



Molecular Characterization of *Plasmodium relictum* in Four Common Bird Species in India

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ABSTRACT

The increasing emergence of wildlife diseases with the possibility to ecological threats as well as domestic animals and human health has prompted the importance of understanding disease dynamics and associated risks in biological conservation. The present study was undertaken from North Eastern part of India from January, 2017 to March, 2018 to identify and molecularly characterize *Plasmodium relictum* based on *cyt b* gene in various species of wild birds (*Upupa epops*, *Passer domesticus*, *Pycnonotus cafer*, *Bubulcus ibis*). The birds were captured by netting system. After blood was collected from wing veins, birds were released from the cages. Blood samples were examined after staining with Giemsa stain. The positive samples were used for amplification of *cyt b* gene of *Plasmodium relictum*. Cloning and sequencing of the amplified products of each samples was performed separately. Out of 120 birds examined, 25 were found positive for *P. relictum* based on morphology and subsequently confirmed by PCR which selectively amplified 712bp of *P. relictum*. Based on sequences and phylogenetic analysis of *cyt b* gene, 8 isolates of *P. relictum* were identified. The obtained complete nucleotide sequences of *cyt b* gene from *P. relictum* revealed 100% identity among themselves while the other sequences registered in Gen Bank showed 95% to 99% similarity. In the phylogenetic analysis all the isolates of *P. relictum* formed a separate clade with high bootstrap values. It can be inferred from the study that *P. relictum* is fairly common in wild birds and the *cyt b* gene is highly conserved among different isolates. It seems that *cytb* could provide suitable genetic markers for discrimination and genetic characterization of *P. relictum*. This is the first attempt of genetic characterization of *P. relictum* from India.

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Authors' Contribution

PB, SG, AD and SSA collected the blood samples. Parasite identification and molecular analysis was done by GP and AS. GP and SKB critically supervised the whole experiment and drafted the manuscript.

Key words

Plasmodium relictum, *cyt b* gene, Molecular characterization, India

INTRODUCTION

Plasmodium relictum infects a wide variety of domestic and wild birds including chickens, ducks, partridges, canaries, pigeons etc (Bensch *et al.*, 2009; Marzal *et al.*, 2014; Drovetski *et al.*, 2014). It may be lethal to the species which have not evolved resistance to parasites such as penguins and naïve birds which often suffer from severe disease and mortality during infection (Ilgūnas *et al.*, 2016). The parasites are easily distinguishable in blood films because of its distinct morphological characteristics. Mature stages typically have predominant nuclei and cytoplasm, numerous pigment granules and markedly

influence the position of host cell nuclei causing lateral shifts in their positions. Microscopic examination of blood films was main diagnostic tool in 20th century worldwide (Atkinson, 2008). Microscopy may reveal the presence of trophozoites, merozoites and gametocytes in erythrocytes.

Several molecular methods are used for detecting avian malarial parasites in blood samples. Most are based on amplification of conserved fragments of 18S rRNA or mitochondrial cytochrome-b gene (Jarvi *et al.*, 2002; Tattiyapong *et al.*, 2016; Valkiūnas *et al.*, 2018). Partial sequences of *cyt b* gene have been successfully used for molecular characterization of parasites and it proved to be excellent molecular marker for disease diagnostics (Elsasser *et al.*, 2009).

Despite wide spread distribution of *P. relictum* in birds, no study has been done in India to characterize the *cyt b* gene and also about the number of copies it

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possesses. Therefore, the objective of this study is to identify comprehensive morphological features of blood stages of the parasites and the genetic diversity of the targeted gene. This will give new directions for future avian malaria research.

MATERIALS AND METHODS

Collection of blood samples

Field work was carried from various states of North Eastern India from January, 2017 to March 2018. Sixty three common hoopoes (*Upupaepops*), twenty seven house sparrows (*Passer domesticus*), twenty two red vented bulbuls (*Pycnonotus cafer*) and eight egrets (*Bubulcus ibis*) were caught with mist nets and large stationary traps. The blood samples were taken by puncturing the wing vein. Blood films were prepared immediately, air dried, fixed in methanol and stained with Giemsa's stain following standard procedure (Soulsby, 1982). About 50µl of whole blood was taken in EDTA coated vials and stored at ambient temperature in the field and then stored at -20°C in the laboratory until further use.

Isolation of genomic DNA from blood samples

Microscopically positive blood samples (25) for *Plasmodium relictum* were subjected to genomic DNA isolation. DNA was isolated from 10µl of EDTA anticoagulated whole blood of 25 positive samples. Twenty hoopoe, two from house sparrow, two from red vented bulbul and one from egret using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer's protocol and stored at -20°C for downstream use.

PCR amplification and purification

The *Plasmodium cyt b* gene specific primer set (PlasF: GAGAATTATGGAGTGGATGGTG and PlasR: GTGGTAATTGACATCCWATCC) was used in this study to amplify the partial sequence of *cyt b* gene with minor modifications. The PCR reaction was carried out in 25µl of 1X PCR green buffer (Thermo Scientific, USA) containing 0.2 µl of Dream Taq DNA polymerase, 10 pmol of each primer and 0.2 mM concentration of each deoxyribonucleoside triphosphate, with 3µl of template DNA. Amplification was performed using a C1000 thermal cycler (BioRad, USA) under following conditions: initial denaturation at 94°C for 5 min, followed by 35 amplification cycles (94°C for 30 sec, 55°C for 45 sec and 72°C for 1 min) and a final extension at 72°C for 10 min. Nested PCR was done to amplify 712 bp gene fragment of *cyt b* region following the same thermal cycling conditions. The PCR amplicons were checked by electrophoresis on 1.5% low melting agarose gel and purified by gel extraction by using Qiaex II gel extraction kit (Qiagen, Germany) following

manufacturer's protocol.

Cloning and sequencing

For cloning of *cyt b* gene, the purified PCR products from each sample were separately ligated into pTZ57R/T TA cloning vector (Thermo Scientific, USA) and incubated at 4°C for overnight. The plasmid DNA constructs were transformed into competent DH5α *Escherichia coli* cells using Ins TAclone PCR cloning kit (Thermo Scientific, USA) according to manufacturer's protocol. The transformed cells were plated immediately on pre-warmed LB agar plates supplemented with ampicillin (100µg/ml), X-gal (30µg/ml) and IPTG (0.5mM/ml) for the development of blue white colonies. The positive clones were confirmed by colony PCR using specific primers. The stab cultures of two positive clones per sample containing the desired gene were custom sequenced from the Department of Biochemistry, Delhi University, South Campus, India. The fragments were sequenced at least twice to reduce possibility of sequencing errors.

Sequence analysis

All the newly generated sequences of *cyt b* gene of *Plasmodium relictum* isolates were compared with each other and with published sequences in the nucleotide database in Gen Bank using the BLAST programme of the National Centre for Biotechnology Information and aligned by Clustal W.

Phylogenetic analysis

Phylogenetic analysis of *P. relictum* of the eight isolates collected from different geographical areas of NE region of India were done independently with the sequences of *cyt b* genes. A total of twenty gene sequences including eight newly generated sequences were used for analysis. Phylogenetic analysis was done by using the MEGA6.0 software (Tamura *et al.*, 2013) with the maximum likelihood method. The nucleotide substitution model for best fit to the data set was evaluated in MEGA6.0. Tamura-Nei model (TN93) with gamma distribution (TN93+G) was found to be the model of choice for phylogenetic analysis of *cyt b* gene. The phylogeny was analysed using 1000 bootstrap replications. *Plasmodium falciparum* species from human was used as out group species to root the tree.

Prevalence study

The prevalence of *Plasmodium relictum* was recorded according to age and species of birds. The prevalence (P) was estimated according to standard method (Thrusfield, 2007) and by the formula as given below:

$$P = \frac{\text{No. of infected birds during specified period}}{\text{Total birds surveyed}} \times 100$$

Statistical analysis

The upper and lower limits of the confidence intervals for the infection values in each species were calculated by the following formula (Bawm *et al.*, 2016)

$$\hat{p} \pm z \cdot \sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$$

where, P-hat is dividing the numbers of events by the number of trials; z-score is the table value and n is the number of samples

Ethical statement

All animal experiments were carried out strictly as per the guidelines issued by ARRIVE and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and was approved by Institutional Animal Ethics Committee with reference No. CVSc/CAU/IAEC/no. 6641, dtd, Selesih, the 25th, April, 2016.

RESULTS

Parasitic identification and prevalence

Microscopic examination of the Giemsa stained thin smear revealed that 25 (21%) out of 120 blood samples were positive for *Plasmodium relictum* (Table I). The trophozoite, schizont stages were observed in the cytoplasm of the red blood cells (Fig. 1C). From morphological characteristics, the parasites were identified as *P. relictum*. *P. relictum* can be distinguished from other plasmodial parasites of birds by the presence of round or irregular gamonts inside RBC with fine and pinpoint pigment granules. Out of 63 hoopoes 20 (16.66%) were found positive for *P. relictum*, 02 (1.66%) of 27 blood samples of house sparrow, 02 (1.66%) of 22 red vented bulbuls and 01(0.83) of 08 egrets also showed positive (Table II). The prevalence of *P. relictum* was found higher in adult (15.83%) birds than sub-adults (5%).

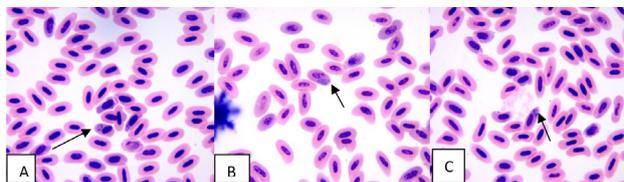


Fig. 1. Trophozoite of *P. relictum* in the erythrocytes.

PCR amplification and cloning of *cyt b* gene

The microscopic finding of *Plasmodium* spp. in red blood cells was confirmed by PCR. The amplified products of *cyt b* gene were checked by ethidium bromide stained agarose gel (1.5%) electrophoresis which showed ~ 712 bp size of the desired gene. The PCR products were purified,

cloned into pTZ57R/T TA cloning vector and confirmed by colony PCR (Fig. 2). Subsequently all isolates were custom sequenced.

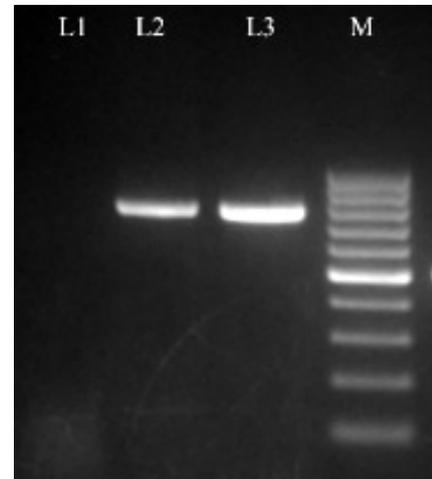


Fig. 2. Identification on 1.5% agarose gel of 712 bp products of amplification of the *cyt b* gene of *P. relictum* after colony PCR; L1–Negative; L2, L3–Positive amplification (712bp); M–100bp DNA ladder.

Sequence analysis of newly generated *cyt b* region

Eight sequences of the *cyt b* gene (712bp) generated in this study were submitted to GenBank (Table II). Sequence similarity searches in BLAST showed all isolates of the targeted genes have high similarity with USA and Germany isolates (Fig. 3). A closer comparison of the newly generated 8 isolates had 100% identity among themselves and 99.6% with USA (DQ659566) and 99.3% with Germany (MF189958) isolates, respectively. These 8 new sequences did not show any difference in nucleotide position.

Table I. Overall prevalence rate of *Plasmodium relictum* in birds.

Total number of birds examined	Number of birds infected (%)	Range
120	25 (21)	
Hoope	20 (16.66)	12.07-21.26
House sparrow	02 (1.66)	0.09-3.24
Red vented bulbul	02 (1.66)	0.09-3.24
Egret	01 (0.83)	0.29-1.95
Young		
Sub adult	06 (5)	2.31-7.69
Adult	19 (15.83)	11.32-20.33

		Percent Identity																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
Divergence	1	█	100.0	100.0	100.0	100.0	100.0	100.0	100.0	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	1	MH373249 <i>P. relictum</i> Mizoram
	2	0.0	█	100.0	100.0	100.0	100.0	100.0	100.0	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	2	MH373250 <i>P. relictum</i> Mizoram
	3	0.0	0.0	█	100.0	100.0	100.0	100.0	100.0	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	3	MH373251 <i>P. relictum</i> Manipur
	4	0.0	0.0	0.0	█	100.0	100.0	100.0	100.0	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	4	MH373252 <i>P. relictum</i> Manipur
	5	0.0	0.0	0.0	0.0	█	100.0	100.0	100.0	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	5	MH373253 <i>P. relictum</i> Tripura
	6	0.0	0.0	0.0	0.0	0.0	█	100.0	100.0	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	6	MH373254 <i>P. relictum</i> Tripura
	7	0.0	0.0	0.0	0.0	0.0	0.0	█	100.0	97.0	97.0	65.9	65.7	99.6	96.9	90.0	99.3	99.3	64.9	94.0	82.7	7	MI1373255 <i>P. relictum</i> Nagaland
	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	█	97.8	97.8	65.9	65.7	99.6	96.9	98.0	99.3	99.3	64.9	94.8	82.7	8	MH373256 <i>P. relictum</i> Nagaland
	9	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	█	62.4	32.9	33.1	51.0	61.9	56.2	74.7	62.9	33.2	63.2	56.4	9	AF254975 <i>P. relictum</i> Sweden
	10	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	0.0	█	52.8	53.0	81.5	97.4	89.8	97.6	97.6	53.2	95.2	83.4	10	AB308046 <i>P. relictum</i> Japan
	11	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	3.0	3.0	█	99.8	99.6	96.9	97.7	100.0	100.0	97.7	93.9	83.1	11	AF495571 <i>P. relictum</i> Sweden
	12	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	2.8	2.8	0.2	█	99.4	97.1	97.9	99.8	99.8	97.9	94.2	83.3	12	AY831748 <i>P. relictum</i> Sweden
	13	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	3.0	2.4	0.4	0.6	█	97.2	98.5	99.7	99.7	62.4	94.7	82.7	13	DQ659566 <i>Plasmodium</i> sp. USA
	14	3.2	3.2	3.2	3.2	3.2	3.2	3.2	3.2	5.5	2.7	3.2	3.0	2.8	█	77.0	97.1	97.1	44.6	87.2	83.4	14	GQ141593 <i>Plasmodium</i> sp. USA
	15	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.5	1.9	2.3	2.1	1.5	2.6	█	98.4	98.4	56.9	95.5	83.6	15	KT363870 <i>P. relictum</i> Spain
	16	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	27.8	2.4	0.0	0.2	0.3	2.6	1.6	█	18.4	8.1	17.1	16.6	16	KY653774 <i>P. relictum</i> USA
	17	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	6.1	2.4	0.0	0.2	0.3	2.6	1.6	0.0	█	44.0	87.3	83.4	17	MF189958 <i>P. relictum</i> Germany
	18	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.6	2.3	2.3	2.3	2.1	1.9	3.0	2.1	2.3	2.3	█	94.4	83.5	18	MG724747 <i>P. relictum</i> Lithuania
	19	5.4	5.4	5.4	5.4	5.4	5.4	5.4	5.4	11.8	4.9	6.3	6.1	5.5	5.0	4.6	4.9	5.1	5.9	█	83.6	19	AY099029 <i>P. gallinaceum</i> USA
	20	19.7	19.7	19.7	19.7	19.7	19.7	19.7	19.7	26.3	18.8	19.2	18.9	19.7	17.4	18.5	17.1	17.4	18.6	18.6	█	20	AJ298787 <i>P. falciparum</i> India

Fig. 3. Distance matrix of *cyt-b* gene sequence of *P. relictum* with the available sequences from GenBank.

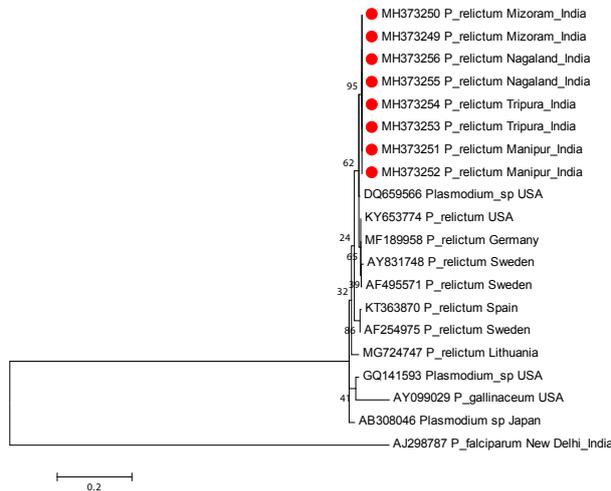


Fig. 4. Phylogenetic tree of *P. relictum* isolates including the outgroup (*P. falciparum*).

Phylogenetic relationship

The phylogenetic tree based on maximum likelihood method with Tamura-Nei model for distance calculation

based on the 712 bp region of *cyt b* gene is shown in Figure 4. *P. relictum* from those areas were compared with *P. gallinaceum* and *P. falciparum*. *Pasmodium relictum* in this research was located in one clade which showed that they are genetically closer to *Plasmodium gallinaceum* than to *P. falciparum*. Isolates from Spain (KT363870), Sweden

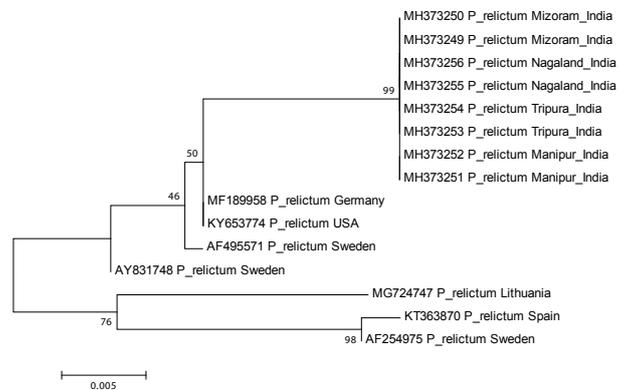


Fig. 5. Neighbour joining tree of *P. relictum* sequences without an out group.

Table II. Species of *Plasmodium relictum* from which cyt b sequences were used for phylogenetic analysis together with their location/host and GenBank accession number.

Species	Region of origin	Host	GenBank accession no.
<i>P. relictum</i>	Mizoram	Hoopoe (<i>Upupa epops</i>)	MH373249*
<i>P. relictum</i>	Mizoram	House sparrow (<i>Passer domesticus</i>)	MH373250*
<i>P. relictum</i>	Manipur	Red vented bulbul (<i>Pycnonotus cafer</i>)	MH373251*
<i>P. relictum</i>	Manipur	Cattle egret (<i>Bubulcus ibis</i>)	MH373252*
<i>P. relictum</i>	Tripura	Cattle egret (<i>Bubulcus ibis</i>)	MH373253*
<i>P. relictum</i>	Tripura	Red vented bulbul (<i>Pycnonotus cafer</i>)	MH373254*
<i>P. relictum</i>	Nagaland	Hoopoe (<i>Upupa epops</i>)	MH373255*
<i>P. relictum</i>	Nagaland	House sparrow (<i>Passer domesticus</i>)	MH373256*
<i>Plasmodium</i> sp.	USA	Red-billed quelea (<i>Quelea quelea</i>)	DQ659566
<i>P. relictum</i>	Japan	Culexfuscanus	AB308046
<i>P. relictum</i>	Lithuania	Common canary (<i>Serinus canaria</i>), European goldfinch (<i>Carduelis carduelis</i>), Zebra finch (<i>Taeniopygia guttata</i>), Budgerigar (<i>Melopsittacus undulatus</i>)	MG724747
<i>P. relictum</i>	Sweden	Sudan golden sparrow (<i>Passer luteus</i>)	AF495571
<i>P. relictum</i>	Sweden	Eurasian blackcap (<i>Sylvia atricapilla</i>)	AY831748
<i>P. relictum</i>	Sweden	Great reed warbler (<i>Acrocephalus arundinaceus</i>)	AF254975
<i>P. relictum</i>	USA	African penguin (<i>Spheniscus demersus</i>)	KY653774
<i>P. relictum</i>	Germany	Carrion crow (<i>Corvus corone</i>)	MF189958
<i>Plasmodium</i> sp.	USA	Tufted titmouse (<i>Baeolophus bicolor</i>)	GQ141593
<i>P. relictum</i>	Spain	House sparrow (<i>Passer domesticus</i>)	KT363870
<i>P. gallinaeum</i>	USA	Red jungle fowl (<i>Gallus gallus</i>)	AY099029
<i>P. falciparum</i>	New Delhi, India	<i>Homo sapiens</i>	AJ298787

*New sequences generated in this present study.

(AF254975) formed separate clade in the neighbour joining tree with high bootstrap support (86%) by removing *P. falciparum* (AJ298787) from the analysis as out-group species. Isolates from Lithuania (MG724747) and Sweden (AY831748) was found distantly related with the present isolates. Another neighbour joining tree with the different *P. relictum* isolates from the present investigation was constructed to check the intra-species variation in relation to each other, if any (Fig. 5).

DISCUSSION

On the basis of morphology, the parasites were identified as *Plasmodium relictum*. However, the microscopic identification is not sufficient to differentiate different species of *Plasmodium* of birds. It requires extensive skills and experience, especially during early and chronic stages of infection (Silveira *et al.*, 2009; Silveira *et al.*, 2013). In the present study, all four species of wild birds were found infected with *P. relictum* with varying intensity. Several studies have revealed a lack

of host specificity in *Plasmodium* infection (Zhang *et al.*, 2014; Silva-Iturriza *et al.*, 2012; Beadell *et al.*, 2009; Waldenström *et al.*, 2002). This means that host switching between avian species is more likely to occur in *Plasmodium*. Adult birds were found more infected than sub-adult groups, probably because of the time between an infection being transmitted to a nestling and parasites entering the nestling stage (Valkiūnas, 2005). After infections, individuals either become ill or die during the active phase. Alternatively, they may develop an effective immune response and clear the infection. As a result, older birds become less susceptible as they grow old. On the other hand, an age dependent deterioration of the immune system lead to a late-life increase in infection compared to sub-adult groups (Palacios *et al.*, 2007; Palacios *et al.*, 2011). No other haemoparasites were detected in the four selected species of birds. However, we only sampled from NE states of India, so additional studies are needed from other locations of India to determine haemoprotozoan diversity.

The microscopic findings of *Plasmodium relictum*

in RBCs of four species of wild birds was confirmed by PCR. PCR was found very sensitive to detect the infection in birds. This result was similar to the study reported by Tattiyapong *et al.* (2016) and Krams *et al.* (2012) who also reported sensitivity of PCR during the detection of haemoparasites infection.

The 712bp PCR amplicon from all positive samples were cloned and subsequently sequenced. The sequences of 8 samples showed clear sequencing patterns. These sequence data were then deposited in NCBI Gen Bank database under accession numbers: MH373249-MH373256. Sequence similarity between the partial *cyt b* gene fragments of all isolates was 100% with no nucleotide substitution. The multiple nucleotide sequence alignments of *P. relictum* showed that the *cyt b* gene in the study region was highly conserved.

On the basis of sequence homology and phylogenetic analysis of *cyt b* gene all isolates of *Plasmodium* sp. were confirmed. The phylogenetic analysis revealed that all *P. relictum* isolates were clustered in the same clade and separated from *P. falciparum*. Similar studies have been reported from different geographical locations (Tattiyapong *et al.*, 2016; Silveira *et al.*, 2013; Murata *et al.*, 2008; Chen *et al.*, 2015). No intra-specific variation was observed among different isolates collected from North-East India suggesting that all isolates were identical. To date, mitochondrial fragments of *cyt b* gene has not been characterized from *Plasmodium* sp. of birds from India. Here we have presented the partial sequence of *cyt b* gene of *Plasmodium relictum*. Neighbour joining tree shows strong support for *Plasmodium* and apicomplexan clade (95% bootstrap support).

In conclusion, we morphologically and genetically characterized *P. relictum* from four species of wild birds in NE region of India. The present study demonstrates that *P. relictum* is fairly common in wild birds and has the conserved gene sequence encoding for cytochrome oxidase b. Along with the microscopic examination, this report first describes the molecular characterization of *P. relictum* of wild birds from NE region of India. It also highlights the importance of isolating and characterizing other *Plasmodium* mitochondrial genes which would provide additional information into codon usage and insights about the evolution of apicomplexan parasites. However, further studies with larger number of samples from various wild birds as well as vectors involved in different geographical areas covering other parts of India should be carried out for determining the status and the genetic diversity of avian malaria parasites in India.

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Statement of conflict of interest

The authors declare that there is no conflict of interest

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