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Short communication

Wild boar as a potential reservoir of zoonotic tick-borne pathogens

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ABSTRACT

The wild boar (*Sus scrofa*) population has increased dramatically over the last decades throughout Europe and it has become a serious pest. In addition, the common habitat of wild boar and of the tick, *Ixodes ricinus*, indicates the potential of wild boar to play a role in epidemiology of epizootic and zoonotic tick-borne pathogens, including *Anaplasma phagocytophilum*. In Europe, epidemiological cycles and reservoirs of *A. phagocytophilum*, including its zoonotic haplotypes, are poorly understood. In this study, we focused on detection and further genetic characterization of *A. phagocytophilum* and piroplasmids in 550 wild boars from eleven districts of Moravia and Silesia in the Czech Republic.

Using highly sensitive nested PCR targeting the *gro*EL gene, the DNA of *A. phagocytophilum* was detected in 28 wild boars (5.1 %) representing six unique haplotypes. The dominant haplotype was found in 21 samples from 7 different districts. All detected haplotypes clustered in the largest clade representing the European ecotype I and the dominant haplotype fell to the subclade with the European human cases and strains from dogs and horses. Nested PCR targeting the variable region of the 18S rRNA gene of piroplasmids resulted in one positive sample with 99.8 % sequence identity to *Babesia divergens*.

The presence of these two pathogens that are primarily circulated by *I. ricinus* confirms the local participation of wild boar in the host spectrum of this tick and warrants experimental studies to address wild boar as a reservoir of zoonotic haplotypes of *A. phagocytophilum*.

1. Introduction

The wild boar (*Sus scrofa*) population has increased dramatically over the last decades throughout Europe and it has become a serious pest. After the Second World War decline due to overhunting, the population has grown steadily since mainly due to favorable management (Maillard et al., 2010), landscapes structural changes (Morelle et al., 2016), and climate change (Markov et al., 2019). Together with the highest reproductive rate among ungulates (Bieber and Ruf, 2005; Holland et al., 2009) and very limited natural mortality caused mostly by extreme weather conditions, diseases and predation by wolves (*Canis lupus*) (Jędrzejewski et al., 1992; Massei et al., 2015; Nores et al., 2008; Okarma et al., 1995), wild boar population has grown along with its negative impacts including (i) damage to crop and amenities (Lombardini et al., 2017), (ii) reduction in plant and animal abundance and richness (Oja et al., 2017), and (iii) a reservoir of epizootic and zoonotic diseases such as bovine tuberculosis, trichinellosis or African swine fever (ASF) (Gavier-Widén et al., 2015; Gortázar et al., 2007). In the Czech Republic, the rise of wild boar population is demonstrated by the 1800 % increase in number of hunted animals between years 1982–2012 ("State veterinary Administration," 2017). Ongoing epidemic ASF outbreak and its large-scale monitoring across Europe (Cwynar et al., 2019) has resulted in hundreds of thousands of blood samples collected in various European regions. Such massive sampling and DNA

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repositories offer a unique opportunity to examine wild boar as a sentinel for pathogens.

The wild boar occurs throughout a wide spectrum of habitats, ranging from semi-arid environments to marshes, forests and alpine grassland. In the past several years, wild boar have also colonized the urban and periurban environment in several European cities (Licoppe et al., 2013). *Ixodes ricinus* is a dominant tick in many ecosystems of Central Europe and can feed on more than 300 vertebrate species (Rizzoli et al., 2014), including counting wild boar. This tick can transmit a wide range of pathogens including *Borrelia* spp., *Anaplasma* spp., *Francisella* spp., *Rickettsia* spp., *Babesia* spp., tick-borne encephalitis virus and several others (Boulanger et al., 2019). The role of wild boar as a host for ticks is described from many parts of the world (Lim et al., 2020; Masatani et al., 2017; Merrill et al., 2018), but the relevant data from Europe are missing. In addition to *I. ricinus* (Honig et al., 2017; Silaghi et al., 2014; Skotarczak et al., 2008), studies occasionally also report wild boar infestations by *Dermacentor reticulatus* (Kazimírová et al., 2018) and *D. marginatus* (Maioli et al., 2012; Ortuño et al., 2006).

Studies on tick-borne pathogens in wild boar in Europe are scarce. Bacteria responsible for Lyme borreliosis in the *B. burgdorferi* sensu lato group were repeatedly reported absent in the European wild boar population, along with other tick-borne pathogens including *Francisella* spp., *Rickettsia* spp., and *Neoehrlichia* spp. (Kazimírová et al., 2018; Pereira et al., 2016; Silaghi et al., 2014; Skotarczak et al., 2008).

Anaplasma phagocytophilum is the most studied tick-borne pathogen in wild boar across Europe. Prevalence in wild boar varies from zero in Spain and Italy (de la Fuente et al., 2005; Portillo et al., 2011; Torina et al., 2008) to 28.0 % in Slovakia (Kazimírová et al., 2018). The evidence of *A. phagocytophilum* based on different methods and genes of interest was reported also from Belgium (Nahayo et al., 2014), Portugal (Pereira et al., 2016), Slovenia (Strasek Smrdel et al., 2009), Romania (Kiss et al., 2014), Poland (Michalik et al., 2012), and Germany (Silaghi et al., 2014) listed in order of increasing prevalence from 1.0 %–12.5 %. The extensive serological survey on 224 wild boars in Slovenia with 69.6 % seroprevalence of *A. phagocytophilum* (Zele et al., 2012) supported the potential role of wild boar in the endemic life cycle of this pathogen. *Anaplasma phagocytophilum* is a genetically polymorphic pathogen and circulating genetic variants differ in host range, clinical manifestation, and zoonotic potential. The range of genes including *ankA*, *msp4,* and *groEL* and the multi-locus approach were used to describe the different strains and the population structure (Bown et al., 2009; Chastagner et al., 2014; Huhn et al., 2014; Jaarsma et al., 2019; Jahfari et al., 2014; Scharf et al., 2011). These studies report clear delineation of strains from deer and other wild ruminants while dogs, horses, hedgehogs, and wild boar have been proposed as a reservoir of strains causing human granulocytic anaplamosis (HGA) (Huhn et al., 2014; Michalik et al., 2012; Scharf et al., 2011; Smrdel et al., 2012; Strašek Smrdel et al., 2015). In Europe, HGA remains overlooked and underdiagnosed, mainly because of mild or asymptomatic cases (Matei et al., 2019). Since the initial case in 1997 in Slovenia (Petrovec et al., 1997), about 100 cases of HGA were confirmed, which is in contrast with reported seroprevalence 16.3 % in a healthy population and supports mostly mild and asymptomatic, thus unnoticed infections (Wang et al., 2020).

Piroplasmids represent an important veterinary threat and possible risk to humans. The extensive knowledge on *Babesia* in deer species (Hrazdilová et al., 2020) is in contrast with missing data and unclear role of similarly abundant wild boar population. Molecular studies across Europe, targeting the 18S rRNA gene, report no findings of piroplasmids in wild boar populations in Hungary, Slovakia, Germany and Portugal (Hornok et al., 2018; Kazimírová et al., 2018; Pereira et al., 2016; Silaghi et al., 2014). The only findings of piroplasmids in wild boar were several reports of unspecified *Theileria* sp. in Italy and Portugal (Pereira et al., 2016; Tampieri et al., 2008; Zanet et al., 2014) and a single report of *B. bigemina* in Italy (Zanet et al., 2014). The etiological agent causing porcine babesiosis in domestic pigs reported from Sardinia, Italy (Zobba et al., 2011) was proven to be absent in a wild boar population in the same area (Zobba et al., 2014). The only two described *Babesia* species infecting pigs (including wild boar), *B. trautmanni* and B. *perroncitoi*, were detected mostly in 1990s based on morphology without any molecular data (Penzhorn, 2006).

In this study, we took an advantage of the extensive wild boar sampling campaign originally conducted to detect ASF and provide data on the prevalence and further genetic characterization of *A. phagocytophilum* and piroplasmids in wild boar from eleven districts on Moravia and Silesia in the Czech Republic.

2. Materials and methods

Samples of the wild boar DNA were acquired following the outbreak of ASF in the district Zlín of the Czech Republic in 2018. During eleven months, 9755 of wild boars were hunted by local hunters and examined for ASF by State Veterinary Institutes in Jihlava and Olomouc. The DNA was isolated from blood by the automatic system QIAsymphony SP (Qiagen, Germany) and the MagNA Pure 96 System or the MagNA Pure LC 1.0 Instrument (Roche, Switzerland) and aliquots were provided for detection of tick-borne pathogens. Each sample was labeled with the information on locality (nearest village), date of sampling, and age category (piglet, yearling, adult). Based on the locality, 50 samples were chosen from each of 11 available districts (Fig. 1A) resulting in the total 550 samples analyzed for presence of *A. phagocytophilum* and piroplasmids.

All PCRs were conducted with 2x PCRBIO Taq Mix Red (PCR Biosystems, UK), first round reactions in nested protocols were done in the total volume of 15.0 μL using 2.0 μL of the template DNA. In the second round, we used 1.0 μL of the first round reaction as a template in the total volume of 25.0 μL. Details for each reaction are provided (Table S1). *Anaplasma phagocytophilum* was detected by nested PCR amplifying 407 bp of the *groEL* gene. For positive samples, the protocol amplifying 1297 nt of the GroESL operon was used. The detection of piroplasmids was done by the highly sensitive nested PCR targeting the variable region of the 18S rRNA gene.

PCR products were visualized on 1.5 % agarose gel with the Midori Green Advance (Nippon Genetics Europe, Germany), products of the expected size were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taiwan) and sequenced by the Macrogen capillary sequencing services (Macrogen Europe, the Netherlands) using the amplification primers. Obtained sequences were edited using the Geneious 9.1.2 software (Kearse et al., 2012) and identity of amplicons was confirmed by BLASTn analysis. Unique haplotypes were identified by ALTER (Alignment Transformation Environment) (Glez-Peña et al., 2010).

The dataset for subsequent phylogenetic analyzes was compiled from the GenBank sequences of the *groEL* gene longer than 1000 nt of *A. phagocytophilum*, representing all ecotypes. Due to an uneven length of sequences, the alignment was calculated in two steps by MAFFT algorithm, using “Auto” strategy for sequences >1000 nt and function –add for implementing sequences <1000 nt to the alignment. The phylogenetic tree was generated by the maximum likelihood method by IQTREE v. 1.6.5 (Nguyen et al., 2015). The best-fit evolution model was chosen based on the Bayesian information criterion (BIC) computed by implemented ModelFinder (Kalyaanamoorthy et al., 2017). Branch supports were assessed by the ultrafast bootstrap (UFBoot) approximation (Minh et al., 2013) and by the SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010). The tree was visualized and graphically edited using FigTree v1.4.1 and Inkscape v0.91.

3. Results

The DNA of *A. phagocytophilum* was detected in 28 wild boars (5.1 %). The number of positive animals per 50 tested animals in each district ranged from 0 in Zlín (ZL) up to 5 in Nový Jičín (NJ). The prevalence slightly increased with age; from piglets (6/175, 3.4 %), yearlings (15/

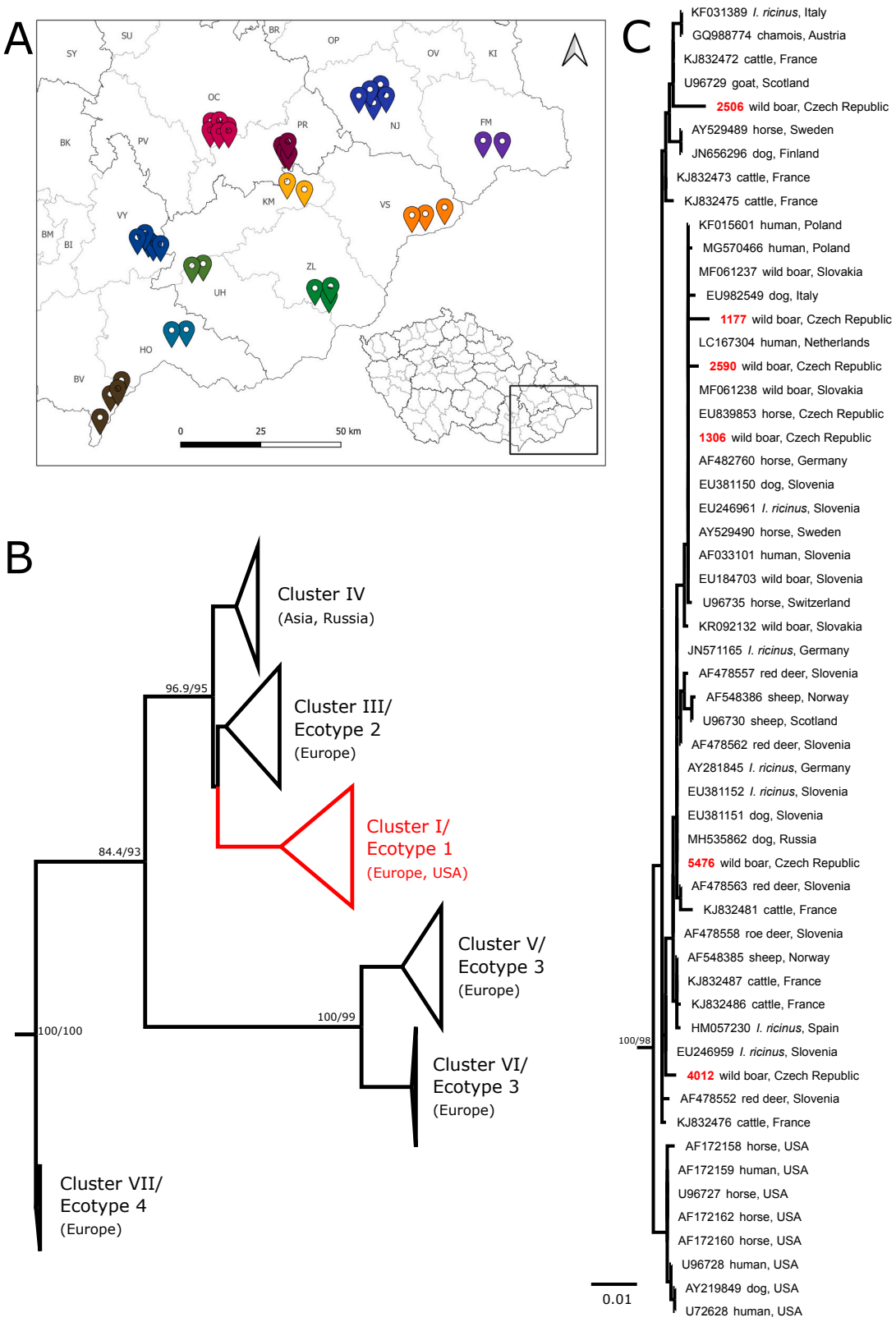

Fig. 1. A) Map of sampling areas across eleven districts of Moravia and Silesia in the Czech Republic. District abbreviation: BV – Břeclav, FM – Frýdek Místek, HO – Hodonín, KM – Kroměříž, NJ – Nový Jičín, OL – Olomouc, PR – Přerov, UH – Uherské Hradiště, VS – Vsetín, VY – Vyškov, ZL - Zlín B) Schematic representation of the maximum likelihood phylogenetic tree based on the *groEL* gene sequences of *A. phagocytophilum* longer than 1000 nt representing all ecotypes. The highlighted clade representing the Ecotype I is displayed in details; bootstrap values (SH-aLRT/UFB) above the 80/95 threshold are displayed; all sequences included in the analysis are listed in Table S2. Five sequences of *A. platys* used as an outgroup are not displayed. C) detailed view of the clade representing the Ecotype I/Cluster I; sequences acquired from the GenBank database are marked by their accession number, host and country of origin. Sequences from this study are highlighted in red and marked by the number of a respective haplotype. The scale bar indicates the number of nucleotide substitutions per site (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

270, 5.6 %) to adults (2/22, 9 %), but without statistical significance (χ^2 (1, N = 467) = 1.88, p = .39). From 28 samples, 18 long (>1150 nt) and 10 short (300–400 nt) sequences of the *groEL* gene were obtained, representing 6 unique haplotypes (deposited to GenBank under accession numbers MT498612-7). The main haplotype (represented by sample no. 1306) was detected in 21 samples from 7 different districts. The second most abundant haplotype (no. 5476) originated from three samples from three different districts; remaining haplotypes were represented by one sample each. Distances between haplotypes ranged from 0.25 % (3 SNPs/1210 nt) between haplotypes 1306 and 5476 to 2.19 % (8 SNPs/366 nt) between haplotypes 1117 and 2506. Nearest hits for each haplotype available in the GenBank database are listed in Table 1.

In phylogenetic analyses (Fig. 1B), all detected haplotypes clustered in the largest clade representing the European ecotype I (Jahfari et al., 2014) closely related to the USA isolates and formed the Cluster I (Jaarsma et al., 2019). Three haplotypes (1306, 2590 and 1177) fell to the subclade with the European human cases and strains from dogs and horses. The other three haplotypes were scattered among strains mostly from cattle and red deer (Fig. 1C).

Nested PCR targeting the variable region of the 18S rRNA gene of piroplasmids resulted in an amplicon of the expected size, approx. 560 bp, in 25 out of 550 samples tested (4.5 %). However, sequencing confirmed the piroplasmid DNA only in a single sample from district Přerov (PR, Fig. 1A) with 99.8 % identity to *B. divergens* from a red deer from the Czech Republic (acc.no. MG344780). PCR targeting the mitochondrial COX1 gene (Hrazdilová et al., 2020) to confirm the identity of detected *Babesia* sp. was negative. Remaining amplicons were identified by sequencing and BLAST analyses with 100 % identity as *Sarcocystis miescheriana* in 21 samples (3.8 %), and *Eimeria polita* and *Cystoisospora suis* in two and one sample, respectively.

4. Discussion

In the human dominated landscape, in addition to rodents, the medium-sized and large mammals including foxes, roe deer, and wild boar serve as hosts for ticks and may play a role in the ecology of tick-borne pathogens. The overpopulation of wild boar in Europe raises concerns in several aspects, including the involvement of these abundant hosts in maintenance of tick populations, shift of natural transmission cycles of pathogens, and spillover of pathogens in humans and domestic animals (Rizzoli et al., 2014).

The overall prevalence 5.1 % of *A. phagocytophilum* in this study is in accordance with the report of 6.0 % prevalence from wild boar in the Czech Republic detected by qPCR targeting the 16S rRNA gene (Hulínská et al., 2004) and also with the data from the forested temperate areas of Europe reporting prevalences 2.7–12.0 % (reviewed in Stuen et al., 2013). These results are in stark contrast to data from syntopic and similarly abundant free-ranging ruminants (several deer species, chamois, mouflon, etc.) where *A. phagocytophilum* was detected in >50 % of animals tested (Stuen et al., 2013). The low prevalence of *A. phagocytophilum* in wild boar in Europe implies participation of these animals in circulation of this pathogen (Strasek Smrdel et al., 2009; Zele et al., 2012) but indicates their rather limited role as a reservoir of *A. phagocytophilum* when compared to that of cervids (Jaarsma et al., 2019).

In our study, we followed the classification of *A. phagocytophilum* based on partial *groEL* sequences as introduced by Jahfari et al. (2014) and extended by Jaarsma et al. (2019). All six detected haplotypes belonged to the ecotype I/cluster I. This clade is associated with the broadest host range and *I. ricinus* as the vector with the distribution across the Western Palearctic and Nearctic regions (Jaarsma et al., 2019). Importantly, ecotype I includes all genetically characterized strains detected from the human cases in Europe (Matei et al., 2019). The BLAST analyses of haplotypes detected in the Czech Republic confirmed the dominance of the genotype previously associated with several human cases in Slovenia (Smrdel et al., 2012), Poland (Welc-Falęciak et al., 2018, 2014) and the Netherlands (Jahfari et al., 2016). The same haplotype was found in three wild boars in the Czech Republic in 2003 near Znojmo (Petrovec et al., 2003) and recently in Slovakia (Kazimírová et al., 2018). Furthermore, this haplotype was also detected in dogs and horses of different geographical origins, which is in concordance with previous studies using various genes and multi-loci classification (Chastagner et al., 2014; Huhn et al., 2014; Michalik et al., 2012; Scharf et al., 2011; Strašek Smrdel et al., 2015). The epidemiological cycles of *A. phagocytophilum* are poorly understood, while red deer have been suggested as the reservoir of non-zoonotic haplotypes of the ecotype I and rodents as a reservoir of other ecotypes (Dugat et al., 2015), the most likely candidates for a reservoir of zoonotic haplotypes remain wild boar.

From the set of 550 wild boar samples from 11 districts of the Czech Republic, we demonstrated that the wild boar population has negligible importance in circulation of piroplasmids, with only a single *B. divergens* positive individual using the highly sensitive nested PCR protocol. Previous studies reporting absence of piroplasmids across Europe were done on a low number of samples (<100) and using qPCR or simple PCR protocols for detection (Hornok et al., 2018; Kazimírová et al., 2018; Silaghi et al., 2014). The only exception was the study in Portugal reporting 3 out of 65 wild boars positive for *Theileria* sp. with the 98.0–99.0 % identity to *T. capreoli* (Pereira et al., 2016). Two European studies on a larger number of samples were done in Italy, using simple and semi-nested PCR found two *B. bigemina* and two *Theileria* sp. positive individuals out of 257 tested (Zanet et al., 2014) and three *Theileria* sp. out of 117 (Tampieri et al., 2008). Application of methods with the highest possible sensitivity targeting the 18S rRNA gene appears to be the only reliable approach to detect the DNA of piroplasmids in samples with low parasitemia and prevalence (Hrazdilová et al., 2019).

Presence of the two pathogens that are primarily circulated by *I. ricinus* confirms the local participation of wild boar in the host spectrum of this tick. Even though the prevalence of *A. phagocytophilum* is not high, the dominance of the zoonotic haplotype in these animals together with their abundant population in Europe warrants experimental studies to address wild boar as a reservoir of this pathogen. Numbers of wild boar sampled within ASF monitoring, together with the fact that its

Table 1
Unique haplotypes of the *A. phagocytophilum groEL* gene detected in a wild boar population and their nearest BLAST hits. District abbreviation: FM – Frýdek Místek, HO – Hodonín, KM – Kroměříž, NJ – Nový Jičín, OL – Olomouc, PR – Přerov, UH – Uherské Hradiště, VS – Vsetín, VY – Vyškov.

Haplotype no.	No. of samples	Districts	GenBank
1306 (1206 nt)	21	FM, KM, NJ, OL, PR, UH, VY	100 % AF033101 (human, Slovenia), AY529490 (horse, Sweden), AF482760 (horse, Germany)
5476 (1205 nt)	3	FM, NJ, UH	100 % AY281823 (*I. ricinus*, Germany)
1177 (366 nt)	1	PR	99.45 % MN093180 (*I. ricinus*, the Netherlands), MG570466 (human, Poland), MF061238 (*S. scrofa*, Slovakia), KT970680 (dog, Italy), KU712132 (*V. vulpes*, Germany), …
2506 (366 nt)	1	HO	99.18 % MT025713 (*I. ricinus*, Italy), MN093256 (*I. ricinus*, the Netherlands), MK069963 (*C. elaphus*, Norway), KU712128 (*C. nippon*, Germany), KJ832471 (horse, France), …
2590 (366 nt)	1	VS	99.73 % MN093180 (*I. ricinus*, the Netherlands), MG570466 (human, Poland), MF061238 (*S. scrofa*, Slovakia), KT970680 (dog, Italy), KU712132 (*V. vulpes*, Germany), …
4012 (366 nt)	1	HO	100 % MK069706 (*C. elaphus* Norway)

diagnostics is mostly based on examination of DNA samples, make the wild boar promising sentinels for vector-borne and other pathogens. If properly managed and shared, these DNA samples can be used for the Europe-wide monitoring of a range of pathogens.

CRediT authorship contribution statement

Kristýna Hrazdilová: Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Paulina Maria Lesiczka:** Investigation. **Jan Bardoň:** Resources. **Šárka Vyroubalová:** Resources. **Bronislav Šimek:** Resources. **Ludek Zurek:** Conceptualization, Writing - review & editing. **David Modrý:** Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101558.

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