonkey.

2.2.4.4. Genetic inter-relationships between the Cameroonian populations based on the different *pfcrt* haplotypes

To determine the genetic inter-relationships between the populations sampled from Cameroon in term of *pfcrt* haplotypes, a NJ phylogenetic tress was constructed using the computer program MEGA version 4.0 (Tamura et al., 2007). The multiple DNA sequences alignment of the different consensus for each type of *pfcrt*-haplotype found in the present study has been utilized as the baseline for the construction of the phylogenetic tree.

2.2.4.5. Tests for genetic associations

In order to ascertain associations between pairs of SNPs in the *pfcrt* and *pfmdr1* genes (SNPs present in coding region) as well as between the two genes SNPs, LD was estimated between each pair of SNPs in six Cameroonian populations using Haploview computer program (Barrett et al., 2005). The LD coefficient (D') was used as a measure to ascertain Linkage disequilibrium between pair of SNPs. The value of D' equal to one (D'=1) is considered as

statistically significant LD between pair of SNPs, whereas D' equal to zero (D'=0) considered as non-significant LD between pair of SNPs.

Furthermore, in order to strengthen the LD analysis performed between both the genes (to find out if these genes are somehow correlated), a paired t-test and a Pearson correlation coefficient were conducted with the help of software GraphPad prism (version 5.0).

CHAPTER 3: RESULTS

3. RESULTS

The present study has been designed with the main focus on the molecular diagnosis of malaria infection in an African country, Cameroon in order to survey at some extent the dynamics of malaria parasites species within the country. For this, the most malaria endemic part of Cameroon (southern part) has been targeted: six big district hospitals in five distinct regions. Diverse techniques, all restricted in the molecular approach area have been applied.

The second attention of this project was drawn on the evolutionary pattern of the two most common genes involved in antimalarial drugs resistance in *P. falciparum* species: *pfcrt* and *pfmdr1* genes. Therefore, these genes have been PCR-amplified and sequenced in Cameroonian *P. falciparum* isolates distributed in five regions (Figure 3.1). The DNA sequences data generated have been then scanned for the presence of SNPs, genetic marker used in this study for the estimation of genetic diversity of both the genes in Cameroon. On the basis of their genetic diversity data, many statistical analyses have been performed in order to infer their evolutionary pattern.

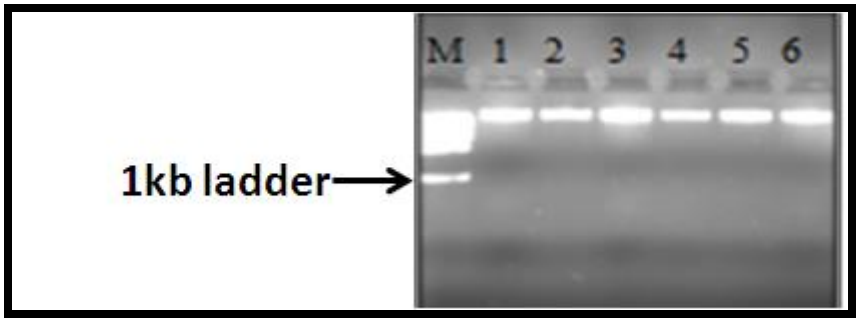
The results of all the techniques done are presented and discussed below.

3.1. Molecular diagnosis of malaria

3.1.1. Genomic DNA isolation of malaria parasite

Genomic DNA of malaria parasites was successfully isolated from the total of 545 symptomatic malaria samples collected. For checking if effectively DNA has been isolated from the blood spots, electrophoresis was conducted with approximately 8-10 µl of the eluted DNA was loaded into agarose gel 1% wells. The Figure 3.1 represents the successful DNA isolation after electrophoresis.

Figure 3.1. Gel picture of the DNA samples after DNA isolation



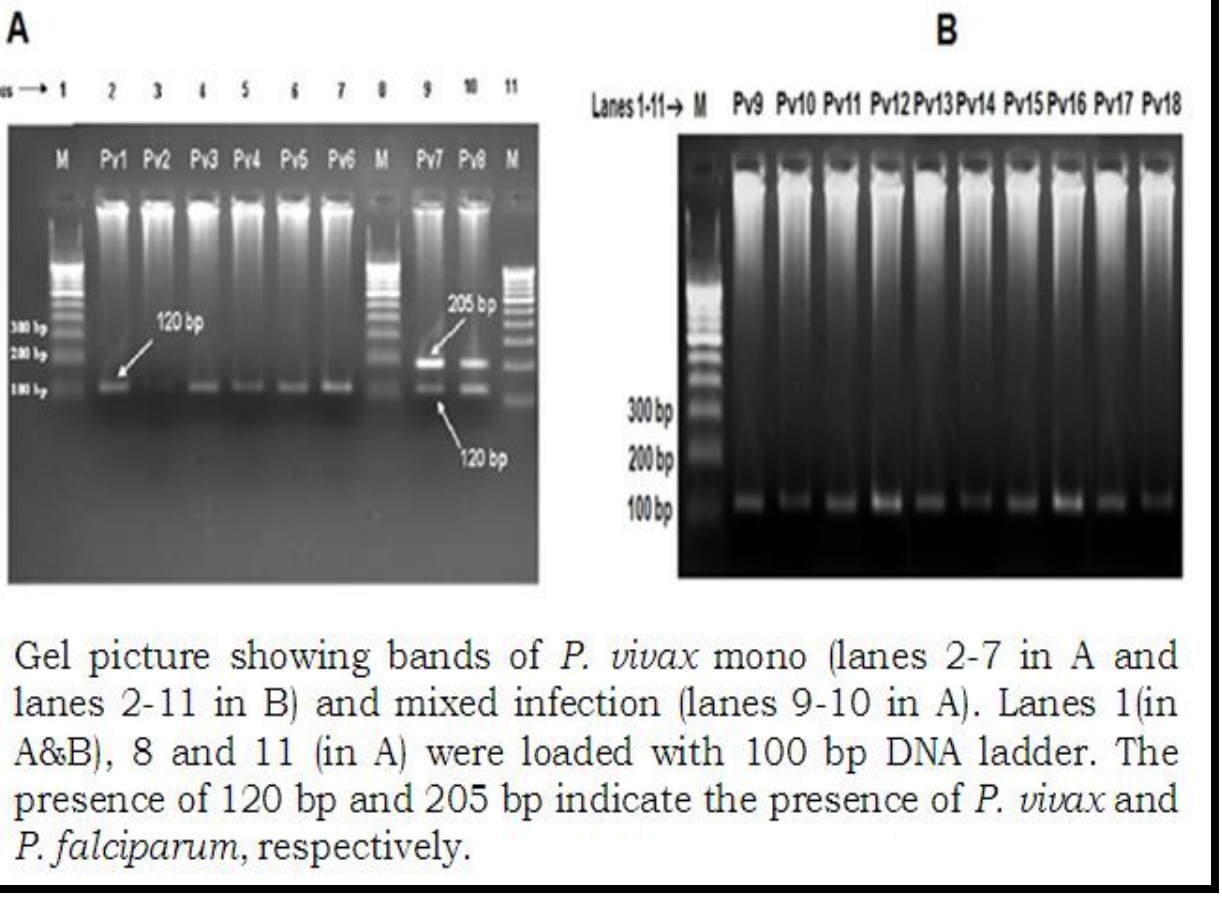
M (the marker) represents 1kb ladder

3.1.2. PCR diagnostic assay of malaria infection based on the 18S rRNA gene

The 18S region of the ribosomal RNA for each species (*P. falciparum*, *P. vivax*, *P. malariae and P. ovale*) was successfully amplified from the 545 isolated DNA samples. Out of this number, 244 (around 45%) were found to be malaria positive distributed as follow: 226 (~93%) patients infected with *P. falciparum*, 16 (~6%) with *P. vivax* and 2 (~1%) were infected with *P. falciparum* and *P. vivax*. No case of *P. malariae* as well as *P. ovale* was detected in the present

study. The Figure 3.2A and 3.2B represent the different bands obtained after PCR amplification of the 18S rRNA especially for the *P. vivax* cases single and mixed malaria parasite infection (*P. falciparum* + *P. vivax*).

Figure 3.2. Gel picture of the 18S region of rRNA PCR products



Gel picture showing bands of *P. vivax* mono (lanes 2-7 in A and lanes 2-11 in B) and mixed infection (lanes 9-10 in A). Lanes 1(in A&B), 8 and 11 (in A) were loaded with 100 bp DNA ladder. The presence of 120 bp and 205 bp indicate the presence of *P. vivax* and *P. falciparum*, respectively.

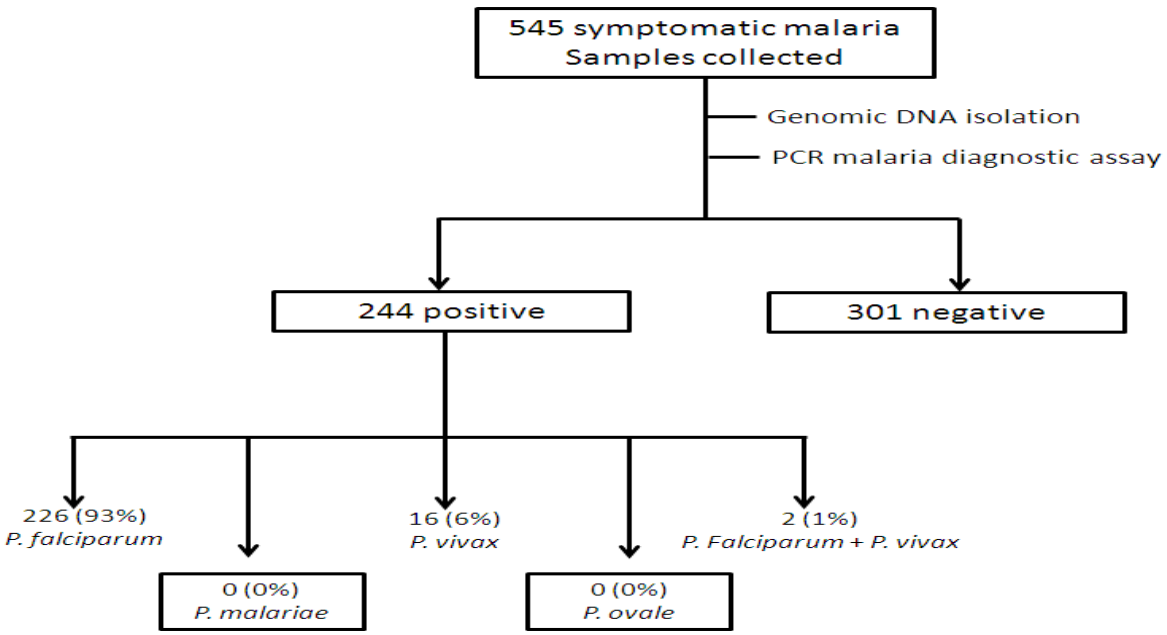
The details of patients considered in this study and the detailed results on the prevalence of different malaria parasites in Cameroon are depicted in the Table 3.1. The summary of the PCR diagnostic assay targeting the 18S rRNA gene is represented by the Figure 3.3.

Table 3.1. Patient details and the differential malaria species infection dynamics in Cameroon

Sampling sites (n)	Gender (n)	Age Range (Min-Max)	Number of infected patients		
			Pf	***Pv***	***Pf + Pv***
Ebolowa (60)	Male (37)	(7 months - 56 years)	36	0	1
	Female (23)	(1 month - 82 years)	21	1	1
Douala (52)	Male (25)	(3 weeks - 46 years)	25	0	0
	Female (27)	(9 months - 80 years)	25	2	0
Bertoua (25)	Male (14)	(5 months - 52 years)	13	1	0
	Female (11)	(1 year - 50 years)	10	1	0
Yaounde (29)	Male (11)	(6 months - 64 years)	10	1	0
	Female (18)	(1 years - 78 years)	18	0	0
Kye-ossi (35)	Male (17)	(1 year - 70 years)	17	0	0
	Female (18)	(1.7 year - 65 years)	18	0	0

***Pf*:** *P. falciparum* ***Pv*:** *P. vivax* **n:** sample size
Min: Minimum **Max:** Maximum

Figure 3.3. Summary of the malaria infection survey in native Cameroonians



3.1.3. Sequencing of the 18S region of the rRNA gene

In order to reconfirm the novel detection *via* PCR of *P. vivax* infection in native Cameroonians, the 18S region of the rRNA successfully PCR amplified was sequenced for samples with *P. vivax* mono-infection and mixed parasitic infection found in this study. The DNA sequences of the *P. vivax* (mono- and mixed) 18S rRNA gene and the *P. falciparum* (from the mixed infections) 18S rRNA gene were aligned with the homologous sequences retrieved (3D7 and *SAL*-1) from NCBI website (http://www.ncbi.nlm.nih.gov/) after BLAST search using the MegAlign module of the DNAStar (Madison, USA) computer program. We found 98-100% similarity between the DNA sequences generated in the present study and sequences of *P. vivax* and *P. falciparum* reported earlier (Figure 3.4 and Figure 3.5), re-confirming correct detection of single infections of *P. vivax* as well as mixed infection due to these two species. After editing, the sequences were deposited in Genbank for the cases of *P. falciparum* with the accession numbers KC428741 to KC428742. The sequences of 18S rRNA for the *P. vivax* cases were deposited in the EMBL-bank of the European Nucleotide Archive (ENA) and their accession numbers are HF945436 to HF945443.

Figure 3.4. DNA sequence alignment for the Cameroonian *P. falciparum* 18S rRNA gene

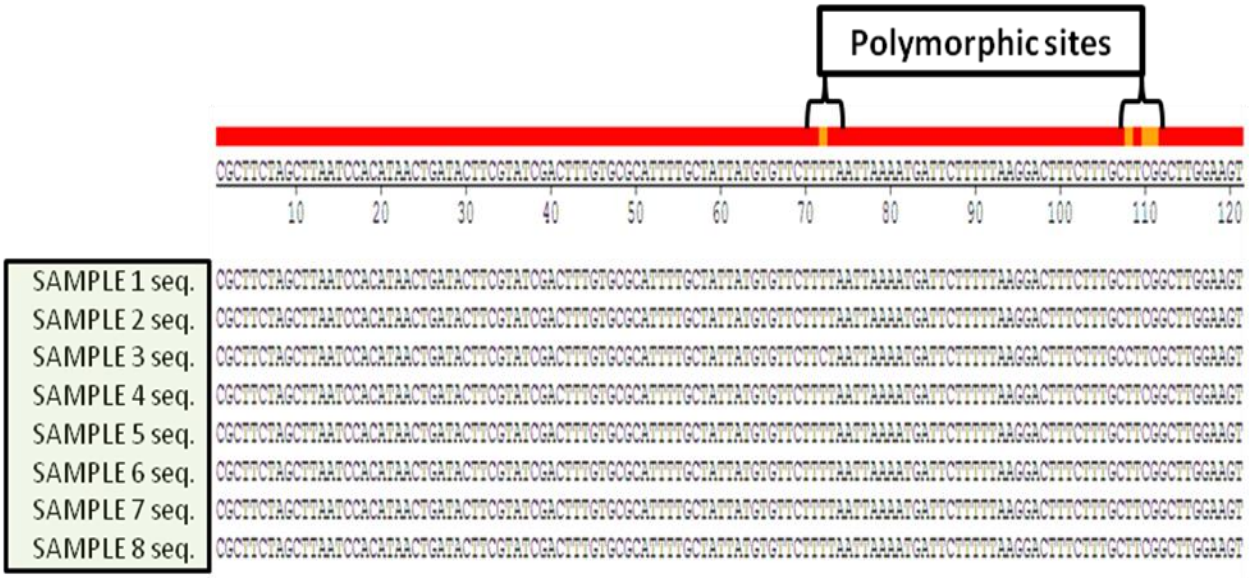
```
3D7     TTTAAACTGGTTTGGGAAAACCAAATATATTATATATTTTGCTTTGTTCAAAATAAGGTT  60
Pf 7    TTTAAACTGGTTTGGGAAAACCAAATATATTATATATTTTGCTTTGTTCAAAATAAGGTT  60
Pf 8    TTTAAACTGGTTTGGGAAAACCAAATATATTATATATTTTGCTTTGTTCAAAATAAGGTT  60

3D7     TTCTAATAAATTATGTTTTTATCAGATATGACAGAATCTTTTTTAAAATCTCTTCAATAT  120
Pf 7    TTCTAATAAATTATGTTTTTATCAGATATGACAGAATCTTTTTTAAAATCTCTTCAATAT  120
Pf 8    TTCTAATAAATTATGTTTTTATCAGATATGACAGAATCTTTTTTAAAATCTCTTCAATAT  120

3D7     GCTTTTATTGCTTTTGAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGAC  180
Pf 7    GCTTTTATTGCTTTTGAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGAC  180
Pf 8    GCTTTTATTGCTTTTGAGAGGTTTTGTTACTTTGAGTAAAATTAAGTGTTCATAACAGAC  180

3D7     GGGTAGTCATGATTGAGTTCATTGT  205
Pf7     GGGTAGTCATGATTGAGTTCATTGT  205
Pf8     GGGTAGTCATGATTGAGTTCATTGT  205
```

Figure 3.5. Multiple DNA sequences alignment for the Cameroonian *P. vivax* 18S rRNA gene



After editing and alignment of the sequences, they have been scanned for the presence of SNPs. Although the DNA sequences of the 18S rRNA gene of Cameroonian *P. falciparum* yielded 100% similarity with the reference sequence, for *P. vivax*, we found a novel SNP at the 72nd nucleotide position in one of the mixed-infected patients (Figure 3.6) by comparing with the reference sequence SAL-1 (GenBank accession number U03079.1).

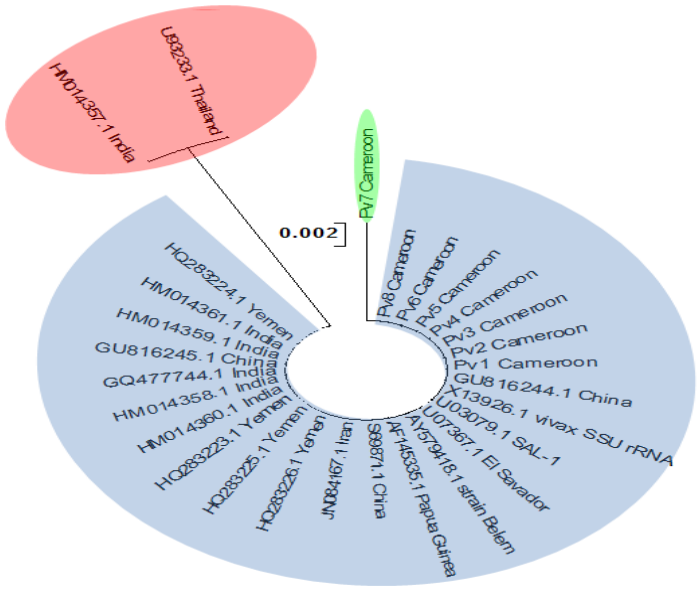
Figure 3.6. Novel mutation found in the Cameroonian *P. vivax* 18S rRNA gene

The Single Nucleotide Polymorphism (SNP) in Pv7 is indicated in red and the representative chromatogram showing a clear pick of "C" is shown below

In order to infer genetic inter-relationships of the Cameroonian *P. vivax* with global isolates at this species-specific 18S rRNA gene, a NJ phylogenetic tree was constructed using MEGA software version 4.0 (Tamura *et al.*, 2007) (Figure 3.7). The NJ tree formed three clades; while almost all the Cameroonian isolates were monophyletic with isolates from majority of the worldwide *P. vivax*

isolates (the largest clade), the mixed infected Cameroonian isolate (Pv7, that contains the SNP, see above) formed an entirely separate clade. Two isolates (one from India and the other from Thailand) formed another clade in the reconstructed phylogenetic tree (Figure 3.7).

Figure 3.7. NJ phylogenetic tree based on the *P. vivax* 18S rRNA gene



3.1.4. PCR-amplification, sequencing and sequences data analysis of the promoter region of the human *Duffy* gene from the Cameroonian *P. vivax* isolates

Since *P. vivax* species is reported to be infecting *Duffy*-negative Africans (Mendes et al., 2011), it was interesting to check if this is true for Cameroonians as well. The human *Duffy*-gene was successfully PCR-amplified from all the 18 isolates found to be infected with *P. vivax* in the present study. The gel picture of the PCR products is depicted in the Figure 3.8.

The PCR products were sequenced (Figure 3.9). The sequences generated were then edited, aligned and scanned for the presence of the T-33C mutation sitting in the promoter region of the human *Duffy* gene. Accordingly, the sequences were cautiously checked for the presence of double peaks at the nucleotide position -33 (sign of heterozigosity also known as partial *Duffy*-positive). A single peak of nucleotide T signifies the patient is homozygous *Duffy*-positive whereas a single peak of nucleotide C signifies the patient is homozygous *Duffy*-negative (Figure 3.9). The newly generated *Duffy* gene sequences were deposited in GenBank with accession numbers KJ534648 to KJ534655. Interestingly, all the eighteen patients infected with either only *P. vivax* or mixed infection with *P. falciparum* were found to harbor the T-33C mutation (Figure 3.9), signifying the fact that all the eighteen Cameroonians were homozygous *Duffy* negative.

3.1.5. PCR-amplification, sequencing and sequences data analysis of the *P. vivax* multidrug resistance gene 1 (*pvmdr1*) and the *pfmdr1* gene from the *P. vivax* mono- and mixed-infected patients

A fragment of 543 bp of the *pvmdr1* gene was successfully PCR-amplified (Figure 3.10A) and sequenced in the *P. vivax* mono-infected samples as well as in the mixed-infected samples. Similarly, a fragment of 510 bp of the *pfmdr1* gene was successfully PCR-amplified (Figure 3.10B) and sequenced in the two mixed infected cases.

Figure 3.8. Gel picture of the PCR products of the Cameroonian human *Duffy* gene

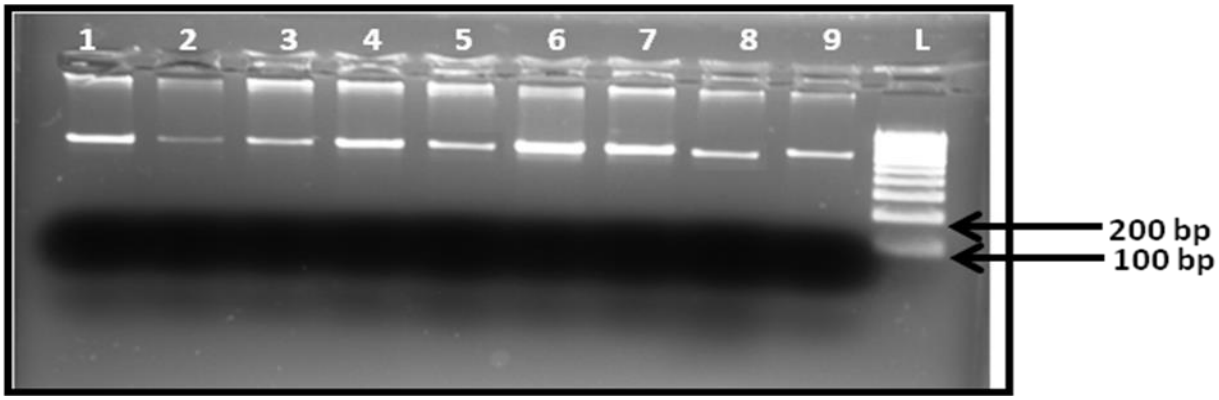


Figure 3.9. Representation of the C-mutation in the Cameroonian *Duffy* gene

D. Pos	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**T**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv1	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv2	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv3	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv4	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv5	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv6	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv7	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC
Pv8	AACCTGATGGCCCTCATTAGTCCTTGGCTCTTA**C**CTTGGAAGCACAGGCGCTGACAGCCGTCCC

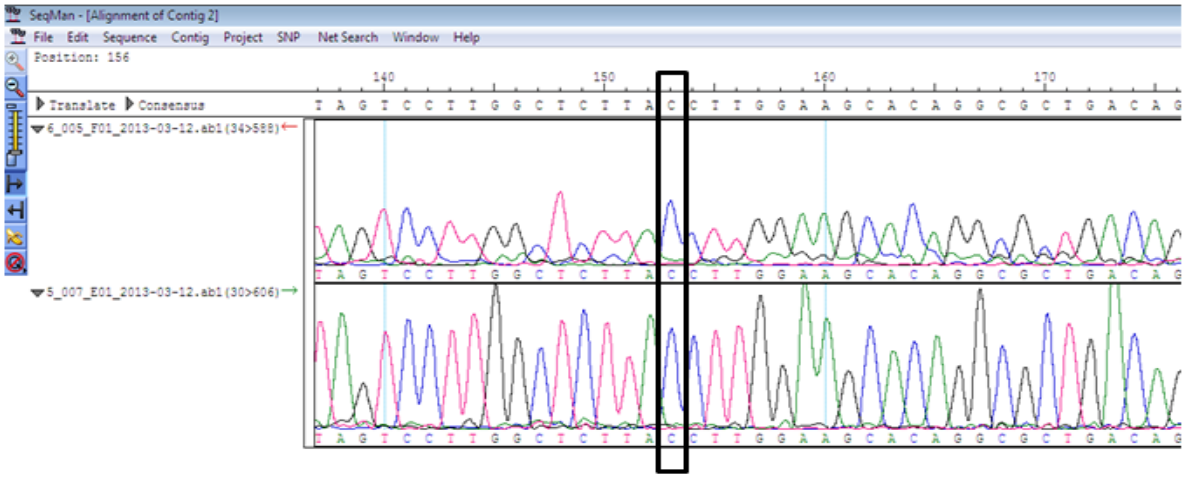
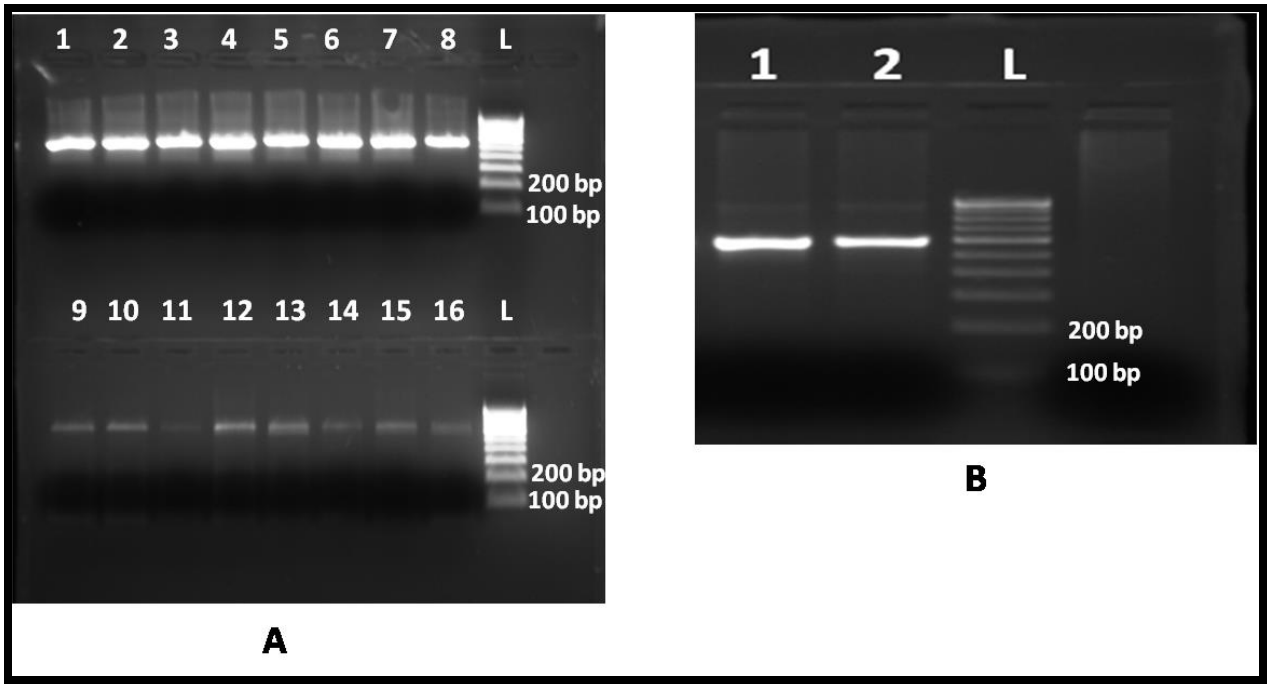


Figure 3.10. Gel picture of the PCR products of the *pvmdr1* (A) and *pfmdr1* (B) genes.

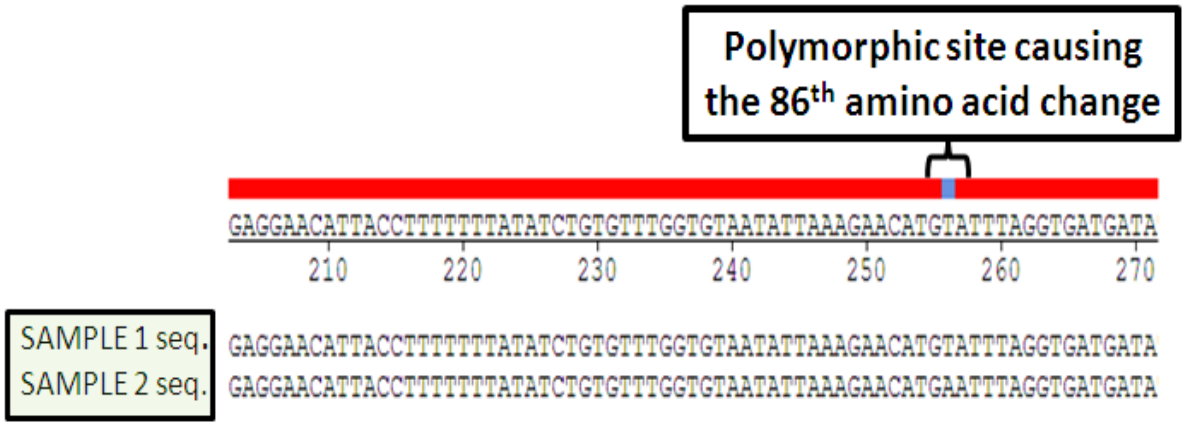


Apart from the presence of four nucleotide substitutions (three non-synonymous and one synonymous), multiple sequences alignment for the *pvmdr1* gene with the respective reference sequence (Figure 3.11) revealed perfect sequence homology between the reference *pvmdr1* gene of the SAL-1 strain and the *pvmdr1* sequences of Cameroonian *P. vivax*. For the *pfmdr1* gene too, apart from a single non-synonymous substitution (at the 86th amino acid position) in one of the two Cameroonian *P. falciparum* isolates from the mixed parasitic infections with *P. vivax*, perfect sequence homology was observed when compared with the reference 3D7 *P. falciparum* strain. The results are presented in the Figures 3.11 and 3.12. The newly generated sequences of the *pvmdr1* and *pfmdr1* genes (for the mixed infected cases) were deposited in the

GenBank with accession numbers from KJ534638-KJ534645 and KJ534646-KJ534647, respectively.

Figure 3.11. DNA sequences alignment for the *pvmdr1* gene.

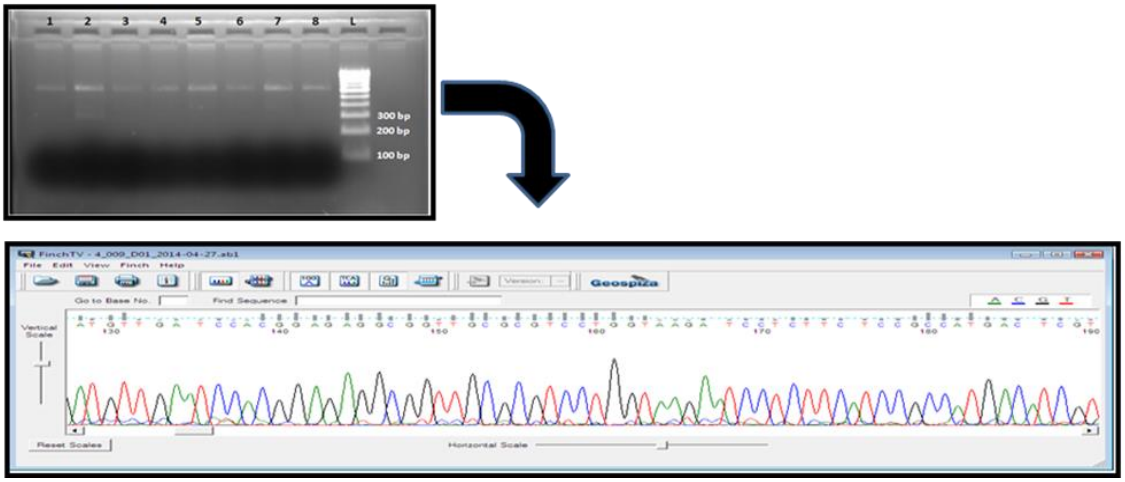
Figure 3.12. DNA sequences alignment for the *pfmdr1* gene in the mixed infected samples.



3.1.6. PCR-amplification, sequencing and sequences data analysis of the *P. vivax* circumsporozoite gene (*pvcsp*) from the *P. vivax* infected patients

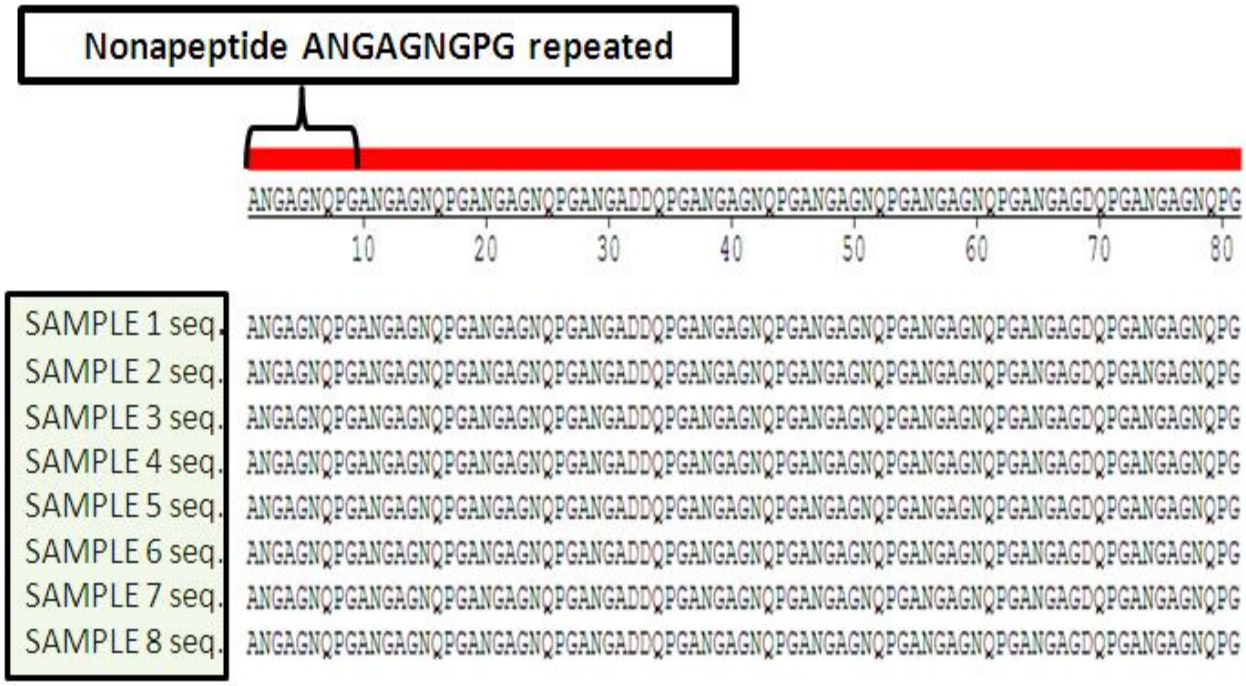
With some evidences of *P. vivax* infection in Cameroonians using PCR diagnostic approaches followed by DNA sequencing and sequence alignments of the 18S rRNA and the *pvmdr1* genes, it was for a great interest not only to reconfirm *P. vivax* infection, but also to know the type of *P. vivax* strain present in these *P. vivax* infected Cameroonians. For this, a portion of the *pvcsp* gene covering the majority of the repeats region was successfully PCR-amplified (Figure 3.13) and sequenced in the *P. vivax* isolates. Multiple sequences alignment with the reference sequence from SAL-1 retrieved from the GenBank (accession number GU339059.1) showed that the sequences generated from Cameroon display the nonapeptide repeats ANGA(G/D)(N/D)QPG, characteristic of the VK247 variant (Figure 3.14). The results therefore not only revalidated the observation of *P. vivax* infection in native Cameroonians, but also indicated that the VK247 type is circulating in Cameroon. The newly generated sequences of the *pvcsp* gene were deposited in the GenBank (accession numbers KM099676 to KM099683).

Figure 3.13. Representative PCR-products of the *pvcsp* gene and the DNA sequence chromatogram



M= 100bp ladder

Figure 3.14. DNA sequences alignment for the *pvcsp* gene



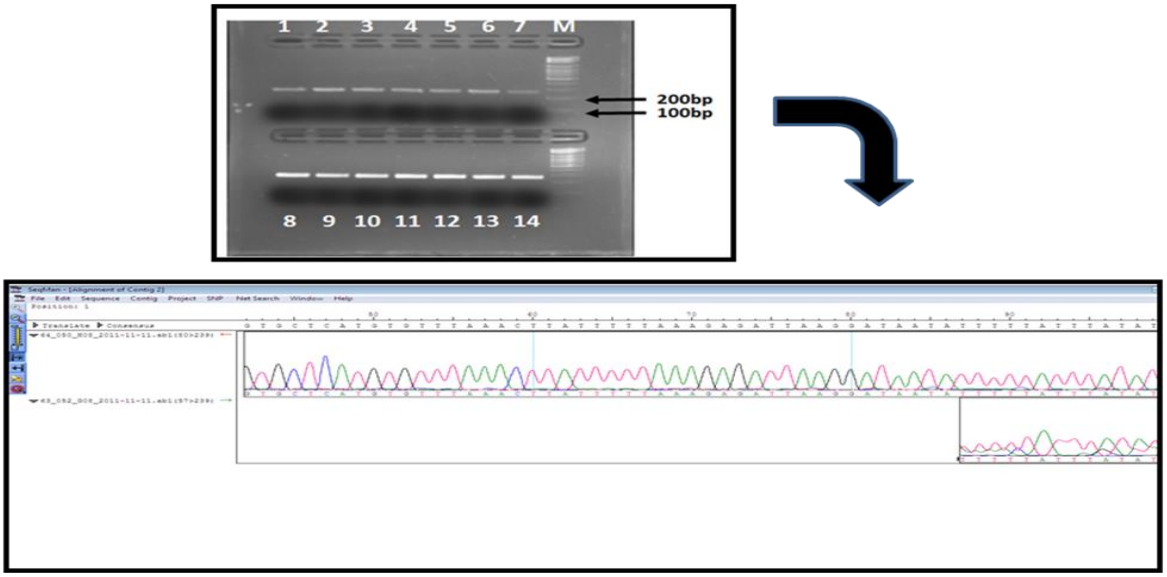
3.2. Evolutionary genetics of the two antimalarials drug resistance genes: *pfcrt* and *pfmdr1*

3.2.1. PCR amplification, sequencing and multiple DNA sequences alignment

A fragment of 264 bp of the *pfcrt* gene was successfully PCR-amplified and sequenced from 180 isolates in total from the five Cameroonian regions sampled. The Figures 3.15 and 3.16 represent the gel picture of the *pfcrt* PCR products and the multiple DNA sequences alignment of the generated sequences with the reference sequence of 3D7 strain retrieved from Genbank (Accession number AL844506.2), respectively.

A fragment of 603 bp of the *pfmdr1* gene was successfully PCR-amplified from the same 180 Cameroonian isolates used for the amplification of the *pfcrt* gene. However, only a coverage of 510 bp was generated after sequencing in four populations from three regions (Ebolowa in the South region, Douala in the Littoral region, Bertoua in the East) out of six populations sampled and 561 bp in the two remaining populations (Yaounde in the centre and Kyeossi, the city in the border of Cameroon, Gabon and Equatorial Guinea). The Figures 3.17 and 3.18 represent the gel picture of the *pfmdr1* PCR products and the multiple DNA sequences alignment of the generated sequences with the reference sequence of 3D7 strain retrieved from Genbank (Accession number AL844504.1), respectively.

Figure 3.15. Representative PCR products of the *pfcrt* gene and the DNA sequence chromatogram



M represents a 100bp ladder

Figure 3.16. DNA sequences alignment for the *pfcrt* gene

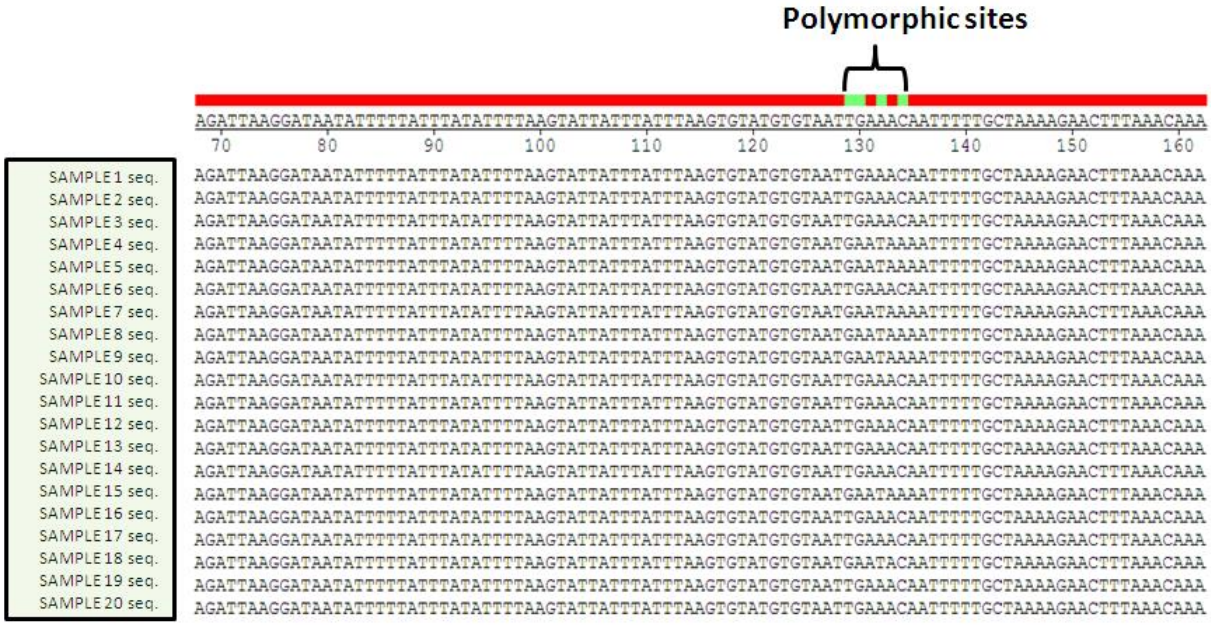
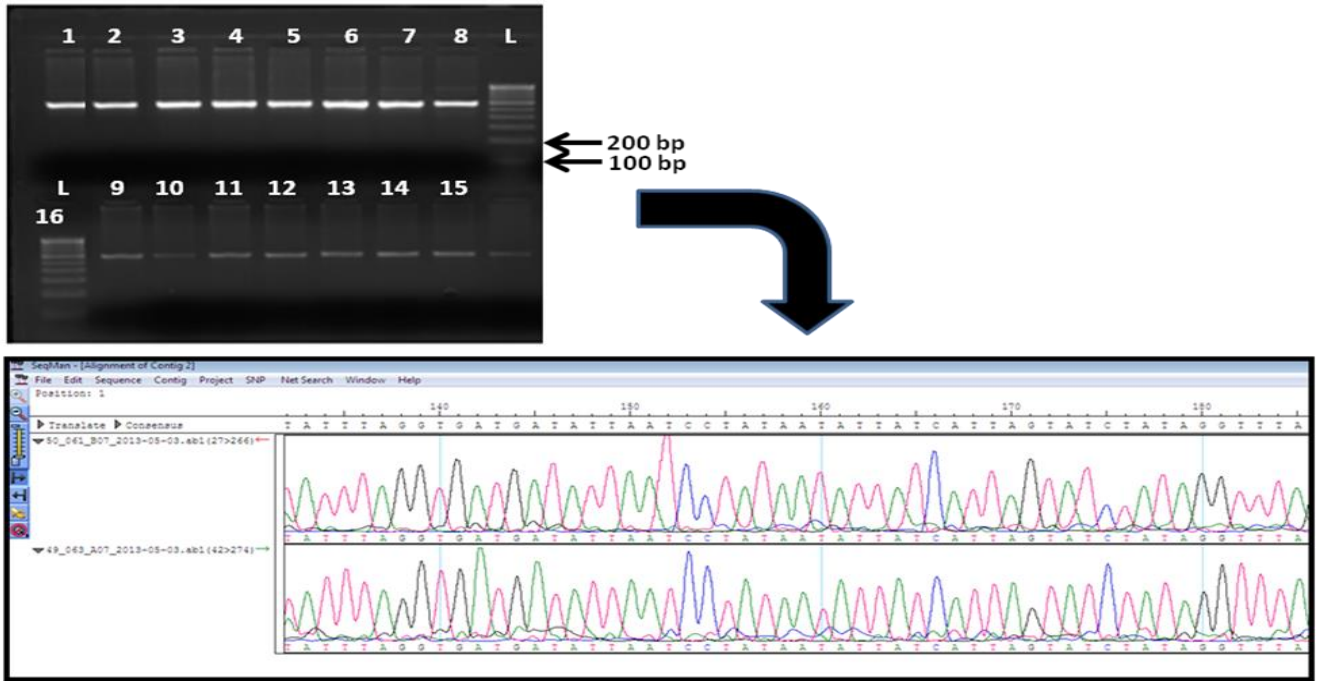
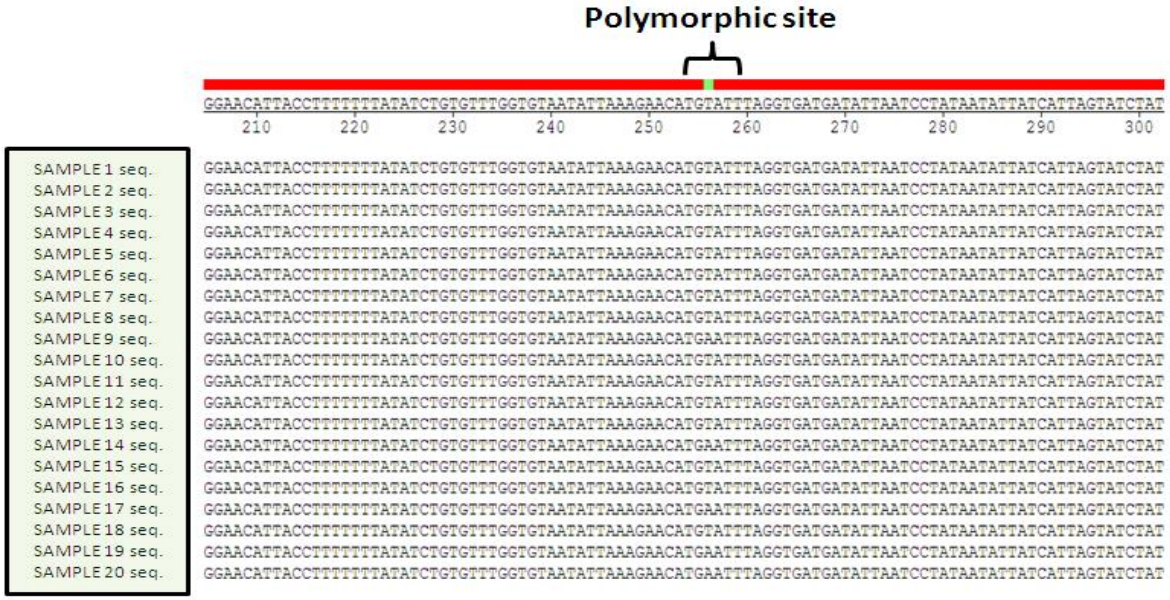


Figure 3.17. Representative PCR products of the *pfmdr1* gene and the DNA sequence chromatogram



L is a 100 bp ladder.

Figure 3.18. DNA sequences alignment for the *pfmdr1* gene

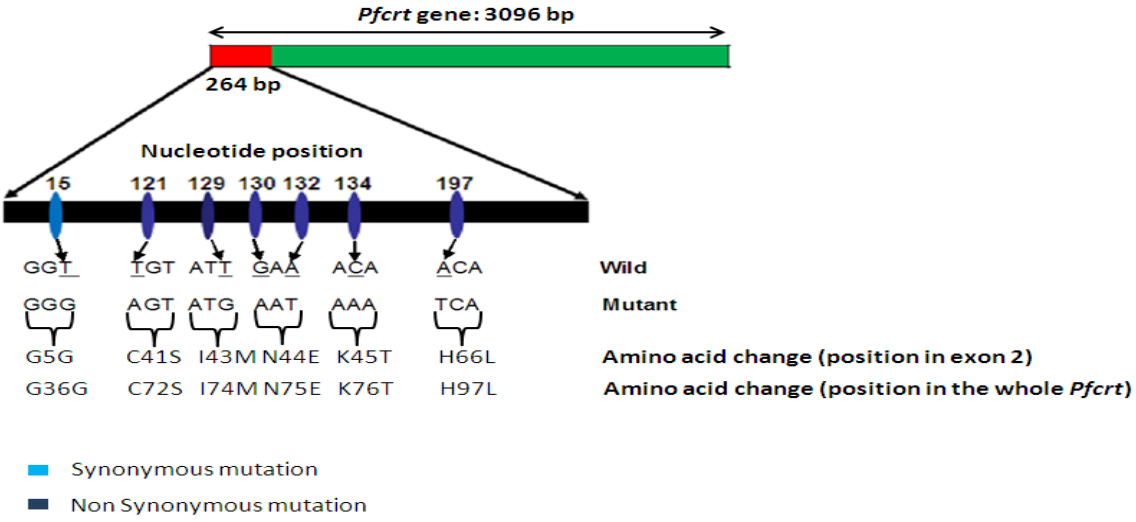


3.2.2. DNA sequences data analyses

3.2.2.1. Ascertainment of the Single Nucleotide Polymorphisms (SNPs)

After multiple DNA sequences alignment of the respective sequences of the *pfcrt* and *pfmdr1* genes along with the reference sequences from 3D7 strain, the number of SNPs was recorded for each gene. Thus, multiple sequence alignment of the 180 DNA data for the second exon of the Cameroonian *pfcrt* gene revealed the presence of seven point mutations (six non synonymous and one synonymous, Figure 3.19), out of which two (one non synonymous and one synonymous) were completely new (never reported in Cameroon so far). The six different non synonymous mutations code for the amino acids located at the different positions 72, 74, 75, 76 and 97 of the whole *pfcrt* gene as commonly known.

Figure 3.19. Genetic polymorphism of the *pfcrt* gene



Based on the amino acids change at the positions 72nd, 73rd, 74th, 75th and 76th, different haplotypes for the *pfcrt* gene in Cameroon were generated and their frequency of distribution estimated in the different regions sampled. As a result, seven different haplotypes (Figure 3.20) including the wild type $C_{72}V_{73}M_{74}N_{75}K_{76}$ were found. It is known that the CVMNK haplotype represents CQS parasites, and in the present study this haplotype was found to be distributed in all the five locations of Cameroon in variable frequencies (Figure 3.21). In total, 65 isolates (36%) were of CVMNK type - meaning about one third of *P. falciparum* isolates are still sensitive to CQ in Cameroon. Out of the rest 115 *P. falciparum* isolates (64%) bearing the CQR-*pfcrt* genotype, a major proportion (86.9%) was of CVIET type. The rest 13.1% *P. falciparum* isolates bearing CQR-*pfcrt* genotypes were segregated into five different haplotypes (SVMNT, 5.2%; SVMET, 1.7%; CVMDT, 3.5%; CVMET, 0.9% and CVMNT, 1.7%, Figure 3.21). Out of the five locations of Cameroon, two population samples from Douala were found to bear all the seven *pfcrt* haplotypes including the CVMNK (Figure 3.21), representing highest haplotype diversity of the *pfcrt* gene than the rest four locations. However, in Douala the CVMNK haplotype was present in a fairly low proportion (27.4%) in comparison to other four population samples. The SVMNT haplotype which is considered to be the fittest one in a CQ environment in comparison to other haplotypes (Sa et al., 2009; Sa et al., 2010) was found only in Douala. Since our data set is based on DNA sequencing, the nucleotide composition of the SVMNT haplotype could further be determined. Interestingly, all the six parasite isolates bearing the

SVMNT haplotypes were found to be of S(agt)VMNT type. Also, the other four CQR-*pfcrt* haplotypes were found in Douala in comparatively higher frequency than in other four Cameroonian locations.

Figure 3.20. Representation of sequence chromatogram of the seven *pfcrt* haplotypes

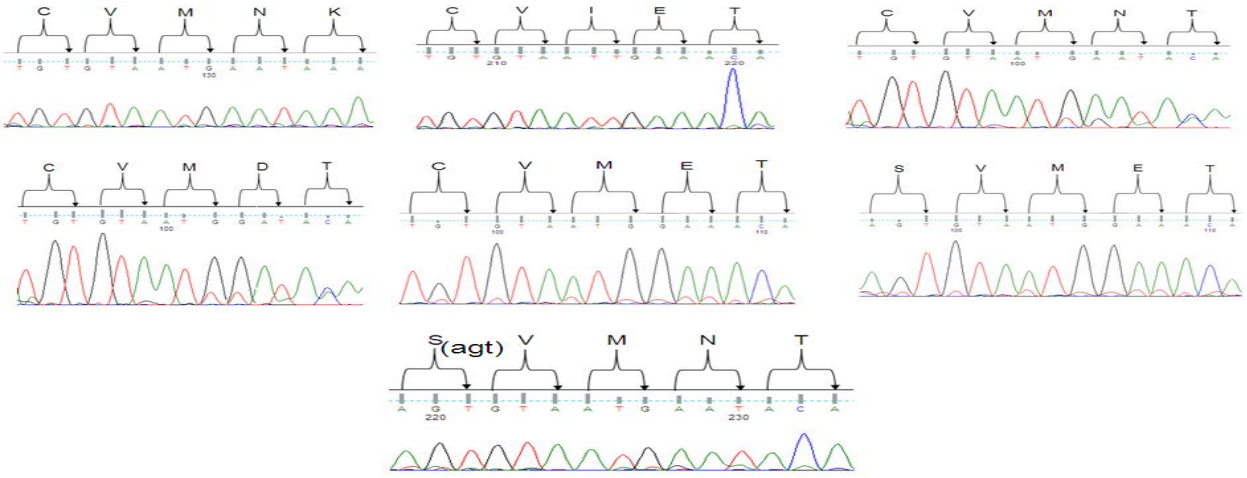
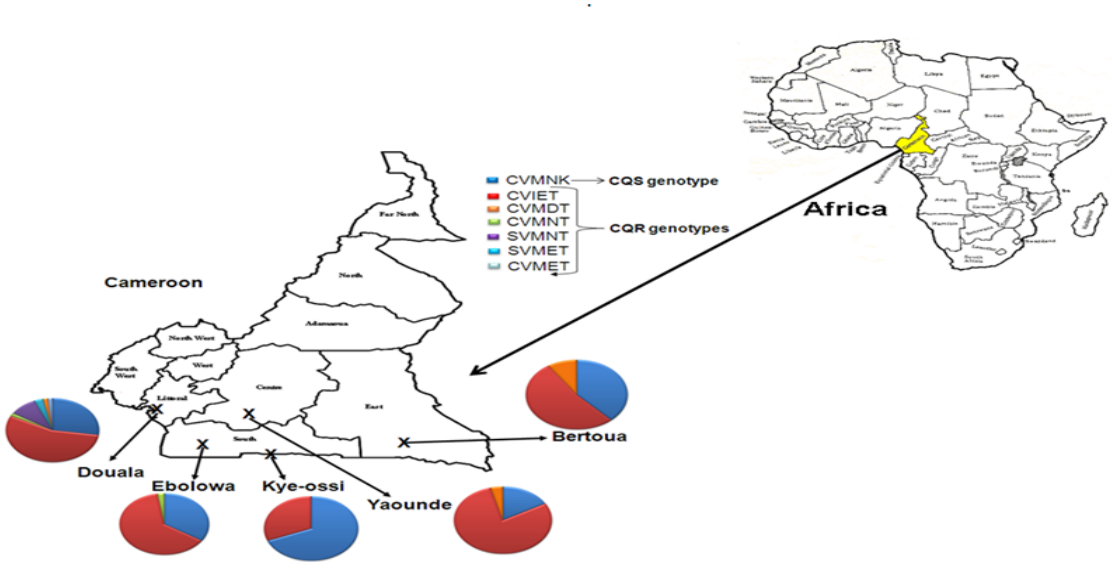


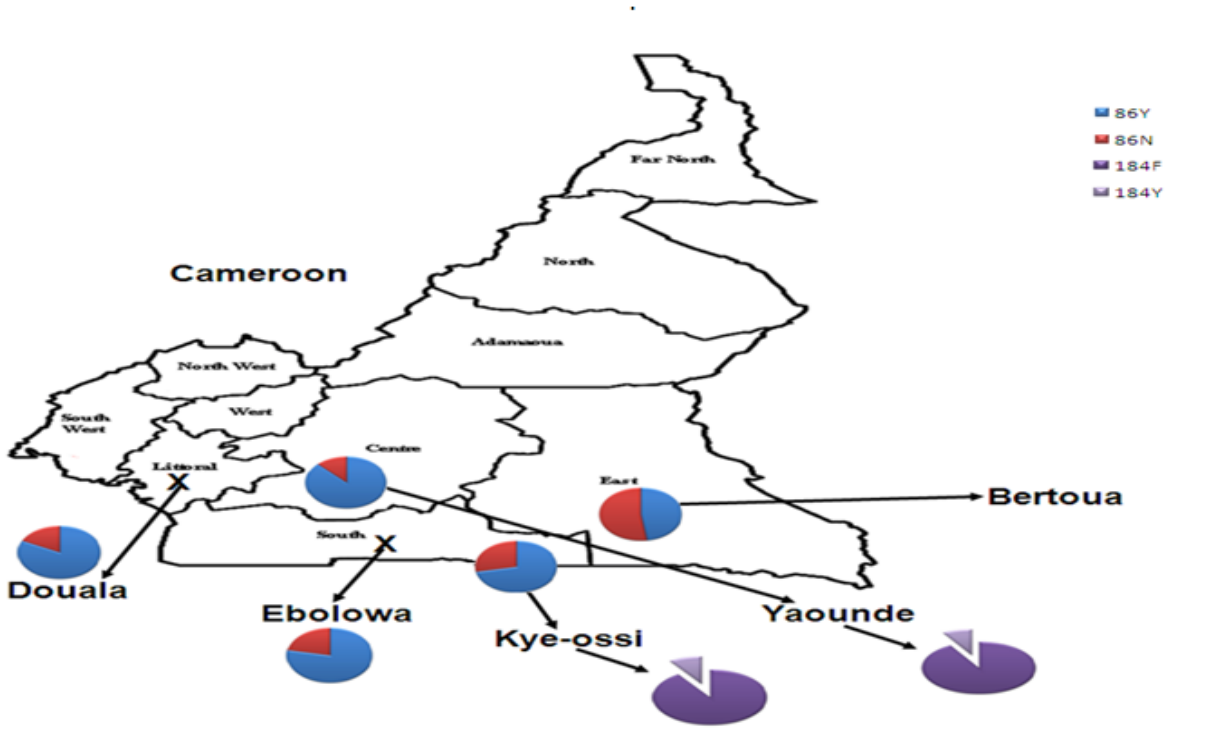
Figure 3.21. Repartition of the *pfcrt* haplotypes in the sampled sites



The pie chart against each sampling locations indicates the frequency of each of the seven *pfcrt* haplotypes found in the respective location

Concerning the *pfmdr1* gene, several mutations have been detected. In total, 20 mutations were found in the Cameroonian *pfmdr1* gene. Out of these 20 polymorphic loci, 11 were non-synonymous (at nucleotide positions: 50, 61, 80, 86, 87, 97, 114, 116, 160, 169 and 184) and 9 were synonymous (at the nucleotide positions: 44, 56, 59, 72, 92, 124, 129, 147 and 151). Out the five mutations in the *pfmdr1* gene (86th, 184th, 1034th, 1042nd and 1246th) known to be correlated with the CQR, the 86th mutation was hypothesized to be the major change associated with CQR in Africa while the triple substitution implicating 1034, 1042 and 1246 was recognized as a potential marker for CQR in South America (Foote et al., 1990; Basco et al., 1995). In the present study, out of the 180 isolates genotyped for this mutation, approximately 137 (76.11%) had the tyrosine mutation at that position (means 86Y). Unfortunately for the 184th mutation, sequencing was successful only in two regions out five and in a total of 56 isolates out of 180. About 51 isolates (91.07%) showed the phenylalanine amino acid at that position (184th F mutation). The Figure 3.22 represents the different frequencies of distribution of the 86th and 184th mutations in the sampled areas of Cameroon. In parallel, the Table 3.2 summarizes all the different mutations at the nucleotide and amino acids positions.

Figure 3.22. Repartition of the 86th and 184th mutations in the sampled sites



The pie chart against each sampling locations indicates the frequency of 86th and 184th mutations found in the respective location

Table 3.2. Summary of the mutations recorded in the Cameroonian *pfmdr1* gene

Nucleotide position	130	148	168	177	181	216	238	256	260	276	289	340	346	370	387	441	451	478	507	551
Amino acid position	44	50	56	59	61	72	80	86	87	92	97	114	116	124	129	147	151	160	169	184
Mutation type	S	NS	S	S	NS	S	NS	NS	NS	S	NS	NS	NS	S	S	S	S	NS	NS	NS
Frequency	1	1	1	1	1	1	1	137	1	1	1	1	1	1	1	1	1	1	1	51
Region	Bta	Dla	Bta	Bta	Bta	Yde	Dla	All	Kye	Kye	Bta	Bta	Dla	Kye	Eba	Bta	Eba	Yde	Kye	Yde, Kye

Bta: Bertoua **Dla:** Douala **Yde:** Yaounde **Kye:** Kyeossi

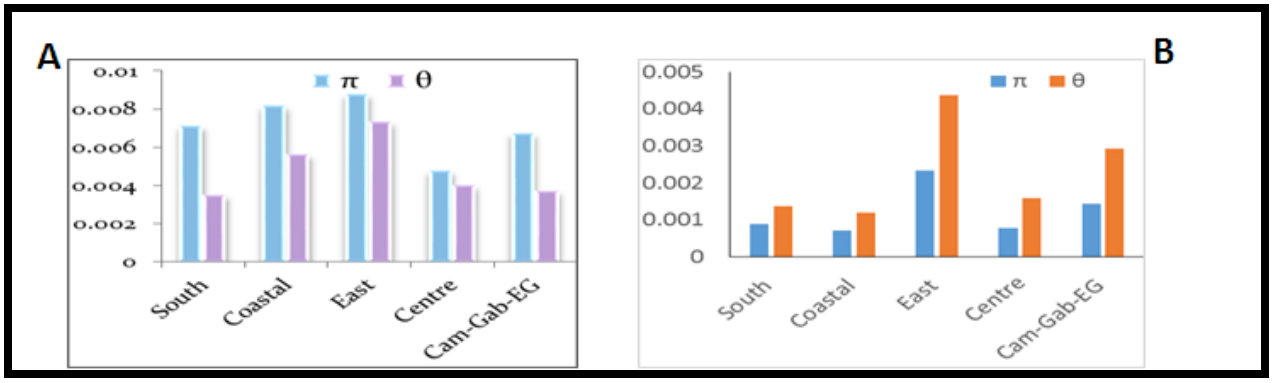
Eba: Ebolowa **S:** Synonymous mutation **NS:** Non Synonymous mutation

3.2.2.2. Nucleotide diversity and tests of neutrality

The nucleotide diversity of the Cameroonian *pfcrt* in the present study was variable (Figure 3.23): highest in Bertoua (the Eastern region of the country) and Douala (Littoral's region); moderate in Ebolowa (South region) and Kyeossi (border between Cameroon, Equatorial Guinea and Gabon) and lowest in Yaounde (Centre's region). Similarly, the haplotype diversity resulting from the ensemble of different mutations found followed almost the same scheme as for the nucleotide diversity (Table 3.3).

Almost comparable variability was observed in the case of the *pfmdr1* gene (Table 13) with the highest diversity denoted in Bertoua similarly as in the case of the *pfcrt* gene (Figure 3.23); the moderate diversity was found in Ebolowa and Yaounde; the lowest in Douala (Figure 3.23).

Figure 3.23. Nucleotide diversity of the *pfcrt* (A) and *pfmdr1* (B) genes in Cameroon



In view to determine whether the data on the Cameroonian *pfcrt* and *pfmdr1* genes fit to a neutral model of molecular evolution, single locus TD, FLF, FLD

and FWH values were calculated in the six Cameroonian sampled populations (Table 3.3 and Table 3.4). These values varied across the populations for both the genes. For the *pfcrt* gene, all the six populations showed a positive value of TD. But with the exception of the population of Ebolowa (TD=2.41921; P<0.05), none of the values was significantly deviating from the neutral model of molecular evolution (Table 3.3). For the others neutrality tests FLF and FLD, almost similar pattern was observed as for TD. However, in the population of Bertoua, all the three tests TD, FLF, FLD showed a negative value. With exception of FLF statistic in Ebolowa population (1.68355; P<0.05), none of the values of FLF and FLD was significantly deviating from the neutral model of molecular evolution (Table 3.3). In the case of the *pfmdr1* gene, apart from New-Bell (from Douala, TD=0.42898; FLF=0.65000; FLD=0.61722), all the three tests of neutrality showed a negative value (Table 3.4). Except in Bertoua where the FLF and FLD values were found to be significantly deviated from the neutral model of molecular evolution (FLF=2.72485; FLD=-2.67733; P<0.05), the three tests were non-significantly deviating from the neutral model (Table 3.4). The FWH test showed a negative value for all the populations except New-Bell (from Douala, FWH=0.90667) for the *pfcrt* gene (Table 3.3). Equally for the *pfmdr1* gene, FWH value was negative in all the populations except in Bertoua (FWH=0.68095) (Table 3.4). However, none of these FWH values was statistically significant for both the genes.

Table 3.3. Haplotype diversity estimates and neutrality tests for the *pfcrt* gene

Populations	Number of haplotypes (h)	Haplotype diversity (Hd)	Nucleotide diversity		Tajima's D (TD)	Fu&Li's F* (FLF*)	Fu&Li's D* (FLD*)	Fay&Wu's (FW-H)	Fu's Fs (Fs)
			Π	θ					
Ebolowa (n=41)	3	0.493	0.00715	0.00354	**2.41921***	**1.68355***	1.02439	-1.47439	4.218
Douala (n=37)	5	0.410	0.00573	0.00544	0.14403	0.36518	0.38699	-3.63964	0.748
Bertoua (n=21)	6	0.695	0.00880	0.00737	0.63011	-0.29382	- 0.60726	-0.45238	2.072
Yaounde (n=24)	3	0.359	0.00480	0.00406	0.50289	1.06309	1.08443	-4.24638	2.032
Kye-ossi (n=32)	2	0.444	0.00678	0.00376	1.98462	1.53953	1.05080	-2.12903	5.671
New-bell (n=25)	7	0.750	0.00866	0.00602	1.31323	0.84425	0.48652	0.90667	-0.355
Douala + Nbell (n=62)	9	0.641	0.00823	0.00565	1.16216	0.13374	-0.39298	-0.87784	-0.508
Pooled pops (n=180)	10	0.534	0.00729	0.00525	0.86468	-0.71737	-1.40039	-1.43948	-0.205

n. Population sample size

***** Significant at the level 5%

Table 3.4. Haplotype diversity estimates and neutrality tests for the *pfmdr1* gene

Population	Number of bp	Number of haplotype (h)	Haplotype diversity (Hd)	Nucleotide diversity		Tajima's D (TD)	Fu&Li's F* (FLF*)	Fu&Li's D* (FLD*)	Fay&Wu's (FW-H)	Fu's Fs (Fs)
				π	θ					
Ebolowa (n=41)	510	3	0.389	0.00088	0.00137	**-0.77982**	**-1.59958**	-1.62762	-0.80244	0.040
Douala (n=37)	510	3	0.287	0.00079	0.00188	-1.41117	-2.21949	-2.11813	-1.13964	-0.232
Bertoua (n=21)	510	4	0.614	0.00233	0.00436	-1.54473	**-2.7248***	**-2.6773***	0.68095	0.458
Yaounde (n=24)	561	5	0.312	0.00099	0.00191	-1.31952	-1.14207	-0.85599	-2.80435	-2.464
Kye-ossi (n=32)	561	6	0.581	0.00170	0.00310	-1.31004	-2.30806	-2.26814	-1.70161	1.700
New-bell (n=25)	510	2	0.333	0.00065	0.00052	0.42898	0.65000	0.61722	-1.00000	0.815
Yaounde (n=24)	510	4	0.308	0.00077	0.00158	-1.27878	-1.54107	-1.35733	-1.21014	-1.936
Kye-ossi (n=32)	510	4	0.504	0.00143	0.00292	-1.43293	**-2.7211***	**-2.7221***	-0.34677	-0.292

n. Population sample size

***** Significant at the level 5%

3.2.2.3. Tests of detection of positive natural selection signature

In order to find out if the Darwinian positive natural selection is playing any role on the evolution of the drug resistant genes *pfcrt* and *pfmdr1* in Cameroon, several statistical tests were conducted. All of them are based on the estimation of the rates of non synonymous mutations per non synonymous sites (dN) versus the synonymous mutations per synonymous sites (dS). This is because the portion of the gene of our interest is a coding region and this approach has been proven to be so far the best way to detect signature of natural selection on coding regions. Thus, it was possible to perform the MK test in two populations out the six sampled populations for the *pfcrt* gene and in four out of six for the *pfmdr1* gene. The two populations (Bertoua and New-Bell) for the *pfcrt* gene exhibited a positive value of the neutrality index and a negative value of alpha (α) (Bertoua: NI=1.333; α=-0.333; New-Bell: NI=5.000; α=-4.000) (Table 3.5). Analogous results were observed in four populations (Ebolowa, Bertoua, Yaounde and Kyeossi) in the case of *pfmdr1* (Table 3.6). None of these results obtained from the MK test was statistically significant in the case of the *pfcrt* gene. Conversely for the *pfmdr1* gene, this test was statistically significant ($P<0.05$) for two populations out of the four (Table 3.6) using the G-test (Yaounde: G-value=5.271; Kyeossi: G-value=4.035).

The second approach used in the present study to detect the signature of positive natural selection was based on the estimation of the Ka/Ks ratio. The result of this test revealed for all the populations for both the genes an omega

(ω) value less than 1 (ω<1) even thought, these results were not statistically significant (P-value<0.05). Similar findings were observed once all the populations were pooled together for the *pfcrt* gene (Table 3.5 and Table 3.6).

Additionally to the MK and the Ka/Ks ratio tests, the codon based Z-test of selection test was conducted using the dN and dS values calculated independently for each population sampled. The hypothesis of positive selection was tested and the P-values of the Z-test for selection were found to be significantly lesser than the level of significance (P<0.05) for the *pfcrt* gene in almost all the populations (Table 3.5) excluding Bertoua (East region). Comparable observation was made when all the populations were pooled together (Table 3.6). In the case of the *pfmdr1* gene, the P-values of the Z-test for selection were found to be higher than the level of significance (P>0.05), meaning not significant in any of the populations (Table 3.6).

Table 3.5. Summary of tests of selection for the *pfcrt* gene

Populations	Mac Donald test		Z-test				Ka/Ks ratio		
	Neutrality index (NI)	Alpha (α= 1-NI)	dN	dS	P HA: dN>dS	P HA: dN<dS	Ka	Ks	ω
Ebolowa (n=41)	n.d	n.d	1.888	0.000	**0.047***	1.000	0.02885	0.03571	0.804
Douala (n=37)	n.d	n.d	0.007	0.000	**0.038***	1.000	0.02885	0.03571	0.804
Bertoua (n=21)	1.333	-0.333	0.010	0.002	0.09	1.000	0.02885	0.03571	0.804
Yaounde (n=24)	n.d	n.d	0.006	0.000	**0.05***	1.000	0.02885	0.03571	0.804
Kye-ossi (n=32)	n.d	n.d	0.009	0.000	**0.05***	1.000	0.02885	0.03571	0.804
New-bell (n=25)	5.000	-4.000	0.011	0.001	**0.05***	1.000	0.01440	0.03593	0.395
Douala + Nbell (n=62)	1.500	-0.500	0.010	0.001	**0.042***	1.000	0.02885	0.03571	0.804
Pooled pops (n=180)	0.600	0.400	0.009	0.000	**0.05***	1.000	0.02885	0.03571	0.804

n.d. not defined **dN.** Number of Non synonymous mutations **HA.** Hypothesis Alternative

n. Population sample size ***** Significant at the level 5% **P.** Probability value

dS. Number of Synonymous mutations

Table 3.6. Summary of tests of selection for the *pfmdr1* gene

Populations	Mac Donald test			Z-test				Ka/Ks ratio		
	Neutrality index (NI)	Alpha (α= 1-NI)	G-test: G-value	dN	dS	P HA: N>dS	P HA: dN<dS	Ka	Ks	ω (Ka/Ks)
Ebolowa (n=41)	3.000	-2.000	0.440	0.001	0.001	1.000	0.477	0.0050	0.0596	0.08389
Douala (n=37)	n.d	n.d	n.d	0.001	0.000	0.060	1.000	0.0050	0.0596	0.08389
Bertoua (n=21)	6.000	-5.000	2.263	0.002	0.004	1.000	0.219	0.0025	0.0598	0.04181
Yaounde (n=24)	24.000	-23.000	**5.271***	0.001	0.001	0.353	1.000	0.0068	0.0719	0.09456
Kye-ossi (n=32)	10.667	-9.667	**4.035***	0.002	0.002	0.464	1.000	0.0068	0.0719	0.09456
New-bell (n=25)	n.d	n.d	n.d	0.001	0.000	0.159	1.000	0.0050	0.0596	0.08389

n.d. not defined **dN.** Number of Non synonymous mutations **HA.** Hypothesis Alternative

n. Population sample size ***** Significant at the level 5% **P.** Probability value

dS. Number of Synonymous mutations

With a view to determine specifically in which amino acids the natural selection is acting (positively or negatively), the PAML test was conducted using Bayesian approaches. Therefore, several models of the program were run. The test under the model M0 gave an omega value lesser than one (ω<1) in all the populations as well as in the pooled population for the *pfcrt* gene (Table 3.7). Interestingly, the models M2a and M8 used to find out positively selected loci in a given gene revealed on the basis of the Naïve Empirical Bayesian approach (NEB), the presence of positive selected sites in the *pfcrt* gene (Table 3.7). These sites were the amino acids sitting at the positions 3, 41, 43, 44, 45, 66 and 69 (this, considering only the second exon of the *pfcrt* gene). Bearing in mind the whole *pfcrt* gene, these positions correspond at the position 34, 72, 74, 97 and 100. Besides, the Bayesian Empirical Bayesian (BEB) approach which has been reported to be in some cases more precise than the NEB approach, revealed only three amino acids sites: 41, 44 and 66 corresponding at the full gene level at positions 72, 74 and 97 found to be positively selected by natural selection.

For the *pfmdr1* gene, the model M0 of PAML test gave an omega value lesser than one (ω<1) in all the populations. The models M2a and M8 detected positively selected sites only in one population (Kyeossi). Based on the NEB approach, no site was found to be selected by natural selection. But based on the BEB approach, a single locus was found to be positively selected by natural selection. This site was the amino acid N for asparagine sitting at the 86th position of the *pfmdr1* gene (Table 3.8).

Table 3.7. PAML parameters estimates for the *pfcrt*- exon 2

Populations	Model Code	Parameter	Estimates of parameters	Positively selected sites	
				NEB	BEB
Ebolowa (n=41)	**Mo (one ratio)**	1	ω = 0.37	Not allowed	Not allowed
	M1a (nearly neutral)	2	P_0= 0.73662 (P_1= 0.26338) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a (positive selection)	4	P_0= 0.85493, P_1= 0.00000 (P= 0.44567) ω_0=0.00000 (ω_1= 1.00000), ω_2= 2.76377	**3G, 43M, 44N, 45K, 69V**	44N
	M7 (beta)	2	P= 0.00500 q= 0.01182	Not allowed	Not allowed
	M8 (beta & ω)	5	P_0= 0.85433 (P_1= 0.14567) P= 0.00500 q= 2.10502 ω= 2.76380	**3G, 43M, 44N, 45K, 69V**	44N
Douala (n=37)	**M0**	1	ω = 0.52046	Not allowed	Not allowed
	M1a	2	P_0= 0.72605 (P_1= 0.27395) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a	4	P_0= 0.85707, P_1= 0.00000 (P= 0.14293) ω_0=0.00000 (ω_1= 1.00000), ω_2= 4044699	**3G, 41C, 43M, 44N, 45K, 66H, 69V**	41C, 44N
	M7	2	P= 0.00500 q= 0.01178	Not allowed	Not allowed
	M8	5	P_0= 0.85707 (P_1= 0.14293) P= 0.00500 q= 17.66404 ω= 4.44698	**3G, 41C, 43M, 44N, 45K, 66H, 69V**	3G, 41C, 44N, 66H
Bertoua (n=21)	**M0**	1	ω = 0.25940	Not allowed	Not allowed
	M1a	2	P_0= 0.77905 (P_1= 0.22095) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a	4	P_0= 0.84701, P_1= 0.00000 (P= 0.15299) ω_0=0.00000 (ω_1= 1.00000), ω_2= 1.83697	**3G, 43M, 44N, 45K, 69V**	44N
	M7	2	P= 0.00500 q= 0.02015	Not allowed	Not allowed
	M8	5	P_0= 0.84700 (P_1= 0.15300) P= 0.00500 q= 14.31020 ω= 1.83696	**3G, 43M, 44N, 45K, 69V**	44N

Populations	Model Code	Parameter	Estimates of parameters	Positively selected sites	
				NEB	BEB
Yaounde (n=24)	**Mo (one ratio)**	1	ω = 0.36445	Not allowed	Not allowed
	M1a (nearly neutral)	2	P_0= 0.73767 (P_1= 0.22095) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a (positive selection)	4	P_0= 0.85761, P_1= 0.00000 (P= 0.14239) ω_0=0.00000 (ω_1= 1.00000), ω_2= 2.84673	**3G, 43M, 44N, 45K, 69V**	44N
	M7 (beta)	2	P= 0.00500 q= 0.01182	Not allowed	Not allowed
	M8 (beta & ω)	5	P_0= 0.85761 (P_1= 0.14239) P= 0.00500 q= 13.97187 ω= 2.84672	**3G, 43M, 44N, 45K, 69V**	44N
Kyeossi (n=32)	**M0**	1	ω = 0.36153	Not allowed	Not allowed
	M1a	2	P_0= 0.73921 (P_1= 0.26079) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a	4	P_0= 0.85922, P_1= 0.00000 (P= 0.14078) ω_0=0.00000 (ω_1= 1.00000), ω_2= 2.90637	**3G, 43M, 44N, 45K, 69V**	44N
	M7	2	P= 0.00500 q= 0.01182	Not allowed	Not allowed
	M8	5	P_0= 0.85921 (P_1= 0.14079) P= 0.00500 q= 4.11002 ω= 2.90640	**3G, 43M, 44N, 45K, 69V**	44N
New-Bell (n=25)	**M0**	1	ω = 0.30080	Not allowed	Not allowed
	M1a	2	P_0= 0.79560 (P_1= 0.20440) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a	4	P_0= 0.86593, P_1= 0.00000 (P= 0.13407) ω_0=0.00000 (ω_1= 1.00000), ω_2= 2.51045	**3G, 41C, 43M, 44N, 45K, 69V**	41C, 44N
	M7	2	P= 0.00500 q= 0.01198	Not allowed	Not allowed
	M8	5	P_0= 0.86593 (P_1= 0.13407) P= 0.00500 q= 1.89397 ω= 2.51046	**3G, 41C, 43M, 44N, 45K, 69V**	41C, 44N

Populations	Model Code	Parameter	Estimates of parameters	Positively selected sites	
				NEB	BEB
Douala + New-Bell (n=62)	**Mo (one ratio)**	1	ω = 0.	Not allowed	Not allowed
	M1a (nearly neutral)	2	P_0= 0. (P_1= 0.) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a (positive selection)	4	P_0= 0., P_1= 0.00000 (P= 0.) ω_0=0.00000 (ω_1= 1.00000), ω_2=	**3G, 43M, 44N, 45K, 69V**	44N
	M7 (beta)	2	P= 0. q= 0.	Not allowed	Not allowed
	M8 (beta & ω)	5	P_0= 0. (P_1= 0.) P= 0. q= ω=	**3G, 43M, 44N, 45K, 69V**	44N
All Populations (n=180)	**M0**	1	ω = 0.26629	Not allowed	Not allowed
	M1a	2	P_0= 0.78890 (P_1= 0.21110) ω_0=0.00000 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a	4	P_0= 0.84961, P_1= 0.00000 (P= 0.15039) ω_0=0.00000 (ω_1= 1.00000), ω_2= 2.08833	**3G, 41C, 43M, 44N, 45K, 66H, 69V**	41C, 44N
	M7	2	P= 0.00500 q= 0.02034	Not allowed	Not allowed
	M8	5	P_0= 0.84960 (P_1= 0.15040) P= 0.00500 q= 17.20291 ω= 2.08834	**3G, 41C, 43M, 44N, 45K, 66H, 69V**	41C, 44N, 66H

Positive selected sites are inferred at P= 95% with those reaching 99% shown in bold. The reference sequence used is the homologous sequence of the chloroquine resistant gene from *Plasmodium reichenowi* retrieved from Genedb with the accession number PRCDC 0707200.

Table 3.8. PAML parameters estimates for the *pfmdr1* gene

Populations	Model Code	Parameter	Estimates of parameters	Positively selected sites	
				NEB	BEB
Ebolowa (n=41)	**M0 (one ratio)**	1	ω = 0.02619	Not allowed	Not allowed
Bonassama (n=37)	**M0**	1	ω = 0.10085	Not allowed	Not allowed
Bertoua (n=21)	**M0**	1	ω = 0.07143	Not allowed	Not allowed
Yaounde (n=24)	**M0**	1	ω = 0.05328	Not allowed	Not allowed
New-Bell (n=25)	**M0**	1	ω = 0.03122	Not allowed	Not allowed
Kyeossi (n=32)	**M0**	1	ω = 0.07024	Not allowed	Not allowed
	M1a	2	P_0= 0.92859 (P_1= 0.07141) ω_0= 0.00513 (ω_1= 1.00000)	Not allowed	Not allowed
	M2a	4	P_0= 0.92847, P_1= 0.04386 (P= 0.02767) ω_0= 0.00508 (ω_1= 1.00000), ω_2= 1.00000	None	86N
	M7	2	P= 0.01131 q= 0.17861	Not allowed	Not allowed
	M8	5	P_0= 0.93258 (P_1= 0.06742) P= 0.01060 q= 0.34262 ω= 1.00000	None	86N

Positive selected sites are inferred at P= 95% with those reaching 99% shown in bold. The reference sequence used is the homologous sequence of the chloroquine resistant gene from *Plasmodium reichenowi* retrieved from Genedb with the accession number PRCDC_0522100.

In order to have more accurateness of analyses performed to detect specific sites evolving on the effect of natural selection, Fast Unbiased Bayesian Approximation test using the web-based interface Datamonkey (http://www.datamonkey.org/; Pond et al., 2005b) was also conducted (FUBAR; Murrell et al., 2013). Fascinatingly, the results depicted the role of positive selection in almost all the amino acids (Tables 3.9 and 3.10) detected by the PAML test for the *pfcrt* gene. For the *pfmdr1* gene, positive selection could be found not only on the amino acid sitting at the 86th position as revealed by the PAML test, but also on the amino acids at the positions 87th, 152nd, 169th and 184th (Figure 3.24) with the strongest emphasis on the 184th position (posterior probability 0.99).

Figure 3.24. Evolutionary analyses of the *pfmdr1* gene using the web-based interface Datamonkey

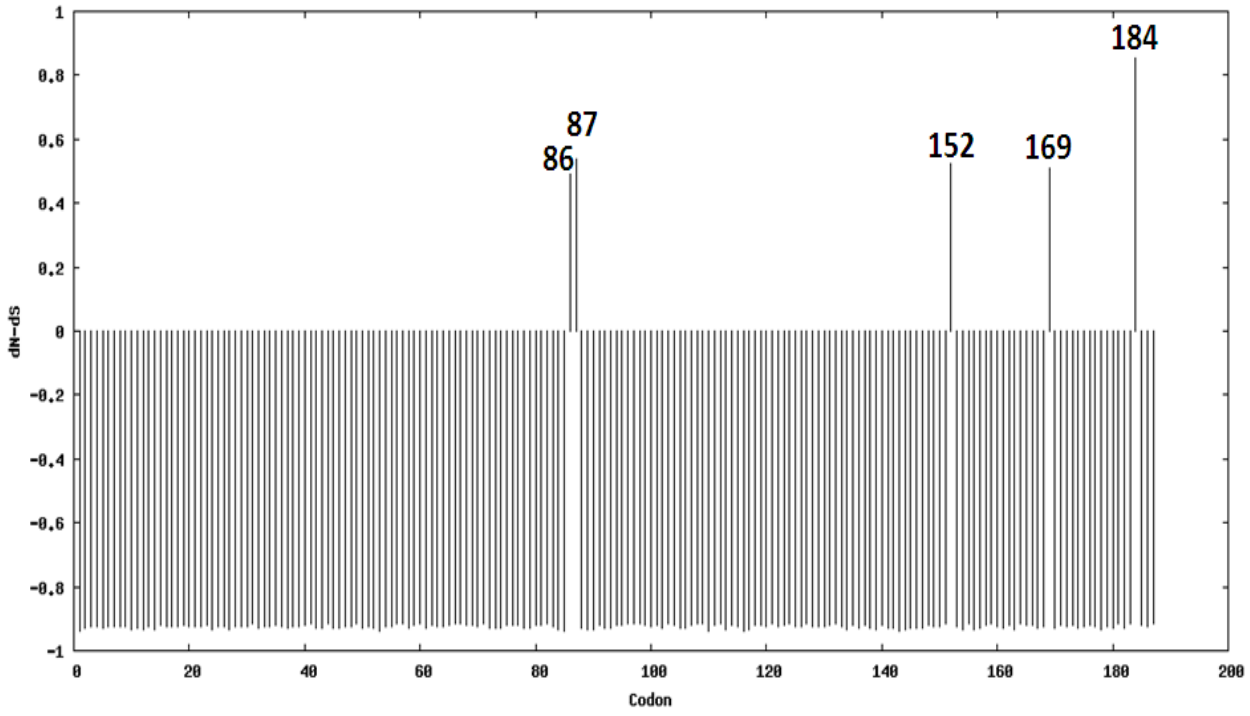


Table 3.9. Evolutionary analyses of the *pfcrt* gene using the web-based interface Datamonkey

Populations	Codons positively selected	E[dS]	E[dN]	Normalized E[dN-dS]	Posterior probability	Bayes Factor
Ebolowa (n=41)	**44 N**	1	5.77	4.77037	0.982963	545.801
Douala (n=37)	**3 G**	1	4.73	3073176	0.996565	97.5585
	41 S	1	4.94	3.94381	0.999709	15221.8
	43 M/I	1	4.69	3.68913	0.947892	80.5033
	44 N	1	4.94	3.94378	0.999703	14901.9
	45 T	1	4.73	3.73123	0.956458	97.3074
	66 L	1	4.69	3.69212	0.9485	81.586
	69 I	1	4.75	3.75379	0.961047	109.292
Bertoua (n=21)	**44 N**	1	3.24	2.24374	0.99129	831.323
Yaounde (n=24)	**44 N**	1	5.62	4.62035	0.98287	531.972
Kyeossi (n=32)	**44 N**	1	6.05	5.05186	0.985664	659.458
New-Bell (n=25)	3 G	1	3.60	2.60207	0.881988	51.9399
	41 S	1	4.07	3.073	0.998609	4987.86
	44 N	1	4.07	3.07282	0.998565	4831.54
	45 T	1	3.61	2.60772	0.883388	52.6467
	69 I	1	3.66	2.66265	0.89699	60.5163

Table 3.10. Analyses using the web-based interface Datamonkey for the *pfcrt* pooled populations

Populations	Codons positively selected	E[dS]	E[dN]	Normalized E[dN-dS]	Posterior probability	Bayes Factor
Douala + New-Bell (n=62)	**41 S**	1	3.07	2.06749	0.997246	1614.11
	44 N	1	3.07	2.06732	0.997189	1659.77
Pooled populations (n=180)	**41 S**	1	2.60	1.59604	0.991932	656.699
	44 N	1	2.60	1.59513	0.991573	628.439

Positive selected sites are inferred at P= 95% with those reaching 99% shown in bold. The reference sequence used is the homologous sequence of the chloroquine resistant gene from *Plasmodium reichenowi* retrieved from Genedb with the accession number PRCDC 0707200.

Codon Position in the alignment.

E[dS] Posterior mean of the synonymous substitution rate at the site.

E[dN] Posterior mean of the non-synonymous substitution rate at the site.

E[dN-dS] Posterior mean of the dN-dS difference

Posterior Pr{dN>dS} Posterior probability for positive selection (dN>dS) at the site.

Bayes Factor{dN>dS} Bayes Factor (posterior odds/prior odds) for positive selection (dN>dS) at the site.

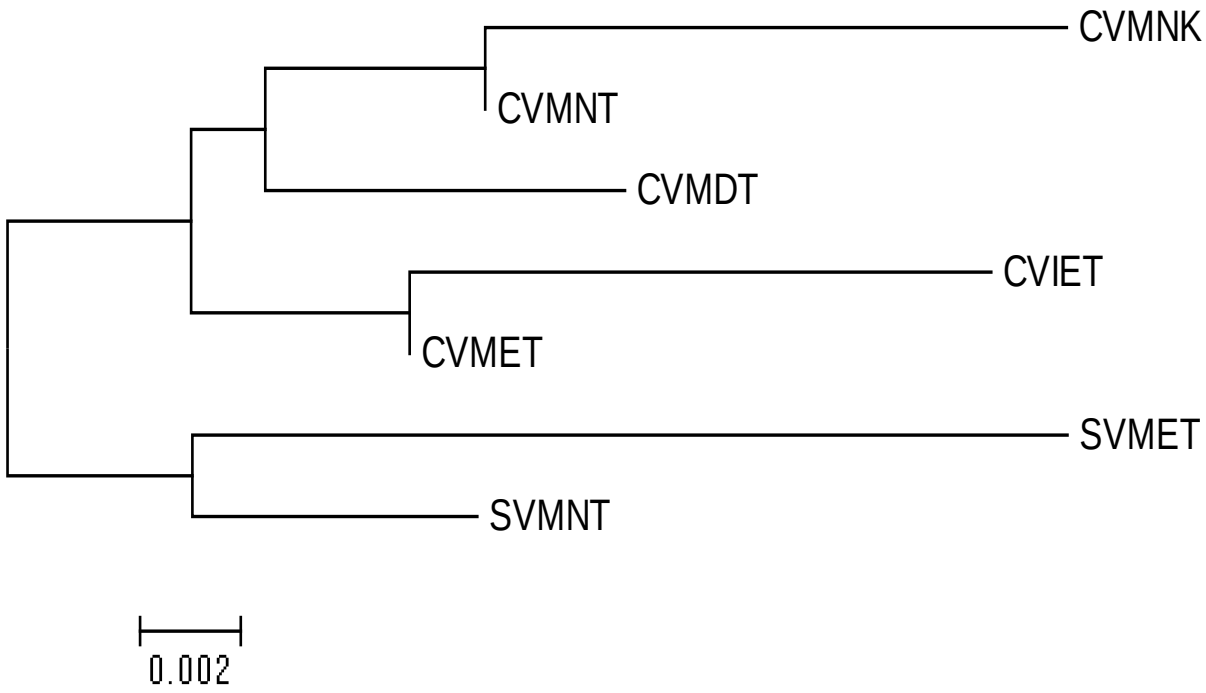
Posterior Pr{dN<dS} Posterior probability for negative selection (dN<dS) at the site.

Bayes Factor{dN<dS} Bayes Factor (posterior odds/prior odds) for negative selection (dN<dS) at the site.

3.2.2.4. Phylogenetic analysis of the *pfcrt* gene

With a view to determine genetic interrelationships between the different CQ-*pfcrt* haplotypes from Cameroon, a NJ phylogenetic tree was constructed (Figure 3.25). The results revealed three clades: one composed of the CQ-sensitive mother haplotype CVMNK and the derived single and double mutants; one composed of the CQ-resistant mother haplotype CVIET and the derived single mutant; and the last clade constituted of the derived CQR haplotypes double and triple mutants.

Figure 3.25. NJ phylogenetic tree of the *pfcrt* haplotypes of Cameroonian *P. falciparum*

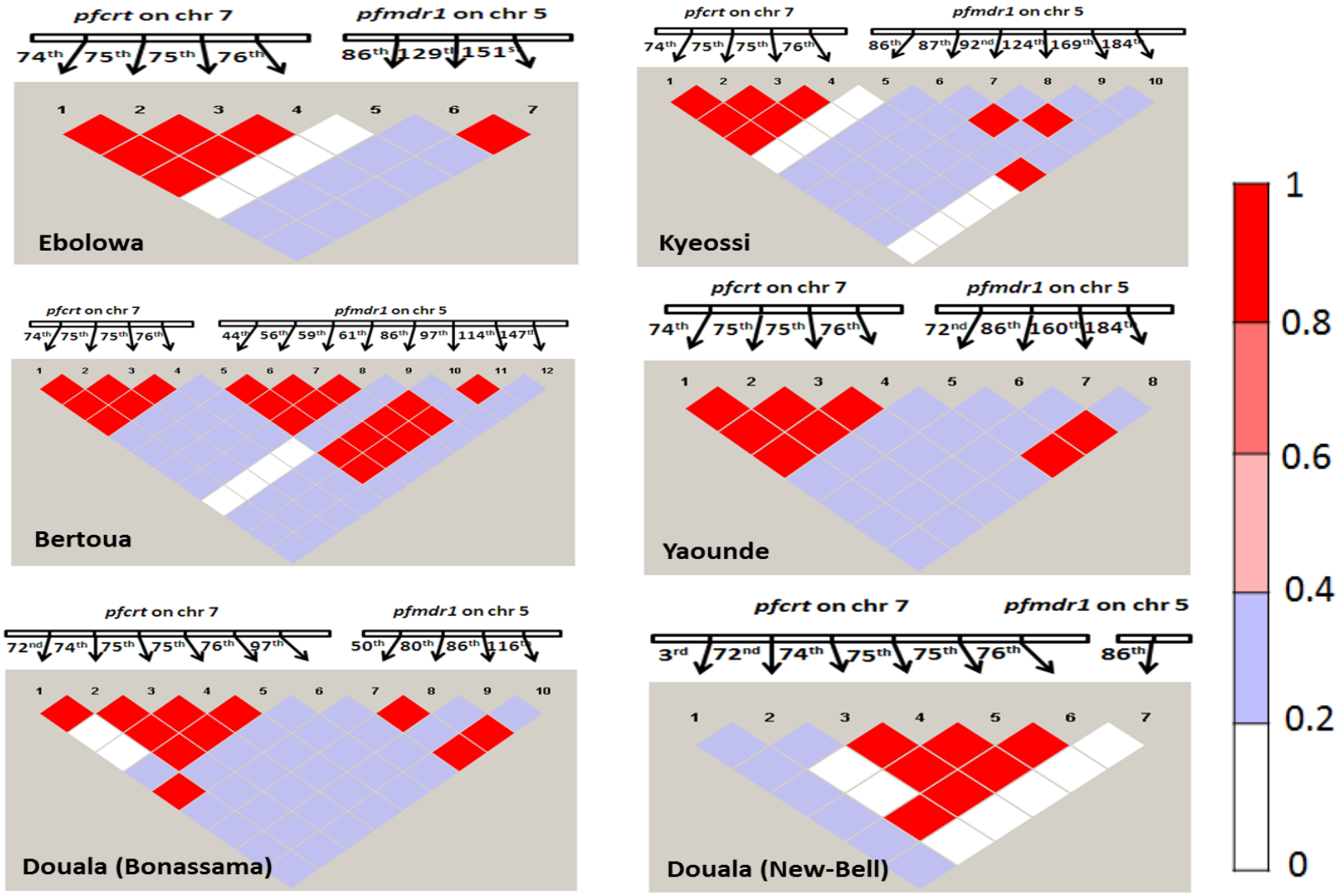


3.2.2.5. Tests for genetic association between SNPs present in both the genes *pfcrt* and *pfmdr1*

In order to find out if there is a possible association between the *pfcrt* and *pfmdr1* genes in the Cameroonian populations, the test of linkage disequilibrium (LD) was performed between SNPs within each gene separately and between both the genes (Figure 3.26). With regard to the intragenic association for the *pfcrt* gene, a strong LD in general between SNPs at the 72nd, 74th, 75th and 76th position was found in all the populations (Figure 3.26). Interestingly, in Bonassama (from Douala) where the novel mutation (H97L) was identified, a strong LD between that SNP and the one sitting at the 72nd position was observed. The results were variable in the case of the *pfmdr1* gene (Figure 3.26). A strong LD was found between several novel SNPs (Figure 3.26). Intriguingly, in Kyeossi and Yaounde where the 184th amino acid was covered, a strong LD was noticed between SNPs at the 86th and 184th positions.

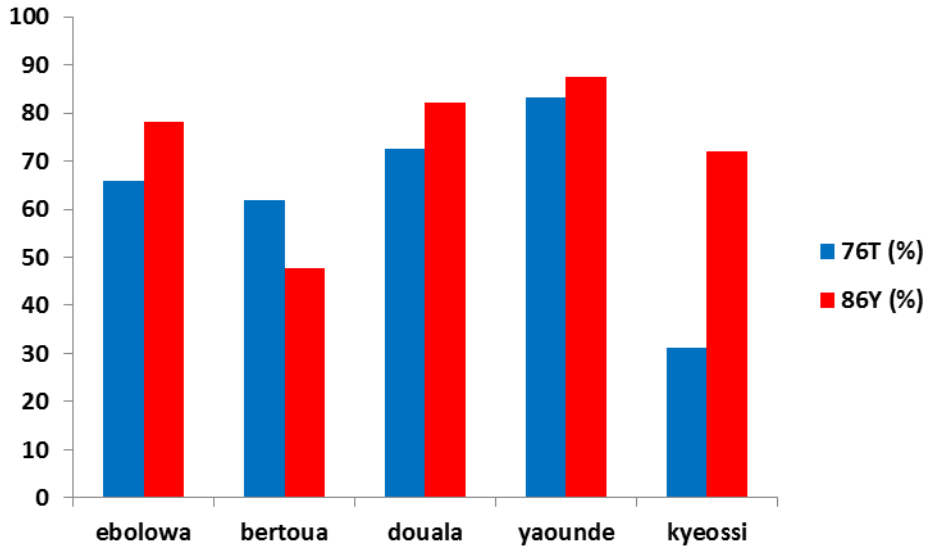
The analysis for the genetic association between the *pfcrt* and *pfmdr1* genes revealed that, apart from Ebolowa, Bertoua, Kyeossi and New-Bell populations where there was absolutely no LD between the 86th SNP in the *pfmdr1* gene and the 76th SNP in the *pfcrt* gene; in Bonassama (from Douala) and Yaounde, there was a very weak LD between these two SNPs (Figure 3.26).

Figure 3.26. LD analysis between the *pfcrt* and *pfmdr1* genes



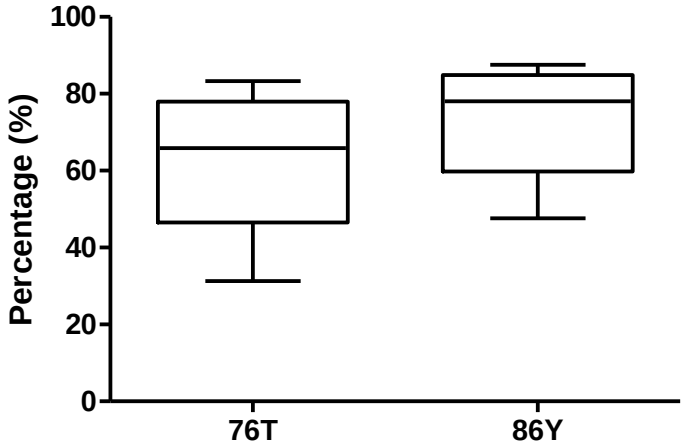
In addition to the LD analysis, the prevalence of the K76T mutation in the *pfcrt* gene along with the prevalence of 86Y in the *pfmdr1* gene were estimated (Figure 3.27) and compared through the paired t-test using the software GraphPad prism (version 5.0). The frequency of each codon mutant seemed to be not different in every population (Figure 3.28).

Figure 3.27. Prevalence of the *pfcrt*-76T and *pfmdr1*-86 Y mutations



Box and whisker plot (Figure 3.28) is a graphical representation of the 2 variables (76T and 86Y frequency) after performing the paired t-test. The whiskers (upper and lower) show the maximum and minimum values. The line in the box represents the arithmetic mean of each variable. This test revealed no statistical difference between the percentage of the mutations 76T and 86Y across the Cameroonian populations sampled in the present study.

Figure 3.28. Box and whisker plot



It was for further interest to know whether there was a relationship between these two codons mutants. Therefore, a Pearson correlation coefficient was performed using the same software as for the t-test. The result (r=0.3805; P=0.2637) showed that there was no association between the codon mutant 76T in the *pfcrt* gene and the codon mutant 86Y in the *pfmdr1* gene.

CHAPTER 4: DISCUSSION

4. DISCUSSION

Accurate diagnosis of malaria parasites holds the key for successful intervention through chemotherapy. To this respect, in recent years PCR-based malaria diagnosis has emerged as a gold standard technique for correct identification of single as well as mixed malaria parasitic infections (Snounou et al., 1993; Johnston et al., 2006; Gupta et al., 2010). Identification of specific malaria parasite infection through PCR habitually is followed by DNA sequencing of the 18S rRNA gene for confirmatory purpose (Gupta et al., 2010a). In many recent studies, some malaria parasite species-specific genes, e.g. *pvcsp*, *pvdhfr*, etc. have often been sequenced as additional markers for further confirmation purpose in different malaria endemic settings (Dhorda et al., 2011; Mendes et al., 2011).

In the present study, PCR diagnostic assays with the 18S rRNA gene revealed out of 244 malaria positive samples, the presence of 226 *P. falciparum*, sixteen *P. vivax* and two mixed infections due to *P. falciparum* and *P. vivax* in Cameroon. The results of DNA sequencing and sequence alignments of the 18S rRNA gene therefore re-confirmed the ability of the molecular diagnostic approach in detecting the single infections of *P. vivax* as well as mixed infection due to these two species by PCR amplification (Snounou et al., 1993; Johnston et al., 2006; Gupta et al., 2010). Although the DNA sequences of the 18S rRNA gene of Cameroonian *P. falciparum* yielded 100% similarity with the reference

sequence, for *P. vivax*, when compared with the reference sequence of the SAL-1 strain, a novel single nucleotide polymorphism (SNP) was found at the 72nd nucleotide position in one of the mixed-infected patients (Figure 3.6). Very similar results following analogous protocols (PCR diagnostic assays followed by DNA sequencing) could determine high incidences of mixed malaria parasite infection in India (Gupta et al., 2010a). Taking together the results of the present study with the study from India (Gupta et al., 2010a), it could therefore be highlighted that nested PCR amplification of the malaria parasite 18S rRNA gene followed by DNA sequencing and sequence alignment with the reference sequences of the respective 18S rRNA genes of malaria parasites could serve as a valuable aid for molecular diagnosis of malaria infection.

DNA sequencing and sequence analyses of the *pvmdr1*, *pvcsp* and *pfmdr1* genes in the Cameroonian *P. vivax* and *P. falciparum* isolates could furthermore validate the results obtained with PCR diagnostic assay. While the four nucleotide substitutions found in the *pvmdr1* gene in Cameroonian *P. vivax* might be population-specific in nature, the observed sequence homology of the *pvmdr1* gene in the present study with the reference *P. vivax* SAL-1 strain reconfirms the presence of *P. vivax* infection in native Cameroonians. The results on the whole on the DNA sequencing and sequence comparison with reference strains of the *pvmdr1* and *pfmdr1* genes therefore are in agreement with the findings with the PCR diagnostic approach followed by DNA sequencing of the 18S rRNA gene (see above). It is thus recommended that associated DNA sequencing and sequence alignments of additional malaria

parasite species-specific genetic markers should be conducted to confirm the observations with PCR diagnostic assay targeting the 18S region of the rRNA gene.

Besides, genotyping of the *pvcsp* gene encompassing the repeats region is often considered to differentiate among three different strains circulating in the global *P. vivax* populations (de Souza-Neiras et al., 2007). To this context, the *P. vivax* VK210 strain type (also called classic type) is known to be distributed in higher frequency in comparison to the VK247 strain in almost all *P. vivax* endemic regions (de Souza-Neiras et al., 2007). However, in some countries (including African countries) where genotyping of the *pvcsp* gene has been performed, such as India, Afghanistan, Angola, Equatorial Guinea, both the *pvcsp* variants (VK210 and VK247) have been found to be circulating in different localities of the same country (Kim et al., 2006; Dhangadamajhi et al., 2010; Mendes et al., 2011). Finding of *P. vivax* strains of only VK247 type in Cameroon in the present study is consequently interesting. Considering the limited number of *P. vivax* samples in the present study coupled with the non-existence of similar studies so far in Cameroon, such a conclusion (on distribution of only VK247 strain in Cameroon) should be dealt with caution; genotyping more number of isolates will ascertain the distribution of specific strain types in Cameroonian *P. vivax*.

The *Duffy*-status of the eighteen Cameroonians infected with *P. vivax* could be ascertained by direct sequencing after PCR amplification of the part of gene

covering the promoter region with the -33rd nucleotide position. Very similar approach has been taken in studies in Indians (Chittoria et al., 2012) and Ethiopians (Woldearegai et al., 2013) in successfully determining the *Duffy* status of humans. Finding of all the eighteen Cameroonians homozygous for the mutated C nucleotide therefore justifies that all the Cameroonians infected with *P. vivax* are homozygous *Duffy*-negative, and that *P. vivax* can infect *Duffy*-negative Cameroonians (Fru-Cho et al., 2014) and in other African countries (Ryan et al., 2006; Menard et al., 2010; Mendes et al., 2011; Wurtz et al., 2011; Woldearegai et al., 2013). The present observation on the *P. vivax* infection in *Duffy*-negative Cameroonians not only corroborate the recent report on *Duffy*-negative native adults from Cameroon by Fru-Cho et al., 2014; but also taken together with similar findings in other African countries, indicate therefore that *P. vivax* might have evolved other, hitherto unknown mechanism(s), to infect *Duffy*-negative humans. Considering the emerging hypothesis on the African origin of *P. vivax* (Liu et al., 2014), it seems highly likely that Africans in general have been selected for the *Duffy*-negative mutation due to long exposure to *P. vivax* infection. However, since the current rate of transmission of *P. vivax* is fairly low in Africa in general, and Cameroon in particular, other host-genetic factor(s) might also be playing significant role in *P. vivax* infection in *Duffy*-negative host genetic background. Furthermore, two out of the five principal malaria vectors of Africa (*An. gambiae* and *An. arabiensis*) responsible for majority of malaria transmission in Cameroon have been reported to have high vectorial capacity to transmit *P. vivax* malaria

parasite (Ryan et al., 2006; Taye et al., 2006; Culleton et al., 2009). Since *P. vivax* infection in asymptomatic native cameroonians has been reported in the south-western part of the country (Fru-Cho et al., 2014) and the fact that the southern part of Cameroon borders Equatorial Guinea where incidences of *P. vivax* infection in indigenous human populations have already been reported (Mendes et al., 2011), it seems imperative that *P. vivax* isolates are capable of successful transmission in these African countries. Although the exact mechanism leading to successful infection of *P. vivax* to *Duffy*-negative Africans has not yet been established, it seems for now that *P. vivax* infection might have been mediated independently (or in combination) of factors like, (i) evolution and spread of specific *P. vivax* strain capable of infecting the *Duffy*-negative humans, (ii) host genetic susceptibility and (iii) vectorial competence. Considering the simple genomic architecture, high mutation rate, less generation time (in comparison to humans and mosquitoes), it seems highly likely that the *P. vivax* strains circulating in sub-Saharan Africa might have evolved to the extent for being able to infect the *Duffy*-negative Africans. However, whether such *P. vivax* strains are specific to Africa or introduced from other *P. vivax* malaria endemic countries is not known. More in-depth sampling of malaria parasites from sub-Saharan Africa and following population genomic studies with multiple putatively neutral SNPs already developed in this malaria parasite (Gupta et al., 2010b; Gupta et al., 2012) could unravel the evolutionary history of African *P. vivax*.

In regard to the second main objective of this study which was to infer evolutionary pattern of the two most common antimalarial drugs resistant genes *pfcrt* and *pfmdr1*, sequencing of the cameroonian *pfcrt* gene revealed in total six different non synonymous mutations coding for the amino acids located at the positions 72, 74, 75, 76 and 97 of the whole *pfcrt* gene. While the changes at the amino acids positions 72, 74, 75 and 76 have already been reported in Cameroon and all the others malaria endemic parts of the globe (Basco et al., 2002; Awasthi and Das, 2013), the change at the 97th position is novel in Cameroon but has already been reported in some Asian countries like India and Thailand (Das Sutar et al., 2011; Setthaudom et al., 2011). Only one isolate (from Douala) out of the 180 in total, harbors this mutation. Similarly for the *pfmdr1* gene, out of the 20 mutations found in the present study, only two have been already reported in Cameroon so far: 86th and 184th mutations (Basco and Ringwald, 2002). Among the remaining 18 novel mutations, only one (the 44th mutation) has already been reported earlier in low frequency in West Africa (based on the *pfmdr1* variant information present on MalariaGen website, http://www.malariagen.net/apps/pf/2.0/). These *pfmdr1* novel mutations are segregated only in three populations out of the six sampled: Ebolowa (one isolate), Bertoua (one isolate) and Kyeossi (two isolates). The findings of many novel mutations within the *pfmdr1* gene coupled with the findings of various new CQ-*pfcrt* haplotypes in Cameroon might signify that both of the genes are under a genetic reconstruction. This hypothesis is further corroborated by the high and variable nucleotide and haplotype diversity

observed across the different populations for both the genes with Bertoua (in the eastern region of the country) harboring the highest diversity for the *pfcrt* and *pfmdr1* genes. In fact, in a previous study done in Cameroon (Mbacham et al., 2010), expression of the markers for resistance: *pfcrt* and *pfmdr1* genes, was found to be distinctive between the southern and the northern regions of the country. It has therefore been hypothesized that genetic structure of malaria parasite in Cameroon might be different from the rest of Africa. The authors have then suggested that, in circumstances of variations across the continent or the sub-regions, a different type of drug policy may be needed probably embracing multiple first line therapies rather than a one drug-strategy-fits-all approach. With respect to this, permanent studies of molecular markers all over Cameroon can help to map local differences in drug resistance.

The key mutations for antimalarial drugs resistance in both the genes *pfcrt* and *pfmdr1* (76T and 86Y mutations respectively), have been denoted in high prevalence in all the Cameroonian populations and this is very scarce. This result is in accordance with the results of a recent investigation made by Menard et al., 2012 on these mutations in Yaounde, a highly urbanized city of Cameroon. In their study, they found high prevalence of these mutants [*pfcrt* 76T (83%) and *pfmdr1* 86Y (93%)] and they have attributed this occurrence to a possible selection of these mutations by the current antimalarial drug artesunate-amodiaquine (AS-AQ). Besides that, it has been hypothesized that AQ has a great tendency to select the CQR-*pfcrt* haplotype SVMNT more likely

than the others CQR-haplotypes (Sa and Twu, 2010). Surprisingly, in the present study, the majority of CQR-*pfcrt* haplotype were of CVIET type and interestingly, the analysis of LD between the *pfcrt* and *pfmdr1* SNPs revealed a very weak or no association between the mutation occurring at the 76th position in the *pfcrt* gene and the one occurring at the 86th position of the *pfmdr1* gene. This is further confirmed by the observation of no significant statistical correlation (P>0.05) between these two mutants after performing the correlation of Pearson test. Even though it has been assumed that the *pfmdr1* gene (86Y mutation) in collaboration with the *pfcrt* gene (76T mutation) control the level of CQR *in-vivo* and/or *in-vitro* (Valderramos and Fidock, 2006; Ferreira et al., 2011), the discussion around this association has always been subjected to a controversy. Some studies in field isolates have found strong correlation between these two genes (Babiker et al., 2001; Mittra et al., 2006; Hatabu et al., 2009; Das Sutar et al., 2011) whereas others found no correlation (Basco et al., 1996; Basco and Ringwald, 1997; Mita et al., 2006; Chauhan et al., 2014). Therefore, combining all the four facts: (i) possible selection of these two mutants by AS-AQ; (ii) selection of SVMNT by AQ as compared to CVIET; (iii) no LD between 76T and 86Y in the present work; (iv) majority of CQR-*pfcrt* haplotypes being CVIET, it seems that the current ACT in use in Cameroon (AS-AQ) might have selected only the 86Y mutant not both. This is supported by the observation of a higher prevalence of 86Y mutant (see above) as compared to 76T mutant. Also, in one of the Cameroonian city sampled (Kyeossi), majority of the *pfcrt* haplotypes was of CVMNK type (the

sensitive one) and approximately 71.9% of individuals harbored the 86Y mutation. Another alternative explanation for this observation of high prevalence of 76T mutant can be the continued presence of CQ in the environment. This is mainly because, it is well known that despite the official withdraw of CQ from the country; still this drug is used by the populations (Mbacham et al., 2005). In fact, the present high percentage of 76T mutant found here might be the result of the drug pressure exerted by CQ on the parasites before the implement of ACTs in Cameroon. As mentioned above, the ACT has been officially adopted in Cameroon in 2004 (WHO, 2008; Menard et al., 2012). However, the effective practice of the use of AS-AQ/artemether-lumefantrine (AL) has been done in 2007 (Sayang et al., 2009). Taking into consideration the fact that the samples used in this study have been collected in 2009 (two years only after the real application of AS-AQ use); plus the fact that it has been shown that once drug pressure (in this case, CQ) is not existing, there is a possible conversion (with time) of CQR strains into sensitive ones with the exception of the SVMNT type like it has been the case in some African countries such Malawi (Kublin et al., 2003) and Kenya (Mwai et al., 2009); the actual observation of high number of 76T mutants seems to be therefore justified. But, the situation can rapidly evolve and change if the SVMNT type detected in the very big cosmopolitan city of Cameroon, Douala spreads and gets fixed.

Parellely, the present study revealed great number of the *pfmdr1* mutant codon 184F (another important mutation in the *pfmdr1* gene) in Yaounde and Kyeossi

where this mutation has been successfully genotyped. Interestingly and surprisingly at a time, in both the populations, a strong LD was found between the mutant 86Y and the mutant 184F. This is interesting since it has been reported in diverse countries (Basco et al. 1996; McCutcheon et al. 1999; Duraisingh and Refour 2005; Das Sutar et al., 2011) including Cameroon (Basco and Ringwald, 1997), that there is no correlation between these two mutations. Most of the studies have revealed in contrast the 86N (wild)-184F (mutant) haplotype and the 86Y (mutant)-184Y (wild) haplotype. The haplotype NF has been shown to be associated with reduced parasite sensitivity to arylaminoalcohol quinolone drugs (such as mefloquine, lumefantrine; Sisowath et al., 2005; Sisowath et al., 2007; Dokomajilar et al., 2006) while the haplotype YY has been linked to decreased sensitivity to 4-aminoquinoline drugs (such as CQ, AQ; Holmgren et al., 2006; Holmgren et al., 2007). Accordingly, selection of NF haplotype has been consistently observed during artemether-lumefantrine (Coartem, Novatis AG, Basel) treatment in several countries including African ones (Sisowath et al., 2005; Sisowath et al., 2007; Dokomajilar et al., 2006; Gadalla et al., 2011). Moreover, it has to be noticed that the implication of the 184F mutant in CQ response was so far demonstrated in the laboratory cultured *P. falciparum* isolates (Foote et al., 1990). Although no such association could be observed in field isolates (Ojurongbe et al., 2007). The findings of statistically significant LD between 86Y and 184F in Cameroon, corroborates the findings of Chauhan et al., 2014 in two populations from *P. falciparum* low endemicity areas in India but with

high genetic diversity. In the present study, Kyeossi is having high nucleotide diversity whereas Yaounde is having low diversity. Considering that Cameroon is one the countries which highly contributes to the global deaths due to malaria (WHO, 2008), plus the fact that these two regions are situated in the southern part of the country (known to be the most endemic part of Cameroon for malaria); it is very strange to see strong LD, high diversity in a high transmission area. This observation is in contrast with the previous hypothesis of strong LD and low genetic diversity (Anderson et al., 2000). But, recent studies have shown that also regions experiencing high recombination rates show signs of ongoing selection at linked sites (Ingvarsson, 2010). Whatever the case might be, it is frightening to see high numbers of 86Y and 184F mutants and more Cameroonian populations should be investigated with a regard on these mutants.

The results on the haplotype diversity of the CQR-*pfcrt* gene revealed the occurrence of S(agt)VMNT haplotype. So far only one African country (Tanzania) has reported the presence of *P. falciparum* isolates bearing the S(agt)VMNT haplotype (Alifrangis et al., 2006). High haplotype diversity of the CQR-*pfcrt* gene indicated in the present study essentially mean that the *pfcrt* gene governing CQ resistance in *P. falciparum* is under massive genetic reconstruction in Cameroon as proposed in India (Das and Dash, 2007). Such genetic reformation might have been propelled by either (i) selection pressure exerted by both misuse of CQ in the field (Das and Dash, 2007) and/or (ii) due to pressure exerted by amodiaquine (AQ), an antimalarial that is a part of the

WHO recommended ACTs for treatment of uncomplicated *P. falciparum* malaria in Cameroon. The second contention is justified by the fact that, AQ has a very similar genetic target (*pfcrt*) as CQ (Menard et al., 2012; Sa and Twu, 2010). Although the S(agt)VMNT haplotype could only be found in Douala, but considering its higher fitness property and Douala being a cosmopolitan city in Cameroon, it can spread to other parts fairly quickly. Whatever the case may be, observations on the high haplotype diversity of the CQR-*pfcrt* haplotypes coupled with appearance of the S(agt)VMNT type is daunting, which can pose a greater challenge to the malaria control program of Cameroon than before. This is true since it has been predicted from previous studies (Sa and Twu, 2010) that the continued use of amodiaquine in combination therapies is hazardous in regions where *P. falciparum* isolates bearing SVMNT haplotype occur.

Menard et al., 2012 have explained the persistence of the *pfcrt* mutant codon (76T in particular) in Cameroon by a feature which consists in a possible association between fitness loss of mutant *P. falciparum* with development of compensatory mechanisms able to maintain mutant parasites even in the absence of drug pressure. Thus, in order to see if any of these two genes is evolving neutrally, several neutrality tests have been performed. For the *pfcrt* gene, apart from the population of Ebolowa where the values of TD and FLF were deviating significantly ($P<0.05$) from the neutral model, all the neutrality tests were not statistically deviating from the neutral model for the rest of Cameroonians populations. For the *pfmdr1* gene, except the population of Bertoua where the FLF and FLD were significantly deviating from the neutral

model, all the neutrality tests were not statistically deviating from the neutral model for the rest of Cameroonians populations as it is the case for the *pfcrt* gene. However, since TD is known to be an indirect approach for detection of natural selection signature, the positive values observed in all the populations for the *pfcrt* gene indicates that *pfcrt* gene might be under a balancing selection. In contrast for the *pfmdr1* gene, the negative values of TD in all the populations with the exception of New-Bell population, indicates that this gene might be under a directional selection (either positive or negative selection). The observation of a negative value of FWH test in almost all the populations for both the genes (even though the test was not statistically deviating from the neutral model of evolution) indicates the presence of high frequency derived variants in the population. This finding signifies that these genes have in the past experienced the effect of positive natural selection. This is true since Genome-wide association studies have identified some drug resistance loci (including the *pfcrt* and *pfmdr1* genes) in *P. falciparum* malaria parasite showing strong signals of positive natural selection (Park et al., 2012). In other hand, the Fs-test which is known to be easily influenced by demography (the second big force of evolution), was not significantly statistically deviating from the neutral theory in the present study. This result is somehow excluding the effect of demography on the *pfcrt* as well as the *pfmdr1* genes in Cameroon.

In order to ascertain precisely if natural selection plays a role on the evolution of the *pfcrt* and *pfmdr1* genes in Cameroon, more robust tests as compared to TD were conducted. The results drawn from the MK test showed for two

populations of the *pfcrt* gene, a positive value of the neutrality index and a negative value of alpha (α). Comparable results were obtained in the four populations for the *pfmdr1* gene. These results signify the presence of purifying selection (negative selection) acting on both the drug resistant genes. However, once pooling all the populations together in the case of the *pfcrt* gene, the results indicate positive value of alpha, meaning the role of positive natural selection signature on the Cameroonian *pfcrt* gene. The results thus corroborate the findings from TD in the case of the *pfmdr1* gene.

The second test which is based on the estimation of the Ka/Ks ratio revealed for all the populations an omega value lesser than one ($\omega<1$) for the *pfcrt* as well as for the *pfmdr1* gene. Similar result was obtained with the pooled population for the *pfcrt* gene. These results suggest a presence of purifying natural selection acting on these genes and substantiate the ones observed after performing the Mac-Donald Kreitman (MK) test.

The codon-based Z-test used for testing the hypothesis of positive natural selection revealed a P-value lesser than the level of significance ($P<0.05$) for the *pfcrt* gene in almost all the populations except in Bertoua (East region). This result indicates the role of positive natural selection on the Cameroonian *pfcrt* gene. This is further supported by the P-value lesser than the level of significance ($P<0.05$) obtained once all the populations are pooled together. The exception of Bertoua population might be due to the low sample size (n= 21) compared to the rest of populations (Table 3.5). In contrast, the P-value not

significantly higher than the level of significance (P>0.05) in the case of the *pfmdr1* gene for all the populations indicates no role of positive natural selection on the evolution of the *pfmdr1* gene in Cameroon. These results confirm the findings from the MK test as well as the test based on the Ka/Ks ratio (see above) for both the genes even though in the case of the *pfcrt* gene, this conclusion is made only for the pooled population.

The PAML test conducted with the aim to find out in which amino acids in particular natural selection is acting, gave an omega value lesser than one (ω<1) under the model M0 for all the populations for the *pfcrt* and *pfmdr1* genes. This is indicating that purifying selection might play a role on the evolution of these genes as demonstrated by the previous tests conducted (see above). However, the models M2a and M8 revealed some positively selected loci in the Cameroonian *pfcrt* gene in all the sampled populations. In general, the amino acids sitting at the positions 3, 41, 43, 44, 45, 66, 69 corresponding once considering the whole *pfcrt* gene at the position 34, 72, 74, 97 and 100 are emphasized as per the NEB approach. But in particular, only three amino acids sites at positions 41, 44 and 66 corresponding at the full gene level at positions 72nd, 75th and 97th are the targets of positive natural selection as per the BEB approach. These results are further reconfirmed by the similar analysis using the web interface Datamonkey. Whatever the case might be, it seems like the block containing the mutations from 72nd till 76th positions plus the 97th position is under positive natural selection, signifying that it is possible that these mutations are not only compensatory to the 76th T mutation

the key of CQR, but they might be working in a synergistic way. This hypothesis seems to be true once looking at the LD analysis; it is clear from the picture that there is a strong LD between these five mutations. For the *pfmdr1* gene, the models of PAML test for detection of positive selected loci have detected only one locus (the 86th N amino acid) to be positively selected by natural selection. This has been detected only in one Cameroonian population: Kyeossi. Similar results were observed with Datamonkey but others sites as 87th, 152nd, 169th and 184th were added with the 184th position having the greatest posterior probability. The findings of positively selected sites in the *pfmdr1* gene only in Kyeossi might be due to the drug pressure. In fact in Cameroon, CQ (previously the first-line therapy for uncomplicated malaria) was officially withdrawn in 2002 and replaced initially by AQ monotherapy. Thereafter, ACTs, notably AS-AQ or AL was gradually introduced in 2004 (Menard et al., 2012). This situation raised the question of the evolution of *P. falciparum* resistance molecular markers in Cameroonian cities.

With a view to find out if there is some genetic inter-relationship between the different *pfcrt* haplotypes found in this study, a NJ phylogenetic analysis has been done. The results demonstrated that the malaria parasite *P. falciparum* seems well structured in term of CQ-*pfcrt* haplotypes. Similar hypothesis has been made in a precedent study (Awasthi et al., 2012). The authors suggested that there is a clear haplotype structure of the parasite across the malaria endemic parts of the world.

In conclusion, the overall results imply that, *Duffy*-negative native Cameroonians are susceptible to *P. vivax* malaria and the most common antimalarial drugs resistant genes *pfcrt*/*pfmdr1* seem to be under genetic reconstruction. While the *pfcrt* gene in Cameroon might experience simultaneously negative natural selection (purifying selection) and positive selection (restricted only to few loci), the *pfmdr1* gene seems to be a under purifying selection with exception of the population of Kyeossi where combination of both the pressures is present. Hypothesis of hitchhiking under positive selection has been proposed for shaping the evolution of the *pfcrt* gene after several studies involving microsatellites present in- and –around it (Lumb et al., 2012; Mallick et al., 2013; Wootton et al., 2002). This might be an alternative justification to the actual observation of selection on the *pfcrt* gene in Cameroon along with strong LD in the block of mutations sitting at the nucleotide position 72nd to 76th.

Whatever the case might be, deep study on the evolution course of these genes in Cameroon is needed for a complete picture about what is going on.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The present study entitled "Molecular diagnosis of malaria infection and evolutionary genetics of drug resistant genes in *Plasmodium falciparum* in Cameroon" aimed:

- To perform molecular diagnosis of malaria infection in Cameroon
- To understand genetic diversity of two drug resistant genes (*pfcrt* and *pfmdr1*) in Cameroonian *P. falciparum*
- To analyse the DNA sequences and infer evolutionary patterns of these two genes in Cameroonian *P. falciparum*

The molecular diagnosis of malaria infection in Cameroon has interestingly revealed and confirmed that *Duffy*-negative native Cameroonians too can, not only be infected by the most prevalent malaria parasite *P. vivax*, but also with both the species: the most dangerous *P. falciparum* and the most prevalent *P. vivax*.

The genetic diversity study of the drug resistant genes *pfcrt* and *pfmdr1* in *P. falciparum* samples from Cameroon showed a high diversity of both the genes, the occurrence of several new CQR-*pfcrt* haplotypes including the fittest one SVMNT. Numerous novel mutations have been detected in these genes and high frequency of the common codons mutants involved into the phenomenon of antimalarial drugs resistance 76T (for the *pfcrt* gene) and 86Y (for the *pfmdr1* gene) is observed in the southern part of the country. Furthermore, with the different statistical analyses performed in order to infer evolutionary patterns of

both the genes, the present study depicts a signature of Darwinian positive natural selection on few loci of the *pfcrt* gene and a purifying selection in general on the *pfmdr1* gene in Cameroon.

How these results are important for the Cameroonian communities? For the malaria control programme of Cameroon? Cameroon, like other African countries is already struggling to control malaria due to *P. falciparum*; additional burden of *P. vivax* mono-infection as well as *P. vivax-P. falciparum* mixed infection is daunting. Furthermore, the cases of *P. vivax* infection were found to be spread in four out of five regions of collection in the southern parts of Cameroon, which are already reeling under high malaria mortality coupled with chloroquine resistance *P. falciparum*. The situation is especially grim considering the infection capabilities of *P. vivax* in *Duffy*-negative Cameroonians and other Africans. Since the current malaria drug policy in Cameroon does not take consideration of either single *P. vivax* infection or mixed parasite infections (WHO, 2013), based on the findings of the present project on detection of native Cameroonians infected with *P. vivax* along with the recent findings from Fru-Cho et al., 2014, on the occurrence of several CQR-*pfcrt* haplotypes, it is urged that apposite measures may be taken to revise the current drug policy and a renewed vector control strategies for malaria treatment and transmission, respectively in Cameroon.

The two genes *pfcrt* and *pfmdr1* are under massive genetic reconstruction in Cameroon. Such genetic reformation might have been propelled by either (i) selection pressure exerted by both misuse of CQ (officially disapproved by the Government) in the field and/or (ii) due to pressure exerted by AQ, an antimalarial that is a part of the WHO recommended ACTs for treatment of uncomplicated *P. falciparum* malaria in Cameroon. Similar large scale DNA sequencing-based surveys of these genes in other parts of Cameroon and in other African countries could not only help in unraveling the extent of diversity of different CQR-*pfcrt* haplotypes, but also would help in choosing appropriate chemotherapeutic combinations for malaria treatment in Africa. Permanent surveillance of these genes appears to be necessary for the monitoring of antimalarial drugs resistance in Cameroon. In other hand, full length sequencing of both the genes in along with flanking regions might provide deep insight about the evolution course of these drug resistant genes in Cameroon.

Since the approach of molecular evolution has so far not yet been applied in Cameroon with the respect of malaria drug resistance, the present study represents therefore the first stone in the advancement of understanding the evolution of malaria drug resistant genes in Cameroon.

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APPENDIX

APPENDIX

1.1. 10X Buffer TBE (Tris-Boric-EDTA)

Tris Base: 108g (PH 8.0)

Boric acid: 55g

0.5M EDTA (PH 8.0: 40 ml

Dissolve and mix the components in 600m1 of water and make up the volume to 1000 ml.

1.2. 1X Buffer TBE (Tris-Boric-EDTA)

10X TBE buffer: 50 ml of

Water: 450ml

Dissolve and mix the components in 300m1 of water and make up the volume 500 ml.

1.3. Agarose gel 2%

Agarose powder: 2g

1X TBE buffer: 100 ml

Dissolve the agarose in 100m1 of 1X TBE buffer and make up the mixture homogenous by using micro oven for 2minutes 30 seconds.

1.4. Agarose gel 1%

Agarose powder: 1g

1X TBE buffer: 100 ml

Dissolve the agarose in 100m1 of 1X TBE buffer and make up the mixture homogenous by using micro oven for 2minutes 30 seconds.

1.5. Ethidium bromide (EtBr)

For a solution of 10mg/ml, 1ml of Water into the stock (in form of powder) contained in the bottle.

1.6. Tris 1M

Tris Base: 3.0285g

Dissolve the base in 10 ml of water and adjust the volume to fa total volume of 25 ml.

1.7. Ethanol 70%

Stock ethanol (95%): 15 ml

Water: 5 ml

Mix the stock solution of ethanol in 5 m1 of water to make a volume of 20 ml.

1.8. TE (Tris-EDTA) buffer

Tris 1M: 0.2 ml

0.5M EDTA (PH 8.0): 0.004 ml

Water: 19.796 ml

Mix the components in 10 m1 of water and adjust the volume to 20 ml.

1.9. 3M Sodium Acetate Buffer (pH 5.2)

Sodium acetate: 40.81 g

Water: 80 ml

Adjust the pH to 5.2 with glacial acetic acid and make up the volume to 100 ml. Sterilize by autoclaving.

LIST OF PUBLICATIONS

- **Ngassa Mbenda HG** and Das A. 2014. Occurrence of multiple chloroquine-resistant *Pfcrt* haplotypes and emergence of the S(agt)VMNT type in Cameroonian *Plasmodium falciparum*. Journal of Antimicrobial Chemotherapy **69**: 400-403.

- **Ngassa MHG**, Awasthi G, Singh PK, Gouado I, Das A. 2014. Does malaria epidemiology project Cameroon as "Africa in Miniature"? Journal of Biosciences **39 (4)**: 727-738.

- **Ngassa Mbenda HG** and Das A. 2014. Molecular Evidence of *Plasmodium vivax* Mono and Mixed Malaria Parasite Infections in *Duffy*-negative Native Cameroonians. PLOS One **9(8)**: e103262.