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Molecular Characterization of Mycobacterial Ribonucleotide Reductase (RNR) and its Implication as a Novel Drug Target

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Abstract

The genus Mycobacterium consists of both pathogenic and non-pathogenic species. The emergence of MDR and XDR tuberculosis and opportunistic infections by non tuberculous mycobacterium in immunosuppressive persons are major concern now a days. The aim of the study was to characterize ribonucleotide reductase (RNR) of Mycobacterium species to produce information about the evolution of the gene which could further be used in targeting RNR as a novel drug target. Here we were analyzed RNR of 23 mycobacterial species. The sequence length of RNR region is about 975 bp. Out of 975 characters 625 (64.10%) are conserved sites (monomorphic) and 350 (35.89%) are variable sites (polymorphic). The total nucleotide diversity (π) is=0.120114 (12.011%). The RNR phylogeny approach in Mycobacterium species provides evidence of several evolutionary lineages evolving from the ancestral polymorphism and fixed in the descendant population. Species of mycobacteria causing tuberculosis or respiratory infection in humans have specific patterns of allele distribution in different motifs, which differentiate them from other opportunistic mycobacterial species. Molecular analysis and structural motif analysis of RNR suggests the occurrence of host-mediated genetic differentiation in mycobacterial species, which requires further wet lab investigations.

Keywords: Mycobacterium, Ribonucleotide reductase, Structural motif, Neutral evolution, Multi drug resistance.

Introduction

Mycobacterium is the genus of actinobacteria, consisting of more than 100 species [1]. The genus includes several pathogenic species viz, *Mycobacterium tuberculosis* causing tuberculosis and *Mycobacterium leprae* causing leprosy in humans. *M. tuberculosis* exhibits extraordinary capabilities to subvert and to resist the bactericidal response of their infected host. These capabilities led the bacillus to colonize one third of the world's population and nearly 1.4 million people are killed annually [2]. The emergence of drug resistance strains is a serious problem for TB control programmes. India and China have largest number of TB cases and the figure is less than one in ten; scale-up is expected in these countries [2]. Another aspect of mycobacterial infection is the emergence of non-tuberculous mycobacterial (NTM) infection in immunosuppressive patients and in healthy individuals which cannot be looked over. XDR-TB has an estimated cure rate of only 30% in patients with an uncompromised immune system compared to a 95% cure rate of drug sensitive tuberculosis [2]. Ribonucleotide reductase (RNR) also known as ribonucleoside diphosphate reductase is a central enzyme in DNA replication system in all organisms. The enzyme catalyzes the formation of deoxyribonucleotides (dATP, dGTP, dCTP and dUDP) from ribonucleotides (ADP, GDP, CDP and UDP) [3] which are further used as precursors of DNA synthesis. The mechanism of action of RNR is strictly conserved in all living organisms [4]. dTDP (deoxythymidine diphosphate) is synthesized by another enzyme (thymidylate kinase) from dTMP (deoxythymidine monophosphate). RNR plays a critical role in regulating the total rate of DNA synthesis so that DNA to cell mass is maintained at a constant ratio during cell division and DNA repair [5].

In the genome of *M. tuberculosis* the RNR is encoded by nrdE (Rv3051c) and nrdF2 (Rv3048c) as well as a putative alternate small subunit encoded by nrdF1 (Rv1981c), which contains key catalytic residues but cannot associate with NrdE to form a functional RNR [6,7]. Apart from the above RNR, the genome of *M. tuberculosis* also contains nrdZ (Rv0570) which encodes a putative class II RNR [8]. The nrdF1gene is unable to substitute for nrdF2 and that the class II RNR, NrdZ, cannot substitute for the class Ib enzyme, NrdEF2 [6]. In this study an attempt has been made to compare the nrdF2 of mycobacterial species to elucidate the evolution of RNR gene as well as the structural differences among them through bioinformatics tools, which might enlighten in adopting a new and safer drug for the cure of tuberculosis and other mycobacterial diseases.

Material and Methods

Dataset

The sequence of nrdF2 of *M. tuberculosis* (H37Rv) was retrieved from NCBI, accession no "AL123456". Homologous sequences were retrieved from the BLAST search of Rv3048c at NCBI. Only the sequences having 95% query coverage and belong to the genus Mycobacterium was taken for further analysis (Table 1).

Nucleotide and haplotype diversity

The alignments of nrdF2 sequences were done using CLUSTAL-X2. Variations in the nucleotides within each sequence were estimated through nucleotide diversity (π), average number of nucleotide differences (Kt) and haplotype diversity (HD). The calculations were

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Mycobacterium species	Accession No	Growth rate	Pathogenic nature
M. tuberculosis	CP003248	Slow	Tuberculosis in human
M. bovis	CP003900	Slow	Tuberculosis in cattle and Opportunistic pulmonary infection in humans, those who are working in cattle shed.
M. africanum	FR878060	Slow	Tuberculosis in human
M. canettii	FO203508	Slow	Lympadenitis in human
M. kansasii	CP006835	Slow	Pulmonary infection resembles with tuberculosis in human
M. paratuberculosis	CP005928	Slow	Johne's disease in cattle and Crohn's disease in humans (In humans it is an opportunistic infection those who are working in cattle shed and are consuming unpasteurized milk)
M. marinum	CP000854	Slow	Infection in fish and opportunistic infection in humans those who are tending home aquaria or bathing in natural pools on the shores
M. liflandii	CP003899	Slow	Nodular and ulcerative skin lesions in *Xenopus* (Frogs)
M. hominissuis	AP012555	Slow	Respiratory infection in pigs and opportunistic infection in humans
M. ulcerans	CP000325	Slow	Buruli ulcer in human
M. avium	CP000479	Slow	Tuberculosis in birds and opportunistic infection in immune compromised humans
M. intracellulare	CP003323	Slow	Pulmonary disease in humans
M indicus pranii	CP002275	Slow	Non pathogen
M. chubuense	CP003053	Rapid	Non pathogen
M. smegmatis	CP001663	Rapid	Non pathogen
M. gilvum	CP002385	Rapid	Non pathogen
M. vanbaalenii	CP000511	Rapid	Non pathogen
M. neoaurum	CP006936	Rapid	Non pathogen
M. rhodesiae	CP003169	Ungrouped	Non pathogen
M. leprae	AL583923	Slow	Leprosy in humans
M. massiliense	CP003699	Rapid	Non pathogen
M. bolletii	CP004374	Rapid	Non pathogen
M. abscessus	CU458896	Rapid	Non pathogen
Arthrobacter species	CP000454	Rapid	--

Table 1: *Mycobacterium* species used in this study with their Genbank Accession number, growth rate and pathogenic forms.

done using DnaSP 5.0 program [9]. The transition/transversion bias (R) was calculated using MEGA 4.0 [10].

Phylogenetic analysis

Phylogenetic analysis was performed by maximum parsimony (MP) method. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in which the initial trees were obtained with the random addition of sequences (10 replicates). Phylogenetic analysis was conducted in MEGA4 [10]. A total of RNR of 23 mycobacterial species were included in the analysis and the RNR sequence of Arthrobacter sp was included in the analysis for rooting the phylogenetic tree.

Structural motif analysis

The retrieved nucleotide sequences were converted into protein sequences using translate tool at ExPASy Bioinformatics Resource portal (http://web.expasy.org/translate) and were saved in FASTA format. The structural motifs of nrdF2 sequences of Mycobacterium spp were generated by MEME Motif discovery tool to identify the similar motifs in each of the sequences. All the settings were set to default, except for the maximum number of Motifs which was increased from three to ten [11].

Results

Nucleotide diversity and haplotype diversity

The sequence length of the "nrdF2" region is about 975 bp. For combined alignment and analysis of "nrdF2", 975 characters were included, of which 625 (64.10%) were conserved sites (monomorphic) and 350 (35.89%) were variable sites (polymorphic). Out of 385 variable sites 278 were parsimony informative. The total nucleotide diversity (π) was=0.120114 (12.011%) and average number of nucleotide difference was K_t= 117.11067. The nucleotide frequencies were 0.216 (A), 0.175 (T), 0.313 (C), and 0.297 (G). The transition/transversion rate ratios were k1=5.879 (purines) and k2= 2.034 (pyrimidines). The overall transition/transversion bias was R=2.287 and the bias were towards transitional mutation (Table 2).

Phylogenetic analysis

A phylogenetic tree was constructed from the aligned dataset of "nrdF2" region, consisting of 24 sequences including Arthrobacter. sp as root. The consensus tree inferred from 5 most parsimonious trees (Figure 1). The consistency index was (0.490105), retention index (0.691549) and composite index was 0.379604 (0.338932) for all sites and parsimonyinformative sites (in parentheses). The MP tree was differentiated into two main clusters A and B with Arthrobacter. sp as root of the tree. Main cluster A further differentiated into 4 sub-clusters A1, A2, A3 and A4. In sub-cluster A1, *M. africanum*, *M. bovis*, *M.tuberculosis, M. canettii* and *M. kansasii* were clustered together with a bootstrap value of 100%. In sub cluster, A2 *M. marinum*, *M. ulcerans* and *M. liflandii* grouped together at a bootstrap value of 100%. In sub-cluster, A3 *M. intracellulare*, *M. indicus pranii*, *M. avium*, *M. avium paratuberculosis*, and *M. avium hominissuis* grouped together at a bootstrap value of 100%. *M. leprae* grouped in subcluster A3 as

	A	T	C	G
A		2.92	5.21	29.05
T	3.59		10.59	4.94
C	3.59	5.93		4.94
G	21.12	2.92	5.21	

Table 2: Maximum composite likelihood estimate of the pattern of nucleotide substitution.

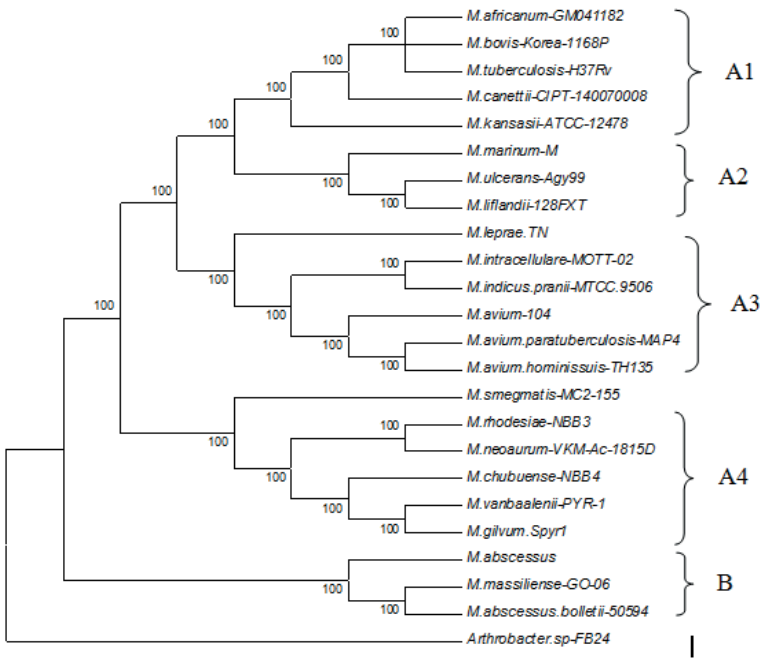


Figure 1: Phylogenetic tree obtained by Maximum Parsimony analysis of RNR region showing phylogenetic relationships among mycobacterial species. The tree is rooted with *Atrhrobacter* sp.

a paraphyletic group. *M. rhodesiae*, *M. neoaurum*, *M. chubuense*, *M. vanbaalenii* and *M. gilvum* were grouped together with a bootstrap value of 100% in subcluster A4, while *M. smegmatis* grouped in the same cluster as paraphyletic group. *M. abscessus*, *M. massiliense* and *M. abscessus bolletii* were clustered in main cluster B at a bootstrap value of 100%.

Structural motif analysis

Structural motif analysis of nrdF2 protein segment resulted in 9 motifs (Motif 1 to 9) of varying size, 5 having 50 amino acids (motif 1, 2, 3, 4 and 5), 2 having 21 amino acid (motif 6 and 7), 2 having 11 amino acids (motif 8 and 9) in length (Figure 2). The most variable motifs are motif 4 and 5. In motif 5 fifteen variable sites were detected whereas in motif 4 seven variable sites. No variation in amino acid sequences were found in motif 8. In Mycobacterium tuberculosis complex cluster specific amino acids were found in 3 positions in motif 2 position 134, in motif 5 position 118 and in motif 9 position 320. Likewise in Mycobacterium avium complex the cluster specific amino acids were found in 6 positions, motif 1 position 208, motif 4 positions 47, 48, 51, 60 and in motif 5 position 219. In case of *M. marinum*, *M. ulcerans* and *M. liflandii* the cluster specific amino acids were found in 5 positions, motif 2 position 134, motif 5 positions 215, 221, motif 7 position 251 and in motif 9 position 318. In pathogenic species of mycobacteria host specific amino acid changes found in the position 208 (F to Y in motif1), 108 (K to R in motif2), 48, 51 and 60 (P to Q, G to A and L to M in motif4), 219 (T to K in motif5) for *M. avium* complex and in position 134 (P to R in motif2), 218, 219 and 221 (A to V, R to T or K to T and Q to A in motif5), 320 (E to D in motif 9) for *M. tuberculosis* complex. In case of *M. marinum*, *M. ulcerance* and *M. liflandi* changes in amino acids were found in positions 134 (P and S in motif2), 214, 215, 217, 219 and 221 (L to A, V to A, D to E, T to R or K to R and Q to T in motif5), 19 (L to V in motif6), 145 and 151 (Q to E and E to D in motif7) and in 318 (E and Q in motif9). In case of *M. leprae*, *M. intracelularae* and *M. indicus pranii* the mutations were either associated with *M. tuberculosis* complex or with *M. avium* complex (Table 3). The amino acid changes in positions 96, 108 and 112, (Y to L, R to K and N to Q in motif2), 56 and 70 (H to G and T to M in motif4), 144 and 148 (M to L and K to R in motif7) were associated with the pathogenic species viz., *M. tuberculosis* complex, *M. marinum*, *M. ulcerance* and *M. liflandi*. In fast growing predominantly non-pathogenic but opportunistic pathogens have some amino acids common to pathogenic mycobacteria in the positions 108, 217, 219, 221, 19, 145 and 148 in different motifs. Motif span, important residues and variable residues were given in (Table 3).

Discussion

The species of genus Mycobacterium are cosmopolitan bacteria and are distributed over a broad eco geographical range. The growth rate of different species of Mycobacterium greatly varies from species to species in artificial medium except *M. leprae* which is not cultivable in any artificial medium. The emergence of MDR [12,13], XDR strains of *M. tuberculosis* [14] as well as the infection of NTMs in immune suppressive individuals is a major concern to the Anti microbacterial therapy. Excluding the infection by predominant species of mycobacteria viz, *M. tuberculosis*, *M. bovis* and *M. leprae* several other species of the genus are being reported as etiologic agent of human pulmonary infection [15]. Nearly one third of known Mycobacterium species have been observed to be associated with disease in humans [16]. The species of NTM associated with human disease are : *M. avium*, *M. intracellulare* [17,18], *M. kansasii* [19], *M. paratuberculosis* [20], *M. scrofulaceum* [21], *M. simiae* [22,23], *M. interjectum* [24], *M. xenopi* [25], *M. szulgai* [26], *M. fortuitum* [27], *M. chelonae* [28], *M. marinum* [29], *M. genavense* [30], *M. ulcerans* [31], *M. smegmatis* [32], *M. thermoresistible* [33], *M. neoaurum* [34], *M. vaccae* [35].

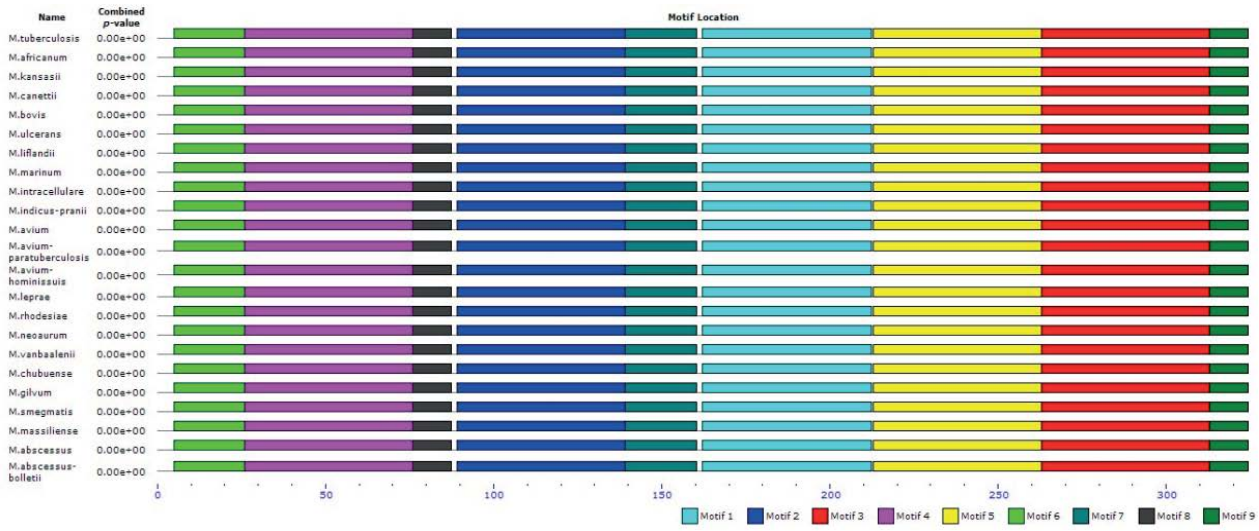
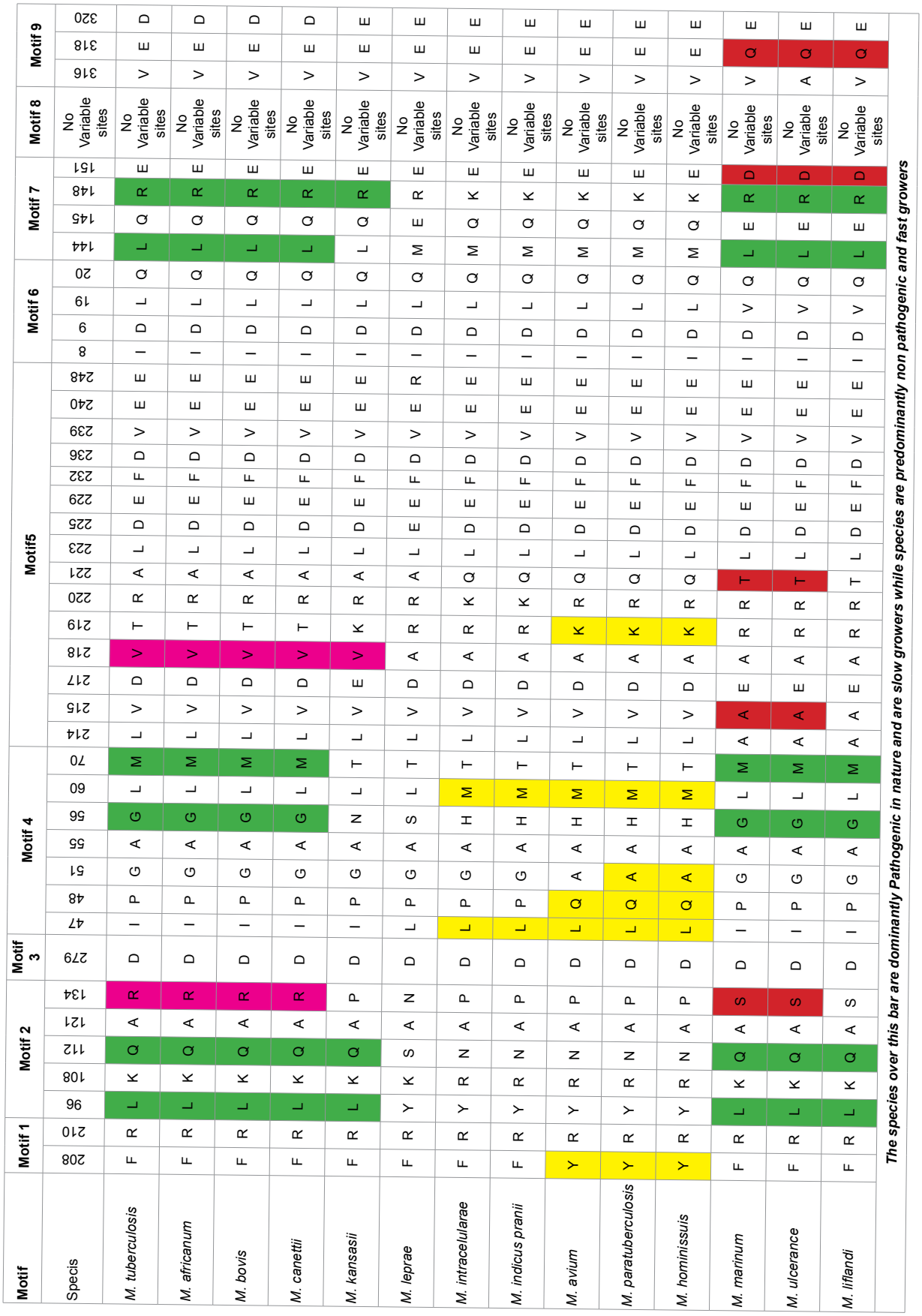


Figure 2: Structural motifs spread in RNR sequence of *Mycobacterium* species.

Presently little information is available on the evolution of RNR in different species of Mycobacterium which governs the central metabolic pathways by directing the DNA replication. In this study we provide an insight into the evolution of RNR in different species of Mycobacterium by combining phylogenetic analysis with structural motif analysis. In this study phylogenetic analysis based on RNR of Mycobacterium species resulted in 5 different sub-clades. We compared the clades with the phenotypic systematic of the genus Mycobacterium to establish the congruence between both methods and found that all the clades were perfectly differentiated on the basis of their growth behaviour. The fast growing species of Mycobacterium were grouped in the sub cluster A4 and main cluster B, whereas the slow growing Mycobacterium were grouped in sub cluster A1, A2 and A3. Our result support the phenotypic systematic described by Stahl and Urbance [36]. From this phylogenetic analysis we also found that the species of Mycobacterium were differentiated on the basis of their host dependency. The species like *M. africanum*, *M. bovis*, *M. tuberculosis M. canettii* and *M. kansasii*, causing human tuberculosis [37-41] were grouped in sub cluster A1. Likewise *M. intracellulare* causing pulmonary tuberculosis in humans [42], *M. avium* causing tuberculosis in poultry and captive birds [43] and also in immune compromise patients/HIV patients [44], *M. avium paratuberculosis* causing tuberculosis in cattle [45], and *M. avium hominissuis* causing pulmonary tuberculosis in pigs, humans and horses [46,47] were placed in sub-cluster A3. Although the species in sub-cluster A3 were caused human pulmonary disease, the prevalence of these opportunistic infections was very low and also these infections are from animal sources. Similarly *M. marinum* causing infection in fishes [48] and *M. liflandii* causing infection in frogs [49,50] were grouped in sub-cluster A2. Exception to the host dependency clustering were species viz, *M. ulcerans*, causing buruli ulcers in humans and it was clustered in sub-cluster A2. This is because *M. ulcerans* is a specialized variants of a common *M. marinum* progenitor that have adapted to live in restricted environments [51].

The RNR phylogeny approach in Mycobacterium species provided evidence of several evolutionary lineages evolving from the ancestral polymorphism and fixed in the descendant populations. The species of Mycobacterium were strongly supported as polyphyletic by the RNR phylogeny. A large number of parsimony informative sites (278) indicated significant genetic diversity between the species of Mycobacterium and this was supported by the higher values for total nucleotide diversity 0.120114 and average number of nucleotide differences (Kt=117.11067). It was confirmed that the bias was towards transitional mutation (Table 2). The higher value of transitional mutations might indicate that some species of Mycobacterium are under selection process, as suggested by Rosenberg et al [52], whilst working with mammalian genome. Exposure of pathogen populations to alternative hosts can cause relaxation or shifts in selection pressure, resulting in greater genetic diversity [53,54]. The high genetic diversity in RNR of Mycobacterium might be due to shift in host. It is evident not only from the clustering pattern but also from the change in amino acid pattern in different structural motifs. This result suggests that the evolutionary trend was driven by variability in the host species or change in environment due to the accumulation of beneficial mutations. This apparent genetic plasticity may also explain the wide host range of the mycobacterial species. However, defying the host-specific clustering pattern discussed earlier, *M. leprae* causing leprosy in human and nine banded armadillos clustered with A3 as paraphyletic group showing RNR genotype difference within the group. From structural motif analysis it was observed that most of the pathogenic mycobacteria have similar motif pattern having small nucleotide differences in the motifs elucidated. *M. avium* group had distinct mutational pattern which might be responsible for the host specific differentiation rather than growth rate (Shown in yellow color in Table 3). Likewise the species of mycobacteria causing tuberculosis or respiratory infection in humans had specific patterns of allele distribution in the amino acid positions 108, 134, 218 and 221 in different motifs, which differentiate them from other opportunistic mycobacterial species (Shown in violet color in Table 3). The pathogenic mycobacterium like *M. marinum* (in fish), *M. ulcerans* (in human opportunistic infection) and *M. liflandii* (in frogs) had specific type of distribution of alleles in different motifs (Shown in red color in Table 3). Human tuberculosis

causing mycobacteria including opportunistic pathogens had same


Motif
Specis
Motif 1
208 210
Motif 2
96 108 112 121 134
Motif 3
279
Motif 4
47 48 51 55 56 60 70
Motif5
214 215 217 218 219 220 221 223 225 229 232 236 239 240 248
Motif 6
8 9 19 20
Motif 7
144 145 148 151
Motif 8
No Variable sites
Motif 9
316 318 320
M. tuberculosis
M. africanum
M. bovis
M. canettii
M. kansasii
M. leprae
M. intracellulare
M. indicus pranii
M. avium
M. paratuberculosis
M. hominissuis
M. marinum
M. ulcerance
M. liflandi
The species over this bar are dominantly Pathogenic in nature and are slow growers while species are predominantly non pathogenic and fast growers

M. abscessus	F	R	Y	K	S	R	P	E	I	Q	N	D	H	L	T	R	V	E	A	K	R	A	I	E	D	Y	E	T	D	E	V	S	V	P	M	E	R	E	No Variable sites	N	E	E
M. massiliense	F	R	Y	K	S	R	P	E	I	Q	N	D	H	L	T	R	V	E	A	K	R	A	I	E	D	Y	E	T	D	E	V	S	V	P	M	E	R	E	No Variable sites	N	E	E
M. bolletii	F	R	Y	K	S	R	P	E	I	Q	N	D	H	L	T	R	V	E	A	K	R	A	I	E	D	Y	E	T	D	E	V	S	V	P	M	E	R	E	No Variable sites	N	E	E
M. vanbaalenii	F	K	Y	R	N	A	P	D	I	P	N	A	H	L	T	L	E	E	A	T	R	Q	L	D	E	F	D	V	E	E	I	D	L	Q	M	Q	K	E	No Variable sites	N	E	E
M. gilvum	F	K	Y	R	N	S	P	D	I	P	N	A	H	L	L	L	A	D	A	T	R	Q	L	D	E	F	D	V	E	E	I	D	L	Q	M	Q	K	E	No Variable sites	V	E	E
M. chubuense	Y	R	Y	R	N	A	P	D	I	P	N	A	G	L	T	Q	Q	E	S	T	K	Q	L	D	E	F	D	V	E	G	I	D	L	Q	M	Q	K	E	No Variable sites	N	E	E
M. smegmatis	Y	R	L	K	N	A	P	D	I	Q	G	D	N	L	T	L	V	E	E	K	K	Q	L	D	E	F	D	V	E	S	I	D	L	Q	M	Q	R	D	No Variable sites	N	E	E
M. neoaurum	Y	K	Y	K	N	A	N	D	I	P	H	D	H	L	T	A	E	E	T	R	K	Q	L	D	E	F	D	T	E	G	I	D	L	Q	M	D	K	E	No Variable sites	V	E	E
M. rodhesiae	Y	R	Y	R	N	S	P	D	I	P	G	D	H	L	T	A	T	A	A	T	Q	A	L	D	E	F	D	V	E	E	I	D	V	Q	M	R	K	E	No Variable sites	N	E	E

Table 3: Amino acid variation in structural motifs of mycobacterial species with their specific positions in different motifs.

type of allele distribution in different locus over a period of time which was fixed in the species level (Shown in green color in Table 3).

Conclusion

The major conclusion from this study is that the RNR sequences of mycobacterial species have unique characteristics in relation to the host species. Variability in the RNR sequence suggests that an evolutionary trend was driven by the encounters of an ancestral population of the pathogen with different host species. The existence of barriers to gene flow such as geographical separation, ecological adaptation or the accumulation of genetic

differences ultimately leads to distinct lineages. In this analysis we found that the accumulation of beneficial mutations over the span of time in mycobacterial species were not due to geographical separation or ecological adaptation but due to the shift and adaptation of new host species. From structural motif analysis it was found that the motifs of different mycobacterial species were of similar type in between the sub-cluster. Thus RNR might be a

useful target for new drug in MDR, XDR and non tuberculosis mycobacterial infection.the nucleotide differences were largely accumulated outside the motifs derived.

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