Rickettsia spp. in rodent-attached ticks in Estonia and first evidence of spotted fever group Rickettsia species Candidatus Rickettsia uralica in Europe.

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Abstract

BACKGROUND: *Rickettsia* spp. are human pathogens that cause a number of diseases and are transmitted by arthropods, such as ixodid ticks. Estonia is one of few regions where the distribution area of two medically important tick species, *Ixodes persulcatus* and *I. ricinus,* overlaps. The presence of the nidicolous rodent-associated *I. trianguliceps* has also recently been shown in Estonia. Although there is no data available in Estonia on human disease caused by tick-borne *Rickettsia* spp., the presence of three *Rickettsia* species in non-nidicolous ticks was also previously reported. The aim of this study was to detect, identify and partially characterize *Rickettsia* species in nidicolous and non-nidicolous ticks attached to rodents.

RESULTS: Larvae and nymphs of *I. ricinus* (*n* = 1004), *I. persulcatus* (*n* = 75) and *I. trianguliceps* (*n* = 117) removed from rodents and shrews caught in different parts of Estonia were studied for the presence of *Rickettsia* spp. by nested PCR. Ticks were collected from 314 small animals of 5 species (*Myodes glareolus* (bank voles), *Apodemus flavicollis* (yellow necked mice), *A. agrarius* (striped field mice)*, Microtus subterranius* (pine voles) and *Sorex araneus* (common shrews)). Rickettsial DNA was detected in 8.7% (103/1186) of the studied ticks. In addition to *R. helvetica* previously found in questing ticks, this study reports the first identification of the recently described *I. trianguliceps*-associated *Candidatus* R. uralica west of the Ural Mountains.

Background

Rickettsia is a genus of small, obligate intracellular gram-negative bacteria. Based on genomic analyses they are classified into four groups: the spotted fever group (SFG), the typhus group, the ancestral group and the transitional group [1]. Some SFG rickettsiae are transmitted by ticks of the family Ixodidae [2], and transmission may occur transovarially as well as transstadially [3, 4]. Several agents of tick-borne rickettsioses are known to circulate in Europe, including *Rickettsia conorii*, *R. massiliae*, *R. slovaca*, *R. raoultii*, *R. monacensis* and *R. helvetica [2, 5], of which the last-mentioned is frequently detected species in* numerous Ixodidae ticks, including *Ixodes ricinus*, *I. persulcatus*, *I. trianguliceps*, and *Dermacentor reticulatus [2, 6, 7]*. Although *R. helvetica* is not believed to be highly pathogenic to humans, there had been several reports from Sweden [8, 9], Netherlands [10], France and Italy [11] which describe rash, mild fever, febrile illness, meningitis and other clinical symptoms associated with this agent in patients. In Estonia, the wide distribution of *R. helvetica,* as well as the presence of *R. monacensis* and *Candidatus* R. tarasevichiae in questing ticks, was shown previously by Katargina *et al.*, but no human cases due to *R. helvetica* infection nor to the other two species had been reported at that time [12].

Research on the circulation of *Rickettsia* spp. is still ongoing as in vectors, which are mainly fleas and ticks, as in their main hosts (small mammals, wild and domestic animals). As this is fairly wide research area, new species are constantly being discovered, e.g. *I. trianguliceps* associated *Candidatus* R. uralica found in Ural in Russia [7]. Also, the wide distribution of some types of vectors increases probability of the prevalence of vector associated pathogens, such as *Candidatus* R. tarasevichae that has been found in China and Europe [12, 13, 14].

Nowadays there are different methods for disease surveillance, including studying sentinel populations for the presence of pathogens in nature. Dogs [15] and cats [16] can be used as sentinels for rickettsiae. However, vectors can also serve as epidemiological sentinels [17].

Recent studies show that *Ixodes* spp. ticks could serve not only as vectors, but also as reservoir host of *R. helvetica*. At the moment there is no clear understanding on whether any mammal species is the host of the *R. helvetica*, while on the other hand rickettsial DNA was found in the blood of wild animals such as rodents, roe deer, wild boar [18] and domestic animals, such as dogs and cats [19]. Moreover, Burri *et al.* [20] reported negative results on *R. helvetica* xenodiagnostic and furthermore low percentage of gaining *Rickettsia* spp. from the positive host had been described by Tommassone *et al.* [21]. It can only be assumed that mammals can be potential hosts, and that they may affect the natural transmission and distribution of rickettsiae.

In the present study, the aim was to investigate the presence of *Rickettsia* spp. in ticks collected from small mammals.

Methods

Sample collection, species identification and DNA extraction.

The study was performed retrospectively on 1186 ticks, which were removed from small mammals. The samples were collected at five sampling sites in Estonia, located in four mainland counties - Järvamaa, Lääne-Virumaa, Tartumaa (collecting in 2013 and 2014), Pärnumaa (collecting was performed only in 2012) – and one island county – Saaremaa (Figure 1). Live-trappings of mice, voles and shrews were carried out once a month during April-November 2012-2014 in natural habitats using Sherman LFA perforated live-traps (Ethical Committee Permission No. 124 by Estonian Ministry of Agriculture). 10 permanent stations (5 traps each within 2 m radius) were placed 100 m apart along a linear transect that randomly intersected different habitats (forest and semi-open). The trappings were performed during the nighttime. The traps were set at 8 p.m. and in the morning at around 8 a.m. traps were checked for animals. Bread was used as main bait method and vegetables served as water replenishment. The trapped animals' species were identified and then executed by cervical dislocation by a specially trained person with accordance to FELASA curriculum. Each animal was individually examined for the presence of ectoparasites, which were then removed, fixed in ethanol and stored at -20°C in separate tubes until further use. Any endangered species caught were immediately released in the habitat. Protective gloves and face masks were worn at all times while handling wild animals for safety purposes.

DNA extraction from ticks was performed with ammonium hydroxide solution according to Moran-Cadenas *et al.* [22]. Tick species were identified by *ITS2* gene-based multiplex PCR assay as previously described by Värv *et al.* [23]. Only ticks identified at the species level by the *ITS2* multiplex PCR were included in this study, and ticks that remained undetermined were omitted.

Rickettsia spp. screening and genospecies detection

All ticks identified at the species level were screened individually by a nested PCR targeting a 667 bp fragment of *Rickettsia* spp. citrate synthase A (*gltA*) gene using primers glt1 – 4 as described by Igolkina *et al.* [7] with subsequent sequencing of all positive samples. For samples identified as *Ca.* R. uralica and randomly selected samples identified as *R. helvetica* by initial screening, additional PCR amplification of ~770 bp fragment of the outer membrane protein B (*ompB*) gene was performed with primers 120-2788F and 120-3599R under conditions described previously [24]. Additionally, a subset of the latter samples was amplified by nested PCR of 834 bp fragment of cell surface antigen 4 (*sca4*) gene with primers sc4-1 and Rj2837r for the primary reaction, and sc4-3 and sc4-4 for the nested reaction, as described by Igolkina *et al.* [7]. PCR products of all positive samples were sent for direct sequencing to the core laboratory of the Estonian Biocentre (Tartu, Estonia), followed by nucleotide sequence alignment using BioEdit v7.2.5 (Ibis Biosciences, USA) and genospecies identification with BLASTN® tools (http://www.ncbi.nlm.nih.gov/BLAST.cgi).

Results

Rickettsia screening and *Rickettsia* species

In this study 1186 ticks were collected from 314 small animals of 5 species: *Myodes glareolus*, *Apodemus flavicollis*, *A. agrarius*, *Microtus subterranius* and *Sorex araneus* (Table 1, Supplementary table 1). A total of 993 *I. ricinus* (924 larvae and 69 nymphs; from all 5 mammal species), 117 *I. trianguliceps* (93 larvae and 24 nymphs; from *My. glareolus* and *A. flavicollis)*, and 76 *I. persulcatus* (64 larvae and 12 nymphs; from *My. glareolus*, *A. flavicollis* and *M. subterranius*) were studied for presence of *Rickettsia* spp. (Table 2).

Rickettsial DNA was detected in 8.7% (103/1186) of the studied ticks, with rates between tick species rates varying from 3.4% (4/117) of *I. trianguliceps* to 10.0% (99/993) of *I. ricinus* and none in *I. persulcatus* (Table 2). As animal samples were not analyzed for *Rickettsia* spp. presence, it is unknown whether ticks acquired the pathogen via transstadial or transovarial transmission, co-feeding or with blood meal.

Rickettsial DNA was detected in ticks from all study sites, with the lowest positivity rates in Tartumaa and Saaremaa (2.6% and 4.8%, respectively) and the highest rate at 19.4% in Pärnumaa.

Rickettsia spp. DNA was detected in ticks collected from 56 out of 314 animals of 3 species – *My. glareolus* (21.8%; 36/165), *A. flavicollis* (13.5%; 19/141) and *S. araneus* (33.3%; 1/3) (Supplementary table 1). The number of ticks analyzed from a single animal varied from 1 to 32, while the rates of *Rickettsia*-positive ticks varied from 4.8% - 100%. The highest positivity rate of rickettsial DNA was observed within ticks from *My. glareolus* caught in Pärnumaa county (23.8%) (Table 1).

According to the partial *gltaA* gene sequencing results there were two *Rickettsia* species detected: *R. helvetica* and *Ca.* R. uralica. *Rickettsia helvetica* DNA was detected in the majority of *Rickettsia* positive tick samples – 97.1% (100/103). It was detected in 9.97% (99/993) of *I. ricinus* and in one out of 117 *I. trianguliceps*. It is noteworthy that the *R. helvetica*-positive *I. trianguliceps* was attached to the same animal (*My. glareolus*) as *R. helvetica*-positive and negative *I. ricinus* (Table 1). *Rickettsia helvetica* DNA was detected in ticks removed from yellow-necked mice, bank voles and common shrews at all studied locations (Table 1).

Another *Rickettsia* species was identified as *Ca.* R. uralica. It was detected in three *I. trianguliceps* ticks removed from two bank voles collected in Pärnumaa and Järvamaa, respectively. The total positivity rate of *Ca.* R. uralica in *I. trianguliceps* amounted to 2.9% (3/117). *Ca.* R uralica was not detected in *I. ricinus* (Table 1, 2).

In order to confirm the species and to reveal possible nucleotide sequence variability within the detected *Rickettsia* species, the partial *ompB* genes of 20 samples (all 3 samples with *Ca.* R. uralica and 17 samples with *R. helvetica*) and the partial *sca4* genes of 9 samples (all 3 samples with *Ca.* R. uralica and 6 samples with *R. helvetica)* were sequenced. All sequenced *R. helvetica* partial gene fragments were identical to each other as well as to those previously detected in questing ticks from Estonia [12]. Sequences of *gltA*, *ompB* and *sca4* gene fragments amplified from all *Ca.* R. uralica positive samples were 100% identical to each other; the *gltA* and *sca4* gene fragments were also 100% identical to initial sequences reported from Siberia (Genbank accession numbers KR150785 and KP747665). *OmpB* gene fragment differed in one nucleotide base, giving 99.9% similarity to the Siberian *Ca.* R. uralica partial *ompB* sequence (Genbank accession number KR150780) [7].

Discussion

In this study, ticks of the generalist species *I. ricinus* and *I. persulcatus*, as well as nidicolous *I. trianguliceps*, attached to small mammals, were analyzed for the presence of vector-borne *Rickettsia* spp. including a species not previously reported in Europe.

Currently there are many studies regarding circulating *Rickettsia* species as in their vectors, tick and fleas, as well as in vector associated mammals, and possible *Rickettsia*-reservoir presence [18, 20, 25, 26, 27]. In the course of screenings of vector arthropods and their hosts, more and more new "Candidatus" *Rickettsia* species are being found [7, 13]. At the moment, little has been studied about the connection between mammals and rickettsiae. Xenodiagnosis studies show negative results for *R. helvetica* [20]. Furthermore, percentage of gaining *Rickettsia* spp. from the positive host is low, as described by Tomassone *et al.* [21]. Additional studies are required to determine the relationship between rodents and rickettsiae, the bacteremia duration, the distribution and natural cycle of *Rickettsia* spp. and their association with different arthropod vectors. To add more, further researches are necessary to find potential reservoir hosts and how Rickettsia maintains in nature.

To our knowledge, this study is the first report on the detection of a newly described species, *Ca.* R. uralica, in Europe. In this study the genospecies was detected only in *I. trianguliceps* ticks removed from voles, which is in agreement with the initial *Ca.* R. uralica report from Siberia designating the specificity of *Ca.* R. uralica to *I. trianguliceps* [7]. The authors claim that the same *Rickettsia* variant was previously detected in *Myodes rutilus* (northern red-backed voles)

and *S. araneus*, which are also present in Estonia. Together with *I. trianguliceps* ticks these small mammals might play a role in the circulation of this *Rickettsia* species in nature. Despite the genetic clustering of this newly-described *Rickettsia* within the spotted-fever group, the pathogenic potential of *Ca.* R. uralica for domestic and wild mammals, pets or humans remains to be studied.

Although spotted fever rickettsioses are known to be emerging diseases spreading across the globe, human case reports due to *R. helvetica* infections are scarce. Serological or molecular tools have been used to detect *R. helvetica* infection in samples from patients with suspected Lyme neuroborreliosis in the Netherlands [10], with unexplained fever following a tick bite in France and Italy [11] and with rash, febrile illness and meningitis in Sweden [8, 9]. *Rickettsia helvetica*, a tick-borne rickettsiae species, is also frequently detected in Europe and Asia [2, 28, 29], being reported to be the prevalent *Rickettsia* species in some of the regions, e.g. in Germany [30], Slovakia [31] and Sakhalin Island in Russia [29]. Estonia belongs to one of the predominant region as well as over 95% of all *Rickettsia* species detected in questing [12] and rodent-attached ticks in the present study were *R. helvetica*. While there are no clinical reports of illness caused by *R. helvetica* in Estonia to date, the detection of this tick-borne pathogens (TBP) at positivity rates within ticks population similar to rates of 23.3% for *Borrelia burgdorferi* s. l. (I. Golovljova and J. Geller, personal communications), suggests that *R. helvetica* should be considered during surveillance for tick-borne diseases in Lyme borreliosis-endemic regions.

Rickettsial DNA was detected in 8.7% of all investigated attached ticks, and in 10,0% of *I. ricinus*, compared to 3,4% in *I. trianguliceps*. High rates of detection of rickettsial DNA in rodent-attached *I. ricinus* were also recently reported from Lithuania [26] where 22.6% of individually tested larvae (maximum likelihood estimation, MLE = 26.5%) were positive for *Rickettsia* spp.

There have been reports of the detection of several TBP, such as *Anaplasma phagocytophilum* [32], *Neoehrlichia mikurensis* and *Babesia microti* [33], *Francisella tularensis* [34] in nidicolous rodent-specialists *I. trianguliceps* ticks removed from small mammals. As reported by Igolkina *et al*. [7], SFG *Rickettsia* was found in 41.2% (14/34) of analyzed *I. trianguliceps* ticks feeding on voles in Western Siberia, which is significantly higher than the results of the current study (3.4%, 4/117). Nevertheless, the role of *I. trianguliceps* in the circulation and maintenance of TBPs is still largely unknown as is its importance and participation in the transmission of pathogens between ticks and rodent hosts.

The absence of rickettsial DNA in rodent-attached *I. persulcatus* larvae (0/64) and nymphs (0/12) could be explained by the relatively small number of *I. persulcatus* covered in the current study. However, several *Rickettsia* species, such as *Ca.* R. tarasevichae (1/530, 0.2%) and *R. helvetica* (8/530, 1.5%) were previously reported in unfed questing *I. persulcatus* ticks in Estonia [12].

We found rickettsial DNA in ticks removed mainly from *My. glareolus* and *A. flavicollis*, but also from several *S. araneus*. Although there are reports on the detection of *R. helvetica* in various small- to large-sized wild mammal samples from Lithuania [35], the Netherlands and Germany [18, 30, 36] and also in *Erithacus rubecula* (European robins) and *Prunella modularis* (dunnocks) from Hungary [37], the significance of these animals in the transmission and maintenance cycle of *Rickettsia* is still debatable [20]. The *Rickettsia* spp. infection rates in ticks, removed from the same animal, varied from 4.8% to 100%, most likely indicating that the ectoparasites might acquire these pathogens not only during blood meals on these animals, but also through previous infected by transstadial, transovarial or horizontal transmission [38]. However, as there were no animal samples tested for the presence of rickettsial DNA in the current study, there is no strict evidence of whether ticks of this study could have acquired the detected *Rickettsia* through feeding.

Surprisingly, 42.7% (44/103) of all *Rickettsia*-positive ticks were removed from rodents caught in Pärnumaa county. Although this region was not covered in the previous study on *Rickettsia* spp. in questing ticks in Estonia [12], a high rate (28%) of *Rickettsia* DNA was also detected in questing ticks in Pärnumaa (M. Vikentjeva, J. Geller, I. Golovljova, unpublished observations). Interestingly, this region has previously not shown such high infection rates with any TBP [39, 40, 41]. However, our longitudinal observations on ticks indicate that the local environment and climate of western coastal Estonia may provide favorable conditions for tick population maintenance and survival, as ticks have always been abundant in these areas (I. Golovljova, unpublished observations).

Conclusion

The results of our study show a higher rate of positivity of *Rickettsia* spp. in ticks from small mammals compared to ones obtained previously in questing ticks. High *Rickettsia* positivity rate in larvae might indicate on a transovarial transmition of *R. helvetica* and possibility of successful co-feeding transmission while feeding on the same host *Rickettsia helvetica* was the most prevalent species, and was most frequently detected in *I. ricinus* ticks, which are considered to be its main vector and the natural reservoir host. This study also provides the first report on the presence of the novel *Rickettsia* species, *Ca*. R. uralica initially reported from Siberian regions of Russia, in Estonian populations of *I. trianguliceps*.

Abbreviations

DNA: deoxyribonucleic acid; ITS2: internal transcribed spacer 2; PCR: polymerase chain reaction; SFG: Spotted fever group; TBP: tick-borne pathogens; qPCR: quantitative polymerase chain reaction.

Declarations

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Availability of data and materials

All additional data associated with this study can be obtained from the corresponding author on reasonable request. Unique sequences of *Candidatus* Rickettsia uralica obtained during this study were submitted to GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MT063090-MT063092.

Authors' contributions

JR and IG study initiation and experimental studies planning; JR sample collection and small mammal species identification; MV and JG DNA extraction, PCRs performing and bioinformatical analysis; MV writing the initial draft of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal experiments were approved by Estonian Ministry of Agriculture permission no. 124 (J. Remm).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Presence of *Rickettsia* spp. in different tick species attached to mice, voles and shrews in Estonian island and mainland counties.

Small mammal collecting county	**Tick species attached to small mammals** № of *Rickettsia* positive ticks/ № of collected ticks (%) {r*ickettsia* species}																
	My. glareolus				*A. flavicollis*				*S. araneus*				*A. agrarius*				*M.*
	IR	*IT*	*IP*	**tot**	*IR*	*IT*	*IP*	**tot**	*IR*	*IT*	*IP*	**tot**	*IR*	*IT*	*IP*	**tot**	*IR*
Järvamaa	21/217 {*Rh*}	2/16 {*Ca*Ru#}	0/1	**23/234 (9,8%)**	2/75 {*Rh*}	0/6	-	**2/81 (2,5%)**	0/10	-	-	**0/10**	-	-	-	**-**	-
Lääne-Virumaa	7/80 {*Rh*}	1/22 {*Rh**}	-	**8/102 (7,8%)**	9/83 {*Rh*}	0/23	0/1	**9/107 (8,4%)**	-	-	-	**-**	-	-	-	**-**	-
Tartumaa	1/23 {*Rh*}	0/18	0/59	**1/100 (1%)**	3/32 {*Rh*}	0/5	0/13	**3/50 (6%)**	-	-	-	**-**	-	-	-	**-**	0/1
Pärnumaa	28/108 {*Rh*}	1/13 {*Ca*Ru#}	0/1	**29/122 (23,8%)**	8/72 {*Rh*}	0/12	-	**8/84 (9,5%)**	7/21 {*Rh***}	-	-	**7/21 (33,3%)**	-	-	-	**-**	-
Saaremaa	8/111 {*Rh*}	0/1	-	**8/112 (7,1%)**	5/140 {*Rh*}	0/1	-	**5/141 (3,5%)**	-	-	-	**-**	0/20	-	-	**0/20**	-
Total	**65/539 (12,1%)**	**4/70 (5,7%)**	**0/61**	**69/670 (10,3%)**	**27/402 (6,7%)**	**0/47**	**0/14**	**27/463 (5,8%)**	**7/31 (22,6%)**	**-**	**-**	**7/31 (22,6%)**	**0/20**	**-**	**-**	**0/20**	**0/1**

* 1 *Rh* positive *IT* from animal with 1 positive and 1 negative *IR*

** All positive from the same animal

None of the IR from the same animal tested positive

My. – Myodes; A. – Apodemus; S. – Sorex; M. – Microtus; IR – Ixodes ricinus; IT – Ixodes trianguliceps; IP – Ixodes persulcatus; tot – total*; Rh – Rickettsia helvetica; Ca*Ru – *Candidatus* Rickettsia uralica

Table 2. *Rickettsia* spp. detection in ticks collected from small mammals.

Small mammal collecting county	Number of ticks infected/tested (%)										*Rickettsia* spp. genospecies (positivity rate comparatively to all positive samples, %) {positivity rate comparatively to all studied samples, %}	
	I. ricinus			*I. persulcatus*			*I. trianguliceps*			Total	*Ca*Ru	*Rh*
	Larvae	Nymphs	**Total**	Larvae	Nymphs	**Total**	Larvae	Nymphs	**Total**			
Järvamaa	21/288 (7,2%)	2/14 (14,3%)	**23/302 (7,6%)**	0/1	-	**0/1**	2/19 (10,5%)	0/3	**2/22 (9,1%)**	**25/325 (7,7%)**	2/25 (8%) 2/325 {0,6%}	23/25 (92%) 23/325 {7,1%)
Lääne-Virumaa	15/151 (9,9%)	1/12 (8,3%)	**16/163 (10,4%)**	0/1	-	**0/1**	1/38 (2,6%)	0/7	**1/45 (2,2%)**	**17/209 (8,1%)**	-	17/17 (1005) 17/209 {8,1%}
Tartumaa	4/52 (7,7%)	0/4	**4/56 (7,1%)**	0/62	0/11	**0/73**	-	0/6	**0/6**	**4/152 (2,6%)**	**-**	4/4 (100%) 4/152 {2,6%}
Pärnumaa	39/186 (21,0%)	4/15 (26,7%)	**43/201 (21,4%)**	-	0/1	**0/1**	0/17	1/8 (12,5%)	**1/25 (4%)**	**44/227 (19,4%)**	1/44 (2,3%) 1/227 {0,4%}	43/44 (97,7%) 43/227{18,9%}
Saaremaa	13/247 (5,2%)	0/24	**13/271 (4,8%)**	-	-	**-**	0/2	-	**0/2**	**13/273 (4,8%)**	-	13/13 (100%) 13/273 {4,8%}
TOTAL	**92/924 (10,0%)**	**7/69 (10,1%)**	**99/993 (10,0%)**	**0/64**	**0/12**	**0/76**	**3/93 (3,2%)**	**1/24 (4.2%)**	**4/117 (3,4%)**	**103/1186 (8,7%)**	**3/103 (2,9%) 3/1186 {0,3%}**	**100/103 (97,1%) 100/1186 {8,4%}**
	Larvae, all tick species total									**95/1081 (9,44%)**		
	Nymphs, all tick species total									**8/105 (7,62%)**		

I – *Ixodes, Rh* – *Rickettsia helvetica*; *Ca*Ru – *Candidatus* Rickettsia uralica

Figures

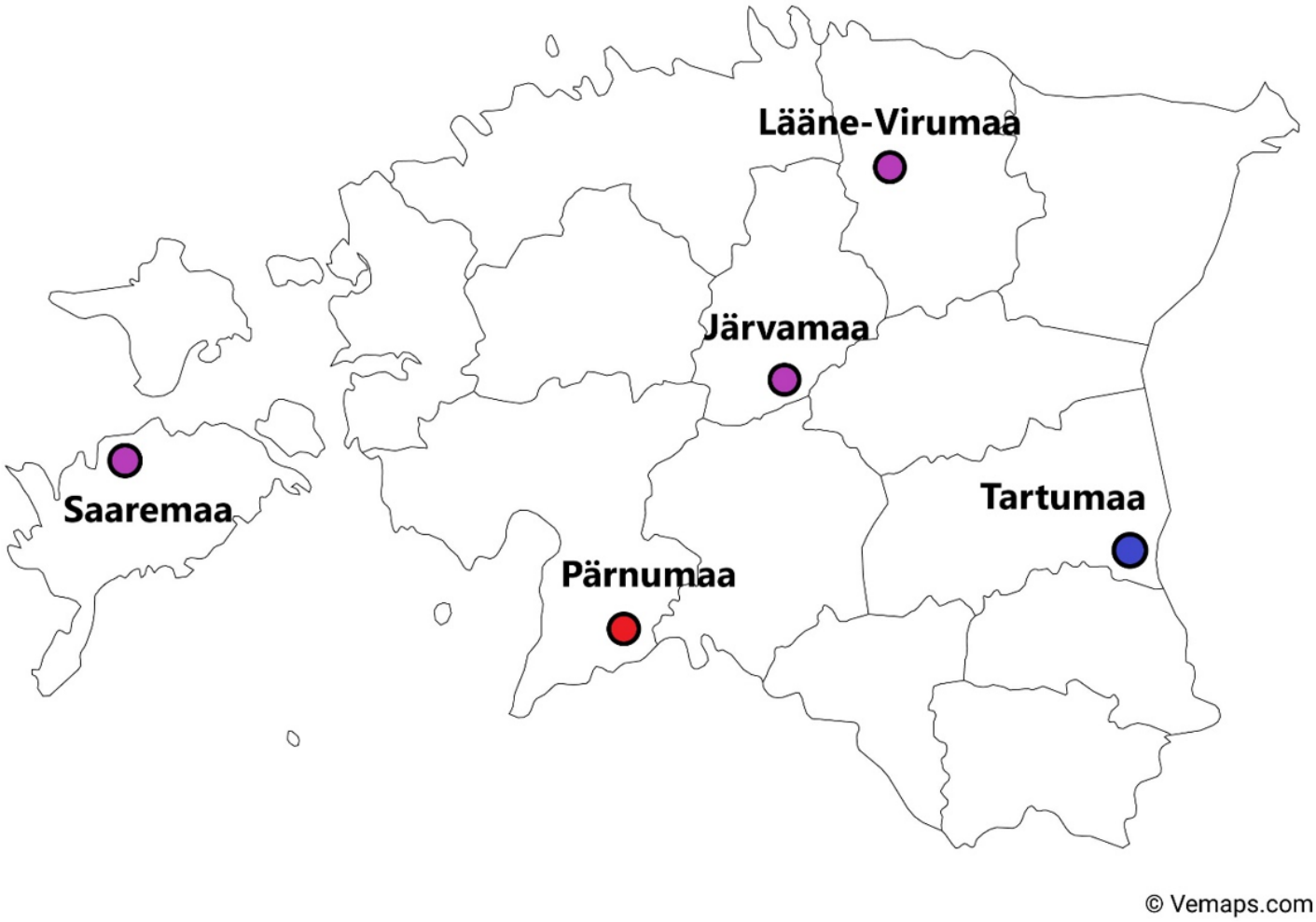

Figure 1

Small mammals trapping sites 2012-2014 in Estonia. Trapping site marks are colored according to study years: blue - trapping in 2013 and 2014, red – trapping in 2012, purple – trapping site from 2012-2014. Precise coordinates of the trapping sites are as follows: Järvamaa (58.7365°; 25.6682°), Lääne-Virumaa (59.2260°; 26.1335°), Tartumaa (58.2493°; 27.3023°), Pärnumaa (58.0687°; 24.8433°) and Saaremaa (58.5075°; 22.4107°).