Appendix

APPENDIX II

- Materials

A. Equipment

1. Micropipettors and tips.

2. Thermal cycler.

3. Apparatus for agarose gel electrophoresis.

4. Photographic equipment and UV transilluminator.

5. Refrigerator (4°C) and freezer (–20°C).

6. Microcentrifuge.

7. Shaker.

8. Test tubes and PCR tubes.

B. Reagents

1. PCR buffer (10X stock): 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 20 m*M* MgCl2 and 1 mg/mL gelatin. Store at 4°C or –20°C.

2. dNTP stock solution: 5 m*M* of each of the four dNTPs: dATP, dCTP, dGTP, and dTTP. Store working stocks at –20°C, and back-up stocks at –70°C.

3. Oligonucleotide primers: A 2.5 μ*M* stock of each store working stocks at–20°C, and back-up stocks at –70°C.

4. AmpliTaq polymerase (Cetus) Store at –20°C.

5. Loading buffer (5X stock): 50 m*M* Tris, pH 8.0, 75 m*M* EDTA, pH 8.0, 0.5% SDS, 30%

w/v sucrose, 10% w/v Ficoll (average mol wt = 400,000), and approx 0.25% w/v of Orange

G dye. If Ficoll is not available, use 40% sucrose. Store at room temperature.

6. TBE buffer (10X stock): 1 *M* Tris, 1 *M* boric acid, 50 m*M* EDTA. A pH of approx 8.3 Store at room temperature.

7. Ethidium bromide solution: 10 mg/mL in water. Extreme care should be taken when handling

This solution as this chemical is highly carcinogenic Store in the dark at 4°C.

8. Distilled Water

APPENDIX II: - WORKING REAGENT MIXTURE

A. Primary PCR mixture:

Reagent	**Stock concentration**	**Working/Final Conc. (Total vol 50ml)**	**Volume (1 rxn 50 µl)**
H2O			28.425
dNTP	2mM	0.5mM	3.125
PCR Buffer(MgCl2)	10x	1x	5
MgCl2	25mM	1.0mM	2.0
Forward Primer	4 uM	0.25 uM	3.125
Reverse Primer	4 uM	0.25 uM	3.125
Thermopol *taq*	5.0U	1.0U	0.2
DNA template			5
Total Volume			50

B. Secondary PCR Mixture:-

Reagent	**Stock concentration**	**Working/Final Conc. (Total vol 50ml)**	**Volume (1 rxn 50 µl)**
H2O			30.425
dNTP	2mM	0.5mM	3.125
PCR Buffer(MgCl2)	10x	1x	5
MgCl2	25mM	1.0mM	2.0
Forward Primer	4 uM	0.25 uM	3.125
Reverse Primer	4 uM	0.25 uM	3.125
Thermopol *taq*	5.0U	1.0U	0.2
DNA template			3
Total Volume			50

APPENDIX III

Protocol :- Extraction protocol

Bench Protocol: Animal Tissues (Spin-Column Protocol)

QIAGEN

Note: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in the *DNeasy Blood & Tissue Handbook*.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If necessary, redissolve any precipitates in Buffers ATL and AL.
- Ensure that ethanol has been added to Buffers AW1 and AW2.
- Preheat a thermomixer, shaking water bath, or rocking platform for heating at 56°C.
- If using frozen tissue, equilibrate the sample to room temperature.

Procedure

1. **Cut tissue (up to 25 mg; up to 10 mg spleen) into small pieces, and place in 1.5 ml microcentrifuge tube. For rodent tails, use one (rat) or two (mouse) 0.4–0.6 cm lengths of tail. Add 180 µl Buffer ATL.**
2. **Add 20 µl proteinase K. Mix by vortexing, and incubate at 56°C until completely lysed. Vortex occasionally during incubation, or place in a thermomixer, in a shaking water bath, or on a rocking platform.**

   Lysis is usually complete in 1–3 h or, for rodent tails, 6–8 h. Samples can be lysed overnight.
3. **Vortex for 15 s. Add 200 µl Buffer AL to the sample. Mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%). Mix again thoroughly.**

   Alternatively, premix Buffer AL and ethanol, and add together.
4. **Pipet the mixture into a DNeasy Mini spin column in a 2 ml collection tube. Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.**
5. **Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x *g*. Discard flow-through and collection tube.**
6. **Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW2. Centrifuge for 3 min at 20,000 x *g* (14,000 rpm). Discard flow-through and collection tube.**

   Remove the spin column carefully so that it does not come into contact with the flow-through.
7. **Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube, and add 200 µl Buffer AE for elution. Incubate for 1 min at room temperature. Centrifuge for 1 min at ≥6000 x *g*.**
   Recommended: Repeat this step for maximum yield.

Genetic diversity of *Plasmodium falciparum* isolated from Yemen based on the Genes of Merozoite Surface Proteins (MSP) 1 and 2.

Nazeh M. Al-abd1,2, Mohammed A. K. Mahdy1,3,§, Abdulsalam M. Q. Al-Mekhlafi1, Nazia B. Abdul-Majid2, Hesham M. Al-Mekhlafi1,3 and Mun Y. Fong 1

1Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

2Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

\Department of Parasitology, Faculty of Medicine and Health Sciences, Sana'a University, Sana'a – Yemen

Email addresses:

NMAA: seaaden@yahoo.com

MAKM: alsharaby9@yahoo.com

AMQA: halkasemi@yahoo.com

NBA: nazia@um.edu.my

HMA: halmekhlafi@yahoo.com

FMY: fongmy@um.edu.my

Abstract

Background: Malaria is a major health problem causing substantial morbidity and mortality in Yemen, with Plasmodium falciparum being the predominant species. In the countries of WHO Eastern Mediterranean region (such as Iran, Pakistan and Sudan), P. falciparum field isolates have been characterized based on their MSP genes. However, no molecular data on the population structure of P. falciparum in Yemen has been reported.

Methods*:* Blood samples were collected from 511 patients with fever. The samples were screened for malaria parasites using Giemsa-stained thick and thin blood films. A total 74 samples had P. falciparum, for which their MSP1 and MSP2 genes were studied using nested PCR.

Results: All the three families (K1, MAD20 and RO33) of MSP1 and the two families (FC27 and 3D7) of MSP2 were detected in this study. 3D7 allelic family was the most frequent (68%), followed by K1 (45%), RO33 (42%), FC27 (42%) and MAD20 (22%). The four allelic families, (MAD20, RO33, FC27 and 3D7), were significantly more prevalent in the hinterland areas as compared to coastland and highland areas ($p < 0.05$) of Yemen. The K1 allele type was most frequent in the highland ($p < 0.02$). The complexity of the infection was significantly ($p < 0.05$) the highest in the hinterland followed by coastland and highland. Urban areas had higher complexity of infection as compared to rural areas ($p < 0.05$). No significant difference was shown in the complexity of falciparum infection between the age groups ($p > 0.05$) nor the different levels of parasitaemia ($p > 0.05$). MSP2 had higher number of alleles than MSP1 (42 vs

18). The highest number of alleles of MSP1 and MSP2 was observed in the coastland and the rural areas.

Conclusions

Significant differences in complexity and the distribution of the family alleles of MSP1 and MSP2 genes between hinterland, coastland and highland areas were observed, reflecting the intensity of malaria transmission between areas. This observation should be taken into consideration in implementing malaria control strategies in Yemen.

Background

Malaria is a major health problem causing substantial morbidity and mortality in Yemen. Within the nine countries in the WHO Eastern Mediterranean region, Yemen has been ranked after Afghanistan, with 60% of population at high risk of malaria and placed among the countries that showed no downward trend in malaria frequency [1, 2]. Plasmodium falciparum is the predominant species which is responsible for more than 90% of the malaria cases, followed by P. vivax and P. malariae [3]. Although Anopheles arabiensis is the main vector of malaria in Yemen, An. culicifacies and An. sergenti have been reported to play important roles in the transmission of malaria [3].

Genetic polymorphism of P. falciparum is an important consideration in vaccination programmes against its infection. The polymorphic regions of merozoite surface protein (MSP) 1 and 2 genes are potential genetic markers that provide information on the multiplicity of P. falciparum infection and level of transmission. Furthermore, the ability of MSP1 and MSP2 to induce strong immune response in human makes them as potential vaccine candidates against the blood stages of the parasite [4, 5].

In the WHO Eastern Mediterranean region, P. falciparum field isolates have been characterized based on the MSP genes in Iran [6, 7], Pakistan [8] and Sudan [9, 10]. However, no available data on genetic diversity of P. falciparum in Yemen. Furthermore, the diversity and complexity of falciparum malaria depend on the level of endemicity. Therefore observations made in holoendemic areas may not be valid for mesoendemic or

hyperendemic areas. Even in areas with similar endemicity, the parasite diversity may be different due to the effect of local conditions [11]. Thus, this study was carried out to determine genetic diversity of P. falciparum in Yemen in order to provide insights for developing effective control measures.

Methods

Study areas and population

The present study was conducted in three governorates in Yemen with a total population of 5.9 million [12]. The selected governorates, Taiz, Hodeidah and Dhamar, represented the mountainous hinterland, coastal areas and highland areas, respectively. Living quarters in the rural communities of the study areas are made of mud or stones and have wooden roofs. Majority of the people work in agriculture, fishing, livestock and handicraft sectors.

The peak time of malaria transmission in the coastal areas occurs in winter (October-April), while in the western mountains, the peak occurs in the summer (May-September). The mountainous hinterland normally shows peak of transmission between October and March. In the highlands areas, which are located at more than 2000 metres above sea level, the transmission occurs throughout the year [3]. Anopheles arabiensis is the main vector in the country but A. culicifacies plays an important role in the transmission of

malaria in the coastal areas. A. sergenti has been reported to be a vector in the mountainous hinterland and highland areas [3].

Study subjects were patients with febrile illness attending hospitals and medical centres from June 2008 to March 2009. Patients participated on a voluntary basis after they were given a clear explanation of the research objectives. If the subjects were children, informed consent was obtained from their guardians. Study protocol was reviewed and approved by the Faculty of Medicine, Sana'a University, Sana'a, Yemen.

Data collection and microscopy

Whole blood from a finger prick was collected from each subject and used to prepare thick and thin blood films. Additional drops of blood were spotted onto Whitman filter paper 3MM (Whatman International Ltd., Maidstone, England), labelled and stored in plastic bags at room temperature until use. Thick and thin blood films were stained with Giemsa stain and examined under microscope at 1000X magnification. Parasitaemia was determined according to the standard techniques and by performing recommended equations [13]. The smear was considered negative after screening 100 high power fields. Specimens positive for P. falciparum were selected for molecular characterization using MSP1 and 2 markers.

DNA extraction and molecular analysis

Parasite genomic DNA was extracted from a blood spot on the filter paper. Briefly, a disc was punched out from the blood spot using a pre-flamed paper puncher and placed in a 1.5 ml centrifuge tubes using a pair of sterile forceps. DNA was extracted using QIAgen DNA Mini Kit blood and tissue (QIAGEN, Cat. no. 51306, Germany) according to the manufacturer's instructions.

Allelic families of MSP-1 (block 2) and MSP-2 (block 3) were analysed using nested PCR assays as mentioned previously [14]. Primary PCR was carried out using primers specific for MSP-1 (block 2) and MSP-2 (block 3). Secondary PCR was carried out using primers specific for allelic families of MSP-1 (K1, MAD20 and RO33) and MSP-2 (FC27 and 3D7/IC). Primary PCR was performed in 50 µL reaction mixture containing 5 µL of DNA template, 1X i-TaqTM buffer free of MgCl2 (iNtRON BIOTECHNOLOGY, Seoul, Korea), 1 mM of MgCl2 (iNtRON BIOTECHNOLOGY, Seoul, Korea), 125 µM dNTP (iNtRON BIOTECHNOLOGY, Seoul, Korea), 0.25 µM of each primer and 1.25 U of i-TaqTM DNA polymerase (iNtRON BIOTECHNOLOGY, Seoul, Korea). Secondary PCR was performed in 25 µL reaction mixture containing 3 µL of DNA template and the same concentrations as the primary PCR. In both amplifications, samples were incubated in the MyCycler thermal cycler (Bio-Rad, Hercules, USA) under the following conditions: initial denaturing step at 95ºC for 5 min, annealing at 58ºC for 2 min and extension at 72ºC for 2 min, followed by 24 cycles of denaturing for 1 min at 94ºC, annealing for 2 min at 58ºC and extension for 2 min at 72ºC, followed by a final

annealing at 58ºC for 2 min and a final extension at 72ºC for 5 min. The PCR products were subjected to electrophoresis in 2% agarose gels and stained with Syber green. The sizes of MSP1 and MSP2 alleles were measured using Quantity One software (Bio-Rad, USA). If the sizes were different by approximately 10 bp, they were considered as the same and categorized as one allele.

Definitions

The detection of a single PCR fragment of an allelic family was considered as an infection with one genotype. Multiple infection of P. falciparum was defined as the presence of more than one genotype of either MSP1 or MSP2 in a single blood sample [15]. The complexity of P. falciparum (multiplicity of infection) is the mean of the frequency of the multiple infections in a single blood sample i.e., the total of genotypes divided by the total number of samples [15]. Complexity and multiple infections were calculated by combining the MSP1 and MSP2 results. Parasitaemia was graded as low (1–999/µl), moderate (1000–9999/µl) and high (>10000/µl).

Statistical analysis

Data were analysed using the SSPS programme for Windows version 11.5 (SPSS Inc., Chicago, IL, USA). The associations between proportions were tested using the $\chi 2$ test. t-test and One Way ANOVA were used to compare between means. Significance was defined as $P < 0.05$

Results

The study was carried out on 511 febrile patients seeking health care. Of these patients, 74 had P. falciparum infection and were selected for the genotyping study. All the three families (K1, MAD20 and RO33) of MSP1 and the two families (FC27 and 3D7) of MSP2 were detected in this study. 3D7 allelic family was the most frequent (68%), followed by K1 (45%), RO33 (42%), FC27 (42%) and MAD20 (22%). The frequencies of mixed infection with more than one family allele were 32% for FC27 + 3D7 and 18% for MAD20 + RO33. The four allelic families (MAD20, RO33, FC27 and 3D7) were significantly more prevalent in hinterland areas as compared to coastland and highland areas. In contrast, K1 allele type was most frequent in the highlands. A total of 55 patients (77.5%) were infected with more than one genotype (table 1). The prevalence of allelic families showed no significant difference between rural and urban areas.

The complexity of multiple infections was defined as the mean of the frequency of multiple infections. The overall complexity found in this study was 2.18. The complexity of the infection showed significant difference between study areas ($F = 14.56$, $p < 0.001$). The highest complexity was registered in hinterland followed by coastland and highland. Urban areas had higher complexity of infection as compared to rural areas ($t = 2.18$, $p < 0.05$). The study results showed no significant difference in the complexity of falciparum infection between the age groups or the different levels of parasitaemia (Table 2).

The distribution of MSP1 and MSP2 alleles in the different geographical areas is summarised in Table 3. MSP2 had higher number of alleles (42) than MSP1 (18). Within the MSP2 allelic families, a total of 24 alleles and 18 alleles were observed in the families 3D7 and FC27, respectively. All MSP1 allelic families had the same number (6) of alleles. The highest number of alleles of the two loci MSP1 (block 2) and MSP2 (block 3) was observed in the coastland and the rural areas.

Discussion

This is the first study which examined the population structure of P. falciparum in Yemen based on MSP1 and MSP2 genes. This study found high proportions of all the allelic families (K1, MAD20, RO33, FC27 and 3D7) with 3D7 being the most frequent and MAD20 the least. Within the families of MSP1 gene, K1-type family was the most frequent and MAD20-type family was the least. Similar distribution of MSP1 allelic families has been reported from French Guiana [11], Kenya [16] and Peru [17]. The distribution of MSP1 allelic families in this study is found not consistent with the situation seen in low endemic areas in Colombia [18], Iran [7], Myanmar [19], Pakistan [8], and Thailand [14], where MAD20 was reported as the dominant allelic family. Analysis of MSP2 (block 3) showed higher prevalence of 3D7/IC allelic family than FC27 allelic family with high proportion of mixed infection with the two families. This frequency is in agreement with previous reports from Myanmar [19] and Pakistan [8].

Although the allelic families of MSP1 and MSP2 were not restricted to geographical location, a significant difference was noted between areas and this could be attributed to the different levels of endemicity in the three locations. Mixed infection was observed in 77.5% of patients and almost all patients from the hinterland areas harbored more than one genotype. Mixed infection rates of almost 50% have been observed in mesoendemic regions [20, 21] and up to 100% in holoendemic regions [10, 22]. In areas with low endemicity, mixed infections can range from 20 -30% [10, 23]. Meiotic recombination during the sexual reproduction stage is a major mechanism for the generation of genetic diversity of P. falciparum. Thus, the high prevalence of mixed infection can enhance cross-fertilization resulting in the generation of novel alleles [24].

The complexity of P. falciparum infection in Yemen was 2.18 which is comparable with the complexity reported previously from hyperendemic areas [4]. The highest complexity was observed in the hinterland areas followed by the coastland and highland areas. These differences were statistically significant, suggesting that endemicity and level of malaria transmission are different in the three locations. The positive association between complexity and endemicity of P. falciparum has been well documented [4, 10, 25, 26] with few exceptions in certain areas where high complexity was reported from low endemic areas [7, 27]. The complexity in the hinterland and the coastland was similar to those observed in hyperendemic areas in Africa [4, 10, 28] while the complexity in the highland was similar to those reported from low transmission areas such as in central and Southeast Asian countries [8, 25, 29]. A world malaria map for the endemicity of P. falciparum in 2007 based on PfPR2-10 placed Yemen with Africa in one region [30, 31].

It was noted that, complexity of falciparum malaria was unexpectedly higher in urban as compared to rural areas. However, this comparison may be biased by the fact that complexity in the urban areas was based on a smaller sample size as compared to that of the rural areas (10 vs 40). Nonetheless, the high complexity in the urban areas may be explained by the massive migration of people from the rural of Yemen to the city. An individual may acquire the infection in the rural areas before moving to the urban areas, and this infection may be overlapped by a new infection. It has been reported that P. falciparum infection can persist for more than 2 years in a semi-immune patient [32]. The increase in the complexity due to the migration between areas of more intense transmission was postulated in a study from Southeast Asia [25].

In the present study, no positive association was observed between complexity and age. Similar findings have also been seen in previous studies [20, 25, 33, 34]. However, a positive association between complexity and age was reported from the rural areas of Burkina Faso [15, 35]. Significant association between complexity and parasite density was documented from southern Mozambique [36], Senegal [24] and Tanzania among infants but not adults [34]. Our study, however, did not indicate any significant association between complexity and parasite density.

MSP1 and MSP2 of the P. falciparum in Yemen had high number of alleles (18 for MSP1 and 42 for MSP2). This allelic variation is higher than those reported in low endemic

areas in Asia [6, 14, 19] and almost similar to those reported in hyper- and holo-endemic areas in Africa [4, 10] and Papua New Guinea [37]. Higher allelic variation was seen in rural areas as compared to urban areas. This could be attributed to the high intensity of the malaria transmission in the rural areas where mosquito breeding sites are abundant in the vicinity of human habitation.

Conclusions

The study indicated high genetic diversity among the P. falciparum population in Yemen. The significant difference in complexity and the distribution of the family alleles between hinterland, coastland and highland areas suggests the difference in malaria transmission between these areas. The high genetic diversity of P. falciparum population in Yemen represents a major challenge for developing malaria control strategies, especially one that is based on vaccination. Furthermore, high genetic diversity may provide a mechanism for the emergence of drug resistance in the P. falciparum population.

Competing interests

No interests

Authors' contributions

NMAA, MAKM, AMQA and FMY designed the study; AMQA dealt with study subjects in the field and carried out the Giemsa microscopy; NMAA carried out the molecular work; MAKM and HMA performed the statistical analysis; MAKM and NMAA interpreted the data; MAKM and NMAA drafted the manuscript; FMY, MAKM, NMAA, HMA and NBA contributed to the revision of the manuscript. All authors read and approved the final manuscript. NMAA, MAKM and FMY are guarantors of the paper.

Acknowledgements

The authors thank all the technical staff in the hospitals and medical centres in the five governorates. The authors also thank all the patients who agreed to participate in this study. The molecular study was carried out at the Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia.

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Tables

Table 1 - Distribution of families of MSP1 and MSP2 in Yemen P. falciparum isolates

Marker	Prevalence n(%)					
	Coastland	Highland	Hinterland	Total	$\chi 2$	P value
MSP1	n= 25	n= 36	n= 13	n= 74		
K1	8 (32)	22 (61)	3 (23)	33 (45)	8.016	< 0.05
MAD20	8 (32)	2 (6)	6 (46)	16 (22)	11.689	< 0.01
RO33	15 (60)	6 (16.7)	10 (77)	31 (42)	19.332	< 0.001
K1 + MAD20	3 (12)	1 (3)	0 (0)	4 (5)	-	-
K1 + RO33	5 (20)	2 (6)	1 (8)	8 (11)	-	-
MAD20 + RO33	6 (24)	1 (2.8)	6 (46)	13 (18)	13.408	< 0.001
K1 + MAD20 + RO33	1 (4)	0 (0)	0 (0)	1 (1.4)	-	-
MSP2	n= 25	n= 36	n= 13	n= 74		
FC27	12 (48)	9 (25)	10 (77)	31 (42)	11.157	< 0.01
3D7/IC	16 (64)	21 (58)	13 (100)	50 (68)	7.786	< 0.05
FC27 + 3D7/IC	10 (40)	4 (11)	10 (77)	24 (32)	19.864	< 0.001
Mixed infection#	18 (72)	24 (72.7)	13 (100)	55 (77.5)	4.634	> 0.05

presence of more than one family of MSP1 or MSP2 in one patient, n = number, figures in the bracket represents the prevalence in percentage

Table 2 - Complexity of falciparum malaria in Yemen

Variables	Mean ± SD	95%CI	Significance
Study area*			
Hinterland	3.23 ± 0.83	2.72 - 3.73	F=14.56, P <0.001
Coastland	2.36 ± 1.15	1.88 - 2.83	
Highland	1.67 ± 0.75	1.41 - 1.92	
Location#			
Rural	2.08 ± 1.06	1.78 - 2.39	T = 2.18, P < 0.05
Urban	2.90 ± 1.10	2.11 - 3.69	
Age*			
< 5	2.5 ± 0.86	1.74 - 2.56	F = 1.014, p > 0.05
5-10	1.9 ± 1.46	2.07 - 2.92	
> 10	2.15 ± 1.14	0.65 - 3.09	
Parasitaemia*			
Low	1.75 ± 0.95	0.23 - 3.27	F = 1.066, p > 0.05
Moderate	2.00 ± 0.73	1.53 - 2.47	
High	2.35 ± 1.21	1.96 - 2.74	

* Difference tested using One Way ANOVA,

Difference tested using t-test.

Table 3 - Distribution of MSP1 and MSP2 alleles of Plasmodium falciparum in different geographical areas in Yemen.

Markers		MSP1		MSP2	
Allelic families	K1	MAD20	RO33	FC27	3D7/IC
Hinterland					
No. of alleles	1	3	3	7	7
Size range (bp)	100	170 - 190	130 – 150	130 – 420	580 – 670
Coastland					
No. of alleles	5	5	4	8	12
Size range (bp)	180 – 270	130 – 230	130 – 170	370 – 720	230 – 800
Highland					
No. of alleles	1	2	4	7	7
Size range (bp)	100	180 – 200	140 – 190	150 – 520	140 - 720
All study areas					
No. of alleles	6	6	6	18	24
Size range (bp)	100 - 270	130 - 230	130 - 190	130 - 720	140 - 800
Rural areas					
No. of alleles	3	5	5	14	18
Size range (bp)	100 – 200	170 – 230	130 – 170	360 – 600	140 - 730
Urban areas					
No. of alleles	3	3	2	5	6
Size range (bp)	100 – 270	130 – 180	130 – 140	130 – 490	150 - 800