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 carboxyterminal. The nucleotide and amino acid 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, GU854123). 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

Hypoderma spp. larvae are the cause of internal myiasis in different animals which is 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 slaughtered 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.

According to Ayaz and Khan, 1998; Ayaz, 1998; Ayaz and Khan, 1999, and Khan et al. (2010), among Hypoderma species affecting wild and domestic animals, Hypoderma bovis (Linnaeus, 1758) and Hypoderma lineatum (De Villers, 1789) are the most important species parasitizing cattle. The morphological methodologies available in the literature for the identification of Hypoderma larvae are limited and the identifications 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
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 farmers 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 MgCl2, 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 theTaqDyeDeoxyTerminator 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.

<table>
<thead>
<tr>
<th>Nucleotide codon position</th>
<th>Number of characters</th>
<th>Synonymous substitutions (%)</th>
<th>Non-synonymous substitutions (%)</th>
<th>Variable sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sequence</td>
<td>640</td>
<td>-</td>
<td>-</td>
<td>67</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt;</td>
<td>213</td>
<td>37.5</td>
<td>62.5</td>
<td>16</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt;</td>
<td>213</td>
<td>0</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt;</td>
<td>214</td>
<td>80.4</td>
<td>19.6</td>
<td>46</td>
</tr>
<tr>
<td>Amino acids</td>
<td>213</td>
<td>-</td>
<td>-</td>
<td>14</td>
</tr>
</tbody>
</table>

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 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup>nucleotide codon position respectively. The final alignment of the sequences included a total of 213 characters, 14 of which were variable characters.

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...
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.

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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