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▣ ORIGINAL ARTICLE

First Molecular Characterization of *Hypoderma actaeon* in Cattle and Red Deer (*Cervus elaphus*) in Portugal

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Abstract: *Hypoderma* spp. larvae cause subcutaneous myiasis in several animal species. The objective of the present investigation was to identify and characterize morphologically and molecularly the larvae of *Hypoderma* spp. collected from cattle (*Bos taurus taurus*) and red deer (*Cervus elaphus*) in the district of Castelo Branco, Portugal. For this purpose, a total of 8 larvae were collected from cattle (n=2) and red deer (n=6). After morphological identification of *Hypoderma* spp. larvae, molecular characterization was based on PCR-RFLP and mitochondrial *CO1* gene sequence analysis. All larvae were morphologically characterized as the third instar larvae (L3) of *H. actaeon*. Two restriction enzymes were used for molecular identification of the larvae. *TaqI* restriction enzyme was not able to cut *H. actaeon*. However, *MboII* restriction enzyme differentiated *Hypoderma* species showing 210 and 450 bp bands in *H. actaeon*. Furthermore, according to the alignment of the mt-*CO1* gene sequences of *Hypoderma* species and to PCR-RFLP findings, all the identified *Hypoderma* larvae were confirmed as *H. actaeon*. This is the first report of identification of *Hypoderma* spp. (Diptera; Oestridae) from cattle and red deer in Portugal, based on morphological and molecular analyses.

Key words: *Hypoderma actaeon*, cattle, red deer, phylogenetic analysis, PCR-RFLP, Portugal

INTRODUCTION

Hypodermosis, one of the most common parasitic diseases in the northern hemisphere [1], is a myiasis caused by warble flies of the genus *Hypoderma* which is common in Bovidae and Cervidae of the Holarctic region. In Europe, red deer (Cervus elaphus) may be infected by *Hypoderma diana* and *Hypoderma actaeon*. *H. diana* is also found in roe deer (*Capreolus capreolus*) which is its main host, but *H. actaeon* seems to be restricted to *Cervus elaphus* [2].

Some epidemiological studies on hypodermosis have been conducted in wild ungulates in central Europe [3,4], France [5], and southern and central Spain [6,7]. In central Spain, an epidemiological study conducted in red deer was based on the inspection of shot animals, during the period of warble detection. All the third instar (L3) were classified as *H. actaeon*, and the global prevalence was 61%. The prevalence in yearling and adult deer shot during the official hunting season was 89% [8]. Despite the life cycle of *Hypoderma* sp. in deer at the western Mediterranean area is not well known, cattle hypodermosis is recognized all over the world [9].

The third instar *Hypoderma* larvae (L3) can be identified on the basis of their morphological characters. These characters are peritreme structure and the spinulation pattern (presence/absence) in their 10th larval segment [2]. More recently, all 4 Hypodermatinae (*H. lineatum, H. bovis, H. diana*, and *H. actaeon*) were accurately characterized by scanning electron microscopy (SEM) by comparing thoracic spines [10]. Additionally, molecular studies on cytochrome *c* oxidase subunit 1 (*CO1*) sequences of the mtDNA has been used as a target gene for the identification studies, allowing the distinction of 5 common *Hypoderma* species together with electron microscopic analysis of key morphological features [11]. Furthermore, *Hypoderma* spp. have been identified in east Turkey by sequence analysis of mt-*CO1* gene and by using PCR-RFLP assay [12].

The identification of *Hypoderma* larvae described in the liter-

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ature is scarce, and it is mainly based on the morphology of the 3rd instars [2]. On the other hand, PCR-RFLP and nucleotide sequences of regions of mt-*CO1* gene have provided precious information on the classification of *Hypoderma* spp. [13]. Nowadays, the mt-*CO1* gene has been extensively used as a target gene in many identification and molecular phylogenetic studies [14], including *Hypoderma* spp. [12,15]. This study aimed to identify morphologically and molecularly *Hypoderma* spp. collected from domestic and wild ruminants in Portugal (cattle and red deer), by morphological characterization, PCR-RFLP analysis, and sequencing analysis of a specific fragment of mt-*CO1* gene.

MATERIALS AND METHODS

Study area

The specimens were collected from hunted red deer (6 larvae) and slaughtered cattle (2 larvae) in the region of Castelo Branco (39°8′N; 7°5′W) and Idanha-a-Nova (39°9′N; 7°2′W) during 2011. Idanha-a-Nova and Castelo Branco are 2 municipalities located at the center of Portugal. Every year in this region, hunting activities are organized to control the population of cervids and wild boars. The samples were collected from shot red deer and slaughtered cattle in an effort of adding some more information about this parasite in domestic and wild ruminants. The 2 municipalities are located at 400 m of altitude in a region with a Mediterranean climate with continental influence. The annual average temperature is 15.3℃, and the annual average precipitation 881 mm. July is usual the driest month, and January is the rainiest month.

Sample collection

Hypoderma spp. naturally infected in red deer (*Cervus elaphus*) and cattle (*Bos taurus taurus*) were found by the palpation of larvae on the back, flanks, and chest, and by the examination of skin, subcutaneous soft tissues, and subcutaneous muscles, to check for gross changes, such as congestion, bleeding, nodules or lumpy lesions, cysts, and abscesses or suppurative lesions. Larvae were collected, washed several times in PBS (pH 7.4) to remove mucosal debris, preserved in 70% ethanol, and stored at -20℃ until analysis.

Morphological identification

The morphological characterization of *Hypoderma* larvae was performed by using the identification key of Zumpt [2]. Morphological characteristics, such as the shape, size, colour, and pattern of spinulation under a stereomicroscope (Olympus SZX16, Olympus Life Science, Tokyo, Japan) were evaluated. The larval bodies were kept at 70% ethanol until molecular procedures.

Genomic DNA extraction

The total gDNA was obtained from the internal tissue of all individual *Hypoderma* larvae using commercial Genomic DNA Purification kit® (K0512, Fermentas, Vilnius, Lithuania) with slight modifications. For instance, prior to isolation of gDNA, the ethanol-fixed *Hypoderma* larvae were washed at least 5 times with saline solution (PBS). The digestion of internal tissues was carried out by the incubation overnight at 56℃ with 200 µl lysis buffer provided by the kit to which 20 µl proteinase-K (20 mg/ml) (Sigma, St. Louis, Missouri, USA) was added. After the digestion step, the protocol of the manufacturer was followed. The pellet was resuspended in 100 µl elution buffer, and gDNA were stored at -20℃ until further use.

PCR

The amplification of fragments of mt-*CO1* gene was done by PCR using conserved specific primers previously described by Otranto et al. [11] (UEA7, 5′-ACAGTTGGAATAGACGTT GATAC-3′ and UEA10, 5′-TCCAATGCACTAATCTGCCATAT-TA-3′) in a total volume of 50 µl containing 5 µl 10× PCR buffer, 5 µl 25 mM $MgCl_2$, 4 µl of each dNTPs, 20 pmol of each primer, 200 ng of template DNA and 1.25 U of Taq DNA polymerase (MBI, Fermentas). The PCR conditions were 2 min at 95℃ (initial denaturation), 40 cycles of 1 min at 95℃, 1 min at 52℃ and 1 min at 72℃ and finally 7 min at 72℃ (final extension). The PCR products were separated on agarose gel (1.4%) and stained with ethidium bromide.

PCR-RFLP

The PCR products were digested at 65℃ overnight with restriction endonuclease *TaqI*, using buffers recommended by the manufacturer (MBI, Fermentas) in 36 µl of the final volume reaction mix, containing 20 µl of PCR product, 10 U enzyme (*TaqI*, 10 U/µl), 4 µl restriction buffer, and 11 µl distilled water. The restriction fragments were separated on 3% agarose gels and stained with ethidium bromide [11].

For *MboII* enzyme, PCR products were digested at 37℃ during 5 hr, using buffers as recommended by the manufacturer (MBI, Fermentas) in a final 31 µl volume reaction mix, con-

taining 10 µl of PCR product, 10 U enzyme (*MboII* 10 U/µl), 2 µl restriction buffer, and 18 µl distilled water. The restriction fragments were separated on 3% agarose gels and stained with ethidium bromide. All gDNA positive control samples (previously sequenced) were provided by the Molecular Parasitology Laboratory of Veterinary Faculty, Firat University, Turkey.

mt-*CO1* sequence and phylogenetic analysis

All the mt-*CO1* PCR products of cattle and deer samples were sequenced. Their nucleotide sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

Multiple sequence alignment and phylogenetic tree construction of the obtained sequence of the mt-*CO1* gene were performed with the use of CLC Main Workbench software (CLC, Aarhus, Denmark) [16]. Unidirectional DNA sequence analysis of the mt-*CO1* gene of all samples were performed. Unreliable ends of the raw sequences were trimmed, and then the phylogenetic tree was built using the neighbour-joining method [17]. Based on pairwise comparisons, sequence differences were calculated using the CLC Main Workbench software. The reliability of the inferred tree was evaluated by bootstrap analysis of 1,000 replicates. Reference sequences of *H. bovis* (KT600293), *H. lineatum* (KP965726), *H. diana* (AF 497763), *H. sinense* (KT600316), *H. tarandi* (AF497764), and *H. actaeon* (AF497765) were also included for phylogenetic analysis.

RESULTS

Morphology

According to the morphological characterization of *Hypoderma* spp. based on the 3rd instar larvae by stereomicroscope, all of the larvae (n = 8) from cattle and red deer were classified as *H. actaeon*. The spiracular plate of *H. actaeon* was recessed, and the ecdysal scar well below the surface of the spiracular plate. The evident "C" shaped structures was raised, and the rima was quite open (Fig. 1).

PCR-RFLP

Furthermore, all the specimens were classified as *H. actaeon* by molecular identification of *Hypoderma* spp. larvae which was carried out by PCR-RFLP and mt-*CO1* gene sequence analysis. Concerning to PCR-RFLP analysis, *TaqI* restriction enzyme was used to discriminate the *Hypoderma* species. However, it was impossible to cut fragments of mt-*CO1* gene by PCR-RFLP while *MboII* restriction enzyme was able to discriminate *Hypoderma* species by the presence of 688 bp bands observed in *H. bovis* and *H. lineatum* (no cut), used as a control, and by the presence of 210 and 450 bp bands of *H. actaeon* (Fig. 2).

mt-*CO1* sequence analysis

Blast analysis of *Hypoderma* spp. sequences in the NCBI was performed and confirmed all the specimens as *H. actaeon*. We found that these sequences matched with mostly mt-*CO1*.

Fig. 1. Morphological identification of *Hypoderma actaeon* larvae. Plate cover by the ecdysal scar. The evident "C" shaped structures is raised and the rima is quite open.

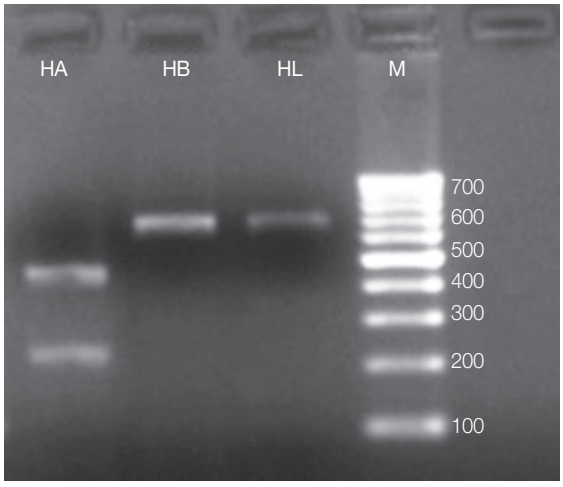

Fig. 2. Molecular differentiation of *Hypoderma* spp. isolates (red deer/cattle) by PCR-RFLP based on *MboII* restriction enzyme. M, Marker-100 bp; HB, *Hypoderma bovis* (688 bp bands); HL, *Hypoderma lineatum* (688 bp); HA, *Hypoderma actaeon* (210 and 450 bp).

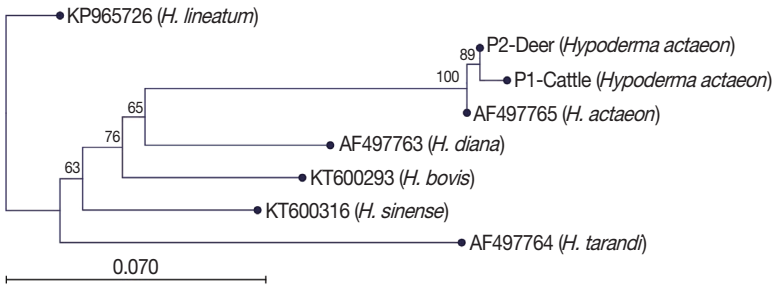

Fig. 3. Phylogenetic tree view of the aligned sequences.

Then, the sequence results were performed to pairwise analysis for genetic distances. The pairwise comparison and phylogenetic tree obtained from the sequences confirmed that all *Hypoderma* larvae were classified as *H. actaeon* (Fig. 3). All sequence results were similar and there were no important variation among the sequences.

DISCUSSION

Hypodermosis shows a worldwide distribution [9] and has been reported in 55 tropical and subtropical countries [18]. This parasitosis is also prevalent in European countries. So far there are limited reports in Portugal despite the high prevalence reported in a neighbour country (i.e. Spain) [19]. Deer hypodermosis is very common in Spain. The prevalence was estimated at 80% [6] and a high intensity of parasite infection was reported (average of 40 larvae) [6,7]. Notwithstanding, more recent studies showed a slight decrease of the prevalence, ranging from 44.8% [20] to 61.0% [8], but the infection intensity remains similar (average of 38.3 larvae) [20].

In wild ungulates, high prevalences of hypodermosis have been reported. In Hungary, 65% of red deer (*Cervus elaphus*) and 98% of roe deer (*Capreolus capreolus*) were infected by *H. diana* while 93% of red deer were infected with *H. actaeon* [4]. *H. tarandi* was found in 97% to 100% of Canadian caribou (*Rangifer tarandus*) [21]. In Europe, *H. actaeon* is recognized as a typical parasite in red deer [2]; however, an incidence of 89% of hypodermosis in red deer caused by *H. diana* was reported in Spain [6]. These species are known as strictly host-specific [2]. A seroprevalence study of bovine hypodermosis was conducted in the area of upper Tagus river of Portugal showing a seroprevalence of 35% [22].

This study describes for the first time the presence of *H. actaeon* in red deer and cattle in Portugal. Regarding the red deer, these findings are in agreement with other studies conducted in Spain where *H. actaeon* was found in red deer and in roe deer (*Capreolus capreolus*) from central Spain [7,23]. It was not possible to confirm the presence of *H. diana* as found in southern Spain [6]. Notwithstanding, the low number of specimens does not allow the exclusion of the possibility of coexistence of these 2 species as it was also observed in Hungary [4].

A limited number of studies based on the morphological identification of *Hypoderma* larvae from cattle and especially red deer (*Cervus elaphus*) are available in the literature. These were mainly based on the morphological features of the 3rd instars [2]. In the present study, all the *Hypoderma* larvae were morphologically identified as *H. actaeon*. Regarding the deer hypodermosis, *H. diana* and *H. actaeon* are the main species that infect deer. These 2 species are morphologically different, since *H. diana* has a distinctive band of spines on the cephalic segment, which are absent in *H. actaeon*. These differences facilitate the identification of these species [10].

Nevertheless, morphological discrimination of *Hypoderma* spp. is not considered an absolutely reliable methodology for identification. *Hypoderma* from different geographical origins may present only slight morphological differences. Similarly, it is very difficult to differentiate *H. lineatum*, *H. bovis*, and *H. actaeon* from cattle and red deer due to the colour and spinulation patterns. Other difficulties in morphological identification might be due to inappropriate preservation and dark brown colour of the larvae. A more reliable method to identify *Hypoderma* larvae is using PCR-RFLP based on mt-*CO1* gene and sequence analysis [24]. The molecular identification based on mt-*CO1* gene has been used to differentiate the 5 common *Hypoderma* spp. (i.e., *H. diana*, *H. bovis*, *H. lineatum*, *H. actaeon*, and *H. tarandi*) [11].

In the present study, a comparative differentiation of *H. bovis* and *H. acteon* was done by using PCR-RFLP based on *TaqI* and *MboII* restriction enzymes. It reflects the significance of PCR-RFLP method for the accurate identification of *Hypoderma*

spp. in cattle and red deer. The molecular characterization of *Hypoderma* spp. (*H. bovis*, *H. lineatum*, and *H. sinense*) was based on mt-*CO1* gene [25]. According to the mt-*CO1* gene sequences analysis and PCR-RFLP findings, all the larvae were molecularly characterized as *H. actaeon* confirming the results of the morphological identification. Our findings on molecular characterization are similar to those reported by Otranto et al. [11,26]. In Portugal, no previous studies based on morphological and molecular characterization of *Hypoderma* spp. are available.

A partial mt-*CO1* gene was successfully used for amplification of the *Hypoderma* spp. The gene segment was sequenced and compared to previously determined sequences of *H. bovis* and *H. lineatum*. The evolutionary rate of different insects (order; Orthoptera) has been determined by using mt-*CO1* gene [27]. The evolutionary rate of different insects (Orthoptera, Diptera, and Hymenoptera) has been determined by using mt-*CO1* gene [14,27]. To define the relationships of *H. sinense* to *H. lineatum* and *H. bovis*, the mt-*CO1* gene was used as a target for sequence analysis. The clear differentiation of *Hypoderma* species (*H. sinense* from *H. lineatum* and *H. bovis*) was done by comparing the mt-*CO1* gene sequences.

In conclusion, our findings allowed us to state confidently that *H. actaeon* is an occurring species in red deer and cattle in Portugal. PCR-RFLP is recommended for species identification as compared to sequencing. This study opens new directions of research on *Hypoderma* spp. in different hosts and different geographical areas of Portugal.

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CONFLICT OF INTEREST

The authors fully declare no financial or other potential conflict of interest.

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