Genetic diversity of *Mycobacterium tuberculosis* in south coastal Karnataka, India, using spoligotyping

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Background & objectives: Despite high occurrence of tuberculosis in India very little information is available about the genetic diversity of *Mycobacterium tuberculosis* isolates prevailing in coastal Karnataka, India. Thus, the present study was undertaken to explore the genetic biodiversity of *M. tuberculosis* isolates prevailing in south coastal region of Karnataka (Udupi District), India.

Methods: A total of 111 Mycobacterial isolates were cultured in Lowenstein Jensen (LJ) medium and after obtaining growth, DNA was extracted and spoligotyping was performed. SITVIT WEB database was used to locate families of spoligotypes.

Results: On analyzing the hybridization results of all 111 isolates on SITVIT WEB database 57 (51.35%) isolates were clustered into 11 Spoligotype International Types (SIT). The largest cluster of 14 (12.61%) isolates was SIT-48 (EAI1-SOM), followed by SIT-1942 (CAS1-Delhi) with 11 isolates (9.9%) and SIT-11 with seven (6.30%). Moreover, 23 isolates (20.72%) had unique spoligotypes and 31 (27.92%) were orphans. Spotclust analysis revealed that majority (67%) of orphan isolates were variants of CAS (37%) and EAI-5 (34%).

Interpretation & conclusions: The present study revealed high biodiversity among the circulating isolates of *M. tuberculosis* in this region with the presence of mixed genotypes earlier reported from north and south India along with certain new genotypes with unique SITs. The study highlights the need for further longitudinal studies to explore the genetic diversity and to understand the transmission dynamics of prevailing isolates.

Key words Genetic diversity - genotyping - *Mycobacterium tuberculosis* - spoligotyping

In 2016, an estimated 10.4 million people developed tuberculosis and 1.67 million died from the disease globally, highlighting the devastation caused by *Mycobacterium tuberculosis* (MTB). India reported the largest number of cases (25%) of the global total[1]. Comparing the share in global burden, the knowledge about the genetic diversity of strains prevailing in India is inadequate. Study on genetic diversity of MTB is important for effectively controlling the emerging drug-resistant strains. Further, it also enables us to understand the transmission dynamics and differentiate between reinfection and reactivation in patients with relapse.

Application of molecular methods such as spoligotyping, restriction fragment length polymorphism (RFLP) and Mycobacterial interspersed repetitive units-Variable number of tandem repeats (MIRU-VNTRs) is useful for understanding the prevailing MTB genotypes and their transmission within the population. Over the years, spacer oligonucleotide genotyping (spoligotyping) has emerged as the most widely used method for MTB genotyping after IS6110-based fingerprinting, which requires large quantities of good quality DNA and has a limitation in isolates having low copies of IS6110 elements[2]. Spoligotyping is based on DNA polymorphism in direct repeat (DR) locus of MTB. The latter contains multiple 36 base pair DRs which are well conserved and are interspersed with non-repetitive spacer sequences which are 34-41 base pairs long[3]. On comparing DR regions of several isolates of MTB, it was observed that the deletions and insertions in DRs occur, but the order of the spacers remains same. Thus, variation in the sequence of spacers is used to determine genetic diversity among isolates by targeting the DR locus for *in vitro* DNA amplification. Spoligotyping is a simple, rapid and sensitive test, and can be performed directly from the clinical samples even if the bacteria are not viable or are in tissues in paraffin-embedded blocks[4].

The population of Udupi district in Karnataka State of India comprises a section of migratory people from other parts of Karnataka. It will be interesting to know about the circulating genotypes of MTB in this geographical area. Thus the present study was aimed to characterize the MTB isolates from pulmonary tuberculosis patients using spoligotyping and to identify the predominant MTB clades prevalent in Udupi district of south coastal Karnataka.

Material & Methods

A cross-sectional epidemiological study was carried out from January to August 2016 in the department of Microbiology, Kasturba Medical College (KMC), Manipal, Karnataka, in association with National JALMA Institute for Leprosy and other Mycobacterial Diseases (ICMR), Agra, after obtaining the approval of Institutional Research and Ethical Committee of KMC, Manipal.

Sample processing & DNA extraction: The stock isolates of MTB obtained from sputum of smear-positive pulmonary tuberculosis patients collected from designated microscopy centres of three *taluks* (Udupi, Kundapura and Karkala) in a stratified sampling technique from Udupi district from September 2011 to August 2014 in our previous study[5], were used. For spoligotyping, the stock isolates were subcultured on Lowenstein–Jensen (LJ) medium (HiMedia, Mumbai) and incubated for four to eight weeks at 37°C. After obtaining the growth on LJ medium, DNA extraction was performed using a commercially available kit from Qiagen (Hilden, Germany) as per manufacturer's instructions.

Spoligotyping: Spoligotyping was carried out by amplifying the whole DR region using the commercially available kit (Mapmygenome, Hyderabad) according to a standardized method using the designated primers' pairs of DRa and DRb[6]. Master cycler gradient 5331 (Eppendorf, Germany) was used for DNA amplification. The amplified PCR products were hybridized with nitrocellulose membrane having covalently linked 43 spacer oligonucleotides following the manufacturer's instructions. MTB H37Rv and *M. bovis* were used as positive control and double-distilled water as a negative control. The hybridized fragments were identified using enhanced chemiluminescence system (GE Healthcare, UK). The spoligotypes were initially reported as 43 digits binary representation of 43 spacers, one (1) was scored for positive hybridization and zero (0) for negative hybridization. The binary codes were converted into octal codes. The octal codes were analyzed using the SITVIT WEB (*http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/*) database to locate the families of spoligotypes[7]. A cluster was defined as two or more isolates with identical spoligotype patterns. However, if the spoligotype pattern in the SITVIT WEB database corresponded to only one isolate, then the pattern was called 'unique'. The spoligotypes that did not match any pattern in the SITVIT WEB database were defined as 'orphan'. The orphans were further analyzed by 'Spotclust', which assigns families based on SpolDB3.0 (*http://tbinsight.cs.rpi.edu/run_spotclust.html*)[8]. The evolutionary relationship of the clinical isolates was analysed based on spoligotyping data by calculating NJ tree dendrogram (Fig. 1) which was based on Jaccard Index and minimum spanning tree (Fig. 2) using MIRU-VNTR*plus* (*http://www.miru-vntrplus.org/MIRU/index.faces*)[9-11]. Discriminatory power of spoligotyping was determined by calculating Hunter Gaston Discriminatory Index (HGDI)[12]. Association between demographic characteristics of the patients with the occurrence of clustered and non-clustered MTB isolates was examined using univariate odds ratio.

Results

A total of 111 clinical isolates of MTB obtained from sputum samples of pulmonary tuberculosis patients were spoligotyped and analyzed. On SITVIT WEB analysis,
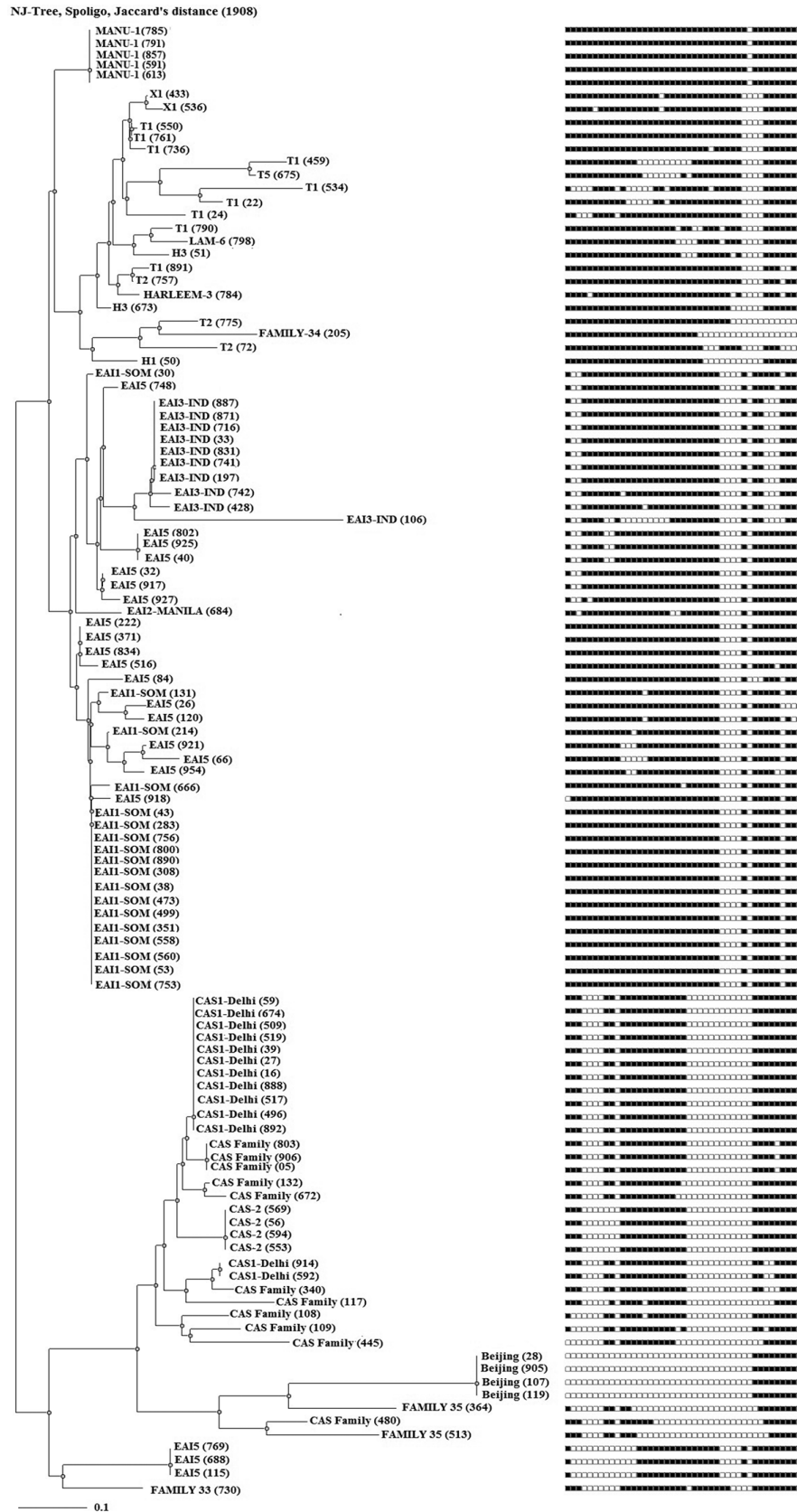
Fig. 1. A spoligotyping-based dendogram generated through N-J tree analysis using Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats plus database showing genetic diversity of 111 clinical isolates from Udupi district, Karnataka, India.
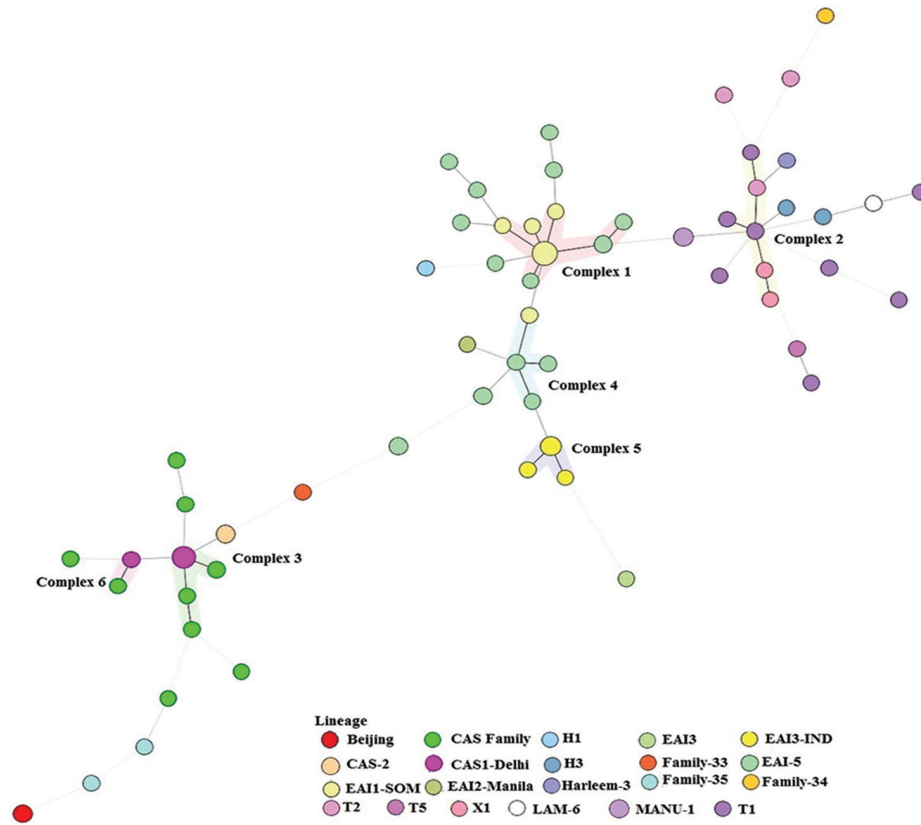
Fig. 2. A minimum spanning tree based on phylogenetic lineages. The structure of the tree is represented by branches (continuous, dashed and dotted lines) and circles representing individual pattern. The length of the branches represents the distance between patterns while the complexity of the lines (continuous, grey dashed and grey dotted) denotes the number of allele/spacer changes between two patterns: solid lines, 1 or 2 or more changes (thicker ones indicate a single change, while the thinner one indicates 2 changes); grey dashed lines represent 3 changes; and grey dotted lines represent 4 or more changes. The size of the circle is proportional to the total number of isolates in our study, illustrating unique isolates (smaller nodes) versus clustered isolates (bigger nodes). The colour of the circles indicates the lineage to which the specific pattern belongs.
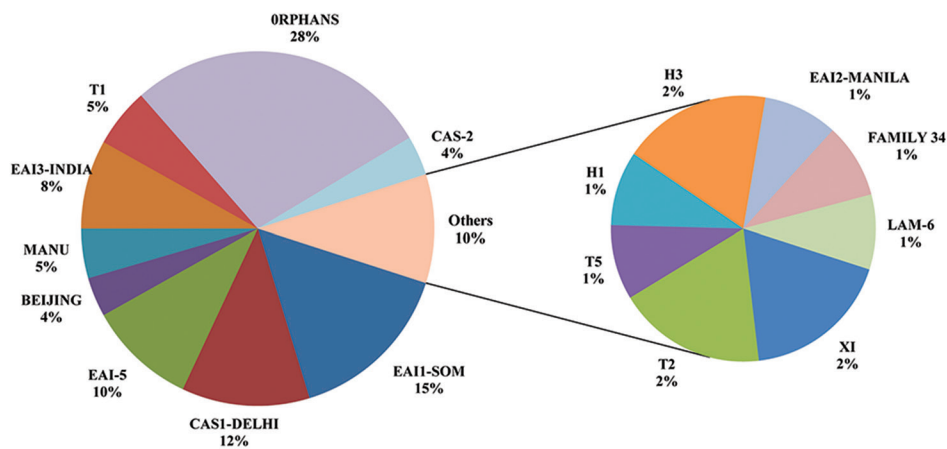
Fig. 3. A pie chart showing the distribution of MTB clades prevalent in Udupi district on the basis of SITVIT WEB analysis.

a total of 57 (51.35%) isolates were clustered into 11 spoligotype international types (SITs) with SIT-48 (EAI1-SOM) being the largest cluster of 14 (12.61%) isolates, followed by SIT-1942 (CAS1-Delhi) and

SIT-11 (EAI3-IND) with 11 (9.9 %) and seven (6.3%) isolates, respectively. Further 23 isolates (20.72%) had unique spoligotypes and 31 (27.92%) were orphans (Table I). The most prevalent clades prevailing in this

Table I. Spoligotypes shared by clinical isolates of *Mycobacterium tuberculosis* evaluated by SITVIT WEB analysis (based on database search August 14, 2016)

SIT number	Octal code	Clade	Spoligotype description	Number of isolates in the present study	Number of isolates reported in India/number of isolates in SITVIT WEB database
SIT 1	000000000003771	Beijing		4	109/5800
SIT 8	400037777413771	EAI-5		3	3/93
SIT 11	477777777413071	EAI3-IND		7	131/436
SIT 19	677777477413771	EAI2-Manila		1	Nil/688
SIT 46	777777770000000	Family-34		1	9/125
SIT 48	777777777413731	EAI1-SOM		14	20/313
SIT 52	777777777760731	T2		1	9/526
SIT 53	777777777760771	T1		2	39/3812
SIT 64	777777607560771	LAM-6		1	2/245
SIT 78	777777777760711	T1		1	5/46
SIT 100	777777777773771	MANU-1		5	25/62
SIT 119	777776777760771	X1		1	13/942
SIT 124	777777777700771	H3		1	1/37
SIT 126	477777777413771	EAI-5		2	27/77
SIT 138	777777777413700	EAI-5		1	9/93
SIT 159	777740017760771	T1		1	Nil/64
SIT 236	777777777413771	EAI-5		3	11/130
SIT 237	777777777700000	T2		1	Nil/87
SIT 256	777777777413671	EAI-5		1	1/51
SIT 288	700377740003771	CAS2		4	35/82
SIT 298	477767777413071	EAI3-IND		1	2/26
SIT 349	777737777413731	EAI1-SOM		1	Nil/18
SIT 490	767776777760771	X1		1	Nil/13
SIT 512	777777707720771	H3		1	1/19
SIT 573	777777663560771	T1		1	Nil/7
SIT 652	477577777413071	EAI3-IND		1	Nil/5
SIT 1182	477777777413731	EAI1-SOM		1	Nil/2
SIT 1251	777767777413731	EAI1-SOM		1	Nil/2
SIT 1268	777760057760771	T5		1	2/6
SIT 1314	703377740003171	CAS1-Delhi		2	3/4
SIT 1369	477777777413671	EAI-5		1	Nil/2
SIT 1626	777777776760771	T1		1	Nil/5
SIT 1942	703377740003771	CAS1-Delhi		11	2/7
SIT 1952	777777774000771	H1		1	1/9

SIT, spoligotype international type

region were EAI1-SOM (15.31%), CAS1-Delhi (12%) and EAI-5 (10%). Beijing (4%) and Harlem (3%) families, which are emerging worldwide and of major concern due to their association with drug resistance, were found less prevalent in this region (Fig. 3). MANU 4.5 per cent (5/111), X1 1.8 per cent (2/111), T2 1.8 per cent (2/111), T1 5.4 per cent (6/111), T5 0.9 per cent (1/111), H1 0.9 per cent (1/111), H3 1.8 per cent (2/111),

EAI2-Manilla 0.9 per cent (1/111) and LAM-6 0.9 per cent (1/111) were the other clades reported in this study (Table I). We further analyzed 31 orphans isolates by Spotclust (SPOLDB3 based) to identify their most probable families. Spotclust analyses revealed that majority (67%) of orphans in this study were variants of clades CAS (37%) and EAI-5 (34%) with 11 and 10 clinical isolates, respectively. One clinical isolate each was found to be the variant of EAI1-SOM, EAI3, T2 and Family 33. Further two clinical isolates were found to be

variants of Family 35, whereas three clinical isolates were found to be variants of T1. The distribution of clades in three *taluks* of Udupi district is shown in Fig. 4. Clinical and demographic characteristics of patients in relation to clustering of MTB isolates are shown in Table II. Two out (1.80%) of 111 patients had multidrug-resistant tuberculosis (MDR-TB) which shared SIT-48 (EAI-SOM) and SIT-1(Beijing) spoligotypes. Further, four (3.60%) isolates were monoresistant to isoniazid, 27 (24.32%) were monoresistant to streptomycin, seven

| Characteristic	Patients with clustered isolates (n=57), n (%)	Patients with unique isolate (n=23), n (%)	OR (95% CI)	*P*
Table II. Clinical and demographic characteristics of patients in relation to clustering of *Mycobacterium tuberculosis* isolates				
Age (yr)				
5-43	32 (56.14)	8 (43.78)	2.4 (0.87-6.55)	0.08
≥44	25 (43.85)	15 (65.22)		
Gender				
Male	45 (78.95)	20 (86.96)	0.56 (0.14-2.21)	0.40
Female	12 (21.05)	3 (13.04)		
Category				
Category new cases (Category I)	43 (75.44)	21 (91.30)	0.29 (0.06-1.40)	0.10
Previously treated cases (Category II)	14 (29.82)	2 (8.70)		
HIV status				
Seropositive	8 (14.03)	4 (19.13)	0.78 (0.21-2.88)	0.70
Seronegative	49 (85.96)	19 (82.60)		
Diabetes status				
Diabetic	5 (8.77)	1 (4.35)	2.12 (0.23-19.17)	0.49
Non-diabetic	52 (91.23)	22 (95.65)		
Smoking status				
Smokers	24 (42.11)	13 (56.52)	0.56 (0.21-1.49)	0.24
Non-smokers	33 (57.89)	10 (43.48)		
Alcohol intake				
Alcoholic	8 (14.04)	6 (26.09)	0.46 (0.14-1.53)	0.19
Non-alcoholic	49 (85.96)	17 (73.91)		
Family history of TB				
Yes	12 (21.05)	2 (8.70)	2.8 (0.57-13.65)	0.18
No	45 (78.95)	21 (91.30)		
Marital status				
Married	35 (61.40)	18 (78.26)	0.44 (0.14-1.36)	0.14
Unmarried	22 (38.60)	5 (21.74)		
Education status				
Uneducated	9 (15.79)	3 (13.04)	1.25 (0.31-5.1)	0.75
Educated	48 (84.21)	20 (86.96)		
TB, tuberculosis; OR, odds ratio; CI, confidence interval				
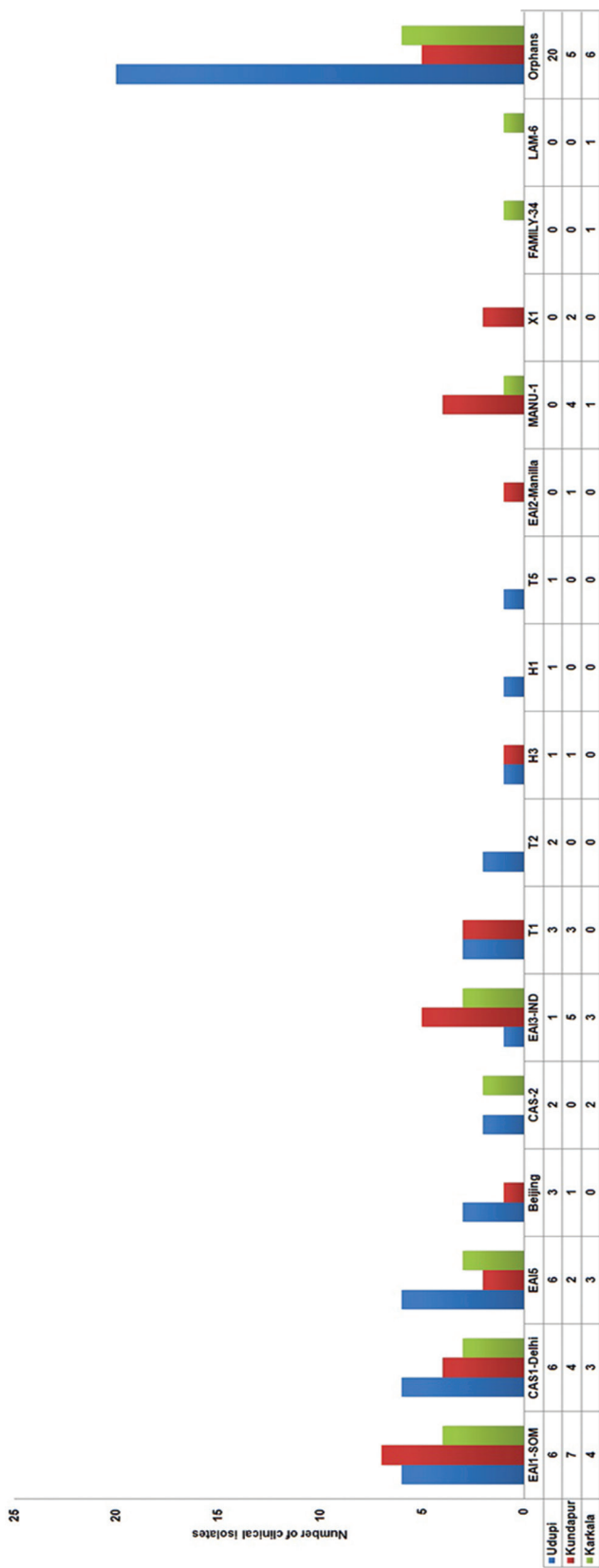
Fig. 4. Distribution of clades in three *taluks* of Udupi district, Karnataka, India.

(6.30%) were polydrug-resistant other than MDR and 71 (63.96%) were sensitive to all first-line drugs. The detailed description of drug resistance in isolates with respect to lineage is given in Table III. Thirteen (11.71%) patients were seropositive for HIV (mean age=41.54, SD=8.10, range: 30-57 yr), SIT-48 was shared by two isolates, one isolate each belonged to SIT-236, SIT-126, SIT-100, SIT-288, SIT-11, SIT-119, SIT-490, SIT-1268, SIT-52 and orphan. Discriminatory power of spoligotyping for 111 clinical isolates of Udupi district based on HGDI was found to be 0.93.

Discussion

The largest cluster in the present study belonged to EAI1-SOM (SIT-48) shared by 14 (12.61%) clinical isolates. EAI1-SOM has earlier been also reported in variable prevalence from south India[13-15]. East African Indian lineages originated in Guinea-Bissau (Africa) and believed to be transmitted to other continents through dispersion of modern humans from that region[16]. EAI1-SOM, a sublineage of EAI originated in Somalia, has been reported from India, with high prevalence in southern regions, whereas prevalence in northern regions of India is reported variably low[17,18]. SIT-1942 (CAS1-Delhi) was the second largest cluster in the present study. CAS1-Delhi lineage supposed to be originated from India was later disseminated to regions such as Saudi Arabia, Kenya, South Africa, Malaysia, Myanmar, Australia, the USA and parts of Europe through frequent migration[19,20]. Beijing, the East Asian lineage originated in China, is frequently reported to be associated with drug resistance and ubiquitously prevalent worldwide[7]. Low prevalence of Beijing has been reported (2.4-14.9%) in most studies in India except Mumbai (18.8%)[21] and Assam (35.4%)[22]. It correlated with the presence of high drug resistance in these geographical regions. In the present study, it was found in about four per cent (4/111) of MTB isolates. One of these isolates, belonging to Beijing lineage, was detected as MDR. The low prevalence of Beijing lineage might be the possible reason for lesser number of multidrug resistance cases in Udupi district. MANU, an ancient lineage believed to be the probable ancestral of CAS and EAI with worldwide prevalence[23], was found to be less prevalent (4.5%) in the present study. It was represented by SIT-100, which was initially reported from Delhi[17]. Some of the unique SITs found in this investigation, such as SIT-349, SIT-298, SIT-46, SIT-52, SIT-119, SIT-64 and SIT-1952, have been reported earlier from north

Drug sensitivity profile	EAI (n=37), n (%)	CAS (n=17), n (%)	MANU (n=5), n (%)	Beijing (n=4), n (%)	Orphans (n=31), n (%)	Others (n=17), n (%)	Total (n=111), n (%)
Pan sensitive	27 (72.97)	9 (52.94)	5 (100)	3 (75)	18 (58.06)	9 (52.94)	71 (63.96)
Rifampicin monoresistance	-	-	-	-	-	-	-
Isoniazid monoresistance	-	3 (17.64)	-	-	1 (3.22)	-	4 (3.60)
Streptomycin monoresistance	8 (21.62)	3 (17.64)	-	-	11 (35.48)	5 (29.41)	27 (24.32)
Ethambutol monoresistance	-	-	-	-	-	-	-
MDR	1 (2.70)	-	-	1 (25)	-	-	2 (1.80)
Poly drug resistance other than MDR	1 (2.70)	2 (11.76)	-	-	1 (3.22)	3 (17.65)	7 (6.30)
Total	37	17	5	4	31	17	111

Table III. Drug susceptibility profiles of clinical isolates with respect to lineage

MDR, multidrug resistant

India[17-20,24-26] and a few such as SIT-256, SIT-138 and SIT-1268 have been reported from south India[14,15,27,28]. Spoligotypes of 31 clinical isolates from the present work did not match with the spoligo patterns available in SITVIT WEB database, suggesting the high evolutionary pressure on the isolates in this region which resulted in emergence of newer spoligotypes. Clustering of isolates was not found significantly associated with any of demographic variables and drug resistance patterns due to limited sample size. IS6110-RFLP analysis was considered as the gold standard for genotyping of MTB[29]. However, MIRU-VNTR typing has now been considered as the new molecular reference tool for being simpler, faster, less DNA demanding and with a similar resolution than RFLP typing[30]. We preferred spoligotyping as MIRU-VNTR typing was an expensive technique for limited resource laboratory like ours.

Although this was one of the first genotyping studies on clinical isolates of MTB in Udupi district of Karnataka, limited number of isolates and the inability to perform MIRU-VNTR typing were the major limitations.

In conclusion, our study showed the presence of highly diverse patterns of spoligotypes in Udupi district. High number of orphans (28%) in this study highlights the presence of a large number of genotypes which are yet to be identified. Longitudinal studies need to be done in this region to understand the transmission dynamics of MTB using different genotyping methods.

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