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Original article

Huge diversity of phleboviruses in ticks from Strandja Nature Park, Bulgaria

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ABSTRACT

The discovery of the first tick-borne phleboviruses associated with severe disease in humans stimulated studies searching for further previously unknown tick-associated viruses. Novel phleboviruses have subsequently been identified in ticks from the USA, Japan and China and recently also from Europe. Here, we investigated the genetic diversity of tick-borne phleboviruses originating from Strandja Nature Park, Bulgaria, a unique primary forest with evergreen plants that was not affected by the last ice ages in the Pleistocene and Holocene.

We found a high genetic diversity of 12 phleboviral sequences in 1542 ticks. The sequences formed five distinct groups and clustered with other tick-borne phleboviruses recently identified in Europe. Although isolation experiments of the detected viruses in cell culture failed, viral RNA copy numbers were stable up to 42 days post infection (dpi) in the supernatant of tick cells whereas they disappeared 14 dpi in that of VeroE6/7 cells. In summary, nearly all tick-associated phleboviruses known to occur in Europe have been detected in one geographic region. Our data show that primary ecosystems in temperate regions are also rich in viral diversity and that this is not only true for tropical regions.

1. Introduction

The genus *Phlebovirus* (family *Phenuiviridae*) contains many viruses that can cause severe disease in humans and animals, e.g. *Rift Valley fever phlebovirus* and *Sandfly fever Naples phlebovirus* (Elliott and Brennan, 2014). The genus can be divided into a monophyletic group of Diptera-transmitted viruses and a paraphyletic group of tick-transmitted viruses. Phlebotomine sandflies, mosquitoes and biting midges mainly transmit diptera-borne phleboviruses. Tick-borne phleboviruses (TBPV) are transmitted by several species of the families Ixodidae and Argasidae. TBPV are subdivided into the Uukuniemi group, the SFTSV group (*severe fever with thrombocytopenia syndrome phlebovirus*, SFTSV), the Bhanja group and the Kaisodi group (Finkeisen et al., 2012; Yadav et al., 2018). While human infections with *Heartland banyangvirus* and SFTSV have been associated with severe disease (McMullan et al., 2012; Yu et al., 2011), infections with viruses of the Uukuniemi and Bhanja groups are mainly asymptomatic (Bouloy, 2011). There was little interest in tick-borne phleboviruses before the discovery of SFTSV and *Heartland banyangvirus*. For several decades *Uukuniemi phlebovirus* was the only known tick-borne phlebovirus. Multiple new phleboviruses have been identified in ticks in recent years (Li et al., 2015; Tokarz et al., 2014). This includes the discovery of novel TBPV sequences in Europe, like Antigone and Lesvos viruses detected in Ixodidae from Greece (Papa et al., 2017, 2016), sequence fragments from three putative viruses, named RiPar, KaMar and AnLuc in Ixodidae from Portugal (Pereira et al., 2016), as well as a group of sequence fragments named Glabbeek/Osterholz which were found in *Ixodes ricinus* ticks from Belgium and Germany (Prinz et al., 2017). Studies on the diversity of TBPV in Bulgaria are scarce. So far, only Bhanja virus has been detected in Bulgaria (Pavlov et al., 1978).

Phleboviruses have a tri-segmented negative-sense RNA genome comprising a small segment (S-segment) encoding the viral nucleocapsid, a medium segment (M-segment) encoding the viral glycoproteins Gn and Gc and a large segment (L-segment) encoding the RNA dependent RNA-polymerase (RdRp). In addition, the S-segment encodes in positive-sense a non-structural protein named NSs (Elliott and Schmaljohn, 2013). This ambisense coding strategy of the S-segment differentiates phleboviruses from most other bunyaviruses (Giorgi et al., 1991). A second non-structural protein (NSm) is encoded in positive-sense on the M-Segment of the Diptera-borne phleboviruses, but

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not by the TBPV. As a further characteristic, phleboviruses contain conserved consensus terminal sequences (Elliott and Brennan, 2014).

Here, we tested hardbodied ticks sampled in a unique ecosystem, the Strandja Nature Park, Bulgaria, for infection with phleboviruses. The Strandja region, located in the southeast of Bulgaria, flanked by the border to Turkey and the Black Sea, represents the last remaining temperate forest with evergreen plants in Europe.

2. Material and methods

2.1. Sampling of ticks and species identification

The Strandja Nature Park belongs to the Burgas district and borders Turkey and the Black sea. It represents the last remaining temperate forest with evergreen plants in Europe that was not reached by the land-ice during the last ice ages in the Pleistocene and Holocene. It has a primeval flora from the Paleogene and Neogene with pontic rhododendron (*Rhododendron ponticum*), oriental beech (*Fagus orientalis*) and various oak species. Settlement and arable land occupies 7% of the nature park and 71% is covered with forest. Five highly protected areas exist within the park, namely Sredoka, Silkosiya, Witanowo, Tisowica and Usunbodschak.

Ticks were collected by flagging from vegetation in sylvatic and grassland habitats, as well as by hand-picking from livestock, like goats and cattle, dogs, as well as from tortoises (*Testudo graeca*, *Testudo hermanni*) and humans at eight sampling sites in the regions of Stoilovo, Silkosiya, Sredoka, Kosti, Bulgari, Sinemorets, Zvezdets and Malko Tarnovo from May to August 2012 (Fig. 1). In addition, ticks were sampled from humans in the Ropotamo Reserve and from injured wildlife treated in the Wildlife Rehabilitation Center of Stara Zagora, located in the center of Bulgaria. Staff of the Rehabilitation center did tick removal from injured wildlife during their routine processes of animal care. Collected ticks were stored individually in liquid nitrogen in the field. Morphological identification of sex and species was performed using a stereo-microscope and taxonomic keys (Babos, 1964; Walker et al., 2003).

2.2. PCR-screening, sequencing and phylogenetic analyses

For molecular screening nymphs and larvae were individually homogenized in 200 µl L-15 medium without additives using 10 ceramic beads and a SpeedMill PLUS homogenizer (Analytik Jena AG). Adult ticks were homogenized in 500 µl medium using 6 steel beads. Pools were generated by merging 100 µl supernatant of 10 homogenized specimens, sorted by species, stage and sampling site (n = 155). Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (Quiagen, Hilden, Germany) and transcribed into cDNA using Superscript™ III reverse transcriptase and random hexamer primers (Invitrogen™ GmbH, Karlsruhe). Pools were tested for phleboviruses by three generic nested-PCRs using Platinum™ *taq* DNA polymerase (Invitrogen™ GmbH, Karlsruhe) amplifying a 521 bp fragment in the first round and fragments of 233 bp, 253 bp, and 501 bp in the nested PCRs of the L-segment. PCR conditions were as followed: 95 °C for 3 min; 10 cycles of 94 °C for 30 s, 55 °C for 20 s with touchdown-steps of 0,5 °C per cycle, 72 °C for 30 s; 40 cycles of 95 °C for 15 s, 50 °C for 20 s, 72 °C for 30 s; and 72 °C for 5 min. Primer sequences are given in Table 1. Individual specimens of virus positive pools containing different tick species, were tested by the above described generic PCR system in order to identify infected tick species. PCR products were Sanger sequenced by Seqlab (Seqlab, Göttingen). Elongation of sequence fragments was performed by nested PCR using sequence specific and generic primers based on related sequences (Table 1). PCR conditions were as followed with respect to primer specific annealing temperatures: 95 °C for 3 min; 45 cycles of 95 °C for 30 s, X°C for 30 s, 72 °C for 1 min; and 72 °C for 5 min. Sequences were compared to the GenBank database using the NCBI Basic Local Alignment Tool (Altschul et al., 1990). Nucleotide and amino acid sequences were analyzed and aligned with other phleboviruses using MAFFT E-INS-I and Geneious v9.1.4. Maximum likelihood phylogenetic analyses were performed using PhyML and the LG substitution model with 1000 bootstrap replicates. Sequences of established and proposed phlebovirus species, as well as sequence fragments of related viruses were selected for the phylogenetic analysis aiming at covering all known tick-borne phlebovirus lineages, with a focus of sequences that have been detected in Europe.

2.3. Virus isolation in cell culture

Virus isolation was performed by inoculating 50 µl of homogenates from phlebovirus positive pools on 4×10^4 Vero E6/7 cells (monkey kidney cell line), on $2{,}5 \times 10^5$ HAE/CTVM8 cells (embryo cells of *Hyalomma anatolicum*) and $1{,}75 \times 10^5$ BDE/CTVM16 cells (embryo cells of *Rhipicephalus* (*Boophilus*) *decoloratus*) seeded in 48-well plates, respectively. For infection of Vero E6/7 cells, culture medium was removed and 150 µl of Gibco™ Dulbecco's Modified Eagle Medium (DMEM) and 50 µl of tick homogenate was added. After 1 h of incubation at 37 °C and 5% CO2, 300 µl DMEM medium containing 2% fetal calf serum was added. For infection of tick cells, 100 µl of cell line specific medium, 50 µl of Gibco™ Leibovitz's L-15 Medium without additives and 50 µl of tick homogenate was added. After 1 h of incubation at 28 °C 300 µl of cell line specific medium was added. For culture conditions and medium composition of tick cell lines see Bell-Sakyi

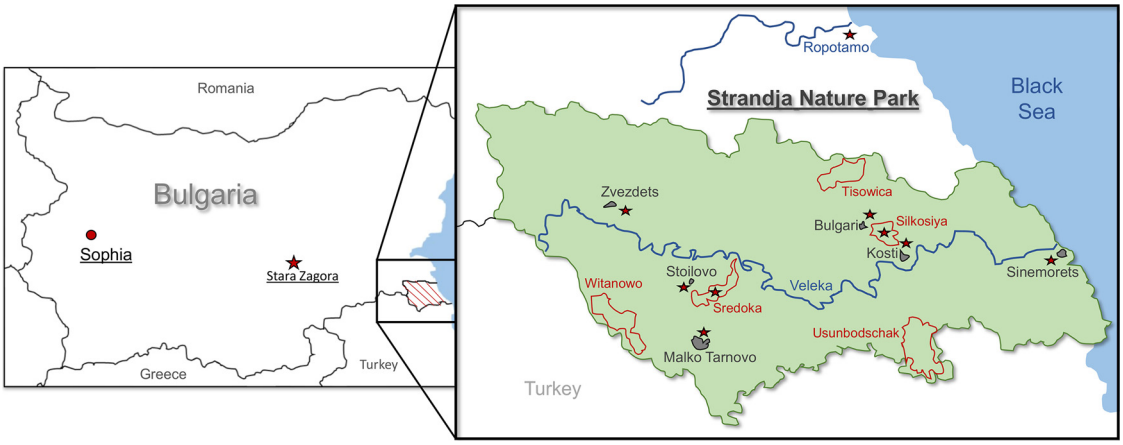

Fig. 1. Map of Bulgaria and the area of Strandja Nature Park. Sampling sites are indicated by a red asterisk, highly protected areas within the Nature Park are framed in red, and villages are marked in grey.

Table 1
Information of primers used for PCR-screeening and sequencing.

Virus	Primer	Sequence	Tm (°C)
Strandja- BG2012-GI	Ph_GI_f464	5‘-TCCTGCAGATCATCATTAACAACC-3‘	53
	Ph_GI_f399	5‘-GCACCCGTTCATTGTAAGAGC-3‘	54
	Ph_GI_f621	5‘-CACTCAAGACCCTTCATGGCG-3‘	55
	Ph_GI_f633	5‘-TTCATGGCGCGTACCGTGGC-3‘	60
	Ph_GI_f508	5‘-GCACCCGTTCATTGTAAGAGC-3‘	54
	Ph_GI_f242	5‘-CACACCACCAAGCTGGCACTAACC-3‘	60
	Ph_GI_f226	5‘-CTTCAACCAAACGCAACACACCACC-3‘	60
	Ph_GI_r254	5‘-GCTTGGTGGTGTGTTGCGTTTGG-3‘	60
	Ph_GI_f445	5‘-TCCTGCAGATCATCATTAACAACC-3‘	58
	Ph_GI_f405	5‘-GCACCCGTTCATTGTAAGAGC-3‘	55
	Ph_GI_f445	5‘-CCCTCCACCACGATGGGCAAAG-3‘	60
	Ph_GI_r156	5‘-CTGGCTCTCTTTCCATGTGTCTCCG-3‘	59
	Ph_GI_f417	5‘-TTCATGGCGCGTACCGTGGC-3‘	60
Strandja- BG2012-GII	Ph_GII_r210	5‘-GATGTGCCGACTGTTTCAATCCTCCC-3‘	60
	Ph_GII_f375	5‘-GGAATGCGGAGATGAAGACCACGG-3‘	60
	Ph_GII_f810	5‘-AGCTCTGATGACTCTGCTGTTC-3‘	54
	Ph_GII_r177	5‘-GCAAGCCCCCATGTTGGTTC-3‘	56
	Ph_GII_f423	5‘-AGGCATATCGAGGAATGGTGGACCC-3‘	60
	Ph_GII_f854	5‘-TGAGAGTCAGGTGAGGCGG-3‘	55
	Ph_GII_r106	5‘-CAATCTTTGAGGACGTGGTGC-3‘	54
	Ph_GII_r251	5‘-TGGTCGTGTGCTGCGTTTGG-3‘	58
Strandja- BG2012-GIII	Ph_GIII_r162	5‘-GCATTGACGAGGTCTTTTGCTTGG-3‘	57
	Ph_GIII_f310	5‘-GAGGATCAAGTTAGATGATGACATTCTGC-3‘	56
	Ph_GIII_f202	5‘-CTTCAACCAGACTCAGCACACGACC-3‘	59
	Ph_GIII_r231	5‘-AGTTTGGTCGTGTGCTGAGTCTGG-3‘	59
Strandja- BG2012-GIV	Ph_GIV_f50	5‘-ACAGAGGCATAACAAGGATG-3‘	49
	Ph_GIV_r64	5‘-TTGTTATGCCTCTGTGGAAG-3‘	50
	Ph_GIV_r78	5‘-TCGTTTTGGCATCCTTGTTA-3‘	50
	Ph_GIV_r54	5‘-TCTGTGGAAGGTCTGTTTTG-3‘	50
	Ph_GIV_f59	5‘-TAACAAGGATGCCAAAACGA-3‘	50
	Ph_GIV_f101	5‘-GACAGCCACTTCTGATGATG-3‘	50
	Ph_GIV_f50	5‘-ACAGAGGCATAACAAGGATG-3‘	49
	Ph_GIV_r146	5‘-TAGATCTCCCTGATGCCGCC-3‘	55
	Ph_GIV_f727	5‘-AGGAAGAACACGAATCTCAAGCC-3‘	55
	Ph_GIV_f808	5‘-TCCTCATCCATCCCTGAAGCC-3‘	55
	Ph_GIV_r97	5‘-GCAAGCAAGTGTTCTGCTCC-3‘	55
Strandja- BG2012-GV	Ph_GV_f377	5‘-CTAGAGGATGAGTTTGCGAC-3‘	50
	Ph_GV_f417	5‘-ATGGAGAAATCACAGTTCCG-3‘	50
	Ph_GV_172	5‘-TGATCCACAGTGTTTTCTGG-3‘	50
	Ph_GV_r116	5‘-TCTTTGGGTTGTTGAGTGTC-3‘	50
	Ph_GV_r75	5‘-ATGGATCTGGCGATTGTTTC-3‘	50
Generic Primer	Ph_F0	5‘-CAACTTGCAACHCTNAARGC-3‘	48-54
	Ph_F1	5‘-TCAARAAGAMNCAACATGGTGG-3‘	50-56
	Ph_F2	5‘-GGACTTAGAGAGATYTAYGTNTTGG-3‘	49-54
	Ph_R1	5‘-TATGCCYTGCATCATYCCWG-3‘	50-54
	Ph_R2	5‘-ACATGRTGACCYTGRTTCCA-3‘	48-55
	Ph_R3	5‘-TGAAGCATGANCCACCYTC-3‘	49-54
	Ph_R4	5‘-GGAAGAARAAYTCTGARTTRTAYTCCA-3‘	49-56

(2004, 1991). The cells were observed daily for signs of cytopathic effect (CPE). Seven days post infection 100 µl of the supernatant from VeroE6/7 cells were passaged three times on fresh cells. Tick cells were incubated for 49 days. Every seven days a 20 µl aliquot of supernatant was taken and stored at −80 °C. The reduced volume was replaced by fresh cell culture medium. The amount of viral genome copies in cell culture supernatant was determined by real-time PCR. Briefly, viral RNA was extracted using the NucleoSpin® RNA Virus Kit (Macherey Nagel, Germany) and cDNA was transcribed using Superscript™ III reverse transcriptase and random hexamer primers (Invitrogen™ GmbH, Germany). The viral genome copy number was determined by real-time PCR using sequence specific primers and probes

(Strandja-B2012-GI: f-5’TGACCTGAGAACCTTCAACCAA-3’, r-5’ GGTGCAGTTCCTTCTTTGTGAAC-3’, TM-5’TCTGATGACCTGAGAACCT TCAACCAAACG-3’;

Strandja-B2012-GII: f-5’-AACAGTCGGCACATCAGATGAC3’, r-5’-GATGAGAGTTAGGGCTAGCTTGG3’, TM-5’-TGAAAACGTTCAACCAAA CGCAGCAC3’;

Strandja-B2012-GIII: f-5‘-CGCATGGAAAGAGAGCAAGAG-3‘, r-5‘-GGTGCCAACAGTCTCAAAACG-3‘; TM-5‘-CTGCCAAGCAAAAGACCTC GTCAATGC-3‘

Strandja-BG2012-GIV: f-5’-CATTCCCTGTTTCCTCATCCAT-3‘, r-5‘-GGAAGCACATCCAGGACAAGA-3’, TM-5’- CCTGAAGCCACGTCCCAG GGC3’

Strandja-BG2012-GV: f-5’TGCCCAGTTACTCTGCAGCTT-3’, r-5’ GGTGAACATTGAGCAGCCTCTT-3’, TM-5’-CAGCCCAAGTGGTGGCCAG TCATC3’). Sequence specific standards were produced by cloning the respective sequence fragments in the pCR®4-TOPO® vector using the TOPO TA Cloning® Kit for Sequencing (Invitrogen™ GmbH, Germany). Plasmids were recovered from cultures using the NucleoSpin Plasmid-Miniprep-Kit (Macherey Nagel, Germany). Plasmid standards in serial 1:10 dilutions were used for quantification.

2.4. Accession numbers

Sequences were deposited in NCBI GenBank under the following accession numbers MK216291 (Strandja-BG2012-GI-strainCT), MK216292 (Strandja-BG2012-GI-strainER), MK216293 (Strandja-BG2012-GI-strainCY), MK216294 (Strandja-BG2012-GI-strainET), MK216295 (Strandja-BG2012-GI-strainEU), MK216296 (Strandja-

Table 2
Sampling data of ticks containing phlebovirus sequences.

Virus	Strain	Tick species	Stage*$_1$	Sampling site	Collected from:	Accession No.
Strandja-BG2012-GI	CT	*Rhipicephalus* sp.	n	Kosti	Dog	MK216291
	ER	*R. bursa*	f	Stoilovo	Goat	MK216292
	CY	*R. sanguineus* (s.l.)	f, m	Stoilovo	Dog	MK261293
	ET	*R. sanguineus* (s.l.)	f	Stoilovo	Dog	MK261294
	EU	*R. sanguineus* (s.l.)	m	Stoilovo	Dog	MK261295
Strandja- BG2012- GII	CS	*H. aegyptium*	m	Stara Zagora	Tortoise	MK261296
	EV	*R. sanguineus* (s.l.)	f, m	Kosti	Dog	MK261297
Strandja-BG2012- GIII	CQ	*R. bursa*	m	Malko Tarnovo	Cattle	MK261298
	CR	*R. bursa*	f	Malko Tarnovo	Cattle	MK261299
	EP	*R. bursa*	m	Malko Tarnovo	Cattle	MK261300
Strandja- BG2012- GIV		*I. ricinus*	n	Sredoka	Vegetation	MK261301
Strandja- BG2012- GV		*Ixodes* sp.	n, l	Silkosiya	Vegetation	MK261302

*$_1$ f = female, m = male, n = nymph, l = larva.

BG2012-GII-strainCS), MK216297 (Strandja-BG2012-GII-strainEV), MK216298 (Strandja-BG2012-GIII-strainCQ), MK216299 (Strandja-BG2012-GIII-strainCR), MK216300 (Strandja-BG2012-GIII-strainEP), MK216301 (Strandja-BG2012-GIV) and MK216302 (Strandja-BG2012-GV).

3. Results

A total of 1542 ticks were sampled from vegetation, livestock, tortoise and humans in Strandja Nature Park, Bulgaria (Fig. 1). Ticks belonged to nine species of five genera, *Rhipicephalus bursa*, *Rh. sanguineus* (sensu lato, s.l.), *Rh. rossicus, Rh. turanicus, Hyalomma marginatum, Hy. aegyptium*, *Dermacentor marginatus*, *Haemaphysalis punctata* and *Ixodes ricinus*. Screening of ticks for infection with phleboviruses revealed twelve positive pools (12/155, 7.74%) collected from six sampling sites (Table 2). The sequences showed the highest identity to different phleboviruses by BLAST analysis and grouped into five genetic groups, provisionally named Strandja-BG2012-GI, Strandja-BG2012-GII, Strandja-BG2012-GIII, Strandja-BG2012-GIV and Strandja-BG2012-GV. One strain per group was elongated by PCR using fragment-specific and generic primers generating sequence fragments covering the third conserved region of the RdRp gene of 1075 bp (Strandja-BG2012-GI), 1146 bp (Strandja-BG2012-GII), 1147 bp (Strandja-BG2012-GIII), 891 bp (Strandja-BG2012-GIV, Motif E missing) and 1135 bp (Strandja-BG2012-GV). Analyses of the conserved motifs PreA, A, B, C, D and E showed that the Strandja strains contained the amino acids highly conserved among phleboviruses (Fig. 2).

The group of Strandja-BG2012-GI was formed by five strains with a pairwise identity of 99% to each other, which were detected in ticks of the species *R. bursa* and *R. sanguineus* (s.l). Strandja-BG2012-GI sequences shared 96% and 85% nucleotide identity with Tick Phlebovirus Anatolia 1 isolate KM59 and Phlebovirus Antigone strain Antigone2-Pella2-Greece-2013, respectively (Dinçer et al., 2017; Papa et al., 2016). Strandja-BG2012-GII included two strains with a pairwise identity of 99%, found in the tick species *H. aegyptium* and *R. sanguineus* (s.l.). Strandja-BG2012-GII sequences showed highest identity of 97% to Tick Phlebovirus ET26 detected in *H. aegyptium* in Turkey (Brinkmann et al., 2018). The group of Strandja-BG2012-GIII consisted of three strains with pairwise identities of 99% detected in *R. bursa* ticks. Highest identity of 98% was found to tick phlebovirus ME17 detected in *R. bursa* from Turkey (Brinkmann et al., 2018). The two further solitary sequences named Strandja-BG2012-GIV and Strandja-BG2012-GV were detected in ticks of the genus *Ixodes*. Strandja-BG2012-GIV showed highest pairwise nucleotide identity of 98% to Leuven phlebovirus CH146 (found in Belgium) and to Norway phlebovirus (found in Norway) (Prinz et al., 2017; Pettersson et al., 2017). Both sequences were detected in ticks of the species *I. ricinus*. Strandja-BG2012-GV showed pairwise nucleotide identities of 79% and 84% to Mukawa virus and Glabeek/Osterholz virus, respectively (Matsuno et al., 2013; Prinz et al., 2017).

Phylogenetic analyses based on nucleotide and translated amino acid sequences of RdRp genes of the Strandja sequences and those of phleboviruses revealed that the Strandja sequences fall into five distinct clades distributed across the phylogeny of tick-borne phleboviruses (Fig. 3). All 12 strains of the five putative species were included in the nucleotide-based tree and only the strain with the elongated sequence information of each species was included in the amino-acid-based tree. Strains of the group Strandja-BG2012-GI clustered together with recently detected phleboviral RdRp sequence fragments from Portugal, Turkey and Greece (AnLuc virus, Tick phlebovirus Anatolia and Antigone virus; Dinçer et al., 2017; Papa et al., 2016; Pereira et al., 2016). Strandja-BG2012-GII sequences clustered with a phlebovirus sequence recently detected in Turkey (tick phlebovirus ET26) and with Bole tick virus from China (Dinçer et al., 2017; Li et al., 2015). Strandja-BG2012-GIII sequences grouped with sequences found in ticks in Turkey and Portugal (tick phlebovirus ME17 and KarMa) (Brinkmann et al., 2018; Pereira et al., 2016). Strandja-BG2012-GIV clustered with a phlebovirus sequence recently detected in ticks from Belgium (Leuven phlebovirus, Prinz et al., 2017) and Blacklegged tick virus detected in Suffolk county, New York, US (Tokarz et al., 2014). Strandja-BG2012-GV clustered with an interesting new clade of tick-associated viruses that share a most recent common ancestor with the mosquito/sandfly-borne (insect-borne) phleboviruses consisting of Mukawa virus detected in Japan, Prignitz virus detected in Germany and Glabbeek virus found in Belgium (Matsuno et al., 2013; Prinz et al., 2017).

All attempts to isolate the five viruses on tick and vertebrate cells failed. Low amounts of viral RNA were detectable in some tick cell culture supernatants (HAE/CTVM8 and BDE/CTVM16) until 42 days post infection (dpi) (Fig. 4). In contrast, viral RNA copies were only measurable until 14 dpi in vertebrate cell culture supernatants.

4. Discussion

Virus discovery studies in ticks have increased markedly after the discoveries of *Severe fever with thrombocytopenia syndrome virus* and *Heartland banyangvirus*. The number of tick-borne phleboviruses has expanded rapidly in recent years (Li et al., 2015; Papa et al., 2017, 2016; Tokarz et al., 2014).

The recent study investigated the genetic diversity of tick-borne phleboviruses in ticks from Strandja Nature Park, Bulgaria. The area was not covered with ice during the Pleistocene and Holocene and contains a unique ecosystem that is more than 2.5 million years old. We detected a high genetic diversity of five putative phleboviruses, provisionally named Strandja–BG2012-GI to –GV, falling into five distinct phylogenetic clades. Interestingly, nearly all major lineages of tick-borne phleboviruses known to occur in distinct regions of Europe where

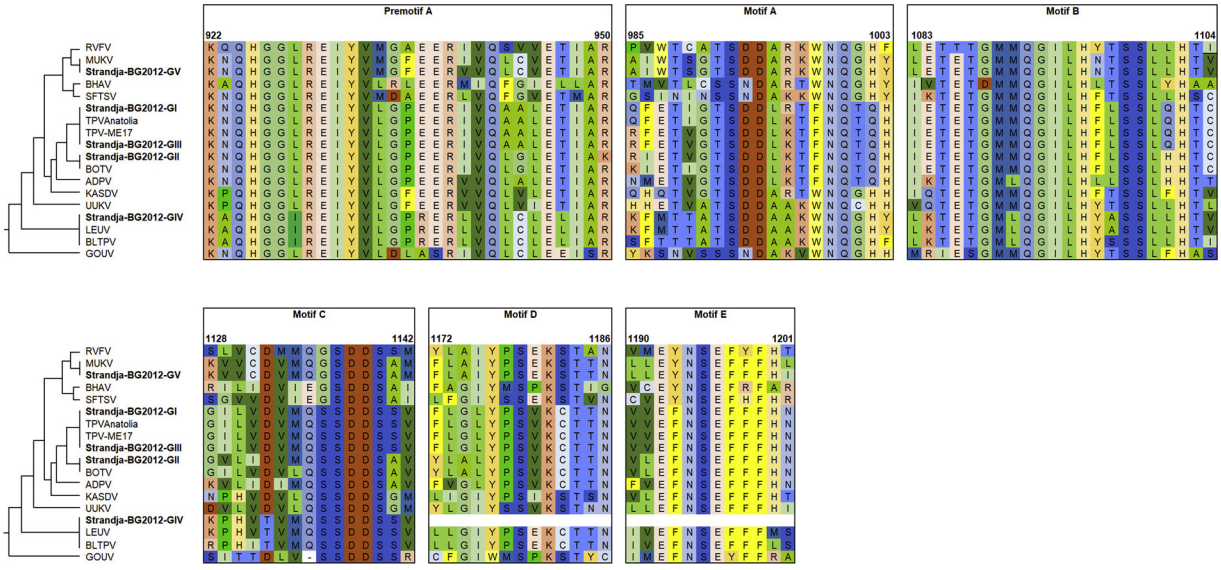

Fig. 2. Alignment of conserved motifs of RdRp proteins of phleboviruses. Amino acid sequences represent Premotif A and motifs A, B, C, D and E. Gaps within the alignment are indicated by a hyphen. None available sequence information is marked by blank sites. ADTV, American dog tick virus; BHAV, Bhanja virus; BOTV, Bole tick virus; BLTPV, Blacklegged tick phlebovirus; GOUV, Goulèako virus; KASDV, *Kaisodi virus*; LEUV, Leuven phlebovirus; MUKV, Mukawa virus; RVFV, *Rift valley fever virus*; SFTSV, *Severe fever with thrombocytopenia syndrome virus*; TPVAnatolia, tick Phlebovirus Anatolia; TPV-ME17, tick Phlebovirus ME17; UUKV, *Uukuniemi virus*.

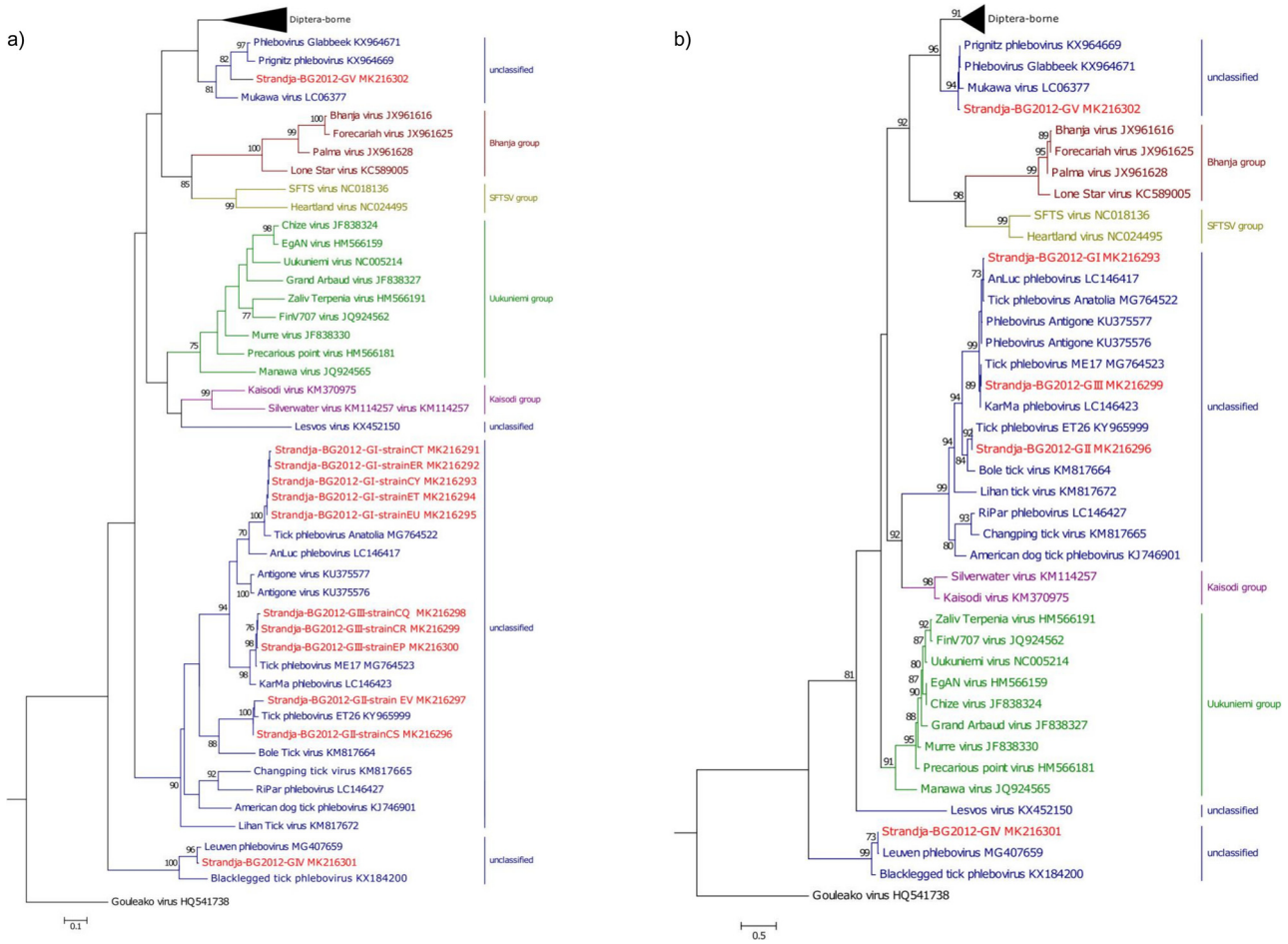

Fig. 3. Phylogentic relationship of Strandja viruses. Maximum likelihood phylogenetic trees based on a 406 nt fragment (a) and a 135 aa fragment (b) of phlebovirus RdRp genes. Trees were constructed using PhyML and the GTR substitution model with Bootstrap values based on 1000 replications. Values > 70% are shown, scale bar indicates nucleotide substitution per site. Newly identified sequences are marked in red.

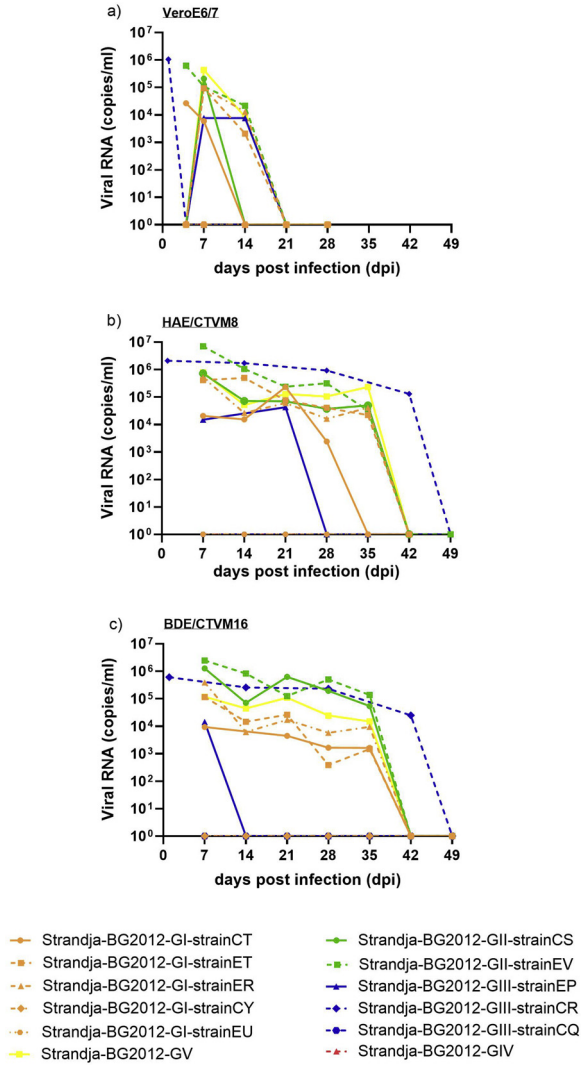

Fig. 4. Virus isolation attempts in cell culture. Number of viral genome copies per ml in cell culture supernatant of VeroE6/7 (a), HAE/CTVM8 (b) and BDE/CTVM16 (c) cells. Cells were infected with 50 µl of phlebovirus positive tick homogenates. Number of genome copies per ml supernatant were measured by RT-PCR on day 1, 7, 14, 21, 28, 35, 42 and 49 after infection in cell culture of cell lines BDE/CTVM16 and HAE/CTVM8 and on day 1, 7, 14, 21, 28 in cell culture of cell line VeroE6/7.

also found in our study region suggesting that biodiverse primary ecosystems in temperate regions also contain a high diversity of pathogens.

No geographic clustering was found for tick-borne phleboviruses originating from Europe. Rather closely related sequences most likely pertaining to one virus species were detected in ticks of the same species or genus suggesting high species specificity. For example, Strandja-BG2012-GI, Antigone and AnLuc sequences were found in *R. sanguineus* (s.l.) ticks, Strandja-BG2012-GIII, tick phlebovirus ME17 and KarMa sequences were all detected in *R. bursa* ticks, Strandja-BG2012-GII, tick phlebovirus ET26 and Bole tick sequences were found in ticks of the genus *Hyalomma*, Strandja-BG2012-GIV and Blacklegged tick phlebovirus in ticks of the genus *Ixodes*, as well as Strandja-BG2012-GV, Mukawa virus and Glabeek, Osterholz sequences in *Ixodes* ticks. Of note, Strandja-BG2012-GII sequences were additionally detected in *H. aegyptium* and *R. sanguineus* (s.l.) ticks. If the virus is able to infect multiple tick genera needs further investigation.

The only tick-borne phlebovirus known to occur in Bulgaria is Bhanja virus (Hubálek, 2009; Pavlov et al., 1978). Bhanja virus is mainly pathogenic to livestock but a few cases of mild febrile illness in humans were reported (Calisher and Goodpasture, 1975). No sequence clustering with the Banja group was detected in this study.

We were not able to isolate any of the detected viruses in cell culture. But interestingly, viral genome copies were detectable in tick cell culture supernatants for up to 42 dpi in contrast to vertebrate cells suggesting that they were either more stable in the supernatant of these cells or limited viral replication took place for a short time period. As tick cells have a very slow growth rate, infectious supernatant was not passaged onto fresh cells but infected cells were incubated for the entire time period of the experiment. This could at least to some extent explain the differences between tick and vertebrate cell cultures. Other explanations for the transient maintenance of viral genome copies in tick cells could be that the detected viruses are adapted to ticks and cannot infect vertebrate cells. However, specific growth factors might be necessary for a successful isolation of the viruses in cell culture. No isolate of the related viruses is available as well preventing further studies on host-range, pathogenicity and serology. Several sandfly-transmitted phleboviruses are associated with disease in humans, some of them also occuring in Europe, like Sandfly Fever Naples virus and Sandfly fever Sicilian virus causing febrile illness, or Toscana virus causing meningitis and meningoencephalitis (Ayhan and Charrel, 2017). In contrast, the number of tick-borne phleboviruses pathogenic for humans is limited to the SFTSV group, containing only two viruses, *Heartland banyangvirus* and *Severe fever with thrombocytopenia syndrome virus*. Both viruses are associated with severe disease in humans, causing symptoms like leukocytopenia, thrombocytopenia and hemorrhagic symptoms with high fatality rates of up to 30% (McMullan et al., 2012; Yu et al., 2011). The diversity of phleboviral sequences in ticks has expanded rapidly in recent years. However, phenotypic virus characterization studies based on infectious virus isolates have been neglected. It thus remains to be studied if any of the newly discovered tick-associated phleboviruses can infect humans and/or animals. Also, complete genome sequencing and analyses would facilitate the development of diagnostic tests to assess the potential public health impact of these viruses.

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