DOI 10.2478/s11687-014-0220-0

HELMINTHOLOGIA, 51, 2: 130 – 140, 2014

Comparative morphological and molecular identification of *Haemonchus* species in sheep

J. VADLEJCH[1*], D. LUKEŠOVÁ[2], J. VAŠEK[3], P. VEJL[3], P. SEDLÁK[3], Z. ČADKOVÁ[1], I. LANGROVÁ[1], I. JANKOVSKÁ[1], O. SALABA[1]

[1]Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 957, 165 21 Prague 6 – Suchdol, Czech Republic, [*]E-mail: *vadlejch@af.czu.cz*; [2]Department of Animal Sciences and Food Processing in Tropics and Subtropics, Institute of Tropics and Subtropics, Czech University of Life Sciences Prague, Kamýcká 165, 165 21 Prague 6 – Suchdol, Czech Republic; [3]Department of Genetics and Breeding, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, Kamýcká 957, 165 21 Prague 6 – Suchdol, Czech Republic

Summary

A combined approach in the determination of *Haemonchus* nematodes from sheep was applied in this trial. Using selected morphological characters 90.2 % females and 84.2 % males of *Haemonchus contortus* and 9.8 % females and 15.8 % males of *Haemonchus placei* were identified. Although cluster analysis based on morphological identification clearly separated two *Haemonchus* species, *H. contortus* was exclusively detected in all specimens using restriction cleavage of the ITS-2 region with *FspB*I endonuclease as well as through the sequencing analysis. Because sheep from both farms have never had contact with other ruminants, and the farmers apply only closed flock turnover, we assume that only *H. contortus* mono-infection occurred on both farms. This opinion is also supported by molecular data. The most striking result of our study was the finding which indicates that the discriminant function is not able to accurately identify *Haemonchus* males at the species level.

Keywords: Barber's pole worm; spicule; discriminant function; PCR-RFLP; sequencing; internal transcribed spacer

Introduction

Haemonchus (large stomach worm, Barber´s pole worm, twisted stomach worm or wire worm) is a pathogenic haematophagous nematode that infecting the abomasum of ruminant hosts. There are two major species that cause economic losses in the ruminant livestock industry worldwide. *Haemonchus contortus* (Rudolphi, 1803) primarily infects the abomasum of domestic sheep (*Ovis aries*), but it can also be found in numerous other ruminants such as goats, llamas, antelopes, cervids, giraffes, and cattle. This nematode is often used as a model organism (Várady *et al*., 2007; 2009; Bártíková *et al*., 2010; Königová *et al*., 2012; Šnábel *et al*., 2012). *Haemonchus placei* (Place, 1893) is a principal parasite of domestic cattle (*Bos taurus*); however, it can infect other ruminant hosts (goats, antelopes, cervids, etc.) including sheep (Hoberg *et al*., 2004; Scott & Sutherland, 2009). There are far fewer articles about this nematode, unlike *H. contortus*.

Because only minor morphological differences between *H. contortus* (*Hc*) and *H. placei* (*Hp*) exist, there was debate in the past on whether they really represented different species. Some authors (Gibbons, 1979; Taylor *et al.*, 2007) synonymized these two parasites, but currently, there is strong evidence that *Hc* and *Hp* are different species. Lichtenfels (Lichtenfels *et al*., 1986; 1988) described morphological differences between appointed species, and Hoberg *et al*. (2004) revealed relationships among thirteen species of *Haemonchus* (*Hc* and *Hp* included) through phylogenetic analysis of twenty-five morphological characters. The cytological (Bremner, 1955) and especially molecular biological characters (Zarlenga *et al*., 1994; Stevenson *et al*., 1995; Blouin *et al*., 1997; Brasil *et al*., 2012) also confirmed that *Hc* and *Hp* are two different species.

Nevertheless, the determination of the *Haemonchus* species discussed above has yet to be adequately resolved. Lichtenfels *et al*. (1994) created a determination key based on surface cuticular ridges (synlophe), spicule length in males and tail length in females. This work provides additional morphological criteria used to identify *Haemonchus* adults to the species level. Unfortunately, the values of these characters often overlap and such determination may be misleading. For differentiation between *Hc* and *Hp*, discriminant function combining three measurements of male spicules (Robert *et al*., 1954; Jacquiet *et al*., 1997) or three measurements of female body (Giudici *et al*., 1999) can be utilized. Nevertheless, these morphological charac-

ters have not yet been verified by molecular methods. Other authors (Zarlenga et al., 1994; Stevenson et al., 1995; Brasil et al., 2012) have identified *Haemonchus* species through molecular data analysis alone, without any morphological confirmation. Relying solely on one type of data (morphological or molecular) can lead to inaccurate interpretation of results.

Accuracy of species identification is essential for certain types of studies, particularly when *Hc* and *Hp* are sympatric in many regions of the world. The aim of this study was to identify *Haemonchus* males and females from sheep, to the species level according to morphological characters and to verify these results using molecular tools. This is the first time this combined approach was used for *Haemonchus* species determination.

Material and methods

Study area, hosts and parasites

Two different types of sheep breeding systems of the Czech Republic were chosen for the purpose of this study. The first sheep farm was located in the area of Southern Bohemia and represented the traditional breeding type. Sheep (Oxford down x Suffolk) remained on the pasture until snow cover, at which time they were moved to a stable. Traditional management system methods with strategic deworming were practiced on this farm. An ecological farm in Middle Bohemia presented the second type of sheep breeding. Sheep (OD x SF) on this farm grazed the pasture most of the year, and spent the winter months in a stable. Ecological farming methods were applied on this farm, and animals had no contact with anthelmintic drugs. The pasture of both farms had never been grazed by other animals. The climate conditions were the same at both locations.

Prior to this experiment, sheep from both study areas were coprologically examined using the Concentration McMaster method (Roepstorff & Nansen, 1998). Trichostrongylid nematode egg positive faecal samples were subjected to coproculture, and obtained infective larvae were identified to the genus level according to van Wyk *et al*. (2004). To minimize morphological variability of nematodes caused by host immune system, only one *Haemonchus* sp. positive adult sheep of the same age from each study area was incorporated in the experiment.

Sheep were necropsied, and their abomasa were collected and promptly transported to the parasitological laboratory at the Czech University of Life Sciences Prague. The abomasum was opened and washed thoroughly, and the abomasal content was placed into a bucket containing physiological saline. The nematodes were obtained by washing the content in a sieve. All adult *Haemonchus* spp. nematodes were collected, washed several times in a PBS buffer (pH 7.4) and stored in 70% ethanol.

Morphological identification

All obtained *Haemonchus* spp. males and females were identified by their morphological characters to the species level according to Lichtenfels *et al*. (1994) and Jacquiet *et al*. (1997). Characters measured in females included body length, distance of cervical papillae from head, oesophagus length, oesophagus length as a percentage of total body length, tail length, and the distance of the right and left phasmid from the distal tip of the tail. Vulval morphology was also recorded. In male nematodes, the following morphological attributes were measured: body, spicule, left and right spicule barb length and gubernaculum. The discriminant function (DF) combining three characters of male spicules, the total length of the spicule (*TL*), and the distance from the tip to the barb of the right (*THr*) and left (*THl*) spicule, was calculated. DF was expressed as $DF = 0.0016\ TL + 0.128\ THr + 0.152\ THl - 9.97$. Species identification was established as follows: $DF < 0.63$ for *Hc* and $0.63 < DF < 3$ for *Hp*. For better observation under a microscope, the male bursa was cut and cleared in phenol-alcohol (80 parts melted phenol crystals and 20 parts absolute ethanol). All specimens were studied using an Olympus BX51 microscope at a total magnification of 200 – 400x, and morphological characters were measured with QuickPHOTO MICRO 3.0 software.

Molecular identification

DNA isolation

All nematode specimens identified using morphological characters were subjected to molecular analysis. Each specimen was rinsed in distilled water and placed into a 0.5 ml tube, into which 300 µl of de-ionized water and 25 µl proteinase K (Machery Nagel) was added. The tubes were heated at 56 ºC in a thermoblock Bio-TDB 120 (Biosan) for one hour. DNA isolation was then carried out using a NucleoSpin Tissue XS (Machery Nagel) according to manufacturer's instructions. The concentration of DNA was determined by a Nanophotometer S-111107AW (Implen). In the end, the DNA concentration of all specimens was diluted at the same level (30 ng/µl).

PCR amplification, restriction analysis and sequencing

For the assessment of genetic variability, the second internal transcribed spacer (ITS-2) of ribosomal DNA was amplified. The polymerase chain reaction (PCR) mixture contained 30 ng of DNA and 0.5 U of Taq polymerase (Roche). The concentration of other components was as follows: 1x KCl PCR buffer [10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3] (Roche), 0.2 mM dNTP, 0.4 µM of each pair of primers NC1F (5´ACGTCTGGTTC AGGGTTGTT 3´) and NC2R (5´TTAGTTTCTTTTCCTC CGCT 3´) (Stevenson *et al*., 1995), 2 mM PCR Enhancer [TMA Oxalate] (Top – Bio) and 0.4 µg/µl BSA. Amplification was carried out in a C1000 thermocycler (Bio-Rad). The temperature profile of PCR included initial denaturation at 95 ºC for 180 s, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at 48 ºC for 30 s, and elongation at 72 ºC for 60 s. PCR was completed with elongation at 72 ºC for 300 s. The quality and specificity of amplicons was verified by electrophoretic separation in 1.5 % agarose gel together with a size standard GeneRuler 100bp DNA Ladder (Thermo Scientific).

Table 1a. Morphometrics of *Haemonchus* females recovered from sheep

No.	Specimen	Body length (mm)	Cervical papillae (μm)	Oesophagus length (mm)	Oesophagus % body	Tail length (μm)	Phasmid right (μm)	Phasmid left (μm)	Vulvar morphology	Morphological determination	Molecular determination	
											PCR-RFLP	sequencing
01	Hc01MB	21.3	481	1.33	6.24	323	231	235	linguiform	*Hc*	*Hc*	*Hc*
02	Hc02MB	21.5	465	1.35	6.28	418	118	123	linguiform	*Hc*	*Hc*	*Hc*
03	Hc03MB	20.4	302	1.35	6.62	390	212	226	linguiform	*Hc*	*Hc*	
04	Hc04MB	20.8	403	1.32	6.35	345	201	205	linguiform	*Hc*	*Hc*	
05	Hc05MB	22.3	464	1.35	6.05	337	158	159	linguiform	*Hc*	*Hc*	
06	Hc06MB	18.4	328	1.31	7.12	366	121	125	linguiform	*Hc*	*Hc*	
07	Hc07MB	22.8	297	1.34	5.88	403	228	230	linguiform + knobbed	*Hc*	*Hc*	
08	Hc08MB	22.9	311	1.35	5.90	404	197	208	linguiform + knobbed	*Hc*	*Hc*	
09	Hc09MB	18.2	359	1.21	6.65	390	183	190	linguiform	*Hc*	*Hc*	
10	Hc10MB	20.8	480	1.28	6.15	400	98	103	linguiform	*Hc*	*Hc*	
11	Hc11MB	14.8	477	1.14	7.70	386	179	186	linguiform	*Hc*	*Hc*	*Hc*
12	Hc16SB	23.9	382	1.57	6.57	495	164	177	linguiform	*Hc*	*Hc*	
13	Hc17SB	14.9	363	1.48	9.93	514	122	126	linguiform	*Hc*	*Hc*	
14	Hc18SB	19.6	288	1.46	7.45	495	142	147	linguiform	*Hc*	*Hc*	
15	Hc19SB	20.6	343	1.54	7.48	511	207	209	linguiform	*Hc*	*Hc*	
16	Hc20SB	17	347	1.24	7.29	414	126	133	linguiform	*Hc*	*Hc*	
17	Hc22MB	24	476	1.36	5.67	510	97	120	smooth	*Hc*	*Hc*	
18	Hc23MB	23	357	1.26	5.48	483	128	149	knobbed	*Hc*	*Hc*	
19	Hc24MB	21.4	362	1.28	5.98	457	217	222	knobbed	*Hc*	*Hc*	
20	Hc25MB	23.1	462	1.34	5.80	472	106	112	smooth	*Hc*	*Hc*	
21	Hc26MB	18.9	289	1.29	6.83	405	233	245	knobbed	*Hc*	*Hc*	
22	Hc27MB	22	324	1.27	5.77	430	154	155	knobbed	*Hc*	*Hc*	
23	Hc28MB	23.4	346	1.35	5.77	416	121	122	knobbed	*Hc*	*Hc*	
24	Hc29MB	16.5	244	1.39	8.42	400	124	136	knobbed	*Hc*	*Hc*	
25	Hc30MB	15	386	1.38	9.20	452	147	151	knobbed	*Hc*	*Hc*	
26	Hc31MB	21.6	285	1.34	6.20	430	113	127	knobbed	*Hc*	*Hc*	
27	Hc32MB	22.7	471	1.28	5.64	400	111	115	knobbed	*Hc*	*Hc*	
28	Hc47SB	19.3	255	1.44	7.46	510	164	174	knobbed	*Hc*	*Hc*	
29	Hc48SB *	15.1	370	1.53	10.13	602	203	207	smooth	*Hp*	*Hc*	*Hc*
30	Hc49SB *	13.8	329	1.45	10.51	486	148	158	smooth	*Hp*	*Hc*	*Hc*
31	Hc50SB *	16.4	408	1.67	10.18	542	165	165	knobbed	*Hp*	*Hc*	*Hc*
32	Hc51SB *	15.3	376	1.39	9.08	565	210	215	smooth	*Hp*	*Hc*	*Hc*
33	Hc52SB	19	345	1.52	8.00	518	153	161	smooth	*Hc*	*Hc*	
34	Hc53SB	22.1	452	1.5	6.79	476	149	150	smooth	*Hc*	*Hc*	
35	Hc54SB	18.5	484	1.27	6.86	451	154	156	knobbed	*Hc*	*Hc*	
36	Hc55SB	19.2	449	1.43	7.45	496	129	139	knobbed	*Hc*	*Hc*	
37	Hc56SB	24.8	463	1.39	5.60	409	139	139	knobbed	*Hc*	*Hc*	
38	Hc57SB	20.3	366	1.22	6.01	483	179	195	knobbed	*Hc*	*Hc*	
39	Hc58SB	21.6	357	1.29	5.97	476	158	163	smooth	*Hc*	*Hc*	
40	Hc59SB	17	414	1.42	8.35	500	194	215	knobbed	*Hc*	*Hc*	
41	Hc60SB	25.5	302	1.49	5.84	488	150	162	knobbed	*Hc*	*Hc*	

Hc *Haemonchus contortus*, Hp *Haemonchus placei*, the asterisk denotes individuals identified by morphological characters as *H. placei*, MB Middle Bohemia, SB Southern Bohemia; cervical papillae and oesophagus length were measured from anterior body part, the oesophagus length was also expressed as percentage of body lenght, phasminds were measured from distal tip of tail, vulvar morphology was scored according to the standard nomenclature

Table 1b. Morphometrics of *Haemonchus* males recovered from sheep

No.	Specimen	Body length (mm)	Spicule length (μm)	Barb length right (μm)	Barb length left (μm)	Gubernaculum (μm)	DF	Morphological determination	Molecular determination	
									PCR-RFLP	sequencing
01	Hc12SB	11.5	475	45	23	246	0.05	*Hc*	*Hc*	
02	Hc13SB	12.6	416	45	25	233	0.26	*Hc*	*Hc*	
03	Hc14SB	16	500	47	22	256	0.19	*Hc*	*Hc*	
04	Hc15SB	13.4	478	47	23	249	0.31	*Hc*	*Hc*	
05	Hc21MB	16.8	451	48	24	251	0.54	*Hc*	*Hc*	
06	Hc33MB	14.4	463	42	27	203	0.25	*Hc*	*Hc*	
07	Hc34MB	15.9	422	47	22	235	0.07	*Hc*	*Hc*	
08	Hc35MB	12.7	436	43	26	206	0.18	*Hc*	*Hc*	
09	Hc36MB	13.1	425	43	25	209	0.01	*Hc*	*Hc*	
10	Hc37MB	14.6	432	42	28	206	0.35	*Hc*	*Hc*	
11	Hc38MB	11.8	405	45	24	200	0.09	*Hc*	*Hc*	
12	Hc39SB *	18	480	48	27	250	1.05	*Hp*	*Hc*	
13	Hc40SB *	16.7	516	52	25	268	1.31	*Hp*	*Hc*	*Hc*
14	Hc41SB	14	491	49	23	220	0.58	*Hc*	*Hc*	
15	Hc42SB	16.6	491	48	23	264	0.46	*Hc*	*Hc*	
16	Hc43SB *	17.1	488	47	25	249	0.63	*Hp*	*Hc*	*Hc*
17	Hc44SB	12.4	532	48	23	261	0.52	*Hc*	*Hc*	
18	Hc45SB	13.5	484	43	25	236	0.11	*Hc*	*Hc*	
19	Hc46SB	15.3	496	46	23	264	0.21	*Hc*	*Hc*	

Hc *Haemonchus contortus*, Hp *Haemonchus placei*, the asterisk denotes individuals identified by morphological characters as *H. placei*, MB Middle Bohemia, SB Southern Bohemia

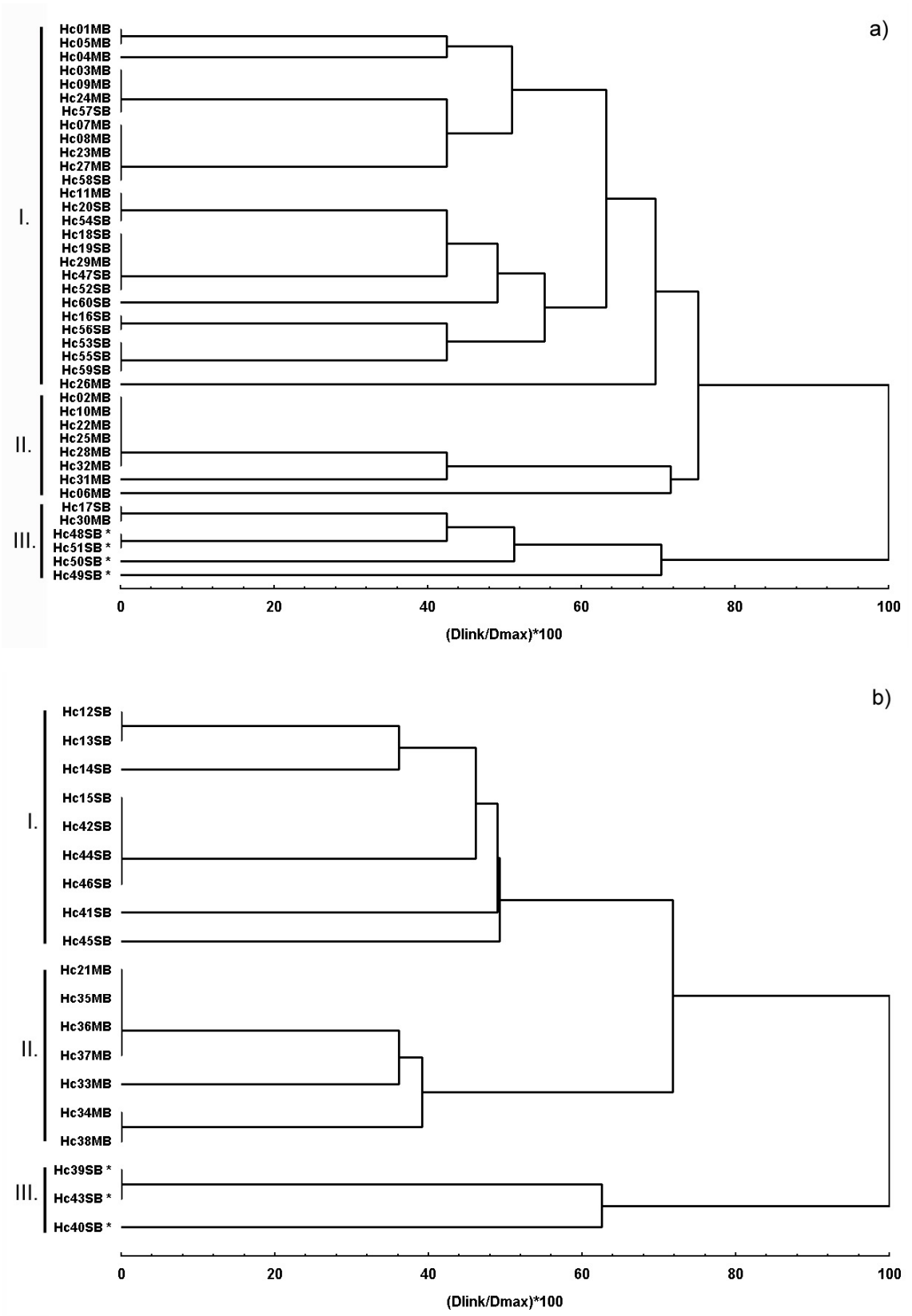

Fig. 1. Cluster analysis of *Haemonchus* females (a) and males (b) based on tertiary data file matrices. The asterisk denotes specimens morphologically identified as *H. placei*. MB denotes Middle Bohemia and SB Southern Bohemia

Molecular identification of all specimens was carried out by the PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) method. Obtained PCR products were subjected to restriction by *FspB*I (*Bfa*I) endonuclease (Thermo Scientific). The reaction mixture (total volume 12 µl) along with 5 units of *FspB*I enzyme further contained 5 µl of PCR product and 1x Tango buffer. Digestion took place at a temperature of 37 ºC for a period of 7 hours. The fragments were separated in 1.5 % agarose gel, stained with ethidium bromide, and documented by a GelDoc XR system (Bio-Rad).
Each amplicon was electrophoretically separated in 1 % agarose gel and subsequently excised with a clean scalpel prior to direct sequencing of PCR products. Extraction of PCR fragments was carried out by a MinElute Gel Extraction Kit (Qiagen) according to manufacturer's instructions. A BigDye Terminator v 3.1 Cycle Sequencing Kit (Life Technologies) was used for bidirectional sequencing reaction. Minimalization of errors was ensured through the sequencing of each amplicon, at least three times from both sides. Products of the sequencing reaction were separated by a genetic analyser ABI310 (Life Technologies).

Data processing and statistical analysis
Forty one *Haemonchus* females and nineteen *Haemonchus* males were individually compared with one another according to their morphological characteristics using cluster analysis. Cluster analysis is not influenced by standard statistical assumptions like homogeneity or normal distribution. However, it can still be affected by several factors. One potential problem could be the different scales of individual morphological features or influential points. We also tried to emphasize which value of the selected morphological character classified specimens as *Hc* or *Hp* species. For this reason, we transformed the original data file into a tertiary data file matrix: file "0" representing *Hc* class; "1" representing *Hp* class; "0.5" designating "intermediate class" (the values for which are morphological characteristics of both species overlapped). Cluster analysis was performed using STATISTICA ver. 9.1 software (StatSoft Inc.). Clustering was based on Euclidean distances, and a UPGMA clustering method was used. Final dendrograms are presented in a standardized form, and the scale is in the percentages of dissimilarities.
Sequencing Analysis Software ver. 5.2.0 (Life Technologies) was used for base calling and quality value (QV) determination. Bases with low quality were removed, and such trimmed sequences were aligned using ClustalW software (Thompson *et al*., 1994). The consensus sequence for every specimen in BioEdit software ver. 7.0.9.0 was created (Hall, 1999), and sequences were deposited in the GenBank under accesion number JX869066 – JX869075.

Results

Morphometric characters of sixty *Haemonchus* specimens are shown in Tables 1a and 1b. Forty one females and nineteen males were identified based on the selected morphological characters to the species level. Thirty-seven specimens of females (90.2 %) were *Haemonchus contortus* and four females (9.8 %) were identified as *Haemonchus placei*. All known types of vulvar morphology were observed in female specimens. The knobbed vulvar process was the most commonly presented type (41.5 %), followed by the linguiform process (39 %); females without a vulvar process were observed in eight out of forty one specimens. Vulvar morphology character was not used for species identification due to its high variability. The identification of male specimens based on selected characters combined with discriminant analysis clearly distinguished the two *Haemonchus* species. Sixteen out of nineteen male specimens were identified as *Hc*, and three specimens (15.8 %) were determined as the species *Hp*. According to the results of morphological analyses, *Hc* and *Hp* are sympatric on the farm in the Southern Bohemia, while sheep on the ecological farm in Middle Bohemia have mono-specific *Hc* infection.
The results of clustering analysis based on morphological characters are shown in the Fig. 1a and 1b. Three main groups including further structured first group are clearly distinguished in the first dendrogram (Fig. 1a). Specimens in group I and II were morphologically classified as *Hc,* whereas group III contained all specimens determined as *Hp.* Similar results are visible in males in the second dendrogram (Fig. 1b). There

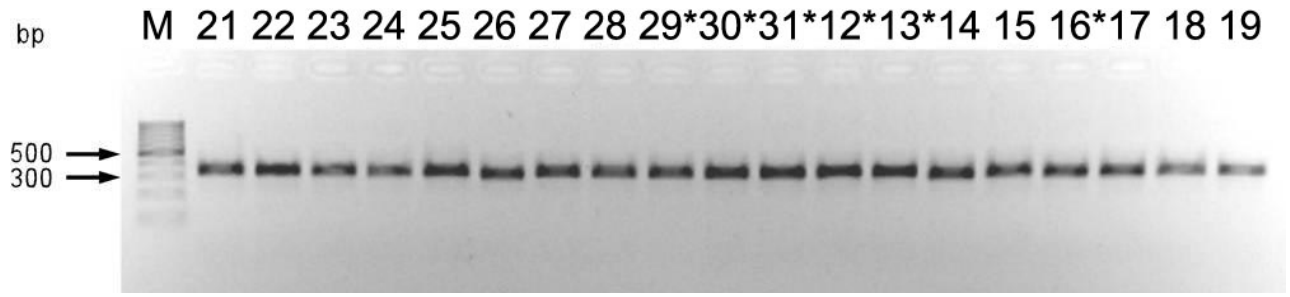

Fig. 2. Representative agarose gel showing results of restriction digestion of *Haemonchus* PCR products with *FspB*I (*Bfa*I) enzyme (Thermo Scinetific). Size standard GeneRuler 100 bp DNA Ladder (M) is indicated in the figure on the left, numbers represent lines with individual specimens (21 – 31 female and 12 – 19 male specimes), and the asterisk highlights specimens (29 – 31, 12, 13 and 16) morphologically identified as *H. placei*. All bands remained unrestricted suggesting *H. contortus* species.

are also three main groups and specimens unambiguously separated due to the fact that the first two groups (I and II) are composed only of individuals morphologically classified as *Hc.* The third group contains *Hp* only.

All studied specimens were concurrently subjected to restriction cleavage of the rDNA ITS-2 region with *FspB*I endonuclease. PCR products of all evaluated specimens remained unrestricted (see Fig. 2.). Because the digestion of the *Hp* ITS-2 PCR products provides two bands while only one unrestricted band occurred in our study, we can assume that all analysed specimens were only *Hc* species. Confirmation of the restriction cleavage results was made by sequencing chosen specimens, which were identified as *Hp* or *Hc* species according to morphological characters. Sequence analysis of size 231 bp ITS-2 region revealed nine point mutations in the specimens (8 biallelic and 1 triallelic) at positions 18, 21, 22, 59, 63, 78, 123 and 196. Four mutations were transition (C/T only) and five mutations were transversion (T/A; T/G or C/G). Alignment of the nucleotide sequences for *Haemonchus* ITS-2 region is shown in Fig. 3.

Discussion

H. contortus and *H. placei* are abomasal nematodes of substantial veterinary importance. They can occur in the same hosts, and they have a similar morphology. For certain purposes, it is necessary to accurately and reliably identify these species.

On the morphological level it is difficult to differentiate *Hc* and *Hp* due to the minor differences between them. There are some morphological features used in the identification of *Haemonchus* females. Lichtenfels (Lichtenfels *et al*., 1986; Lichtenfels *et al*., 1994) proved the pattern of cuticular ridge (synlophe) to be of value for distinguishing between mentioned species. For this purpose, these authors primarly used subventral and subdorsal ridges. However, species misidentification can occur when synlophe of hybrids is used. Intermediate pattern of subventral ridges of *Hc* and *Hp* hybrids were described by Lichtenfels *et al*. (1986). Sublateral ridges are also problematic in the identification process because the cross section rarely displays the standard number of sublateral ridges (Lichtenfels *et al*., 1994). For these reasons and also because work with synlophe is time consuming and requires sufficient skills in morphology, this character is deemed impractical for common identification and was not utilized in this study. Giudicci *et al*. (1999) used the discriminant function (DF) combined with synlophe length, tail length and tail width to discriminate among *Haemonchus* females. This DF was not used in our study due to its synlophe character. Seven other morphological characters according to Lichtenfels *et al*. (1994) were chosen for *Haemonchus* species differentiation. Many intermediate values between *Hc* and *Hp* were recorded, and these values complicated identification.

Three vulvar lobe types were observed in female specimens. The most common type was the knobbed (41.5 %), followed by the linguiform (39 %); the smooth shape was registered in only 19.5 % of specimens. These results are not in accordance with those of certain authors (Jacquiet *et al*., 1998; Thomas *et al*., 2007) who recognized the linguiform vulva as the predominant type in *Haemonchus* females recovered from sheep. All of these authors studied the *Haemonchus* population in Africa, where environmental conditions are different from those in the Czech Republic (Europe). However, Hunt *et al*. (2008) indicated significant differences in vulval lobe types among *Hc* female isolates from different locations of Australia, and in one area, they recorded the same proportion of vulval types as we did in our study. Vulval morphology of *Haemonchus* females can be affected by the nematode ability to establish and develop in a host. This morphological character can be used rather for the characterisation of the nematode population, which occurs in ruminant hosts in certain areas, than for species identification.

Male morphological characters in most nematodes are more reliable and suitable for species identification than their female characters (do Amarante *et al*., 2011). Due to the low variability of spicule morphology (spicule length, spicule barb on the right and left spicule) within species as well as to the substantial differences among species, these features are useful in *Haemonchus* male identification. Lichtenfels *et al*. (1994) used the spicule length for this purpose; they used a 450 µm value as a threshold for differentiation between two species. *Hc* spicules are under 450 µm, and spicule length above 450 µm is typical for *Hp*. In some cases, a single feature is insufficient to distinguish among nematode species. For this reason, the discriminant function (DF) combining three characters of spicule morphology, which had been used by other authors (Jacquiet *et al*., 1997; Riggs *et al*., 2001; Achi *et al*., 2003) to identify *Haemonchus* males, was also applied in our study. The DF revealed the presence of both *Haemonchus* species. The majority of specimens represented *Hc* males (16 of 19 specimens), while *Hp* males were identified in 15.8 % of specimens.

There are certain factors influencing morphological identification of nematodes. The host immune system can regulate nematode female length. There is a significant association between increased IgA activity against larvae and reduced *Hc* female length (Strain & Stear, 2001; Saddiqi *et al*., 2011). Moreover, host breed (Aumont *et al*., 2003), host gender (Gruner *et al*., 2004), diet (Hoste *et al*., 2008) and concurrent parasitic infection (Reinecke *et al*., 1982; Terefe *et al*., 2005) can affect nematode length. Morphological features that detect the length of a specific character used in species identification may be affected as well. This is particularly important in nematodes like *Haemonchus,* which exhibit minor morphological differences. Another factor affecting morphological characters is a fixation medium that can distinctly change nematode morphology (Jacquiet *et al*. 1997). The soft parts of the nematode body and the surface structure (used in female identification) are especially sensitive to the action of formaldehyde, which is still used by most morphologists. For these reasons, it is evident that questionable species identification procedures should be verified by molecular methods.

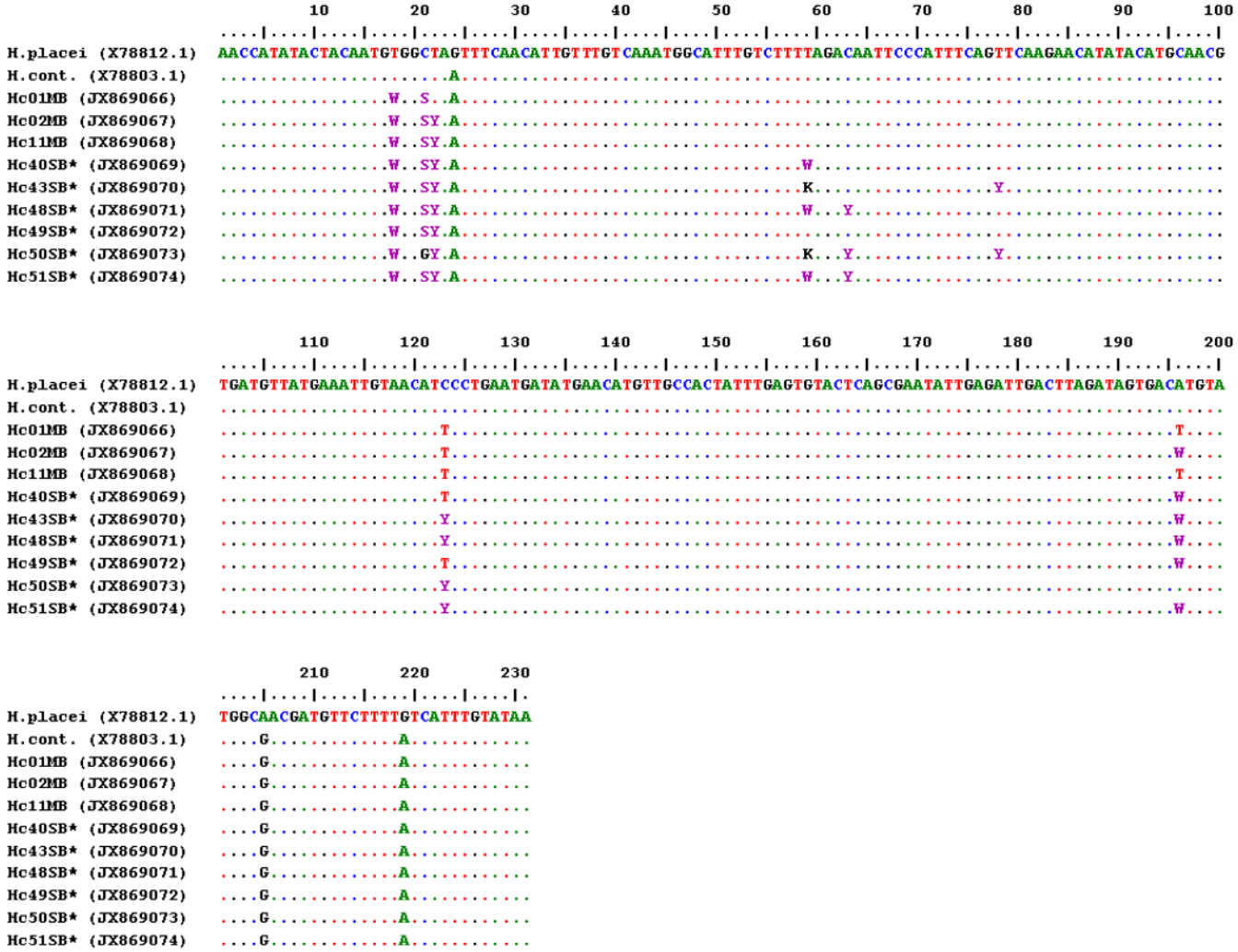

Fig. 3. Alignment of the nucleotide sequences for the ITS-2 region of *Haemonchus* specimens. Identical bases are represented by dots. Nine point mutations at positions 18, 21, 22, 59, 63, 78, 123 and 196 were detected in specimens. Four mutations were transition (C/T only) and five mutations were transversion (T/A; T/G or C/G). Standard ambiguity codes mark nucleotide sites that are polymorphic within specimens (W = A or T, S = C or G, K = G or T, Y = C or T). The asterisk denotes specimens morphologically identified as *H. placei*. MB denotes Middle Bohemia and SB Southern Bohemia region.

Molecular analyses of parasitic species have a broad range of utilizations (Criscione, 2005; Gasser, 2006), of which species identification is one of the most common and crucial. In this study molecular diagnostics of *Haemonchus* spp. was based on findings of Stevenson *et al*. (1995). These authors postulated that *Hc* and *Hp* have three different bases in the sequence of the ITS-2 region at the positions 24, 205 and 219. PCR-RFLP analysis of the ITS-2 region, confirmed by sequencing analysis performed in our study, revealed no variation between the specimens at the previously mentioned positions. For this reason, specimens were molecularly identified as *Haemonchus contortus*. Although alternative methods of molecular identification are possible (Blouin, 1997; Bensch *et al*., 2004), the ITS-2 region for *Haemonchus* species identification was successfully used by many authors (Chilton *et al*., 2001; Troell *et al*., 2003; Brasil *et al*., 2012) in analysing specimens from various parts of the world.

From the results of our study, it is evident that the use of only one approach (preferring morphology) in species identification may lead to inaccurate results, particularly if two or more species are sympatric and have only minor morphological differences. One of the most striking results of this study was the finding that species identification based on widely used discriminant function does not corresponded to molecular identification. For this reason, the DF should be evaluated by molecular tools on a greater number of specimens from various parts of the world in order to confirm its suitability for *Haemonchus* species identification. In conclusion, molecular tools may assist us resolved the ambiguous identification of certain parasitic nematodes.

Acknowledgments

The authors wish to acknowledge thank Mr. Brian Kavalir (Canada) for his assistance in proofreading this manuscript. This study has been funded by the Grant of the National Agency for Agricultural Research of the Czech Republic, No. QI111A199.

Ethical standards

All experiments conducted with laboratory animals comply with the current laws of the country in which they were performed.

Conflict of interest

The authors declare that they have no conflict of interest.

References

ACHI, Y. L., ZINSSTAG, J., YAO, K., YEO, N., DORCHIES, P., JACQUIET, P. (2003): Host specificity of *Haemonchus* spp. for domestic ruminants in the savanna in northern Ivory Coast. *Vet. Parasitol.,* 116: 151 – 158. DOI: 10.1016/S0304-4017(03)00258-9

AUMONT, G., GRUNER, L., HOSTACHE, G. (2003): Comparison of the resistance to sympatric and allopatric isolates of *Haemonchus contortus* of Black Belly sheepin Guadeloupe (FWI) and of INRA 401 sheep in France. *Vet. Parasitol.,* 116: 139 – 150. DOI: 10.1016/S0304-4017(03)00259-0

BÁRTÍKOVÁ, H., SKÁLOVÁ, L., LAMKA, J., SZOTÁKOVÁ, B., VÁRADY, M. (2010): The effects of flubendazole and its metabolites on the larval development of *Haemonchus contortus* (Nematoda: Trichostrongylidae): an in vitro study. *Helminthologia*, 47(4): 269 – 272. DOI: 10.2478/s11687-010-0042-7

BENSCH, S., PÉREZ-TRIS, J., WALDENSTRÖM, J., HELLGREN, O. (2004): Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? *Evolution,* 58(7): 1617 – 1621

BLOUIN, M. S., YOWELL, C. A., COURTNEY, C. H., DAME, J. B. (1997): *Haemonchus placei* and *Haemonchus contortus* are distinct species based on mtDNA evidence. *Int. J. Parasitol.,* 27(11): 1383 – 1387

BRASIL, B. S., NUNES, R. L., BASTIANETTO, E., DRUMMOND, M. G., CARVALHO, D. C., LEITE, R. C., MOLENTO, M. B., OLIVEIRA, D. A. (2012): Genetic diversity patterns of *Haemonchus placei* and *Haemonchus contortus* populations isolated from domestic ruminants in Brazil. *Int. J. Parasitol*., 42: 469 – 479

BREMNER, K. C. (1955): Cytological studies on the specific distinctness of the Ovine and Bovine „strains" of the nematode *Haemonchus contortus* (Rudolphi) Cobb (Nematoda: Trichostrongylidae). *Aus. J. Zool*., 3: 312 – 323

CHILTON, N. B., NEWTON, L. A., BEVERIDGE, I., GASSER, R. B. (2001): Evolutionary relationships of trichostrongyloid nematodes (Strongylida) inferred from ribosomal DNA sequence data. *Mol. Phylogenet. Evol*., 19(3): 367 – 386. DOI:10.1006/mpev.2001.0938

CRISCIONE, C. D., POULIN, R., BLOUIN, M. S. (2005): Molecular ecology of parasites: elucidating ecological and microevolutionary processes. *Mol. Ecol*., 14(8): 2247 – 2257. DOI: 10.1111/j.1365-294X.2005.02587.x

DO AMARANTE, A. F. (2011): Why is it important to correctly identify *Haemonchus* species? *Rev. Bras. Parasitol. Vet*., 20(4): 263 – 268

GASSER, R. B. (2006): Molecular tools--advances, opportunities and prospects. *Vet. Parasitol*., 136(2): 69 – 89. DOI: 10.1016/j.vetpar.2005.12.002

GIBBONS, L. M. (1979): Revision of the genus *Haemonchus* Cobb 1898 (Nematoda: Trichostrongylidae). *Syst. Parasitol*., 1: 3 – 24

GIUDICI, C. J., CABARET, J., DURETTE-DESSET, M. C. (1999): Description of *Haemonchus placei* (Place, 1893) (Nematoda, Trichostrongylidae, Haemonchinae), identification and intra-specific morphologic variability. *Parasite,* 6(4): 333 – 342

GRUNER, L., CORTET, J., SAUVÉ, C., HOSTE, H. (2004): Regulation of *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* worm populations by grazing sheep with differing resistance status. *Vet. Res*., 35(1): 91 – 101. DOI: 10.1051/vetres:2003043

HALL, T. A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser*., 41: 95 – 98

HOBERG, E. R., LICHTENFELS, J. R., GIBBONS, L. (2004): Phylogeny for species of *Haemonchus* (Nematoda: Trichostrongyloidea): considerations of their evolutionary history and global biogeography among Camelidae and Pecora (Artiodactyla). *J. Parasitol*., 90(5): 1085 – 1102

HOSTE, H., TORRES-ACOSTA, J. F., AGUILAR-CABALLERO, A. J. (2008): Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? *Parasite Immunol*., 30(2): 79 – 88. DOI: 10.1111/j.1365-3024.2007.00987.x

HUNT, P. W., KNOX, M. R., LE JAMBRE, L. F., MCNALLY, J., ANDERSON, L.J. (2008): Genetic and phenotypic differences between isolates of *Haemonchus contortus* in Australia. *Int. J. Parasitol*., 38: 885 – 900. DOI: 10.1016/j.ijpara.2007.11.001

JACQUIET, P., CABARET, J., CHEIKH, D., THIAM, E. (1997): Identification of *Haemonchus* species in domestic ruminants based on morphometrics of spicules. *Parasitol. Res*., 83(1): 82 – 86

KÖNIGOVÁ, A., HRČKOVÁ, G., VELEBNÝ, S., DOLINSKÁ, M., MOLNÁR, L., VÁRADY, M. (2012): Effect of albendazole therapy on susceptible and resistant *Haemonchuscontortus* larvae in Mongolian gerbils (*Meriones unguiculatus*) and distribution of inflammatory cells in the stomach wall. *Helminthologia*, 49 (4): 211 – 220. DOI 10.2478/s11687-012-0040-z

LICHTENFELS, J. R., PILIT, P. A., LE JAMBRE, L. F. (1986): Cuticular ridge patterns of *Haemonchus contortus* and *Haemonchus placei* (Nematoda: Trichostrongyloidea). *Proceedings of the Helminthological Society of Washington,* 53(1): 94 – 101

LICHTENFELS, J. R., PILIT, P. A., LE JAMBRE, L. F. (1988): Spicule lengths of the ruminant stomach nematodes *Haemonchus contortus*, *Haemonchus placei*, and their hybrids. *Proceedings of the Helminthological Society of Washington*, 55(1): 97 – 100

LICHTENFELS, J. R., PILITT, P. A., HOBERG, E. P. (1994): New morphological characters for identifying individual specimens of *Haemonchus* spp. (Nematoda: Trichostrongyloidea) and a key to species in ruminants of North America. *J. Parasitol*., 80(1): 107 – 119

REINECKE, R. K., DE VILLIER, I. L., JOUBERT, G. (1982): The effect of predosing calves with *Trichostrongylus axei* on subsequent challenge with *Haemonchus placei*. *Onderstepoort J. Vet. Res*., 49(3): 159 – 161

RIGGS N. L. (2001) Experimental cross-infections of *Haemonchus placei* (Place, 1893) in sheep and cattle. *Vet. Parasitol.*, 94: 191–197

ROBERTS, F. H. S., TURNER, H. N., MCKEVETT, M. (1954): On the specific distinctness of the ovine and bovine „strains" of *Haemocnhus contortus* (Rudolphi) Cobb (Nematoda: Trichostrongylidae). *Aus. J. Zool*., 2: 275 – 295

ROEPSTORFF, A., NANSEN, P. (1998): Epidemiology, diagnosis and control of helminth parasites of swine. Rome, IT, FAO, 161 pp.

SADDIQI, H. A., JABBAR, A., SARWAR, M., IQBAL, Z., MUHAMMAD, G., NISA, M., SHAHZAD, A. (2011): Small ruminant resistance against gastrointestinal nematodes: a case of *Haemonchus contortus*. *Parasitol. Res.*, 109: 1483 – 1500. DOI: 10.1007/s00436-011-2576-0

SCOTT, I., SUTHERLAND, I. (2009): Gastrointestinal Nematodes of Sheep and Cattle: Biology and Control. Oxford, UK, Wiley-Blackwell, 242 pp.

STEVENSON, L. A., CHILTON, N. B., GASSER, R. B. (1995): Differentiation of *Haemonchus placei* from *H. contortus* (Nematoda: Trichostrongylidae) by the ribosomal DNA second internal transcribed spacer. *Int. J. Parasitol*., 25(4): 483 – 488

STRAIN, S. A., STEAR, M. J. (2001): The influence of protein supplementation on the immune response to *Haemonchus contortus*. *Parasite Immunol*., 23: 527 – 531

ŠNÁBEL, V., KÖNIGOVÁ, A., DOLINSKÁ, M., WOLSTENHOLME, A. J., VÁRADY, M. (2012): Allozyme analysis of *Haemonchus contortus* resistant and susceptible to anthelmintics, with an indication of dipeptidases associated with resistance. *Helminthologia*, 49(3): 128 – 133. DOI: 10.2478/s11687-012-0027-9

TAYLOR, M. A., COOP, R. L., WALL, R. L. (2007): Veterinary Parasitology. 3rd Edition, Oxford, UK, Blackwell Publishing, 874 pp.

TEREFE, G., YACOB, H. T., GRISEZ, C., PREVOT, F., DUMAS, E., BERGEAUD, J. P., DORCHIES, P., HOSTE, H., JACQUIET, P. (2005): *Haemonchus contortus* egg excretion and female length reduction in sheep previously infected with *Oestrus ovis* (Diptera: Oestridae) larvae. *Vet. Parasitol*., 128(3 – 4): 271 – 283. DOI:10.1016/j.vetpar.2004.11.036

THOMAS, N., TESHALE, S., KUMSA, B. (2007): Abomasal nematodes of sheep and goats slaughtered in Awassa (Ethiopia): species composition, prevalence and vulvar morphology. *Helminthologia,* 44(2): 70 – 75. DOI: 10.2478/s11687-007-0006-8

THOMPSON, J. D., HIGGINS, D. G., GIBSON, T. J. (1994): CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*., 22(22): 4673 – 4680

TROELL, K., MATTSSON, J. G., ALDERBORN, A., HÖGLUND, J. (2003): Pyrosequencing analysis identifies discrete populations of *Haemonchus contortus* from small ruminants. *Int. J. Parasitol*., 33(7): 765 – 771. DOI:10.1016/S0020-7519(03)00068-7

VAN WYK, J. A., CABARET, J., MICHAEL, L. M. (2004): Morphological identification of nematode larvae of small ruminants and cattle simplified. *Vet. Parasitol*., 119(4): 277 – 306. DOI:10.1016/j.vetpar.2003.11.012

VÁRADY, M., ČUDEKOVÁ, P., ČORBA, J. (2007): In vitro detection of benzimidazole resistance in *Haemonchus*

contortus: egg hatch test versus larval development test. *Vet Parasitol*., 149(1 – 2): 104 – 110. DOI: 10.1016/j.vetpar.2007.07.011

VÁRADY, M., ČORBA, J., LETKOVÁ, V., KOVÁČ, G. (2009): Comparison of two versions of larval development test to detect anthelmintic resistance in *Haemonchus contortus*. *Vet. Parasitol*., 160(3-4): 267 – 271. DOI: 10.1016/j.vetpar.2008.11.010

ZARLENGA, D. S., STRINGFELLOW, F., NOBARY, M., LICHTENFELS, J. R. (1994): Cloning and characterization of ribosomal RNA genes from three species of *Haemonchus* (Nematoda: Trichostrongyloidea) and identification of PCR primers for rapid differentiation. *Exp. Parasitol*., 78: 28 – 36

RECEIVED MARCH 11, 2013

ACCEPTED APRIL 4, 2014