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Studies on morphology and molecular characterization of oriental cat flea infesting small ruminants by barcoding

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Abstract

The study was carried out to identify the flea species infesting small ruminants in Karnataka state, India. The morphological differentiation of species of genus *Ctenocephalides* was often confused due to loss of specific characters. Therefore, DNA barcoding of COX1 mitochondrial gene was carried and PCR product yielded a specific amplicon at 700 bp. The COX1 sequences of *Ctenocephalides orientis* from sheep were showing 99-100% homology to *C. orientis* from goats whereas, *C. felis felis* from dogs showed 99% homology to *C. felis felis* from cat. The accession number of KX467332 and KX467333 for *C. orientis* from Sheep and goats, KX467334 and KX467335 for *C. felis felis* from dog and cats respectively was allotted by NCBI. In the phylogenetic analysis, *C. orientis* formed a separate cluster to the subspecies of *C. felis* and clustered as sister species with *C. canis*. Thus, supporting *C. orientis* as separate species as previously suggested by morphological and molecular approaches.

Keywords: Small ruminants, *C. orientis*, COX1 gene, Barcoding

Introduction

Small ruminants are the major source of income to the farmers through milk, meat, wool and hide production which has positive impact on their socio-economic life. Presently, India has 65.07 million sheep (*Ovis aries*) and 135.17 million goat (*Capra hircus*) population out of which Karnataka contributes 9.58 and 5.27 million of sheep and goat population respectively. Fleas of the genus *Ctenocephalides* are the important group of ectoparasites infesting small ruminants in tropical and subtropical countries like India causing dermatitis, severe anaemia and deaths especially in young animals. Fleas are important since they act as a vector of zoonotic pathogens such as *Rickettsia felis* and *Bartonella species* [1]. However, small ruminants like sheep and goats do not have their own species of flea [2]. The cat flea, *Ctenocephalides felis* has a wide host range including small ruminants, distributed worldwide and formerly consisted of four sub species viz., *Ctenocephalides felis felis*, *Ctenocephalides felis orientis*, *Ctenocephalides felis strongylus* and *Ctenocephalides felis damarensis*[3]. But, morphological revision of the phallosome (male reproductive organ) proposed for the elevation of *C. orientis* and *C. damarensis* to full species level [4, 5]. However, the morphological differentiation of species of genus *Ctenocephalides* is often confused due to loss of specific characters like legs, head and minute morphological differences like head shape, chaetotaxy and manubrium of clasper. Therefore, molecular characterization is a boon for exact identification and classification of fleas of the genus *Ctenocephalides*. DNA barcoding using COX1 mitochondrial gene as a universal marker is the most reliable method as COX1 mitochondrial gene are in abundance (1000 identical copies per cell), is highly conserved functional domains and variable region [6]. Hence, a barcode of fleas infesting small ruminants, dogs and cats of Karnataka was undertaken.

Materials and Methods**Study area, sample and identification**

The study was carried out during January 2015 to June 2016 in private farms, household pens and nomadic flocks of sheep and goats in Karnataka state located between 11.30° and 18.30° north latitude and 74° and 78.30° east longitude in India. Fleas were collected from both sheep

and goats and also from dog and cats closely associated with these flocks. Initially, animals were restrained and examined in detail. Later fleas were collected from randomly selected infested animals in a flock using fine toothed metal comb and placed in a plastic vial containing absolute ethanol. The collected fleas were brought to the laboratory and identified based on the standard keys [7].

DNA extraction, PCR amplification and Sequencing

The genomic DNA of adult *Ctenocephalides* was extracted using Qiagen DNeasy Blood and Tissue Kit (Germany) as per the manufactures protocol. The COX1 mitochondrial gene of *Ctenocephalides* species viz., *C. orientis* and *C. felis felis* were targeted and subjected for PCR by using Phire animal direct Kit using the universal primers: forward 5'-GGTCAACAAATCATAAAGATA TTGG-3 and reverse 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' [8]. Reaction for the COX1F/COX1R primers were performed in a total volume of 50µl consisting 2X PCR buffer 25 µl, 0.5µl COX1F (20ppm), 0.5 µl COX1R(20ppm), DNA polymerase 1µl, flea DNA, NFW to make final volume 50µl under the following cycling conditions: an initial denaturation stage at 98° C for 5min, then 40 cycles of denaturation at 98° C for 5 sec, annealing at 45° C for 10 sec and 72° C for 30 sec followed by a final extension phase at 72° C for 1 min [9]. Once the PCR reaction were carried out, 1µl of DNA release buffer and gel loading dye were subjected for electrophoresis in 1.5% agarose gel and the images were captured using gel documentation system. The amplified products were sent for DNA sequencing to M/s Chromous Bengaluru, India. The sequences obtained were subjected to Basic Local alignment search tool (BLAST) analysis at NCBI-BLAST. The sequences were edited and submitted to National Centre for Biotechnology Information (NCBI) GeneBank. The BoldSystem V3 was used to generate barcode for the two species of *Ctenocephalides*. The DNA sequences of the both *C. orientis* and *C. felis felis* from the study and other DNA sequences of *Ctenocephalides* species uploaded from GenBank database were assembled and subjected to MEGA 5.0 software for Phylogenetic tree construction. The tree obtained by applying Neighbor-joining (NJ) method with 1000 bootstrap support. The evolutionary history was inferred using the Neighbor-joining method [10]. The optimal tree with the sum of branch length = 0.50258811 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [11]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [12] and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All positions containing gaps and missing data were eliminated. There were a total of 601 positions in the final dataset.

Results and Discussion

Morphological identification

In the present study, two species of fleas in the genus *Ctenocephalides* viz., *Ctenocephalides orientis* and *Ctenocephalides felis felis* were identified morphologically. The oriental cat flea, *C. orientis* being found on small

ruminants whereas cat flea, *C. felis felis* was found on dogs and cats associated with infested small ruminants farms. *C. orientis* (*C. felis orientis*) was previously reported on Goats from Karnataka [13] supporting that *C. orientis* is the dominant flea species found in India. Previously, four subspecies viz., *Ctenocephalides felis felis*, *Ctenocephalides felis orientis*, *Ctenocephalides felis strongylus* and *Ctenocephalides felis damarensis* were classified under the species *C. felis* [14, 15]. However, *C. orientis* and *C. damarensis* as a separate species based on the revision of the morphological characters of the male reproductive organ [4]. The presence of both genal and pronotal combs confirmed the identity of the genus *Ctenocephalides*. In *C. orientis* flea, the anterior most genal spine was shorter than subsequent spine. The genal ctenidium consisted of seven to eight pairs of spines and the pronotal ctenidium has got totally 15 to 16 spines. The length of head was twice as long as height and the frons were elongate and broadly rounded at anterior end (Fig. 1). Metepisternum or lateral metanotal area (LMA) had two bristles. Females had a row of one to 12 short spiniform bristles behind the antennal groove (Fig. 2). Dorsal margin of hind tibia had seven notches with stout and single bristles on third and sixth notches. Chaetotaxy formula of metatibial bristles was 2-2-1-2-2-1-3 (Fig. 3). In males, the manubrium of the clasper was widened at the apex (Fig. 4).



Fig 1: *C. orientis*: a. Elongate and broadly rounded head; b. LMA with two bristles and c. 1st comb is shorter than 2nd comb



Fig 2: *C. orientis* female: a row of small bristles behind antennal fossa

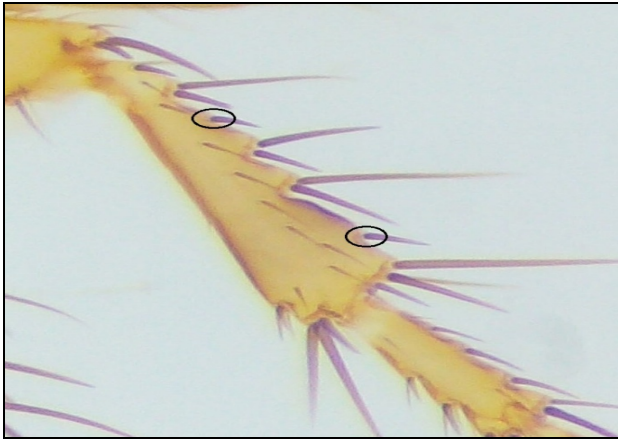


Fig 3: *C. orientis*:seven notches; 3rd and 6th notches with single and stout bristles

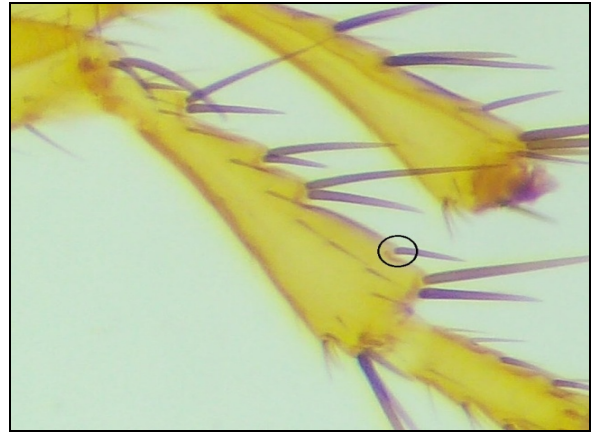


Fig 6: *C. f. felis*. Six notches; 5th notch with single and stout bristle



Fig 4: *C. orientis* male showing widened manubrium

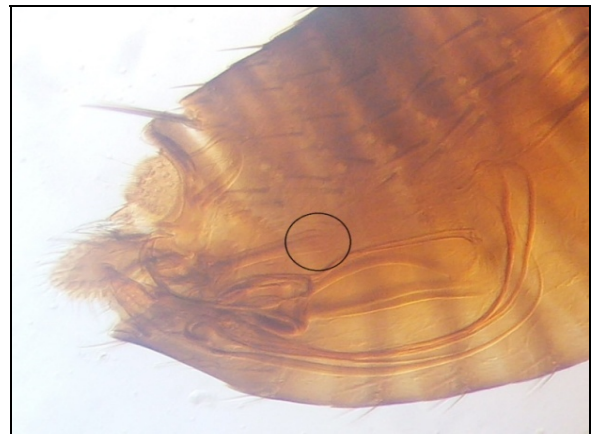


Fig 7: *C. f. felis* male showing straight manubrium

Whereas, *C. felis felis*'s anterior most genal spine was nearly as long as the subsequent spine. The genal ctenidium consisted of seven to eight pairs of spines and the pronotal ctenidium had 15 to 16 spines. The length of the head was twice as long as height and the frons were elongate and pointed at anterior end (Fig.5). Metepisternum or lateral metanotal area (LMA) had one or two bristles. Dorsal margin of hind tibia had six notches with stout and single bristles on fifth notch. Chaetotaxy formula of metatibial bristles was 2-2-2-2-1-3 (Fig.6). In males, the manubrium of the clasper was straight, narrow and slightly widened at the apex (Fig.7).

PCR amplification and sequencing

The PCR targeting the COX1 mitochondrial gene of *Ctenocephalides species viz., C. orientis* and *C. felis felis* using the universal COX1F/COX1R primers yielded a specific amplicon approximately at 700 bp (Fig. 8). Similar amplicon were amplified in *C. f. felis*, *C. f. strongylus* and *C. orientis* from Australia, Africa and South East Asia respectively [16]. The sequences of both forward and reverse direction of *C. orientis* from small ruminants and *C. felis felis* from dogs and cats were obtained in.ab1 file format and.txt format and were submitted to GeneBank, NCBI and the accession numbers was allotted, *C. orientis* from Sheep with KX467332 and goats KX467333. *C. felis felis* from dog with KX467334 and cats KX467335.



Fig 5: General morphology of *C. f. felis*: a'. Elongate and pointed head; b'.LMA with two bristles and c'. 1st comb is as long as 2nd comb

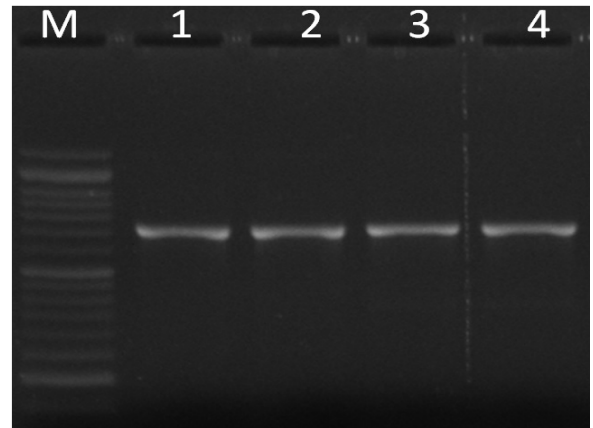


Fig 8: PCR amplification of COX1 mitochondrial gene

In the present study, the COX1 sequences of *C. orientis* from sheep were showing 99-100% homology to *C. orientis* from goats whereas, *C. felis felis* from dogs showed 99% homology to *C. felis felis* from cat as intraspecific variation in DNA sequences of COX1 mitochondrial gene is much lower than interspecific variation^[8].

Phylogenetic analysis

In the present phylogenetic tree obtained by collecting sequences of other fleas from GeneBank using MEGA5.0 software showed that COX1 sequences of *C. orientis* in small

ruminants from India was clustered with *C. canis* from Central Europe whereas, the COX1 sequences of *C. felis felis* in dogs and cats from India was clustered with *C. felis strongylus* from Africa supporting the classification of *C. orientis* as a separate species. The fleas collected from Thailand, morphologically identified as *C. felis orientis* (*C. orientis*) formed sister group to *C. felis* and from central Europe formed a sister clade to dog flea *C. canis* on analysing the COX1 and COX2 sequences of mitochondrial gene^[1,16].

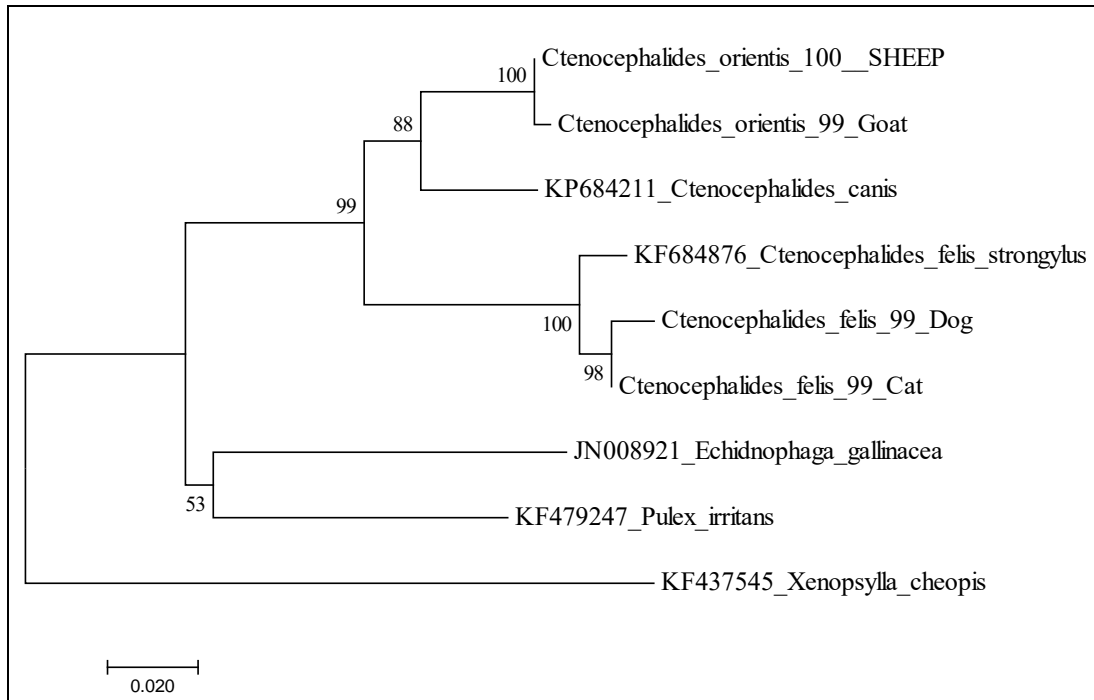


Fig 9: Evolutionary relationships of taxa

Conclusion

The oriental cat flea, *C. orientis* is the species infesting small ruminants of southern India. Morphologically identified *C. orientis* were conformed through molecular method as well. Molecular identification of flea species using barcoding approach is an aid in exact identification and upgrading of current classification of fleas species as DNA sequences contains highly conserved functional and variable region. The phylogenetic analysis of COX1 mitochondrial gene of Oriental rat flea, *C. orientis* and cat flea, *C. felis felis* confirmed the *C. orientis* as a separate species because it forms a separate cluster to the subspecies of *C. felis* and also clustered as sister species with *C. canis*. Thus, considering *C. orientis* as separate species as previously suggested by morphological and molecular approaches.

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