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DNA barcoding of commonly prevalent *Culicoides midges* in South India

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ABSTRACT

DNA barcoding has gained increased recognition as a molecular tool for species identification of insects. Interspecific variation in DNA sequences of some genes is much higher than intraspecific and provides an opportunity to use DNA sequences for species identification. A study was therefore undertaken to barcode 5 commonly prevalent *Culi*COI*des* species in farming regions of Bengaluru districts in Karnataka state. The barcoding of Cytochrome oxidase I (COI) gene of *C. anophelis, C. palpifer, C. huffi, C. innoxius* and *C. circumscriptus* yielded an amplified fragment of 648 bp sequence. Barcode for all 5 species was generated using BoldSystems v3 and submitted to genbank for accession numbers. DNA barcoding enabled exact identification of 5 prevalent species.

Key word: Bold Systems v3, *Culi*COI*des*, Cytochrome oxidase I, DNA barcoding

Culicoides, biting midges, are nematocerous flies which are one of the smallest haematophagous flies measuring from 1 to 3 mm in size. More than 1,400 species of genus *Culicoides* were identified worldwide of which about 96% are obligate blood feeders attacking mammals and birds (Mellor *et al*. 2000). *Culicoides* is a prime vector for various viruses causing bluetongue disease, African horse sickness, epizootic hemorrhagic disease, Akabane, Aino, Chu-zan, and bovine ephemeral fever, vesicular stomatitis, equine encephalosis and schmallenberg viruses, protozoans such as haemoproteus, leucocytozoon, hepatocystis, avian trypanosomes, lizard and avians plasmodia and helminths such as *Onchocerca cervicalis*, dipetalonema and other filarid worms of birds and mammals (Prasad and Bhatanagar 2000).

Culicoides anophelis is a predator of engorged mosquitoes, parasitizing on another dipteran *Anopheles stephensi*. At least 19 mosquito species in the genera *Anopheles*, *Culex*, *Aedes* and *Armigeres* were documented as hosts of *C. anophelis*. The mosquito females infested with this ectoparasite failed to lay eggs and did not survive for long (Ma *et al*. 2013). *C. circumscriptus* is regarded as ornithophilic in Israel, man biting and also feeding on cattle in Thailand. It is also a vector for *Leucocytozoon* in birds (Dasgupta 1995).

The morphological identification of *Culicoides* based on morphological features is tedious, confusing and time-consuming because its size ranges from 1 to 3 mm. Therefore the molecular method of identification is an aid for identification of species. Among the molecular methods DNA barcoding is widely used in species identification and biodiversity research (Kim *et al*. 2012). Cytochrome oxidase I (COI) barcoding sequences can be used to discover cryptic species: closely related and morphologically similar ones (Rivera *et al.* 2009) and is reliable, cost-effective and accessible solution to the current problem of species identification (Hebert *et al.* 2003). Mitochondrial (Mt) DNA is used for DNA barcoding because Mt DNA is much smaller than nuclear DNA and sequencing is easy. COI is used for DNA barcoding and very efficient for species identification, easy to isolate from wide range of organisms, therefore, in the present study COI was used for DNA barcoding. A study was therefore undertaken to barcode commonly prevalent *Culicoides* species in farming regions of Bengaluru districts in Karnataka state in view of their vector potential and importance as pests of animals and man.

MATERIALS AND METHODS

Traps and collection: Flies were collected by using UV-light trap from 11 different farms of cattle, buffalo, sheep and goat in rural and urban districts of Bengaluru. Collections were made during dawn and dusk and the traps were set from 6 pm to 6 am. The light trap was located within 25 m of livestock premises and were suspended from the walls of building at 1.5–2.0 m above the ground level at night.

To 300 ml of clean water 2 drops of detergent was added into the collecting beaker to break the surface tension of the water allowing collected insects to sink into the solution.

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The collected insects were transported to the laboratory in the water-filled collecting beaker and then recovered and preserved in 95% ethanol and kept in the deep freeze at –20°C. Identification of *Culicoides* was initially based upon wing pattern, and confirmed subsequently by mounting several specimens on glass slides after clearing in liquified phenol solution for 12 h (Wirth and Martson 1967).

Morphological identification of *Culicoides* spp. was done (Wirth and Hubert 1989, Glick 1990, Sen and Dasgupta 1959, Dasgupta 1995, Kitaoka 1984, Wirth and Hubert 1961 and Pires *et al.* 2010). The characters for morphological identification of *Culicoides* spp. include wing pattern, presence and distribution of macrotrichia, lengths of flagellar segments 3-15, length of 5 palpal segments, antennal ratio and palpal ratio, distribution of antennal coeloconica, form of sensory pit on third palpal segment, form of whole third palpal segment, proboscis-head ratio, number and form of spermathecae, form of male genitalia, appearance of leg and thorax, number (sometime absence) of teeth of mandible, number of hind tibial comb and largest spine among them (i.e. on third pair of legs), length of body, length and breadth of wing, costal ratio.

PCR primers: COI gene of *Culicoides* was amplified by universal primers COIF/COIR. The published universal primers were synthesized. The details of the primers and their base sequences are given in Table 1. The primers obtained were reconstituted in nuclease free water (NFW) as per the requirements and stored at –20°C.

PCR amplification of COI by using phire animal direct PCR kit: Culicoides species, viz. *C. anophelis, C. palpifer, C. huffi, C. innoxius* and *C. circumscriptus* were subjected for PCR by using Phire animal direct PCR kit by using COIF/COIR primers. Reactions for the COIF/COIR primers were performed in a total volume of 50 µl consisting of 2X Phire animal tissue PCR buffer 25 µl, COIF 0.5 µl (20 pm), COIR 0.5 µl (20 pm), Hot start DNA polymerase 1 µl, insect sample (4 in number), NFW to make final volume 50µl under the following cycling conditions: an initial denaturation stage at 98°C for 5 min; then 40 cycles at 98°C, 5 sec denaturation; 45°C, 10 sec annealing; 72°C, 30 sec extension and a final extension phase at 72°C for 1 min. After completion of PCR reaction, 1µl of DNA release buffer and gel loading dye which were provided in the kit were added and then were subjected to electrophoresis in 1.5% agarose gel and 100 bp DNA ladder was used as marker. The images were captured using gel documentation system. The PCR products were sequenced at a commercial firm.

RESULTS AND DISCUSSION

Morphological identification: Based on morphometry and by different morphological keys (Wirth and Hubert 1989,Glick 1990,Sen and Dasgupta 1959, Dasgupta 1995, Kitaoka 1984). *Culicoides* species, viz. *C. anopheles, C. palpifer, C. huffi, C. innoxius,* and *C. circumscriptus* were found prevalent.

PCR amplification of COI for barcoding of Culicoides *spp.:* Hebert *et al.* (2003) established that the mitochondrial gene cytochrome *c* oxidase I (COI) could serve as the core of a global bioidentification system for animals. *CO1* gene of insects was amplified by PCR, and they yielded specific amplicon of 658bp. In the present study *C. anophelis, C. palpifer, C. huffi, C. innoxius* and *C. circumscriptus* yielded a specific amplicon at 648 bp (Fig. 1) which is in agreement with the above findings. The above results were also in accordance with others (Ander *et al.* 2012, Puente *et al.* 2012, Kim *et al.* 2012). Therefore molecular method of identification was found to be faster with increased sensitivity and specificity. DNA barcoding has been widely used in species identification and biodiversity research (Kim *et al*. 2012) and provide a reliable, cost-effective and accessible solution to the current problem of species identification (Hebert *et al.* 2003).

Sequencing of PCR products: PCR products were sequenced in both forward as well as reverse directions and sequencing results were obtained in .ab1 file format and .txt format. Sequences were then checked for homology online with the bioinformatics tool BLAST (Basic local alignment search tool) from NCBI (National Centre for Biotechnology Information) server which confirmed the specificity of primers. The sequences showing maximum similarity confirmed the species.

The sequences were edited and the allotted accession numbers by genbank, (> *Culicoides anophelis* ACCESSION NO: KF145178, > *Culicoides circumscriptus* ACCESSION

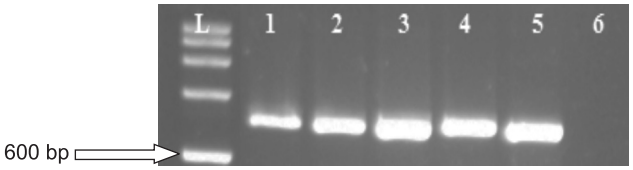

Fig. 1. PCR amplification of COI for barcoding of Culicoides. (L: 100 bp Ladder, Lane 1: *C. innoxius*, Lane 2: *C. huffi*, Lane 3: *C. anophelis*, Lane 4: *C. palpifer*, Lane 5: *C. circumscriptus*, Lane 6: NTC).

Table 1. Nucleotide sequence of COIF/COIR primers

Primer code	Nucleotide sequence	Product size (bp)	Reference
COIF	5'-GGTCAACAAATCATAAAGATATTGG-3'	648-658	Hamada *et al.* (2010), Rivera and Currie (2009), Hebert *et al.* (2003), Pramual *et al*. (2011) Puente. *et al.* (2012), Kim *et al.* (2012)
COIR	5'-TAAACTTCAGGGTGACCAAAAAATCA-3'		

NO: KF145180, > *Culicoides huffi* ACCESSION NO: KF145177, > *Culicoides innoxius* ACCESSION NO: KF145176, *Culicoides palpifer* ACCESSION NO: KF145179). The BoldSystem V3 was used to generate barcode for the five species of *Culicoides*. A project was created at BoldSystems v3; Project Name is VETIP (Veterinary important insects), where all the specimen data and sequences were uploaded. The present aim is at creation of DNA barcode library and automated identification of all eukaryotes based on DNA barcode library.

Identification of 5 different species of *Culicoides* based on morphology were confirmed by DNA barcoding to prove correct identity method. Interspecific variation in DNA sequences of COI genes is much higher than intraspecific and this provided an opportunity to use DNA sequences for exact species identification.

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REFERENCES

Ander M, Troell K and Chirico J. 2012. Barcoding of biting midges in the genus *Culicoides*: a tool for species determination. *Medical and Veterinary Entomology* doi: 10.1111/j. 1365–2915.

Cetre-sossah C, Baldet T, Delecolle J C, Mathieu B, Perrin A, Grillet C and Albina E. 2004. Molecular detection of *Culicoides* spp. and *Culicoides imicola,* the principal vector of bluetongue (BT) and African horse sickness (AHS) in Africa and Europe. *Veterinary Research* **35:** 325–37.

Dasgupta S K. 1995. Morphotaxonomic features and species of Indian *Culicoides* (Diptera: Ceratopogonidae). *Bluetongue: Indian Perspective*. pp.115-88. (Eds) Prasad G and Srivastava R N. CCS HAU press, Hisar.

Glick J I. 1990. *Culicoides* Biting Midges (Diptera: Ceratopogonidae) of Kenya. *Journal of Medical Entomology* **27** (2): 85–195.

Hamada N, Pepinelli M, Glória A M and Luz S L B. 2010. A new black fly species from Brazil, closely related to *Simulium guianense* Wise (Diptera, Simuliidae), revealed by morphology and DNA barcoding. *Zootaxa* **2428**: 22–36.

Hebert P D N, Cywinska A, Ball S L and Dewaard J R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London* B **270**: 313–21.

Kim D W, Yoo W G, Park H C, Yoo H S, Kang D W, Jin S D, Min H K, Paek W K and Lim J. 2012. DNA Barcoding of fish, insects, and shellfish in Korea. *Genomics Information* **10** (3).

Kitaoka S. 1984. Japanese *Culicoides* (Diptera: Ceratopogonidae) and keys for the speciesI and II. *Bulletin of the National Institute of Animal Health* No. **87**: 73–108.

Ma Y, Xu J, Yang Z, Wang X, Lin Z, Zhao Way, Wang, Li X, Shi H. 2013. A video clip of the biting midge *Culicoides anophelis* ingesting blood from an engorged *Anopheles* mosquito in Hainan, China. *Parasites and Vectors* **6**: 326.

Mathieu B, Lie perrin A, Baldet T, Dele´ colle J C, Albina E and Cetre-sossah C 2007. Molecular identification of Western European species of obsoletus complex (Diptera: Ceratopogonidae) by an internal transcribed spacer-1 rDNA multiplex polymerase chain reaction assay. *Journal of Medical and Veterinary Entomology* **44** (6): 1019–25.

Mellor P S, Boorman J and Baylis M. 2000. *Culicoides* biting midges: Their role as Arbovirus vectors. *Annual Review of Entomology* **45**: 307–40.

Nolan D V, Carpenter S, Barber J, Mellor P S, Dallas J F, Mordue (Luntz) A J and Piertney S B. 2007. Rapid diagnostic PCR assays for members of the *Culicoides obsoletus* and *Culicoides pulicaris* species complexes, implicated vectors of bluetongue virus in Europe. *Veterinary Microbiology* **124**: 82–94.

Pages N, Munoz-munoz F, Talavera S, Sarto V, Lorca C and Nunez J I 2009. Identification of cryptic species of *Culicoides* (Diptera: Ceratopogonidae) in the subgenus *Culicoides* and development of species-specific PCR assays based on barcode regions. *Veterinary Parasitology* **165**: 298–310.

Prasad G and Bhatnagar P K. 2000. *Culicoides* : *Biology and transmission of viruses*. *V National Training programme Compendium of Acarines and Insects of Veterinary and Medical Importance*. On Centre of Advanced Studies, Department of Parasitology, Veterinary College, Bangalore, pp. 34–38.

Pramual P, Wongpakam K, Adler P H. 2011. Cryptic biodiversity and phylogenetic relationships revealed by DNA barcoding of Oriental black flies in the subgenus *Gomphostilbia* (Diptera: Simuliidae). *Genome* **54**: 1–9.

Pires G A, Ramilo D, Diaz S, Meireles J, Boinas F and Fonseca I P D. 2010. Investigating morphological structures of *Culicoides* from *obsoletus* complex by using scanning electron microscopy and composed optical microscopy**.** Microscopy: Science, Technology, Applications and Education. Pg 792–802.

Puente J M D L, Martinez J, Ferraguti M, Nuez A M D L, Castro N and Figuerola J. 2012. Genetic characterization and molecular identification of the bloodmeal sources of the potential bluetongue vector *Culicoides obsoletus* in the Canary Islands, Spain. *Parasites and Vectors* **5**: 147.

Rivera J and Currie D C. 2009. Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). *Molecular Ecology Resources* **9**: 224–36.

Sen P and Dasgupta S K. 1959. Studies on Indian *Culicoides* (Diptera: Ceratopogonidae). *Annals of Entomological Society of America* **52**: 617-30.

Stephan A, Clausen P H, Bauer B and Steuber S. 2009. PCR identification of *Culicoides dewulfi* midges (Diptera: Ceratopogonidae) potential vectors of bluetongue in Germany. *Parasitological Research* **105**: 367–71.

Wirth W W and Marston N. 1967. A method for mounting small insects on microscope slides in canada balsam. *Annals of Entomological Society of America* **61**: 783–84.

Wirth W W and Hubert A A. 1961. New species and records of Taiwan *Culicoides* (Diptera Ceratopogonidae). *Pacific Insects* **3** (1): 11–26.

Wirth W W and Hubert A A. 1989. The *Culicoides* of Southeast Asia Diptera (Ceratopogonidae). *Member American Entomological Institute*. no. 44.