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Research Note

Morphological and molecular genetic analysis of *Synhimantus (Synhimantus) laticeps* (Rudolphi, 1819) (Nematoda, Acuariidae) from the barn owl (*Tyto alba*) and the common kestrel (*Falco tinnunculus*) in AustriaD. EBMER¹, H.-P. FUEHRER¹, B. EIGNER¹, H. SATTMANN², A. JOACHIM^{1*}

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Summary

In the framework of the biodiversity initiative and barcoding project “Austrian Barcode of Life” (ABOL) post mortem examinations of the gastro-intestinal tracts of different species of wild birds were carried out and several adult helminths were retrieved. In the gizzard of two barn owls (*Tyto alba*) and one common kestrel (*Falco tinnunculus*) acuariid nematodes belonging to the species *Synhimantus (Synhimantus) laticeps* (Rudolphi, 1819) were discovered. This report illustrates the identification of this parasitic nematode by morphometric comparison and scanning electron microscopic photographs. Furthermore, genetic identification of individual parasites based on a fragment of the mitochondrial *cytochrome c oxidase subunit I* (COI) gene and the nuclear 18S ribosomal RNA gene was carried out. This report constitutes the first COI-based DNA barcoding of *S. (S.) laticeps* and its first record in the barn owl (*Tyto alba*) in Austria.

Keywords: *Synhimantus*; Acuariidae; birds of prey; barn owl; morphology

Introduction

Synhimantus (Synhimantus) laticeps (Rudolphi, 1819) is a parasitic nematode of the order Spirurida, belonging to the family Acuariidae (for a review on the genus, see Cram, 1927; Chabaud, 1975). Most of the representatives of the superfamily Acuarioidea parasitize the upper digestive tract of birds (Anderson, 2000). After a first description as *Spiroptera laticeps* in the esophagus of the rough-legged buzzard (*Buteo lagopus*) by Rudolphi (1819), Siebold (1837) described this species as *Spiroptera fallax* in the gizzard of the barn owl (*Tyto alba*). Morphologically, two cutaneous cordons which are recurrent and anastomosing in pairs and the conspicuous tricuspid cervical papillae (deirids), both located in the cervical region on each lateral surface, characterize nematodes of this species (Cram, 1927). Males of *S. (S.) laticeps* are mainly characterized by their dissimilar spicules (Cram, 1927), especially by the complex shape of the left spicule (Kotremba, 1978; Etche-

goin *et al.*, 2000). The left spicule can reach three (Etchegoin *et al.*, 2000) to four (Acosta *et al.*, 2008) times the length of the right one. The eggs of all acuarioids are oval, thick-shelled and contain a first stage larva upon excretion (Anderson, 2000). Male individuals parasitizing the gizzard of the barn owl (*T. alba*) can reach a body length of 3.37 – 6.24 mm, females in this host can be 4.71 – 5.00 mm long (Etchegoin *et al.*, 2000). However, male specimens inhabiting the gizzard of the common kestrel (*F. tinnunculus*) were reported to be 6.83 – 10.60 mm long (Acosta *et al.*, 2008), and females can reach a body length of 12.50 – 15.95 mm (Kotremba, 1978). The host range of *S. (S.) laticeps* includes various species of birds of prey and owls (Cram, 1926; Furmaga, 1957; Yamaguti, 1961). Esophagus, gizzard and the glandular stomach were reported as localisations of the adult worms (Furmaga, 1957). This species was reported from Europe, Africa, Asia, Australia, North and South America (Cram, 1926; Smogorzhevskaya, 1990; Etchegoin *et al.*, 2000). More recently *S. (S.) laticeps* was reported

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from the barn owl (*T. alba*) in Italy (Santoro *et al.*, 2012), in Spain (Sanmartin *et al.*, 2004), in the Netherlands (Borgsteede *et al.*, 2003) and in Argentina (Etchegoin *et al.*, 2000). Parasite findings in the common kestrel (*F. tinnunculus*) were frequently reported for Southern Europe (Santoro *et al.*, 2010; Acosta *et al.*, 2008; Sanmartin *et al.*, 2004), Turkey (Aşti *et al.*, 2017), Germany (Krone *et al.*, 2000) and Austria (Kotremba, 1978). Knowledge about the life cycle of *S. (S.) laticeps* is currently limited. Due to the fact that adult stages mainly occur in birds of the order Accipitriformes, Falconiformes and Strigiformes it was hypothesized that small vertebrates could be involved as intermediate hosts (Cram, 1927). Anderson (2000) suggested a possible development of acuariid nematodes from terrestrial hosts in a broad variety of arthropod intermediate hosts, e.g. isopods, grasshoppers, beetles and diplopods. In the present study we report on the morphological and genetic analysis of *Synhimantus (S.) laticeps* isolated from the organs of two barn owls (*T. alba*) and one common kestrel (*F. tinnunculus*) from Burgenland, Austria.

Material and Methods

During the period between 1978 and 2015 a total of 168 dead wild birds were acquired by the Natural History Museum Vienna. The animals were mostly collected from eastern Austria. The viscera, which in most cases included the heart and digestive tract, were extracted by staff of the museum and placed into preserving jars. In the context of the DNA barcoding project "Austrian Barcode of Life" (www.abol.ac.at) a parasitological dissection of the organs was carried out. In 39 of 168 examined hosts helminths were detected. The present work deals with the examination of the organs of two barn owls (*T. alba*) and one common kestrel (*F. tinnunculus*) found in Burgenland, Austria, between 2008 and 2014. Nematodes were found under the lining of the gizzard. They were extracted using dissection needles, preserved in 75 % ethanol and stored in the refrigerator at 4 °C. For morphological identification, six specimens were cleared and mounted in glycerol, placed on glass slides and covered with coverslips. They were assessed according to their characteristic structures using a light microscope (Leitz Diaplan). Morphological features were measured and photographed. The determination of the nematodes followed established morphological keys (Cram, 1927; Chabaud, 1975). In preparation for the scanning electron microscopic examination four specimens were dehydrated in alcohol (80, 85, 90, 95 and 100 % ethanol, one hour each). After storage in 100 % ethanol for 24 hours and an air drying step on blotting paper for 48 hours specimens were sputter coated with platinum (Leica EM SCD500) and photographed with a scanning electron microscope (JEOL JSM 6610LV) at 15 kV with 0.2 nA beam current. For molecular genetic identification, five individuals of *S. (S.) laticeps* (three females and one male) from the barn owl (*T. alba*) and one male individual from the common kestrel (*F. tinnunculus*) were used. DNA was isolated with a DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). PCR was carried out with an Eppendorf

Mastercycler pro S (Eppendorf AG, Hamburg, Germany), targeting two different loci. On the one hand, a part of the *mitochondrial cytochrome c oxidase subunit I (COI)* gene was amplified with primers previously used in a broad spectrum of metazoan invertebrates, LCO1490 (5'-GGTCAACAAATCATAAAG ATATTGG-3') and HC02198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994). The profile of the PCR was composed of one cycle of initial denaturation for 2 min at 94 °C, 40 cycles of denaturation at 94 °C, annealing at 45 °C and elongation at 72 °C for 1 min each and one cycle of a final elongation at 72 °C for 5 min. Secondly, primers targeting the *ribosomal 18S (r18S) RNA* gene were used amplifying two overlapping fragments (F23: 5'-ATTCCGATAACGA GCGAGA-3' and UNI3: 5'-TGATCCTTCTGCAGGTTAC-3'; F24: 5'-AGRGGTCAAATYCGTGGAC-3' and R13: 5'-GGGCATCACAG ACCTGTTA-3'). The amplification protocol was the same as for *COI* except for a higher denaturation temperature (95 °C), 30 cycles of denaturation and a higher annealing temperature (50 °C) (Honisch, 2008). The PCR products were checked by agarose gel electrophoresis. Positive samples were sequenced (LGC Genomics, Germany). The sequences were edited with the free software Genedoc (<http://www.psc.edu/>) and the obtained barcode sequences were compared to reference sequences using the Barcode of Life Data Systems (<http://www.boldsystems.org>) and the nucleotide BLAST of GenBank® (<http://www.ncbi.nlm.nih.gov/genbank>). The obtained sequences were submitted to GenBank®. The *COI*-based DNA-barcode was published in the Barcode of Life Data Systems as well. Remaining specimens of *S. (S.) laticeps* were stored in 75 % ethanol and deposited in the Third Zoological Department of the Natural History Museum Vienna, Vienna, Austria.

Results

During the parasitological dissection 20 specimens of *S. (S.) laticeps* in total were recovered under the gizzard lining of a female barn owl (*T. alba*), 18 specimens from the gizzard of a male barn owl (*T. alba*) and two specimens from the gizzard of a common kestrel (*F. tinnunculus*).

Morphological description of *S. (S.) laticeps*: The body of *S. (S.) laticeps* showed a yellow whitish color and the cuticle had fine transverse stripes (Fig. 1/A). On the lateral side of the anterior part of the body two pseudolabia were visible. Each of them had a small process which tapered conically. On both sides at the base of the pseudolabia a pair of cephalic papillae was formed (Fig. 1/A, 1/B). Arising from the lateral margins of the pseudolabia two cutaneous cordons were marked. Initially the cordons extended caudally. After reaching a distance of 283 (234 – 350) µm from the anterior part of the body they turned and recurred for about one half of their length and anastomosed in pairs on each lateral surface. Deirids with a diameter of 13.7 (11.3 – 16.3) µm were located in the cervical region posteriorly to the bend of the cordons on each side and revealed a very typical tridentate shape (Fig. 1/C).

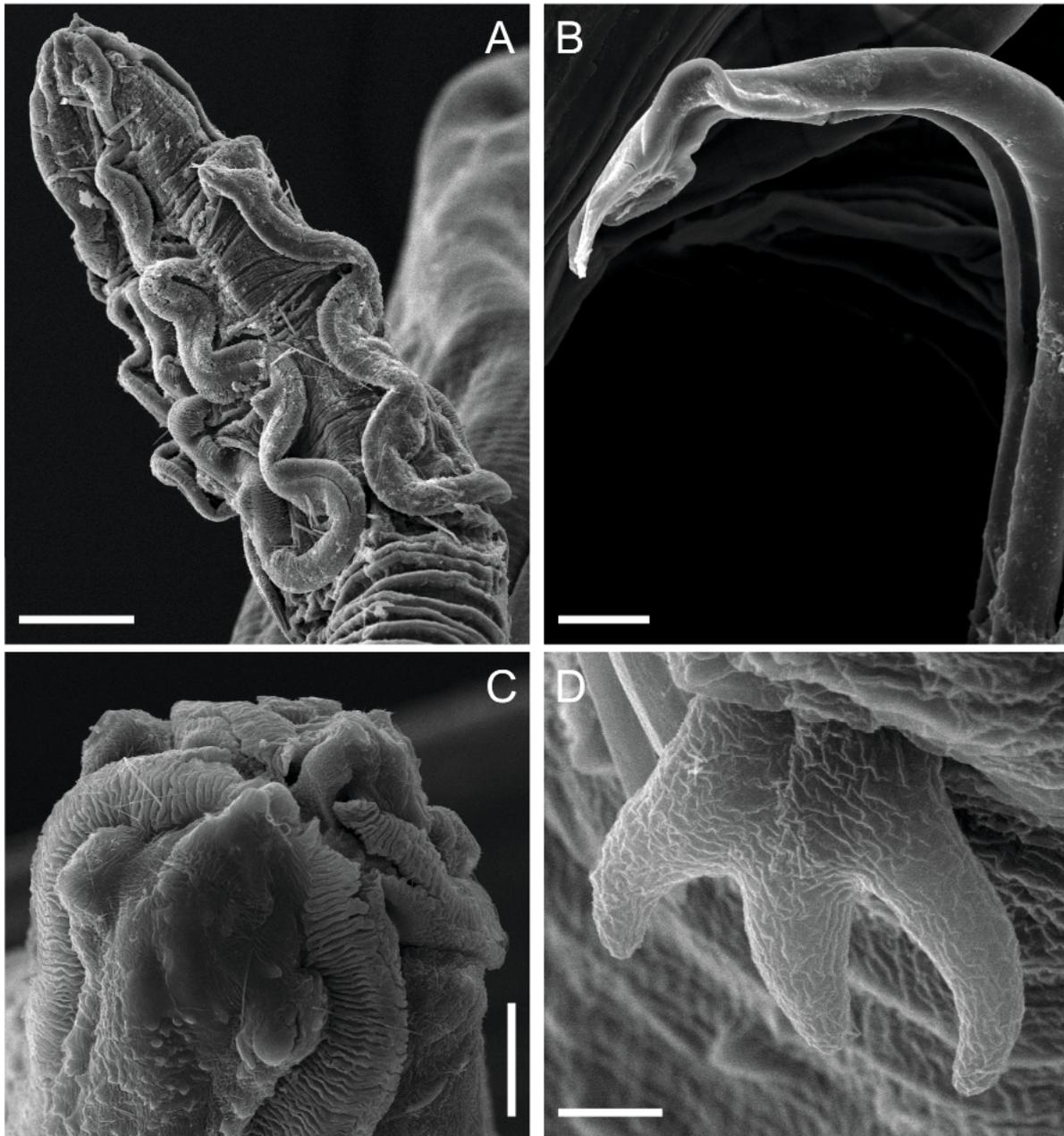


Fig. 1. *Synhimantus (Synhimantus) laticeps*, scanning electron micrographs. (A) Lateral overview of the anterior part of the body a female; B) lateral view of the distal end of the left spicule; C) apical view of the two pseudolabia and the arising cordons of a female specimen; D) deirids showing the typical tridendate shape, lateral view; Scale bars: A = 50 μm ; B = 20 μm ; C = 10 μm , D = 2 μm .

Females (measurements of five specimens; see Table 1): The body length was 7.74 (6.97 – 8.97) mm, the width of the body 250 (222 – 298) μm . The length of the buccal capsule was 211 (163 – 284) μm , the muscular esophagus was 490 (462 – 516) μm ; the glandular esophagus was 2.31 (2.10 – 2.57) mm long. The length of the cordons was 296 (260 – 350) μm , the width of the cordons (widest part) 199 (182 – 211) μm . Larvated eggs contained in the

uterus were 37.3 (36.0 – 39.2) x 23.9 (21.9 – 25.8) μm . The distance of the vulva from the anterior part of the body was 4.72 (4.14 – 5.24) μm , the location of the vulva was near mid-body.

Males (measurements of four specimens; see Tab. 2): The body length was 5.71 (5.25 – 6.19) mm, the width of the body was 161 (146 – 186) μm . The length of the buccal capsule was 169 (158 –

Table 1. Comparison of measurements (in μm unless indicated otherwise) of female specimens of *Synhimantus (Synhimantus) laticeps*.

References	Present study	Etchegoin et al. (2000)	Kotremba (1978)
Host species	<i>Tyto alba</i>	<i>Tyto alba</i>	<i>Falco tinnunculus</i>
Geographical distribution	Austria	Argentina	Austria
Number of specimens (n)	5	2	n.a.
Length [mm]	7.74 \pm 0.74 (6.97 – 8.97)	4.85 (4.71 – 5.00)	12.40 – 15.95
Maximum width	250 \pm 31.3 (222 – 298)	215 (208 – 221)	340 – 590
Buccal capsule	211 \pm 59.3 (163 – 284)	108 (105 – 110)	230 – 410
Muscular esophagus	490 \pm 19.7 (462 – 516)	405 (375 – 435)	730
Glandular esophagus [mm]	2.31 \pm 0.20 (2.10 – 2.57)	2.11 (1.88 – 2.33)	3.05 – 3.20
Deirids (diameter) ¹	15.3 \pm 0.92 (14.5 – 16.3)	14 (12 – 15)	–
Vulva from anterior end ¹ [mm]	4.72 \pm 0.55 (4.14 – 5.24)	–	6.90 – 9.40
Cordons: length	296 \pm 42.2 (260 – 350)	146 (143 – 150)	–
Cordons: width	199 \pm 11.4 (182 – 211)	–	–
Eggs: length ²	37.3 \pm 1.08 (36.0 – 39.2)	35.6 (34.5 – 36)	36 – 42
Eggs: width ²	23.9 \pm 1.36 (21.9 – 25.8)	20 (18 – 21)	18 – 27

¹Three individuals were measured; ²Two eggs from each female individual in the present study were measured.

Table 2. Comparison of measurements (in μm unless indicated otherwise) of male specimens of *Synhimantus (Synhimantus) laticeps*.

References	Present study		Etchegoin et al. (2000)	Acosta et al. (2008)	Kotremba (1978)
	<i>Tyto alba</i>	<i>Falco tinnunculus</i>	<i>Tyto alba</i>	Spain	<i>Falco tinnunculus</i>
Host species					
Geographical distribution	Austria	Austria	Argentina	Spain	Austria
Number of specimens (n)	3	1	6	10	n.a.
Length [mm]	5.86 \pm 0.34 (5.51 – 6.19)	5.25	4.47 (3.37 – 6.24)	8.89 (6.83 – 10.60)	8.90 – 10.90
Maximum width	162 \pm 21.0 (146 – 186)	158	145 (130 – 165)	237 (145 – 278)	220 – 290
Buccal capsule	170 \pm 18.2 (158 – 191)	165	107 (80 – 129)	215 (193 – 243)	220
Muscular esophagus	549 \pm 49.7 (508 – 604)	468	388 (309 – 420)	723 (554 – 932)	790
Glandular esophagus [mm]	1.94 \pm 0.13 (1.86 – 2.09)	1,579	1.96 (1.75 – 2.13)	2.50 (1.80 – 3.36)	1.80 – 2.60
Deirids (diameter)	12.1 \pm 1.06 (11.3 – 12.8)	13.1	11.6 (11 – 12)	–	–
Cordons: length	278 \pm 26.9 (247 – 294)	234	149 (143 – 150)	360 (309 – 431)	–
Cordons: width	151 \pm 9.50 (141 – 160)	106	–	–	–
Right spicule	158 \pm 22.5 (142 – 184)	148	194 (180 – 221)	202 (183 – 241)	180 – 200
Left spicule	579 \pm 111 (454 – 667)	622	554 (520 – 600)	796 (750 – 839)	680 – 790
Spicule length ratio	3.7 \pm 0.70 (3.2 – 4.5)	4.2:1	2.9:1	3.2:1 – 4.2:1	–

191) μm , the muscular esophagus was 5329 (468 – 604) μm , the glandular esophagus was 1.85 (1.58 – 2.09) mm. The length of the cordons was 267 (234 – 294) μm , their width (widest part) was 140 (106 – 160) μm . The length of the left spicule was 590 (454 – 667) μm , that of the right spicule 156 (142 – 184) μm . The spicule ratio was 3.8:1 (3.2:1 – 4.5:1). The distal shape of the left spicule exhibited a centered furrow. Towards the end of the spicule both lateral margins spread in asymmetric ways (Fig. 1/D). At the posterior end of the body four pairs of pre-cloacal pedunculated papillae and five pairs of post-cloacal pedunculated papillae were formed.

Molecular genetic identification: Amplicons of the expected sizes (529 bp for *COI* and 875 bp for the complete 18S RNA fragment) could be obtained by PCR with the DNA of the specimen isolated from the common kestrel (*F. tinnunculus*). The obtained sequences were submitted to GenBank® (*COI* accession number: KY348789; 18S accession number: KY249248) and BOLD (*COI* sequence ID: BPHA335).

Discussion

The morphology of the isolated specimens was in good accordance with the original description and the characterization of *S. (S.) laticeps* by Cram (1927), Furmaga (1957) and Kotremba (1978). Regarding scanning electron microscopic photographs of *S. (S.) laticeps* from the barn owl (*T. alba*) by Etchegoin *et al.* (2000), similarities especially in the morphology of the shape of the deirids and the left spicule were clearly recognizable. Comparison with morphometric data from previous studies, however, revealed some differences. The female specimens from the Austrian barn owl (*T. alba*) were considerably longer than specimens from this host reported by Etchegoin *et al.* (2000) and the ratio of the buccal capsule and the cordon length to the total body length differed, whereas the ratio of the muscular and glandular esophagus to the total body length was similar. Independently of different host species the diameters of the deirids were generally very constant. The measurements of the eggs varied only slightly in different host species. The characteristic structures of our male specimens from the common kestrel (*F. tinnunculus*) were slightly smaller compared to reference data from the same host species published by Acosta *et al.* (2000). However, the ratio of the length of the muscular esophagus, the glandular esophagus and the cordons to the total body length was very similar. Osche (1955) reviewed the classification of the genera *Dispharynx* and *Synhimantus*. Species of the genus *Dispharynx* are mainly characterized by their non-anastomosing cordons (Cram, 1927). However, Osche (1955) pointed out that some species of the genus *Synhimantus* can feature cordons which do not anastomose. Using the example of *Synhimantus robertdolfusi* he showed that the manifestation of anastomosis of the cordons can also depend on the sex of the individuals. He demonstrated an age-dependent development of the shape and the anastomosing of the cordons using the example

of the third and fourth larval stages of *Dispharynx soricis*. In his conclusion he even suggested a synonymization of the two genera. In other publications some authors considered *Dispharynx* as a subgenus of *Synhimantus* (Chabaud, 1975; Zhang *et al.*, 2004; Acosta *et al.*, 2008). In the context of a large investigation of the parasite fauna of indigenous owls in Austria 182 owls were examined by Kutzer *et al.* (1982). Specimens of *S. (S.) laticeps* were found in the long-eared owl (*Asio otus*) and the related species *Synhimantus (Dispharynx) falconis* was reported from the long-eared owl (*Asio otus*), the Eurasian eagle-owl (*Bubo bubo*) and the tawny owl (*Strix aluco*). Four barn owls (*T. alba*) were negative for endoparasites (Kutzer *et al.*, 1982). Our findings constitute the first record of *S. (S.) laticeps* in the barn owl (*T. alba*) in Austria. Kutzer *et al.* (1980) examined the incidence of endo- and ectoparasites in birds of prey in Austria. Beside the common buzzard (*Buteo buteo*) and the Eurasian sparrowhawk (*Accipiter nisus*), findings of *S. (S.) laticeps* were reported in the common kestrel (*F. tinnunculus*). According to several reports from Germany (Krone, 2000; Honisch, 2008), Southern Europe (Sanmartin *et al.*, 2004; Acosta *et al.*, 2008; Santoro *et al.*, 2010) and Turkey (Aşti *et al.*, 2017) the common kestrel (*F. tinnunculus*) constitutes a common host species of this parasite. During the molecular analysis of tail tissue of lacertid lizards with nematode-specific 18S ribosomal RNA gene primers, Perera *et al.* (2013) received sequences of various genera of nematodes, including representatives of the genus *Synhimantus*. Based on their findings and the presumption that the sequences could be derived from larval nematode stages, they discussed the possible role of reptiles as paratenic hosts. For piscivorous birds, Anderson (2000) mentioned the encapsulation of the infective third-stage larvae of acuarioids in the intestine or on the mesenterium of paratenic hosts, such as frogs or fish. The possible transfer of the third-stage larvae between paratenic host species was considered (Anderson, 2000). The food range of the common kestrel (*F. tinnunculus*) includes small mammals, especially voles. In warmer areas the birds could be more dependent on insects and lizards as food source (Cramp and Simmons, 1980). During a previous study on phylogenetic relationships of nematodes from birds of prey in Germany a genetic analysis of *S. (S.) laticeps* based on a fragment of the 18S ribosomal RNA gene was carried out and the obtained sequence was submitted to GenBank® (Accession number: EU004818) (Honisch and Krone, 2008). Another sequence of this gene of an individual parasitizing in the Eurasian sparrow hawk (*A. nisus*) in the Netherlands was published in GenBank® in 2015 (Accession number: KP861914) (Schreven *et al.*, 2015). The two sequences represent the only entries of *S. (S.) laticeps* in GenBank® and showed a similarity of 99 % with the sequence of the present study. Comparison of the obtained *COI* DNA barcode determined in the present study to reference sequences in GenBank® and the Barcode of Life Data Systems, however, revealed no concordant results. Therefore, this study presents the first records of *COI* barcode sequences of this species.

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