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DNA barcoding of flies commonly prevalent in poultry farms of Bengaluru District

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Abstract
DNA barcoding has been widely used in species identification and biodiversity research because it has been shown that in many groups, including insects, interspecific variation in DNA sequences of some genes is much higher than intraspecific and this provided an opportunity to use DNA sequences for species identification. Cytochrome oxidase I (COI) barcoding sequences can be used to discover cryptic, closely related and morphologically similar species. DNA barcoding has gained increased recognition as a molecular tool for species identification in various groups of organisms. A study was, therefore, undertaken to barcode five fly species prevalent in poultry farms in and around Bengaluru districts in Karnataka state. The barcoding of COI gene of *Musca domestica, Chrysomya megacephala, Hydrotaea capensis, Hermetia illucens* and *Sarcophaga ruficornis* yielded an amplified fragment of 658 bp sequence. Barcode for all 5 species was generated using Bold_Systems v3 and submitted to GenBank and accession numbers were obtained. In the present study, identification of five different fly species based on morphology was also confirmed by DNA barcoding to prove their correct identity.

Keywords: DNA barcoding, COI, Bold Systems v3, flies, poultry farms, Karnataka

Introduction
India's poultry industry represents a major success story. It is one of the fastest growing segments of the agricultural sector today in India with annual growth rates of 5.57 and 11.44 percent in egg and broiler production, respectively. While agricultural production has been rising at the rate of 1.5-2 percent per annum over the past two to three decades, poultry production has been rising at the rate of around 8-10 percent per annum, with an annual turnover of US$ 7500 million [1]. The sector is providing direct or indirect employment to 6.5 million people. It accounts for about one percent of India's GDP and 11.70 percent of the GDP from the livestock sector. Livestock population of India is among the highest in the world, it contributes approximately 4% to GDP and 27% to agricultural GDP [2]. South India accounts for majority of total poultry production and consumption in the country [3]. The high growth has placed India at 3rd position after China and USA with a production of 59.8 billion eggs and 5th after USA, China, Brazil, and Mexico with regard to meat production [4].

One of the significant problem in poultry farms all over India is the fly menace. Several species of flies are found associated with poultry production facilities such as houseflies and their relatives like flesh flies, blow flies, bottle flies, filter flies, soldier flies and vinegar or fruit flies. These flies cause annoyance, discomfort and are also harmful to human and animal health capable of transmitting many diseases to human and animals. In view of the severity of the problem, these flies need to be studied and effective control measures have to be evolved.

Insects are usually identified by morphological methods, which is a tedious, time-consuming, needs a specialist, holotype comparison, difficult to identify large number of insects in a short period of time, and requires taxonomic expertise. The morphological similarity in females of certain species complex of insects is a challenge for identification. Therefore, molecular method of identification is being advocated for identification, which is faster and has increased sensitivity and specificity. One such molecular method is DNA barcoding [5, 6, 7] and this approach was employed for confirmation of morphologically identified insects and insects based on their fragments. The mitochondrial gene cytochrome *c* oxidase I (COI) could serve as the core of a global bio identification system for animals [7]. COI gene of insects was amplified by PCR and yielded specific amplicon of 658bp. Many authors have done COI barcoding by using universal primers by amplifying 658bp length of gene [8] and used COI barcode to identify morphologically identified *Chrysomya* species (*C. flavifrons, C. latifrons, C.*

megacephala, C. nigripes, C. rufifacies, C. safranea, C. semimetallica, C. varipes and *C. incisuralis*) from East Coast of Australia [9]. Identification of various flies belonging to Calliphoridae and Muscidae has been done in Germany [10] and for identifying *Lucilia sericata* from India [11], to identify sand flies in Peru [12], *Sarcophaga tibialis* and *S. cultellata* from Spain [13]. Identification of immature life stages of a forensically important flesh fly (Diptera: Sarcophagidae) was done [14]. The identification of *C. imicola, C. oxystoma, C. peregrinus*, *C. anophelis, C. palpifer, C. huffi, C. innoxius* and *C. circumscriptus* [15] was made and sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia [16]. Identification of flies belonging to Calliphoridae and Muscidae in Iberian Peninsula [17] and neotropical sand flies (Diptera, Psychodidae, Phlebotominae) in Brazil [18].

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 fly species in poultry farms of Bengaluru districts in Karnataka state in view of their importance as pests of poultry, animals and man.

Materials and methods

Eleven different poultry farms in Bengaluru were selected and flies were collected from 2013-2014, one year period by using sweep net, different flies were preliminarily identified based on wing pattern, color of flies, head and thorax pattern and separated. Some adult flies were dry preserved to preserve their natural colour and bristles and the rest were preserved in 70% ethanol. The date of collection, season, species, and region of collection was recorded. Of the different flies some were stored in 95% ethanol at -20 ^{0}C in the deep freezer for molecular studies and larvae were collected by handpicking from poultry manure of the different larvae. Larvae of different flies were separated and stored in 70% ethanol, some were taken and stored in 95% ethanol at -20 ^{0}C in the deep freezer for molecular studies. The adult flies and larvae were confirmed subsequently by mounting several specimens on glass slides after clearing in liquefied phenol solution for 24 hr [19].

Morphological identification:

Morphological identification of adult flies was done by using keys [20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32]. The characters for morphological identification of fly included length of fly, eyes, antennae, wing pattern, and colour of flies, thorax and abdominal pattern.

Morphological identification of larvae was done by using keys [33, 34, 35, 36, 37, 38, 39, 40]. Basic morphological features of larva used for identification purpose included length of larvae, integument, spines, cephalopharyngeal skeleton, respiratory structures such as anterior and posterior spiracles.

Molecular identification:

Morphologically five different flies were identified by using keys [20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40], *viz*., *M. domestica, C. megacephala, H. capensis, H. illucens* and *S. ruficornis.* All flies from different farms were subjected for barcoding. For barcoding, cytochrome c oxidase subunit I mitochondrial gene was targeted and amplified by using universal primers.

DNA extraction: The genomic DNA of adult flies and larvae were extracted Qiagen "DNeasy Blood and Tissue kit" (Germany) as per the manufacturers protocol, procured from M/s J J BIOTECH, Bengaluru.

PCR primers: COI gene of flies was amplified by universal primers LCO1490/HCO2198. 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: The amplification reactions were carried out with composition of PCR mix for species specific amplification of COI of flies (Table 2) in 0.2ml PCR tubes using a programmable thermal cycler as detailed below cycling conditions [7] (Table 3).

Table 1: Nucleotide sequence of CO1F/CO1R primers

Primer code	Nucleotide sequence	Product size (bp)
LCO1490 HCO2198	5'-GGTCAACAAATCATAAAGATATTGG-3 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	658

Table 2: Composition of PCR mix for species specific amplification of CO1 of flies

Contents	Quantity
10X Taq buffer	5µl ($Mgcl_2$-15mM)
dNTP's mix	1µl (2.5mM each)
Taq DNA polymerase (1unit/ul)	1µl (1unit)
LCO1490	2µl (20pm)
HCO2198	2µl (20pm)
Template	5µl
NFW to make final volume	50µl

Table 3: PCR conditions for the amplification of CO1 of fly spp.

Initial denaturation	Denaturation	Annealing	Extension	Final extension	Stop reaction
95 ^{o}C 5min	95 ^{o}C 1min	45 ^{o}C 1min	72 ^{o}C 1min	72 ^{o}C 10min	4 ^{o}C 5min
	Repeated for 30 cycles				

After completion of PCR reaction, 5µl of amplified products along with 6X gel loading dye 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.

Sequencing of PCR products

The PCR products were sent to M/s Eurofins Pvt. Ltd,

Bengaluru, India, for sequencing, where 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 by using the available bioinformatics tool BLAST (Basic local alignment search tool) from NCBI (National centre for Biotechnology information) server.

Phylogenetic analysis

All the 29 DNA sequences were clustered by Clustal w for pairwise and multiple alignment and further subjected to phylogenetic tree construction by applying Neighbour-Joining approach with 1000 bootstrap support in MEGA v4.0 software.

Results

Morphological identification of adult flies revealed *M. domestica*, *C. megacephala, H. capensis, H. illucens* and *S. ruficornis* to be prevalent.

PCR amplification of COI of morphologically identified flies for barcoding viz *M. domestica, C. megacephala, H. capensis, H. illucens, S. ruficornis* were amplified by using universal primers LCO1490 and HCO2198 they yielded specific amplicon of 658bp (Fig. 1).

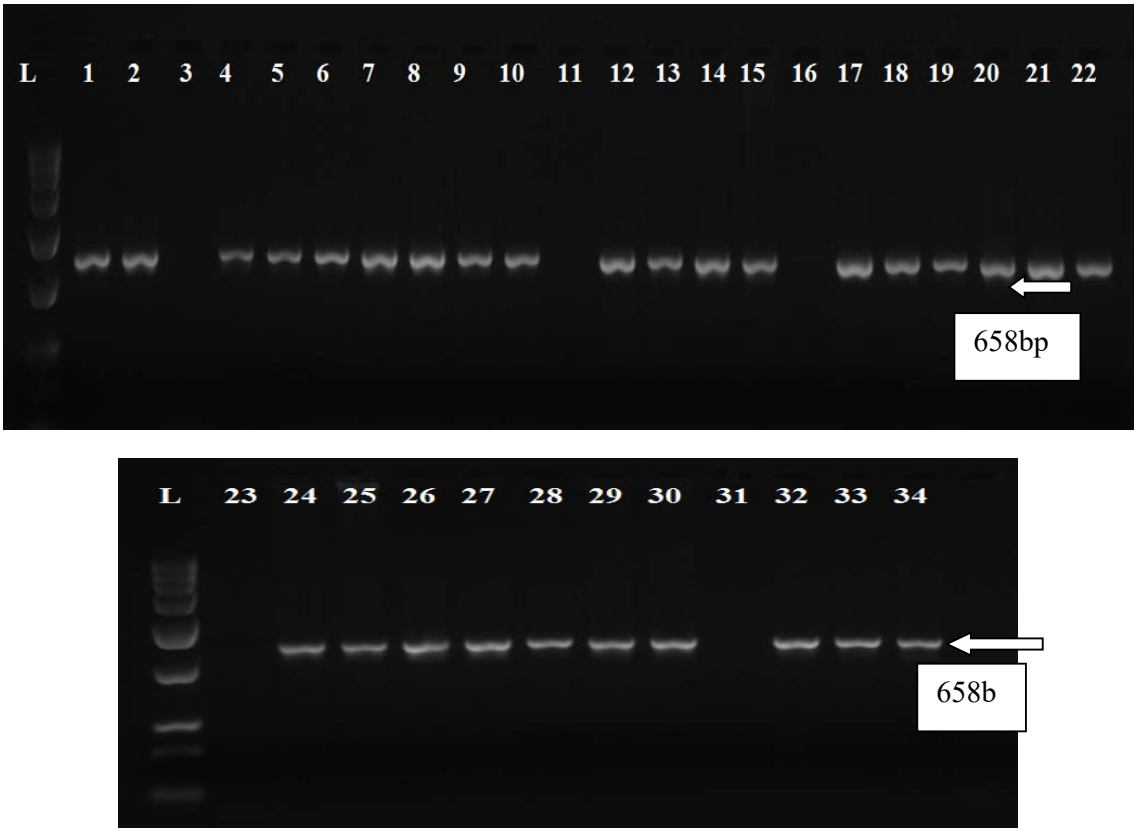

Fig 1: PCR amplification of COI gene for barcoding of flies (L: 100 bp Ladder, Lane 1: *C. megacephala*, Lane 2: *C. megacephala*, Lane 3: NTC-no template control, Lane 4: *M. domestica,* Lane 5: *M. domestica,* Lane 6: *C. megacephala*, Lane 7: *C. megacephala*, Lane 8: *M. domestica,* Lane 9: *M. domestica,* Lane 10: *C. megacephala*, Lane 11: NTC, Lane 12: *C. megacephala*, Lane 13: *M. domestica,* Lane 14: : *M. domestica,* Lane 15: *M. domestica,* Lane 16: NTC, Lane 17: *Hy. capensis,* Lane 18: *C. megacephala*, Lane 19: *M. domestica,* Lane 20: *M. domestica,* Lane 21: *M. domestica,* Lane 22: *M. domestica,* Lane 23: NTC, Lane 24: *M. domestica,* Lane 25: *M. domestica,* Lane 26: *M. domestica,* Lane 27: *M. domestica,* Lane 28: *M. domestica,* Lane 29: *M. domestica,* Lane 30: *M. domestica,* Lane 31: NTC, Lane 32: *He. illucens,* Lane 33: *M. domestica,* Lane 34: *S. ruficornis*

Sequencing of PCR products

The PCR products were sent to Euro-fins Ltd. India for sequencing, where 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 by using the online freely available bioinformatics tool BLAST (Basic local alignment search tool) from NCBI (National centre for Biotechnology information) server which confirmed the specificity of primers. All the sequences for particular species under BLAST system were confirmatory. The nucleotide sequence data obtained were edited and then subjected for BLAST with NCBI nucleotide sequence data library.

Sequences were edited and submitted to GenBank and accession numbers were allotted and for those species barcode was generated using BoldSystems v3. The accession numbers allotted were >A-2_ KX230656 *Chrysomya megacephala,* >A-3_ KX230657 *Chrysomya megacephala,* >A-4_ KX230658 *Musca domestica,* >A-5_ KX230659 *Musca domestica,* >A-7_ KX230660 *Chrysomya megacephala,* >A-8_ KX230661 *Chrysomya megacephala*, >A-10_ KX230662 *Musca domestica,* >A-11_ KX230663 *Musca domestica,* >A-14_ KX230665 *Chrysomya megacephala,* >A-15_ KX230666 *Chrysomya megacephala,* >A-16_ KX230667 *Musca domestica,* >A-17_ KX230668 *Musca domestica*, >A-18_ KX230669 *Musca domestica,* >A-19_ KX230670 *Hydrotaea capensis,* >A-21_ KX230671 *Chrysomya megacephala,* >A-22_ KX230672 *Musca domestica*, >A-23_ KX230673 *Musca*

domestica, >A-24_ KX230674 *Musca domestica*, >A-26_ KX230676 *Musca domestica*, >A-27_ KX230677 *Musca domestica*, >A-29_ KX230678 *Musca domestica*, >A-30_ KX230679 *Musca domestica*, >A-31_ KX230680 *Musca domestica*, >A-32_ KX230681 *Musca domestica*, >A-34_KX230682 *Musca domestica*, >A-35_ KX230683 *Musca domestica*, >A-37_ KX230684 *Hermetia illuscens*, >A-38_ KX230685 *Musca domestica*, >A-40_ KX230686 *Sarcophaga ruficornis*.

Phylogenetic analysis

Tree clustered into three major clade, first clade consisting of *M. domestica*, second clade consisting of *H. capensis* and *S. ruficornis*, whereas third clade consisting of *C. megacephala*. *H. illucens* clustered as an outgroup (Fig. 2).

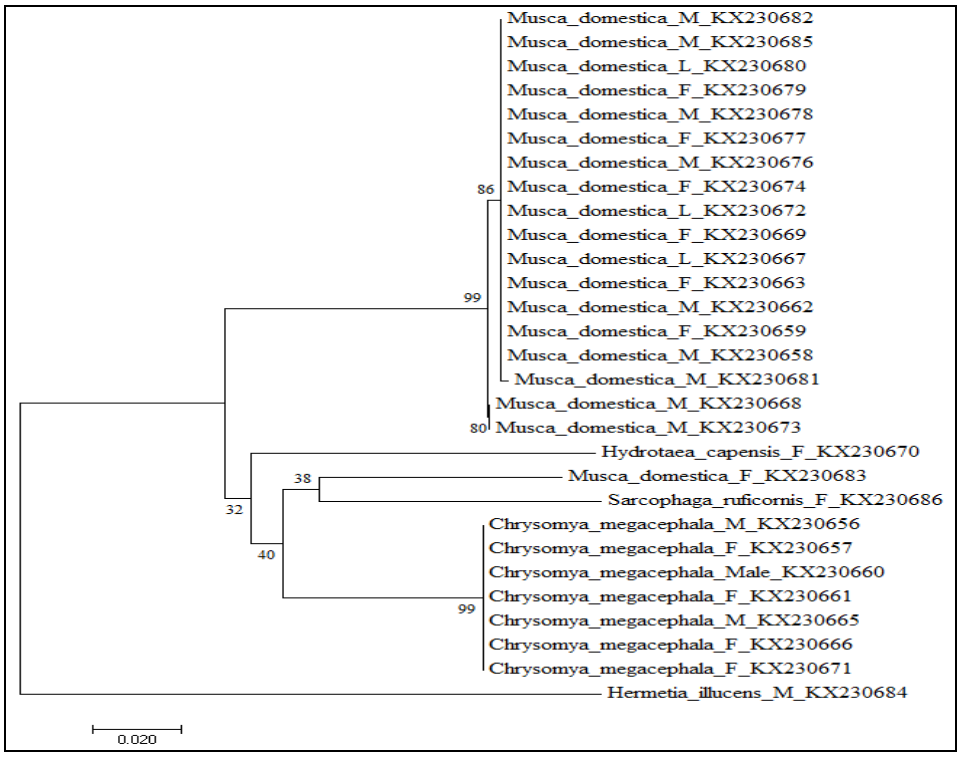

Fig 2: Phylogenetic analysis of flies from *Musca*, *Hydrotaea*, *Sarcophaga* and *Chrysomya* genus

Discussion

The morphological identification based on various morphological features is tedious and time-consuming and needs a specialist, holotype comparison is time consuming and difficult to identify large number of insects in a short period of time, requires taxonomic expertise and morphological similarity in females of certain species complex of insects is a challenge to identify. Therefore, molecular method of identification was advocated for identification, which is found to be faster and had the advantage of sensitivity and specificity.

DNA barcoding has been widely used in species identification and biodiversity research (Kim *et al*., 2012) [5] because it has been shown that in many groups, including insects, interspecific variation in DNA sequences of some genes is much higher than intraspecific and this provided an opportunity to use DNA sequences for species identification. Cytochrome c oxidase subunit I (COI) barcoding sequences can be used to discover cryptic, closely related and morphologically similar species. DNA barcoding has gained increased recognition as a molecular tool for species identification in various groups of organisms **(**Rivera *et al.,* 2009) [6] and this provides a reliable, cost-effective and accessible solution to exact species identification (Hebert *et al.,* 2003) [7]. In the present study all the flies, which were identified morphologically and were confirmed by DNA barcoding.

A DNA barcode is a short sequence from standardized portions of the mitochondrial genome (a 658 bp of mtCOI). The concept for barcoding is to study genetic variation between species, which exceeds variation within species. MtDNA is used for DNA barcoding because MtDNA is much smaller than nuclear DNA, sequencing is easy. MtDNA has fast mutation rate with a significant variation between species & less variation among species. It is less prone to insertions, deletions and other large scale of rearrangements, which spoils the generation of barcode. It is very stable with little or no degradation in museum specimens, easy alignment having short segments with less cost to sequence.

COI is used for DNA barcoding because this is the most popular gene used worldwide and very efficient for species identification, highly conserved at DNA sequences level-within species, alignment process is not difficult- protein coding region, easy to isolate from wide range of organisms, therefore, in the present study CO1 was used for DNA barcoding.

Mitochondrial gene cytochrome *c* oxidase I (COI) could serve as the core of a global bio-identification system for animals. COI gene of animals, insects were amplified by PCR, they yielded specific amplicon of 658bp. In the present study COI of all flies yielded an amplified fragment of 658bp which is in agreement with Hebert *et al.,* 2003 [7]. The 658 bp of COI sequence of *M. domestica, C. megacephala, H. aenesence, H. capensis, H. illuscence* and *S. ruficornis* after BLAST analysis confirmed the species identified based on morphology. In accordance with the present study various authors used CO1

barcoding by using universal primers by amplifying 658bp length of gene such as Nelson (2008) [9] used COI barcode to identify morphologically identified *Chrysomya* species (*C. flavifrons, C. latifrons, C. megacephala, C. nigripes, C. rufifacies, C. safranea, C. semimetallica, C. varipes* and *C. incisuralis*) from East Coast of Australia. Boehme *et al*. (2012) [10] used it for identifying various flies belonging to Calliphoridae and Muscidae in Germany. Bhaskaran and Sebastian (2015) [11] used the method for identifying *L. sericata* from India. Nzelu *et al*. (2015) [12] used it to identify sand flies in Peru. Arnaldos *et al*. (2015) [13] used COI barcoding for identification of *S. tibialis* and *S. cultellata* from Spain. Meiklejohn *et al*. (2013) [14] for identification of immature life stages of a forensically important flesh fly (Diptera: Sarcophagidae). Archana *et al*. (2014) [15] reported the identification of *C. imicola, C. oxystoma, C. peregrinus*, *C. anophelis, C. palpifer, C. huffi, C. innoxius* and *C. circumscriptus.* Gutierrez *et al*. (2014) [16] identified sand fly species (Diptera, Psychodidae, Phlebotominae) in Colombia. Rolo *et al*. (2013) [17] used it for identifying flies belonging to Calliphoridae and Muscidae in Iberian Peninsula. Pinto *et al*. (2015) [18] identified neotropical sand flies (Diptera, Psychodidae, Phlebotominae) in Brazil.

Conclusion

In present study, identification of five different fly species was confirmed by morphological as well as by DNA barcoding to prove their correct identity. Even the immature stages of the fly, *viz*., larvae were identified up to species level by using barcoding as morphological species identification at any life stage is very challenging. 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

1. Rajesh Mehta, Nambiar RG. The poultry industry in India. Poultry in the 21st Century, 1-61.
2. Ali MM. Emerging Prospective of Indian Livestock: A Study on Poultry Industry. Asian Journal of Managemental Sciences. 2015; 4(1):33-39.
3. Abroader Consultancy India Pvt. Ltd., 2015.
4. Central Avian Research Institute, Izatnagar, 2011.
5. Kim DW, Yoo WG, Park HC, Yoo HS, Kang DW, Jin SD. *et al*. DNA Barcoding of Fish, Insects, and Shellfish in Korea. Genomics Inform. 2012; 10(3):206-211.
6. Rivera J, Currie DC. Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). Molecular Ecological Resource. 2009; 9:224-236.
7. Hebert PDN, Cywinska A, Ball SL, Dewaard JR. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B. 2003; 270:313-321.
8. Jalali SK, Ojha R, Ankita Gupta and Venkatesan T. Identification of a calliphorid Fly, *Pollenia Rudis* (Diptera: Calliphoridae) based on its fragments through DNA barcoding. International Journal of Agriculture, Environment and Biotechnology. 2015; 8(4): 991-994.
9. Nelson LA. Molecular identification and thermal attributes of forensically important blowflies (Diptera: Calliphoridae: Chrysomya). University of Wollongong thesis collections. University of Wollongong. 2008.
10. Boehm P, Amendt J, Zehner R. The use of COI barcodes for molecular identification of forensically important fly species in Germany. Parasitol Research. 2012; 110:2325-2332.
11. Bhaskaran PKP, Sebastian CD. Molecular barcoding of green bottle fly, *Lucilia sericata* (Diptera: Calliphoridae) using COI gene sequences. Journal of Entomology and Zoology Studies. 2015; 3(1):10-12.
12. Nzelu CO, Cáceres AG, Jiménez MJA, Rosas MFL, Trujillano HHY, Caipo DVL. DNA barcoding for identification of sand fly species (Diptera: Psychodidae) from leishmaniasis-endemic areas of Peru. Acta Tropica. 2015; 145:45-51.
13. Arnaldos MI, Ruiz C, Torres B, Begoña I, García MD, Mora DG, *et al*. DNA barcoding of two forensically important fleshly species (Diptera: Sarcophagidae) from spain and notes on barcoding success within genus Sarcophaga meigen, 1826. *Ciencia forense*. 2015; 12:73-90.
14. Meiklejohn KA. Taxonomy and systematic of Australian Sarcophaga s.l. (Diptera: Sarcophagidae). University of Wollongong thesis collections. University of Wollongong. 2012.
15. Archana M, D' Souza PE, Jalali SK, Renukaprasad C, Ojha R. DNA barcoding of commonly prevalent Culicoides midges in South India. Indian Journal of Animal Sciences. 2014; 85(1):37-39.
16. Gutierrez MLC, Rafael J, Vivero Iva ND, Lez V, Charles H, Porter Uribe S. DNA Barcoding for the Identification of Sand Fly Species (Diptera, Psychodidae, Phlebotominae) in Colombia. PLoS ONE. 2014; 9(1):1-9.
17. Rolo EA, Oliveira ARAB, Catarina G, Dourado AB, Farinha AAB, Rebelo MTAB, *et al*. Identification of sarcosaprophagous Diptera species through DNA barcoding in wildlife forensics. Forensic science International. 2013; 228:160-164.
18. Pinto IDS, Chagas BDD, Rodrigues AAF, Ferreira AL, Rezende. DNA Barcoding of Neotropical Sand Flies (Diptera, Psychodidae, Phlebotominae): Species Identification and Discovery within Brazil. Plose one. 2015, 1-18.
19. Wirth WW, Marston N. A method for mounting small insects on microscope slides in canada balsam. Annals of Entomological Society of America. 1967; 61:783-784.
20. White R S, Aubertin D, Smart J. The fauna of British India including the remainder of the oriental region. Diptera (Calliphoridae). Taylor and Francis, LTD, London. 1940; 4.
21. Sabrosky CW. The Muscid Genus Ophyra in the Pacific Region (Diptera). Proc. Hawn. Ent. Soc. 1949; 8(3):423-432.
22. James MT. The soldier flies or stratiomyidae of California. Bulletin of the California insect survey volume 6, no. 5. University of California press Berkeley and Los Angeles California Cambridge University press London, England, 1960.
23. Van Emden FI. The fauna of India and the adjacent countries. Diptera (Muscidae). Manager of publications,

Government of India, Delhi. 1965; 7(1).
24. Zumpt F. Myiasis in man and animals in the Old World: A textbook for physicians, veterinarians and zoologists. Butterworth and co. (publishers) LTD. London, 1965.
25. Fonsseca D. Handbooks for the identification of British insects. Diptera Cyclorrhapha Calyptrate section (b) Muscidae. Royal Entomol Soc London, 1968.
26. Walker A. The Arthropods of Humans and Domestic Animals- a guide to preliminary identification. 1st edition. Chapman and Hall, London. 1994.
27. Triplehorn CA, Johnson NF. Borror and Delong's Introduction to the study of Insects. 7th edition. Thomson Brooks/Cole, Australia, Asia, United states, 2005.
28. Carvalho CJBD, Patiu CADM. Key to the adults of the most common forensic species of Diptera in South America. Revista Brasileira de Entomol. 2008; 52(3):390-406.
29. Whitworth T. Keys to the Blow Fly Species (Diptera Calliphoridae) of America, North of Mexico. Forensic entomology. The Utility of Arthropods in Legal Investigations second edition. Editors: Jason H. Byrd & James L. Castner. Chapter 19. CRC Press Taylor & Francis Group. Boca Raton London New York, 2010.
30. Szpila K. Key for identification of third instars of European and Mediterranean blowflies (Diptera, Calliphoridae) of forensic importance. Jens Amendt M. Lee Goff Carlo P. Campobasso Martin Grassberger. J. Amendt *et al*. (eds.), Curr Concept Forensic Entomol. 2010, 43-56.
31. Ramaraj P, Selvakumar C, Ganesh A, Janarthanan S. Report on the occurrence of synanthropic derived form of *Chrysomya megacephala* (Diptera: Calliphoridae) from Royapuram fishing harbour, Chennai, Tamil Nadu, India. Biodiversity Data Journal. 2014; 2:1-12.
32. Irish ST, Lindsay, Wyatt N. Key to adults of Afrotropical species of the genus Chrysomya Robineau-Desvoidy (Diptera: Calliphoridae). African Entomology. 2014; 22(2):297-306.
33. Holloway BA. Identification of third-instar larvae of flystrike and carrionassociated blowflies in New Zealand (Diptera: Calliphoridae). New Zealand Entomology. 1991; 14(1):24-28
34. Wells JD, Byrd JH, Tantawi TI. Key to Third-Instar Chrysomyinae (Diptera: Calliphoridae) from Carrion in the Continental United States. Journal of Medical Entomology. 1999; 36(5):638-641.
35. Sukontason KL, Sukontason K, Piangjai S, Boonchu N, Chaiwong T, Roy. Larval morphology of *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) using scanning electron microscopy. Journal of Vector Ecology. 2003; 28(1):47-52.
36. Sukontason K, Sukontason KL, Klun RN, Sripakdee D, Piangjai S. Differentiation of the Third Instar of Forensically Important Fly Species in Thailand. Annals of Entomological Society of America. 2004; 97(6):1069-1075.
37. Brink SL. Key diagnostic characteristics of the developmental stages of forensically important calliphoridae and sarcophagidae in central southafrica. The thesis is submitted in accordance with the requirements for the Philosophae Doctor degree in the Faculty of Natural and Agricultural Sciences Department of Zoology and Entomology at the University of the Free State. 2009, 1-257.
38. Thyssen PJ. Keys for Identification of Immature Insects. Jens Amendt M. Lee Goff Carlo P. Campobasso Martin Grassberger Editors Current Concepts in Forensic Entomology, chapter 2. Springer Dordrecht Heidelberg London New York. 2010, 25-42.
39. Szpila K. Key for identification of third instars European and Mediterranean blowflies (Diptera, Calliphoridae) of forensic importance Third instars. Jens Amendt M. Lee Goff Carlo P. Campobasso Martin Grassberger. J Amendt *et al*. (eds.), Current Concept Forensic Entomology. 2010, 43-56.
40. Velasquez Y, Magana C, Sanchez AM, Rojo, S. Diptera of forensic importance in the Iberian Peninsula: larval identification key. Medical and Veterinary Entomology. 2010; 24:293-308.