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Molecular Detection and Differentiation of *Hypoderma* Species (Diptera: Oestridae) Affecting Cattle in Pakistan

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Abstract.- *Hypoderma* spp. larvae are the cause of internal myiasis in different animals which is characterized by subcutaneous warbles. These are mostly differentiated at species level based on the morphology of third stage larvae. Therefore, the present study was conducted to amplify the most variable part of the *cox1* gene of two *Hypoderma* spp. (*i.e.*, *Hypoderma bovis* and *H. lineatum*) retrieved in Pakistan by PCR. This part encodes the region spanning from external loop 4 (E4) to carboxylic terminal. The nucleotide and amino acidic identity within the *cox1* gene of the two *Hypoderma* spp. were 89.5% and 93.4%, respectively. Particularly, the amino acid sequences of the two *Hypoderma* spp. herein analyzed were identical to that available in GenBank™ (*H. bovis*, GU984818 and *H. lineatum*, GU584123). The mean nucleotide composition was A=32.7%, C=16.6%, G=11.5% and T= 39.2%. The overall transition/transversion (Ts/Tv) ratio (R) was 1.008. In addition, the molecular analysis of PCR products was also performed. Nucleotide sequence changes and inter-specific variations will help in genetic differentiation of the examined species providing information for rapid molecular identification of *Hypoderma* spp.

Keywords: *Hypoderma bovis, Hypoderma lineatum*, Oestridae, mitochondrial DNA and sequencing.

INTRODUCTION

Hypodermosis are myiases characterized by the presence of warbles under the skin of wild and domestic ruminants both in developed and developing countries. Hypodermosis has become one of the most common parasitic diseases in many countries of the northern hemisphere. In Pakistan, the hypodermosis is endemic and much diffused among cattle, buffalo, sheep and goat (Khan *et al*., 1994; Ayaz, 1998; Ayaz and Khan, 1999).In another study, Khan *et al*. (2006) reported that the prevalence of hypodermosis was higher in slaughter house versus field, cattle versus buffaloes, males versus females, and young versus old animals in Dera Ghazi Khan and Rajan Pur districts of southern Punjab (Pakistan). Ahmed *et al*. (2013) reported that the animals of local breeds, extensive grazing system and primoinfested showed higher infestation rate in Potowar region of Pakistan.

Among *Hypoderma* species affecting wild and domestic animals, *Hypoderma bovis* (Linnaeus, 1758) and *Hypoderma lineatum* (De Villers, 1789) mainly parasitize cattle. The morphological methodologies available in the literature for the identification of *Hypoderma* larvae are limited and the identificationis mainly based on the morphology of third instars (James, 1947; Zumpt, 1965). Colwell *et al*. (1998) differentiated the third instars of four *Hypoderma* species (*i.e*., *H. actaeon*, *H. diana*, *H. bovis* and *H. lineatum*) by a comparative description of thoracic spines using scanning electron microscopy.

Additionally, molecular studies on partial cytochrome c oxidase subunit 1 (*cox1*) of mitochondrial DNA (mtDNA), frequently used as a DNA barcode, allowed distinguishing five common *Hypoderma* species (*i.e., H. actaeon*, *H. bovis*, *H. diana*, *H. lineatum* and *H. tarandi*), together with SEM analysis of key morphological features (Otranto *et al*., 2003). Balkaya *et al*. (2010) identified the *Hypoderma* spp. in east Turkey on the basis of mitochondrial *cox1* sequence analysis and PCR-RFLP. The aim of this paper is to present the molecular identification of two *Hypoderma*

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specimens coming from the same geographical area (Pakistan) and parasitizing the same host (cattle), by amplifying a specific fragment of *cox1* gene.

MATERIALS AND METHODS

Larvae collection and morphological identification

Current study was conducted in district Chakwal of Punjab, Pakistan. These districts are located in the northern part of the Punjab. The topography of the land is mainly hilly. Cattle, buffaloes, sheep and goat rising are a major agricultural enterprise of the fanners in this area. The larvae of warble flies were extracted from the backs of naturally infested cattle and buffaloes using the method described by Scholl and Barrett (1986). Two third stage larvae (L3) of *Hypoderma* spp. were collected together with all relevant information. The collected larvae were examined for morphological characteristics like shape, size, colour and pattern of spinulation under a stereomicroscope (Leica MS5; Leica, Heidelberg, Germany) to confirm their identity by morphological keys (James, 1947; Zumpt, 1965) and stored in 70% ethanol for further processing.

Genomic DNA extraction, PCR and sequencing

Genomic DNA was isolated from larval dried internal organs using DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). The most variable part of the cox1 gene which is responsible for encoding the area spanning from external loop 4 (E4) to carboxylic terminal, was amplified by PCR using specific UEA7 primers
(5'-TACAGTTGGAATAGACGTTGATAC-3')
and UEA10
(5'-TCCAATGCACTAATCTGCCATATTA-3')
originally designed by Zhang and Hewitt (1996). The molecular analysis was conducted for the partial *cox1* gene. The PCR amplification was carried out in a total volume of 50 µl, including ~100 ng of genomic DNA, 10 mM Tris HCl, pH 8.3 and 50 mM KCl, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 50 pmol of each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA).

The following amplification protocol was employed in a thermal cycler (2720, Applied Biosystems): 95°C for 10 min (for polymerase activation), followed by 40 cycles each of 95 °C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min and 10 sec (extension), followed by 7 min at 72°C (final extension). Negative controls (no DNA template) were included in all PCR reactions which were run in a Thermal Cycler 2720 (Applied Biosystems, Foster City, CA, USA).

Amplicons were resolved in ethidium bromide-stained agarose (Gellyphor, EuroClone, Milan, Italy) gels (1.5%) and sized by comparison with Gene RulerTM 100-bp DNA Ladder (MBI Fermentas, Vilnius, Lithuania) as molecular marker. Gels were photographed using Gel Doc 2000 (Bio-Rad, Hercules, CA, USA). Amplicons produced were purified using Ultrafree-DNA spin columns (Millipore, Bedford, MA, USA) and then sequenced directly using the*Taq*DyeDeoxyTerminator Cycle Sequencing Kit (v.2, Applied Biosystems, Foster City, CA, USA) in an ABI-PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were then determined from both strands using the same primers as used for the primary PCR.

The similarities among the primary nucleotide sequences for each *Hypoderma* species and that of *H. lineatum* (GU584123) were determined by BLASTX analysis (Altschul *et al.*, 1997) and alignment using the ClustalW program (Larkin *et al.*, 2007). The level of sequence differences (D) was made using the formula D= 1 - (M/L) (Chilton *et al.*, 1995), where M is the number of alignment positions at which the two sequences had a base in common, and L was the total number of alignment positions over which the two sequences were compared.

RESULTS

The profiles of the amplicons here examined showed specific patterns of 480bp in size (Fig. 1). Sample 1 was 100% homologous to *Hypoderma bovis* (Accession Number: GU984818; Balkaya *et al.*, 2010), whereas sample 2 resulted 99.7% homologous to *Hypoderma lineatum* (Accession Number: GU584123; Weigl *et al.*, 2010).

The mean nucleotide composition was A=32.7%, C=16.6%, G=11.5% and T= 39.2%. The

Table I.- Percentage of synonymous and non-synonymous substitutions and variable sites occurring in total sequences at different codon positions of *cox1* gene and variable, informative and singleton sites of amino acids calculated for the two *Hypoderma* species herein analysed.

Nucleotide codon position	Number of characters	Synonymous substitutions (%)	Non-synonymous substitutions (%)	Variable sites
Total sequence	640	-	-	67
1st	213	37.5	62.5	16
2nd	213	0	100	5
3rd	214	80.4	19.6	46
Amino acids	213	-	-	14

overall transition/transversion (Ts/Tv) ratio (R) was 1.008. The molecular data of *cox1* gene for the two species is given in Table I**.** Out of the total variable sites, synonymous substitutions and non-synonymous substitutions were 37.5 % vs 62.5%, 0.0% vs 100% and 80.4 % vs 19.4% at 1st, 2nd and 3rdnucleotide codon position respectively. The final alignment of the sequences included a total of 213 characters, 14 of which were variable characters.

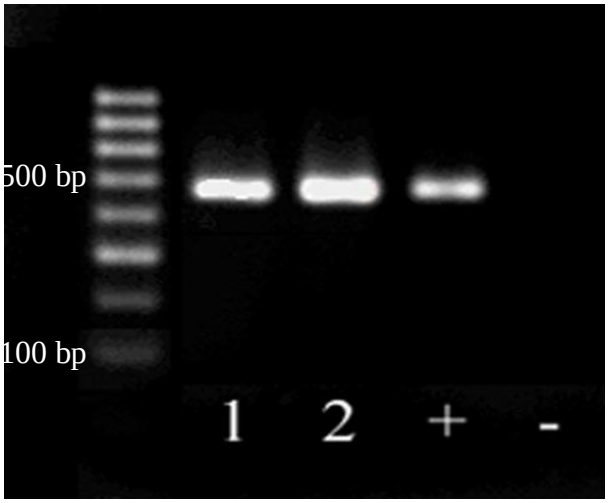

Fig. 1. Electrophoretic patterns of PCR amplification from mtDNA carried out using primers UEA7-UEA10: 100-bp ladder Gene Ruler™- MBI Fermentas (lane 1), samples 1 and 2 (lanes 2-3), positive and no-DNA control (lanes 4-5).

The BASTX analysis confirmed the monophyly of species here analyzed separating sample 1 from sample 2 with a high level of sequence differences. Sequence differences between the two species were due to insertions/deletions and to substitution events in certain areas (Fig. 2).

DISCUSSION

The present study revealed the usefulness of this method for a clear discrimination of the most widely distributed *Hypoderma* spp. in cattle. Particularly, the molecular identification of those morphologically similar species which sometimes parasitize the same host (*e.g.*, *H. bovis* and *H. lineatum*) could overcome the difficulties linked to the identification of both larvae and adult flies.

The *cox1* sequences of both species did not display false stop codons or frameshift mutations causing insertions or deletions. The nucleotide and amino acidic identity within the *cox1* gene of the two *Hypoderma* spp. were 89.5% and 93.4%, respectively (Figs. 2-3). Particularly, the amino acid sequences of the two *Hypoderma* spp. herein analyzed were identical to that available in GenBank™ (*H. bovis*, GU984818 and *H. lineatum*, GU584123). The overall A + T content was 71.2% and 72.5% for *H. bovis* and *H. lineatum*, respectively, with the highest bias of adenine and thymine detected at the third codon position (mean value =91%), according to the lower selection and mutational pressure (Jermiin *et al*., 1994). Balkaya *et al*. (2010) performed morphological identification and molecular characterization of *Hypoderma* species on the basis of *cox1* sequence analysis and PCR-RFLP. Two hundred and twenty three (28.6%) out of 778 cattle were seropositive for hypoderma antibodies. All positive cattle were female of a local breed. Seven out of 10 *Hypoderma* larvae were morphologically classified as third instar larvae (L3) of *H. bovis* and 3 were classified

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Sample1     TACAGTTGGA ATAGACGTTG ATACCAAGGCTATTTTACTTTCAGCACAAATAATTATTGCTGTACCAACT
Sample2     .......... .......... ....ACGA.. .TA...TACA ......AC.. .......... ..........

Sample1     GGAATTAAAA TTTTCAGATG ATTAGCAACT TTACATGGAA CACAAATCAA CTACTCTCCT GCAACTTTAT
Sample2     .......... ....T..... .C........ C......... .G.....T.. T......... ..........

Sample1     GATCTTTAGG GTTTGTTTTT TTATTTACAG TTGGAGGATT AACTGGAGTA ATTTTAGCTA ATTCATCTAT
Sample2     ....A..... A......... .......... .A........ .......... .......... ..........

Sample1     TGATATTATT TTACATGATA CATATTATGT AGTAGCTCAC TTCCATTATG TTTTATCTAT AGGAGCTGTA
Sample2     .......... .......... .......... .........T. .T..C.... .......... ..........

Sample1     TTTGCCATTA TAGCTGGATT CATTCATTGA TTTCCATTAT TTACAGGATT AACATTAAAT GTTAAACTAT
Sample2     ..C..T..C. ....A..... T......... .....TC... .......... ...T...... A.A...T...

Sample1     TAAAAAGCCA ATTTGTCATT ATATTTTTAG GAGTAAATTT AACCTTTTTC CCTCAACACT TTTTAGGGTT
Sample2     ......AT.. ....A.T... .......... .......C.. ...T...... ........T. .......AC.

Sample1     AGCTGGCATA CCTCGACGTT ATTCTGATTA TCCAGATGCT TATACCACAT GAAATGTAAT TTCAACTATT
Sample2     ...A..A... ..A.....A. .......C..C......... ..C....... .......... ......A..A

Sample1     GGATCATCAA TTTCTCTTTT AAGTATTTTA TTATTTCTAT TTATTATTTG AGAAAGATTA CTATCACAAC
Sample2     ..T....... .....A.A.. ...A..C... ..T...A... .......... ....G..... A.........

Sample1     GACAAGTATT ATTTCCTATC CAATTAAATT CATCAATTGA ATGATTACAA AATACACCAC CCTCTGAACA
Sample2     .......... ...C.....T .......... .......... ....C..... .......... .A........

Sample1     CTCTTATTCT GAACTCCCAT TATTAACTAA TTTCTAATAT GGCAGATTAG TGCATTGGA
Sample2     T......... .......... .......... .......... .......... .........
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Fig. 2. Alignment (5'–3') of *cox1* gene of the two species examined *Hypodermabovis* (sample 1) and *Hypodermalineatum* (sample 2). Grey are the sequences of the oligonucleotide primers used in the PCR assay, bold and underlined is the noncoding sequence. Identical bases are indicated by a dot. The complete genes included in the alignment are indicated between the lines.

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Sample1    TVGMDVDTKAILLSAQMIIAVPTGIKIFSW LATLHGTQIN YSPATLWSLG FVFLFTVGGL
Sample2    ........R.YFT..T.............. .......... .......... ..........

Sample1    TGVILANSSIDIILHDTYYVVAHFHYVLSM GAVFAIMAGF IHWFPLFTGL TLNVKLLKSQ
Sample2    ............................... .......... .......... ...M....N.

Sample1    FVIMFLGVNLTFFPQHFLGLAGMPRRYSDY PDAYTTWNVI STIGSSISLL SILLFLFIIW
Sample2    .I............................. .......... ..M.....M. ...F.M....

Sample1    ESLLSQRQVLFPIQLNSSIEWLQNTPPSEHSYSELPLLTNF*YGSLVHW
Sample2 .  G.M.........................................
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Fig. 3. Alignment (5'–3') of amino acid sequences of *cox1* gene of the two species examined *Hypodermabovis* (sample 1) and *Hypodermalineatum* (sample 2). Identical amino acids are indicated by a dot.

as L3 of *H. lineatum*. The TaqI restriction enzyme was used to differentiate the *Hypoderma* spp. on the basis of the 438 and 250 bp bands for *H. bovis* and the 488 and 200 bp bands for *H. lineatum* resulting from PCR-RFLP. According to the alignment of the mitochondrial CO1 sequences of the *Hypoderma* species and the PCR-RFLP results, all examined larvae were classified as *H. bovis*.

Samuelsson *et al*. (2013) examined carcasses for muscle and tissue parasites, and recorded warble larvae infestations concurrent with the hunting of musk oxen. DNA extracted from samples of larvae

was amplified targeting 579 bp of the COI gene, and subsequently sequenced to be confirmed as *H. tarandi*.

Similarly in the region like China, Otranto *et al.* (2004) reported that cattle and yak hypodermosis in China is caused by *Hypoderma bovis* and *H. lineatum*, with prevalence reaching up to 98-100% of the animals and maximum intensities exceed 400 warbles for each animal. The molecular characterization of the most variable region of the mitochondrial cytochrome oxidase I gene and of the ribosomal 28S gene has been performed for the third-stage larvae collected from cattle and yaks in China and identified (on the basis of the spinulation on the ventral side of the 10th segment) as *H. bovis*, *H. lineatum*, and *H. sinense*.

The results here reported also provide new data into the inter-specific variability within genus *Hypoderma*.

CONCLUSION

The present study revealed the usefulness of this method for a clear discrimination of the most widely distributed *Hypoderma* species in cattle. Particularly, the molecular identification of those morphologically similar species which sometimes parasitize the same host (*e.g.*, *H. bovis* and *H. lineatum*) could overcome the difficulties linked to the identification of both larvae and adult flies. The results here reported also provide new data into the inter-specific variability within genus *Hypoderma*.

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