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Inter-Simple Sequence Repeat (ISSR) Polymorphism of Tuberculosis Patients from Mile Four Hospital, Abakaliki, Nigeria

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Abstract: Tuberculosis (TB) is a serious health issue in Ebonyi State and Nigeria at large. The knowledge of genetic diversity and population structure of TB patients in Ebonyi State is still inadequate. Therefore, this work tends to assess the genetic diversity and population structure in association with tuberculosis population using Inter-simple sequence repeat (ISSR) markers. A total of 25 samples were collected, in which 21 were from TB patients and 4 from NTB patients as controls. DNA extraction was done using Cetyltrimethylammonium bromide (CTAB) method followed by Polymerase chain reaction (PCR) with ISSR primers. Analyses of dendrogram, principal component analysis, genetic diversity and population structure were computed with the scored matrix data. Six ISSR markers were polymorphic. The polymorphic information content (PIC) values ranged from 0.2635 to 0.8158 with a mean of 0.6156. The genetic diversity ranged from 0.1504 to 0.8352 with a mean of 0.6156, while the major allele frequency ranged from 0.2800 to 0.9200 with a mean of 0.5018. The allele count ranged from 1 to 23, while the frequency ranged from 0.0400 to 0.9200. The values of number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H) and Shannon's information index (I) from the entire population were 2.000±0.000, 1.8562±0.1011, 0.4595±0.0330 and 0.6517±0.0350, respectively while that of the six ISSR markers ranges from 1.3200-2.0000, 1.1184-1.9091, 0.0456-0.4571 and 0.1388-0.6611, respectively. From the six ISSR marker data, the number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) ranged from 2 to 25 and 32% to 100% respectively. A dendrogram of the 25 TB and non- NTB clustered them into nine major groups. This study revealed high diversity among TB patientsatgene loci also probed, but did not show any clear association between TB susceptibility and the gene loci.

Keywords: Polymorphic, Population Structure, Genetic Diversity, ISSR Markers, Dendrogram, Association

1. Introduction

Over the years, tuberculosis (TB) has remained a major public health problem both in Nigeria and the world at large. It has been an unprecedented world's most infectious deadly killer disease with about 4500 lives lost per day. Unfortunately, Nigeria is far worse hit by this global epidemic in Africa. Currently she ranks seventh in the world and second in Africa [1].

Tuberculosis is a chronic infectious disease; a pathogenic bacterial disease that is caused by a rod-shaped bacterium *Mycobacterium tuberculosis* [2]. This bacterium especially

Mycobacterium bovis or *Bovis tuberculosis* affects human beings and other higher animals like cattle [2]. The followings are the predisposing or risk factors of tuberculosis: HIV infection, malnutrition, alcoholism, silicosis, cigarette smoking, diabetes melittus, corticosteroids, infliximab, overcrowding and genetic susceptibility [3-6].

In the developing world Tuberculosis is a serious health issue, its etiological mechanisms is not clear and this hinders the development of effective strategies for the treatment of the disease. The worldwide resurgence of TB has been a very big challenge for health institutions and researchers over the

years. According to World Health Organization (WHO) report in 2014, 8.6 million people developed TB disease while 1.3 million people died from TB disease as at 2012 and in this as many as 450,000 new TB cases were caused by multi drug resistant *Mycobacterium tuberculosis* strain and some are being caused by total drug resistant [7]. They also estimated that up to two billion individual are currently infected with TB [8]. The WHO global TB report in 2015 reveals that TB is responsible for the deaths of 1.5 million people and generated 10.4 million cases of which 100,000 were drug resistant tuberculosis [9]. In 2017, about 10 million people developed TB in 2017, there were 1.6 million deaths which among them were 300,000 HIV positive people, out of this 10 million people who fell ill with TB in 2017, only 6.4 million were officially recorded by national reporting systems, leaving 3.6 million people undiagnosed or detected but not reported [8]. Houben and Dodd in their work "The Global Burden of latent TB" reported that almost one in four people in the world are infected with TB bacteria [10]. In 2017, WHO reported that Nigeria is among the three countries that topped most in TB incidence [1].

Despite significant progress over the last decades, TB remains the world's deadliest infectious disease and requires acceleration of efforts to end its epidemic in Nigeria and the whole world. Although it is curable, some of the bacterium that causes TB is becoming resistant to TB drugs, Patients adherence to therapy is another difficult task because most regimes require taking the drug daily for six months even to two years for complicated cases. Because of this mismanagement of TB drugs which causes resistant to the main drugs, there may be a future TB epidemic with restricted treatment options, it is vital that TB control be managed properly and new tools developed to prevent, treat and diagnose the disease. Therefore an association study of candidates with TB need to be conducted so as to examine the association of predicted genetic materials with TB infections to allow identification of the gene for its susceptibility which could pave way to developing a protocol for suppressing the gene.

2. Materials and Method

2.1. Study Area

The research was carried out at the TB unit of Mile Four Hospital (MFH) Abakaliki, Ebonyi State, Nigeria. Mile Four Hospital is located in Abakaliki, Ebonyi State Nigeria at latitude $6.3^0 1^1$N and longitude $8.1^0 3^1$E. Other units in Mile Four Hospital includes; Leprosy Unit, Gynaecology Unit, Peadiatrics and General medicine unit. The patients in this hospital came from Ebonyi, Enugu, Abia and Anambra states.

2.1.1. Experimental Design
This study was designed to assess the genetic diversity of Tuberculosis patients using Inter-simple sequence repeat markers. Both TB patients' blood and the blood of people that are not with active TB were used. The study population includes a total of 25 individuals (21 TB patients and 4 non TB patients).

2.1.2. Ethical Consideration
Ethical clearance was collected from Ebonyi state University with reference number EBSU/DRIC/UREC/VOL. 04/056 and mile four hospital. Written informed consent was obtained from all participants who willingly accepted to participate in the study.

2.2. Sample Collection and Analysis

Five millilitres of blood sample were collected by the nurses in the hospital through vein puncture from each consenting individual into a sterile container with ethyl-diamine–tetraacetric acid (EDTA) anticoagulants. Each sample was labeled with the date of collection and laboratory identity number. Samples were transported to the laboratory immediately in cold box with frozen ice packs to achieve condition of about 4-8°C. The molecular analysis was carried out in Biotech Research Centre in Ebonyi State University, Abakaliki, Ebonyi State, Nigeria.

2.2.1. Analysis of Genetic Diversity Using ISSR Marker Techniques
Genomic DNA was extracted from blood samples of 21TB patients and 4 Non TB individuals using CTAB method of DNA extraction procedure. From the blood sample, 250 μL of whole blood was pipetted and 3ml of whole blood was also pipetted, 500 μL of extraction buffer was added in a sterile 1.5 ml microcentrifuge, It was thoroughly mixed and vortexed. Then it was incubated in water bath at 60°C for 15 minutes, brought to room temperature and equal volume of phenol was added, chloroform and iso-amyl alcohol was also added in the ratio of 25:24:1. It was well mixed by vortexing and centrifuged at 13000 rpm for 10min. It then was removed from the centrifuge and 450 ul of the supernatant was transferred into new sterile 1.5ml tube. 400 μL of ice-cold isopropanol was added for precipitation of the DNA and it was mixed and incubated for 2 hours at -20°C. After the incubation, it was transferred to the centrifuging machine and centrifuged at 14000 rpm for 15 min to sediment the DNA. The supernatant was decanted gently ensuring that the pellets were not disturbed. 500$_s$μL of 70% ethanol was added to the pellets and centrifuged at 13000 rpm for 5 min to wash the pellet. The ethanol was decanted and air-dried the DNA at room temperature. The pellets were suspended in 100 μl of nuclease free water for further use or storage.

DNA quality was assessed by using 1.5% agarose gel for electrophoresis. It was then visualized under UV light.

2.2.2. PCR Amplifications
PCR amplification was done using PCR machine and ISSR makers

The PCR consisted of 2.0 μL of 100ng/μL DNA, 2.5μL of 10 x buffer (Bioline, Taunton, USA), 1.5 μL of 50mM $MgCl_2$ (Bioline, Taunton, USA), 2.0 μL of 2.5 mMdNTPs (Bioline, Taunton, USA), and 0.2 μL 500U DNA Taq polymerase (Bioline, Taunton, USA), 1.0 μL of 10ppm each primer per ISSR primer and was made up to total volume of 25uL by adding 15.8 μL DEPC-treated water (Invitrogen Corporation, USA). The PCR cycling profile for the reaction consist of an initial step at 94°C for 5 min., 35 cycles of 94°C for 30s,

55°C for 1min, and 72°C for 1 min., and 8-min final extension at 72°C. 5μL of the amplicon was electrophoresed in 1.5% agarose gel containing 0.5mg/ml ethidium bromide.

Gel electrophoresis was done using agarose gel and electrophoresis apparatus while the image was viewed and captured using transilluminator UV light. 1.5% Agarose Gel was prepared using Biology dictionary.net/gel electrophoresis method. The following buffers and solutions were used; Agarose solution, Ethidium bromide and electrophoresis buffer and DNA samples and ladders were used for the nucleic acids and oligonucleotides.

Table 1. List of primers used for this study.

S/N	Primer name	Sequence (5´-3´)
1.	ISSR889	D^{c}B^{d}D^c ACA CAC ACA CAC AC
2.	ISSR827	ACACACACACACACACG
3.	ISSR856	ACACACACACACACACY^{a}A
4.	UBC826	ACACACACACACACACC
5.	ISSR888	BDB CAC ACA CACACACA
6.	ISSR816	CACACACACACACACAT
7.	ISSR890	VHVGTGTGTGTGTGTGT
8.	ISSR836	AGA GAG AGA GAG AGA GY^a
9.	ISSR901	CACACACACACACACARY
10.	ISSR808	AGAGAGAGAGAGAGAGC
ISSR Data Analysis		

The ISSR data was analyzed by scoring one (presence of alleles) and O (absence of alleles). The data achieved from the scoring of the ISSR amplicons were used for phylogenetic reconstruction using Unweighted Pair Group Mean Arithmetic (UPGMA) and dissimilarity index in Jaccard's option (Jaccard, 1908) were used for the analysis. The analysis was conducted using NTSYSpc software version 2.02.

The genetic diversity, allele frequency and PIC were computed using Power Marker (Version 3.25). Genetic diversity and population structure analyses of the accessions were analyzed using POPGENE software version 1.32.

3. Results

3.1. ISSR Polymorphism

To access the level of genetic diversity in 25 samples of TB and NTB population studied, a total of ten ISSR primers were used to study the genetic diversity among the human population identified to be TB and non-TB individuals. The ten primers produced bands that were used in the analyses. The ten (10) ISSR primers amplified a total of 155 alleles. The amplified alleles from each primer ranged from 3-24, with a mean of 15.500 (Table 2). Polymorphic information content values ranged from 0.1450-0.8158 with a mean value of 0.5872. The ISSR markers such as ISSR 889, 827, 856, 826, 890, and 901 were found to be polymorphic while ISSR 888, 816, 836 and 808 were monomorphic with the least of PIC values. The genetic diversity ranged from 0.1504-0.8352 with a mean value of 0.6156 while major allele frequency spanned from 0.2800-0.9200 with a mean value of 0.5018.

Table 2. Allele frequency, number of alleles, gene diversity and polymorphic information content values generated from twenty five TB and Non TB patients using ten ISSR markers.

Marker	Major allele frequency	No. of obs.	Allele No	Gene diversity	PIC
ISSR 889	0.4000	25.0000	24.0000	0.7392	0.7010
ISSR 827	0.6000	25.0000	23.0000	0.5952	0.5618
ISSR 856	0.3600	25.0000	24.0000	0.7872	0.7623
UBC 826	0.2800	25.0000	24.0000	0.8352	0.8158
ISSR 888	0.8400	25.0000	3.0000	0.2816	0.2635
ISSR 816	0.7600	25.0000	3.0000	0.3904	0.3531
ISSR 890	0.3600	25.0000	22.0000	0.7808	0.7521
ISSR 836	0.9200	25.0000	10.0000	0.1504	0.1450
ISSR 901	0.2800	25.0000	18.0000	0.7936	0.7625
ISSR 808	0.6800	25.0000	4.0000	0.4576	0.3834
Mean	0.5018	25.0000	15.500	0.6156	0.5872

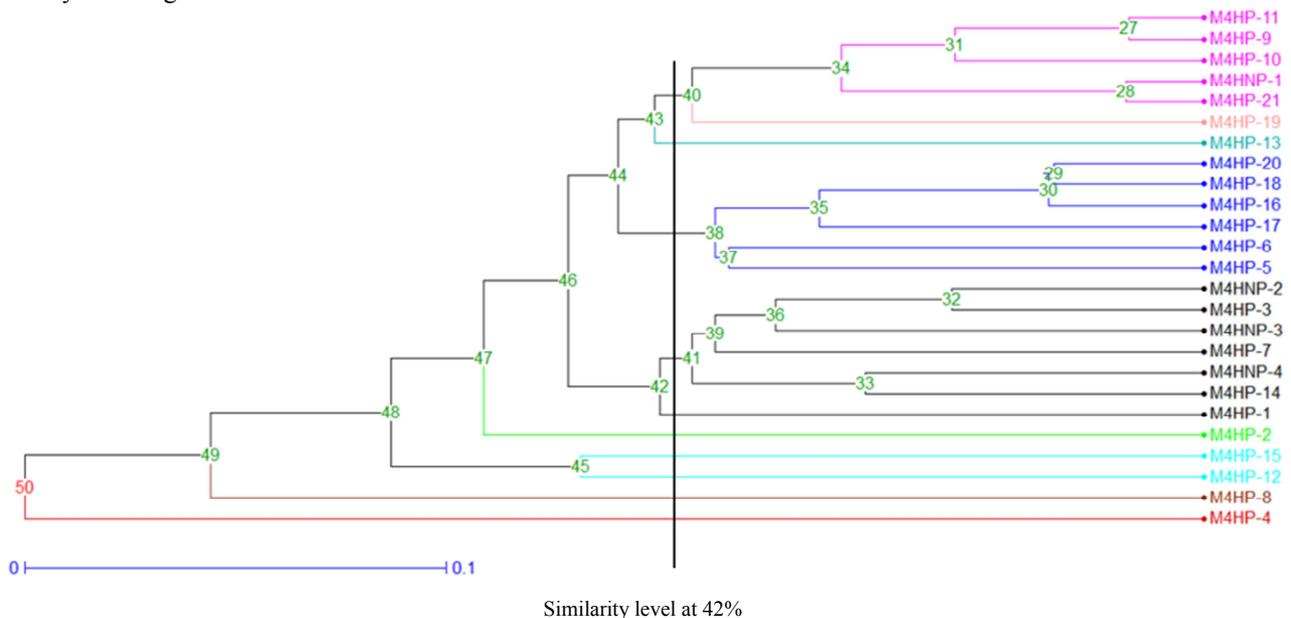PIC=Polymorphic information content

Similarity level at 42%

Figure 1. Dendrogram of tuberculosis and non-tuberculosis populations as determined by ten Inter-simple sequence repeat (ISSR) markers.
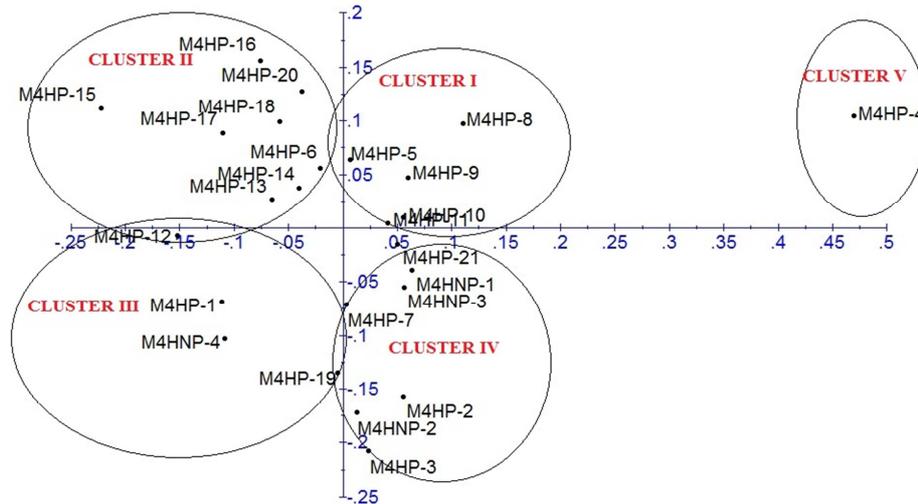
Figure 2. Principal component analysis of tuberculosis and non-tuberculosis patients as revealed by ten inter-simple sequence repeat markers.

Table 3 represented the allele, allele count and frequency obtained from each of the ISSR loci in the genotypes. The allele count spanned between 1 and 23 while the frequency ranged from 0.0400 and 0.9200.

The ISSR markers revealed the highest genetic diversity in the TB sample from Enugu State population compared to the other populations, with number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (H), and Shannon's information index (I) values of 2.000, 1.9007, 0.4739 and 0.6668, respectively with a sample size of 1 (Table 4), while the genetic diversity in Anambra State population was found to be the lowest, with corresponding values of 2.000, 1.8207, 0.4455 and 0.6363. The degree of genetic diversity of these samples were ranked in a decreasing order as Enugu>Ebonyi>Abia>Anambra populations. From the ISSR markers, the mean values of Na, Ne, H and I from the entire population were 2.000, 1.8562, 0.4595 and 0.6517 with standard deviations of 0.000, 0.1011, 0.0330 and 0.0350, respectively.

Table 3. *Allele, allele count and frequency values generated from twenty five Tuberculosis and NON Tuberculosis patients using ten Inter Simple Sequence Repeat markers.*

Marker	Allele	Count	Frequency
ISSR 889	0/0/0/1	1	0.0400
ISSR 889	0/0/1/0	4	0.1600
ISSR 889	0/0/1/1	6	0.2400
ISSR 889	0/1/1/0	10	0.4000
ISSR 889	0/1/1/1	3	0.1200
ISSR 889	1/1/1/0	1	0.0400
ISSR 827	0/1/1/0/0/1/0	4	0.1600
ISSR 827	0/1/1/1/0/1/0	15	0.6000
ISSR 827	0/1/1/1/0/1/1	1	0.0400
ISSR 827	0/1/1/1/1/1/0	3	0.1200
ISSR 827	1/1/1/1/0/1/0	1	0.0400
ISSR 827	1/1/1/1/1/1/0	1	0.0400
ISSR 856	0/0/0/0/1	1	0.0400
ISSR 856	0/0/1/0/0	6	0.2400
ISSR 856	0/0/1/1/1	1	0.0400
ISSR 856	0/1/1/0/1	3	0.1200
ISSR 856	0/1/1/1/0	1	0.0400
ISSR 856	1/0/1/0/1	1	0.0400

Marker	Allele	Count	Frequency
ISSR 856	1/1/1/0/1	1	0.0400
ISSR 856	1/1/1/1/0	1	0.0400
ISSR 856	1/1/1/1/1	1	0.0400
UBC 826	0/0/0/0/1	2	0.0800
UBC 826	0/0/1/0/1	2	0.0800
UBC 826	0/0/1/1/1	2	0.0800
UBC 826	0/1/1/0/1	3	0.1200
UBC 826	0/1/1/1/1	4	0.1600
UBC 826	1/0/1/1/1	1	0.0400
UBC 826	1/1/1/0/1	7	0.2800
UBC 826	1/1/1/1/1	4	0.1600
ISSR 888	0/0/0/1	2	0.0800
ISSR 888	1/1/0/1	2	0.0800
ISSR 888	1/1/1/1	21	0.8400
ISSR 816	0/0/1	2	0.0800
ISSR 816	0/1/1	4	0.1600
ISSR 816	1/1/1	19	0.7600
ISSR 890	1/0/0/0	5	0.2000
ISSR 890	1/0/0/1	3	0.1200
ISSR 890	1/0/1/0	2	0.0800
ISSR 890	1/1/0/0	1	0.0400
ISSR 890	1/1/0/1	4	0.1600
ISSR 890	1/1/1/1	9	0.3600
ISSR 836	0/0/0	1	0.0400
ISSR 836	0/1/1	23	0.9200
ISSR 836	1/1/1	1	0.0400
ISSR 901	0/0/0	3	0.1200
ISSR 901	0/1/0	5	0.2000
ISSR 901	0/1/1	3	0.1200
ISSR 901	1/0/0	1	0.0400
ISSR 901	1/1/0	7	0.2800
ISSR 901	1/1/1	6	0.2400
ISSR 808	0/0	17	0.6800
ISSR 808	1/0	7	0.2800
ISSR 808	1/1	1	0.0400

Population Structure and Genetic Diversity of TB Population from Mile Four Hospital Abakaliki

From the genetic differentiation using ISSR markers, the mean values of total gene diversity (Ht), gene diversity within population (Hs), coefficient of gene differentiation or gene diversity among the population (Gst) and level of gene flow (Nm) were 0.4515, 0.3869, 0.1580 and 2.6647, respectively. This is shown in Table 5. The values of Ht, Hs,

Gst and Nm from the sample collected from patients from Enugu State ranged from 0.000-0.4739, 0.000-0.4000, 0.0000-0.1559 and 0.0000-2.7072 with mean values of 0.4739, 0.4000, 0.1559 and 2.7072, respectively. The values of Ht, Hs, Gst and Nm from the population of TB and NTB collected from patients from Ebonyi State ranged from 0.4506-0.4898, 0.3429-0.4327, 0.0578-0.2533 and 1.4737-8.1538 with mean values of 0.4634, 0.3899, 0.1582 and 3.0452, respectively. Those collected from patients from Anambra State had the values of Ht, Hs, Gst and Nm from the population ranged from 0.3367-0.4800, 0.2857-0.4408, 0.0816-0.1837 and 2.2222-5.6250 with mean values of 0.4455, 0.3853, 0.1368 and 1.0373, respectively. Those collected from patients from Abia State had the values of Ht, Hs, Gst and Nm from the population ranged from 0.4200-0.4898, 0.3102-0.4082, 0.1667-0.2614 and 1.4126-2.5000 with mean values of 0.4549, 0.3592, 0.2141 and 1.9563, respectively.

Table 4. *Genetic diversity parameters generated from 25 samples from Tuberculosis and Non-Tuberculosis patients using ten ISSR markers.*

Sample name	Location	Population size	Na	Ne	H	I
M4HP-1	Ebonyi	17	2	1.8491	0.4592	0.6518
M4HP-2	Ebonyi		2	1.6897	0.4082	0.5983
M4HP-3	Ebonyi		2	1.7896	0.4412	0.6332
M4HP-5	Ebonyi		2	1.9429	0.4853	0.6784
M4HP-6	Ebonyi		2	1.876	0.4669	0.6597
M4HP-7	Ebonyi		2	1.9429	0.4853	0.6784
M4HP-8	Ebonyi		2	1.8202	0.4506	0.6429
M4HP-11	Ebonyi		2	1.9007	0.4739	0.6668
M4HP-13	Ebonyi		2	1.96	0.4898	0.6829
M4HP-15	Ebonyi		2	1.8491	0.4592	0.6518
M4HP-16	Ebonyi		2	1.8491	0.4592	0.6518
M4HP-18	Ebonyi		2	1.9007	0.4739	0.6668
M4HP-21	Ebonyi		2	1.9007	0.4739	0.6668
M4HNP-1	Ebonyi		2	1.876	0.4669	0.6597
M4HNP-2	Ebonyi		2	1.8202	0.4506	0.6429
M4HNP-3	Ebonyi		2	1.8491	0.4592	0.6518
M4HNP-4	Ebonyi		2	1.9007	0.4739	0.6668
Total			34	31.7167	7.8772	11.1508
Mean			2	1.8657	0.4634	0.6560
				Ne	H	I
M4HP-4	Anambra	5	2	1.5077	0.3367	0.5196
M4HP-9	Anambra		2	1.9231	0.48	0.673
M4HP-10	Anambra		2	1.9231	0.48	0.673
M4HP-14	Anambra		2	1.96	0.4898	0.6829
M4HP-19	Anambra		2	1.7896	0.4412	0.6332
Total			10	9.1035	2.2277	3.1817
Mean			2	1.8207	0.4455	0.6363
M4HP-20	Enugu	1	2	1.9007	0.4739	0.6668
Total			2	1.9007	0.4739	0.6668
Mean			2	1.9007	0.4739	0.6668
M4HP-12	Abia	2	2	1.7241	0.42	0.6109
M4HP-17	Abia		2	1.96	0.4898	0.6829
Total			4	3.6841	0.9098	1.2938

Table 5. *Genetic differentiation in the populations of TB and Non-TB patients using ten ISSR markers.*

Sample name	Location	Population size	Ht	Hs	Gst	Nm
M4HP-1	Ebonyi	17	0.4592	0.3673	0.2000	2.0000
M4HP-2	Ebonyi		0.4082	0.3592	0.1200	3.6667
M4HP-3	Ebonyi		0.4412	0.3837	0.1304	3.3333
M4HP-5	Ebonyi		0.4853	0.4000	0.1758	2.3445
M4HP-6	Ebonyi		0.4669	0.4000	0.1434	2.9878
M4HP-7	Ebonyi		0.4853	0.4245	0.1253	3.4899
M4HP-8	Ebonyi		0.4506	0.3592	0.2029	1.9643
M4HP-11	Ebonyi		0.4739	0.3918	0.1731	2.3881
M4HP-13	Ebonyi		0.4898	0.4163	0.1500	2.8333
M4HP-15	Ebonyi		0.4592	0.3429	0.2533	1.4737
M4HP-16	Ebonyi		0.4592	0.3592	0.2178	1.7959
M4HP-18	Ebonyi		0.4739	0.3837	0.1904	2.1267
M4HP-21	Ebonyi		0.4739	0.4000	0.1559	2.7072
M4HNP-1	Ebonyi		0.4669	0.4000	0.1434	2.9878
M4HNP-2	Ebonyi		0.4506	0.4082	0.0942	4.8077
M4HNP-3	Ebonyi		0.4592	0.4327	0.0578	8.1538
M4HNP-4	Ebonyi		0.4739	0.4000	0.1559	2.7072

Sample name	Location	Population size	Ht	Hs	Gst	Nm
Total			7.8772	6.6287	2.6896	51.7679
Mean			0.4634	0.3899	0.1582	3.0452
Sample name	Location	Population size	Ht	Hs	Gst	Nm
M4HP-4	Anambra	5	0.3367	0.2857	0.1515	2.8000
M4HP-9	Anambra		0.4800	0.3918	0.1837	2.2222
M4HP-10	Anambra		0.4800	0.4408	0.0816	5.6250
M4HP-14	Anambra		0.4898	0.4408	0.1000	4.5000
M4HP-19	Anambra		0.4412	0.3673	0.1674	2.4862
Total			2.2277	1.9264	0.6842	17.6334
Mean			0.4455	0.3853	0.1368	1.0373
M4HP-20	Enugu	1	0.4739	0.4	0.1559	2.7072
Total			0.4739	0.4	0.1559	2.7072
Mean			0.4739	0.4	0.1559	2.7072
M4HP-12	Abia	2	0.4200	0.3102	0.2614	1.4126
M4HP-17	Abia		0.4898	0.4082	0.1667	2.5000
Total			0.9098	0.7184	0.4281	3.9126
Mean			0.4549	0.3592	0.2141	1.9563
Over all mean			0.4595	0.3869	0.158	2.6647
St. Dev			0.0011	0.0014		

Ht=total gene diversity; Hs=gene diversity within population; Gst=coefficient of gene differentiation or gene diversity among the population; Nm=level of gene flow.

Table 6 Genetic diversity within 21 Nigerian Tuberculosis and Non Tuberculosis patients using inter-simple sequence repeat markers.

Marker	Na	Ne	H	I	NPL	PPL%
ISSR 889	2.000	1.6598 (0.197)	0.3886 (0.0791)	0.5742 (0.089)	25	100
ISSR 827	2.000	1.8805 (0.159)	0.4637 (0.0564)	0.6551 (0.061)	25	100
ISSR 856	2.000	1.7712 (0.289)	0.4180 (0.1113)	0.6031 (0.124)	25	100
UBC 826	2.000	1.8443 (0.194)	0.0456 (0.0712)	0.6408 (0.078)	25	100
ISSR 888	2.000	1.9091 (0.176)	0.0472 (0.0678)	0.6611 (0.075)	25	100
ISSR 816	2.000	1.8659 (0.191)	0.4571 (0.0707)	0.6475 (0.978)	25	100
ISSR 890	2.000	0.7296 (0.292)	0.4016 (0.1267)	0.5807 (0.580)	24	96
ISSR 836	1.9600 (0.2)	1.6729 (0.150)	0.3951 (0.0839)	0.5777 (0.121)	24	96
ISSR 901	1.88 (0.3317)	1.5841 (0.315)	0.3396 (0.1557)	0.5016 (0.213)	22	88
ISSR 808	1.3200 (0.47)	1.1184 (0.1895)	0.0849 (0.1301)	0.1388 (0.209)	2	32

Standard deviations are in parentheses; NPL=number of polymorphic loci; PPL=percentage polymorphic loci; Ne=effective number of alleles; H=Nei's gene diversity, I=Shannon's information index.

From the ten ISSR marker data, the number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) ranged from 2-25 and 32%-100%, respectively as shown in Table 6. The Na, Ne, H and I spanned from 1.3200-2.0000, 1.1184-1.9091, 0.0456-0.4571 and 0.1388-0.6611, respectively. Their standard deviation ranges were 0.2000-0.4761, 0.1502-0.3154, 0.0564-0.1557 and 0.0615-0.9781, respectively.

3.2. Hierarchical Clustering TB and Non TB Population in Mile Four Hospital, Abakaliki

A dendrogram of the 25 TB and NTB using UPGMA procedure clustered them into nine major groups (Figure 1). Group I consists of M4HP-11, M4HP-9, M4HP-10, M4HNP-1 and M4HP-21, M4HP-19 with 40% bootstrap value. They contained genotypes from places such as Ebonyi, Enugu, Anambra and Abia States. The genotype M4HP-19 is the most genetically isolated one in group I with genetic distance of 40%. Group II had only M4HP-13 with 43% bootstrap value and it was collected from a patient from Ebonyi State. Group III clustered M4HP-20, M4HP-18, M4HP-16, M4HP-17, M4HP-6, M4HP-5 with 38% bootstrap value and was collected from a patient from Enugu, Ebonyi and Abia State.

Group IV contained M4HNP-2, M4HP-3, M4HNP-3, M4HP-7, M4HNP-4 and M4HP-14 with a bootstrap value of 41% and they were sampled from Anambra and Ebonyi States. Group V had 42% of genetic distance and clustered M4HP-1. Group VI had only M4HP-2 with a value of 47%. Group VII clustered together M4HP-15 and M4HP-12 with a value of 45%. Group VIII had a total of genetic distance of 49% and contained only M4HP-8. Group IX had a total of 50% of its genetic distance and clustered only M4HP-4. The dissimilarity maximum value was 0.7097 while that of the minimum was 3.5714×10^{-2} at 95% percentile with the edge length sum of 3.0373.

Principal component analysis of the generated amplicons resulted to five clusters (Figure 2). Each cluster is a representative of unique accession.

4. Discussion

The results of the study showed that six primers used were highly polymorphic. The six ISSR markers that were polymorphic in this study were in agreement with the work of Bonnet and Branchard on their work titled 'Non anchored Inter Simple Sequence Repeat Markers' they found out that ISSR

markers were proved to be polymorphic in the assessment of genetic diversity [11]. The percentage of polymorphism observed among the study group was expected because the genotypes studied were from different individual that came from different States.

On the PIC value, most of the markers (seven) can be used as an informative in population – genetic analyses, this is in conformity with Botstein and his group on their works on the construction of a genetic – linkage map in man using restriction fragment length polymorphisms. According to them, the markers showing PIC values higher than 0.5 are normally considered as a good source of information in population genetics analyses [12]. The result of the population structure and genetic diversity showed that the mean values of total gene diversity (Ht) was 0.4515, that of gene diversity within population (Hs) was 0.3869, coefficient of gene differentiation (Gst) or gene diversity among the population was 0.1580 and the level of gene flow (Nm) was 2.6647. The high gene flow among populations detected in this study pointed towards the possibility of instances of more than one isolated populations possessing unique genotypes not found in other populations this is because Kumar and his group said that in population genetics, the value of gene flow was normally less than one migrant per generation into a generation, the value of GST among the population was greater than 0.25 and this generally was regarded as the threshold quantity beyond which significant population occurs [13]. The value of GST in this study was lower than that of the threshold quantity beyond which significant population occurs; this was in contrary with the work of Kumar on the assessment of genetic diversity of *Podophyllumhexandrum* using ISSR markers. In their study, The GST value was higher than that of the threshold quantity [13]. In assessing the population diversity of the study group, the ones from Enugu state demonstrated the highest values of Ht and Hs followed by those from Ebonyi state, and then Abia and lastly Anambra but GST was higher in the group from Abia state followed by Ebonyi State, Enugu and then Anambra. NM was higher in Ebonyi state followed by those from Enugu, then Abia and lastly Anambra state. The number of polymorphic loci (NPL) and percentage polymorphic loci (PPL) ranged from 2-25 and 32%-100%, respectively. The high level of percentage polymorphic loci (PPL) greater than 70% suggests that there was high degree of genetic variation among TB patients for ISSR marker, such high level of percentage polymorphic loci was also reported by Kumar and his group in their study on the assessment of genetic diversity of *Podophyllumhexandrum* using ISSR markers [13]. Their PPL were mostly 100% and few 80%. The percentage polymorphism obtained from this present study was quite high indicating the higher informative nature of the ISSR markers used.

The number of observed alleles (Na) is 2.0000 in all the samples tested. The maximum number of effective alleles (Ne) (1.9007) was found in population from Enugu and the lowest number (0.8207) was detected in population from Anambra state. Based on the Nei's gene diversity (H) and

Shannon's information index (I) values, Population from Anambra state showed a slightly lower genetic diversity. The analysis of genetic polymorphism obtain with ISSR markers demonstrated that the highest percentage of polymorphic loci (PPL) was 100%, largest number of polymorphic loci (NPL) was 25 and the highest gene diversity (H=0.6363) and Shannon's information index (0.6668) were found in population from Enugu state. Therefore, the sample individuals from Enugu state could be considered to have the highest genetic diversity. This may be as a result of different strains of TB diseases among the patients.

Variation in the numbers of alleles at each marker locus serve as a measure of genetic variability. More than 80% of the markers exhibited three or more alleles per locus.

The effective number of allele at each locus provides information on predominant alleles. Since allelic diversity in this work was high, it can be concluded that the genetic diversity is sufficiently high. However, a higher number of alleles for each locus showed that the marker used were appropriate to analyze diversity.

The result of the dendrogram revealed that some of the clustering pattern of the groups showed the existence of definite pattern of relationship among TB patients, geographical origins and genetic diversity. It was obviously demonstrated that some groups were made up of only TB patients. Some clusters were made up of genotypes mostly from the same geographical region while others had genotypes from two or more areas, hence the number of entries varied from cluster to cluster. The clustering showed genetic relatedness among the individuals and the genetic distance varied among groups/clusters. The principal component analysis clustered them into five clusters with each cluster being so unique; four of the clusters are closely related and have genetic distance of 0.15 and below while one isolated itself and had a genetic distance of 0.5. This divergence may have been caused by mutation or introduction of other materials in the person's body.

The NTB's clustering together with TB patients showed no variation between Non TB and TB patients, but the vast majority of the Non TB patients were grouped into a single group which indicates high similarity among the individuals.

In all, the presence of familial clustering with a genetic distance less than 50% in the dendrogram and 0.5 in the PCA shows that the TB patients though coming from different geographical locations are genetically related. The familiarity or closeness of the cluster indicates that there is a susceptibility gene somewhere in the chromosomal region.

Conclusively, this work has shown that this was the first known report on the use of ISSR markers for analyzing genetic diversity of people suffering from infectious diseases like TB, the result could not be compared with any other previous findings in Nigeria since none had been done to the best of my knowledge.

5. Conclusion and Recommendation

Based on the polymorphic feature and genetic diversity, it

is evident from the data that since the allelic diversity is high, the genetic diversity is therefore high and based on the criterion for selecting ISSR markers, the markers used is highly polymorphic and it is very useful for the evaluation of genetic diversity within and among population. ISSRs have proved to be efficient in analyses of the levels of genetic differentiation and diversity in mammals and other organisms.

Also the clustering of both TB and Non TB patients in one cluster equally showed that the gene of an individual should not be considered as the main predisposing factor for susceptibility to TB disease.

Due to the limitations in the number of studied populations, further research with more population needs to be analysed to be able to evaluate the genetic diversity and population structure among populations.

References

[1] World Health Organization (2018). *"World Tuberculosis Day"* Global Tuberculosis report 287 pp.

[2] Davies, P. (2006). ″A possible link between vitamin D deficiency and impaired host defense to Mycobacterium tuberculosis″. *Tubercle.* 66 (4): 301–306.

[3] Anthony, H. (2005). *TB/HIV*: a Clinical Manual (seconded.). Geneva: *World Health Organization*. 75 pp.

[4] Möller, M. and Hoal, E. (March 2010). "Current findings, challenges and novel approaches in human genetic susceptibility to tuberculosis". *Tuberculosis.* 90 (2): 71–83.

[5] Nicas, M., Nazaroff, W. and Hubbard, A. (2005). "Toward understanding the risk of secondary airborne infection: emission of respirable pathogens". *Journal of Occupational and Environmental Hygiene.* 2 (3): 143–154.

[6] Restrepo, B. (2007)."Convergence of the tuberculosis and diabetes epidemics: renewal of old aquaintances". *Clinical Infectious Diseases.* 45 (4): 436–438.

[7] World Health Organization (2015). "*Tuberculosis Factsheet"* Global tuberculosis report Geneva: 104pp.

[8] World Health Organization (2016). *"Tuberculosis Factsheet"* Global Tuberculosis report Geneva 206 pp.

[9] World Health Organization (2015). World Health Organization international TB publication. *Global Report* 1126 pp.

[10] Houben, C. and Dodd, E. (2016) Burden of latent TB infections, *A Re-estimation using Mathematical Modeling* 45: 451-554.

[11] Bornet, B. and Branchard, M. (2012). 'Non anchored inter simple sequence repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting', *Plant Molecular Biology Reporter* 19; 209–215.

[12] Botstein D, (1980) Construction of a genetic-linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics* 32 (3): 314-31.

[13] Kumar, A., Gaura, G., Gandham, R., Panigrahi, M., Ghosh. S., Saravanan, B., Bhushan, B., Tiwari, A., Sulabh, S., Priya, B., Asaf, V., Gupta, J., Wani, S., Sahu, A. and Sahoo, A. (2017). Global gene expression profile of peripheral blood mono nuclear cells challenged *Theileria annulata* in cross bred and indigenous cattle. *Infection, Genetics and Evolution; journal of molecular epidemiology and evolutionary genetics of infectious diseases* 47: 9–18.