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A Novel Model Approach for Detecting Wild Animals through Blood Fed Mosquitoes: Scope in Indirect Wildlife Sampling and Wildlife Forensics

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Abstract - In this study we detected the wild animals through blood fed female mosquitoes collected in a controlled environment i.e. a zoological park situated at Dehradun, India and explained its scope in indirect wild animal sampling in fields and in wildlife forensics. The eighteen blood-fed mosquitoes were collected from Malsi Deer Park, Dehradun on a single day and DNA was isolated from the individual blood filled midgut of the mosquitoes. The three mitochondrial genes (Cytochrome b, 16S rRNA and 12S rRNA) were amplified on the DNAs from the blood collected from each mosquito. After DNA sequencing the seven animal species were detected from eighteen blood samples representing mainly the mammals and birds. The two species detected were not present in the zoological park, hence, it validates this approach and proves that it can be applied in fields. The approach is novel in a way that it is non-invasive in nature and has implications in detecting the wild animals indirectly and in wildlife forensics.

Key words - ***Novel, wild animals, blood fed mosquitoes, wildlife, forensics***

I. Introduction

The mosquitoes and leeches have been used for the detection of the host species from the blood fed by them in the past. However, the benefits of these efforts were confined to only some places and with limited applications [1, 2 and 3]. Here we describe a model approach for the indirect sampling of wild animals though blood fed mosquitoes which has far and more effective implications in wildlife and forensics. The idea for this approach has arisen from the rationale of an autogenous type of reproduction which requires the host blood for the maturation of eggs in mosquitoes. As only the female mosquitoes have the blood feeding mouth parts these can be sampled and stored, before the

digestion of blood in midgut, until used in the laboratory to identify the species and obtain other informations from the host blood using DNA technology. It is evident that different species of mosquitoes (*Anopheles*, *Aedes* and *Culex*) feed on a wide array of vertebrate hosts including amphibians, reptiles, birds, mammals and even fishes [4 and 5]. So, we exploited this blood feeding habit of mosquitoes to detect the host's blood species and its implications in indirect wildlife sampling and wildlife forensics.

We sampled the blood fed mosquitoes from Malsi Deer Park, a mini zoological park surrounded by Malsi Reserve Forest in Dehradun, Uttarakhand, India and isolated the DNA from blood obtained from each of them individually. After DNA sequencing we found the species of different classes of vertebrates, kept in the zoological park. Although this is a model approach and has to be tested in fields, but no doubts it holds a promising approach for identifying species through blood fed mosquitoes with the implications in the field of wildlife for indirect animal sampling and in wildlife forensics. The novelty and limitations of this model approach have also been described in the discussion section.

II. Material and methods

Study area, collection and storage of mosquitoes: Eighteen mosquitoes were collected from the resting sites, near to the enclosures of different wild animals in the Malsi Deer Park, Dehradun, Uttarakhand on a single day in July, 2014 according to the method described by Vipin *et al.* [6] (Fig. 1). The GPS coordinates and elevation of mosquito collection sites were recorded using GPS. The mosquitoes were preserved in 95% ethanol individually in separate eppendorf tubes and taken to the Wildlife Forensic and Conservation Genetics Cell, Wildlife Institute of India, Dehradun and stored at -40^{0}C until used for DNA isolation. The mosquitoes were identified up to genus level morphologically using pictorial mosquito identification key developed by Centre for Disease Control, USA (http://www.cdc.gov/nceh/ehs/docs/pictorial_keys/mosquitoes.pdf).

Isolation of genomic DNA, PCR amplification, DNA sequencing and species identification: The freezed mosquitoes having blood in their midgut were screened and allowed to thaw for 10 minutes in sterile glass petridishes. An individual mosquito was placed on a glass slide and put under the stereo microscope (Leica-microsystems). The intestine of each mosquito was dissected out with the help of two needles, putting one at the thorax and pulling down the last segment of abdomen with second under the stereo microscope. The taken out midgut having blood was ruptured with the help of a sterilized needle and the blood was carefully collected with the help of 0.2-10.0 µl pipette (Gilson, Germany). The genomic DNA was isolated as per our earlier described method [6].

The PCR amplification was done by amplifying partial fragments of cytochrome b (472 bp; [7]), 16S rRNA (550 bp; [8]) and 12S rRNA (450 bp; [9]) genes of mitochondrial DNA in a 20 µl reaction volume containing 1Unit Taq polymerase enzyme, 10µM of each primer, dNTPs, 1.5 Mm $MgCl_2$, PCR buffer, BSA, double distilled water and 100 ng of genomic DNA. The cycling conditions were initial denaturation at 94^{0}C at 05 min followed by 35 cycles for 40 sec at 94^{0}C, 45 sec at 53^{0}C, and 45 sec at 72^0 and 10 min at 72^{0}C with final extension on a thermal cycler (Applied Biosystems, USA). Amplification products were visualized by electrophoresis (5 µl) on a 2% agarose gel containing ethidium bromide.

Amplified PCR products were purified using QIAquick® PCR Purification Kit (QIAGEN, Germany). Cycle sequencing PCR was performed for these purified PCR products with their respective primers following the suggested composition of master mixture from Applied Biosystems. Cycle sequencing PCR products were purified using "BigDye terminator clean-up" method. These products were then subjected for sequencing on 3130 Genetic Analyzer (Applied Biosystems, USA).
DNA sequence analysis: The cyt b, 16S rRNA and 12S rRNA gene sequences were used for BLAST search on National Centre for Biotechnology Information (NCBI), USA for species identification. The cyt b, 16S rRNA and 12S rRNA gene sequences of generated in this study could not be submitted to GenBank because of their small size (<200 bp). Interested readers may request us for these sequences.

III. Results

Isolation of genomic DNA, PCR and species identification: From a total of eighteen mosquitoes collected the seventeen were found to be of genus *Culex* and one was of *Anopheles* genus. A high quality genomic DNA was obtained from all mosquitoes ($OD_{260/280}$ = 1.8-2.0) except one (mosquito ID 17). The DNA yield per sample range between 0.8-1.0 µg. Six, three and sixteen samples showed amplification with cyt b, 16S rRNA and 12S rRNA genes, respectively. Seven species of wild animals (*Axis axis, Bubo bubo, Gallus gallus, Lophura leucomelanos, Milvus migrans, Panthera pardus and Strix leptogrammica*) covering mammals and birds were detected from the blood taken out from seventeen mosquitoes. One sample resulted in *Anopheles minimus*. The details of the mosquito samples with resulted wild animal species have been shown in Table 1. All but two species (*Lophura leucomelanos and Milvus migrans*) detected from the host blood of mosquitoes were present in the Malsi Deer Zoological Park, Dehradun at the time of collection of mosquitoes.

Discussion and scope of animal sampling through blood fed mosquitoes in wildlife and wildlife forensics

The earlier attempts to identify the animal species using blood fed leeches and mosquitoes had limitations like the use of leeches to detect species is confined to the area of a pond or the habitats of their occurrence, some studies have used the primers which were designed to detect only a handful of domestic mammals and some have used blood from mosquitoes to detect only the infectious diseases [1, 2 and 3]. This model approach did not require designing of any species specific primer as it uses universal mitochondrial primers and can identify animal species present at least within a radius of 3-5 km^2. The approach is novel in many ways like to identify and discover new mammalian, avian, amphibian, reptilian or even endangered and vulnerable species, distribution in relation to sex, individual identification, population genetic structure, phylogeny and addressing human-wildlife conflicts. Moreover the approach is non-invasive in nature which has taken the advantage from natural host-parasite interactions.

Scope of detecting host species from blood fed mosquitoes in

Indirect assessment of biodiversity pattern and sex determination in wild and surrounding areas: The biodiversity, in and around, of a forested area can be determined indirectly through detection of wild animals including herbivores, carnivores, birds, reptiles and amphibians from the blood taken out from the blood fed mosquitoes collected from in and around the forested areas using aspirators or light traps. The light traps modified to capture mosquito species i.e. having the source of humidity and CO2 as attractants may be installed at identified locations in the forest during night times and in the morning blood fed mosquitoes can be screened and stored for the further use. However, to capture the mosquito species which are day active the traps have to be modified accordingly by simply switching of or removing the source of light from it. Though the mosquitoes require the blood proteins for the maturation of eggs from warm blooded animals [5] but they do feed on amphibians and reptiles also (Heatwole and Shine, 1976). The sex of the identified species can be determined through PCR using species specific sex determination primers.

Individual identification: Though the conditions of multiple blood meals have been reported in different mosquito species but if we keep apart a general counted percentage (10-40%) of such mosquitoes mentioned in several past studies [2] we can still expect a minimum of 60% mosquitoes with single host blood meal through which DNA can be extracted and the individual of the species can be identified. In our own experiment of hand-feeding of ten *Anopheles stephensi* mosquitoes in one attempt, in artificial conditions, it was found that 1-3 mosquitoes were incompletely blood fed. Which means they still have the chances of feeding on some other hosts, however, 70-90 % of mosquitoes fed completely until the blood oozes out from the rectum (Vipin *et al.*, unpublished). All these data indicates that 90-60% of blood fed mosquitoes contain blood from single host which can be used in population and phylogenetic studies.

Population and phylogenetic studies: The individuals identified of the same species from these mosquitoes, collected from areas separated by some barriers can be used for population genetic or phylogenetic studies. This methodology may also act as an additional aid to the direct and dedicated sampling for genetic studies.

Diagnosis of diseases in wild animals: The blood in mosquitoes may also contain the antibodies against pathogens, viruses and bacteria in host blood which can be detected through enzyme-linked immunosorbent assay (ELISA) [3]. Hence, the mosquitoes collected can be used to indirectly detect the spread of any disease in wild animal populations and the disease eradication measure can then be taken for the conservation of the species.

Wildlife forensics: In addition of the direct biological evidences (blood stains, saliva, hairs, scats and urine) the blood-fed mosquitoes can also be used in collection of indirect forensic evidences in human-wildlife conflicts. The blood fed mosquitoes after feeding completely on a host usually prefer to rest in suitable resting sites within the average range of 106 meters [10]. This habit can be exploited for proving the involvement of a conflict animal in an attack on human or live-stock. Most of the carnivores after killing and devouring of their prey have the habit to rest for some time. If the nearby area of actual site of the conflict i.e. near the carcase can be searched for blood-fed mosquitoes the species and individuality of the attacking or conflict animal could then be identified from the blood taken out from the mosquito. The identification of conflict animal is important while dealing human-wildlife conflicts because targeting a different and innocent animal in place of real one may further worsen the existing situations.

Effects of vector biology on implications of this model approach

The proposed approach may prove to be best applicable at the equatorial region of the world because this is the region where a maximum biological diversity of animals is found and so is the presence of mosquitoes. However, the biology of the mosquitoes i.e. favourable environmental conditions (temperature and humidity), availability of breeding sites, average lengths of the day and night, digestion of blood, place, time of sampling and species, range and height of the flight may have some effects on the implications of proposed model approach in the following different ways.

The effect of temperature and humidity: The geographical regions where any of the following i.e. the average temperature or the level of relative humidity is below or above the 27^0C and 80%, respectively, provide unfavourable environment conditions for the mosquitoes to survive [11]. In those regions the approach would be less effective. The extremes of the season of a year i.e. summer and winters will have a temporary effect; also the weather across the globe never remains the same.

Availability of breeding sites: The availability of water bodies like ponds, lakes, small water pockets near streams and the tree holes provide natural breeding sites for mosquitoes. The sampling of blood fed mosquitoes after the monsoon season greatly increase the effectiveness of this approach as breading sites are formed in plenty.

Season, time, place and species: In adding to the availability of breeding sites the season, time and place plays an effective role in designing the best mosquito sampling strategies. The average length of the light and darkness i.e. short-day (10 hour light and14 hour dark) greatly affects the blood feeding nature in mosquitoes. As the length of the days which becomes short in autumn provides best time for their survival. The dusk and dawn is the most appropriate time in a day to catch the mosquitoes during which they remain the most active. After having a blood meal their belly becomes swollen and they prefer to rest in a nearby area which can be a shadowy, cool and moist resting sites like under leaves of bushes and grasses, tree holes and abandoned man-made structures in forested areas. The identification of mosquito species may help greatly in deciding the appropriate time of their collection in fields, because aedes mosquitoes remain active during the day times while anopheles and culex are active at night times ((http://www.who.int/water_sanitation_health/resources/vector007to28.pdf).

Digestion of blood in mosquito: The digestion of blood in mosquitoes is a very critical factor for the effectiveness of this proposed approach. The average time for digestion of blood in a mosquito midgut may be around 30 hrs after having a blood meal [12]. So, the time available to collect the mosquito to get the blood for the identification of host species through DNA analysis is 30 hrs. However, if the mosquitoes are immediately preserved in 95% ethanol in the fields or at -40^0C in a laboratory, the DNA can be extracted at any desired time.

Range and height of the flight: The average range and height of the flight of mosquitoes have very significant implication for this kind of study for animal sampling and in wildlife forensics. It is known that the average range of flight of a mosquito is between 3-5 km in one step [13 and 14]. Though, the blood fed mosquitoes prefer to rest in a nearby area, however, it cannot be ruled out that a blood fed mosquito has the capacity to travel 3-5 km in one step. This implies that the host species detected from the mosquito's blood may be present anywhere within the radius of 3-5 km from the site where the blood fed mosquito was collected. The occurrence of *Lophura leucomelanos and Milvus migrans* inside the Zoological Park indicated that either the mosquitoes have fed on these species within the range of 3-5 km of Park, the surrounding forested area of the Park i.e. the Malsi Reserve Forest is known to have the presence of these two species, and have rested inside the zoo or these two species have visited the zoo and got fed by mosquitoes. Also the mosquito from which *Axis axis* was detected had been collected from the resting site near the cage of *Bubo bubo* which was situated at a distance of 65 meters from the cage of *Axis axis*, this explains that after feeding on *Axis axis* the mosquito had flown 65 meters to find the resting place. So, there are equal chances of both, means either the blood fed mosquitoes or the *Lophura leucomelanos and Milvus migrans* have come inside the Park. However, the detection of these two non-housed species validates this study and proves that it can be applied effectively in the fields for indirect detection of wild animal species. If the terrain of the fields is hilly then the areas between the heights of 300-600 meters should be searched for blood fed mosquitoes, because this is the preferred range of the height of the presence of mosquitoes [14]. These two factors may help in indirectly detecting the wild animals, present at remote places and in providing the indirect evidences of the presence of a wild animal, specifically a carnivore, in human-wildlife conflicts.

Future perspective of use of other blood parasites in indirect animal detection and other uses

Other than female mosquitoes and leeches the animals which feed on blood of other species may prove to be very potential source for indirect animal sampling/ detection in different habitats and for detecting diseases. Among them are the fleas which serves as the host for *Yersinia pestis*, the blood from fleas can give the information about the host rodent species which can be managed for the control of plague. The blood from animal mites and ticks can give the information on the diversity of their hosts present in that area. The oxpeckers, vampire finches and vampire bat represent a different category of blood parasites for indirect detection of host animal species and diagnosis of any disease in them through the blood they feed on them. The lampreys can be targeted for the indirect detection of animal diversity in marine ecosystems. At this point the blood parasite species mentioned in this paragraph are only seen as potential targets for indirect animal sampling and diagnosis of diseases, however, the feasibility of their use, effects of their biology on this approach and their sampling strategies need to be explored first before moving ahead in this direction.

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Table 1. Details of species detected from eighteen mosquitoes.

S. No.	Species detected from blood in mosquito	Mosquito ID	Cage IDs of the species detected from blood in mosquitoes	Cage coordinates	Collection site elevation (meters)
1	*Axis axis* (Chital)	2	1	N30 23 21.6 E78 04 32.0	872
2	*Bubo bubo* (Eurasian eagle owl)	4 and 14	2	N30 23 22.7 E78 04 34.1	861
3	*Gallus gallus* (Red jungle fowl)	3, 5, 8, 11 and 13	3	N30 23 23.2 E78 04 33.6	867
4	*Lophura leucomelanos* (Kalij pheasant)	10	Not housed inside the zoo	-	-
5	*Milvus migrans* (Black kite)	1,15 and16	Not housed inside the zoo	-	-
6	*Panthera pardus fusca* (Leopard)	7 and 12	4	N30 23 22.2 E78 04 33.1	866
7	*Strix leptogrammica* (Brown wood owl)	6 and 9	5	N30 23 22.7 E78 04 34.1	861
8	*Anopheles minimus*	18	-	-	-

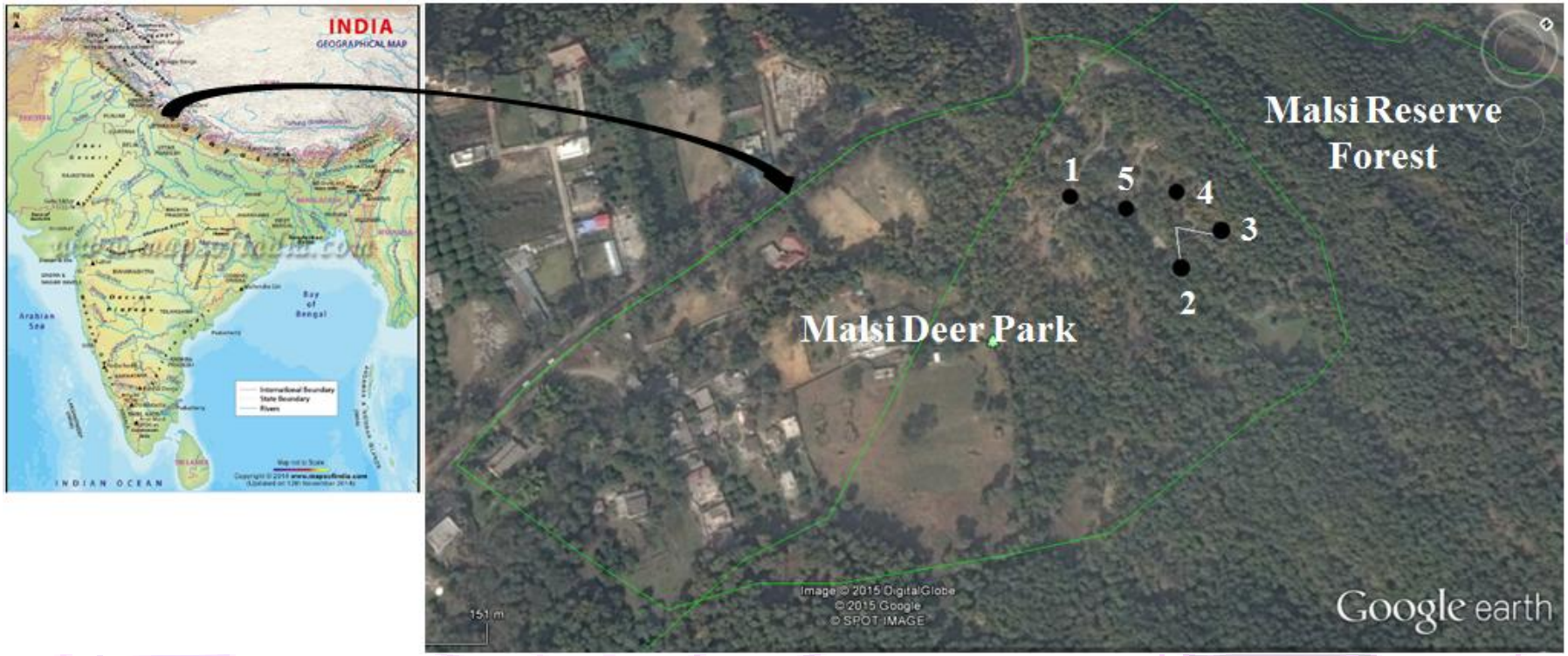

Figure 1. Map showing the mosquito collection sites near the cage locations (Black dots) of five species housed in Malsi Deer Park, Dehradun (Maps modified from http://www.mapsofindia.com and https://earth.google.com).