Whole Mitochondrial Genome Sequence of an Indian *Plasmodium falciparum* Field Isolate

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**Abstract:** Mitochondrial genome sequence of malaria parasites has served as a potential marker for inferring evolutionary history of the *Plasmodium* genus. In *Plasmodium falciparum*, the mitochondrial genome sequences from around the globe have provided important evolutionary understanding, but no Indian sequence has yet been utilized. We have sequenced the whole mitochondrial genome of a single *P. falciparum* field isolate from India using novel primers and compared with the 3D7 reference sequence and 1 previously reported Indian sequence. While the 2 Indian sequences were highly divergent from each other, the presently sequenced isolate was highly similar to the reference 3D7 strain.

**Key words:** *Plasmodium falciparum*, malaria, whole genome sequencing, mitochondrial genome, India

Malaria is a vector-borne infectious disease, endemic to many of the tropical and subtropical countries of the globe including India. Approximately half (273 million) of the high risk population outside Africa resides in India [1]. *Plasmodium falciparum* malaria is the leading cause of deaths and accounts for about 50% of malaria cases in India [2]. The problem is further compounded by high virulence and emergence of drug resistance in *P. falciparum*, and till now no effective vaccine is available. The primary hurdle to design an effective vaccine that would work in all malaria endemic populations is highly observed genetic diversity in *P. falciparum* field isolates, as this parasite uses the genetic diversity to fight against the anti-malarial drugs and host immunity [3]. Therefore, the analysis of within-species genetic diversity is very important for understanding evolutionary processes both at the population and the genetic level which will not only enlightens the origin, historical migration, and demography of different populations, but also inform if new parasite genotypes of high virulence and drug resistance are emerging and spreading to different populations. Such understanding on the long run will definitely be of help in devising effective population-based control measures.

To this respect, mitochondrial (*mt*) genome has served as an ideal marker to understand evolutionary history of many model and non-model organisms and has been a marker of choice for reconstructing historical patterns of population demography and phylogenetic studies [4]. Several characteristics features; such as high mutation rate [5], maternal inheritance [6], and lack of recombination [7] have made the *mt* genome an ideal extra-nuclear genome to reconstruct evolutionary histories of the species. In malaria parasites, *mt* genome is of particular relevance, due to (i) its small size (~6 kb), (ii) haploid, and (iii) contains 3 protein-coding genes, cytochrome c oxidase I (*cox1*), cytochrome c oxidase III (*coxIII*) and cytochrome b (*cytb*) [8].

All these 3 genes are essential for a range for cellular processes; like membrane potential maintenance, heme and co-enzyme Q biosynthesis, and oxidative phosphorylation [9]. Most importantly, the *cytb* gene of *P. falciparum* mitochondria is a potential target for an antimalarial drug, atovaquone [10]. Moreover, the *mt* genome of parasites evolves neutrally and shows no signs of recombination or selection [11], hence the whole genome behaves as a single locus and all sites share a common genealogy, which makes it ideal for studying within-species variations and phylogenetic analysis. While *mt* genome sequences have been reported from many malaria endemic countries of the world [11], only 1 complete *mt* genome sequence of *P. falciparum* isolate originating from unknown location in India has so far been reported [12]. Considering *P. falciparum* malaria is widespread in India, lack of *mt* genome se-
quence information from multiple Indian isolates has debarred us in unravelling the net diversity of Indian *P. falciparum*. To fill this gap in knowledge and to initiate population genomic studies of Indian *P. falciparum*, we herewith report the whole mt genome sequence of a single Indian field isolate, and the results of preliminary comparative genomic studies between the existing Indian data [12] and the reference sequence (3D7 isolate).

Finger-pricked blood sample of a microscopically diagnosed *P. falciparum* infected malaria patient from Bilaspur (Chhattisgarh state, India) was spotted (2-3 spots) on Whatmann filter paper, dried, and brought to the laboratory in New Delhi. Genomic DNA was extracted from these dried blood spots using QIAamp mini DNA isolation kit (Qiagen, Hilden, Germany). Since both *P. falciparum* and *Plasmodium vivax* are endemic to India, we used the isolated genomic DNA to perform PCR diagnostic assay to identify mixed infection following the nested PCR with genus and species-specific primers based on 18S rRNA gene [13]. The study was approved by the Ethics Committee of the National Institute of Malaria Research (NIMR), India, and written informed consent of the patients have been obtained.

To sequence the whole mt genome of the present *P. falciparum* isolate originated from Bilaspur (referred as Blsp1), we checked for PCR amplification using the sets of primers reported to sequence the Indian *P. falciparum* isolate PfPH10 [12]. However, we could not PCR-amplify any of the fragments of the mt genome of the Blsp1 isolate with repeated attempts. Therefore, we used the whole mt genome sequence information of the published 3D7 isolate, downloaded from the NCBI (www.ncbi.nlm.nih.gov/) database with GenBank accession no. AY282930, and chopped the whole mt genome into 19 different DNA fragments. In order to sequence the whole mt genome of *Plasmodium falciparum* along with annealing temperatures of the respective fragments (Table 1), a min extension at 72°C followed by 5 min final extension at 72°C. Successfully amplified PCR products were further purified by incubating with

| S. no. | Primer name | Primer sequence (5’ to 3’) | Annealing temperature (°C) |
|-------|-------------|-----------------------------|-----------------------------|
| 1     | M_1F        | TGCTATTGGATTCACCAACTGGTCC   | 63.7                        |
|       | M_1R        | GTCTGCTATGGAAGTGTGA          |                             |
| 2     | M_2F        | TGTTACTATGCAACAGAACAT        | 63.2                        |
|       | M_2R        | GCCTGGGATTATATCCACTCT        |                             |
| 3     | M_3F        | GGGTATTCCGACTGGTGCTCT       | 63.7                        |
|       | M_3R        | CAACAGTACAGCGTGGAACA         |                             |
| 4     | M_4F        | AGGAGCAAACAGTGCTCAG         | 63                          |
|       | M_4R        | GGCAATTTGGAATATGCCTG         |                             |
| 5     | M_5F        | ACTTCTTCTGCGCATTTGTG        | 63.7                        |
|       | M_5R        | GCATCATGTATGAGTGCATG         |                             |
| 6     | M_6F        | TTGTAAGAGTTGCAAAAAGTTCTC    | 60.4                        |
|       | M_6R        | GCACATCTATGTTGATG           |                             |
| 7     | M_7F        | CAGAATAAAAAACTTTCCTGGAATAGG | 61.8                        |
|       | M_7R        | AAGTAGGGATGACTGCT           |                             |
| 8     | M_8F        | CGCACTCTGAAAATATAGGA        | 61.8                        |
|       | M_8R        | CATGAGGCTCTGGAATTAAGA       |                             |
| 9     | M_9F        | GAACGCTTATAAGCGCTGAC        | 52.7                        |
|       | M_9R        | AGTCGACCTCGTCCACAC          |                             |
| 10    | M_10F       | CCGGATTATCGCGAGGAGT         | 52.8                        |
|       | M_10R       | CAGGATCTCGAAAATACCCA        |                             |
| 11    | M_11F       | CCGGTTTTAACCTGCGAGGAT       | 62.6                        |
|       | M_11R       | GCATCACTATTGCGACGAT         |                             |
| 12    | M_12F       | CGGCTACAAAGATTTTCTGAGGA    | 62.6                        |
|       | M_12R       | GTCTATTGTTATGCAGCAATAGA    |                             |
| 13    | M_13F       | GCATTTCAGATTTCTTTCTGAT      | 62.6                        |
|       | M_13R       | AAACATCTGTTGTATAGCGACTT     |                             |
| 14    | M_14F       | CACACTTAAATTAAACCCACTTGCCA | 62.6                        |
|       | M_14R       | GGACTACTTACACATTGTCCTCA     |                             |
| 15    | M_15F       | TTGCTATTCCATGGGCTCAAA       | 62.6                        |
|       | M_15R       | CGACTGTGTTACCTGGAACA        |                             |
| 16    | M_16F       | TCACATCTTGATAGCTGTAGG       | 62.6                        |
|       | M_16R       | GGAAGATGCGATCTGACGTCA       |                             |
| 17    | M_17F       | TTACGCTCCCAAGGAAACAAAA      | 62.6                        |
|       | M_17R       | GAGGGTTTTGCGGAAAAATCTA      |                             |
| 18    | M_18R       | GGGATTTGCGGAGAAGAAGAGA      | 62.6                        |
|       | M_18R       | GGAAGTAGGAAATGAGTGTTG       |                             |
| 19    | M_19F       | CCGGGTCTAAAACCTCCCTCAA      | 62.6                        |
|       | M_19R       | AGAACAGTCTGGCTGGAAGT        |                             |
Exonuclease-I and Shrimp Alkaline Phosphatase (Fermentas, Life Sciences, Burlington, Ontario, Canada) and DNA sequencing was performed in an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, California, USA), an in-house sequencing facility of NIMR. All the 19 PCR-amplified fragments were sequenced in both forward and reverse directions (2x coverage) and each DNA sequence was individually edited and assembled using the EditSeq and MegAlign modules of the Lasergene (DNASTAR, Madison, Wisconsin, USA) computer program. All the 19 finally edited sequences were manually assembled to form a single whole mt genome sequence and aligned with the 3D7 reference sequence and the published Indian P. falciparum sequence (PfPH10) [12] using the MEGA v5.0 computer program [14] to ascertain nucleotide differences, if any, by comparing the mt genome sequences of 3 different isolates. Further, to understand the phylogenetic interrelationship among the 2 Indian (Blsp1 and PfPH10), the reference 3D7, and other 21 Plasmodium species, Neighbor-joining (NJ) phylogenetic tree was constructed using the MEGA v5.0 computer program [14] with 100 bootstrap replicates.

For phylogenetic analysis, the whole mt genome sequences of Plasmodium species infecting primates (GenBank no. AB354573, AB434919, AB434920, AB354574, AB354572, AV722799, AB354575, NC_007232, AB434918, NC_002235), rodents (GenBank no. AB379663, AB599931, AB558173), birds (GenBank no. AB599930, AB250415, AB302215), Lizard (GenBank no. NC_009961) and humans (GenBank no. M76611, NC_007243, AB354570, AB354571, AY282930), were downloaded from the NCBI web database (www.ncbi.nlm.nih.gov) and aligned using MEGA v5.0 computer program [14]. The whole mt genome sequence of the Blsp1 isolate has been deposited in GenBank public domain sequence database with accession number KJ144901.

Using the 19 novel primer-pairs (Table 1) designed in the present study, we could successfully sequenced the whole mt genome of a single P. falciparum field isolate (Blsp1) from an endemic locality of India with 2x coverage and compared with the whole mt genomes of 2 other isolates (3D7 and the previously reported PfPH10 isolate from India) [12]. A detailed list of novel primers designed in the present study is provided in Table 1, and the approximate locations of each primer-pair in the circular mt genome of P. falciparum is presented in Fig. 1. While the total length of the whole mt genome from 2 Indian P. falciparum isolates (PfPH10 and Blsp1) was similar in size of 5,967 bp (Fig. 1), surprisingly, the alignment of these 2 isolates revealed nucleotide differences in 22 positions; 2 of these were in coxIII gene, 5 in cox1 and, 7 in cyt b genes (Table 2), suggesting very high amount of variation in the mt genome in Indian P. falciparum. However, when the presently sequenced mt genome from Blsp1 isolate was aligned with the 3D7 isolate, only 1 nucleotide difference could be observed between these 2 genomes (Table 2).

This observation was in contrast to an earlier report on high sequence variation in mt genome between the worldwide and Indian isolates involving the PfPH10 isolate [12]. In any case,

**Table 2.** Alignment showing variations in the 3 mt [2 Indian (Blsp1 and PfPH10) and 1 reference (3D7)] genomes of Plasmodium falciparum isolates

| Nt Positions | 208 | 223 | 230 | 510 | 615 | 1122 | 1339 | 2175 | 2768 | 3330 | 3433 | 3444 | 3764 | 3766 | 3868 | 3985 | 4352 | 4353 | 4420 | 4640 | 4759 | 4952 | 5485 |
|-------------|-----|-----|-----|-----|-----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 3D7         | G   | A   | G   | C   | G   | A    | T    | T    | T    | A    | A    | C    | A    | T    | T    | T    | T    | T    | T    | T    | T    | T    | T    |
| Blsp1       |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| PfPH10      | A   | T   | A   | G   | T   | C   | G   | C   | A   | G   | A   | C   | C   | G   | T   | G   | T   | A   | A   | G   | C   | C   | A   |

Nt = nucleotide.
*Joy et al. (2003). †Present study. ‡Sharma et al. (2001).
the present observation on low mitochondrial genome diversity between a single *P. falciparum* isolate (Blsp1) and the reference 3D7 corroborates earlier opinion on the overall low variations among *P. falciparum* mt genomes [11]. Furthermore, the observation of 22 nucleotide changes between just 2 *P. falciparum* mt genomes from India (Table 2) reflects many-fold higher diversity in India, compared to only 30 SNPs found in 100 worldwide *P. falciparum* isolates of African, Asian, Papua New Guinean, and American origins [11]. Such observed high sequence variability between the Blsp1 and PfPH10 isolates might be the reason of failure for PCR amplification with primer information used to amplify and sequence the PfPH10 isolate [12]. This argument is further justified by the fact that, by using the 3D7 isolate (with only 1 nucleotide change), we could successfully amplify the Blsp1 isolate, justifying high sequence similarities. In order to nullify the role of PCR and sequencing errors, we have re-amplified and re-sequenced all the 19 DNA fragments of Blsp1 isolate using a different PCR thermal cycler (total 4x coverage), but the observed results were not different from the previously sequenced data. This observation essentially means that neither any inaccuracy in the PCR nor in sequencing techniques have contributed to our observed results.

With the whole mt genome sequence of a second *P. falciparum* isolate (Blsp1) in hand showing high sequence differences, we were interested to understand evolutionary interrelationships between the 2 Indian mt genomes with the reference 3D7 isolates by constructing NJ phylogenetic tree (Fig. 2). We have also included the published whole mt genome sequences of different *Plasmodium* species infecting an array of organisms. The tree topologies of the NJ phylogenetic tree justifies the evolutionary patterns of *Plasmodium* species according to their respective hosts [15,16]. For the *P. falciparum* isolates infecting humans, the Blsp1, 3D7, and C10 (GenBank accession no. M76611) form a single clade, whereas the PfPH10 isolate was placed away from this clade, justifying high genetic differentiation of this Indian isolates from the rest of *P. falciparum* isolates (Fig. 2). Whatever the case may be, the high sequence similarity between the whole mt genome sequences of the Blsp1 and 3D7 isolates justify the notion that intra-specific mt genome variations are in fact minimal [11] and therefore mt genomes remain conserved among the phylum Apicomplexa [8] as well as in *Plasmodium* species infecting different hosts (both humans and non-humans) [17], possibly due to very low recombination rate and uni-parental (maternal) inheritance [6].

However, the observed high variability between the mt genomes of 2 Indian isolates suggests high genetic diversity in Indian *P. falciparum* [2] which can be further validated by sequencing isolates from more Indian populations. Such study would not only fill the gap of the existing knowledge about the worldwide mt genome diversity but also help to bring out important and so far not-fully-resolved evolutionary history of global *P. falciparum*. Moreover, whole mt genome sequence comparisons in multiple *P. falciparum* isolates from all over India would also inform the extent of genetic diversity of the cytb gene that is considered to be the target of an effective antimalarial, atovaquone [10]. As this antimalarial is currently not used in India, the knowledge of the extensive diversity through population genomic studies of mt genome of Indian *P. falciparum* would possibly help in deciding whether to incorporate atovaquone in malaria control programs in India.
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CONFLICT OF INTEREST

We have no conflict of interest related with this study.

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