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Investigation on haplotypes of ixodid ticks and retrospective finding of *Borrelia miyamotoi* in bank vole (*Myodes glareolus*) in Switzerland

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ABSTRACT

The current status of tick species, important tick-borne bacteria and protozoan parasites is well-documented in Switzerland. However, reports on the genetic diversity and geographical relationships of tick species in this country appear to be in part lacking or outdated. Thus, the aim of this study was to collect ticks from various host species in southern Switzerland, to compare them in a geographical context and to screen in these samples rare tick-borne pathogens hitherto not reported or having low prevalence in Switzerland.

In 2019–2020 altogether 177 ixodid ticks were collected from the vegetation, as well as from humans ($n = 17$), dogs ($n = 23$), cats ($n = 41$), red deer ($n = 8$), a European rabbit and a European hedgehog at 25 locations in three cantons of south Switzerland. Tick species were identified morphologically, followed by DNA extraction and comparison of mitochondrial haplotypes with molecular-phylogenetic methods. Tick DNA extracts, as well as sixty-two rodent liver or spleen tissue DNA extracts (representing six species) available from 2005 to 2006 were screened for trypanosomes, *Occidentia massiliensis* and *Borrelia miyamotoi*.

Morphologically, three tick species were identified: *Ixodes ricinus* ($n = 170$), *Rhipicephalus sanguineus* sensu lato ($n = 6$) and *I. hexagonus* ($n = 1$). In contrast to companion animals (dogs, cats) immature ticks (larvae and nymphs) predominated on humans, which was a highly significant association ($P < 0.0001$). Molecular comparison of the cytochrome *c* oxidase subunit I (*cox*1) gene with GenBank data established the species as *R. sanguineus* sensu stricto and confirmed *I. hexagonus*, both showing 99.8–100% sequence identity to conspecific ticks from northern Italy. Seventy-nine specimens morphologically identified as *I. ricinus* revealed high 16S rRNA gene haplotype diversity and represented two phylogenetic groups. Two *I. ricinus* haplotypes from Switzerland belonged to the same haplogroup with *I. inopinatus* from Spain, Germany and Austria as well as with *I. ricinus* reported from a broad geographical range of Europe (including Italy, the Netherlands, Poland, Latvia and Sweden). All 141 tick DNA extracts (from five *R. sanguineus* s.l., 135 *I. ricinus* and one *I. hexagonus*) and 62 rodent tissue DNA extracts were negative for trypanosomes and *O. massiliensis*. However, *B. miyamotoi* was identified in a bank vole (*Myodes glareolus*) and three ticks by sequencing.

From Switzerland, this is the first report of tick haplotypes that are phylogenetically closely related to *I. inopinatus*. However, based on their morphology, both specimens are considered as *I. ricinus*. These results highlight the importance that the identification of *I. inopinatus* should be based on coherent morphologic and molecular properties. This is also the first report of rodent-borne *B. miyamotoi* in Switzerland. Taking into account the year of collection (2005), in a chronological order this might be the first indication of *B. miyamotoi* in any rodent species in Europe.

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1. Introduction

In Europe, hard ticks (Acari: Ixodidae) are considered as the most important vectors of pathogens with high veterinary-medical importance (Jongejan and Uilenberg, 2004). While in the era of climate change the increasing significance of ticks and tick-borne diseases has long been recognized on this continent (Parola and Raoult, 2001), not all regions are equally affected. The most dramatic emergence of newly discovered tick species and/or tick-borne pathogens has been witnessed in northern Europe (Mysterud et al., 2017), but relevant changes are also evident in the Mediterranean region in southern Europe (Efstratiou and Karanis, 2019), highlighting the importance of studies monitoring this situation.

Switzerland occupies an intermediate position between western, central and southern Europe. Although it has a temperate climate, this shows considerable variations within the country, ranging from cooler weather in and north of the Alps to warmer summer and milder winter in its southernmost canton (Ticino) which is closest to the Mediterranean region (Spinedi and Isotta, 2004). Accordingly, the tick fauna of Switzerland reflects the simultaneous presence of species with diverse ecological needs, including cold tolerant tick species (such as *Ixodes ricinus*: Dautel and Knülle, 1997) as well as thermophilic ones in the south (i.e., *Rhipicephalus sanguineus* sensu lato and *R. turanicus*: Aeschlimann et al., 1965; Bernasconi et al., 2002). In addition, migratory birds may import exotic tick species (Papadopoulos et al., 2002), and may also facilitate genetic exchange between populations of indigenous tick species within and outside Switzerland (Lommano et al., 2014).

In general, the occurrence and prevalence rates of tick-borne bacteria and protozoan parasites are well-documented and have recently been updated several times in Switzerland (Casati et al., 2006; Boretti et al., 2009; Lommano et al., 2012; Hofmann-Lehmann et al., 2016; Oechslin et al., 2017; Pilloux et al., 2018). However, investigations on the genetic diversity and geographical relatedness of tick species in this country appear to be outdated. For instance, Switzerland was not included in the pan-European survey of *I. ricinus* populations (Noureddine et al., 2011), and regional studies focusing on this tick species date back to around two decades ago (Delaye et al., 1997; de Meeûs et al., 2002).

The primary aim of the present study was to compensate for the above mentioned scarcity of the haplotype-related data on tick species occurring in Switzerland. Therefore, ixodid ticks were collected from various host species in the southern part of the country, and consequently analyzed molecularly and phylogenetically based on mitochondrial markers. Special emphasis was laid on investigating the possible presence of *Ixodes inopinatus* which was originally described as a Mediterranean tick species (Estrada-Peña et al., 2014), but more recently has also been reported from northern central Europe (Hauck et al., 2019).

It was also within the scope of this study to attempt the detection of rare tick-borne pathogens which have not hitherto been reported in ticks or rodents in Switzerland. In the pathogen screening assays not only recently collected ticks, but rodent tissue DNA extracts (available from 2005 to 2006) were also analyzed retrospectively. Target pathogens included *Borrelia miyamotoi* (Spirochaetales: Spirochaetaceae), a relapsing fever group spirochaete transmitted from rodent and bird reservoirs (Wagemakers et al., 2017) to humans via the bite of hard ticks. *Borrelia miyamotoi* is associated with species of the *I. ricinus* complex (in Europe mainly with *I. ricinus*) as vectors, in which it is maintained transovarially, unlike Lyme disease spirochaetes (Siński et al., 2016). It was reported in ticks (Lommano et al., 2012; Oechslin et al., 2017) but was not found in rodents in Switzerland (Burri et al., 2014).

Tick and rodent tissue DNA extracts were also screened for *Occidentia massiliensis* (Rickettsiales: Rickettsiaceae) and trypanosomes (Kinetoplastida: Trypanosomatidae). The rationale for this was that recently *O. massiliensis* (originally isolated and described from soft ticks: Mediannikov et al., 2014) has been found in hard ticks (Hornok et al., 2022), and the occurrence of *Trypanosoma* sp. has been recognized in *I. ricinus* in Switzerland (Aeschlimann et al., 1979) and in Slovakia (Luu et al., 2020).

2. Materials and methods

2.1. Sample collection and identification

Between August 2019 and August 2020, altogether 177 ticks were collected from cats ($n = 41$), dogs ($n = 23$), humans ($n = 17$), red deer ($n = 8$), a European rabbit and a European hedgehog, as well as from the vegetation at 25 locations in three cantons of southern Switzerland (Ticino, Geneva, Vaud: Supplementary Fig. 1). Ticks were stored in 70% ethanol, and their species were morphologically identified using taxonomic keys (Estrada-Peña et al., 2014; 2017). Pictures of selected ticks were made with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan).

In addition, 62 rodent tissue (51 liver, 11 spleen) DNA extracts, available from 2005 to 2006 (Willi et al., 2007) and stored at -20 °C, were analyzed retrospectively. Sample sources included two *Arvicola terrestris*, 11 *Apodemus flavicollis*, 27 *Apodemus* sp., 10 *Myodes glareolus*, three *Microtus agrestis*, two *Microtus arvalis*, as well as seven *Mus musculus* from the canton Grisons, Switzerland. Rodent species were identified according to Hausser (1995).

2.2. DNA extraction

Tick analyses are summarized in Supplementary Fig. 2. In total, 141 morphologically intact ticks (five *R. sanguineus* s.l., 135 *I. ricinus* and one *I. hexagonus*) were selected for individual DNA extraction. Ticks were disinfected on their surface with sequential washing for 15 s in three Petri dishes (containing 10% NaClO, tap water or distilled water, respectively), then air-dried. DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction, including an overnight digestion in tissue lysis buffer and Proteinase K at 56 °C. A negative control (tissue lysis buffer) was also processed in each set of tick samples to monitor cross-contamination. The success of DNA extraction from ticks was checked by measuring DNA concentration with a Thermo Scientific Multiscan GO Spectrofotometer (Thermo Fisher Scientific Oy, Vantaa, Finland). Conditions and controls of DNA extraction from rodent tissues are described elsewhere (Willi et al., 2007).

2.3. Molecular taxonomic analyses of ticks

The cytochrome *c* oxidase subunit I (*cox*1) gene was chosen for the molecular comparison of *R. sanguineus* s.l. ($n = 5$) and *I. hexagonus* ($n = 1$) with GenBank data, taking into account availability of relevant sequences from northern Italy (Pistone et al., 2017; Hornok et al., 2017). A 710-bp-long fragment of this gene was amplified with a conventional PCR using the primers LCO1490 (forward: 5′ -GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (reverse: 5′ -TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Integrated DNA Technologies, IDT, Leuven, Belgium) (Folmer et al., 1994) as reported (Hornok et al., 2016).

The 16S rRNA gene was chosen for the molecular-phylogenetic analyses of *I. ricinus*, because this target gene has corresponding sequences for *I. inopinatus* (Estrada-Peña et al., 2014) and for comparison with other studies (Noureddine et al., 2011; Paulaskas et al., 2016; Jaenson et al., 2016; Chitimia-Dobler et al., 2018; Hauck et al., 2019). The method used in this study amplifies an approx. 460-bp-long fragment of the 16S rRNA gene of Ixodidae (Black and Piesman, 1994) with the primers 16S+1 (5′- CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′) and 16S-1 (5′-CCG GTC TGA ACT CAG ATC AAG T-3′) (IDT) as reported (Hornok et al., 2016). This PCR was performed with 79 DNA extracts of *I. ricinus*, including ticks collected from cats (24 ticks), dogs (12 ticks), deer (16 ticks), humans (20 ticks), a European rabbit (one tick) and the vegetation (five ticks). The origin of one tick was unknown.

In the *cox*1 and 16S rRNA gene PCRs sequence-verified DNA from *R. sanguineus* "dog96" (Licari et al., 2017) served as positive control.

2.4. Molecular screening for Borrelia miyamotoi (Spirochaetales: Spirochaetaceae)

An approx. 730-bp-long fragment of the glycero-phospho-diester phospho-diesterase (*glpQ*) gene of *B. miyamotoi* was amplified with the primers glpQ-BM-F2 (5′-ATG GGT TCA AAC AAA AAG TCA CC-3′) and glpQ-BM-R1 (5′-CCA GGG TCC AAT TCC ATC AGA ATA TTG TGC AAC-3′) (IDT) (Hovius et al., 2013; Szekeres et al., 2015). The reaction volume of 25 µl contained 5 µl of extracted DNA, and 20 µl of reaction mixture including 0.2 µl (1 unit) HotStarTaq Plus DNA polymerase (QIAGEN, Hilden, Germany), 0.5 µl (200 µM) PCR nucleotide mix (QIAGEN), 0.5 µl (1 µM) of each primer and 2.5 µl of 10 × Coral Load PCR buffer (15 mM $MgCl_2$ included) (QIAGEN). Touchdown PCR was used for the amplification, during which an initial denaturation step at 95 °C for 5 min was followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s (annealing temperature was decreased -1 °C/cycle) and extension at 72 °C for 1 min. The first 10 cycles were followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min; final extension was performed at 72 °C for 10 min, in a GenePro Thermal Cycler TC-E-48D (BIOER Technology Co., Ltd, Hangzhou, China). Sequence-verified DNA of *B. miyamotoi* from *I. ricinus* nymph "M19" (Szekeres et al., 2015) served as positive control.

2.5. Molecular screening for Occidentia massiliensis (Rickettsiales: Rickettsiaceae)

This PCR is based on a primer pair (Hornok et al., 2022) which matches the heat shock chaperonin protein encoding *groEL* gene of *O. massiliensis*, amplifying a 656-bp-long part of the gene. The primers Om-groELf1 (5′-AAA AAA GAA ATG TTA GAA GAT ATT GC-3′) and Om-groELr2 (5′-GTA CGT ACW ACT TTA GTT GG-3′) (IDT) were used in a reaction volume of 25 µl, which included 5 µl of extracted DNA, and 20 µl of reaction mixture containing 0.2 µl (1 unit) HotStarTaq Plus DNA polymerase (QIAGEN, Hilden, Germany), 0.5 µl (200 µM) PCR nucleotide mix (QIAGEN), 0.5 µl (1 µM) of each primer and 2.5 µl of 10 × Coral Load PCR buffer (15 mM $MgCl_2$ included) (QIAGEN). For amplification, an initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 5 min (performed in a GenePro Thermal Cycler, BIOER). Sequence-verified DNA of *O. massiliensis* from *Africaniella* (formerly *Amblyomma*) *transversale* "Amb2" (Hornok et al., 2022) served as positive control.

2.6. Molecular screening for trypanosomes (Kinetoplastida: Trypanosomatidae)

DNA samples were also screened with a conventional PCR that amplifies an approx. 900-bp-long fragment of the 18S (SSU) rRNA gene of trypanosomes and related kinetoplastids. The primers 609F (forward: 5′-CAC CCG CGG TAA TTC CAG C-3′) (da Silva et al., 2004) and 706R (reverse: 5′-CTG AGA CTG TAA CCT CAA-3′) (IDT) (Ramírez et al., 2012) were used as described (Szőke et al., 2017). Sequence-verified DNA of *Trypanosoma corvi* "II." (kindly provided by Jan Votýpka) served as positive control.

2.7. Negative controls, sequencing and phylogenetic analyses

In all PCRs non-template reaction mixture served as negative control. Extraction controls and negative controls remained PCR negative in all tests.

Purification and sequencing of the PCR products (in one direction with the forward primer, repeated when necessary) were done by Biomi Ltd. (Gödöllő, Hungary). Quality control and trimming of sequences were performed with the BioEdit program, then alignment with GenBank sequences by the nucleotide BLASTN program (https://blast.ncbi.nlm.nih.gov). Representative sequences were submitted to GenBank (*cox*1: MW135447-MW135447, 16S rRNA: MW130924-MW130953, *glpQ*: MW139976). Sequences from other studies (retrieved from GenBank) included in the phylogenetic analyses had nearly 100% coverage with sequences from this study. This dataset was resampled 1000 times to generate bootstrap values. An unrooted phylogenetic tree was made in the present study because it is beneficial to show the clustering of related sequences (Kinene et al., 2016), in accordance with the aims of this study. Phylogenetic analyses were conducted with the Maximum Likelihood method and Jukes-Cantor model by using MEGA version 7.0.

2.8. Statistical analysis

Infestation prevalence data according to tick developmental stages (number of immature ticks [larvae and nymphs] vs number of adults compared between host species), as well as finding of haplotypes on cats (vs other hosts) were analyzed by Fisher's exact test. Differences were regarded significant when $P < 0.05$. For the comparison of tick abundance data, meteorological seasons were taken into account (spring: March to May; summer: June to August; autumn: September to November; winter: December to February).

2.9. Ethical permission

Ticks collected from animals were provided by veterinarians (who removed them during animal care) and by licensed hunters (who collected ticks *post mortem* from game animals killed for other purposes). Ticks from human subjects were provided for this study on a volunteer basis, after removal by the infested persons themselves. Written consents were obtained from the organizers of tick collection from humans (M. Pasche, D. Guscetti) to use these ticks for scientific purposes. Rodent samples were taken from a previous study (Willi et al., 2007). Thus, no vertebrate animals were killed or restrained for the purpose of tick collection, therefore no ethical permission was needed.

3. Results

3.1. Tick species and their molecular-phylogenetic analyses

Morphologically, three tick species were identified: *I. ricinus* ($n = 170$), *R. sanguineus* s.l. ($n = 6$) and *I. hexagonus* ($n = 1$) (Table 1). *Ixodes ricinus* was found on five host species, i.e., cat, dog, human, red deer and European rabbit, but *I. hexagonus* and *R. sanguineus* s.l. only on one host species, European hedgehog and dog, respectively.

Regarding the developmental stages of *I. ricinus*, only one nymph occurred on dogs, while as many as four larvae and eight nymphs on cats, although this was a non-significant association (25 adults and one immature tick on dogs vs 61 adults and 12 immature ticks on cats: $P = 0.17$). On the latter host, adults included three mating pairs. However, in contrast to companion animals (dogs, cats) immature ticks (larvae and nymphs) predominated on humans (14 immatures and six adults), which was a highly significant association ($P < 0.0001$).

Ixodes ricinus was collected in all four seasons (including winter in case of cats only). Adults of this tick species were active year-round. All three mating pairs were collected in May–June (Table 1). The presence of nymphs on their host or on the vegetation was associated with the spring and the summer. *Ixodes ricinus* larvae were found only during summer (Table 1).

Considering results of molecular taxonomic analyses, two *R. sanguineus cox*1 gene haplotypes were identified from the same dog, differing in one nucleotide. Comparison (Blast alignment) showed that both haplotypes from southern Switzerland had 99.7% (585/587bp) and

Table 1
Hosts, species and stages of ticks from Switzerland (2019–2020) according to month of collection.

Origin Generic name	Tick Number of hosts	Species	Total number	Stage or sex (month of collection)			
				Female	Male	Nymph	Larva
Human	17	*Ixodes ricinus*	20	2 (June), 4 (July)	-	4 (May), 6 (June), 2 (July)	2 (August)
Dog	23	*Rhipicephalus sanguineus* s.l.	6	-	-	6 (August)	-
		Ixodes ricinus	26	1 (April), 7 (May), 6 (June), 3 (July), 2 (August), 3 (September), 1 (October), 1 (November)	1 (April)	1 (June)	-
Cat	41	*Ixodes ricinus*	74	5 (February), 4 (March), 10 (April), 14 (May), 10 (June), 7 (August), 1 (September), 3 (October), 2 (December)	2 (February), 2 (May) ⚥, 2 (June) ⚥ ⚥	1 (March), 1 (April), 1 (May), 2 (June), 3 (August)	4 (June)
Red deer	8	*Ixodes ricinus*	29	1 (June), 3 (July), 17 (September)	8 (September)	-	-
Rabbit	1	*Ixodes ricinus*	7	-	-	-	7 (August)
Hedgehog	1	*Ixodes hexagonus*	1	1 (June)	-	-	-
Vegetation	4 occasions	*Ixodes ricinus*	14	1 (July)	-	1 (June)	12 (July)

Marks: ⚥ Mating with a female.

Abbreviation: s.l. - sensu lato.

99.8%–100% identities (629–630/630bp) with *R. sanguineus* sensu stricto from southern France (GenBank: MH630346) and northern Italy (KX757904), respectively. *Ixodes hexagonus* collected in southern Switzerland was genetically most closely related to conspecific ticks reported from northern Italy, meaning 100% (631/631bp) identity with the corresponding sequence (MG432679).

Molecular analysis of 79 *I. ricinus* specimens revealed high haplotype diversity, i.e., in total 30 different 16S rRNA sequences (Supplementary Table 1). Regarding the countries of origin with the closest sequence matches in GenBank, most *I. ricinus* 16S rRNA haplotypes were shared between Switzerland and Slovakia (i.e., 16 out of 30), followed by France (12 out of 30) (Supplementary Table 1). Ticks from hosts with multiple ticks infesting them (up to five *I. ricinus* specimens per host) showed the presence of different 16S rRNA haplotypes on the same host individual in the majority of cases (10 out of 13).

An *I. ricinus* female collected from a cat (in Malvaglia, Ticino) and an *I. ricinus* larva from a rabbit (sampled in Chironico, Ticino) had 99.2% (360/363bp and 361/364bp) sequence identities with *I. inopinatus* (KM211789) and were assigned to haplogroup "184-AG" because of AG bases at positions 184/185 in their 16S rRNA gene sequences, similarly to *I. inopinatus*. In case of the larva, pictures were made prior to DNA extraction and these confirmed morphologically its identity as *I. ricinus*, i.e., marginal dorsal (md) setae measured approximately 3–4 times the length of the 5th scutal seta (sc5), similarly to *I. ricinus* (Supplementary Fig. 3). All other 77 specimens identified morphologically as *I. ricinus* had CT bases at positions 184/185 in their 16S rRNA gene sequences and were therefore assigned to haplogroup "184-CT". The separation of these two haplogroups was also confirmed phylogenetically (Fig. 1). Both "184-AG" haplotypes from Switzerland clustered together with the two originally described haplotypes of *I. inopinatus* (KM211789, KM211790), as well as with others recently identified under this species name in Germany (KY569416-KY569417) and Austria (KY569418). In addition, cluster "184-AG" contained several other haplotypes which were morphologically identified as *I. ricinus* in previous studies but had AG bases at positions 184/185 in their 16S rRNA gene sequences. The latter samples originated from north Africa and further European countries, including Tunisia, Spain, Italy, the Netherlands, Poland, Latvia and Sweden (Fig. 1).

3.2. Molecular screening of rare pathogens in ticks and rodents

All tick DNA extracts ($n = 141$) and rodent tissue DNA extracts ($n = 62$) were PCR negative for trypanosomes and *O. massiliensis*.

However, three *I. ricinus* ticks collected in this study were PCR positive for *B. miyamotoi*, amounting to a prevalence of 2.2% in *I. ricinus* (three out of 135 ticks). The *glpQ* sequence of *B. miyamotoi* could be amplified from all three ticks and showed 100% identity with each other and with several sequences in GenBank, including strain EU1 (KJ003844). These PCR-positive ticks included two *I. ricinus* females (removed from dogs on two occasions, in June, 2020 in Versoix, canton Geneva), as well as an *I. ricinus* nymph from a cat (sampled in June 2020 in Givrins, canton Vaud). Thus, all three ticks which contained the DNA of *B. miyamotoi* originated in the southwestern "corner" of Switzerland.

In addition, the *glpQ* sequence of *B. miyamotoi* was also successfully amplified from a rodent liver (bank vole, *M. glareolus*). This sequence had 100% identity (648/648bp) with the above tick-derived sequence. The relevant bank vole originated in eastern Switzerland (Malans, canton Grisons) and was sampled in October, 2005.

4. Discussion

During this study, four tick species were collected and analyzed from the points of view of their seasonality, host associations, molecular-phylogenetic properties and contents of tick-borne pathogens. To extend the scope of the latter, rodents were also included, on account of their significant roles as hosts of immature ticks and reservoirs of tick-borne pathogens.

Regarding the seasonality of *I. ricinus* in Switzerland, the questing activity of adults usually lasts from mid-March to mid-October (Herrmann and Gern, 2015), while immature stages feed on rodents from late February to early November (Pérez et al., 2012; Herrmann and Gern, 2015). However, here the presence of *I. ricinus* females was also noted during winter months (December and February) on cats, unlike on dogs. The most likely explanation for the winter activity of *I. ricinus* adults is that in the study period Switzerland experienced the mildest winter in the history of its meteorological records (Pisani and Glatthard, 2020). Differences between cats and dogs may be related to their mode of keeping, i.e. cats are frequently allowed to roam freely and thus have more occasions to contact questing ticks on the vegetation.

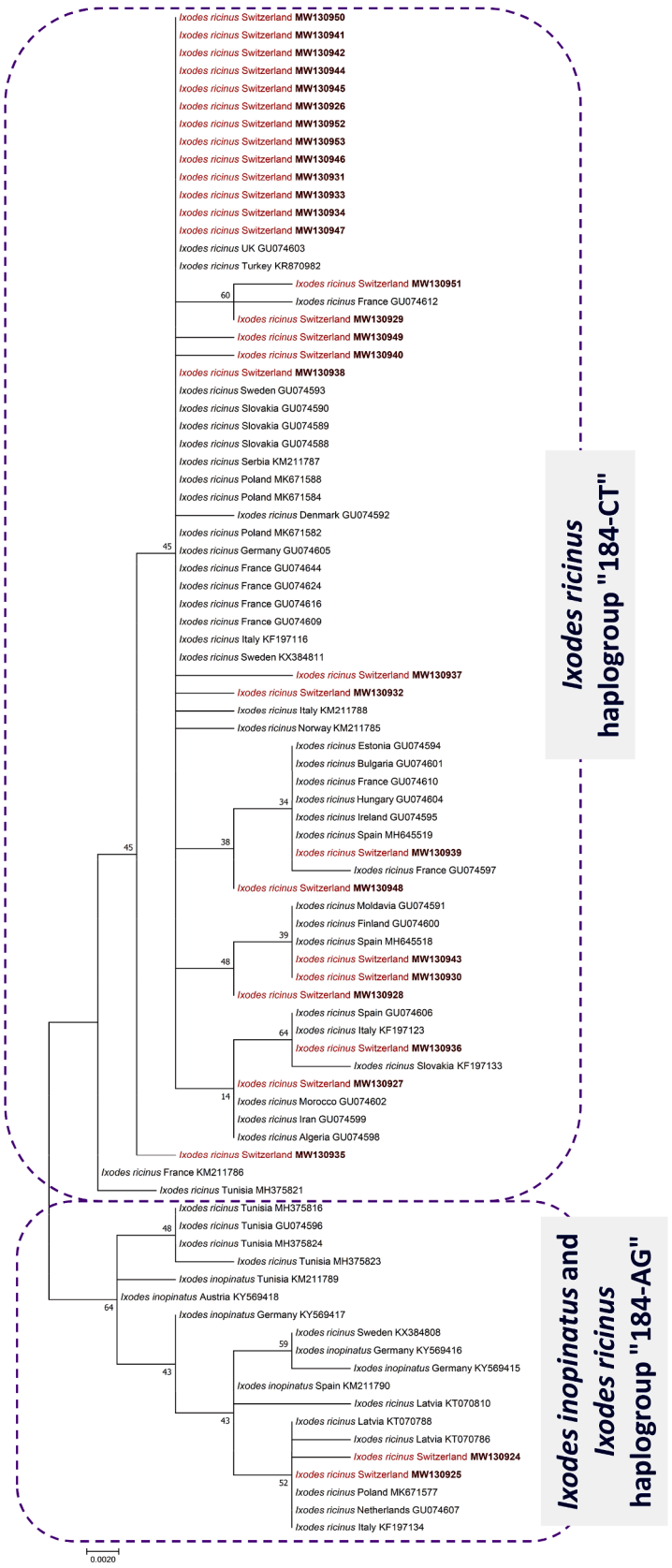

Fig. 1. Maximum Likelihood phylogenetic tree of *Ixodes ricinus* and *I. inopinatus* 16S rRNA gene sequences from Europe. Tick species names are followed by the country of origin and GenBank accession numbers. The sequences from this study are indicated with red color and bold accession numbers. The scale-bar indicates the number of 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.)

Human infestation with ticks in southern Switzerland was shown here to be associated with immature ticks (particularly nymphs), which can be attributed to their smaller size (i.e., prior to engorgement frequently remaining unnoticed). This confirms similar data from other countries (e.g., Wilhelmsson et al. 2013).

During the molecular analyses an extremely high genetic diversity of *I. ricinus* was found, with 30 different 16S rRNA haplotypes. In contrast, no small-scale population genetic divergence was found between local *I. ricinus* populations in Switzerland (Delaye et al., 1997). Interestingly, when populations of *I. ricinus* were analyzed previously for their relationships within Europe based on phenotype characteristics, specimens from Switzerland almost completely aligned with others from only one country, Slovakia (Estrada-Peña et al., 1996). This was confirmed by the present results, because the highest number of 16S rRNA haplotypes from Switzerland were shared with Slovakia. A possible explanation for this phenomenon is the frequent transportation of ticks by birds between these two countries. Among birds, the European robin (*Erithacus rubecula*) was reported to be the main host of *I. ricinus* larvae and nymphs in Switzerland (Papadopoulos et al., 2002), and this bird species is known for its southwest to northeast migratory route in Europe, including flyway connection between Switzerland and Slovakia (Hornok et al., 2012).

On the other hand, *R. sanguineus* and *I. hexagonus* are rarely transported by birds (Papadopoulos et al., 2002), therefore, on a continental level, reduced gene flow and higher rate of geographical structuring can be anticipated between their populations. The occurrence of these two tick species in southern Switzerland is well-documented (Bernasconi et al., 1997; 2002), but their taxonomic analyses have not been reported. Based on ticks collected during the present study, comparisons of the *cox*1 gene indicated that *R. sanguineus* s.s. and *I. hexagonus* in southern Switzerland are genetically most closely related to conspecific ticks reported from northern Italy. Although the sample number was low, this may reflect "haplotype confluence" of their populations between the regions of southern Switzerland and northern Italy, most likely due to ground-bound dispersal events.

Two *Ixodes* haplotypes identified in this study clustered together with *I. inopinatus* which was originally thought to be a Mediterranean tick species (Estrada-Peña et al., 2014), but later it was also discovered with low abundance in other parts of Europe (e.g. Chitimia-Dobler et al. 2018, Hauck et al. 2019). Already prior to its description, genetic analysis of microsatellites of *I. ricinus* ticks collected in Switzerland showed absence of differentiation at the local scale (even between specimens separated by the Alps) but marked difference between Switzerland and Tunisia (de Meeûs et al., 2002). Later on, *I. inopinatus* was described as a new species based (in part) on samples collected in Tunisia. Nevertheless, until now haplotypes aligning with this tick species have not been reported in Switzerland.

According to the present results, ticks from haplogroup "184-AG" appear to be rare in southern Switzerland, because these were found only in two cases. Both haplotypes had AG bases at position 184/185 in the 16S rRNA gene (similarly to the originally described specimen of *I. inopinatus*: KM211789). Phylogenetic analysis of 16S rRNA gene sequences performed here, including data from this as well as other studies and GenBank, suggest the pan-European presence of *I. ricinus* haplogroup "184-AG", i.e., in regions of Scandinavia (Sweden), Baltic and Benelux countries (Latvia and the Netherlands, respectively), as well as central Europe (Poland) and southern Europe (Italy).

Considering the latter regions, all ticks included in the phylogenetic analysis here, were morphologically identified as *I. ricinus* (e.g., Paulaskas et al. 2016, Jaenson et al. 2016) well after the description of *I. inopinatus* (Estrada-Peña et al., 2014). However, some ticks providing sequences in haplogroup "184-AG" were identified morphologically as *I. inopinatus* (KM211789, KM211790: Estrada-Peña et al., 2014; KY569415-KY56941: Chitimia-Dobler et al., 2018). Thus, according to the above, identification of *I. inopinatus* should be based on coherent morphologic and molecular properties, and not exclusively on molecular data (as in Hauck et al. 2019). In addition, since there was no clear geographical pattern in the 16S rRNA phylogenetic tree (Fig. 1), the present findings confirm the lack of consistent genetic structuring of *I. ricinus* populations within Europe (Noureddine et al., 2011).

All tick and rodent tissue DNA extracts were PCR negative for trypanosomes and *O. massiliensis*. Thus, PCR-negativity of all ticks and rodents evaluated here from southern Switzerland suggest that these have low or no relevant epidemiological significance in the studied region.

The prevalence of *B. miyamotoi* in *I. ricinus* collected in Switzerland was reported to be 1 to 2.5% in other studies (Lommano et al., 2012; Oechslin et al., 2017), which corresponds to the present finding (2.2%). Interestingly, the latter study reported the highest prevalence of tick-borne *B. miyamotoi* in western Switzerland (Basel, Neuchâtel), which coincides with the geographical aspects of the present findings, i.e. PCR positivity of ticks was detected only in the western part of southern Switzerland. While in the present study *B. miyamotoi*-infected ticks were removed from two dogs and a cat, these pet animals are only known to be susceptible to soft tick-borne relapsing fever spirochaetes (Elelu, 2018).

In addition to PCR-positive ticks, the sequence of *B. miyamotoi* was successfully amplified here from the liver of a bank vole. Natural infection of the bank vole with *B. miyamotoi* was reported in countries neighboring Switzerland (e.g., Cosson et al. 2014). However, in a previous study carried out in Switzerland on *B. miyamotoi* in rodents (including the bank vole), blood samples remained negative for this spirochaete (Burri et al., 2014). Therefore, to the best of our knowledge, this is the first finding of *B. miyamotoi* in a rodent in Switzerland. Taking into account the year of collection (2005), in a chronological order this might be the first indication of *B. miyamotoi* in any rodent species in Europe. The bank vole (*M. glareolus*) was experimentally shown to be a competent reservoir of *B. miyamotoi*, with the ability to cause infection of tick larvae feeding on them (Burri et al., 2014). Thus, the present finding contributes to our knowledge on the natural epidemiological role of this rodent species in Switzerland.

In conclusion, it was demonstrated here for the first time that 16S rRNA haplotypes phylogenetically close to *I. inopinatus* occur in Switzerland. However, species identification should be based on coherent morphologic and molecular properties, and according to the morphologic criteria reported in the original description of *I. inopinatus* (Estrada-Peña et al., 2014), both tick specimens of its haplogroup collected in Switzerland are considered as *I. ricinus*. This is also the first report of rodent-borne *B. miyamotoi* in Switzerland, which originates from 2005 and therefore, in a chronological order, might be the first indication of *B. miyamotoi* in any rodent species in Europe.

CRediT authorship contribution statement

Sándor Hornok: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Julie Daccord:** Data curation, Investigation. **Nóra Takács:** Methodology, Visualization, Investigation. **Jenő Kontschán:** Methodology, Software. **Barbara Tuska-Szalay:** Methodology, Visualization, Investigation. **Attila D. Sándor:** Data curation, Investigation. **Sándor Szekeres:** Methodology, Software. **Marina L. Meli:** Methodology, Software. **Regina Hofmann-Lehmann:** Conceptualization, Supervision, Writing – review & editing.

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