

Hard Ticks Parasitizing European Ground Squirrel, *Spermophilus citellus* (L., 1766) (Rodentia: Sciuridae) in Serbia

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Abstract: Specimens of the endangered species *Spermophilus citellus* (European ground squirrel) from populations inhabiting 12 localities in Vojvodina (Serbia) were inspected for the presence of attached hard ticks. A total of 1009 ticks were found on 151 infested animals. With respect to the level of infestation, over 60% of the squirrels were found with five or less ticks attached, while the distribution of the number of animals with a higher level of infestation tended to follow the 80/20 rule. A notably higher level of infestation was detected in males as compared to females. Nymphs were the predominant developmental stage of ticks parasitizing the inspected European ground squirrel specimens. The majority of removed ticks belonged to the nidicolous species *Ixodes laguri* (79%), while the remaining ticks were identified as *Haemaphysalis concinna*. In seeking molecular confirmation of morphological identification of *Ixodes* specimens, COI gene sequences of *I. laguri* were obtained and deposited in GenBank.

Keywords: ticks, *Spermophilus citellus*, *Ixodes laguri*, *Haemaphysalis concinna*, enzootic cycles

Introduction

Ticks are obligatory haematophagous ectoparasites of terrestrial vertebrates including amphibians, reptiles, birds and mammals. Ticks can cause direct effects on their hosts during the feeding process but they are much more important as transmitters of causative agents of infectious diseases (bacterial, viral and protozoan). JONGEJAN & UILENBERG (2004) indicated that the variety of pathogens transmitted by ticks is greater than in any other group of arthropod vectors. These ectoparasites are important for both human and veterinary medicine. Recently, 17 human pathogens were mentioned as proven to be transmitted by ticks (DANTAS-TORRES et al. 2012), while the list of tick-borne pathogens of veterinary importance is even longer (JONGEJAN & UILENBERG 2004). Tick-borne pathogens in ecosystems persist

in natural enzootic cycles that include vertebrate reservoirs in addition to the tick vectors. The role of reservoirs in these cycles is usually to maintain and multiply the pathogens, while the vectors spread them vertically and horizontally in terms of time and space. For successful prevention and control of tick-borne diseases, it is of crucial importance to identify all components of the enzootic cycle, including vertebrate hosts of different tick developmental stages, in the area of interest.

The European ground squirrel, *Spermophilus citellus* (L., 1766) (Rodentia: Sciuridae), is a medium-sized rodent species that lives in open habitats with short grass, such as steppes, dry meadows and pastures (RUŽIĆ 1978, PETROV 1992). It lives in colonies, in long complex burrow systems, with each

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individual having its own burrow system (RUŽIĆ 1978). Behavioural characteristics make *S. citellus* an exquisitely suitable host of ticks in the mentioned ecosystems. Data concerning ticks parasitizing *S. citellus* are scarce. *Ixodes laguri* is the most common tick species associated with the European ground squirrel, while *Haemaphysalis sulcata* and soft ticks (*Argas bureschi*) have also been reported as ectoparasites of this mammal (PESHEV 1955, BERON 1974, MIHALCA et al. 2012).

Spermophilus citellus is distributed in South-eastern and Central Europe, and its range is divided into two main geographic areas by the Carpathian Mountains (MITCHELL-JONES et al. 1999, WILSON & REEDER 2005). In Serbia, the distribution of *S. citellus* is divided. The majority of these animals live north of the Sava and Danube Rivers (in the Vojvodina Province), while a few small and isolated mountain populations exist in the south-eastern part of the country (PETROV 1992). Habitat loss and fragmentation during the last decades has led to a dramatic decline in the number of populations throughout the whole range of *S. citellus*. As a consequence, this species is included in the International Union for Conservation of Nature (IUCN) Red List as vulnerable (COROIU et al. 2011). It was recently reported (ŘÍČANOVÁ et al. 2011) that small and highly fragmented populations from the Czech Republic suffered from low genetic diversity and an increased level of inbreeding, which can have a further impact on the adaptive variation and ability of individuals to respond to the influence of pathogens and illnesses. Previous studies showed that populations of *S. citellus* in Serbia are viable with a low level of inbreeding (ĆIROVIĆ et al. 2008, ĆOSIĆ et al. 2008, 2013), but pronounced fluctuations in density and the existence of barriers between populations caused by habitat fragmentation point to the need for constant monitoring.

The purpose of this study was to investigate the fauna of hard ticks in Serbian populations of *S. citellus* with reference to both the presence of ticks as ectoparasites of *S. citellus* and the possible role of ground squirrels in natural enzootic cycles of tick-borne pathogens. The obtained data are discussed and compared with previously published data concerning tick-borne pathogens detected in hard ticks from Serbia (MILUTINOVIĆ et al. 2008, TOMANOVIĆ et al. 2010, 2013, RADULOVIĆ et al. 2011).

Materials and Methods

Tick collection and processing of material

Ground squirrels were captured during the summer months (July-early September) as a part of continu-

ous monitoring of populations in Vojvodina (the northern province of Serbia). Live cage traps were baited with fresh fruit. The sex of all animals was recorded, as well as the precise place of capturing. During the period 2007-2013, all of the captured ground squirrels were thoroughly inspected for the presence of ticks in all developmental stages. No animal was injured during this research. All captured animals were released back in the same place at dusk. Ticks were collected from 151 animals at 12 localities (Fig. 1, Table 1). All detected ticks were removed with tweezers, put in separate tubes with 70% ethanol and transported to the Laboratory for Medical Entomology of the Institute for Medical Research in Belgrade for identification. Morphological identification of ticks was performed using a standard taxonomic key (POMERANCEV 1950). Determination of species, sex and developmental stage was performed in the laboratory.

Molecular systematic analysis

In order to confirm morphological identification of *Ixodes* species, which was based on available incomplete taxonomic keys, randomly selected tick samples were chosen for further molecular analysis.

Genomic DNA was extracted from individual ticks using the Genomic DNA Purification Kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. Amplification of the internal transcribed spacer 2 (ITS2) region and barcoding cytochrome c oxidase I (COI) sequences was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Vilnius, Lithuania) and previously described primers and conditions (RADULOVIĆ et al. 2010, ĆAKIĆ et al. 2012). Products proven to be positive after agarose gel electrophoresis underwent sequencing, which (including primer walking) was performed by Macrogen Inc. (Amsterdam, the Netherlands). Representative

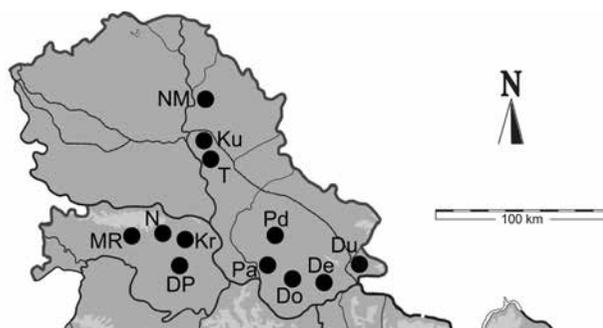


Fig. 1. Distribution of collected ticks from the northern part of Serbia (Vojvodina). MR (Mala Remeta), N (Neradin), Kr (Krušedol), DP (Donji Petrovci), Pd (Padi-na), Pa (Pančevo), Do (Dolovo), De (Deliblato), Du (Dupljaja), T (Taraš), Ku (Kumane), NM (Novo Miloševo)

Table 1. Number, species and developmental stages of collected ticks per localities.

Locality	Host		Ticks (N)								
			I. laguri				H. concinna				
	sex	N	larvae	nymph	female	male	larvae	nymph	female	male	
Krušedol (K)	M	3			5						
	F	8		7	7						
Σ		11		7	12						
Neradin (N)	M	16	15	86	7			10			
	F	23	1	74	10			12			
Σ		39	16	160	17			22			
Donji Petrovci (DP)	M	17		112	10			78			
	F	11		27	10			39			
Σ		28		139	20			117			
Mala Remeta (MR)	M	18	1	124	9			34			
	F	11	2	134	9	1		38			
Σ		29	3	258	18	1		72			
Okolina Pančeva (OP)	M	3		2	6			1			
	F	7		7	8						
Σ		10		9	14			1			
Dolovo (Do)	M	1			1						
	F	1	1								
Σ		2	1		1						
Deliblato (De)	M							1			
	F	1									
Σ		1						1			
Padina (P)	M	7		44	4	1					
	F	6		22	3						
Σ		13		66	7	1					
Dupljaja (Du)	M	2	1	3	6						
	F	5		11	3						
Σ		7	1	14	9						
Novo Miloševo (NM)	M	4		3	1						
	F	1			1						
Σ		5		3	2						
Taraš (T)	M										
	F	2		2							
Σ		2		2							
Kumane (K)	M	2	4	4	2						
	F	2	1	4							
Σ		4	5	8	2						
ΣΣ		151	26	666	102	2	0	213	0	0	
Total number of ticks:			796				213				
			1009								

sequences were deposited in the GenBank database under the following accession numbers: COI sequences--KM096850-KM096859; and ITS2 sequences--KM096860-KM096863.

Phylogenetic analysis was done with “Phylogeny.fr” software (DEREEPER et al. 2008) set

to default parameters, with 100 replications for determining bootstrap values.

Statistical analysis

The chi-square test was performed using MS Excel

2010, in addition to which average values with statistical errors were also calculated. According to the level of infestation, *S. citellus* specimens were arbitrarily divided into three groups, as follows: lightly infested (1-5 ticks per animal), moderately infested (6-10) and highly infested (>10).

Results

A total of 1009 tick specimens were collected from 151 infested individuals of *S. citellus*, giving a mean intensity of infestation of 6.675 ± 0.700 ticks per infested animal. Males appeared to be more infested, with an average of 7.849 ± 0.957 ticks per animal, in comparison with 5.577 ± 1.006 ticks per animal detected in females. The majority of animals (around 64%) had a low level of infestation, while almost equal numbers of animals were found with moderate and high infestation levels (Table 2). From 30 highly infested animals (≥ 10 ticks attached), a total of 670 ticks were detached. The distribution of male *S. citellus* individuals according to the infestation level was shifted toward moderate and high levels in comparison to the distribution of females, the difference being statistically significant ($p=0.006$). Specimens discovered with a very high infestation level had > 20 ticks per individual (12/151, $\approx 8\%$), with up to 48 and 49 ticks found on two females. A total of 371 (37%) ticks were found on 12 highly infested animals.

Two species of hard ticks were found to parasitize *S. citellus* in Serbia, namely *Ixodes laguri* and *Haemaphysalis concinna*. Out of 1009 ticks collected, 796 individuals belonged to the species *I. laguri* (78.69%), while 213 were identified as *H. concinna* (21.11%). Classical morphological determination was sufficient to identify *H. concinna* specimens, while determination of species of individuals belonging to the genus *Ixodes* was more demanding, mostly due to the significant number of immature individuals, as well as partial damage to tick mouthparts incurred during the collection process. Phylogenetic analysis based on COI and ITS2 sequences suggested that all tested ticks of the genus *Ixodes* belong to the same species, as all sequences obtained in this study segregated in a separate cluster clearly apart from other clusters present (Figs. 2a, 2b). There were no available COI or ITS2 sequences of *I. laguri* previously deposited in the GenBank database to be used in the phylogenetic analysis. However, the sequences obtained from our samples were grouped in a cluster close to the COI sequence of *Ixodes redikorzevi* (Fig. 2a), a species closely related to *I. laguri*. Nymphs appeared as the

predominant developmental stage of collected ticks (Fig. 1). All collected *H. concinna* individuals were nymphs, while 83.67% of collected *I. laguri* ticks belonged to this developmental stage. In addition to nymphs, 26 (3.27%) larvae, two (0.25%) males and 101 (12.81%) females of *I. laguri* were also collected (Table 1). Out of 151 infested *S. citellus* individuals, 109 were infested only with *I. laguri* and six only with *H. concinna*, while 36 animals were jointly infested with both tick species. Mixed infestation with both tick species, *I. laguri* and *H. concinna*, was detected on 10 of 12 highly infested animals.

Discussion

Ground squirrels represent good candidates for tick hosts. Their biology is in line with the needs of both exophilic and nidicolous tick species. The feeding behaviour of ground squirrels makes them exposed to exophilic ticks questing in vegetation, while several nidicolous tick species have been recorded in burrows of different ground squirrel species (PESHEV 1955, WHITAKER 1972, BERON 1974, USLU et al. 2008, MIHALCA et al. 2012, ANSTEAD et al. 2014). Our results are in agreement with previous reports indicating *I. laguri* as the most common nidicolous tick species parasitizing on *S. citellus* (PESHEV 1955, MIHALCA et al. 2012). Surprisingly, in the present study only one exophilic tick species was reported on *S. citellus*. This could be explained by the fact that the sampling was conducted during the national program of *S. citellus* monitoring, which is carried out during the period from July to early September every year. Spring is the reproductive season of ground squirrels and any disturbance, as well as the catching of animals, is avoided at this time of year. On the other hand, in studying hard tick population dynamics, we previously found that the activity of ticks in Serbia reaches peaks during the spring and in late autumn (MILUTINOVIĆ 1992, MILUTINOVIĆ & RADULOVIĆ 2002, MILUTINOVIĆ et al. 2006). Our unpublished data indicate that the seasonal maximum in activity of the species *H. concinna* is shifted toward the summer months in ecosystems suitable as habitats of *S. citellus*. The same can be concluded from the report of HORNOK & FARKAS (2009), who described the distribution and peak activity of questing ticks in different biotopes in Hungary. Moreover, parasitizing of *S. citellus* by other exophilic tick species, especially during the spring months, cannot be ruled out.

There are very few data on the role of ground squirrels in enzootic cycles of the causative agents of vector-borne diseases. Some species are involved in

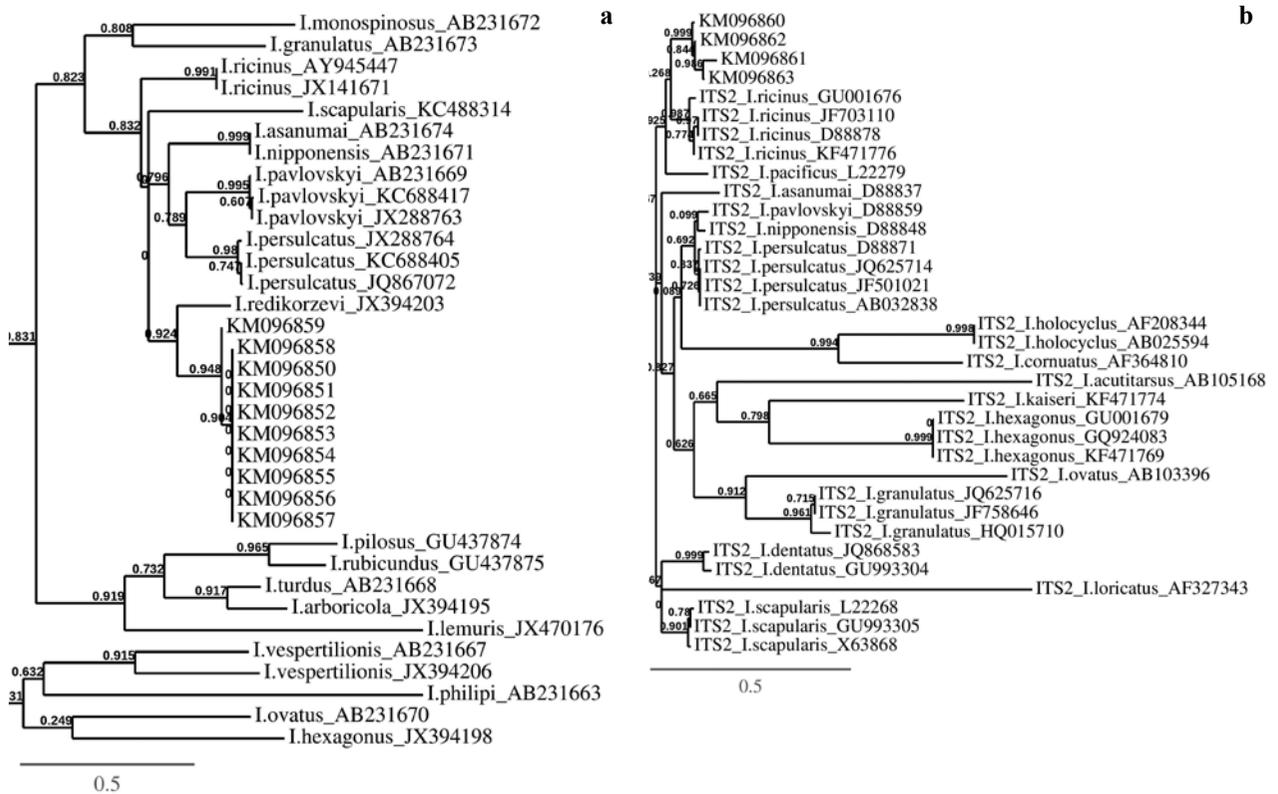


Fig. 2. Phylogenetic analysis based on cytochrome c oxidase I (COI) (a) and internal transcribed spacer 2 (ITS2) (b) sequences of *Ixodes* sp. ticks obtained in this study (accession numbers KM096850-KM096863) and those previously deposited in GenBank.

Table 2. Distribution of the number of European ground squirrels based on the level of tick infestation.

Sex of European ground squirrel	Number of animals with different level of tick infestation		
	Low (1-5 ticks per animal)	Medium (6-10 ticks per animal)	High (>10 ticks per animal)
Male	37	18	18
Female	59	10	9
TOTAL	96	28	27

the maintenance of the flea-borne pathogen *Yersinia pestis*, the causative agent of plague (EISEN & GAGE 2008), while North American species are an important component for the maintenance of an enzootic cycle of the Colorado tick fever virus, which is transmitted by the tick *Dermacentor andersoni* (CAREY et al. 1980, CIOTA & KRAMER 2010). To the best of our knowledge, there are no data about the role of *S. citellus* in enzootic cycles of tick-borne pathogens. In any event, association with both exophilic and nidicolous ticks suggests that *S. citellus* could be included in an enzootic cycle of tick-borne pathogens through some bridge vectors and maintain it in association with nidicolous species, as was previously described for several burrowing mammals (MAUPIN et al. 1994, GERN et al. 1997). Several tick-borne pathogens, including *Anaplasma phagocytophilum*, *A. ovis*, *Babesia canis* and *Coxiella burnetii*, were recently detected in questing adult *H. concinna* ticks

from Serbia (TOMANOVIĆ et al. 2013). It is important to note that ticks for the mentioned study were collected in localities ecologically similar to habitats of *S. citellus*.

Our results show predominance of the nymphal developmental stage in comparison with larvae and adults in tick parasitism on *S. citellus*. In general, this can be attributed to coincidence of the sampling period with the seasonal peak in activity of the nymphal instar (HONZAKOVA et al. 1980, MENG et al. 2014). In addition, *H. concinna* is a generalist tick species, with adults mostly feeding on rabbits and large mammals. On the other hand, *I. laguri* is a specialist, which can explain the presence of all three developmental stages parasitizing *S. citellus*.

WOOLHOUSE et al. (1997) empirically identified a widely applicable relationship in epidemiology and parasitology, postulating that 20% of the host population contributes to at least 80% of the transmission

potential. This relationship, also known as the 80/20 rule, explains the observed aggregation of ticks on their hosts, which has further implications for the establishment and persistence of tick-borne pathogens (PERKINS et al. 2003, HARRISON & BENNETT 2012). Despite collecting of samples at the time of year when tick activity is very low in the studied localities (MILUTINOVIĆ 1992, MILUTINOVIĆ & RADULOVIĆ 2002, MILUTINOVIĆ et al. 2006), the aggregation of ticks on *S. citellus* individuals recorded in our study tends to follow this rule. However, the majority of inspected *S. citellus* individuals had a low level of infestation, indicating that ticks as ectoparasites do not represent a risk for populations of *S. citellus* in Serbia. The higher level of infestation recorded in male specimens of *S. citellus* is probably a result of sexual differences observed in the behaviour of ground squirrels: males are more active and occupy

larger ranges, especially during the mating season (MICHENER & MCLEAN 1996). The predominance of joint infestation by *I. laguri* and *H. concinna* in highly infested animals, as well as the higher proportion of *H. concinna* nymphs found on male specimens of *S. citellus* (around 58%), supports this statement.

To conclude, our results show that *S. citellus* populations in Serbia have the potential to be a part of the maintenance of enzootic cycles of tick-borne pathogens. In future studies, the presence of tick-borne pathogens in both species of ticks removed from *S. citellus* should be studied, taking care not to endanger populations of this vulnerable species of rodent in the process.

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