Genetic diversity of *Plasmodium falciparum* in Grande Comore Island

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Research

Keywords: Malaria, SNPs, msp1, msp2, Comoros.

Posted Date: May 10th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-26560/v1

Version of Record: A version of this preprint was published at Malaria Journal on September 3rd, 2020.
See the published version at https://doi.org/10.1186/s12936-020-03384-5.

Abstract

Background Despite several control interventions resulting in a considerable decrease in malaria prevalence in the Union of the Comoros, the disease remains a public health problem with high transmission in Grand Comore compared to neighboring islands. In this country, only a few studies investigating the genetic diversity of *Plasmodium falciparum* have been performed so far. For this reason, this study aims to examine the genetic diversity of *P. falciparum* by studying samples collected in Grande Comore in 2012 and 2013, using merozoite surface protein 1 (*msp1*), merozoite surface protein 2 (*msp2*) and single nucleotide polymorphism (SNP) genetic markers.

Methods A total of 151 positive rapid diagnostic test (RDT) samples from Grande Comore were used to extract parasite DNA. Allelic families K1, Mad20 and RO33 of the *msp1* gene as well as allelic families IC3D7 and FC37 of the *msp2* gene were determined by using nested PCR. Additionally, 50 out of 151 samples were genotyped to study 24 SNPs by using high resolution melting (HRM).

Results Two allelic families were predominant, the K1 family of *msp1* gene (55%) and the FC27 family of *msp2* gene (47.4%). Among 50 samples genotyped for 24 SNPs, 42 (84%) yielded

Introduction

Despite several interventions to prevent, control and eliminate malaria, 219 million cases and 435,000 deaths occurred worldwide in 2018, mainly in children under 5 years and pregnant women in Africa [1]. Among the five species that infect humans, *Plasmodium falciparum* remains the most dominant as well as fatal in a majority of Africa. It is associated with 95% of malaria-associated deaths [2, 3]. Many factors contribute to this severity of *P. falciparum* infections, such as the level of acquired immunity of the individual and genetic characteristics of the parasite [4].

The genetic diversity of *P. falciparum* remains an obstacle to achieve malaria elimination. In particular, this is due to the ability of the parasite to modify its genome by expressing a variety of proteins to evade host defenses. Among these proteins are merozoite surface protein-1 (MSP1) and merozoite surface protein-2 (MSP2). The genes coding for MSP1 and MSP2 have been widely used as genetic markers to determine allelic polymorphisms of *P. falciparum* [5–7]. Furthermore, *P. falciparum* genome is characterized by substitutions of single nucleotides occurring at specific positions in the genome, single nucleotide polymorphisms (SNPs). At the moment, approximately 112,000 SNPs in *P. falciparum* genome are known and considered useful molecular markers to study genetic diversity. In contrast to *msp1* and *msp2*, SNPs can be used for barcoding to identify and characterize genetic diversity of *P. falciparum* [8–10].

In the Comoros archipelago, which is composed of four islands in the Indian Ocean between Madagascar and the African coast, malaria remains a major public health problem with high transmission and mortality rate among children under five years old [2]. Since 2004, Comoros has dramatically increased deployment of intervention strategies, including insecticide-treated bed nets, the treatment of patients

with artemisinin-based combination therapy and the introduction of Intermittent Preventive Treatment (IPT) in pregnant women. These strategies have resulted in a significant decrease in malaria infection, from 54078 cases in 2004 to 1072 in 2015 in Comoros [11]. However, extensive interventions against malaria might as well result in changes in the population structure of Plasmodium species [12]. Consequently, an estimation of genetic diversity in local *P. falciparum* isolates can provide important information of how to improve strategies to reach malaria elimination.

An earlier study, published in 2010, revealed a significant diversity of *P. falciparum* strains in the Union of Comoros [13]. Since this study, to our knowledge, no other studies have been conducted in Comoros investigating genetic diversity of the parasite [14]. Furthermore, the aim of the present study is to characterize the genetic diversity of *P. falciparum* in Grande Comore using *msp1*, *msp2* and SNPs. Additionally, genotyping of *msp1* and *msp2* was used to determine the multiplicity of infection (MOI).

Methods

Study site

Samples were collected in 2012 and 2013 in three sites of Grande Comore. Malaria transmission varies within these three sites: in Moroni it's hypoendemic, in Mitsamiouli it's mesoendemic, and in Mbeni (**Figure 1**) it's hyperendemic [13]. Rapid diagnostic test (RDTs) results that were positive for *P. falciparum* were collected from health centers. RDTs that were negative or invalid were excluded from this study. All RDTs were stored at room temperature until they were used for DNA extraction.

DNA extraction

Parasite DNA stored on the nitrocellulose membrane of RDTs was extracted according to the protocol developed by Cnops et al. [15]. The following RDTs were used in Grande Comore by the time of the present study: CareStart™ Malaria HRP2/pLDH Combo RDT (Access Bio, Inc., Somerset, NJ) and Standard Diagnostics Bioline Malaria Ag Pf/Pan test (SD Bioline/Abbott, Yongin-si, Gyeonggi-do, Republic of Korea) 15/. DNA extraction was performed using QIAmp DNA or Blood Mini kit (Qiagen) following the manufacturer's instructions.

Genotyping of *msp1* and *msp2*

Msp1 and *msp2* genes were amplified by nested PCR as described in earlier studies [16, 17]. PCR products were analyzed by gel electrophoresis on 2% agarose gels and visualized by ultraviolet trans-

illumination (BioradGel Doc™ XR + System with Image Lab). A 100 bp DNA ladder marker (Quick Load) was used to determine the size of PCR.

Genotyping of SNPs

Twenty-four SNPs of *P. falciparum* were genotyped by using high-resolution melting (HRM) as previously described [18]. These SNPs were performed with a high minor allele frequency. The cycling and melting condition for SNPs were as follows: 95°C denaturation for 2 min followed by 55 cycles of 95°C for 5 s and 56°C for 30s. Then a pre-fusion cycle of 5 s each at 95°C and 37°C, followed by a melt from 35 to 90°C at 0.30°C/s.

Statistical analysis

The Student's t-test was used to compare the allelic frequencies of *msp1* and *msp2* genes in three regions of the Grande Comore. The MOI was calculated by averaging number of fragments detected in *msp1* or *msp2* genes by the number of samples. The significance threshold was fixed at $\alpha = 0.05$.Genetic diversity of msp1 and msp2 genes of the 3 populations was determined by the number of alleles per locus and by the Nei unbiased expected heterozygosity index (He) using GENETIX software version 4.05. MEGA X version 10.1 has been used for sequence alignment and to calculate genetic diversity.

Results

Genotyping of *msp1* and *msp2*

Parasite DNA was extracted from 151 RDTs that yielded positive bands for *P. falciparum*. All 151 samples were successfully genotyped: 25 from Moroni, 49 from Mitsamiouli, and 77 from Mbeni. Among them, 21 individual alleles were found, including 12 alleles for *msp1* gene and 9 alleles for *msp2* gene.

For *msp1*, 7 K1 type alleles (150–300 bp), 3 MAD20 type alleles (190–230 bp), and 2 RO33 type alleles (150 and 200 bp) were observed (**Figure 2**). The most frequent K1, RO33, MAD20 type alleles were 200 bp (60/132), 150 bp (17/18), and 200 bp (34/64) fragments, respectively. For *msp2*, 3 FC27 type alleles (350–450 bp) and 6 IC3D7 type alleles (450–700 bp) were obtained. For FC27, the most frequent type allele was 400bp fragments, and the most frequent type allele for IC3D7 was 500 bp fragments (**Figure 3**).

In all three regions, the K1 family was predominant (55%) for *msp1* gene (**Table 1**). It was more frequent in Mbeni (71.1%) compared to Moroni (50%; $p = 0.047$) and Mitsamiouli (30.6%; $p < 0.01$) (**Figure 4**). There was no significant difference of K1 frequency between Moroni and Mitsamiouli ($p > 0.05$). However,

MAD20 *msp1* allelic family was more frequent in Mitsamiouli (22.4%) compared to Moroni (19.2%; p = 0.55) and Mbeni (9.6%; p = 0.02). RO33 *msp1* allelic family was poorly represented (2.4–4.0%) in all three sites.

For *msp2* gene, IC3D7 allelic family was predominant in Moroni (65%). Furthermore, the frequency was lower in both Mitsamiouli (39%; p = 0.056) and Mbeni (31.6%; p = 0.0012). The FC27 family was found to be dominant in Mbeni (60.5%), in comparison to Moroni (20%; p = 0.0012) and Mitsamiouli (36%; p = 0.013).

In Moroni a total of 10 (40%) samples showed polyclonal infection with at least two clones, resulting in mean multiplicity of infection (MOI) of 1.40 **(Table 2).** In Mitsamiouli, 26 (53%) samples showed polyclonal infections with at least two clones, (MOI=1.57) and in Mbeni 26 (33.8%) samples were polyclonal (MOI=1.35). No significant difference of MOI between Moroni and Mitsamiouli (p = 0.21) using a non-parametric test. In Mbeni, 26 (33.8%) samples were polyclonal, and mean MOI was 1.35. The difference of MOI was significant between Mbéni and Mitsamiouli (p = 0.02) but no significant difference between Moroni and Mbeni (p = 0.6).

Genotyping of SNPs

In this study, 50 out of 151 (33%) samples were genotyped for 24 SNP markers. Among these, 42 samples (1 from Moroni, 17 from Mitsamiouli, and 24 from Mbeni) and 21 SNPs yielded interpretable results (**Additional file 1: Table S1**). Altogether, 36 (85%) different genotypes were obtained. Six (15%) isolates grouped into two clusters, defined as a group of parasites with identical nucleotide sequences: one cluster consisting of 2 (5%) parasite populations from Mitsamiouli (defined by the barcode TACCXCGACXTAATAAGAXGG) and another cluster consisting of 4 (10%) samples from Mbeni (defined by the barcode TATTCCGTTGCCACTCGATTG). The X symbol indicates that there is no amplification. Moreover, among 36 genetically unique parasites, several isolates had a strong linkage since they only differed from each other by a few nucleotides (**Additional file 2: Figure S1**).

Allelic frequency

Data on allelic frequency showed that several nucleotides predominated at the study sites. At P2 position, adenine (A) predominated in all sites. At P5 position, cytosine (C) predominated. At positions P21 and P24, adenine (A) and guanine (G) were seen in the majority of isolates collected in Mitsamiouli and Moroni, respectively. The lowest of minority alleles frequency (MAF) was found in position 20 with 11.9% (**Additional file: Table S1**).

Comparison of clonality between SNPs and *msp*

To investigate if there is an association between barcode and msp (clonality), SNPs (clusters and unique barcodes) were classified as monoclonal or polyclonal. Analysis showed that all clusters were polyclonal. Among unique barcodes, 7 (24.1%) were monoclonal, and 29 (75.9%) were polyclonal.

Genetic diversity

Genetic diversity (He) was estimated from msp and SNPs. For msp genes, the highest mean diversity was observed for Moroni (He= 0.84) and Mbeni (He = 0.83) **table 3**. There was no significant difference between Mbeni and other sites (each p = 0.99). The mean genetic diversity for all sites was 0.83 (table 3). For SNPs, He was determined at Mitsamiouli and Mbeni**.** The highest genetic diversity was found at Mitsamiouli (He = 1) and the lowest at Mbeni (He = 0.41).

Otherwise, He values were compared using both methods (SNPs, Msp) in both sites. The msp gene had He values higher in Mbeni (the difference was significant, p <0.01) and in both sites but no significant difference (p = 0.53). However, in Mitsamiouli, SNPs had higher He but no significant difference (p = 0.8) (**Table 4**).

Discussion

The Union of Comoros has entered the phase of malaria elimination after the implementation of several strategies, such as the massive use of insecticide impregnated bed nets (ITNs), artemisinin-based combination therapy (ACT) for confirmed malaria cases, intermittent preventive treatment in pregnant women (IPTp), and mass drug administration (MDA) with artesunate-mefloquine and primaquine. Genetic diversity of *P. falciparum* can be a source of antimalarial drug resistance. However, this has so far received limited attention and needs to be further investigated. In Grande Comore genotyping of *msp1* and *msp2* genes revealed a high allelic polymorphism. For 12 alleles in the *msp1* gene, the K1 family was dominant (55%). For 9 alleles in the *msp2* gene, the FC27 family was most represented (47.4%) were found. These results are similar to those obtained in Madagascar [19] and in Grande Comore in isolates 2013–2016 group [14]. Interestingly, the results in the present study differ from those observed in Grande Comore in isolates 2006–2007 group, where the RO33 family was predominant [14].

Fragment analysis of *msp1* and *msp2* allelic families using agarose gel electrophoresis used in the present study has limitations due to difficulties in defining specific alleles with accuracy for each isolate. To alleviate this drawback, 24 SNPs were used to determine the genetic diversity of *P. falciparum* in Grande Comore for the first time. These 24 SNPs are able to identify different parasites with a high minor allele frequency. [18]. After SNP genotyping, it was found that 85% of isolates are genetically unique. This results strongly suggest that there is a high level of genetic diversity of *P. falciparum* on the island (He = 0.6). The results of SNP genotyping confirm those of *msp1* and *msp2* markers (He = 0.82, p = 0.03).

The comparison of results from the studies concerning msp and barcode showed that all clusters were polyclonal. Similarly, among isolates with unique barcodes a majority (78.9%) were polyclonal as well. Although data obtained in the present study suggests the barcode of 21 SNPs cannot predict mixed

infections. MOI from msp remain the most powerful tool for determining P. falciparum infections. The average MOI found in this study for all sites was 1.36 for msp1 and 1.05 for msp2. These results are similar to those obtained in Grande Comore in isolates 2013–2016 group suggested that a decreasing malaria transmission intensity on this island [13].

Moreover, the considerable level of genetic diversity is found in this study despite trends in decreased malaria transmission in Comoros. Indeed, it has been established that genetic diversity of *P. falciparum* is more frequent in areas of high transmission [20]. This observation may give an explanation for the high malaria persistence in Grand Comore compared to neighboring islands, despite implication of malaria eliminating strategies such as MDA. This hypothesis is supported by the fact that Grande Comore, compared to three other islands in the Union of Comoros, solely accounted for 99.4% of malaria cases in 2014 [21]. Indeed, in Grande Comore, there are a large number of water cisterns and ablution basins servingas mosquito breeding site [2]. Therefore, local vector control interventions such as larval source management may be relevant to imply further.

Conclusion

Clinical isolates of *P. falciparum* showed high genetic diversity in Grande Comore. This might be one of the reasons for the high malaria transmission in comparison to neighboring islands, despite the implication of several malaria elimination strategies. Further studies on genetic diversity are essential to determine the impact of past interventions. Additionally, they may contribute to the adoption of future strategies on how to achieve malaria elimination in the Union of the Comoros.

Abbreviations

A: adenine; ACT: artemisinin-based combination therapy; C: cytosine; G: guanine; HRM: high-resolution melting; MOI: multiplicity of infection; msp1: merozoite surface protein-1, msp2: merozoite surface protein-2, RDT: rapid diagnostic test; SNP: single nucleotide polymorphisms; HRM: high resolution melting; ITNs: insecticide impregnated bed nets: MDA: mass drug administration. IPTp: intermittent preventive treatment in pregnant women.

Declarations

Availability of data and material

All data generated or analysed during this study are included in this article and are available from the corresponding author.

Acknowledgements:

We thank the sample collection teams in Comores: Mohamed Abdou Said Fazul,Miradji Maamoune, Mbaye Toyb. We also thank Baba Dieye for critical reading of the manuscript and Amy K. Bei, Rachel F. Daniels for analysis results.

Authors' contributions:

N.P.M performed experiments and wrote the manuscript. A.D.A. and M.S conceived and designed the study. H.B analyzed, edited and reviewed the manuscript. S.R contributed materials and analysis tools and offered experimental advice. C.K.D and L. B contributed to data analysis and reviewed the manuscript. M.S gave constructive advice and reviewed the manuscript. L.B A.D.A and S.M. supervised the research, wrote, read, and approved the final manuscript. All authors read and approved the final manuscript.

Funding

Not applicable

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests:

The authors declare that they have no competing interests.

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Tables

Table 1. Genetic diversity of *P. falciparum msp* genes in Grande Comore

Gene	Allelic family	n (%)
msp1	K1	87 (55%)
	MAD20	24 (15.2%)
	RO33	5 (3.2%)
	K1/MAD	36 (22.8%)
	K1/RO33	5 (3.2%)
	MAD/RO33	0 (0%)
	K1/MAD20/RO33	1 (0.63%)
	Total K1	129 (81.64%)
	Total MAD20	61 (38.6%)
	Total RO33	11 (7%)
msp2	FC27	65 (47.4%)
	IC3D7	53 (38.7%)
	FC27/IC3D7	19 (13.8%)
	Total FC27	84 (61.3%)
	Total IC3D7	72 (52.5%)

There was a total of 158 *msp1* and 137 *msp2* fragments, respectively.

Table 2. Multiplicity of infection of *msp1* and *msp2* genes from Moroni, Mitsamiouli and Mbeni

Sites	Moroni	Mitsamiouli	Mbeni	all sites
Endemicity	Hypoendemic	Mesoendemic	Meso to hyperendemic	
	n=25	n=49	n=77	n=151
MOI				
msp1	1.46	1.46	1.29	1.36
msp2	1	1.06	1.06	1.05
msp1+msp2	1.4	1.57	1.35	1.43

Table 3: Genetic diversity of Plasmodium falciparum at the 3 sites studied in Grande Comore

Moroni		Mitsamiouli		Mbeni		all sites	
N	He	N	He	N	He	N	He
34	0.85	74	0.82	103	0.81	211	0.84
30	0.83	67	0.85	102	0.76	199	0.82
64	0.84	141	0.84	205	0.78	410	0.83

Table 4: Comparison of genetic diversity using msp and SNPs

	Mitsamiouli		Mbeni		All sites	
	N	He	N	He	N	He
msp1+msp2	67	0.79	79	0.79	146	0.82
SNPs	17	1	24	0.41	41	0.61
P-value		0.8		**0.0001**		0.53

Figures

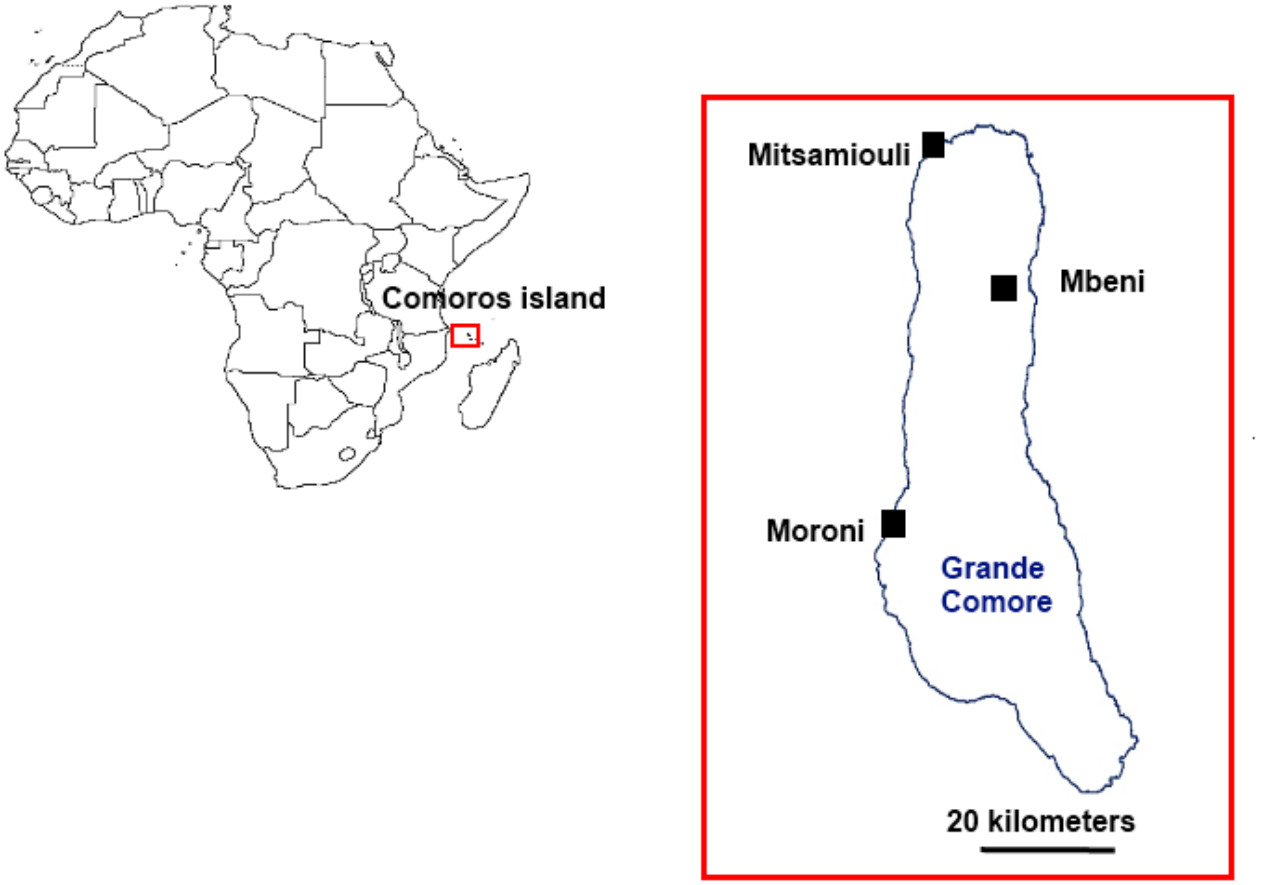


Figure 1

Map of Grande Comore Island showing the study sites

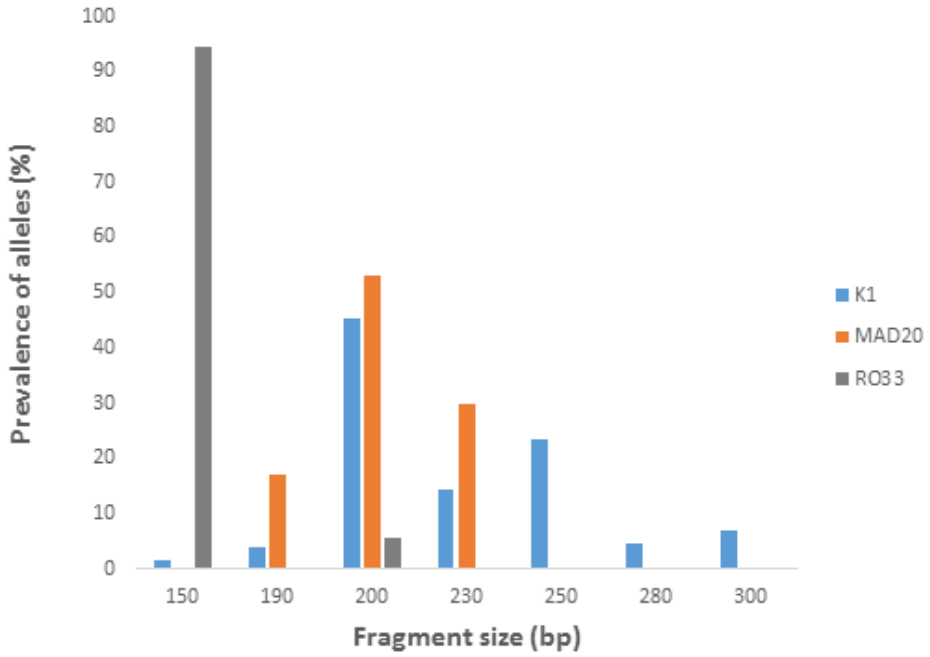


Figure 2

The frequencies of K1 (blue), MAD20 (orange), and RO33 (gray) msp1 alleles are shown in the figure. The total number of K1 MAD20 and RO33 alleles are 134, 64 and 18, respectively.

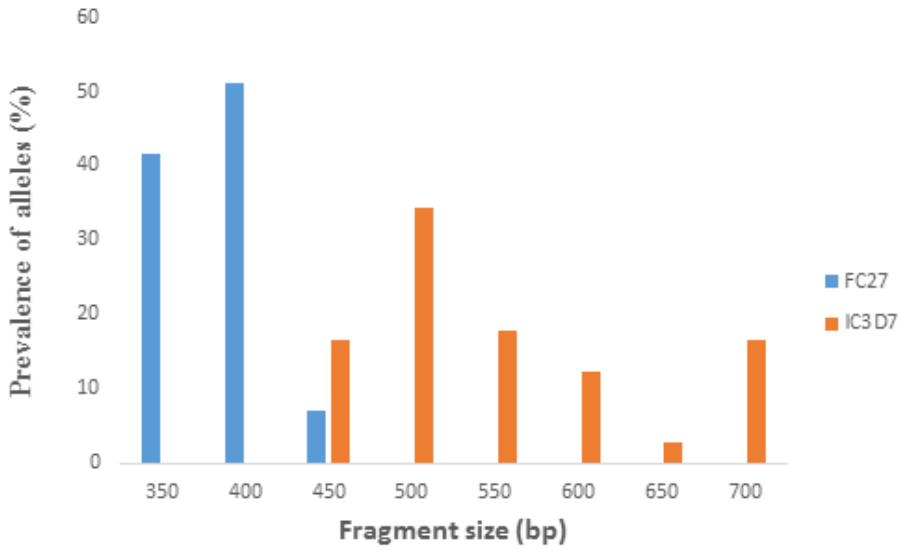
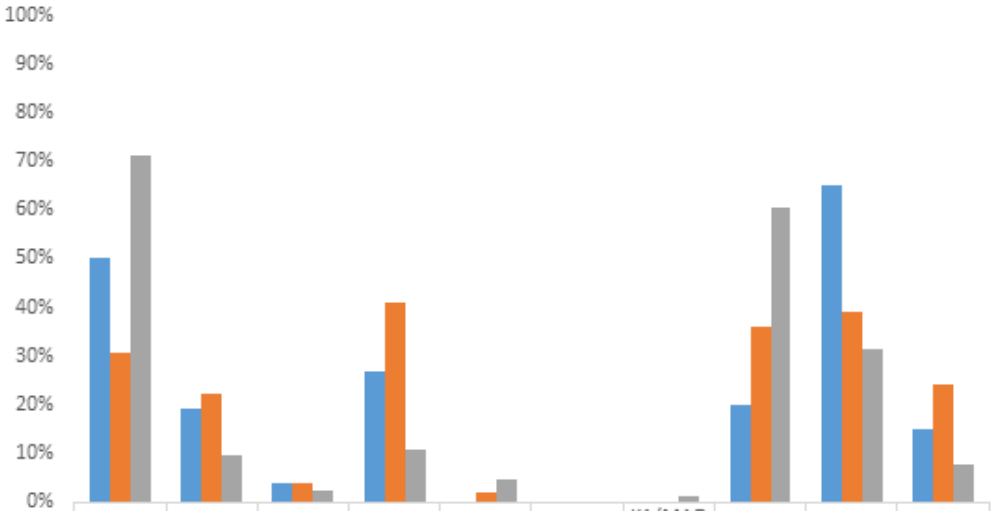


Figure 3

The frequencies of FC27 (orange) and IC3D7 (red) msp2 alleles are shown in the figure. The total number of FC27 and IC3D7 alleles are 84 and 73 respectively.



	K1	MAD	RO	K1/MAD	K1/RO	MAD/RO	K1/MAD/RO	FC	IC	FC/IC
	msp1							msp2		
Moroni	50%	0.19%	0.04%	27%	0%	0%	0%	20%	65%	15%
Mitsamiouli	0.31%	0.22%	4%	0.41%	2%	0%	0%	36%	39%	24%
Mbéni	0.71%	0.10%	0.02%	0.11%	0.05%	0%	0.01%	0.61%	0.32%	0.08%

Figure 4

Allelic frequencies of msp1 and msp2 genes in 3 regions of Grande Comore.

Supplementary Files

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- Additionalfile2.png
- Additionalfile1.xlsx