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First report on Molecular Characterization of Oestrus ovis in sheep from India

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Abstract

The objective of the present study is to characterize the dipteran larvae species infesting the sheep being maintained at SRRC, Mannavanur, by means of COI gene based PCR. During the last week of May 2021,post mortem examination of the skull of an Avikalin male sheep (20 months old) revealed the presence of larvae in its nasal sinuses. The larvae were washed in PBS (pH 7.2) and preserved in 70% alcohol. Total genomic DNA was isolated from the larvae using an initial step of grinding with liquid Nitrogen in a sterile mortar and pestle. Using the isolated genomic DNA from the larvae as a template, Cytochrome c oxidase subunit I (COI) gene based PCR was employed using the primers designed based on the COI gene of reference isolate of *Oestrus ovis* available in the GenBank. Full length COI gene (1534 bp) gene of *Oestrus ovis* in sheep from South India was targeted in the PCR experiment. The pTZ57R/T vector was used for the cloning of the PCR amplified fragment and the confirmed recombinant plasmid was subjected to sequencing experiments. In addition to morphological examination, based on COI gene based PCR, eventual sequencing experiments and BLAST analysis, it was confirmed that the larvae in the nasal sinuses of sheep from South India were *Oestrus ovis*. The South Indian isolate of *Oestrus ovis* is sharing 100% sequence identity both at nucleotide and amino acid levels with that of *O.ovis* from Spain. The North Indian isolate of *O.ovis* (from Jammu) exhibited 92 & and 99% identity at respective nucleotide and amino acid levels with South Indian isolate. With other members of the subfamily Oestrinae, the share of per cent nucleotide and amino acid identities of South Indian *O.ovis* ranged from 85–86% and 95–96%, respectively. *O.ovis* from South India was grouped with the other members of Oestrinae from different geographical areas of the globe in the analysis of phylogenetic tree based on COI amino acid sequences. Based on the research findings, it is concluded that *Oestrus ovis* is the dipteran species infesting the sheep at Mannavanur, Tamil Nadu, India. To our knowledge, this is the first report on full length nucleotide sequences of COI gene of *O.ovis* in sheep from Indian subcontinent.

Introduction

Infestation of live vertebrates (humans and/or animals) by the larvae of the flies belonging to the order Diptera is called as Myiasis (Zumpt 1965). Since time immemorial, the flies responsible for myiasis are considered as devastating creatures leading to huge losses in animal husbandry, which would be reflected as low milk yield, poor body weight, infertility and negligible hide quality (Zumpt 1965).

The order Diptera consists of true flies, which are discriminated from other insects by the possession of a single pair of functional wings with a reduced hind wing, termed halters (McAlpine 1989).

Within the order Diptera, there are two suborders: the Nematocera and the Brachycera. The flies within the suborder Nematocera seldom cause accidental myiasis. The flies causing specific myiasis are grouped under Muscomorpha or Cyclorrhapha, which is an infraorder within the suborder Brachycera .More precisely, the flies responsible for facultative myiasis are classified under Calyptratae, which is a subsection falling under the section Schizophora within the infraorder Muscomorpha or Cyclorrhapha (Francesconi and Lupi 2012).

The larvae of the flies grouped under the Genus *Oestus* (Order-Diptera; subsection – Calyptratae ; superfamily - Oestroidea ; Family – Oestridae ; subfamily – Oestrinae) are responsible for Oestrosis, which is nothing but a nasal myiasis, leading to a serious menace among sheep & goats and rarely in other animal species (Horak 2005).

Sheep nasal bot fly is nothing but the adult female fly, *Oestrus ovis*, having the swarming behavior around the heads of the animals and being the viviparous in nature, usually lay the already hatched larvae in the nostrils of sheep (Francesconi and Lupi 2012).

Owing to the obligatory nature, the newly deposited first stage larvae tend to wander actively in the nasal cavity and fix to the mucous membrane (Scala et al. 2002). Eventually, due to the process of two moultings, third stage larvae would be formed. There would be irritation and mechanical damage to the nasal sinuses of the host due to the process of wandering and growth of the larvae. The health of the affected animals would get impaired due to the clinical problems such as dyspnoea, profuse nasal discharge and restlessness, which are the consequences of the damage to host nasal sinuses by the unique larval behavior (Dorchies et al. 1998). Further, the secretory and excretory proteins of the larvae would induce local as well as systemic immune responses leading to the aggravation of the situation (Angulo-Valadez et al.2011).

Often, most of the mild cases are asymptomatic, which could result in the symptoms of generalized infestation such as, emaciation; so that reduction in animal production and economic loss would eventually ensue (Papadopoulos et al. 2006). Upon the sneezing activity of the host, the third-stage larvae would be expelled onto the ground leading to the completion of the life cycle but the retained third stage larvae in the nasal sinuses would result in the death of the host animal species, which is a consequence of the formation of septic sinusitis (Ahaduzzaman et al. 2015).

In endemic areas, the larvae of *O.ovis* are of public health importance, which is characterized by the cases of ocular affections in humans (Rao et al. 2018) and the cases are prevalent among shepherds and farmers (Dunbar et al. 2008).

There are many research works pertaining to the etiology, taxonomy, biology, immunology, treatment and control aspects of *Oestrus ovis* (Karademir et al. 2020). Traditional Identification of *Oestrus* species by the usage of morphological features of larvae and adults is cumbersome and upto the level of species of these parasites, molecular methods are warranted. Indeed, there are a very few data available about the molecular phylogenetic studies on *O. ovis* populations (Moreno et al. 2015).

Molecular markers based on mitochondrial DNA such as the gene encoding for the cytochrome c oxidase subunitI (COI) and the nuclear 28S (rDNA) as well, are widely used in population genetics and phylogenetic analyses for the members of dipteran families (Otranto et al. 2005; Nelson et al. 2012) .

The PCR (polymerase chain reaction) studies employing both mitochondrial COI gene and nuclear 28S rDNA gene sequences have been used to characterize and analyse the phylogenetics of bot flies, *Oestrus*

spp., (Diptera, Oestridae), from domestic and wild Bovidae hosts (Moreno et al.2015).

The standard COI gene based PCR was used for the Molecular Characterization and Phylogenetic Analyses of *Oestrus ovis* larvae causing Human Naso-pharyngeal Myiasis (Karademir et al. 2020).

Otranto et al. (2003) targeted the highly variable part of the mitochondrial cytochrome oxidase subunit I (COI) gene (the carboxyl terminal of the gene ranging from external loop 4 to its end) in PCR experiments for the molecular characterization of Oestridae species of dipteran flies responsible for obligate myiasis.

Metwally et al. (2021) employed COI gene based PCR for the molecular characterization of *Oestrus ovis* L. (Diptera, Oestridae) in Sheep and Goats from Riyadh, Saudi Arabia.

In India, *O. ovis* is prevalent in sheep (Allaie et al. 2016 ; Prabhu et al. 2019) and goats (Madhu et al.2019; Lakshmanan et al.2019). Godara et al. (2010) recorded an aberrant migration of *O.ovis* larvae into the mandibles of a non- descript goat in India. The larvae of *O.ovis* would also cause parasitic encephalitis in Sirohi Goat (Shivasharanappa et al.2011).

Further, from Indian subcontinent, there is no scientific documentation about molecular and phylogenetic characterization of *O. ovis* lineages other than a single GenBank record of partial mt-CO1 sequence of an isolate from Jammu (GenBank accession: ON912055.1). The area of the present study, i.e., Mannavanur comes under Southern part of India where as the place Jammu comes under Northern part of India.

Due to the economically important parasitosis in small ruminant husbandry and documentation of numerous cases of human ocular infections across the nation (Sreejith et al.2010; Dutta Majumder et al.2019), it is imperative to develop a baseline information about the molecular phylogenetics of *O.ovis* populations in India, from the epidemiological point of view.

Southern Regional Research Centre (SRRC), one of the regional centre's ICAR- Central Sheep and Wool Research Institute (ICAR-CSWRI), is located at Mannavanur, a village within Palani Hills and is about 32 kms away from Kodaikanal town, Dindigul district, Tamil Nadu. SRRC is well known for the maintenance of synthetic woolen breeds of Sheep (Bharat Merino and Avikalin) as well as meat breeds of rabbits (White Giant and Soviet Chinchilla) (Nagarajan et al.2019).

Semi intensive system of management is being followed for sheep. On everyday, during morning hours, graziers use to take the sheep into the pasture area nearing the reserve forest area and bring back to SRRC during the evening hours (Nagarajan 2020). Upon post-mortem examination of the sheep at SRRC, Mannavanur having the history of natural death, the dipteran larvae were noticed in the nasal sinuses of some of the animals. So far, the baseline information about the taxonomy, species and molecular data of the dipteran larvae infesting the nasal sinuses of the sheep at SRRC, Mannavanur, has not been documented.

In order to address the above said issues, the present researchable problem was therefore formulated with the following objectives.

1. Characterization of the species of *Oestrus* infesting sheep at SRRC, Mannavanur by employing mitochondrial cytochrome c oxidase subunit I (COI) gene based Polymerase Chain Reaction (PCR).
2. Cloning of PCR amplified products into a suitable vector for the purpose of sequencing experiments and
3. Bioinformatics analysis of the COI nucleotide sequences of dipteran larvae from Sheep at SRRC, Mannavanur, obtained out of sequencing experiments, with that of different dipteran flies from various geographical areas available in the NCBI database.

Materials and methods

Area of the study

Southern Regional Research Centre is commonly known as SRRC and is one of the regional centres of ICAR-Central Sheep and Wool Research Institute, located at Mannavanur, Kodaikanal, Tamil Nadu, Mannavanur is a farming village containing about 1500 families coming under Dindigul district, Tamil Nadu, India is one of the most important tourist centres of Kodaikanal. SRRC is located about 35 kms away in the west direction from Kodaikanal at an altitude of 2030 metres. The Coordinates for this farming village are 10°12'45"N and 77°20'38"E.

Collection of the dipteran larvae from the sheep

Southern Regional Research Centre (SRRC) is well known for the maintenance of exotic woollen sheep breeds namely, Bharat Merino and Avikalin. During the last week of May 2021, an Avikalin male sheep (20 months old) died suddenly. Upon Post mortem examination, the nasal sinuses of the dead sheep were having dipteran larvae (Fig. 1). The larvae were washed in in phosphate buffered saline (pH 7.2) and preserved in 70% alcohol or the purpose of genomic DNA isolation.

Identification of the species of the dipteran larvae by Polymerase Chain Reaction

The larvae preserved in 70% alcohol were subjected to total genomic DNA isolation. The larvae taken out of 70% alcohol were ground to make a fine powder using liquid Nitrogen in a sterile mortar and pestle. The larvae in powder form was then used for the isolation of total genomic DNA isolation using Invitrogen™ PureLink™ Genomic DNA Mini Kit as per the manufacturer's specifications provided for Mammalian Tissue and Mouse/Rat Tail Lysate. The purity and concentration of the genomic DNA were approximately checked by running 0.8% agarose gel electrophoresis.

Reaction volumes for the PCR of 50 µl were used and contained 5 µl of 10X buffer with 15 mM $MgCl_2$, 10 mM of each dNTPs, 100 pmol of each oligonucleotide primer, 100 ng of larval DNA sample and 3U Taq DNA polymerase. The primers used for the amplification of cytochrome c oxidase subunit I (COI) gene of *Oestrus sp;* The forward primer, OOCOX-F (24 mer): 5' TCGCGACAATGGCTT TTTTCTACA 3' and reverse primer, OOCOX-R (25 mer): 5' AGTTATTTAGTAAGG GTAATTCTGA 3'. The primer sequences were

designed based on cytochrome oxidase subunit 1 (COI) gene of *Oestrus ovis* reported in NCBI Accession No. NC_059851.1.

As per the conventional PCR, a first step of initial denaturation of the template at 94°C for 5 min in a thermal cycler (BioRad, USA) was included for the reaction mixture. Cycling conditions for PCR were 35 cycles of 60 s at 94°C, 60 s at 55°C and 60 s at 72°C, followed by a final extension for 10 min at 72°C. The total genomic DNA isolated from the blood of sheep was included as a negative control in the PCR.

Cloning and sequencing of COI gene of Oestrus sp.

Resultant PCR products were separated on 1.2% agarose gels containing ethidium bromide (10 mg/ml), and visualised under UV(ultra violet) light. The PCR products were purified using QIAquick Gel Extraction Kit and cloned into pTZ57R/T vector (Fermentas) using the protocol according to manufacturer’s instructions. The plasmids were transformed into *Escherichia coli* DH5α. Colonies harbouring the recombinant plasmid were inoculated into LB (Luria Bertani) broth containing Ampicillin (50 µg /ml) and incubated at 37°C overnight with horizontal shaking. The plasmid DNA was extracted from culture using QIAprep Spin Miniprep Kit. The recombinant plasmids were confirmed by PCR using the gene specific primers. The sequencing of three positive clones was carried out in both directions using Sanger sequencing method by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India and the firm was instructed to use both M13 forward and M13 Reverse primers for the sequencing experiments, since the said cloning vector consisted of the above said universal primer sequences in its backbone.

Sequence and phylogenetic analysis

Using BLAST (Biological Local Alignment Search Tool) software of NCBI (Altschul et al.1990), the nucleotide sequences provided by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India, were analysed. Upon BLAST search, the top most sequences displaying 100% alignment with the nucleotide sequences of the present study were COI gene of *Ostrus ovis* from Spain (GenBank Accession No. NC_059851.1).

The determined nucleotide sequences of COI gene of *Oestrus ovis* from Sheep at SRRC, Mannavanur, India were then submitted to GenBank and the accession No. ON000070 was obtained. Further, the resultant nucleotide sequences of COI gene of *Oestrus ovis* from SRRC, Mannavanur, India were then assembled and drawn the alignment with that of 29 dipteran species published earlier in the GenBank (Table 1) using Clustal W algorithm in Clustal X2.1 software tool (Larkin et al.2007). Pairwise nucleotide sequence identities among the 30 dipteran species were also computed using Clustal X2.1(Larkin et al.2007).

Functional motifs in the protein encoded by COI gene of *Oestrus ovis* were predicted by using the software Scanprosite, which uses standalone prosite patterns (De Castro et al. 2006; Sigrist et al. 2013).

The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones et al. 1992). Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

Results and Discussion

The aim of the present study is to characterize the dipteran larvae infesting sheep at SRRC, Mannavanur by means of molecular phylogenetic analysis.

The area of the present study falls under subtemperate zone, which is located about 2030 metres above mean sea level and is receiving an annual rainfall of 1055 mm throughout the year. The grazing area of sheep is invaded by grass species including Kikuyu (*Pennisetum clandestinum*) and speargrass (*Heteropogon contortus*) (Swain et al.2004).

At SRRC, Mannavanur, the cases of sheep infested with *Oestrus ovis* larvae are mild and asymptomatic and the larvae in the nasal sinuses of sheep are noticed only upon post-mortem inspection. So far, the typical systemic cases of Oesrosis have not been observed among sheep at SRRC, Mannavanur. Numerous factors including the host susceptibility, chrobinoology of *O. ovis* in a particular geographical region and system of animal management would be influencing the responses of the host to oestrosis and larval burden (Sotiraki and Hall 2012). Very recently, Bello et al. (2022) concluded that there is no correlation between the larvae infestation intensity & the clinical signs of Oestrosis and ELISA is better when compared to PCR technique for the diagnosis of *O.ovis* in live animals.

In addition, the oral administration of commercial preparation of Ivermectin (HITEK^R : Each ml contains 0.800 mg of Ivermectin : Dose – 25 ml/100 Kg body weight) for the treatment of gastrointestinal parasitism among sheep at SRRC, Mannavanur, is a periodical practice and this could be the reason for the absence of typical clinical cases of Oestrosis among sheep in the present study. Bello et al. 2022) reported that in sheep, oral drenching of with Closantel at a dose rate 10 mg/kg and subcutaneous injection with ivermectin at a dose rate of 0.2 mg/kg could be effective in the control of oestrosis.

As per the published report (Soulsby 1987), the dipteran larvae in the nasal sinuses of sheep at SRRC, Mannavanur was identified as the maggot of *O.ovis* based on the possession of 10 segments with a dark transverse band on the dorsal surface of each segment and triangular posterior spiracle with radiating slits (Fig. 1). Additionally, the maggots were also onfirmed as the third stage larvae of *O.ovis* as there were spines on the ventral aspect (Allaie et al.2016). As per the findings of Metwally et al. (2021), it was found that third instar larvae (L3) of *O.ovis* in Sheep and Goats from Riyadh, Saudi Arabia would be yellow in colour while immature stage and light brown in colour while mature stage. The larvae were consisting of broad transverse blackish bands on the dorsal side. There was a presence of variable number of small denticles on the dorsal side of the second segment .The following segments were plain with a rough, leather like skin pattern, which was clearly observed only on the darkened parts. The ventral side of the larvae was characterized by the presence of series of strong spines on the segments and the spines were haphazardly located on the segment but the spines were following a regular pattern in the

succeeding segments. The preanal bulge was naked where as the postanal bulge were having fewer spines. Further, the posterior peritremes were circular in shape having a central button and devoid of a clear-cut suture. There was a channel in the posterior peritremes of *O.ovis* larvae. It is therefore recommended that it is essential to carry out a detailed morphological examination of the larvae of *Oestrus ovis* in sheep from SRRC, Mannavanur in the future.

Further, there are three stages in the development of Oestridae larvae. The most frequently observed larvae by veterinary inspectors at slaughterhouses are L3 larvae. It is very easy to separate the internal tissues of L3 from the chitin rich external cuticle and therefore, there would be a huge quantity of larval tissues available for genomic DNA isolation. Additionally, when compared to first (L1) and second (L2) stage larvae, fully developed L3 would be having low amount of enzymes which are having the inhibitory activities in the downstream processing of the genomic DNA (Otranto et al.2003). In the present study also, the yield and quantity of the genomic DNA extracted from the sheep maggots were found to be good and eventually, the amplicons obtained out of PCR experiments were also having a single intense band (around 1600 bp size) while visualizing the agarose gel under UV light after running electrophoresis. It is therefore concluded that in the present study, the stage of dipteran larvae utilized for genomic DNA extraction and PCR experiments could undoubtedly be the third stage larvae of *O.ovis*.

Insect mitochondrial cytochrome oxidase I (COI) genes are known for their huge size, carriers of both conserved and variable regions and the hot spots for mutations. Owing to the said genetic features, COI genes would be beneficial in the accurate calculation of the rate of evolution among the insect species (Lunt et al.1996). Therefore, COI gene based PCR was used for the molecular data analysis of the dipteran larvae collected from the nasal sinuses of sheep in the present study.

Since there is no published report about the molecular characterization of *O.ovis* from Indian sheep, the present study is the first report of its own kind.

As per the GenBank Accession No. NC_059851.1 representing the complete mitochondrial genome of *O.ovis*, in the present study, the size of the PCR amplified fragment specifying cytochrome c oxidase subunit I (COI) gene of *O.ovis* from Sheep at SRRC, Mannavanur, Tamil Nadu, India is 1534 bp and it is nothing but the full length nucleotide sequence of COI gene for *O.ovis* (covering the full length coding sequence of COI gene of *O.ovis* – from 1499 bp to 3032 of GenBank Accession No. NC_059851.1).

As reported earlier [16], in the present study, the COI gene of *O.ovis* was also having high AT content (69%) (A: 30% 475; T: 39% 568; G: 13% 212; C: 18% 279). In a recent study on molecular analysis of *O.*ovis larvae in Small ruminants from Saudi Arabia, it was found that the overall GC content of 23 mtCOI gene sequences was 35.6% (i.e., 64.4% AT content) (Metwally et al.2021).

While looking for the type II restriction enzyme sites within the nucleotide sequences of both COI gene of *O.ovis* from Mannavanur (South Indian isolate ; GenBank Accession No. ON000070.1) & Jammu (North Indian isolate; GenBank Accession No. ON912055.1) using the GenScript Restriction Enzyme Map Analysis Tools (free software available in the public domain), it was found that the common hexabase

cutter (Sac I – GAGCTC) was having only one cutting site at the position 857/862 in the former sequence, where as the latter sequence was having two cutting sites for Sac I at positions 257/262 & 857/862. Similarly, the COI gene sequences of Mannavanur isolate was having one cutting site for the restriction enzymes, EcoRV (GATATC) and Xho I (CTCGAG) at positions 1181/1184 and 896/897, respectively but no recognition site for the said two six base cutters was present in COI gene sequences of Jammu isolate from India. This interesting feature of restriction enzyme analysis could be explored for the differentiation of large number of specimens of *O.ovis* isolates from different geographical areas of India.

The COI gene sequences of *Oestrus ovis* from Sheep at SRRC, Mannavanur, India, obtained out of sequencing experiments by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India, were compared to that of 29 different dipteran species available in the NCBI database. In the present study, the alignment of last 90 amino acids at carboxyl terminal ranging from the amino acid position 421 to amino acid position 510 of COI protein of *Oestrus ovis* from Mannavanur with that of other dipteran species is shown in Fig. 2. The above said amino acid alignment region is falling within the hypervariable part of the COI protein corresponding to the region from external loop 4 (E4) to the carboxyl terminal (–COOH) of myiasis-causing Oestridae (Otranto et al.2003).

Similar to the findings discussed in earlier report (Otranto et al.2003), in the present study also, the amino acid alignment revealed that the presence of five most conserved residues in COI protein within the subfamily of Oestrinae (i.e. K-405, L-450, I-464, F-493 and N-510) was noticed. But in case of *Rhinoestrus usbekistanicus* (GenBank Accession No. NC_045882.1), the amino acid Lysine (K) was replaced by the amino acid Serine (S) at position 405 (Supplementary Fig. 1).

The sequence analysis of the current study revealed that the recently published *Ostrus ovis* sequence from Jammu has 99% sequence identity with our sequence. At position 118, amino acid Thr appears in Oestrinae subfamily compared to Lys in *Calliphora, Haematobia* and *Stomoxys* family, whereas it was replaced by Asn in other members included in this study. Similarly, at position 253, Ser was replaced by Pro in *Oestrus ovis* sequence published from Jammu compared to other sequences that were included in this study, which were conserved with Ser. At position 268, Ser was found to be conserved in all reported *Oestrus* species, however, it was substituted with Ala in a few species like *Rhinoestrus, Cephenemyia, Muscina, Gasterophilus* and *Culicoides*, whereas it was replaced with Thr in *Phlebotomus*. Similarly, at position 332, amino acid Tyr/Ser was replaced by Leu in *Culicoides* species and *Ostrus ovis* sequence of Jammu, whereas it was replaced by Phe in *Rhinoestrus* and *Cephenemyia* species. At positions 335 and 339, either Ala/Thr was replaced by Ser or vice versa. At position 483, the residue Pro was replaced by Thr in all the *Ostrus ovis* sequences, whereas it was replaced by Ser in case of *Rhinoestrus* sps. At position 499, Ala was replaced by Ser in sequence of Oestrinae subfamily members, *Glossina, Hhypoderma* and *Gasterophilus species*, whereas in case of *Muscina* by Thr (Supplementary Fig. 1).

As per the published report (Schwartz et al.2019), the amino acid Ser substituted with either Pro or Ala occur only in the conserved position, whereas Ser substituted with either Asn or Gly occur at less conserved position. According to the above said report, the occurrence of Ser with Ala substitution (in

most cases) at position 44, 253, 268, 335, 339 and 499 could highly be conserved, where as substitution of Ser with Asn or vice versa at position 487 might be less conserved. However, substitution of Pro to Thr/Ser or vice versa at position 483 could be important for functional phosphorylation (Supplementary Fig. 1).

While searching for the functional motifs within the protein encoded by COI gene of *O.ovis* using the Scanprosite software, the presence of cytochrome oxidase subunit I signature and profile was observed in the region ranging from 1–510 (Fig. 3). Apart from the family signature, the protein sequence also possesses Heme-copper oxidase catalytic subunit, copper B binding region signature (WFFGHPEVYILILPGFGMISHIISQESGKKETFGSLGMIYAMLAIGLLGFIVWAHH) in the region ranging from 234–289 (Fig. 4) .

As far as the sequence analysis of 30 COI gene sequences pertaining to different dipteran spcies in the present study is concerned, it was observed that COI gene of *O.ovis* from sheep at Mannavanur, Tamil Nadu, India (South India), exhibited 100% sequence identity both at nucleotide and amino acid levels with that of *O.ovis* from Spain (GenBank Accession No. NC_059851.1). *O.ovis* from Jammu, India (North India) (Gen Bank Accession No. ON912055.1) shared 92 and 99 percent identity with *O.ovis* from Mannavanur at nucleotide and amino acid level, respectively. With other members of Oestrinae, the range of nucleotide and amino acid identity of *O.ovis* from South India was 85–86% and 95–96%, respectively. *Hypoderma lineatum* (GenBank Accession No. NC_013932.1) was having 81% identity at nucleotide level and 90% at amino acid level with *O. ovis* from South Indian. On the other hand, with *Gasterophilus intestinalis* (GenBank Accession No. NC_029834.1), the South Indian isolate of *O.ovis* was sharing 80% and 90% at nucleotide and amino acid levels, respectively. The COI gene of the members belonging to the subfamilies Caalliphorinae, Chrysomyinae and Lucilinae members were displaying 85 & 92% identity at nucleotide and amino acid levels with that of South Indian isolate of *O.ovis* (Table 1).

Phylogenetic relationship was constructed employing MEGA-X on the multiple sequence alignment of COI amino acid sequences of 30 dipteran flies. In the phylogenetic analysis, the nucleotide sequences having the size of more than 1000 bp of COI gene of the dipternan flies (including faculatative myiasis causing flies as well as biting flies) were only retrieved from the NCBI database and some of the nucleotide sequences were full length COI gene. There were only three number of nucleotide sequences (more than 1000 bp) representing the COI gene of *O.ovis* in the GenBank database.

Other than Cuterebrinae, the COI gene sequences (more than 1000 bp) belonging to the members of the other three subfamilies Oesstrinae, Gasterophilinae and Hypodermatinae were included in the molecular analyses of the present study. Since the nucleotide sequences representing the COI gene of members of the subfamily Cuterebrinae available in the NCBI database were less than 1000 bp, no COI gene sequences of the said subfamily within Oestridae family was included in the molecular phylogenetics of the present study.

Phylogenetic analysis based on amino acid sequences ruled out the close relationship between *O.ovis* from South India and other oestrid flies within the subfamily Oestrinae from different geographical area

across the world (Fig. 5).

Based on the phylogenetic tree, it was found that COI gene could clearly differentiate the species in all three subfamilies (Oestrinae, Hypodermatinaedae and Gasterophilinae of estridae (except Cuterebrinae) (Fig. 5). Further, the branches of the subfamilies Gasterophilinae and Hypordematinae were forming the clusters and divided from the subfamily Oestrinae (Fig. 5). This finding is also consistent with the earlier reports on Oestrinae species from Turkey (Karademir et al.2020), Spain (Moreno et al.2015) and Italy (Otranto et al.2003), where in, partial COI nucleotide sequences were used in the phylogenetic analysis and there was a clear cut cluster formed by the members of the subfamily Oestrinae & deviating from the members belonging to the other three subfamilies within the family Oestridae. Interestingly, the members of the subfamilies, Calliphorinae, Chrysomyinae and Lucilinae within the family Calliphoridae were grouped and clustered separately in the present phylogentic tree analysis (Fig. 5) and is agreeing with the recent report (Nasser et al. 2021).

In the present study, the positions of *Oestrus ovis* and *Rhinoestrus usbekistanicus* in the phylogenetic tree (Fig. 5) are supporting the findings of the earlier report (Aleix-Mata et al. 2021), where in the said two Oestrinae species could be sister groups as per the phylogenetic tree analysis involving the whole mitochondrial genome of the 13 Oestridae species.

Even though *O. ovis* is the usual parasite of small ruminants across the continents, it is also known to infest other ruminants including Old World camels (Fekry et al.1997), New World camels (Gomez-Puerta et al.2013), the Siberian ibex (*Capra ibex sibirica*), argali (*Ovis ammon*), bighorn sheep (*O. canadensis*), Barbary sheep (*Ammotragus lervia*) and the European mouflon (*O. orientalis musimon*) as accidental hosts (Moreno et al.1999). The grazing area of sheep SRRC, Mannavanur, is co-grazed by cattle, buffaloes and horses, which are belonging to the villagers of the local area. In addition to the domestic animal species, the area of the present study is also a habitat for wild animals such as Indian Guar, Deer, wild pig and red dogs (Nagarajan 2020). Owing to the co-sharing of both domestic and wild animals, the area of the present study is speculated to have the richness of dipteran flies (belonging to the superfamilies, Muscoidea and Oestroidea), which are responsible for facultative myiasis in the total fauna population.

Conclusions

Based on the research findings obtained out of the present study, it is concluded that

1. The dipteran larvae obtained from the nasal cavity of Sheep at SRRC, Mannavanur, Palani Hills, Tamil Nadu, India, is the larval stages of *Oestrus ovis*

2. *Oestrus ovis* is a sister to *Rhinoestrus usbekistanicus.*

Future Prospective

From the future prospective point of view, the following issues pertaining to *Oestrus ovis* need to be extensively addressed in order to develop a complete data about the dipteran flies causing facultative myiasis in the area of the present study.

1. Seasonal prevalence

2. Analysis of large number of specimens

3. The accidental hosts including both domestic and wild ungulates

4. Comparison with the other oestrid flies infesting both domestic & wild bovine species, equines and cervines

5. Human ocular infections, if any and

6. Development of appropriate control strategies.

Declarations

Ethical Approval

All animal experiments were carried out as per the approval from the Institute Animal Ethical Committee of SRRC, Mannavanur, Tamil Nadu, India.

Competing Interests

The authors declare that they have no competing interests.

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Tables

Table 1 is available in the Supplementary Files section.

Figures

Fig.1

Figure 1

The larvae of *Oestrus ovis* present in the nasal sinuses of the sheep

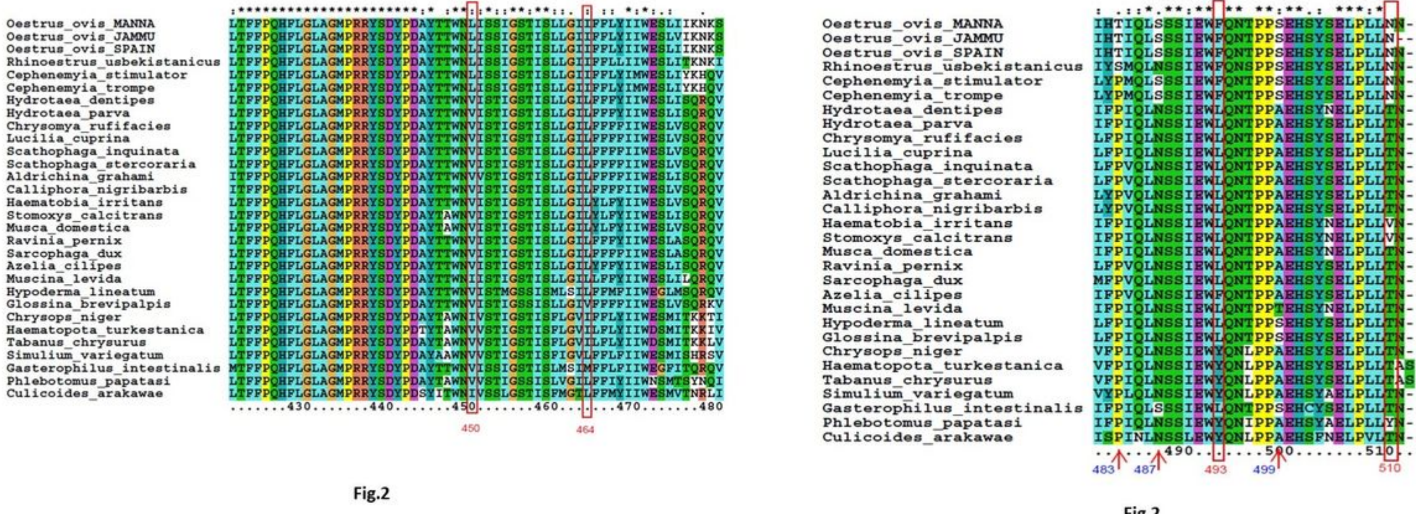
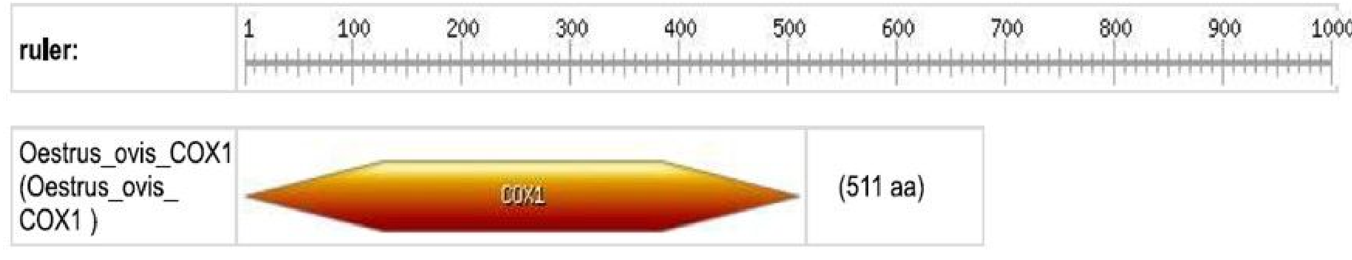


Figure 2

Alignment of last 90 amino acid sequences (from 421 to 510 at Carboxyl terminal) of COI gene of *Oestrus ovis* from Mannavanur with that of different dipteran flies from various geographical areas of the world .The upward arrows indicate the conserved amino acid positions.

Upper case represents match positions, lower case insert positions, and the '-' symbol represents deletions relative to the matching profile.



PS50855 **COX1** *Cytochrome oxidase subunit I profile* :

1 - 510:	**score =** 115.048

Fig.3

Figure 3

Position Cytochrome oxidase subunit I signature in COI protein of *Oestrus ovis*

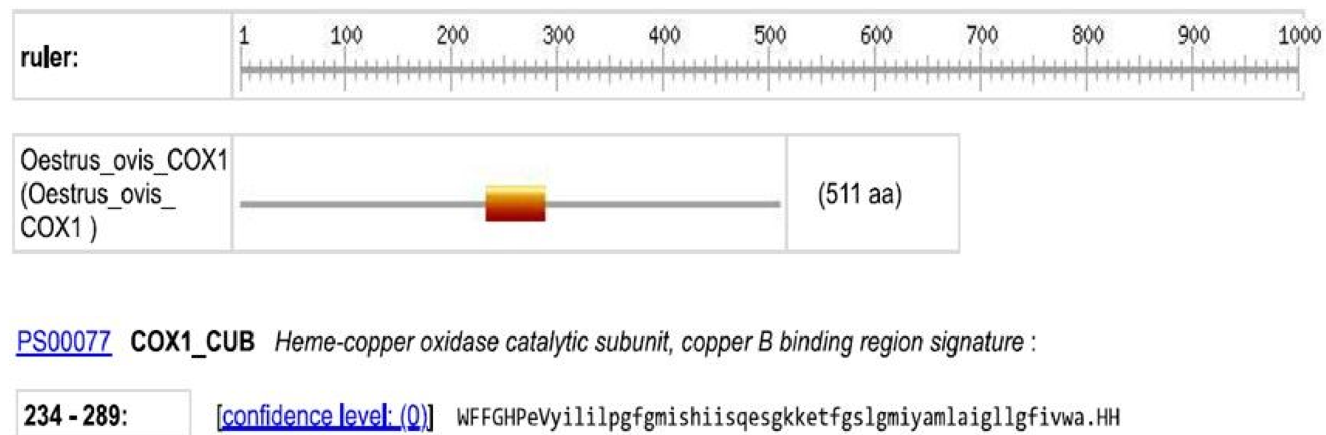


Fig.4

Figure 4

Position of Heme-copper oxidase catalytic subunit, copper B binding region signature COI protein of *Oestrus ovis*

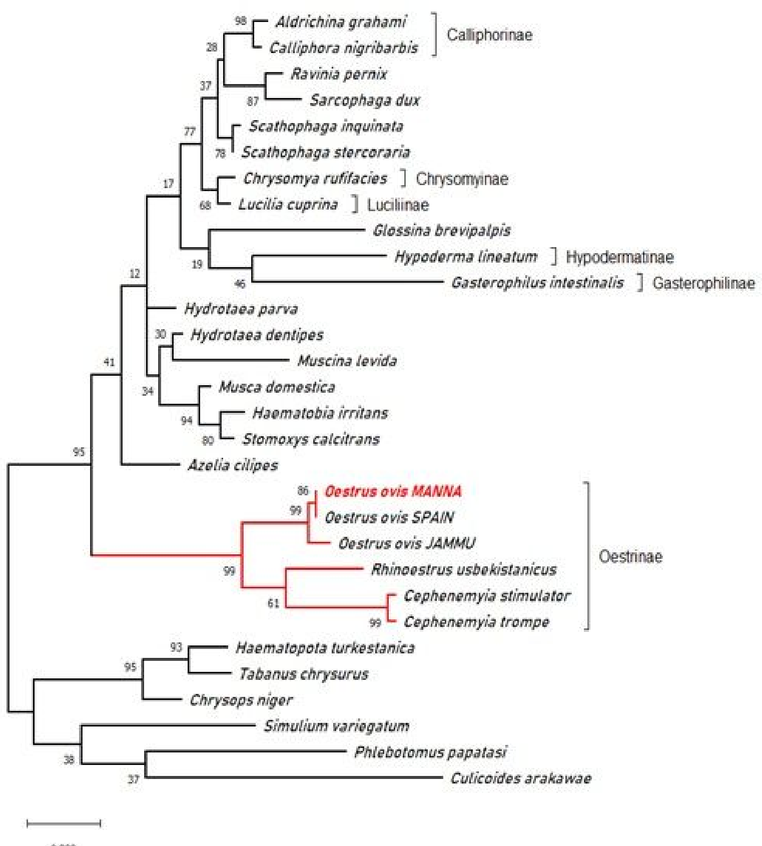


Fig.5

Figure 5

Phylogenetic tree based on amino acid equences of COI I gene of *Oestrus ovis* from Mannavanur with that of different different dipteran flies cestodes from from various geographical areas of the world . The evolutionary history was inferred by using the Maximum Likelihood method and and JTT matrix-based model [32]. The tree with the highest log likelihood (-4027.05) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 30 amino acid sequences. There were a total of 512 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [33]

Supplementary Files

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