PREVALENCE AND MOLECULAR CHARACTERIZATION OF MAJOR *Culicoides* SPECIES FOUND IN SELECTED PARTS OF TAMIL NADU

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ABSTRACT

Culicoides are the biting midges that are involved in the transmission of bluetongue, an economically important disease of small ruminants particularly sheep. The prevalence of Culicoides species in and around sheep farms of selected districts of Tamil Nadu was investigated by setting up UV- LED CDC traps. A total of 1787 specimens of Culicoides were collected, out of which Culicoides imicola was found to be in abundance. The other species included Culicoides oxystoma, Culicoides peregrinus, Culicoides brevitarsis and Culicoides fulvus. It was found that female midges outnumbered the male population in all the identified species. The overall percentage distribution of pigmented, non-pigmented, blood fed and gravid forms were observed to be 25.73%, 25.49%, 38.95% and 9.81% respectively. Morphological identification was complemented by molecular characterization using barcodes obtained by amplification of the mitochondrial eytochrome oxidase gene. The results of BLAST analysis revealed that all the other four species grouped with their respective species clusters except C. oxystoma which showed a higher degree of genetic divergence.

Key words: Culicoides species, Prevalence, DNA barcoding, COI gene, Sheep farm.

INTRODUCTION

Bluetongue is an infectious, non-contagious disease of ruminants especially sheep. The disease is transmitted between its vertebrate hosts, most commonly by the biting midges of the genus *Culicoides* which comes under the family *Ceratopogonidae*

These vectors are found on all large land masses ranging from the tropics to the tundra, except the regions of Antarctica and New Zealand. More than 1400 species of *Culicoides* of veterinary importance had been identified globally, out of which 63 species are identified from different geographical regions of India (Archana *et al*., 2014). These midges are of great

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concern because they not only act as vector of bluetongue, but also of several viral, protozoan and filarial diseases of animals and birds.

Based on vector competence studies, putative bluetongue virus vector species known to occur in India are *C. oxystoma*, *C. peregrinus*, *C. imicola*, *C. actoni*, *C. brevitarsis, C. dumdumi* and *C. fulvus* (Purse *et al.,* 2014). The vector borne transmission of bluetongue virus is influenced by ambient temperature, humidity and total seasonal rainfall (Mullens *et al.,* 1995; Mellor *et al.*, 2000). The availability of blood meal from a target host species and the propensity of a particular bluetongue serotype are known to influence the species of *Culicoides* involved in transmission of the disease. Thus, vector surveillance studies form an integral part of bluetongue disease transmission and prediction of outbreaks.

The ultra violet, Light Emitting Diode of Centers for Disease Control and prevention (UV-LED CDC) light suction trap is recommended for surveillance of *Culicoides* in southern India. Reference key to identify *Culicoides species* was proposed by several authors including Bellis *et al.* (2014) and were based on morphological patterns of wings, legs and antennae. Unidentified and cryptic species paved way for applicability of molecular techniques like DNA barcoding adopted by Meyer (2008) and further improvisations as attempted by Ander *et al*. (2013) and Harrup *et al.* (2016) clarified the disputes in grouping closely resembling species into their respective clusters.

The present study was undertaken to investigate the prevalence of different *Culicoides species* in and around sheep farms located in selected districts of Tamil Nadu and characterize the vector species involved using morphological and molecular techniques.

MATERIALS AND METHODS

Collection of *Culicoides* species

Culicoides midges were collected by using UV- LED CDC light suction traps set up at sheep farms. The sites chosen included sheep farms at Arasankulam village of Tirunelveli district, Kaanchriamkulam of Madurai district, Chennimalai of Erode district and Livestock Farm Complex, Tamil Nadu Veterinary and Animal Sciences University, Madhavaram Milk Colony of Chennai district. Logistics of setting up of light suction traps like distance of light trap from nearby dung yard and water sources, climatic conditions such as temperature of the farm, relative humidity of the farm and previous rainfall reported were recorded to determine the influence of these parameters on the prevalence of the vector.

Screening of the catch for morphological identification

Aliquots of collected catch were screened under dissection Stereo zoom microscope in watch glass. *Culicoides species* were segregated from the non *Culicoides species* based on the typical wing pattern. As per the reference key published by Bellis *et al.* (2014), specimens were identified at species level based on wing, head, thorax and abdomen pattern.

The gender and physiological status of the specimens were also determined based on the morphological gender-based characteristics proposed by IBVNET group in its website (IBVNET, 2020). Males were identified by the presence of heavily pigmented pedicel, plumose antenna, longer wings, narrow abdomen with prominent genitalia, while females were characterized by shorter pedicel, broader wings with dark markings and stout abdomen with a tapered apex bearing a pair of small rounded cerci visible below the ninth tergum. Female *Culicoides* were further divided into four categories based on abdominal pigmentation status as follows: pigmented, non-pigmented, blood fed and gravid.

Non-destructive method of DNA extraction

The total DNA was extracted from *Culicoides* midges using nondestructive method described by Harrup *et al.* (2016). Briefly, species-wise collected *Culicoides* (n=10) were subjected to digestion by overnight incubation with 200µl of DXT tissue digest reagent (Qiagen, USA - Catalogue No.950183) and 10µl 1% Proteinase K in a micro centrifuge tube. Nucleic acid in the lysate was precipitated with sodium acetate and ethanol followed by resuspension in 50µl of nuclease free water for further use.

Amplification of cytochrome oxidase (COI) gene by PCR

Amplification of the fragment of the mitochondrial COI gene barcoding region was achieved by polymerase chain reaction using the LCO - HCO primer set (Sense 5′-GGT CAA CAA ATC ATA AAG ATATTG G -3’ and Antisense 5′-TAA ACT TCA GGG TGA CCA AAAAAT CA-3′).The PCR reaction was performed in a total volume of 20 µl reaction mix consisting of 4µl nuclease free water, 10µl Top Taq Master Mix (Qiagen,USA - CatalogueNo.200403), 1µl of each primer (20µM) and 4 µl of template DNA (approximately 5-30 ng). The PCR cycling conditions were as follows: an initial denaturation step at 94^0 C for 3 minutes followed by 40 cycles of 94^0 C for 30 seconds, 46^0 C for 30 seconds, 72^0 C for 1 min, and a final extension step at 72^0C for 10 min. PCR products were electrophoresed on 1.5% agarose gels along with 100 bp DNA ladder and the amplicons were visualized under UV transilluminator.

Sequence analysis of COI gene of *Culicoides species*

The consensus sequences of both forward and reverse sequences of mitochondrial COI gene of the identified *Culicoides* species were generated using Bioedit v.7.2.8 software. The consensus sequences were subjected to BlastN similarity search in GenBank (Zhang *et al.*, 2000) database for *Culicoides* species confirmation. Other COI gene sequences of *Culicoides* species of similar regions were accessed from GenBank database for sequence comparison. The percent identity matrix and phylogenetic analysis was executed using Mega 6 software programme.

Statistical analysis

The statistical analysis was executed by the methods described by Snedecor and

Cochran (1984). The data generated with respect to percentage distribution of the *Culicoides* vector in the selected districts and gender pattern were subjected to Chi - square test using SPSS software and significant differences were indicated at 1% and 5% ($P< 0.01$ and $P< 0.05$).

RESULTS AND DISCUSSION

Choice of the site for setting up of light suction traps

In this study, light suction traps were set up at sheep farms of four districts namely Tirunelveli, Erode, Madurai and Chennai at the geographical co-ordinates mentioned in Table 1. The districts were chosen based on the severity of the disease recorded. Among them, Tirunelveli and Erode are endemic for bluetongue with severe outbreaks, Madurai with moderate disease occurrence and Chennai with rare occurrence, if at all in the mild form (Krishnamohan Reddy *et al*., 2016).

Choice of the light suction traps used

We preferred to use the UV - LED CDC type trap over the Onderstepoort Veterinary Institute (OVI) trap fitted with fluorescent UV bulb, since the count, diversity, sex and status of the *Culicoides* species caught by these traps are proportional to the intensity of the UV light emitted by the trap and the power of the fan. The portability and increased power efficiency of LED-based light suction traps also helped during the logistical challenge of limited electricity availability and frequent power failure during monsoon. Also, LED-based light suction traps provided an advantage of increase in the number of night-collections to four consecutive days with one battery charge and the option of photo switch, for switching off during the light hours of the day.

Table 1. Parameters recorded at the time of setting up of light traps with regard to site location

Parameters	Tirunelveli	Erode	Madurai	Chennai
Month of Trap setting	November	December	November	December-January
Previous rain reported	October	October	October	October
Farm location	7°43 21''N 47'29''E	8°23 18''N 34'19''E	6°19 19''N 27'04''E	7°32 13''N 29'18''E
Distance between farm and trap	8m	8m	8m	8m
Height of trap from ground level	4m	4m	4m	4m
Distance between trap and dung pit	15m	12m	10m	12m
Distance between traps and nearby water sources	25m	25m	20m	30m
Temperature of the farm	34.8°-35.9°C	34.6°-36.2°C	32.7°-34.7°C	33.8°-34.9°C
Relative Humidity of the farm	81%	84%	83%	85%
Total number of light hours of the day during trap setting	10h	10h	12h	12h
Time of trap setting	5:30 p.m.	5:30 p.m.	5:30 p.m.	5:30 p.m.
Time of trap removal	7:30 a.m.	7:30 a.m.	7:30 a.m.	7:30 a.m.
Number of animals housed	350 sheep	300 sheep	240 sheep	150 sheep
Construction material of farm roof	Asbestos sheet	Asbestos sheet	Asbestos sheet	Asbestos sheet

Table 2. Prevalence of *Culicoides* species in different districts based on the catch obtained using light suction traps

Districts	*C. imicola*	*C. oxystoma*	*C . peregrinus*	*C. brevitarsis*	*C. fulvus*	Unknown species	Total	Non Culicoides insects
Tirunelveli	322	102	38	14	3	2	481*	252*
Erode	481	101	19	7	2	2	612*	283*
Madurai	241	87	46	20	1	1	396*	213*
Chennai	210	45	23	15	4	1	298*	197*
Total	1254	335	127	55	10	6	1787*	921*

*Chi square value of 9.32 on comparison of total *Culicoides* with total non *Culicoides* at different districts
*Significant (P<0.05)

Influence of the parameters observed for setting up of light suction traps

The location and logistics of setting up of light suction traps at a farm premise are important criterion for vector studies. The parameters recorded at the time of setting up of light traps were listed in Table 1. It could be observed that traps were set up at a distance of 8 m from the animal holding and at 4 m height from the ground level. Along with the above distance parameters, 10-12 hours of day light was found to yield better catch of *Culicoides* in our earlier studies. Taking into consideration that high moisture levels are important for the development and survival of *Culicoides* as documented by Losson *et al.* (2007), traps were set up at 15m distance from the dung pit and 25m from the nearby water sources, that act as breeding places for *Culicoides*. Also, the recorded relative humidity of 81-85% at the farm sites favoured high moisture content of the ecosystem required by *Culicoides*.

Vector abundance and competence are markedly influenced by climatic conditions like temperature, season, rainfall, humidity and wind velocity (Mullens *et al.,* 1995). Rainfall plays an important role in the prevalence of *Culicoides species* (Udupa, 2001). In this study, traps were set following an outbreak during the post rainy days of October to January. The abundance of *Culicoides* (n = 1787) trapped was found to be higher when compared to that of non *Culicoides* species (n = 921), amounting to 65.99% of the catch.

The tropical temperature of India was shown to influence the total catch, diversity of the *Culicoides species* involved and viral replication in the host (Bhoyar *et al.,* 2009). Earlier studies by Ilango (2006) stated that the highest activity of *Culicoides* midges was found to vary between 13°C and 35°C. Similarly, Losson *et al.* (2007) has shown that adult *Culicoides variipennis* did not survive the harsh winter season, while *C. obsoletus* survived at minimum temperatures ranging between 6°C and 12°C. It was also documented by Mellor *et al.* (2000) that the virus can replicate at a temperature above 15°C in the vector and that the intensity of replication mounted with increasing temperature. In the present study, the recorded day temperature ranged from 32.7º - 36.2ºC. Thus it could be postulated that the reason behind the large catch of *Culicoides* in this study could be due to a combination of factors like ideal temperature, post rainy season and

Table 3. Percentage distribution of the trapped *Culicoides* species based on abdominal pigmentation status

Species	Male^{a*}	Female ^{a**}				
		Pigmented^{b**}	Non pigmented ^{b**}	Bloodfed ^{b**}	Gravid ^{b**}	Total
C.imicola	35 (2.79%)	248 (19.77%)	362 (28.87%)	542 (43.22%)	67 (5.34%)	1254
C.oxystoma	35 (10.44%)	128 (38.21%)	39 (11.64%)	76 (22.69%)	57 (17.01%)	335
C. peregrinus	26 (20.47%)	36 (28.35%)	18 (14.17%)	19 (14.96%)	28 (22.05%)	127
C.brevitarsis	12 (21.82%)	17 (30.91%)	6 (10.91%)	11 (20.00%)	9 (16.36%)	55
C.fulvus	2 (20.00%)	1 (10.00%)	1 (10.00%)	3 (30.00%)	3 (30.00%)	10
	Total				**1781**	

Chi square value of 112.91oncomparison between male and female *Culicoides* represented by 'a' and among pigmented, non-pigmented, blood fed and gravid represented by 'b' in the column head

** Significant (P<0.01)

high relative humidity that are the typical features of a tropical climate.

Morphological identification of *Culicoides species*

A total catch of 2708 specimens were obtained in this study. However, the biting midges of *Ceratopogonidae* family that are closely related to the non-biting *Chironomidae* family needs to be differentiated. *Culicoides* midges (n=1787) in the total catch obtained were distinguished from other insects (n=921) by the presence of biting mouthparts in the female, the closed and flat wings with typical venation pattern (two well developed radial cells, a medial vein which forks distally to the r-m cross-vein and absence of r4 and r5 region) and their short fore-legs as per the keys of Wirth and Hubert (1989). Accordingly, the count of *Culicoides* species was observed to be 481, 612, 396 and 298 in Tirunelveli, Erode, Madurai and Chennai respectively, whereas non *Culicoides species* accounted to 252, 283, 213 and 197 respectively in the corresponding districts and were listed in Table 2.

Species -wise prevalence of *Culicoides* at different collection sites

Morphological characterization of the mounted specimens following the reference keys published by Bellis *et al.* (2014) revealed that five species of *Culicoides* namely *Culicoides imicola, Culicoides oxystoma, Culicoides peregrinus, Culicoides brevitarsis* and *Culicoides fulvus* were more prevalent in the selected sheep holdings of Tamil Nadu. The rest of the midges were classified as unknown species.

The percentage distribution of total *C. imicola, C. oxystoma, C. peregrinus, C. brevitarsis* and *C. fulvus* trapped in all the four districts amounted to 70.17%, 18.74%, 7.10%, 3.07% and 0.55 % respectively. The abundance of *C. imicola* is well substantiated by its status of being a pan-continental species as proposed by Onyango *et al.* (2015). The order of abundant species is also consistent with the findings of Harrup *et al*. (2016) who recorded that *C. oxystoma* was more abundant than *C. peregrinus* and *C. brevitarsis*. However, a contrasting observation of*C. oxystoma* being the most abundant species in Gujarat was documented by Dadawala *et al.* (2012).

It could be seen that *C. imicola,* the most abundant species in our study correlated with that of Udupa (2001) in Tamil Nadu and accounted to 66.94, 78.59, 60.85 and 0.24% of the total *Culicoides* catch in Tirunelveli, Erode, Madurai and Chennai respectively.

Percentage distribution of the trapped *Culicoides* species based on gender and abdominal pigmentation

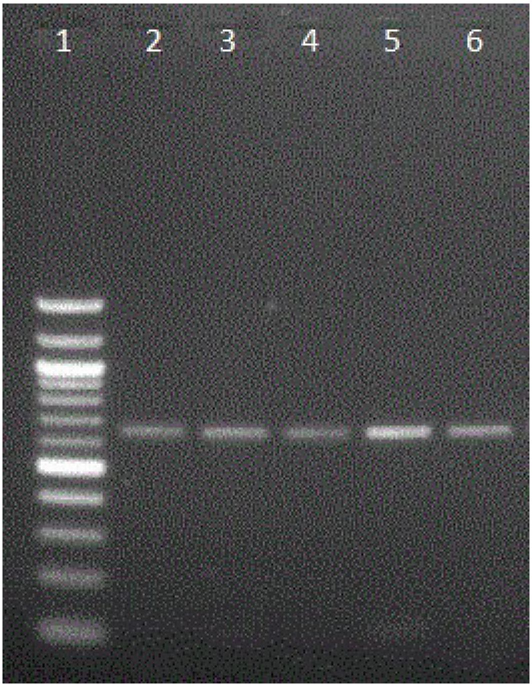
Our observations on gender distribution of the midges presented in Table 3 revealed that the prevalence of female *Culicoides* (78.18 – 97.21%) was found to be much higher than the males midges (2.79– 21.82%) in any given catch. This preponderance nature of female *Culicoides* is in accordance with the observations of Satheesha *et al*. (2006) and Archana *et al.* (2014), reporting a female - male percentage of 93.16 vs. 6.84% and 75.7 - 99.25 vs. 24.3 - 0.75% and 90.90 vs 9.10% respectively.

The percentage distribution of female *Culicoides species* based on abdominal pigmentation is presented in Table 3. It could be observed that the overall percentage of pigmented, non-pigmented, blood fed and gravid forms were 25.73, 25.49, 38.95 and 9.81% respectively. However, Harrup *et al.* (2016) recorded a different distribution pattern of 51.9% non-pigmented, 18% pigmented, 20.4% blood fed and 0.6% gravid forms, although in both these studies the traps were set up in Tamil Nadu only. This variation in abdominal pigmentation could be due to the fact that the latter study was conducted during rainy season while our study was undertaken during post rainy days leading to changes in the climatic conditions at the trap site, influencing the availability of breeding niches and blood meal from the host species.

Females are obligate blood feeders meant for egg production and oviposition, while males are non-blood feeders that usually die after mating. This could explain the importance of vector competence of female midges, especially the gravid and pigmented forms outnumbering the male midges.

4.6 Molecular characterization of *Culicoides* species by DNA barcoding

In the present study, attempts were made to characterize the *Culicoides* species both morphologically and by molecular method. Hence, the nondestructive method of DNA extraction was carried out so that the same specimens could be used for mounting thereby preserving the morphological characteristics. In order to differentiate and identify *Culicoides* at species level, the DNA barcode sequence assay based on the deep interspecies differences in mitochondrial COI gene proposed by Meyer (2008) was used. Amplification of partial COI gene using LCO/HCO primers yielded amplicons of 658bp in all the five species identified and is represented in Fig. 1. The sequences obtained by bidirectional sequencing for *C. imicola, C. oxystoma, C. peregrinus, C. brevitarsis* and *C. fulvus* are available in GenBank with accession numbers MW_164979 to MW_164983 respectively.



Lane 1:100 bp DNA ladder; Lanes 2 to 6: Amplicons of 658 bp of *Culicoides imicola, Culicoides oxystoma, Culicoides peregrinus, Culicoides brevitarsis* and *Culicoides fulvus* respectively

Fig. 1. Agarose gel electrophoresis (1.5% gel) showing amplicons of mitochondrial COI gene of *Culicoides* species

The BLAST analysis yielded homology of 100% to Indian specimens of *Culicoides imicola*, although tolerable intra species differences lie within a range of 0 to 9% for this species. Similarly, the observed minimum homology for *C. peregrinus, C. brevitarsis* and *C. fulvus* with the already published sequences were 98.18, 95.41 and 99.79% respectively with a query coverage of almost 100%. These values are well within the tolerable intra species differences that range between 1.8 to 2.3, 0 to 5.6%, as proposed by Ander *et al*. (2013) for these species respectively.

Phylogenetic tree using an unrooted tree model constructed for grouping the midges by species-wise clusters is presented in Fig. 2. It could be observed that all the four identified species except *C. oxystoma* fall into the defined species cluster. Though *C. oxystoma* clustered close to its ancestral types, our sequence data showed a branching away from the other intra species specimens used for analysis. Our sequence of *C. oxystoma* showed a slightly lesser homology range of 88.59% at the maximum to 87.54% at the minimum when BLAST analysed with sequences of other Indian specimens. The less homology of *C. oxystoma* had been earlier documented by Dadawala *et al.* (2012) at intra species level and is attributable to the high genetic divergence of the species. However, the tolerable intra species difference for this species lies within the range of 0 to 5.8%. An earlier study by Bakhoum *et al.* (2013) had proved the usefulness of barcoding in resolving the ambiguity of genetic divergence of two species namely *C. schultzei* and *C. oxystoma*, though they appeared to be morphologically similar.

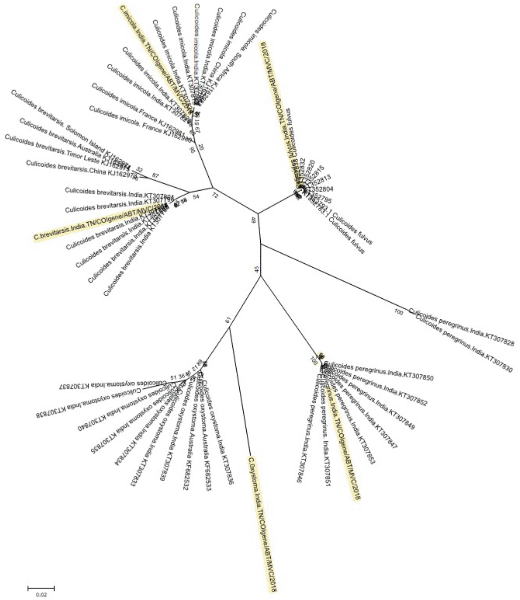


Fig. 2. Phylogenetic analysis of the COI gene of five identified *Culicoides* species into their respective clusters

It can be concluded that the prevalence and diversity of *Culicoides species* in a given geographical location is influenced by climatic conditions and host species and that morphological typing of vectors are better complemented by molecular tools like DNA barcoding.

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