Molecular epidemiology of Powassan virus in North America

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Powassan virus (POW) is a tick-borne flavivirus distributed in Canada, the northern USA and the Primorsky region of Russia. POW is the only tick-borne flavivirus endemic to the western hemisphere, where it is transmitted mainly between *Ixodes cookei* and groundhogs (*Marmota monax*). Deer tick virus (DTV), a genotype of POW that has been frequently isolated from deer ticks (*Ixodes scapularis*), appears to be maintained in an enzootic cycle between these ticks and white-footed mice (*Peromyscus leucopus*). DTV has been isolated from ticks in several regions of North America, including the upper Midwest and the eastern seaboard. The incidence of human disease due to POW is apparently increasing. Previous analysis of tick-borne flaviviruses endemic to North America have been limited to relatively short genome fragments. We therefore assessed the evolutionary dynamics of POW using newly generated complete and partial genome sequences. Maximum-likelihood and Bayesian phylogenetic inferences showed two well-supported, reciprocally monophyletic lineages corresponding to POW and DTV. Bayesian skyline plots based on year-of-sampling data indicated no significant population size change for either virus lineage. Statistical model-based selection analyses showed evidence of purifying selection in both lineages. Positive selection was detected in NS-5 sequences for both lineages and envelope sequences for POW. Our findings confirm that POW and DTV sequences are relatively stable over time, which suggests strong evolutionary constraint, and support field observations that suggest that tick-borne flavivirus populations are extremely stable in enzootic foci.

INTRODUCTION

Powassan virus (POW; family *Flaviviridae*, genus *Flavivirus*) is the sole North-American representative of the tick-borne encephalitis serological complex of the flaviviruses. POW comprises two genetic lineages including the prototype lineage (POW, lineage I) and a second lineage that was first described in the late 1990s [deer tick virus (DTV), lineage II] (Telford *et al.*, 1997). In nature POW and DTV appear to perpetuate in relatively distinct transmission cycles, with the former maintained by *Ixodes cookei* and/or *Ixodes marxi* and medium-sized mammals, and the latter maintained by *Ixodes scapularis* and white-footed mice (Artsob, 1988; Ebel *et al.*, 2000). Whereas POW has been considered to be a minor public-health concern due to the relative host-specificity of its arthropod vector, DTV could be a greater threat since it is mainly associated with the tick vectors that have driven the ongoing epidemics of Lyme disease, human babesiosis and human granulocytic anaplasmosis (Rodgers & Mather, 2007; Daniels *et al.*, 1998; Falco *et al.*, 1995). DTV has been

isolated from ticks in several regions of North America (Brackney *et al.*, 2008; Ebel *et al.*, 1999; Telford *et al.*, 1997). It is considered a distinct lineage of POW because of high nucleotide and amino acid sequence identity (84 and 93 %, respectively) (Kuno *et al.*, 2001) and strong serological cross-reactivity (Beasley *et al.*, 2001). POW has been isolated from ticks and/or seropositive animals in Canada, the USA and the Primorsky region of Russia (Artsob, 1988; Leonova *et al.*, 2009). Although data from field-based studies of POW and DTV are scant, two recent reports demonstrated that infection rates in ticks in a DTV focus in northern Wisconsin were remarkably stable over a 10-year period (Brackney *et al.*, 2008; Ebel *et al.*, 1999), and that infections within ticks were surprisingly genetically homogeneous, exhibiting little or no quasispecies structure (Brackney *et al.*, 2010). Therefore, POW and DTV appear to be similar to many other tick-borne agents in that they are nidal infections that are maintained in highly stable enzootic foci for long periods of time. Paradoxically, human disease due to infection by POW and/or DTV appears to be increasing. Human cases have increased markedly between the periods 1958–1998 and 1999–2007 (Hinten *et al.*, 2008), and a recent fatality was unequivocally

attributed to DTV (Tavakoli *et al.*, 2009). These observations raise the possibility that these agents may be an emerging burden to public health in regions of North America where they are enzootic. Accordingly, we sought to determine whether the apparent emergence of tick-borne flavivirus disease in North America may be driven by increases in the virus population size, reasoning that the well-documented expansion in the population of deer ticks that has occurred in recent decades may have consequently increased the population size, or sizes, of their pathogens. In particular, full genome sequences were determined and the phylogenetic relationships of POW and DTV from North America and Russia were reconstructed. We then estimated the divergence time between lineages and tested for evidence of positive selection. Finally, population dynamics were inferred under a Bayesian framework, thus shedding light on the mechanisms influencing the epidemiology of POW in North America.

RESULTS

Phylogenetic analyses

To assess the phylogenetic relationships of POW, we used 11 full-genome, 23 envelope and 22 NS-5 viral sequences. Kyasanur forest disease virus (KFDV) and Alkhurma virus were included as out-groups. Maximum-likelihood topologies were congruent in full-genome, NS-5 and envelope partitions in recovering two supported monophyletic groups that correspond to lineage I POW and DTV (lineage II POW) (Fig. 1a, b). Full genome topology showed that, within lineage I POW, samples were subdivided into Russia–Ontario [maximum-likelihood bootstrap (MLB)=100] and the sample from New York was basal. DTV showed two subclades clustering all samples from Wisconsin (MLB=99) and Massachusetts with Connecticut (MLB=100). Envelope and NS-5 topologies supported these divisions, with DTV from Colorado being basal to the Wisconsin strains, and the isolate of DTV from West Virginia basal to the New England strains.

Substitution rates, divergence times and population size changes

Bayesian skyline plots (BSPs) were generated for envelope (Fig. 2a) and NS-5-encoding sequences (Fig. 2b), using relaxed (uncorrelated log normal) molecular-clock models. Overall, results indicate that the effective number of POW and DTV infections through time was stable over the distant past (years 1100–1950) with recent declines, apparently beginning around 1950. BSPs for envelope and NS-5 partitions were congruent in placing the time to the most recent common ancestor (MRCA) for DTV and POW at around 1000 years before the present (ybp) (Fig. 2). Estimated nucleotide substitution rates were 2.2×10^{-4} substitutions site^{-1} year^{-1} using envelope sequences and 3.9×10^{-5} substitutions site^{-1} year^{-1} using NS-5 sequences.
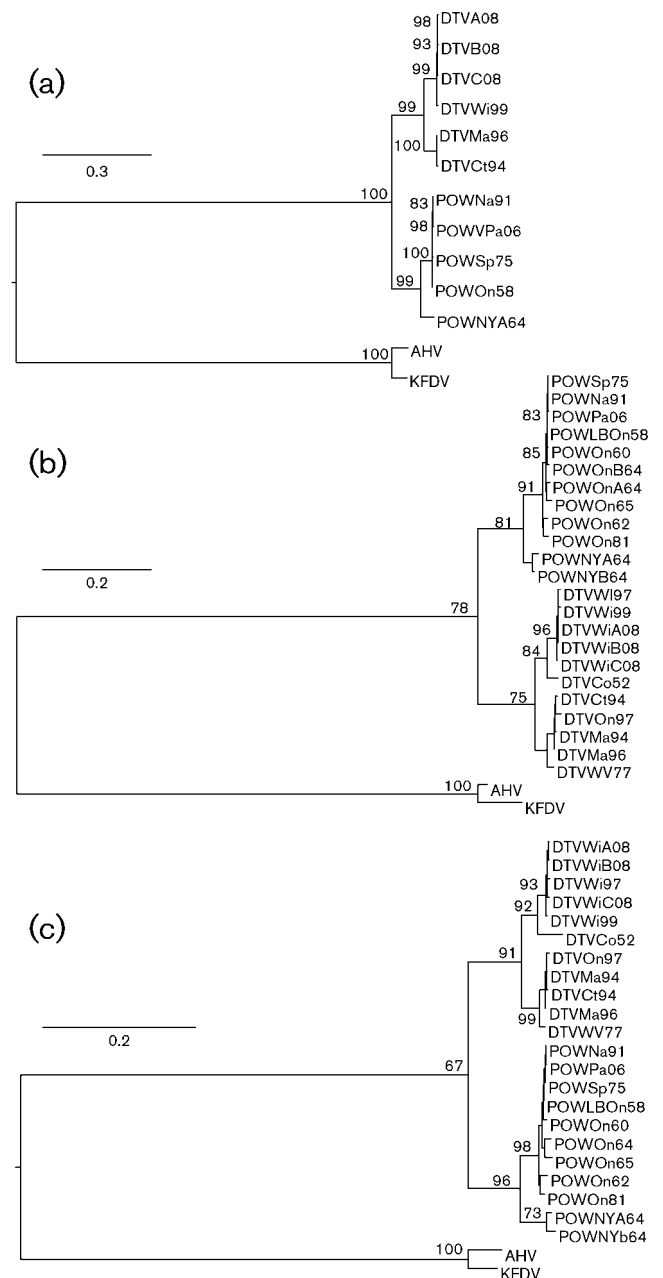

Fig. 1. Maximum-likelihood tree obtained from full coding sequences (10 245 nt) (a), envelope sequences (1491 nt) (b) and NS-5 partial sequences (2709 nt) (c) of POW and DTV strains. Numbers above branches indicate pseudoreplicate bootstrap values.

Using concatenated envelope and NS-5 sequences, we estimated divergence times of POW and DTV lineages under a Bayesian–Markov chain Monte Carlo (BMCMC) framework (Fig. 3). The time to the MRCA of DTV isolates was estimated at 195 ybp [highest posterior density (HPD)=73–382] and for lineage I POW, at 196 ybp (HPD=67–382); the analysis placed the time for the MRCA between the two lineages at approximately 484 ybp (HPD=114–1048).
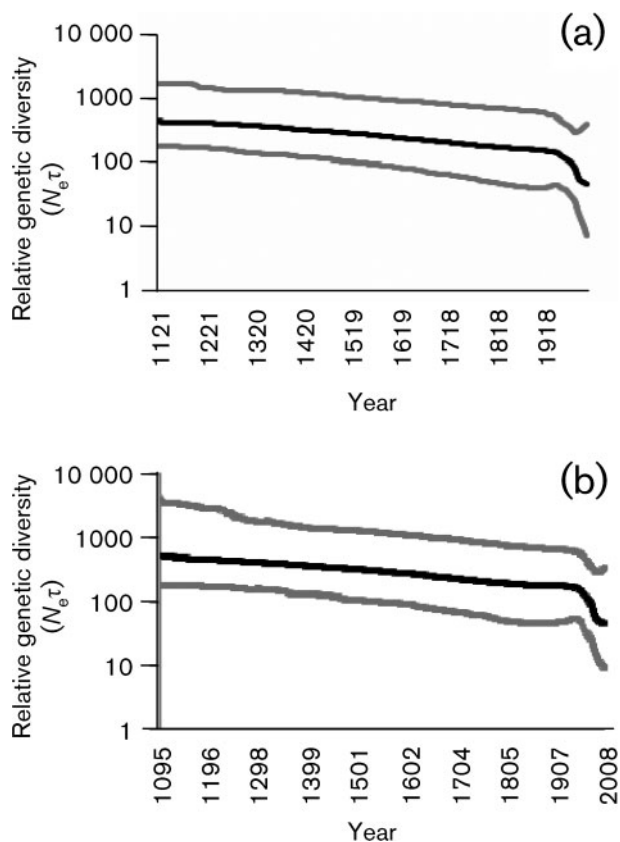
Fig. 2. Population dynamics of POW and DTV depicted using BSPs derived from envelope-encoding sequences (a) and NS-5-encoding sequences (b). The plots show changes in relative genetic diversity, depicted as the effective number of infections ($N_e\tau$; N_e is the effective population size and τ the generation time from infected host to infected host) through time. The thick, solid line is the median estimate of $N_e\tau$, while the 95 % HPD intervals are shown in grey.

Selection analyses in POW strains

Sliding window analysis of dN/dS and π, conducted with complete genomic coding sequences of both POW and DTV, indicated purifying selection throughout the genome,
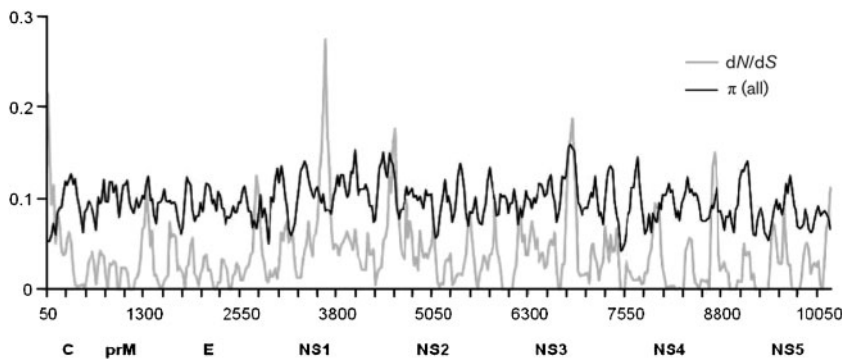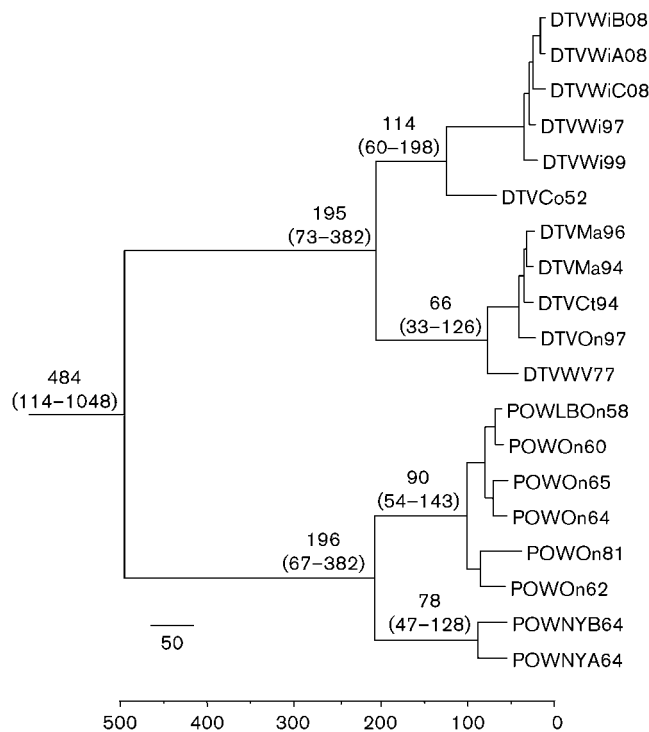

Fig. 3. Maximum clade credibility tree generated from concatenated envelope and NS-5 sequences (4200 nt) of POW and DTV strains. The median node ages and their 95 % HPD values are shown on the major nodes, with the 95 % HPD values shown in parentheses.

with values distributed below 0.3 for dN/dS throughout (Fig. 4). Likelihood-based approaches mostly agreed, showing that most substitutions were silent and probably reflect strong purifying selection acting over the POW genome. The single-likelihood ancestor counting (SLAC), random effects likelihood (REL) and partitioning approach for robust inference of selection (PARRIS) models detected purifying selection throughout the sequences, while the fixed effects likelihood (FEL) model found evidence for positive selection at two different sites in two different NS-5 partitions (one in POW lineage I only, the other evident

Fig. 4. Estimates of dN/dS and genetic diversity (π) across POW and DTV full-genome sequences.

Table 1. Results from selection analyses

Models to test for selection were conducted on the DATAMONKEY and SELECTON servers as indicated. CDS, Coding sequence; NS, not significant.

Partition	DATAMONKEY				SELECTON			M–K test
	SLAC	FEL	REL	PARRIS	MEC (vs M8a)	M8 (vs M8a)	M7	Neutrality index (*P*-value)
NS5 POW	Purifying	Positive (735)	Purifying	No +	Significant	NS	No +	
NS5 DTV	Purifying	Purifying	Purifying	No +	NS	Significant	No +	
NS5 All	Purifying	Positive (383)	Purifying	No +	Significant	NS	No +	2.089 (0.00107)
ENV POW	Purifying	Purifying	Purifying	No +	Significant	NS	No +	
ENV DTV	Purifying	Purifying	Purifying	No +	NS	NS	No +	
ENV All	Purifying	Purifying	Purifying	No +	Significant	NS	No +	1.820 (0.0753)
POW CDS	Purifying	Purifying	Purifying	No +	NS	NS	No +	
DTV CDS	Purifying	Purifying	Purifying	No +	NS	NS	No +	
CDS All	Purifying	Purifying	Purifying	No +	NS	No +	No +	0.753 (0.03539)

in comparisons of the two lineages) (Table 1). Using the SELECTON server (http://selecton.tau.ac.il) we found evidence for positive selection in the envelope and NS-5 sequences using the MEC model and in the DTV NS-5 sequences using the M8 model (Table 1). McDonald–Kreitman (M–K) tests showed significant values for partitions of full-genome sequences ($P=0.035$) and NS-5 sequences ($P=0.001$), but did not conclusively show significant differences from neutral evolution for envelope sequences ($P=0.077$) (Table 1).

DISCUSSION

Our phylogenetic analyses using both partial and whole-genome sequences found support for two distinct lineages of POW, similar to previous findings by Ebel *et al.* (2001) and Kuno *et al.* (2001). The clustering of Russian samples with the prototypic 1958 LB strain from Ontario supports assertions that a strain similar to this was recently imported into Russia, most probably in the past 100 years, potentially from importation of mink for fur farms in the area (Leonova *et al.* 2009). Envelope and NS-5 topologies showed DTV from Colorado basal to the Wisconsin strains and the isolate of DTV from West Virginia basal to the New England strains. Isolates taken from overlapping geographical areas in both Ontario and New England were placed into the two separate lineages (POW and DTV), suggesting that these two partially sympatric lineages exploit different ecological niches, and thus supporting the association of each lineage with a distinct transmission cycle (Ebel *et al.*, 1999).

BSPs, which measure population dynamics based on genetic diversity, show a long, stable history for both POW and DTV, with some evidence of recent declines in these populations. These results support field data that suggest that a stable, persistent virus population is present in the upper Midwest USA (Brackney *et al.*, 2008; Ebel *et al.*, 2000). Although the BSPs show a recent decline in populations of POW, an increase in human cases of

POW encephalitis has been reported over the past 10 years (Hinten *et al.*, 2008). This may be the result of more human contact with areas where the virus is present (Lloyd-Smith *et al.*, 2009). In addition or alternatively, there could be an enhanced awareness of arthropod-borne flaviviruses due to the introduction of West Nile virus (WNV) to the USA in 1999, which lead to increased testing and better diagnosis (Hinten *et al.*, 2008). Genetic diversity of flaviviruses is very low within tick hosts (Brackney *et al.*, 2010), especially compared with mosquito-borne flaviviruses, where intrahost populations have greater genetic diversity (Jerzak *et al.*, 2005). The lack of diversity within POW populations may limit the number of unique genomes available and consequently the adaptability of POW to new ecological niches. Ultimately, this could produce the patterns of strong genetic conservation and population stability that we document in this study. Other factors that may influence population size, as estimated by genetic diversity, could be: the low levels of replication achieved in ticks, resulting in smaller populations generated at this stage of the life cycle; transstadial or transovarial transmission that may occur as part of the viral life cycle, providing a bottleneck for virus transmission; and the potential for co-feeding transmission, which would separate the need for replication in vertebrate hosts from the population dynamics of the virus overall (Ebel & Kramer, 2004; Nuttall *et al.*, 1994). Finally, our sampling effort might not be sufficient to detect very recent (i.e. in the past 30 years) changes in virus population size that would be associated with the well-documented expansion in the populations of their main vector, *I. scapularis*. In sum, our results are concordant with previously published data which suggest that populations of POW (and DTV), like tick-borne encephalitis and other tick-borne pathogens, are very stable in nature (Blaskovic & Nosek, 1972; Goethert & Telford, 2009; Gresikova & Calisher, 1989).

Estimated nucleotide substitution rates ranged from 2×10^{-4} to 4×10^{-5} substitutions site^{-1} year^{-1}, with NS-5-derived estimates approximately one $\log_{10}$ lower than

those derived from envelope sequences. Nucleotide substitution estimates for the segmented negative-sense RNA virus transmitted by ticks, Crimean–Congo haemorrhagic fever virus (family *Bunyaviridae*, genus *Nairovirus*), found a rate of 5.8×10^{-5} for the L segment, which encodes the RNA-dependent RNA polymerase, and 1.09×10^{-4} for the S segment, which encodes the structural nucleocapsid protein, although the authors concluded this could be a result of greater taxon sampling for the S segment, which is not true of our dataset (Carroll *et al.*, 2010). Nucleotide substitution rates for other vector-borne flaviviruses, such as KFDV and tick-borne encephalitis, tick-borne flaviviruses related to POW, or the mosquito-borne flaviviruses yellow fever virus, St Louis encephalitis virus, and WNV fall within this range (Baillie *et al.*, 2008; Bryant *et al.*, 2007; Mehla *et al.*, 2009; Snapinn *et al.*, 2007). The nucleotide substitution rates estimated for POW and DTV are lower than the typical estimate of approximately 10^{-3} substitutions site^{-1} year^{-1} found for RNA viruses (Jenkins *et al.* 2002). However, vector-borne RNA viruses have been found, overall, to display lower mutation rates than other RNA viruses, probably because of the requirement for replication in two distinct cell types (vertebrate and invertebrate), which may lead to stronger evolutionary constraint and lower overall accumulation of mutant types (Jenkins *et al.*, 2002). Although the mutation rates are lower than expected for RNA viruses, they are 20-fold higher than those found in a recent study of POW using only the Russian and LB sequence data (Leonova *et al.*, 2009). This difference may be due to a restricted focus of Russian transmission, or because the strain of POW in Russia is closely related to the LB strain because it was only recently introduced there from North America, thus leading to lower genetic diversity in those samples (Leonova *et al.*, 2009). The apparently non-native Russian focus may prove a useful laboratory, over time, for studying the actual rate of divergence in a new viral focus in nature, and provide an interesting contrast to literature concerning the similar importation of mosquito-borne WNV into North America (Ebel & Kramer, 2009; Beasley *et al.*, 2003; Bertolotti *et al.*, 2007). In any case, our data show that nucleotide substitution rates for POW are similar to those of other flaviviruses and are variable along the length of the genome.

Divergence times were estimated to assess the likelihood that DTV and POW diverged recently as a result of expanding deer-tick populations. Using the BMCMC approach and concatenated complete NS-5 and envelope sequences (approximately 4000 nt total), we estimated the MRCA for POW from DTV at approximately 484 ybp (95 %, HPD=114–1048). This estimate agrees overall with previous studies based on an approximately 2000 nt sequence comprising portions of envelope, NS-3 and NS-5 sequences (Charrel *et al.*, 2005). Previously, greater genetic diversity was found within the DTV lineage, leading researchers to speculate that this may be the older lineage (Ebel *et al.*, 2001). Our divergence estimates show a nearly

simultaneous radiation of DTV and POW approximately 200 years ago, with no indication that one clade is ancestral to the other. Divergence of DTV and POW may have occurred well before the current expansion of deer-tick populations, and must have been driven by other factors.

We observed strong purifying selection, and some evidence of positive selection, in the sequences we studied. An indicator of positive selection has previously been detected in partial POW-envelope alignments, but not in NS-5 alignments, using an M–K test (Ebel *et al.*, 2001). We found significant signals for positive selection occurring in full-genome sequences and NS-5-encoding sequences using M–K tests, and approaching significance for envelope-encoding sequences. The likelihood models we employed were also able to identify positive selection in certain sites. However, we were unable to consistently detect specific codons that appeared to be subject to positive selection using multiple programs. All methods that were capable of doing so detected purifying selection at multiple sites. Our results indicate that most substitutions in POW and DTV genomes were synonymous, indicating that strong purifying selection limits the accumulation of genetic changes in POW virus populations. Similar results have been found in other arthropod-borne flaviviruses (Baillie *et al.*, 2008). Despite some incongruence in the methods identifying positive selection, we suggest that this process is acting on POW and DTV genomes.

METHODS

Virus strains. RNA samples were generated from three isolates of ticks collected in Wisconsin in 2008 (Brackney *et al.*, 2008), one isolate from the Nantucket field station in 2000 (Ebel *et al.*, 2000), wicf9901 from Wisconsin in 1999 (Ebel *et al.*, 1999) and 64-7062 in 1964 (Whitney & Jamnback, 1965) (Table 2).

RNA extraction, RT-PCR and sequencing. RNA was extracted using an RNeasy Protect Mini-kit (Qiagen), according to the manufacturer's protocol, from 100 µl viral stock and eluted into 100 µl double-distilled H₂O. RT-PCR was conducted using Superscript III One-Step RT-PCR with Platinum *Taq* (Life Technologies) using the following conditions: 55 °C for 30 min (reverse transcription), 95 °C for 15 min (initial denaturation) and 40 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Primers amplifying approximately 1 kb segments were used (sequences available on request). PCR products were purified using StrataPrep PCR purification kits (Stratagene), and sequenced with the primers used for RT-PCR, yielding greater than twofold coverage, by the University of New Mexico DNA Research Services. Sequences were assembled using SeqMan (DNASTAR), and aligned with CLUSTAL W in BioEdit (Hall, 1999). The full genome sequences from the POW and DTV strains sequenced for this study have been deposited in GenBank and have been assigned accession numbers HM440558–HM440563. Other previously published sequences used in the study were obtained from GenBank. Sequences were tested for evidence of recombination using GARD (genetic algorithm for recombination assessment) which is available at http://datamonkey.org.

Phylogenetic analysis. Maximum-likelihood trees were generated in PAUP* version 4 (Swofford, 2002), considering all characters as

Table 2. Isolates of POW used in this study

All locations are in the USA unless indicated otherwise.

Code	Strain	Collection location	Year	Source	Genbank accession no.
DTVWiA08		Spooner, WI	2008	*I. scapularis*	HM440560
DTVWiB08		Spooner, WI	2008	*I. scapularis*	HM440561
DTVWiC08		Spooner, WI	2008	*I. scapularis*	HM440562
POWPa06	Part/2006	Partizansk, Russia	2006	Human	EU543649
DTVWi99	wicf9901	Chippewa Falls, WI	1999	*I. scapularis*	HM440558
DTVWi97	DTV-SPO	Spooner, WI	1997	*I. scapularis*	AF310921, AF310938
DTVMa96	NFS001	Nantucket, MA	1996	*I. scapularis*	AF310918, AF310947, HM440559
DTVCt94	DTV-CT	Connecticut	1994	*I. scapularis*	AF310919, AF311056
DTVMa94	DTV-IPS	Ipswich, MA	1994	*I. scapularis*	AF310947, AF310918
POWNad91	Nad-1991	Nadezdinsk, Russia	1991	Human	EU670438
POWOn81	t18-23-81	Ontario, Canada	1981	*I. cookei* on *Marmota* sp.	AF310909, AF310943
DTVWV77	12542	West Virginia	1977	*Vulpes* sp.	AF310920, AF310949
POWSp75	Sp-9	Spassk, Russia	1975	*Dermacentor silvarum*	EU770575
POWOn65	m11665	Ontario, Canada	1965	Tick	AF310910, AF310937
POWNYA64	64-7062	New York	1964	Ticks on *Marmota* sp.	AF310915, AF310944, HM440563
POWNYB64	64-7483	New York	1964	Unknown	AF310916, AF310945
POWOn64A	M8998	Ontario, Canada	1964	Unknown	AF310911, no NS-5
POWOn64B	1982-64	Ontario, Canada	1964	*Marmota monax*	AF310913, AF310939
POWOn62	1247-62	Ontario, Canada	1962	*Tamiasciurus hudsonicus*	AF310914, AF310942
POWOn60s	m1409	Ontario, Canada	1960s	Tick	AF310912, AF310940
POWOn58	LB	Powassan, Ontario, Canada	1958	Human brain	NC003687
DTVCo52	791A-52	Colorado	1952	*Dermacentor andersonii*	AF310922, AF310950

unordered with four possible states (A, C, T, G). We employed heuristic searches with ten random stepwise additions of sequences and tree bisection–reconnection branch swapping. Node support was evaluated with 1000 non-parametric bootstrap pseudoreplicates (Felsenstein, 1985), using the search conditions described above. Analysis was performed with 11 full-genome sequences.

Divergence times, substitution rates and population size changes. Bayesian phylogenetic analyses were performed to investigate the timescale of diversification of POW and DTV lineages using BEAST version 1.5.1 (Drummond & Rambaut, 2007). For these analyses, 24 sequences or partial sequences including ENV and NS-5 (4000 nt) were included. To assess the extent of temporal structure in the sequence data, we performed a regression analysis of tree root-to-tip genetic distance against sampling date using the program Path-O-Gen, (http://tree.bio.ed.ac.uk/software/pathogen/) based on a neighbour-joining tree. Regression analysis showed sufficient temporal structure in the viral sequences of this study (having the range 1958–2008) to estimate rates and dates. Analyses were performed by comparing uniform rates across branches (strict clock) and uncorrelated relaxed-clock assumptions. We used the uncorrelated log-normal assumption after comparing the marginal posterior distribution with the prior distribution of the standard deviation of the uncorrelated log-normal distribution (Suchard *et al.*, 2001).

The Hasegawa, Kishino and Yano model of nucleotide substitution was used with γ-rate heterogeneity among sites as selected by comparison of Akaike information criterion scores. Two independent MCMC chains were run for 50 000 000 steps with sampling every 5000 steps, following a discarded burn-in of 5 000 000 steps of the posterior samples. The samples from the two runs were combined, and the convergence of the chain, sampling and mixing was confirmed by inspection of the MCMC samples using the program TRACER version 1.5 (http://beast.bio.ed.ac.uk/Tracer).

Selection and recombination detection. Alignments of POW sequences were tested for possible breakpoints using GARD (Kosakovsky Pond *et al.*, 2006), which is available at http://datamonkey.org (Kosakovsky Pond & Frost, 2005a). Models of nucleotide substitution were calculated using MODELTEST (Posada & Crandall, 1998). Non-synonymous to synonymous rate ratios were estimated in HyPhy (www.hyphy.org), using the maximum-likelihood phylogeny and the general time-reversible model of nucleotide substitution. Tests for positive selection were conducted on the DATAMONKEY server using the following methods: SLAC, FEL, REL and PARRIS. SLAC counts the number of non-synonymous changes per non-synonymous site (dN) and tests whether it is significantly different from the number of synonymous changes per synonymous site (dS). FEL and REL estimate ratios of non-synonymous to synonymous changes for each site in an alignment, but FEL fixes estimates of branch lengths and substitution rate parameters for each site independently, whereas REL allows rate variation in both synonymous and non-synonymous rates and the nucleotide substitution model (Kosakovsky Pond & Frost, 2005b). PARRIS is able to take both recombination and differences in synonymous evolution rate into account (Scheffler *et al.*, 2006). Additionally, we tested for selection using three models from the SELECTON server: MEC, M7 + β and M8. DNAsp (Librado & Rozas, 2009) was used to calculate M–K tests, the ratio of the mean number of non-synonymous (dN) to synonymous (dS) nucleotide substitutions (dN/dS) and the genetic diversity (π). Analyses were performed using full coding sequences.

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