IDENTIFICATION OF 12 YEARS OLD MUSEUM SAMPLES OF *ISOMYIA TIBIALIS*, *COSMINA PRASINA* AND *COSMINA LIMBIPENNIS* (CALLIPHORIDAE: RHINIINAE)

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ABSTRACT

Mitochondrial deoxyribonucleic acid (mtDNA) is a prudent and effective tool in identification and phylogenetic analysis of various dipterans insects. The most intermittently used gene is Cytochrome oxidase subunit I (COI) which is immensely informative and considered valuable for predicting divergence. In the present study 180 bp region of COI gene has been successfully amplified for 12-13 years old, dried museum samples, namely *Isomyiatibialis* (Villeneuve,1927), *Cosminaprasina*(Brauer and Bergenstamm, 1889)and*Cosminalimbipennis*(Macquart,1848)belonging tosubfamily Rhiniinae. DNA sequencing was performed and finally phylogenetic tree was constructed for deciphering phylogenetic interrelationship. DNA in such old samples usually becomes highly fragmented and shows inter strand crosslinks, which further hamper the amplification process, thus this short fragment of COI gene may prove to be a valuable identification and phylogenetic marker.

Keywords: Cytochrome oxidase subunit I (COI); Mitochondrial DNA Typing; Rhiniinae; museum samples; identification; phylogenetic analysis

No. of Tables: *3* ***No. of Figures:*** *1* ***No. of References:*** *4*

INTRODUCTION

Insects are diverse, vivid, proficient creatures, which are cosmopolitan in their distribution. Among these, the dipterans represent one of the largest insect orders. Rhiniinae is now categorised as a subfamily in forensically important Calliphoridae family (Bharti, 2011). In the present study, an attempt has been made to sequence 3 different species belonging to two genera. The challenge with the old and dried museum samples is that, the DNA gets highly fragmented and the samples despite of proper preservation do get infected with certain bacteria and fungi. Thus it is a tedious task to extract DNA and successfully sequence such samples. Worldwide, valuable alpha and beta level of taxonomic work, based on morphological and genitalial attributes have been undertaken on these flies. Many foreign authors have also been known to contribute significantly in the molecular field pertaining to the family Calliphoridae but this subfamily has remained completely ignored. This is a first attempt to sequence and molecularly analyse members of this subfamily. Rhiniinae is an important subfamily of family Calliphoridae. Members of this group are metallic to dull grey-brown and have glossy occiput. Epistome is strongly projecting and thoracic squama is bare. The most striking diagnostic feature of genus *Cosmina* is the absence of presuturalacrostrichals and that of genus *Isomyia*is the presence of black setae in the upper part of mesopleuron.

MATERIALS AND METHODS

Specimens The three species *Isomyiatibialis*, *Cosminalimbipennis* and *Cosminaparsina*were chosen for the present work. These were approximately 12 -13 years old. These flies were killed using ethyl acetate vapours and the samples were dried, preserved in insect boxes using naphthalene powder. The samples were old and degraded, so extraction of DNA was a challenging task. Morphological description of these flies is as under:

Table 1:

Name	Frons	Epistome	Genae	Facial carina	Parafrontalia	Thorax	Chaetotaxy
Isomyiatibialis	reddish brown	Dull golden,bare and projecting below vibrissae	Metallic purplish with silver dusting				
Cosminalimbipennis	Reddish brown Face shining dark grey,blakpalpi,vertical	Shining black	Shining brown	absent	Ash coloured with black spots	Dull greenish with coppery reflections, silver dusted	Acrostichals0+2, dorsocentrals 2+4, intra alars 1+2, presutral present, humeral 1, posthumeral

	bristles present					with black spots	2,supraalar 3
Cosmina parsina	Golden reddish Face yellowish,palpiorange, vertical bristles absent	Yellow with golden tomentum	Golden with golden hair	weak	Brown	Dull greenish with coppery reflections, silver dusted	Acrostichals0+2, dorsocentrals 2+4,intra alars 1+2, presutral present, humeral 3, posthumeral 3, supraalar 3

DNA extraction: The old museum samples were first rehydrated and then washed in distilled water to remove any foreign DNA or microbes. These samples were then dissected using sterilized surgical blade. Only legs were dissected out and used for DNA isolation. The tissue was crushed using liquid nitrogen. Then cell lysing (0.01M EDTA, 0.03 M Tris- HCl , 0.01 M sucrose) solution and proteinase K was added and incubated overnight at 55°C in 1.5ul eppendorf tubes. These were then subjected to centrifugation at 13,000 rpm for 7 minutes. After this, the pellet containing the debris was discarded and the supernatant was carefully procured and used for further extraction(Kambhapati and Rai, 1991). Equal proportions of isopropanol and chilled ethanol approximately double in volume to the supernatant were added. To this supernatant 1ul of glycogen solution (Qiagen) was added which is a vital ingredient for the precipitation of traces of DNA. These were then left in the deep freeze for DNA precipitation for overnight. Then these were centrifuged at 13,000 rpm for 10minutes. The supernatant was discarded and pellet was washed with ethanol and air dried. PCI treatment was done for further purification. This purified pellet was then dissolved in TE and stored at -20°C.

Amplification

A specific region of COI gene of mitochondrial DNA was chosen as the target for amplification. Amplification was the most challenging part as optimum annealing temperature was required to be set for every sample. All polymerase chain reactions were performed using Bio-rad T100 TM thermal cycler. The thermal cycler conditions were the following: initial denaturation at 98°C for 2 minutes followed by 40 cycles at 98°C for 30 seconds, annealing at 55°C for 40 seconds, elongation at 72°C for 1minute and final elongation at 72°C for 7 minutes. 50µl PCR cocktail constituted of Phusiontaq DNA polymerase enzyme 1U/50µl reaction, 5X Buffer 10µl, 10pm dNTP, 50Mm $MgCl_2$ 1µl, 10pm primers 1ul each and MQ water (Thermo Fisher Scientific, India). The primers used were modified universal primers (Junqueira*et al*., 2002).

Electrophoresis

PCR products were detected by gel electrophoresis in 1.2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

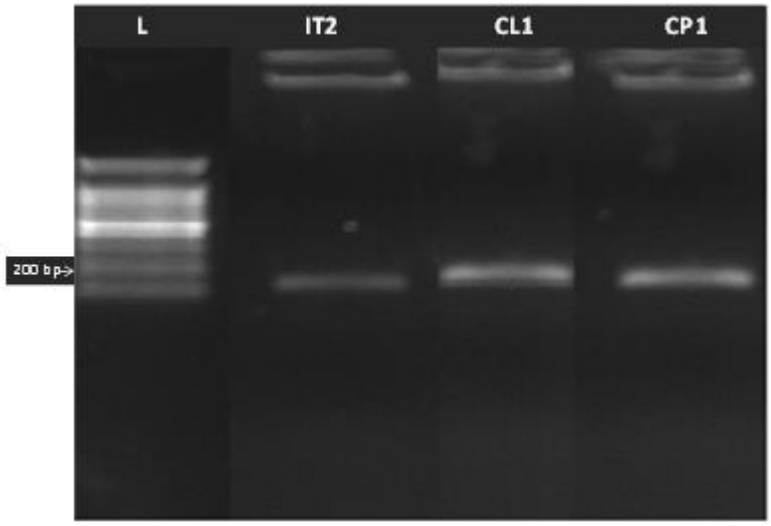

Fig 1:*Gel image of successfully amplified PCR products of 14 years old blow fly species namely; IT- Isomyiatibialis; CL- Cosminalimbipennis; CP- Cosminaparsina and L- Ladder*

Table 2: *Primers used*

Name	Sequence
COI –F	TTGATTTTTTGGTCATCCAGAAGT
COI- R	ACTGTAAATATATGATGAGCTCA

Sequencing

>Seqit1 [organism= Isomyiatibialis]
ATAAAGTTTTCCTTAAAATCGGTATTTTCTCCGAGATTTTGCGATTTTCTCAAGAAAGCGCCTTTTCGGATACTCTTCAA
TGGTATTCGCAACGGTTCTTATTGGTTTCTTAGGTTTCATGGTATGAGCTCATCACATGTTTACATTATA
>Seqit2 [organism= Isomyiatibialis]
GTTATTGGCTGGAAGCAAGATCAAGACAGTTGCGATACCTGGAAGAGATCCGAAAAGGCGCTTTCTTGAAAA
AATCGCAAAAATTTCGGAGAAAATACCGAAAGCCGGCAATACCAAGATGTAAACTTCTGGATGTCCAAAGAA
TCAAAATTTAGT
>Seqcl1 [organism= Cosminalimbipennis]
CACTTACAGCTTCGGATATCTCCCATATTGTAACTTACTACTCCGGAAAAAAAGAACCATTTGGATACATAGGTAT
GGTCTGAGCTATGATATCAATTGGCTTCCTAGGGTTTATCGTGTGAGCTCATCATATATTTACAGAAAA
>Seqcl1 [organism= Cosminalimbipennis]
CTAAGGAGCCCTTTGATATCATAGCTCAGACCATACCTATGTATCCAAATGGTTCTTTTTTTCCGGAGTAGTAAGTTA
CAATATGGGAGATTATTCCGAAGCCTGGTAGGATAAGAATATAAACTTCTGGATGACCAAAAAATCAAAGAA
>Seqcp1 [organism= Cosminaparsina]
GAAATTTCCCTTGCCCTTTGGCATCATCTCGGCGATAGTGCCGACTTTTGCCAGAAGCCGCTGTTTGGTTATGCAT
CAATGGTGTATGCAACGTCAAGTATCGCGCTGCTGTCATTTTTGGTGTGAGCTCATCATATATTTACAGTAATC
>Seqcp1 [organism= Cosminaparsina]
TTCGCCCACCGCCGATCTTTGACGTTGCATACACCATTGCATGCATAACCAAACAGCGGCTTTCTGGCAAAAG
TATTGAAATATAGCCGAGATGATGCCAAAGGCAGGCAAAATCCTGATGTACACTTCTGGATGACCAAAAAATC
AAAGTA

Table 3:

Sample	Reference	Protein translation
Isomyiafulvicornis	Seqif1	IKFSLKSVFS PRFCDFLKKA PFRILFNGIR NGSYWFLRFH GSSSHVYII
Isomyiafulvicornis	Seqif2	YWLEARSRQL RYLEEIRKGA FLKKSQKFRR KYRKPAIPRC KLLDVQRIKI
Cosminalimbipennis	Seqcl1	TYSFGYLPYC NLLLRKKRTI WIHRYGLSYD INWLPRVYRV SSSYIYRK
Cosminalimbipennis	Seqcl2	SLIFWSSRSL YSYPTRLRNN LPYCNLLLRK KRTIWIHRYG LSYDIKGLL
Cosminaparsina	Seqcp1	NFPCPLASSR RCRLLPEAA VWLCINGVCN VKYRAAVIFG VSSSYIYSN
Cosminaparsina	Seqcp2	YFDFLVIQKC TSGFCLPLAS SRLYFNTFAR KPLFGYACNG VCNVKDRRWA

RESULT AND DISCUSSION

The present study pertains to the use of small *COI* marker for the identification of old dried pinned museum samples. The old samples generally loose the intact DNA as the tissue and cells decompose and thus only small amount of fragmented DNA is available for amplification. The author has been able to successfully amplify and sequence small region of COI gene for three species of family Calliphoridae, namely *Isomyiafulvicornis, Cosminalimbipennis*and*Cosminaparsina*. Sequencing results showed thatthis primer has worked satisfactorily well forthese old museum samples. The attempt was to try if this small fragment of *COI* gene holds some signature sequences specific for identification. The results shows that this small fragment does provide small percentage of match to the species but the percentage match is not very significant thus the author suggests cloning of these small fragments in a suitable vector.

REFERENCES

Bharti, M. 2011 "An updated checklist of blowflies (Diptera: Calliphoridae) from India." Halteres

Junqueira, A.C.M, Lessinger, A.C and Azeredo-Espin, A.M.L 2002 Methods for the recovery of mitochondrial DNA sequences from museum specimens of myiasis causing flies.Medical and Veterinary Entomology, (16)pp. 39-45

Srinivas Kambhampati andKaramjit S. Rai.1991Mitochondrial DNA variation within and among populations of the mosquito *Aedesalbopictus* ,*Genome*, 34(2): 288-292, 10.1139/g91-046

Tamura, Koichiro, *et al.*2013"MEGA6: molecular evolutionary genetics analysis version 6.0." *Molecular biology and evolution* 30.12: 2725-2729.