Veterinary Research Communications (2023) 47:2165–2171
https://doi.org/10.1007/s11259-023-10079-0

BRIEF REPORT

Molecular identification of *Sarcocystis* species in sika deer (*Cervus nippon*) of free-ranging populations in Germany and Austria

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Received: 25 November 2022 / Accepted: 30 January 2023 / Published online: 8 February 2023

Abstract

In this study, for the first time, *Sarcocystis* species were identified molecularly in sika deer (*Cervus nippon*) that form free-ranging populations in several countries of Europe. Diaphragm muscle samples from 151 deer from 10 populations in Germany and Austria were examined for sarcocysts. By one-gram methylene-blue staining, sarcocysts were recorded in samples of 114 animals (75.5%) which originated from all populations. Sarcocysts were more often ($p < 0.0001$) recorded in yearling and adult deer than in calves. Infection intensity was generally low with ~ 70% of the sarcocyst positive deer harbouring ≤ 10 sarcocysts per 1-gram diaphragm muscle. Based on *cox1* sequence comparison, 10 species of *Sarcocystis*, all previously reported parasitizing cervids, were identified: *S. elongata*, *S. entzerothi*, *S. hjorti*, *S. iberica*, *S. japonica*, *S. linearis*, *S. morae*, *S. pilosa*, *S. silva* and *S. truncata*. The prevailing *S. hjorti* was detected in sika deer of all 10 populations. The identification in sika deer of *S. hjorti*, *S. iberica*, *S. elongata*, *S. linearis*, *S. morae* and *S. silva* constitutes new host records. With the additional species records of this study, the highest number of *Sarcocystis* species, at least 16, was identified in this host.

Keywords *Sarcocystis* · Sika deer · Free-ranging · Molecular identification · *cox1* · Europe

Introduction

Sika deer (*Cervus nippon*) are native to the mainland of eastern Asia and the adjacent Japanese islands. Because their taxonomy is still somewhat confused, in a rather pragmatic approach two subgroups or types are distinguished: the smaller 'Japanese' sika deer and the larger 'mainland' sika deer with each comprising several subspecies. Starting about 150 years ago, sika deer were imported into Europe and, following later translocations and releases, developed after World War II into currently well-established free-ranging populations in several countries in Europe. These populations are thought to be essentially of sika deer with Japanese roots with some of them showing substantial increase and tendency of expansion (Groves 2006; McCullough et al. 2009; Apollonio et al. 2010; Putman et al. 2022).

Sarcocystis species (Apicomplexa: Sarcocystidae) are parasitic protozoa of mammals, birds, and reptiles which form cysts mainly in muscles or central nervous system of the intermediate host and develop sporocysts in the intestine of their definitive host (Gjerde 2013). So far, seven *Sarcocystis* species have been identified in the musculature of farmed 'mainland' type sika deer in Lithuania (*S. entzerothi*, *S. frondea*, *S. nipponi*, *S. ovalis*, *S. pilosa*, *S. taeniata*, *S. truncata* – Prakas et al. 2016, Rudaitytė-Lukošienė et al. 2018). Likewise, seven species have been identified in native sika deer in Japan (*S. gjerdei*, *S. japonica*, *S. matsuoae*, *S. ovalis*, *S. pilosa*, *S.* cf. *taeniata*, *S.* cf. *tarandi* – Abe et al. 2019a, b). The *Sarcocystis* species composition of sika deer forming free-ranging populations outside of their natural range has not been studied yet. In this study we therefore investigated the *Sarcocystis* species diversity in sika deer naturalized in Germany and Austria where sarcocysts previously were detected by histology in approximately one third of the animals (Rehbein et al. 2022).

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Materials and methods

Sampling

For this study, diaphragm muscle samples of 138 sika deer were collected in the nine populations in Germany and Austria that were sampled for the recently reported *Sarcocystis* prevalence survey (Rehbein et al. 2022) and of 13 sika deer in the 'Schwansen' population (Schleswig-Holstein, Germany) (Fig. 1). The 'Schwansen' population derived from the nearby 'Ostangeln' population following migration of some animals in the late 1950's (Rehbein 2010). The sika deer sampled (eight to 22 animals per population; Table 1) were harvested according to the hunting regulations during the years of 2018/19 and 2019/20. Age and sex information of the animals was provided by the hunters; however, neither sex nor age information, no age information or no sex information was available for seven, 11 and 10 animals, respectively. In all localities sika deer share the habitat with roe deer (*Capreolus capreolus*). In the locations 'Ostangeln', 'Schwansen', 'Hüttener Berge' and 'Oberpfälzer Wald' there are also fallow deer (*Dama dama*) and in 'Arnsberger Wald', 'Oberpfälzer Wald' and 'Ostrong' sika deer share the habitat with red deer (*Cervus elaphus*) (Rehbein 2010; Rehbein et al. 2022). Until examination, the muscle samples were kept frozen (~-20 °C).

Microscopic examination

Sarcocystis infection prevalence and intensity were assessed on the basis of 1-gram methylene-blue stained muscle samples as described previously (Prakas et al. 2019). Sarcocysts were morphologically differentiated by their cyst wall appearance in fresh-squashed preparations under a light microscope (Rudaitytė-Lukošienė et al. 2021). Attempts were made to isolate sarcocysts from the muscle tissues with two fine preparation needles from all samples which were sarcocyst positive by methylene-blue staining. Unfortunately, cyst isolation from some samples was not successful and molecular analysis of isolated cysts failed in several other cases. If morphologically distinct sarcocysts were detected in one and the same animal's sample, efforts were made to isolate them. However, isolated cysts appeared morphologically similar in almost all cases; sarcocysts showing different wall protrusions in one and the same sample were observed only in three cases.

Overall, 95 sarcocysts were extracted and subjected to molecular analysis. Eighty-eight sarcocysts from 85 sika deer were identified; identification of seven cysts failed.

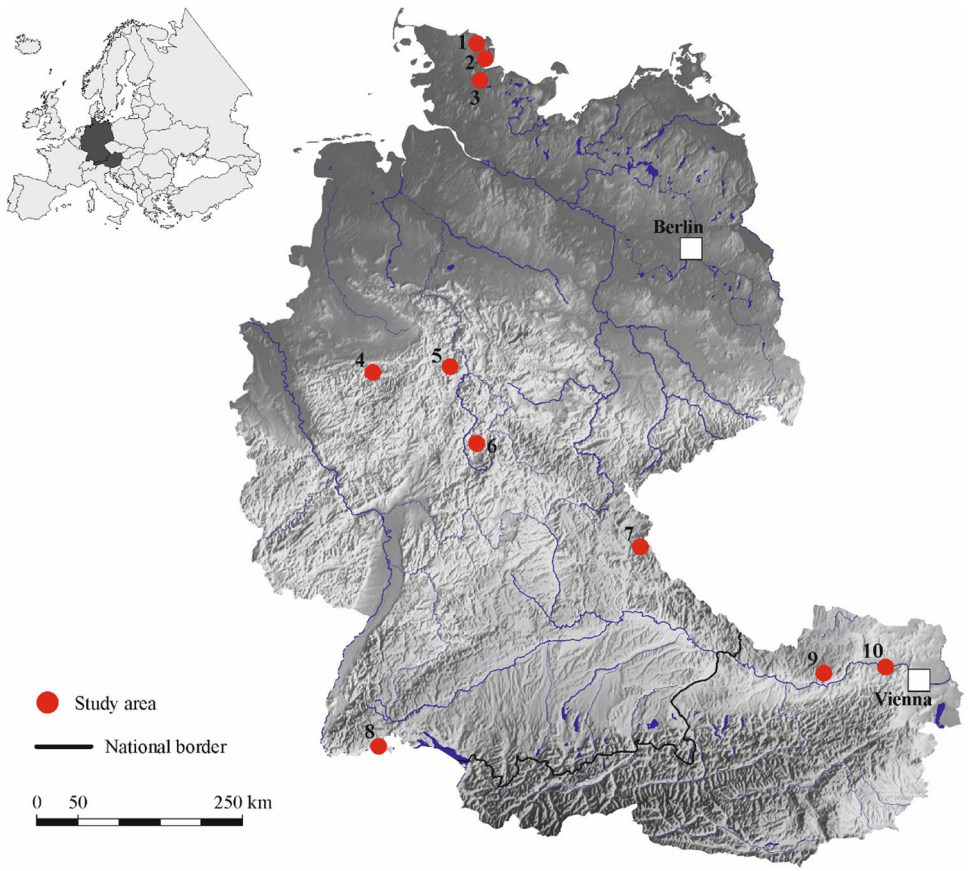

Fig. 1 Map of Germany and Austria showing location of free-ranging sika deer populations sampled (study localities): 1 = 'Ostangeln', 2 = 'Schwansen', 3 = 'Hüttener Berge'; 4 = 'Arnsberger Wald'; 5 = 'Weserbergland'; 6 = 'Schlitzer Land' 7 = 'Oberpfälzer Wald'; 8 = 'Hochrhein'; 9 = 'Ostrong'; 10 = 'Tullner Donauauen'

Table 1 Summary of the results of the examination of 1−gram methylene−blue stained diaphragm muscle samples for sarcocysts (sarcocyst prevalence and intensity) of 151 free−ranging sika deer from ten populations in Germany (DEU) and Austria (AUT)

Sika deer population (locality)	Detection of sarcocysts in 1 gram diaphragm tissue			
	Calves (<1 year[1])	Yearlings (1 − <2 years[2])	Adults (≥2 years)	All sika deer
	Number of sarcocyst positive deer/number of deer examined Number of sarcocysts in 1 gram diaphragm tissue (range)			
1, Ostangeln (DEU)	2/10 (0 – 6)	4/4 (3 – 15)	6/6 (3 – 8)	12/20 (0 – 15)
2, Schwansen (DEU)	3/9 (0 – 10)	−−−	3/4 (0 – 3)	6/13 (0 – 10)
3, Hüttener Berge (DEU)	0/6 (0 – 2)	3/6 (0 – 15)	5/5 (3 – 8)	8/17 (0 – 15)
4, Arnsberger Wald (DEU)	3/5 (0 – 29)	5/5 (2 – 5)	8/11 (0 – 12)	16/21 (0 – 29)
5, Weserbergland (DEU)	6/7 (0 – 53)	3/4 (0 – 63)	4/4 (1 – 21)	13/15 (0 – 63)
6, Schlitzer Land (DEU)	1/1 (2)	4/4 (3 – 28)	3/3 (3 – 17)	8/8 (2 – 28)
7, Hochrhein (DEU)[3]	1/2 (0 – 20)	3/3 (4 – 11)	3/3 (1 – 5)	16/18 (0 – 49)
8, Oberpfälzer Wald (DEU)[4]	−−−	7/7 (5 – 32)	1/1 (28)	9/9 (5 – 32)
9, Ostrong (AUT)	1/2 (0 – 4)	2/2 (1 – 12)	3/4 (0 – 6)	6/8 (0 – 12)
10, Tullner Donauauen (AUT)[5]	2/3 (0 – 23)	3/3 (8 – 34)	9/9 (3 – 16)	20/22 (0 – 34)
Total	**19/45 [42.2%]**[a]	**34/38 [87.2%]**[b]	**45/50 [90.0%]**[b]	**114/151 [75.5%]**

[1] Estimated age at sampling 5 to 8 months

[2] Estimated age at sampling 13 to 20 months

[3] No age information for 1 animal

[4] No age information for 10 animals

[5] No age information for 7 animals

[a,b] Prevalence values with different superscript character differ significantly ($p < 0.0001$) in Fisher's test

Molecular analysis

The GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) was used to extract genomic DNA from individual sarcocysts according to the manufacturer's instructions. All isolates were used for PCR amplification of partial *cox1* sequences using SF1/SR8D or SF1/SR11 primer pairs (Gjerde 2013; Gazzonis et al. 2019). For PCR reactions, the DreamTaq PCR Master Mix (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) was used according to the manufacturer's instructions. The PCR cycling conditions and sequencing were carried out as previously described (Rudaitytė-Lukošienė et al. 2021). The obtained sequences were manually checked and compared using BLAST (http://blast.ncbi.nlm.nih.gov/). Eighty-eight obtained *cox1* sequences of *Sarcocystis* species from sika deer were deposited in GenBank under the accession numbers OP617366–OP617453.

Statistical analysis

Associations between sarcocyst positivity by examination of 1-gram muscle samples (prevalence of infection) and age (group) and sex were assessed using contingency tables and Fisher's exact test. Sarcocyst counts in 1-gram muscle samples (intensity of infection) were analysed using the Kruskal-Wallis test. All testing was two-sided, and level of significance for all analyses was set at $p < 0.05$.

Results

Sarcocysts were detected in 75.5% (114/151) of the sika deer examined with prevalence ranging from almost 50–100% among the populations (Table 1). Sarcocyst positivity differed significantly ($p < 0.0001$) between calves (19/45, 42.2%), yearlings (34/38, 87.2%) and adult deer (45/50, 90.0%). Sarcocysts were significantly ($p < 0.0001$) more

Table 2 Identification and genetic variation of *Sarcocystis* species isolated from free−ranging sika deer in Germany (DEU) and Austria (AUT)

Sarcocystis species (number of isolates)	GenBank acc. no.	locality (country)	Sequence similarity, %.		
			Intraspecific – Comparison with sequences obtained in the present study	Intraspecific – Comparison with sequences available in GenBank	Interspecific – Comparison with most closely related species
S. hjorti (60)	OP617366- OP617425	OA, SW, HB, AW, WB, SL, OW, HR (DEU); OR, TD (AUT)	99.1–100	98.8–100	*S. pilosa* 95.4–96.6
S. entzerothi (10)	OP617426- OP617435	WB, SL, OW (DEU); OR, TD (AUT)	99.3–100	98.5–100	*S. matsuoae* 94.0–94.6
S. iberica (5)	OP617436- OP617440	AW, OW, TD (DEU, AUT)	99.0–100	99.0–100	*S. venatoria* 95.4–96.3
S. truncata (5)	OP617441- OP617445	SW, AW, SL, TD (DEU, AUT)	99.5–99.9	98.5–100	*S. japonica* 96.0–97.0
S. elongata (2)	OP617446- OP617447	SW (DEU)	99.9	98.5–100	*S. tarandi* 96.9–97.8
S. pilosa (2)	OP617448- OP617449	OW (DEU)	99.9	98.5–99.9	*S. hjorti* 95.6–96.3
S. linearis (1)	OP617450	AW (DEU)		98.8–99.2	*S. taeniata* 96.3–97.1
S. morae (1)	OP617451	OA (DEU)		97.8–100	*S. cervicanis* 89.4–89.8
S. japonica (1)	OP617452	TD (AUT)		98.5–99.7	*S. silva* 96.6–97.6
S. silva (1)	OP617453	HB (DEU)		98.3–100	*S. japonica* 96.3–97.3

often recorded in the samples from yearling and adult sika deer than in those from calves; but there was no difference in the prevalence of *Sarcocystis* infection between yearling and adult deer ($p = 1$). No difference ($p > 0.7$) in the prevalence of *Sarcocystis* infection was found between male and female deer (46/58, 79.3% vs. 58/76, 76.3%).

Infection intensity was generally low (median, five sarcocysts per 1 gram of diaphragm tissue) with ~70% and ~90% of the sarcocyst positive animals harbouring no more than 10 or no more than 20 sarcocysts per 1 gram of muscle. Sarcocyst counts differed significantly ($p < 0.001$) between calves, yearlings and adult deer with yearlings and adult deer demonstrating significantly ($p < 0.01$ each) higher intensity of infection than the calves. A marginal statistical difference was noted in the intensity of *Sarcocystis* infection in the diaphragm between total male and female sika deer (8.82 ± 11.46 vs. 5.67 ± 8.59; $p = 0.088$).

By light microscopy, three types of sarcocysts were distinguished. The majority of sarcocysts had hair-like protrusions, while the others had finger-like protrusions or appeared smooth. Based on *cox1* sequence analysis, sarcocysts with hair-like protrusions were assigned to *S. hjorti* ($n = 60$), *S. iberica* ($n = 5$), or *S. pilosa* ($n = 2$), sarcocysts with finger-like protrusions were assigned to *S. entzerothi* ($n = 10$), *S. truncata* ($n = 5$), *S. elongata* ($n = 2$), *S. japonica* ($n = 1$) or *S. silva* ($n = 1$) and smooth walled sarcocysts were assigned to *S. linearis* ($n = 1$) or *S. morae* ($n = 1$) (Table 2). The molecular identification on the basis of *cox1* sequences was accepted as valid because *Sarcocystis* species interspecific and intraspecific genetic variability values did not overlap. Comparing sequences obtained in the present study with sequences of the same species deposited in GenBank, up to 2.2% differences were established.

Sarcocystis hjorti was the predominating species, detected in all populations, while *S. entzerothi*, *S. truncata* and *S. iberica* were identified in five, four and three populations, respectively (Table 2). The remaining six species were detected each in one population. On the basis of the sika deer population, analysis of the samples revealed one to five *Sarcocystis* species per population (Table 2). In the diaphragm samples of three individuals, molecular analysis of the isolated sarcocysts revealed co-infections: *S. elongata/S. hjorti*, *S. entzerothi/S. hjorti* and *S. entzerothi/S. pilosa*.

Discussion

This study, for the first time, reports the identification of species of *Sarcocystis* parasitizing sika deer with Japanese ancestry from populations established outside of their natural range in Germany and Austria. Sarcocysts were found in ~75% of the diaphragm samples (Table 1). High prevalence of *Sarcocystis* spp. was also detected in wild sika deer in Japan (91.2% and 100%) (Matsuo et al. 2014; Abe et al. 2019b) and farmed 'mainland' type sika deer in Lithuania (92.7%) (Malakauskas and Grikienienė 2002). By contrast, the previous survey in free-ranging sika deer in Germany and Austria, based on histological examination of heart and diaphragm muscle collected during the years 2001 to 2006 and 2011 to 2013, found an overall sarcocyst prevalence of 37% with 21% of the diaphragm samples testing positive (Rehbein et al. 2022).

It could not be proved whether prevalence was changed due to differences of examination technique, type of specimens and individuals, and therefore monitoring of the situation is of necessary. In agreement with studies from Japan (Matsuo et al. 2014) and Germany and Austria (Rehbein et al. 2022), prevalence of sika deer *Sarcocystis* infection was positively correlated with the age of the animals.

With a median of five sarcocysts in 1 gram of diaphragm tissue the overall intensity of infection in the sika deer from Germany and Austria is considered low. A three-fold higher intensity was found in farmed 'mainland' type sika deer in Lithuania (median, 16 sarcocysts in 1 gram of diaphragm tissue; Malakauskas and Grikienienė 2002). Also, the present work confirms findings of other studies which recorded an age-related sarcocyst count increase in sika deer (Matsuo et al. 2014; Rehbein et al. 2022) indicating that previous exposure does not result in a robust protective host immune response, at least not under low-intensity of infection conditions.

Based on *cox1* sequence analysis 10 species of *Sarcocystis* were identified in the free-ranging sika deer from Austria and Germany. The origin of the animals as well as the presence of other species of cervids and the fauna of potential definitive hosts in a habitat may have had an important effect on the *Sarcocystis* species composition of the sika deer. The current study reveals a new host record for six *Sarcocystis* species, namely *S. elongata*, *S. hjorti*, *S. iberica*, *S. linearis*, *S. morae* and *S. silva*. *Sarcocystis* species described in sika deer from Japan (Abe et al. 2019b) and in farmed 'mainland' type sika deer from Lithuania (Prakas et al. 2016; Rudaitytė-Lukošienė et al. 2018) were also identified using the *cox1* genetic marker. The composition of species detected in all major studies of *Sarcocystis* spp. in sika deer differed substantially Prakas et al. 2016; Rudaitytė-Lukošienė et al. 2018; Abe et al. 2019a, b; and current study). In addition to a different diversity of *Sarcocystis* species, the frequency of detection of the *Sarcocystis* species differed between studies. Of the 10 *Sarcocystis* species found in current study, *S. hjorti* was the most common. *Sarcocystis taeniata* and *S. pilosa* predominated in farmed sika deer in Lithuania (Rudaitytė-Lukošienė et al. 2018) whereas *S. japonica* was the most common species in native free-ranging sika deer in Japan (Abe et al. 2019b) and appears to be the only strictly intermediate host specific *Sarcocystis* species.

The identification of 10 previously known *Sarcocystis* species in the sika deer with Japanese roots in central Europe of which six represent new host records (*S. hjorti*, *S. iberica*, *S. elongata*, *S. linearis*, *S. morae*, *S. silva*) shows that there are still questions related to the fauna of these parasites in sika deer. At least 16 *Sarcocystis* species (*S. elongata*, *S. entzerothi*, *S. frondea*, *S. gjerdei*, *S. hjorti*, *S. iberica*, *S. japonica*, *S. linearis*, *S. matsuoae*, *S. morae*, *S. nipponi*, *S. ovalis*, *S. pilosa*, *S. silva*, *S. taeniata* and *S. truncata*) have been identified in the host sika deer based on *cox1* sequence analysis Prakas et al. 2016; Rudaitytė-Lukošienė et al. 2018; Abe et al. 2019a, b; and current study). To the best of our knowledge, this is the highest number of *Sarcocystis* species established in one intermediate host species. This particular high diversity of *Sarcocystis* species may potentially be related to a recent adaptation of various cervid *Sarcocystis* spp. to sika deer in situations of close contact to other cervids (e.g., confinement in game parks before release in the wild in the past or use of small, restricted habitats in the wild which are frequently shared by related cervid hosts).

Overall, this study further adds on the observations of an apparently lower intermediate host specificity for *Sarcocystis* species than previously been thought and thus asks for continuing systematic work towards correct identification, definition of distribution and historical links of host–parasite relationships across regions.

Acknowledgements For their invaluable help with the collection of the samples the authors are grateful to Rolf Petersen ('Ostangeln'), Holger Andersch ('Schwansen'), Jan Janzen ('Hüttener Berge'), Renate Assmann ('Arnsberger Wald'); Christoph Kieneke ('Weserbergland'), Meinrad Bender ('Schlitzer Land'), Stefan Bösl ('Oberpfälzer Wald'), Stefan Weißenberger ('Hochrhein'), FM DI Hubert Hofmann ('Ostrong') and FM Dr. Herbert Tiefenbacher ('Tullner Donauauen'), and Mike Heddergott is thanked for creating Fig. 1.

Authors' contributions Petras Prakas: Conceptualization, formal analysis, supervision, writing-original draft and preparation, writing-review and editing. Steffen Rehbein: Conceptualization, formal analysis, investigation, resources, writing-original draft and preparation, writing-review and editing. Eglė Rudaitytė-Lukošienė: formal analysis, investigation, writing-original draft and preparation, writing-review and editing. Dalius Butkauskas: formal analysis, resources, writing-review and editing. All authors read and approved the final manuscript.

Data availability The *cox1* sequences of ten *Sarcocystis* species identified in in free-ranging sika deer from Germany and Austria were submitted to the GenBank database under the OP617366–OP617453 accession numbers.

Declarations

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Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

Conflict of interest The authors declare no conflicts of interest.

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