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Ixodid ticks and tick-borne encephalitis virus prevalence in the South Asian part of Russia (Republic of Tuva)

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ABSTRACT

The most significant processes of arbovirus evolution can be expected to occur in the territories where ticks of different species cohabitate and at the boundaries of virus occurrence, where the probability of the appearance of new virus variants is high due to the possible shift in the main vectors and/or vertebrate hosts. One of the most interesting regions in this regard is the Republic of Tuva. Since most of its territory is covered by mountain ranges and intermountain basins, we were able to study the distribution of vectors and viruses in geographically isolated areas at different altitudes and in various landscapes. From 2008 to 2017, we conducted six expeditions to Tuva and collected 3,077 adult ticks and 24 nymphs. The distribution of tick species was confined to specific landscapes, as follows: *Dermacentor nuttalli* occurred in steppes, *D. silvarum* inhabited forest-steppe areas, and *Ixodes persulcatus* inhabited mixed forests. All three species of ticks were collected on plains and mountain slopes. The range of *D. silvarum* was shown to be lower than 1300 m above sea level (a.s.l.). Only *D. nuttalli* and *I. persulcatus* were collected at higher altitudes. According to our observations, single nymphs of *D. nuttalli* appear on animals one month before larvae appear. This finding confirms the hypothesis that the immature forms of *D. nuttalli* are able to overwinter under favourable conditions. We isolated 9 strains and 3 isolates of tick-borne encephalitis virus (TBEV) from *I. persulcatus*, one strain from *D. nuttalli* and one strain from *D. silvarum*. The TBEV strain from *D. nuttalli* was isolated from the territory inhabited only by *Dermacentor* ticks. All isolated strains belong to the Siberian subtype of TBEV. TBEV was detected in ticks from all the investigated altitudes. There were no statistically significant differences in the virus prevalence between the *Dermacentor* and *Ixodes* ticks. The

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results of our work provide additional support for the hypothesis of the existence of TBEV foci in areas with an absolute dominance of *D. nuttalli*.

1. Introduction

Ixodid ticks are specific vectors of many species of spirochetes, trypanosomes, filarias, rickettsias, and viruses, many of which cause severe human and animal diseases (Balashov, 1998). One of the most common tick-borne arboviruses in northern Eurasia is the tick-borne encephalitis virus (TBEV).

The main TBEV vectors are *Ixodes persulcatus* (Schulze, 1930) and *Ixodes ricinus* (L., 1758). In certain TBEV-endemic areas where *I. persulcatus* and *I. ricinus* are absent or few in number, other ixodid tick species may act as a TBEV vectors, including proven vector – *Haemaphysalis concinna* Koch, 1844 (Hoogstraal, 1966; Kozuch and Nosek, 1980; Voshchakina, 1954), and possible vectors – *Ixodes pavlovskyi* Pom., 1946 (Rar et al., 2017; Romanenko and Kondrat'eva, 2011), *Ixodes ovatus* Neumann, 1899 (Takeda et al., 1998), *Haemaphysalis longicornis* Neumann, 1901, *Haemaphysalis flava* Neumann, 1897, and *Ixodes nipponensis* Kitaoka & Saito, 1967 (Yun et al., 2012). However, without proven transmission capability, the vector competence of these species of ticks is still under discussion (Kahl et al., 2002).

In recent decades, there has been an expansion of the TBEV area to the north. The northern boundary of the TBEV distribution extends from Norway, Sweden, and Finland in Europe to the Far East in Russia (Han et al., 2001; Lindblom et al., 2014; Ruzek et al., 2019; Soleng et al., 2018). Some of the probable causes of this phenomenon could be climate change and anthropogenic factors (Korotkov et al., 2015; Mansfield et al., 2009).

From the north to the south of Eurasia, the forest zones transit into the forest-steppe (cohabitation of *Dermacentor* and *Ixodes* ticks) and steppe zones, which are dominated by *Dermacentor* ticks. In this regard, the definition of the southern boundary of TBEV distribution is complicated because the role of *Dermacentor* ticks in maintaining TBEV foci is not clear. This issue has been studied (Belova et al., 2018, 2017, 2013; Biernat et al., 2014; Kahl and Dautel, 2013; Karbowiak et al., 2015; Wójcik-Fatla et al., 2011). Currently, the southern border of the TBEV area extends through Central and Southern Europe, the European part of Russia, Kazakhstan, Mongolia, China, Korea, and Japan (Frey et al., 2012; Khasnatinov et al., 2010; Lu et al., 2008; Muto et al., 2015; Ruzek et al., 2019; Sun et al., 2017; Süss, 2011; Wu et al., 2013; Yoshii et al., 2017; Yun et al., 2012, 2016).

The most significant processes of arbovirus evolution can be expected to occur in the territories where ticks of different species cohabitate and at the boundaries of virus occurrence, where the probability of the appearance of the new virus variants is high due to the possible shift in the main vectors and/or vertebrate hosts (Moudy et al., 2007). From this point of view, one of the most interesting regions is the Republic of Tuva (Tuva), a federal subject of Russia, which is located in the southernmost part of Siberia. Over 80% of the territory of Tuva is occupied by mountain ranges (the southern part of the Altai-Sayan mountainous country), and less than 20% lies within the intermountain valleys. The presence of mountain massifs makes it possible to study the distribution of ticks and viruses at different altitudes and on geographically isolated territories. There are four landscape zones in Tuva: high-mountain tundra, mountain forest, mountain steppe, and desert steppe. In transition territories of one zone into another, the cohabitant areas of *Ixodes* and *Dermacentor* ticks are formed. One of the features of the Republic of Tuva is a high abundance of *Dermacentor* ticks. The study of isolated tick populations of *I. persulcatus* and *Dermacentor*, as well as their cohabitant areas, will provide information on the ability of *Dermacentor* ticks to independently maintain the TBEV focus.

The initial data on tick distribution in the Republic of Tuva were published in the end of the 1950s (Babenko, 1959; Piontkovskaya et al., 1959). It was previously shown that the fauna of ixodid ticks in Tuva consisted of the following nine species: *I. persulcatus*, *Ixodes crenulatus* Koch, 1844, *Ixodes stromi* Fil., 1957, *Ixodes lividus* (Koch, 1844), *Dermacentor silvarum* Olenev, 1931, *Dermacentor nuttalli* Olenev, 1929, *Dermacentor reticulatus* (Fabr., 1794), *Dermacentor marginatus* (Sulzer, 1776), and *H. concinna* (Burenkova et al., 2008; Filippova, 1997; Galatsevich et al., 2003; Yemelyanova et al., 1962).

In the early 1970s, TBEV was first isolated from Pallas's pika (*Ochotona pallasi*) in south-west Tuva (Danileko et al., 1971). In the 2000s, *Rickettsia raoultii, Rickettsia sibirica, Borrelia garinii, Borrelia afzelii, Borrelia miyamotoi*, and TBEV of the Siberian genotype were isolated from ticks from Tuva (Burenkova et al., 2008; Mel'nikova et al., 2014). Borrelias and rickettsias were identified in *I. persulcatus* and *D. nuttalli*. The isolation of TBEV strains has only been described from *I. persulcatus* ticks. The characteristics of TBEV circulation in geographically isolated territories and the isolation of TBEV from different species of ticks are of particular interest.

The aim of this work was to study *Ixodes* and *Dermacentor* ticks as TBEV carriers and vectors in the Republic of Tuva and their association with the geobotanical landscape.

2. Materials and methods

2.1. Collection of ticks

From 2008 to 2017, we conducted six expeditions. Ticks were collected from the beginning of May to the middle of June, which is the active period of adult *I. persulcatus* (Khizhinskii, 1963) and *Dermacentor* (Yemelyanova et al., 1962) ticks. A total of 3,077 adult ticks of different species, 15 *I. persulcatus* nymphs and 9 *D. nuttalli* nymphs (Table 1) were collected by flagging from vegetation in the highland forests, middle and lower mountains, rivers valleys and floodplains, steppe and forest-steppe zones, as well as in local outdoor recreational areas; and manually from domestic animals, including cows, sheep, horses, and dogs. Ticks were identified using taxonomic keys (Filippova, 1997, 1977). The locations of tick collection, tick species and their abundance are presented in Fig. 1 and Table 1.

2.2. Preparation of tick suspension

Adult ticks were homogenised individually or in pools (three to five individuals) according to species composition, location, and route of collection using the laboratory homogeniser TissueLyser II (QIAGEN, Germany) in medium 199 with Earle's salts (FSBSI Chumakov FSC R & D IBP RAS, Russia). To analyse ticks individually, we added 300 µl of the medium per *I. persulcatus* tick and 500 µl – per *Dermacentor* tick. When the ticks were analysed in pools, the volume of the medium added was calculated according to the species and the number of ticks in the pool, as follows: for each *I. persulcatus* tick, 150 µl of medium was added; for each *Dermacentor* tick, 200 µl of medium was added.

2.3. Detection of TBEV in tick suspension by PCR

RNA was isolated using TRI reagent (Sigma-Aldrich, USA) according to the manufacturer's protocol. RNA was diluted in the required amount of water and used for reverse transcription.

Reverse transcription was carried out with M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol with random hexamer primers (SYNTHOL, Russia).

PCR was performed according to the previously described procedure, using primers specific for the coding regions of E protein, Kgg19

Table 1
Number of ticks at collection points.

Point on the map	*I. persulcatus*		*D. nuttalli*		*D. silvarum*	
	flag (tick/flag-hour)	animals	flag (tick/flag-hour)	animals	flag (tick/flag-hour)	animals
I. Tuva basin steppe province						
1. Khemchik dry steppe district						
4		1♂		3♂, 5♀		
5			2♂, 8♀ (3)	1♂		
6			1♀ (0.6)			
7				1♂	1♀ (1)	
2. Central Tuva meadow steppe district						
11			2♂, 1♀ (12)			
12		7♀				14♂, 11♀
13	18♂, 31♀ (14)					
14	2♂ (0.7)					
15			2♂, 1♀ (8)			
20	6♂, 3♀ (2.4-4)		7♂, 10♀ (2.5-12.6)		6♂, 6♀ (2.9)	
21			1♂, 3♀ (1.25)	22♂, 4♀		
22			8♂, 15♀ (3.8)	30♂, 15♀		
23	4♂, 5♀ (2.35)					
24			4♂, 4♀ (2.6)			
25			134♂, 192♀ (158.1)			
26			8♂, 16♀ (32.4)			
28	2♂, 2♀ (2)			2♂, 9♀		23♂
29	1♂ (1.5)					
3. Turan-Uyuk larch meadow steppe district						
16			47♂, 61♀ (25)		2♀ (2)	
18	18♂, 15♀ (1.2-8)		3♀ (1)			
II. West Sayan mountain taiga province						
1. Kurtushibinsk steppe-heath cedar larch district						
17	10♂, 11♀ (3.3-5.3)					
19	75♂, 69♀, 2NN (2.7-8.8)		1♀ (0.25)			
3. Tannu-Ola tundra steppe-heath larch district						
10	56♂, 57♀ (25.1)					
27	24♂, 16♀ (8.9)					
30	3♂, 7♀ (1-1.5)					
III. East Sayan mountain taiga province						
1. Todzha cedar larch district						
36	1♂ (1.67)	1♀	2♂, 3♀ (1.8)	3♀	2♂, 6♀ (2)	11♀
2. East Tuva larch mountain tundra district						
34	198♂, 149♀ 13NN (48.8-82)				1♀ (1)	
35		1♂, 1♀				
IV. South-Eastern Altai mountain steppe province						
Mongun-Tayga mountain tundra deserted district						
1				191♂, 178♀		
2			21♂, 66♀ (101), 4NN			
3			7♂, 22♀ (38.7)	40♂, 34♀ 5NN		
V. Ubsunur plain lowland-steppe province						
2. Ubsunur deserted steppe district						
8			4♂, 25♀ (14.5)	107♂, 60♀		
9			50♂, 121♀ (36.5)	105♂, 83♀		
31			7♂, 8♀ (12)	5♂, 5♀		
32			46♂, 64♀ (2.2-26.3)	83♂, 66♀		2♂
33			48♂, 103♀ (12.8-31.4)	2♂, 17♀		
Total	**418♂, 365♀, 15NN**	**2♂, 9♀**	**400♂, 728♀ 4NN**	**592♂, 479♀ 5NN**	**8♂, 16♀**	**39♂, 22♀**

NN - nymphs.

and Kgg31 (Romanova et al., 2006), followed by electrophoretic analysis.

2.4. Detection of TBEV in tick suspension by real-time PCR

RNA was isolated using RIBO-prep (AmpliSens, Russia) according to the manufacturer's protocol. cDNA was synthesized from RNA using the Reverta-L RT kit (AmpliSens, Russia) according to the manufacturer's instructions. The kit uses M-MLV reverse transcriptase and random hexamer primers. The cDNA was amplified by real-time PCR using the AmpliSens® TBEV, B.burgdorferi sl, A.phagocytophillum, E.chaffeensis / E.muris-FRT kit (AmpliSens, Russia).

2.5. Infection of cell lines

One-day-old cultures of pig embryo kidney (PEK) cells and Vero cells were infected with tick suspensions that were PCR-positive for the presence of TBEV RNA. One hundred microliters of tick suspension was added to each well of a 24-well panel (cell culture plate 24-well, Costar) with the cell culture and incubated for 1 h at 37 °C. Then, 1 ml of a maintenance medium consisting of medium 199 (for PEK cell culture) or DMEM medium (for Vero cell culture), 2% bovine serum (FBS, Gibco), and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) was added and incubated at 37 °C for 7 days. The infected culture supernate was harvested immediately after the appearance of the cytopathogenic effect (CPE) or on the 7th day after infection in the absence of CPE.

2.6. Isolation of TBEV in mice

Two-day-old ICR mice (FSBSI Scientific Center of Biomedical Technologies of Federal Medical Biological Agency, "Stolbovaya" branch) were injected intracerebrally with 10 μl of virus-containing fluid (infected cell culture supernate, tick suspension or 5% mouse brain suspension). The mice were observed for signs of disease for 14 d and were sacrificed when signs of disease occurred. The mice were maintained according to the international guidelines for animal husbandry, including the recommendations of CIOMS, 1985 and the FELASA Working Group Report, 1996–1997; Chumakov IPVE ethics committee decision #19 from 01.09.2016.

2.7. Sequencing

Viral RNA from the tick suspensions and the culture supernate of infected cells was isolated with TRI Reagent LS (Sigma-Aldrich, USA) according to the manufacturer's protocol. Reverse transcription was performed with virus-specific primers (Kgg30, Kgg32; Supplementary Table 1) and M-MLV reverse transcriptase according to the manufacturer's protocol (Promega, Madison, WI). Viral genomic cDNA was amplified by PCR using overlapping sets of TBEV-specific primers (Kgg65–TBE1095 r, Kgg35–Kgg26, Kgg16–Kgg30, and Kgg37–Kgg38; Supplementary Table 1). Sequencing was carried out in both directions directly from PCR-amplified DNA on the ABI PRISM 3730 (Applied Biosystems) sequencer using ABI PRISM® BigDye™ Terminator v. 3.1. Genomic sequences were assembled using SeqMan software (DNAstar, USA).

2.8. Phylogenetic analysis

RNA sequences of 44 strains of TBEV from GenBank of all genotypes and the 10 strains described in this article were used in the phylogenetic analysis. Among the strains of the Siberian genotype, sequences were taken from the territories that were geographically near to the

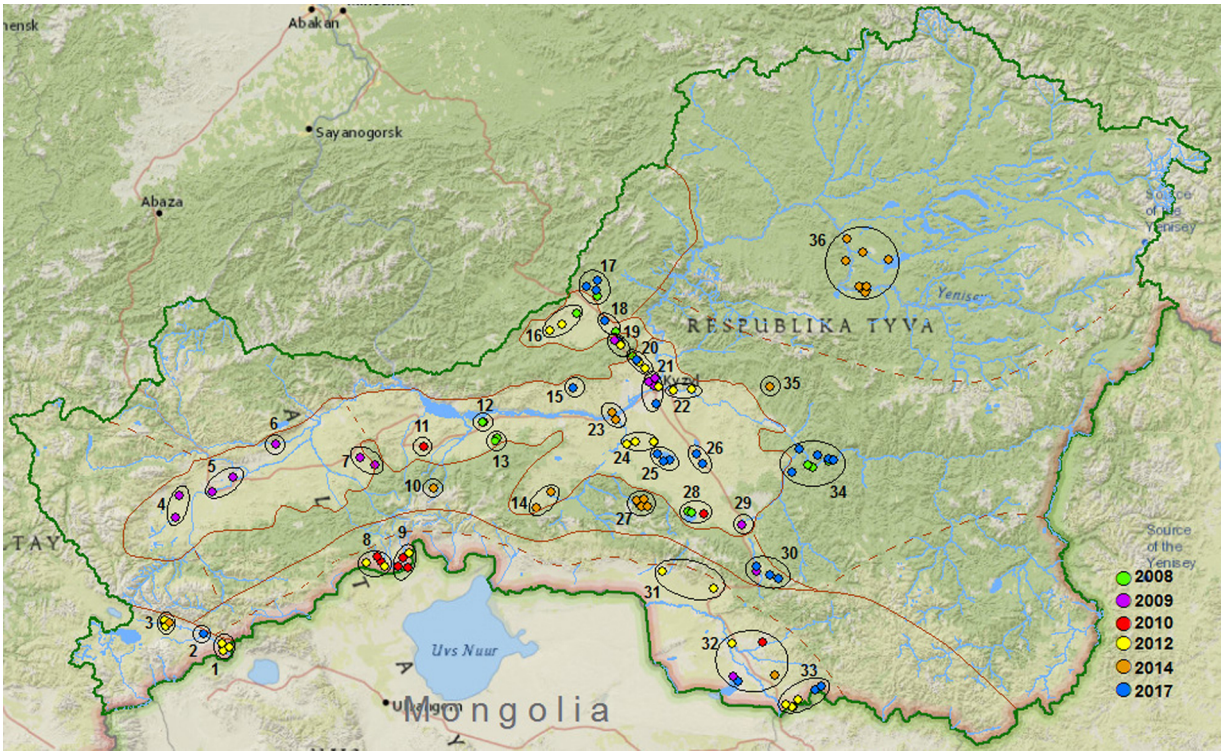

Fig. 1. Locations of Tick Collections in the Republic of Tuva.
2008 – collection in May; 2009 – collection in May; 2010 – collection in April – May; 2012 – collection in May; 2014 – collection in June; 2017 – collection in May – June

Republic of Tuva. The nucleotide sequences of the genome coding region of protein E were aligned using Clustal-X 2.0.11. Phylogenetic analysis was conducted using the neighbour-joining method using MEGA 6.0 with 1000 bootstrap replications.

2.9. Creation of the thematic maps

Thematic maps were created at Sechenov University in the ArcGIS 10 program of ESRI Company (No. EFL268386118) by integrating the locations of tick collection from GPS, maps from https://services.arcgisonline.com/ArcGIS/services, and monograph data (Maskaev et al., 1985).

3. Results

3.1. Collection of ticks

The Republic of Tuva is a mountainous region of Russia where only 20% of the territory is occupied by intermountain valleys. Approximately half of Tuva's territory is occupied by mountain forests (cedar, larch, and pine), and at approximately at the 2200 m a.s.l., forests are replaced by mountain tundra and, more rarely, meadows. Steppe vegetation predominates in the basins, but there are also areas of pine forests.

The climate of Tuva is sharply continental and is characterised by moderate to insufficient humidity. Rainfall distribution in the region is extremely sporadic, ranging from 150 mm per year in the basins to 1000 mm in the mountains. Winter is frosty and calm, and there is little snow in the basins. The average temperature in January ranges from −28 °C to −35 °C. Summer is moderately warm in the mountains and hot in the basins. The temperature in July averages between 15 °C and 20 °C. The duration of winter is from November to April. The snow cover is set at the end of October and reaches 15–20 cm in the valleys, and up to 2 m in the mountains. The snow thaws in the middle of April in the basins, and in May in the mountains. Spring (April to May) is short, clear, windy, and dry (http://gov.tuva.ru/region/geography/).

In the mid-1980s, a geobotanical subdivision of the Republic of Tuva was conducted (Maskaev et al., 1985), according to which Tuva's territory was divided into 5 provinces and 12 districts (Fig. 2, Appendix A).

To describe ixodofauna, we relied on natural geographical obstacles restricting the migration pathways of animal hosts. Tick collection locations (points) were sorted into groups according to the geographic location and geobotanical subdivision of Tuva (Fig. 1, Table 1).

The central part of the Republic is occupied by the Tuva Basin (Fig. 2; I.), and belongs to the Tuva Basin steppe province. There are three districts (described below) in the province. Ticks were collected in all districts.

In the Khemchik dry steppe district (Fig. 1, Table 1, points 4, 5, 6, and 7), which is located within the Khemchik Basin, we collected ticks by flagging (11 *D. nuttalli* and 1 *D. silvarum*) and from animals, including cows (*Bos taurus taurus*) and horses (*Equus* spp.) (10 *D. nuttalli* and 1 *I. persulcatus*). *D. nuttalli* were collected in the steppe region and *D. silvarum* was collected on the forest border at the western foothill of the Adar-Dash Ridge. The altitude of collection locations ranged from 800 to 1500 m a.s.l.

In the Central Tuva meadow steppe district (Fig. 1, Table 1, points 11–15, 20–26, 28, and 29, the altitude 550–1000 m a.s.l.), which is located in the Ulugh-Khem Basin, we collected 74 *I. persulcatus* ticks, 408 *D. nuttalli*, and 12 *D. silvarum* by flagging. In addition, we collected 7 *I. persulcatus*, 82 *D. nuttalli*, and 48 *D. silvarum* from animals (cows, horses, dogs (*Canis lupus familiaris*), sheep (*Ovis aries*)).

In the Turan-Uyuk larch meadow steppe district (Fig. 1, Table 1, points 16 and 18), which is occupied by the Turan-Uyuk Basin, we collected by flagging 33 *I. persulcatus* ticks, 111 *D. nuttalli*, and 2 *D. silvarum*. The altitude of the collection locations ranged from 700 to 930 m a.s.l.

West Sayan mountain taiga province is divided into three districts

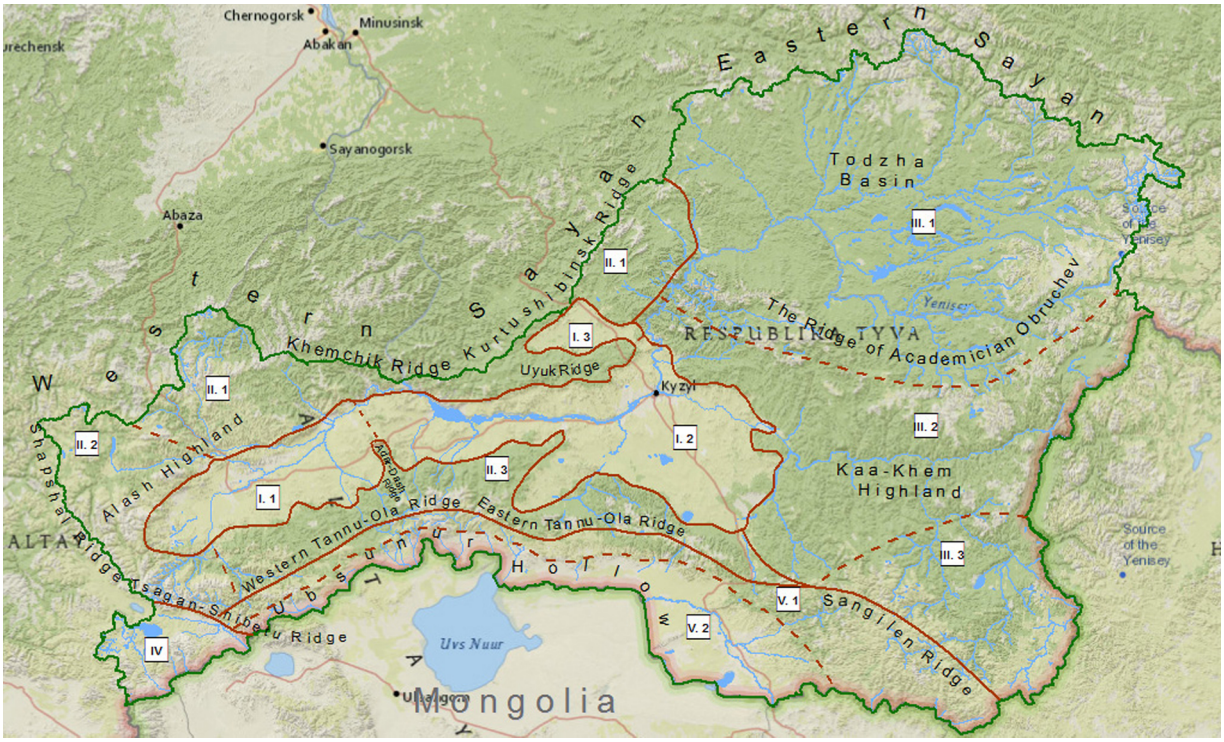

Fig. 2. The Scheme of Geobotanical Subdivision of the Republic of Tuva.
I — Tuva basin steppe province, district: I.1 — Khemchik dry steppe, I.2 — Central Tuvinian meadow steppe, I.3 — Turan-Uyuk larch meadow steppe; II — West Sayan mountain taiga province, district: II.1 — Kurtushibinsk steppe-heath cedar larch, II.2 — High Khemchik larch steppificated mountain tundra, II.3 — Tannu-Ola tundra steppe-heath larch; III — East Sayan mountain taiga province, district: III.1 — Todzha cedar larch, III.2 — East Tuva larch mountain tundra, III.3 — Sangilen larch mountain tundra; IV — South-Eastern Altai mountain steppe province, Mongun-Taiga mountain tundra deserted district; V — Ubsunur plain lowland-steppe province, district: V.1 — South Tannu-ola Sangilen steppe, V.2 — Ubsunur deserted steppe.
Border: Province
District
Border of the Republic of Tuva
State Border of the Russian Federation

(Fig. 2; II.). Ticks were collected in the northern and southern districts.

Kurtushibinsk steppe-heath cedar larch district occupies the northern slopes of the Western Sayan, the Uyuk Ridge, and the eastern part of the Alash Highland. In this district, ticks were collected in the forest located to the north of the city of Turan at an altitude of 870–1663 m a.s.l. (Fig. 1, Table 1, point 17) and in the forest and along the road in open terrain on the eastern slope of the Uyuk Ridge at an altitude of 1150–1300 m a.s.l. (point 19). A total of 165 *I. persulcatus*, 1 *D. nuttalli*, and 2 nymphs of *I. persulcatus* were collected by flagging. Nymphs were collected in places where vegetation was practically absent (no grass at all).

Tannu-Ola tundra steppe-heath larch district occupies the northern macroslopes of the mountain system of the Western and Eastern Tannu-Ola. In this territory, 163 *I. persulcatus* were collected by flagging (Fig. 1, Table 1, points 10, 27, and 30). The altitude of the collection locations ranged from 900 to 1000 m a.s.l.

The East Sayan Mountain taiga province (Fig. 2; III.) occupies the eastern part of the Republic of Tuva. Ticks were collected in the central and northern parts of the province.

The northern part of the province is represented by the Todzha cedar larch district (Fig. 2; III.1.). The collection of ticks in this district was carried out in June, when the main peak of tick activity had already passed (Fig. 1, Table 1, point 36). We collected one *I. persulcatus*, 5 *D. nuttalli*, and 8 *D. silvarum* by flagging and one *I. persulcatus*, 3 *D. nuttalli*, and 11 *D. silvarum* from animals: bullock (*Bos taurus*), dog, horse, sheep. *Dermacentor* ticks were collected in forest meadows and abandoned arable land. The altitude of the collection location was 950–1050 m a.s.l.

The central part of the province is represented by the East Tuva larch mountain tundra district (Fig. 2; III.2.). We collected 347 adult *I. persulcatus*, 13 nymphs of *I. persulcatus* and 1 adult *D. silvarum* by flagging. In addition, we collected 2 adult *I. persulcatus* ticks from a dog (Fig. 1, Table 1, points 34, 35). Nymphs were collected in places where vegetation was practically absent. The altitude of the collection location was 800–900 m a.s.l.

The southeastern Altai mountain steppe province is represented by only one district, the Mongun-Taiga mountain tundra deserted district (Fig. 2; IV.), which is located in the south-western part of Tuva and is separated from the rest of the Republic by the Tsagan-Shibetu Ridge. Only *D. nuttalli* were collected in this part of Tuva: 116 ticks were collected by flagging; and 443 ticks were collected from animals (horses, sheep, cows) (Fig. 1, Table 1, points 1, 2, and 3). This ratio in the ticks collected from vegetation and animals is because the district is an animal husbandry area. Therefore, the ticks were predominantly collected from animals. In April 2014, 5 nymphs of *D. nuttalli* were removed from ground squirrels (*Spermophilus undulatus* (Pallas, 1778)). In April 2015, 4 nymphs of *D. nuttalli* were captured near the ground squirrel burrows. The altitude of the collection location was in the range of 1630–2100 m a.s.l.

The Ubsunur plain lowland-steppe province occupies the southern macroslopes of the Western and Eastern Tannu-Ola Ridges, the south-western plateau of the Sangilen Highland, and the northern part of the Ubsunur Hollow (Fig. 2; V.). We collected ticks in the Ubsunur deserted steppe district (Fig. 2; V.2.), which coincides with the northern part of the Ubsunur Hollow. There, we collected only *Dermacentor* ticks: 476 by flagging and 533 from animals (calf, cow, sheep, lamb, horse, goat

(*Capra hircus*)) (Fig. 1, Table 1, points 8, 9, 31, 32, and 33). The altitude of the collection locations was in the range of 1000–1200 m a.s.l. The Western and Eastern Tannu-Ola Ridges are a natural obstacle from the north.

D. nuttalli occurs in steppes with poor shrub vegetation, which are the main areas for grazing. These ticks inhabit intermountain depressions, where most of the desert and steppe regions of Tuva are located, and *D. nuttalli* can also be found on the mountain ranges where there are no larch and coniferous forests. *D. nuttalli* is the only representative of the ixodofauna that was collected by flagging in the southern part of Tuva in the Ubsunur Hollow. Ticks were collected in different years and in different parts of the hollow (Fig. 1, Table 1). The number of ticks varied according to location, and the maximum number in our collection was 36.5 individuals per flag-hour; from certain animals (e.g. cow), more than 100 individuals were removed.

The situation was slightly different in the Tuva Basin because there are places with coniferous-deciduous forests inhabited by other tick species besides *D. nuttalli*. The maximum number of *D. nuttalli* in the Tuva Basin was 158.1 individuals per flag-hour. *D. nuttalli* ticks were encountered at altitudes ranging from 570 to 2000 m a.s.l. According to the Tuva Anti-Plague Station staff, *D. nuttalli* ticks can be found above 2400 m a.s.l.

D. silvarum is confined mainly to the forest-steppe regions of Tuva. Specific habitats, as described earlier (Burenkova et al., 2008), are headland and river valleys (Fig. 1; points 7 and 12), shrub meadows near settlements (Fig. 1; points 12 and 16), forest outliers and grassy larch groves (Fig. 1; point 20). We also noted that *D. silvarum* can sometimes be found in the taiga in open areas of spruce-birch, larch-birch, and larch forests (Fig. 1; points 34 and 36). The maximum number of *D. silvarum* collected was 2.9 individuals per flag-hour, and these ticks were observed along the roadside (point 20). Most of the ticks were collected from domestic animals (Fig. 1; points 12 and 36). *D. silvarum* ticks were collected up to 1200 m a.s.l. and were not observed at higher altitudes.

According to our data, *I. persulcatus* reached its maximum abundance in the subzone of mixed forests, preferring wetted biotopes with small open spaces (points 27 and 34). This species also occurred on the descents, at the foot of the slopes (point 18), along the slopes and small rivers valleys (point 10), on drained roadsides with sandy cover overgrown with grass and even on roadsides and glades (point 18 and 19). The number of ticks in such places varied from 8 to 82 individuals per flag-hour. Elsewhere, the number of *I. persulcatus* ticks was as low as 1–2 individuals per flag-hour. Some of the specimens were collected in the steppe with no forest nearby, which was likely the result of ticks being carried by birds or mammals. Ticks of this species were collected at altitudes of 550–1576 m a.s.l.

3.2. Detection of tick-borne encephalitis virus

Ticks collected both from vegetation and animals were analysed for the presence of TBEV RNA in pools or at the individual level. TBEV-positive ticks collected from animals were detected only among *Dermacentor* ticks (Table 2).

Viral RNA was detected in ticks that were collected in the following districts: Ubsunur deserted steppe (points 9 and 32), East Tuva Mountain tundra larch (point 34), Kurtushibinsk steppe cedar larch

Table 2
Tick-borne encephalitis virus prevalence[a].

Point on the map	Tick species	Altitude, m a.s.l.[b]	Number of analyzed ticks (total)	Number of analyzed pools/ individual ticks		Number of TBEV-positive pools/ individual ticks		TBEV strain	GenBank access. no.	MIR[c] (%)
				vegetation	animal	vegetation	animal			
4	*I. persulcatus*	950 – 1050	1		0/1		0			0[d]
	D. nuttalli		8		0/8		0/3			37.5[d]
5	*D. nuttalli*	811 – 854	11	0/11		0/6				54.5[d]
9	*D. nuttalli*	1520	359	42/0	25/3	2/0	1/2			1.4
10	*I. persulcatus*	914	113	29/0		1/0		TV14-T20214	MH746802[e]	0.9
13	*I. persulcatus*	712	49	10/0		2/0		TV08-T2540 TV08-T2546	MH614278[e] KU052690[f]	4.8
17	*I. persulcatus*	900	21	4/5		0/1		TV08-T2513	MH614281[e]	4.76
18	*I. persulcatus*	799 - 900	33	9/1		1/0		TV17-T24686	MH614282[e]	3.0
	D. nuttalli		3	1/0		0				0[d]
19	*I. persulcatus*	1538,7	144	40/45		2/1		TV09-T4929 TV17-T24679	MH614279[e] MH614285[e]	2.1
	D. nuttalli		1	0/1		0				0[d]
20	*I. persulcatus*	968 – 1067	9	4/1		0/1		TV08-T2510	MH614277[e]	11.1[d]
	D. nuttalli		17	6/1		0				0[d]
	D. silvarum		12	4/2		0				0[d]
28	*I. persulcatus*	1031	4	0/4		0/1				25.0[d]
	D. silvarum		23		6/0		2/0	TV08-T2515	MK411539[e]	8.6
32	*D. nuttalli*	1087 - 1566	205	20/5	29/15	12/4	1/0	TV17-T25121/1	MH614283[e]	8.3
	D.silvarum		2		0/2		0			0[d]
34	*I. persulcatus*	800	348	73/12		4/0		TV08-T2508 TV08-T2460 TV17-T24714 TV17-T24615	MH614276[e] MH614280[e] MH614286[e] MH614284[e]	1.2

[a]the Table shows only the points of tick collection where TBEV was detected.
[b]above sea level.
[c]MIR (minimal infection rate) $= \frac{\text{Number of TBEV positive pools + number of TBEV positive individual ticks}}{\text{Number of analyzed ticks (total)}}$.
[d]hypothetical data, due to the small amount of analysed ticks.
[e]genotype was determined during the present work.
[f]Chernokhaeva et al., 2016.

(points 17 and 19), Tannu-Ola tundra steppe larch (point 10), Khemchik dry steppe (points 4 and 5), Central Tuva meadow steppe (points 13, 20, and 28), and the Turan-Uyuk larch meadow steppe (point 18) (Fig. 1, Table 2). TBEV RNA was not detected in ticks from Mongun-Taiga mountain tundra deserted and Todzha cedar larch districts. From the Mongun-Taiga district, 560 *D. nuttalli* were analysed. The absence of viral detection in such a large number of ticks could indicate that there is no TBEV circulation in that district at all; alternatively, the lack of detection may be because the infection rate of ticks is less than 0.2%. From the Todzha district, only 29 individuals were analysed, and the fact that we did not detect TBEV RNA in these ticks may be due to the small number of collected ticks; alternatively, the lack of detection may be because the infection rate is less than 3.5%.

In certain districts, the number of analysed ticks was insufficient to calculate the virus prevalence. In this regard, the infection rate of ticks was estimated only for those points where more than 20 ticks were analysed.

At almost all the points where a sufficient number (more than 20 individuals) of *I. persulcatus* was collected, we detected TBEV RNA in ticks. The virus prevalence of *I. persulcatus* ranged from 0.9% at point 10 to 4.8% at point 17 (Table 2).

D. silvarum in numbers greater than 20 individuals were collected at the points 12 (25 ticks) and 28 (23 ticks), where all ticks were removed from sheep and cows, respectively. TBEV was not detected in ticks from point 12. During the analysis of ticks from point 28, RNA was detected in two pools from the six analysed (8.6%). All these ticks were removed from one animal.

The prevalence of TBEV was not significantly different between *D. silvarum* and *I. persulcatus* ticks both within the Central Tuva meadow steppe district and in Tuva in general (chi-square test: 0.296 and 0.147, respectively; $p > 0.05$).

D. nuttalli ticks were collected in almost all districts. Infected individuals were identified in the Khemchik dry steppe district (points 4 and 5) and the Ubsunur deserted steppe district (points 9 and 32). Sufficient numbers of individuals to determine virus prevalence were collected only at points 9 and 32, where ticks were collected from vegetation and animals. Among the ticks collected from point 9, we detected 5 samples containing TBEV RNA (1.4%): 2 pools of ticks collected from vegetation (out of 42 analysed), and 1 pool and 2 individual ticks from animals (out of 25 and 3 of analysed, respectively). It should be noted that the 3 positive ticks mentioned above were removed from different animals. The TBEV infection rate of ticks at the point 32 was 8.3% (Table 2). Out of the 29 pools analysed and the 15 individual ticks collected from animals, only 1 pool was TBEV-positive. The infection rate of *D. nuttalli* was not significantly different from that of *I. persulcatus* and *D. silvarum* (chi-square test: 0.067 and 0.263, respectively; $p > 0.05$).

A difference was not observed between the infection rate of *I. persulcatus* ticks in the areas of cohabitation with *Dermacentor* ticks and in the areas of absolute dominance of *I. persulcatus* ticks. Furthermore, we did not observe differences between the infection rates of ticks from different provinces.

TBEV was detected in ticks at all investigated altitudes. A correlation between the infection rate of ticks and the altitude of the collection location was not observed. The highest point where TBEV was detected in *Dermacentor* ticks and *I. persulcatus* ticks was 1566 m a.s.l. (point 32) and in 1506 m a.s.l. (point 19), respectively.

3.3. Isolation of tick-borne encephalitis virus

We used the following definition of the virus strain: a virus that was passaged several times, which resulted in a virus population with stable properties under given laboratory conditions. By “isolate”, we mean the primary material containing viral RNA fragment with the identified nucleotide sequence, without strain isolation.

To isolate strains, samples that were positive according to PCR and real-time PCR were used for the intracerebral infection of two-day-old ICR mice and the infection of PEK and Vero cell cultures (Table 3). Fourteen PCR-positive samples of *I. persulcatus* were used to isolate the virus, and 9 strains (64.3% of isolation) and 3 isolates were obtained. We used 31 PCR-positive samples to isolate viruses from *D. nuttalli* as a result, only 1 strain of TBEV (3% isolation) was isolated (Table 3). All these strains were isolated from hungry ticks that were collected by flagging. We detected TBEV in *D. silvarum* ticks only in 2 pools out of the 6 analysed; these ticks were removed from an animal at the point 28. From one of these pools, we isolated a TBEV strain (Table 2).

Table 3
Passage history of positive samples.

Tick species	Number of positive samples in PCR or Real-Time PCR	Passages, number of obtained strains or isolates / number of studied samples		
		PEK/Vero	Mice	PEK/Vero+Mice
I. persulcatus	PCR – 14	-	3/5	9/9[a]
D. nuttalli	PCR – 19	0/9	-	0/8
	RT-PCR – 12	0/5	-	1/7
D. silvarum	PCR – 2	-	-	1/2

[a]3 out of 9 are isolates that do not replicate in PEK, Vero cells and mice.

For the phylogenetic analyses, we used RNA sequences of 44 GenBank entries of TBEV and 10 strains isolated in Tuva, one of which was described earlier (Chernokhaeva et al., 2016). The analysis was performed on the genome coding region of E protein fragments (1290 bp). All strains described in this article as well as four strains isolated in 2011 (Mel’nikova et al., 2014) belong to the Siberian subtype of TBEV and form one monophyletic group (Fig. 3). The other three strains isolated in Tuva by the same authors form another monophyletic group together with the strains isolated in the Kemerovo region.

4. Discussion

Previously, it was stated that the following nine species of ixodid ticks are distributed in the Republic of Tuva: *I. persulcatus*, *I. crenulatus*, *I. lividus*, *I. stromi*, *D. silvarum*, *D. nuttalli*, *D. reticulatus*, *D. marginatus*, and *H. concinna*. (Babenko, 1959; Filippova, 1997; Getta, 1957; Yemelyanova et al., 1985, 1962). During our expeditions, only the following three species of ticks were collected: *I. persulcatus, D. silvarum*, and *D. nuttalli*. Since *I. crenulatus*, *I. lividus*, and *I. stromi* are nest parasites, it is impossible to collect them by flagging. *D. reticulatus*, *D. marginatus*, and *H. concinna* ticks were not observed during our expeditions.

The Republic of Tuva is enclosed by mountain ranges from neighbouring regions, and only in the south does the steppe part of the Republic transition into the steppes of Mongolia. The distribution of ticks in Tuva is sporadic. Thus, in the forest zone, *I. persulcatus* is the most epidemiologically significant TBEV vector. In the forest-steppe and steppe zones, on the plains and the slopes of the mountains, all three species of ticks can be found, but the *Dermacentor* ticks are predominant. *D. silvarum* was not observed at altitudes higher than 1300 m a.s.l (highest point 51.296732, 91.973457, point 7). Above that altitude, only *D. nuttalli* (highest point 50.426985, 90.145055; 2016 m a.s.l.; point 1) and *I. persulcatus* (highest point 51.946460, 94.124137; 1539 m a.s.l.; point 19) were found. It was noted that in the Republic of Altai, which is located to the west of Tuva, the critical altitude for *D. silvarum* was 1321 m a.s.l.; for *I. persulcatus*, it was 1500 m a.s.l.; and for *D. nuttalli*, it was 2383 m a.s.l. (Shchuchinova et al., 2015). Our data for the upper altitude limits of tick habitats in Tuva are very similar to those recorded in Altai.

Generally, the number of ticks is highly variable in different geographic locations and can be affected by anthropogenic activity. In the

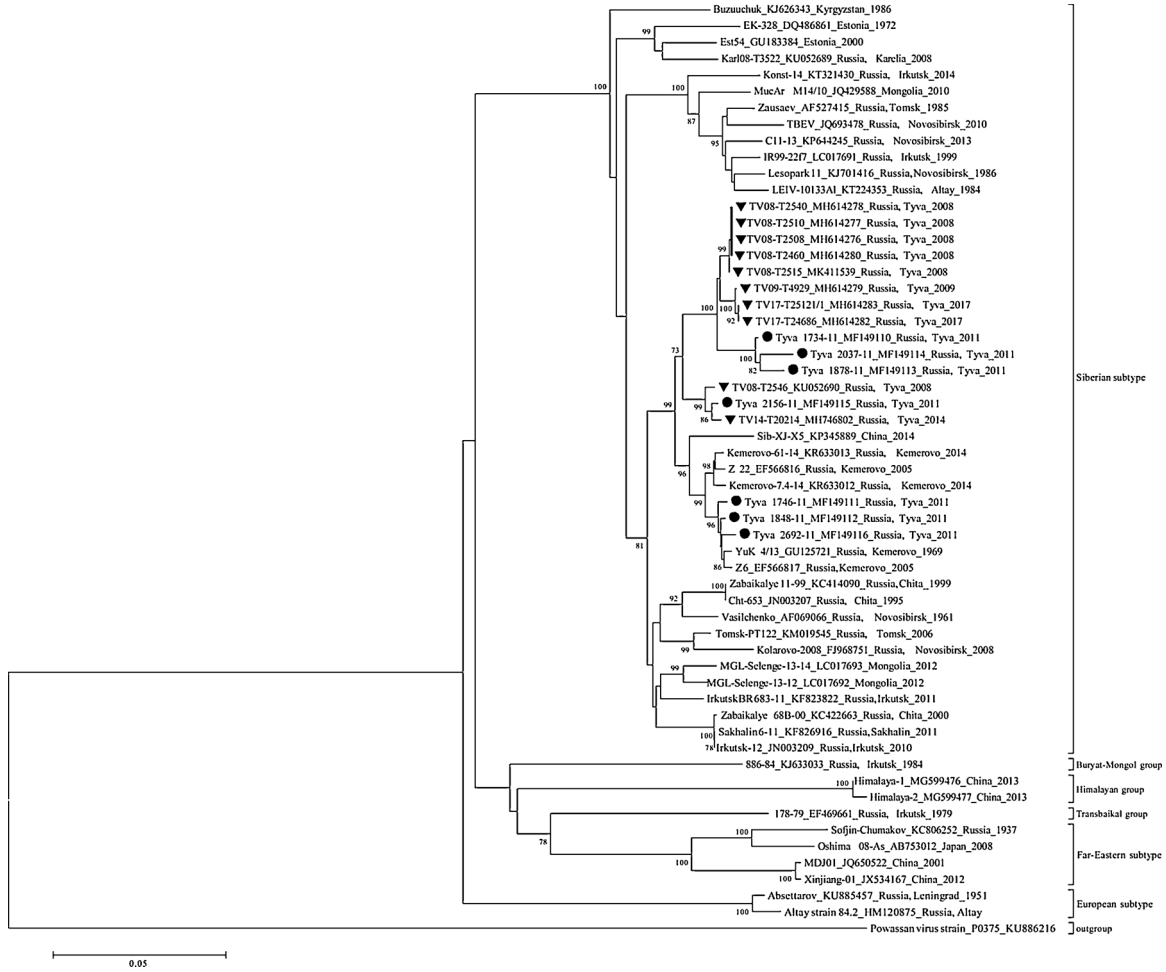

Fig. 3. Phylogenetic Tree of Tick-Borne Encephalitis Virus.
RNA sequences of representatives of all TBEV genotypes were included in the phylogenetic analysis. Phylogenetic trees were constructed using E protein fragments (1290 bp) in MEGA 6.0 with the neighbour-joining method (1000 bootstrap replications). Bootstrap values (> 70%) are shown at the branches.
● Mel'nikova et al., 2014
▼ Strains from the present work

middle of the 20th century, large-scale deforestation was carried out throughout the Republic of Tuva. At the end of the 20th century, a vast amount of arable land was abandoned and has gradually become overgrown with weeds and bushes, which led to the spread of virus hosts and vectors. A portion of the indigenous population of Tuva leads a nomadic lifestyle and constantly migrates through the territory with herds of horses, sheep, and cattle (cows, yaks). Due to the indifferent attitude of the local people to *Dermacentor* ticks and the low level of acaricide treatment, more than one hundred ticks can be observed feeding on a single animal. All of the above-mentioned factors may contribute to the sharp increase in the number of *Dermacentor* ticks.

TBEV RNA was detected in *I. persulcatus*, *D. silvarum*, and *D. nuttalli* ticks collected both from vegetation and animals. It is important to address the possibility that the engorged and partially engorged ticks removed from animals could acquire the TBEV from a bloodmeal. In our study, only 7 out of the 80 analysed samples of *D. nuttalli* from animals contained TBEV RNA (Table 2). Moreover, most of the TBEV-positive ticks were removed from different animals. The same results were obtained during the analysis of *D. silvarum* ticks from animals. From a single cow, 23 *D. silvarum* ticks were removed and were divided into 6 pools. Two out of six pools were TBEV-positive. This observation suggests that the feeding of an infected tick on a large mammal does not necessarily lead to the infection of other ticks simultaneously feeding on the same animal. It should be also mentioned that non-viremic TBEV transmission during co-feeding of ticks has only been described in small mammals (Labuda et al., 1993). Thus, we calculated the TBEV infection rate of ticks from animals in the same manner as the infection rate of ticks from vegetation.

There were no significant differences between the infection rate of *Dermacentor* ticks and *I. persulcatus* (Table 2), which differs from the global data. In the Republic of Altai, the infection rate of *D. silvarum* and *D. nuttalli* is significantly higher than that of *I. persulcatus*. (Shchuchinova et al., 2015). In the Zhiguliovsk Reserve, which is located in the central part of Russia and is inhabited by three species of ticks (*I. persulcatus*, *D. reticulatus*, and *D. marginatus*), the abundance and TBEV infection rate of *Dermacentor* ticks were higher than those of *I. persulcatus* (4.3% vs. 1.4%, respectively) (Morozov et al., 2009). In Poland, the prevalence of TBEV in *I. ricinus* was 1.6%, whereas in *D. reticulatus*, it was 10.8% (Wójcik-Fatla et al., 2011).

In the Republic of Tuva, the highest points where TBEV was identified were 1539 m a.s.l. for *I. persulcatus*, 1520 m a.s.l. for *D. nuttalli*, and 1031 m a.s.l. for *D. silvarum*. According to our data, the virus prevalence in ticks does not depend on altitude. This finding is inconsistent with the data from the neighbouring Republic of Altai, where it was shown that with the increase in the altitude, the virus prevalence in ticks decreases (Shchuchinova et al., 2015). In Central Europe, TBEV was observed at altitudes up to 1140 m a.s.l., and TBE cases were also registered at an altitude of 1500 m a.s.l. (Danielová et al., 2010; Holzmann et al., 2009).

By analysing ticks for TBEV infection, we detected 14 positive

samples of *I. persulcatus*, 31 of *D. nuttalli*, and 2 of *D. silvarum*. Out of these samples, we obtained 9 TBEV strains from *I. persulcatus* and one strain each from *D. nuttalli* and *D. silvarum*. Three isolates were also obtained from *I. persulcatus*, which we could not establish under laboratory conditions. The isolation efficiency of strains from TBEV-positive ticks of different species varied: for *I. persulcatus*, 9 strains out of 14 positive samples were isolated (64.3%), for *D. nuttalli*, 1 strain out of 31 positive samples were isolated (3%), for *D. silvarum*, 1 strain out of 2 positive samples were isolated. We could not estimate the isolation efficiency of TBEV strains from *D. silvarum* due to an insufficient number of TBEV-positive samples. The isolation efficiency of TBEV strains from *I. persulcatus* was significantly higher than that from *D. nuttalli* (chi-square test 20.804, $p < 0.001$). This phenomenon may be due to the lower virus titre in *D. nuttalli* ticks compared with *I. persulcatus* ticks, which is supported by the fact that we could not sequence fragments of the TBEV genome from PCR-positive samples of *D. nuttalli*. However, it was previously shown that under laboratory conditions, the titre of TBEV in *Dermacentor* ticks during persistent infection is higher than that in *Ixodes* ticks (Belova et al., 2018, 2017). Another potential explanation may be that different properties of the virus adapted to reproduction in various tick species (Belova et al., 2018, 2017; Chunikhin and Dzhivanian, 1977; Romanova et al., 2007).

In our study, TBEV RNA was detected in *Dermacentor* ticks, some of which were collected in the territories where these ticks predominate (points 9 and 32). Cases of tick-borne encephalitis (TBE) in the steppe regions have been recorded in Tuva; however, there is no well-documented evidence of a patient becoming ill after a *Dermacentor* tick bite. The role of *Dermacentor* ticks in maintaining virus circulation in natural foci has been actively discussed (Alekseev and Chunikhin, 1991; Belova et al., 2018, 2017, 2013; Földvári et al., 2016; Kahl and Dautel, 2013; Karbowiak et al., 2015; Naumov et al., 1981; Zhmaeva and Pchelkina, 1967). The initial assumptions about the possible role of *Dermacentor* ticks as a TBEV vector were made in the 1960s. These assumptions were based on the records of TBE cases in the forest-steppe regions of Siberia in 1959–1960, where *D. reticulatus* predominated and *Ixodes* ticks were absent (Netsky and Shaiman, 1964). Our observations show that *D. nuttalli* ticks quite often bite people, and the first TBE cases after tick bite are recorded from the beginning of April, when *I. persulcatus* is practically absent. Stable TBEV circulation in natural foci depends on the ability of the virus to replicate and persist for a long time in the vector as well as on the effectiveness of vertical and horizontal transmission among vectors. The capacity for *D. marginatus* and *D. reticulatus* to harbour the TBEV for more than 80 d has been experimentally confirmed (Belova et al., 2017; Nosek and Kozuch, 1985). Transstadial survival and transovarial transmission of TBEV were established for *D. reticulatus* ticks (Karbowiak et al., 2016; Zhmaeva and Pchelkina, 1967). According to the experimental data, *D. reticulatus* and *D. marginatus* can acquire, support replication, and harbour viruses for a long time. The efficient transstadial survival of TBEV was shown as well (Belova et al., 2017, 2013). Additionally, the possibility of TBEV transmission among *D. marginatus* ticks by co-feeding of infected and uninfected ticks on the same animal has been confirmed (Alekseev and Chunikhin, 1991). Taken together, these findings indicate the potential of *Dermacentor* ticks to successfully maintain TBEV circulation under experimental conditions.

However, it is known that the immature forms of *Dermacentor* ticks are not able to overwinter, unlike the immature forms of *Ixodes* ticks (Balashov, 1998). The overwintering of immature ticks is considered to be important for the successful maintenance of virus circulation. Most *Dermacentor* species are characterised by the feeding of immature and adults ticks on different host species. This fact suggests that the effectiveness of TBEV transmission for *Dermacentor* ticks is limited by the rate of transovarial transmission and the transstadial survival of the virus (Kahl and Dautel, 2013). On the one hand, due to the high number of *D. nuttalli* ticks and the ability of certain *Dermacentor* species to transmit TBEV transovarially and transstadially, *D. nuttalli* ticks might be able to support the focus on their own. On the other hand, in 1962, the ability of immature forms of *D. nuttalli* to overwinter was hypothesised. The basis for this hypothesis was the appearance of single nymphs on animals one month prior to the appearance of larvae (Yemelyanova et al., 1962). In addition, in April 2014, we removed *D. nuttalli* nymphs from ground squirrels, and in April 2015, we collected several nymphs near the ground squirrels' burrows. Previously it was thought that immature ticks of Eurasian *Dermacentor* species cannot overwinter (Balashov, 1998). However, our finding is consistent with the hypothesis that immature forms of *D. nuttalli* are able to overwinter in the microclimate conditions of the ground squirrels' burrows. These finding may also support the hypothesis that the beginning of feeding of adult ticks is in January and February, since the time from the beginning of the feeding of adult ticks to the development of the nymph ranges from 67 to 130 d (Balashov, 1998). The confirmation or refutation of these hypotheses requires further experimental studies.

Thus, the present data on virus detection in the areas with an absolute predominance of *Dermacentor* ticks together with the hypothetical ability of the immature forms to overwinter under favourable conditions support the possibility of *Dermacentor* ticks to self-support TBEV focus.

Special attention should be paid to the risk of infection and disease of people in the TBEV foci where *Dermacentor* ticks predominate or cohabitate with *Ixodes* ticks. If the risk of TBE infection is mainly determined by factors such as the vector abundance, virus prevalence in vectors, and the aggressiveness of the ticks towards humans, then the risk of disease depends on the characteristics of the human population (genetic susceptibility, immune status, etc.) (Yudin et al., 2018), the virus dose obtained from the tick bite and the properties of the viral population (e.g., neuroinvasiveness). The species of ticks in turn, can affect both the risk of infection and the risk of disease in people in the TBEV focus. We showed that the adaptation of TBEV to *I. ricinus* and *D. reticulatus* by sequential parenteral passages leads to the appearance of variants with reduced neuroinvasiveness in laboratory mice (Belova et al., 2017, 2015). The appearance of such variants in the natural viral population can lead to the risk of infection in the natural focus remaining high but with a low risk of the disease. The difficulty in virus isolation from *Dermacentor* ticks (in GenBank, there is a small number of such isolates) indirectly confirms hypothesis. The final answer to the questions of whether the TBEV foci can be supported solely by *Dermacentor* ticks and what is the epizootic and epidemic potential of such foci warrants further research.

5. Conclusion

During the expeditions to the Republic of Tuva, we collected by flagging and removal from cattle and domestic animals ixodid ticks of the following three species: *I. persulcatus*, *D. nuttalli*, and *D. silvarum*. The distribution of these species was confined to specific landscapes, as follows: *D. nuttalli* occurred in steppes, *D. silvarum* – in forest-steppe areas, and *I. persulcatus* – in mixed forests. TBEV of the Siberian subtype circulates in the territory of Tuva in all tick species. The present data support the hypothesis of the ability of immature forms of *D. nuttalli* to overwinter under favourable conditions, as well as the possibility of the existence of TBEV foci in locations with an absolute dominance of *D. nuttalli*.

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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.2019.04.019.

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