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Multiple transmissions of tick-borne encephalitis virus between Japan and Russia

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Tick-borne encephalitis (TBE) is a zoonotic disease causing meningitis, encephalitis, and meningoencephalitis. Tick-borne encephalitis virus (TBEV) is an etiological agent of TBE. From an analysis of five distinct sequences of Japanese TBEV, it has been proposed that Japanese TBEV was transmitted from Russia to Japan on just a single occasion 260–430 years ago. Here thirteen distinct nucleotide sequences encoding the entire region of the envelope protein for Japanese TBEV were analyzed. It is shown, from the phylogenetic analysis, that Japanese TBEV belongs to the Far Eastern subtype, which is known to be highly pathogenic. Japanese TBEV was divided into three groups, and TBEV was inferred to have been transmitted between Japan and Russia at least three times, which were estimated to have occurred several hundred years ago. These results indicate that TBEV has not only been endemic but also transmitted multiple times to Japan.

Key words: tick-borne encephalitis virus, transmission, evolutionary rate, divergence time

INTRODUCTION

Tick-borne encephalitis (TBE), which was first recognized in 1931 (Schneider, 1931), is a zoonotic disease causing meningitis, encephalitis, and meningoencephalitis. TBE is endemic to the geographical areas ranging from Europe through Siberia to the Far East, and has been called Central European encephalitis (CEE) in Europe and Russian spring-summer encephalitis (RSSE) in the Far East.

Tick-borne encephalitis virus (TBEV), which was first isolated in 1937 (Zilber and Soloviev, 1946), is an etiological agent of TBE. TBEV is a member of the genus *Flavivirus* in the family *Flaviviridae*. The virion of TBEV is about 50 nm in diameter and enveloped. The genome of TBEV is a non-segmented, single-stranded, and positive-sense RNA of about 11,000 nucleotides (nt), which consists of a 5'-untranslated region (UTR) (about 130 nt), a 3'-UTR (400–700 nt), and an open reading frame encoding a polyprotein of about 3400 amino acids (aa). The polyprotein is co- and post-translationally cleaved into capsid (C, 112 aa), membrane precursor (prM, 168 aa) (prM is further processed into membrane [M, 75 aa]), envelope (E, 496 aa), non-structural 1 (NS1, 352 aa), NS2A (230 aa), NS2B (131 aa), NS3 (621 aa), NS4A (149 aa), NS4B (252 aa), and NS5 (903 aa) proteins by cellular and viral proteases (Mandl et al., 1989).

From a phylogenetic analysis, TBEV has been classified into three subtypes; the European subtype, which causes CEE, the Far Eastern subtype, which causes RSSE, and the Siberian subtype (Ecker et al., 1999). The Far Eastern and Siberian subtypes are more closely related to each other than to the European one (Grard et al., 2007). Rodents are reservoirs of TBEV. A tick species, *Ixodes ricinus*, which is distributed across Europe and the Middle East, is the main vector for the European subtype, and *I. persulcatus*, which is distributed from Eastern Europe to Japan, is the main vector for the Far Eastern and Siberian ones. TBEV is transferred between ticks by co-feeding, where infected and non-infected ticks feed simultaneously in close proximity on the same individual rodent and TBEV replicated at the skin around the former tick is transferred to the latter tick through migratory cells (Labuda et al., 1993). The life cycle of ticks is 2–5 years, during which they feed only three times and the infectivity and virus titre of TBEV is maintained at a low level. Humans are infected by TBEV through a bite of an infected tick or the consumption of infected goat's milk. Annually, about 3000 people in Europe and about 11,000 people in Russia are infected with TBEV (Gritsun et al., 2003). The case fatality rate is 1–2% for the patients infected with the European and Siberian subtypes of TBEV and about 30% for those infected with the

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Far Eastern one (Ecker et al., 1999). Purified, concentrated, and formaldehyde-inactivated whole virion vaccines are available for prophylaxis against TBEV, although they are not licensed in Japan (Barrett et al., 2004).

In Japan, TBEV was first isolated in 1993 (Takashima et al., 1997). From a phylogenetic analysis including five distinct sequences of Japanese TBEV, it was found that Japanese TBEV made a single cluster, which was closest to the Far Eastern subtype (Hayasaka et al., 1999). The rate of synonymous substitution was estimated to be (2.9–4.9) $\times$ 10^{-4} per site per year (Hayasaka et al., 1999; Jenkins et al., 2002; Hanada et al., 2004). From these observations, it has been proposed that Japanese TBEV belonged to the Far Eastern subtype and was transmitted from Russia to Japan on just a single occasion, which was estimated to have occurred 260–430 years ago (Hayasaka et al., 1999). The divergence time of the Far Eastern and Siberian subtypes was also estimated to be 1700–2100 years ago.

Table 1. Sequence data used in the present study

Strain	Geographical origin	Source	Year of isolation	Subtype	INSD accession number	Reference
Miz416/97	Hokkaido, Japan	*Clethrionomys rufocanus*	1997	Far Eastern	AB237184	N.A.[a]
Kam586/97	Hokkaido, Japan	*C. rufocanus*	1997	Far Eastern	AB237185	N.A.
Kam588/97	Hokkaido, Japan	*C. rufocanus*	1997	Far Eastern	AB237186	N.A.
Kik629/97	Hokkaido, Japan	*C. rufocanus*	1997	Far Eastern	AB237187	N.A.
Miz660/97	Hokkaido, Japan	*C. rufocanus*	1997	Far Eastern	AB237188	N.A.
Oh696/97	Hokkaido, Japan	*C. rufocanus*	1997	Far Eastern	AB237189	N.A.
Oh701/97	Hokkaido, Japan	*C. rufocanus*	1997	Far Eastern	AB237191	N.A.
Kita987/99	Hokkaido, Japan	*C. rufocanus*	1999	Far Eastern	AB237192	N.A.
Senzhang	China	N.A.	1953	Far Eastern	AY174188	N.A.
Oshima 5-10	Hokkaido, Japan	Dog blood	1995	Far Eastern	AB001026	Hayasaka et al., 1999
Oshima 3-6	Hokkaido, Japan	Dog blood	1995	Far Eastern	AB022291	Hayasaka et al., 1999
Oshima I-1	Hokkaido, Japan	*Ixodes ovatus*	1996	Far Eastern	AB022292	Hayasaka et al., 1999
Oshima A-1	Hokkaido, Japan	*Apodemus speciosus*	1995	Far Eastern	AB022293	Hayasaka et al., 1999
Oshima C-1	Hokkaido, Japan	*C. rufocanus*	1996	Far Eastern	AB022294	Hayasaka et al., 1999
KH98-2	Khabarovsk, Russia	*I. persulcatus*	1998	Far Eastern	AB022295	Hayasaka et al., 1999
KH98-5	Khabarovsk, Russia	*I. persulcatus*	1998	Far Eastern	AB022296	Hayasaka et al., 1999
KH98-10	Khabarovsk, Russia	*I. persulcatus*	1998	Far Eastern	AB022297	Hayasaka et al., 1999
Sofjin-HO	Primorskii Kray, Russia	Human brain	1937	Far Eastern	AB022703	Hayasaka et al., 1999
VL99-m11	Vladivostok, Russia	*I. persulcatus*	1999	Far Eastern	AB049345	Hayasaka et al., 2001
KH99-m9	Khabarovsk, Russia	*I. persulcatus*	1999	Far Eastern	AB049346	Hayasaka et al., 2001
D1283	Khabarovsk, Russia	Human brain	1998	Far Eastern	AB049347	Hayasaka et al., 2001
N132	Vladivostok, Russia	*I. persulcatus*	1979	Far Eastern	AF091013	Ecker et al., 1999
RK1424	Latvia	*I. persulcatus*	1977	Far Eastern	AF091016	Ecker et al., 1999
T-blood	Perm, Russia	Human blood	1939	Far Eastern	AF091019	Ecker et al., 1999
Aina	Irkutsk, Russia	Human cerebrospinal fluid	1963	Siberian	AF091006	Ecker et al., 1999
IR99-1m1	Irkutsk, Russia	*I. persulcatus*	1999	Siberian	AB049348	Hayasaka et al., 2001
IR99-1m4	Irkutsk, Russia	*I. persulcatus*	1999	Siberian	AB049349	Hayasaka et al., 2001
IR99-2m3	Irkutsk, Russia	*I. persulcatus*	1999	Siberian	AB049350	Hayasaka et al., 2001
IR99-2m7	Irkutsk, Russia	*I. persulcatus*	1999	Siberian	AB049351	Hayasaka et al., 2001
Vasilchenko	Novosibirsk, Russia	Human blood	1961	Siberian	AF069066	Gritsun and Gould, 1998
Zausaev	Siberia, Russia	Human brain	1985	Siberian	AF527415	Gritsun et al., 2003

[a]Not available.

The purpose of the present study was to conduct a phylogenetic analysis of TBEV to better understand the origin and evolution of Japanese TBEV. For this purpose, it is important to use as many Japanese TBEV as possible in the phylogenetic analysis. In the present study, the analysis was conducted using all sequences of Japanese TBEV available in the International Nucleotide Sequence Database (INSD).

MATERIALS AND METHODS

Sequence data Thirteen distinct nucleotide sequences of Japanese TBEV, five of which (Oshima 3-6, Oshima 5-10, Oshima A-1, Oshima C-1, and Oshima I-1) have been analyzed in a previous study (Hayasaka et al., 1999), were retrieved from the INSD (DNA Data Bank of Japan release 69). These sequences encoded the entire region of the E protein (1488 nt). The year of isolation for the viral strain was available for all of these sequences. To conduct a phylogenetic analysis of Japanese TBEV, sequences of the Far Eastern subtype were retrieved from the INSD, because Japanese TBEV was likely to belong to the Far Eastern subtype. Sequences of the Siberian subtype were also retrieved to be used as an outgroup. Only those sequences of the Far Eastern and Siberian subtypes for which the year of isolation for the viral strain was known were used for the analysis so that the evolutionary rate and divergence times could be estimated. The 31 sequences analyzed in the present study are listed in Table 1.

Data analysis Multiple alignment of the 31 sequences of Japanese TBEV and the Far Eastern and Siberian subtypes encoding the entire region of the E protein was made using the computer program CLUSTAL W (version 1.83) (Thompson et al., 1994) and contained no gaps. The phylogenetic tree for these sequences was constructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) with the p distance using all nucleotide sites (1488 nt). The p distance is known to be useful for constructing reliable phylogenetic trees when large numbers of closely related sequences are analyzed (Nei and Kumar, 2000; Takahashi and Nei, 2000). However, the one-parameter (Jukes and Cantor, 1969), two-parameter (Kimura, 1980), and TrN (Tamura and Nei, 1993) models were also used for estimating the evolutionary distances. When the pattern of nucleotide substitution for the entire coding region was examined by MODELTEST (Posada and Crandall, 1998), the symmetrical (SYM) model (Zharkikh, 1994) with Γ distribution for the rate heterogeneity among sites (Γ shape parameter [α] = 0.26) and the general time reversible (GTR) model (Rodriguez et al., 1990) with Γ distribution and invariable sites were found to be the fittest to the data using the hierarchical likelihood-ratio test (hLRT) and the Akaike information criterion (AIC) (Akaike, 1974), respectively. Therefore, the TrN model with Γ distribution (α = 0.26) was also used for estimating the evolutionary distances. Credibility for each interior branch of the phylogenetic tree was assessed by the bootstrap method with 1000 resamplings (Felsenstein, 1985). MEGA (version 3.1) (Kumar et al., 2004) was used for conducting these analyses.

The evolutionary rate of TBEV and the times at the interior nodes of the phylogenetic tree were estimated by using the molecular clock. Since the molecular clock usually holds for synonymous substitution (Nei and Kumar, 2000), the four-fold degenerate sites (229 sites) were extracted from the multiple alignment. The rate and the times were estimated simultaneously by the maximum likelihood (ML) method using TIPDATE (Rambaut, 2000), which is implemented in PAML (version 3.15) (Yang, 1997). The HKY (Hasegawa et al., 1985) model was assumed as the pattern of nucleotide substitution. To test whether the molecular clock held, the log-likelihood ($\ln L$) value was computed under the assumption of molecular clock or rate heterogeneity among branches. The null hypothesis of the molecular clock was tested by the LRT under the assumption that twice the difference in the $\ln L$ value ($2\Delta\ln L$) followed a χ^2 distribution with a degree of freedom of 28 (χ^2_{28}). In addition, to test whether the evolutionary rate was constant among sites, the $\ln L$ value was computed under the assumption that the rate was constant or followed a Γ distribution among sites. The null hypothesis of rate constancy among sites was tested by the LRT under the assumption that $2\Delta\ln L$ followed χ^2_1.

RESULTS

The phylogenetic tree constructed by the NJ method with the p distance for the 31 sequences of Japanese TBEV and the Far Eastern and Siberian subtypes is shown in Fig. 1. All of Japanese TBEV belonged to the Far Eastern subtype. Interestingly, Japanese TBEV was divided into three groups; Oshima 3-6, Oshima 5-10, Oshima A-1, Oshima C-1, Oshima I-1, Kik629/97, Miz416/97, Miz660/97, Oh696/97, and Oh701/97 made a single cluster, Kam586/97 and Kam588/97 made a single cluster, and Kita987/99 made a cluster with Russian (and Latvian) TBEV. All of these clusters were supported with 100% bootstrap probability. The same topology was obtained when the one-parameter, two-parameter, and TrN models were used instead of the p distance, except that the positions of KH98-2 and KH99-m2, which were associated with a small bootstrap probability (43%), were exchanged. According to the parsimony principle, the topology indicated that TBEV was transmitted between Japan and Russia at least three times, with five equally parsimonious scenarios; (1) the ancestor of the Far Eastern subtype existed in Russia, and TBEV was trans-

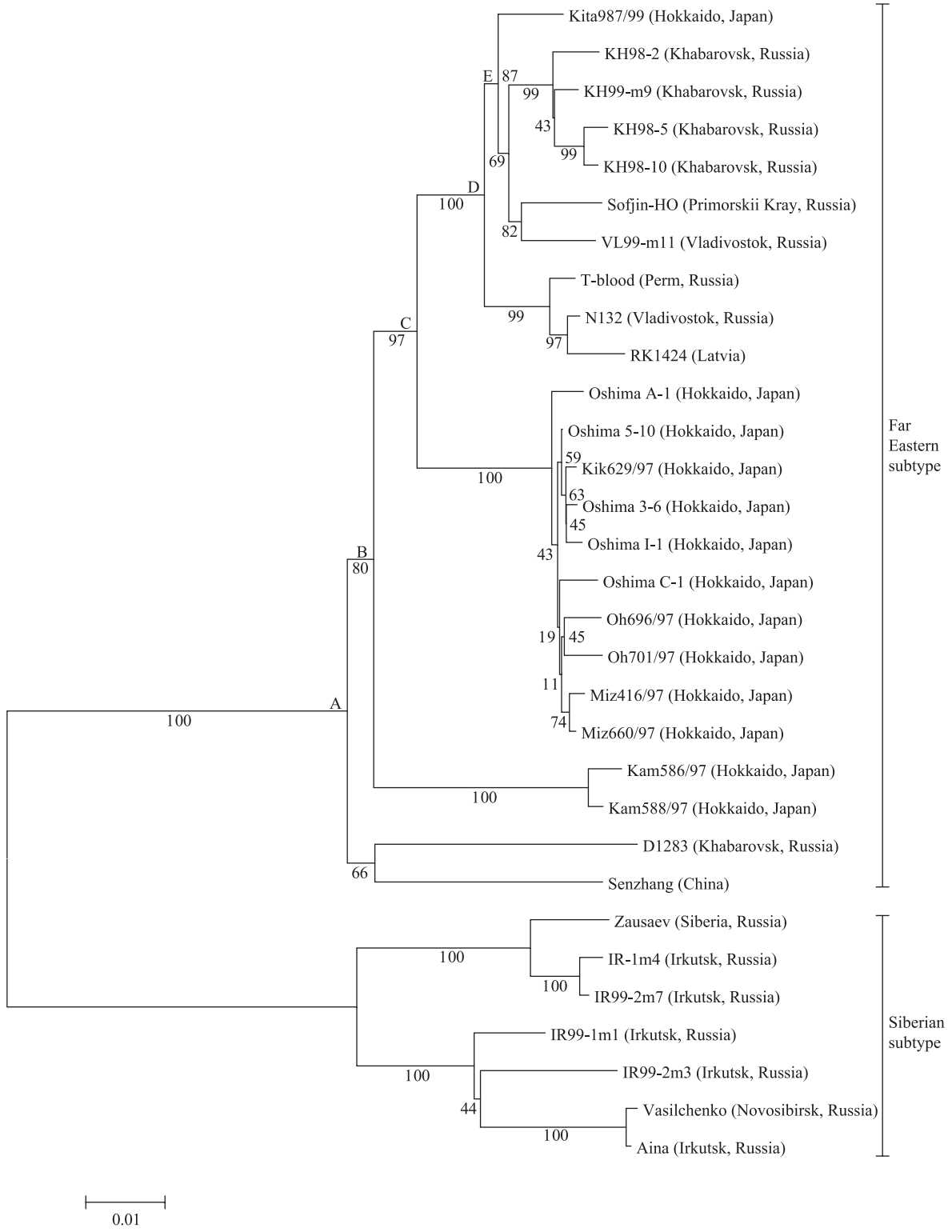

Fig. 1. Phylogenetic tree of the 31 sequences of Japanese TBEV and the Far Eastern and Siberian subtypes constructed by the NJ method with the p distance for all nucleotide sites of the E gene (1488 nt). The geographical origin for the viral strain is indicated in the parentheses after the strain name. The number attached to each interior branch indicates the bootstrap probability. A, B, C, D, and E are labels for the interior nodes. The scale bar indicates the number of nucleotide substitutions per site.

mitted from Russia to Japan at the interior nodes labeled as B, C, and E in Fig. 1, (2) the ancestor existed in Russia, and TBEV was transmitted from Russia to Japan at A, and from Japan to Russia at D and E, (3) the ancestor existed in Russia, and TBEV was transmitted from Russia to Japan at A, from Japan to Russia at C, and from Russia to Japan at E, (4) the ancestor existed in Japan, and TBEV was transmitted from Japan to Russia at A, D, and E, and (5) the ancestor existed in Japan, and TBEV was transmitted from Japan to Russia at A and C, and from Russia to Japan at E.

Although it is difficult to decide which of the five possible scenarios is the most likely, it is interesting to estimate the times at interior nodes A, B, C, D, and E and the divergence of the Far Eastern and Siberian subtypes. The rate of synonymous substitution and divergence

Table 2. Rate of synonymous substitution and divergence times estimated using the phylogenetic tree in Fig. 1

Model	α	ln*L*	Rate ($\times 10^{-4}$ per site per year) ± standard error	Divergence of the Far Eastern and Siberian subtypes (B.C.) ± standard error	A[a] (A.D.) ± standard error	B[a] (A.D.) ± standard error	C[a] (A.D.) ± standard error	D[a] (A.D.) ± standard error	E[a] (A.D.) ± standard error
HKY	N.A.[b]	−1640.23	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HKY + Γ	1.68	−1628.59	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HKY + clock	N.A.	−1658.19	1.72 ± 1.37	132 ± 1709	1251 ± 590	1363 ± 504	1508 ± 387	1718 ± 210	1759 ± 182
HKY + Γ + clock	1.73	−1647.01	1.62 ± 1.44	793 ± 2502	1149 ± 747	1299 ± 617	1455 ± 479	1698 ± 249	1734 ± 226
TrN	N.A.	−1631.97	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
TrN + Γ	1.93	−1622.68	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
TrN + clock	N.A.	−1650.08	1.76 ± 1.38	158 ± 1704	1259 ± 575	1376 ± 486	1514 ± 376	1722 ± 203	1764 ± 175
TrN + Γ + clock	1.98	−1641.14	1.68 ± 1.45	707 ± 2358	1175 ± 704	1324 ± 579	1470 ± 452	1706 ± 236	1744 ± 211
GTR	N.A.	−1623.93	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GTR + Γ	2.08	−1615.86	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GTR + clock	N.A.	−1641.56	1.74 ± 1.39	153 ± 1718	1255 ± 584	1368 ± 498	1510 ± 384	1720 ± 207	1762 ± 179
GTR + Γ + clock	2.16	−1633.89	1.65 ± 1.44	661 ± 2335	1171 ± 713	1317 ± 590	1465 ± 460	1704 ± 240	1742 ± 214

[a]Label for interior node in Fig. 1.
[b]Not applicable.

Table 3. Rate of synonymous substitution and divergence times estimated under the assumption that the positions of KH98-2 and KH99-m9 were exchanged in the phylogenetic tree in Fig. 1

Model	α	ln*L*	Rate ($\times 10^{-4}$ per site per year) ± standard error	Divergence of the Far Eastern and Siberian subtypes (B.C.) ± standard error	A[a] (A.D.) ± standard error	B[a] (A.D.) ± standard error	C[a] (A.D.) ± standard error	D[a] (A.D.) ± standard error	E[a] (A.D.) ± standard error
HKY	N.A.[b]	−1641.81	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HKY + Γ	1.65	−1629.73	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HKY + clock	N.A.	−1660.01	1.78 ± 1.38	63 ± 1605	1274 ± 554	1383 ± 473	1523 ± 364	1725 ± 198	1765 ± 172
HKY + Γ + clock	1.69	−1648.38	1.68 ± 1.45	716 ± 2367	1175 ± 704	1322 ± 581	1471 ± 451	1706 ± 236	1740 ± 214
TrN	N.A.	−1633.84	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
TrN + Γ	1.88	−1624.11	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
TrN + clock	N.A.	−1652.19	1.82 ± 1.39	85 ± 1600	1283 ± 540	1395 ± 457	1529 ± 354	1729 ± 192	1769 ± 167
TrN + Γ + clock	1.93	−1642.80	1.73 ± 1.46	629 ± 2225	1200 ± 662	1346 ± 544	1486 ± 426	1713 ± 223	1749 ± 200
GTR	N.A.	−1625.80	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GTR + Γ	2.03	−1617.31	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GTR + clock	N.A.	−1643.68	1.80 ± 1.39	82 ± 1611	1278 ± 548	1387 ± 468	1524 ± 361	1727 ± 195	1767 ± 170
GTR + Γ + clock	2.10	−1635.57	1.71 ± 1.44	584 ± 2201	1196 ± 670	1339 ± 554	1482 ± 432	1711 ± 226	1747 ± 203

[a]Label for interior node in Fig. 1.
[b]Not applicable.

times estimated by using the HKY model with the phylogenetic tree in Fig. 1 are summarized in Table 2. When the rate was assumed to be constant among sites, $\ln L = -1658.19$ and $\ln L = -1640.23$ under the assumption of molecular clock or rate heterogeneity among branches, respectively, and the molecular clock

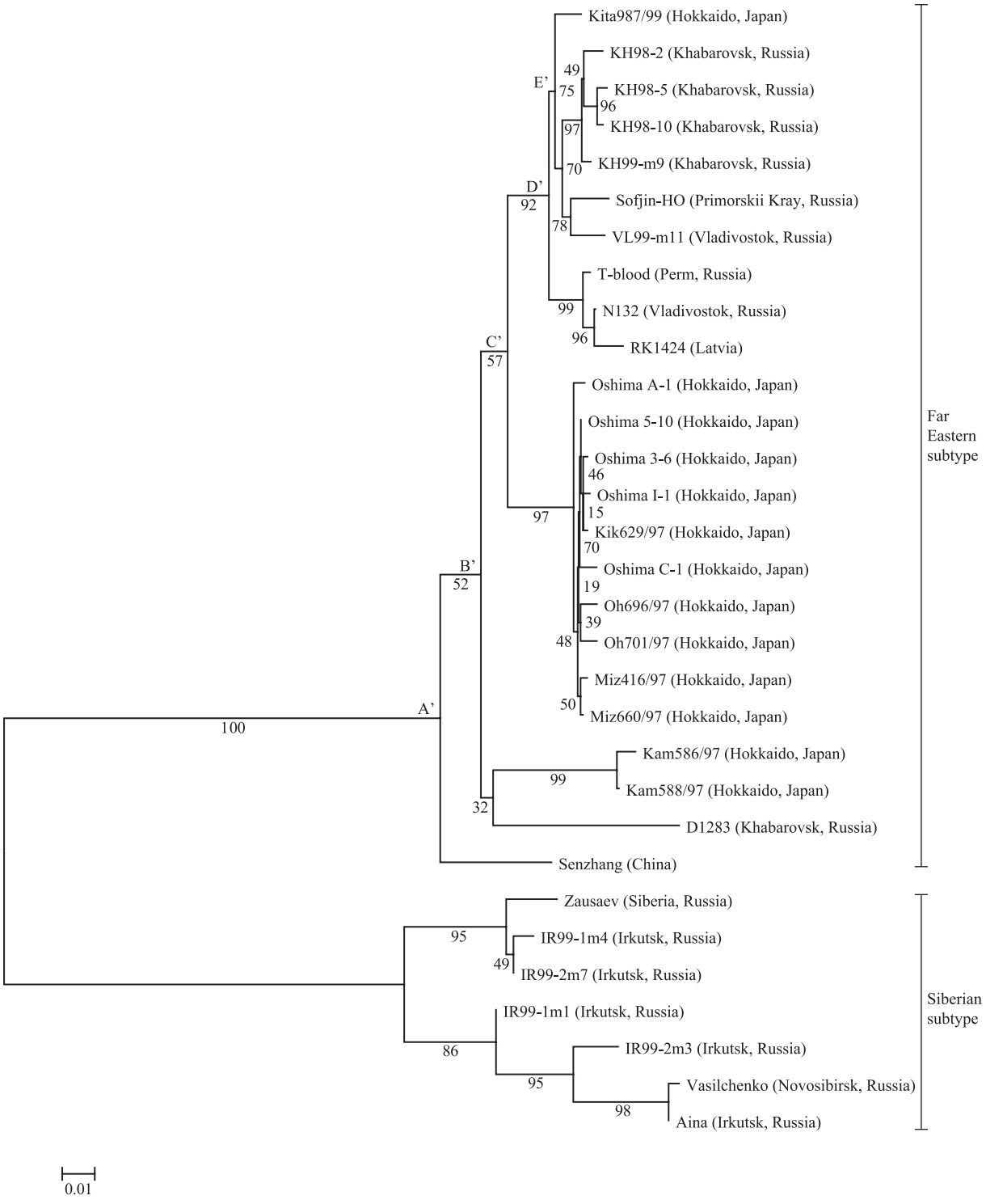

Fig. 2. Phylogenetic tree of the 31 sequences of Japanese TBEV and the Far Eastern and Siberian subtypes constructed by the NJ method using the TrN model with Γ distribution ($\alpha = 0.26$) for all nucleotide sites of the E gene (1488 nt). The geographical origin for the viral strain is indicated in the parentheses after the strain name. The number attached to each interior branch indicates the bootstrap probability. A', B', C', D', and E' are labels for the interior nodes. The scale bar indicates the number of nucleotide substitutions per site.

was not rejected ($2\Delta\ln L = 35.92$; $p = 0.14$). Similarly, when the rate was assumed to follow a Γ distribution among sites, $\ln L = -1647.01$ and $\ln L = -1628.59$ under the assumption of molecular clock or rate heterogeneity among branches, respectively, and the molecular clock was not rejected ($2\Delta\ln L = 36.83$; $p = 0.12$). In contrast, rate constancy among sites was rejected under the assumptions of both molecular clock ($2\Delta\ln L = 22.37$; $p = 2.25 \times 10^{-6}$) and rate heterogeneity among branches ($2\Delta\ln L = 23.28$; $p = 1.40 \times 10^{-6}$).

These results indicate that the rate of synonymous substitution and divergence times can be reliably estimated under the assumptions that the molecular clock holds and the rate follows a Γ distribution among sites. Under these assumptions, the average rate of synonymous substitution was estimated to be 1.62×10^{-4} per site per year (Table 2). The absolute years at interior nodes A, B, C, D, and E and the divergence of the Far Eastern and Siberian subtypes were estimated to be about 1150 A.D., 1300 A.D., 1450 A.D., 1700 A.D., 1750 A.D., and 800 B.C., respectively. Taking into account the five possible scenarios for the transmissions of TBEV between Japan and Russia, these results indicate that TBEV has been endemic to Japan at least from 1300 A.D. For reference, under the assumption of rate constancy among sites, the rate of synonymous substitution was estimated to be 1.72×10^{-4} per site per year, and the absolute years at interior nodes A, B, C, D, and E and the divergence of the Far Eastern and Siberian subtypes about 1250 A.D., 1350 A.D., 1500 A.D., 1700 A.D., 1750 A.D., and 150 B.C., respectively. However, caution should be exercised with these estimates, because the variances were relatively large. It should be noted that, when the pattern of nucleotide substitution for the four-fold degenerate sites was examined, the TrN model with Γ distribution and equal base frequencies and the SYM model with Γ distribution were found to be the fittest to the data using the hLRT and the AIC, respectively. However, similar results were obtained when the TrN and GTR (which contained the SYM model) models were used instead of the HKY model (Table 2). Similar results were also obtained under the assumption that the positions of KH98-2 and KH99-m9 were exchanged in the phylogenetic tree in Fig. 1 (Table 3).

The topology of the phylogenetic tree constructed by the NJ method using the TrN model with Γ distribution ($\alpha = 0.26$) (Fig. 2) was slightly different from that in Fig. 1, suggesting slightly different scenarios for the transmissions of TBEV between Japan and Russia. However, Japanese TBEV was divided into the same three groups and TBEV was inferred to have been transmitted between Japan and Russia for a similar number of times (at least three) as in Fig. 1. The estimates of the rate of synonymous substitution and divergence times (Table 4) were also similar to those in Tables 2 and 3.

Table 4. Rate of synonymous substitution and divergence times estimated using the phylogenetic tree in Fig. 2

Model	α	ln*L*	Rate ($\times 10^{-4}$ per site per year) ± standard error	Divergence of the Far Eastern and Siberian subtypes ± standard error	A'[a] (A.D.) ± standard error	B'[a] (A.D.) ± standard error	C'[a] (A.D.) ± standard error	D'[a] (A.D.) ± standard error	E'[a] (A.D.) ± standard error
HKY	N.A.[b]	−1649.53	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HKY + Γ	1.62	−1637.10	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
HKY + clock	N.A.	−1667.85	1.92 ± 1.38	101[c] ± 1369	1320 ± 477	1378 ± 442	1564 ± 307	1742 ± 171	1782 ± 146
HKY + Γ + clock	1.67	−1655.75	1.78 ± 1.45	531[d] ± 2080	1222 ± 621	1284 ± 578	1508 ± 395	1720 ± 211	1756 ± 188
TrN	N.A.	−1642.27	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
TrN + Γ	1.76	−1634.19	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
TrN + clock	N.A.	−1660.49	1.93 ± 1.40	55[c] ± 1414	1324 ± 478	1374 ± 448	1563 ± 310	1742 ± 172	1782 ± 147
TrN + Γ + clock	1.89	−1650.62	1.82 ± 1.45	477[d] ± 1996	1240 ± 594	1299 ± 555	1517 ± 380	1724 ± 204	1762 ± 180
GTR	N.A.	−1633.98	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GTR + Γ	1.95	−1624.95	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GTR + clock	N.A.	−1651.63	1.92 ± 1.39	53[c] ± 1415	1322 ± 479	1365 ± 455	1558 ± 314	1740 ± 174	1781 ± 149
GTR + Γ + clock	2.04	−1643.00	1.80 ± 1.44	439[d] ± 1972	1239 ± 598	1294 ± 561	1513 ± 385	1722 ± 206	1760 ± 182

[a] Label for interior node in Fig. 2.
[b] Not applicable.
[c] A.D.
[d] B.C.

DISCUSSION

In a previous study, Japanese TBEV was classified into only one group, and was inferred to be transmitted from Russia to Japan on just a single occasion, which was estimated to have occurred 260–430 years ago (Hayasaka et al., 1999). These results were obtained by analyzing only five distinct sequences of Japanese TBEV (Oshima 3-6, Oshima 5-10, Oshima A-1, Oshima C-1, and Oshima I-1). However, in the present study, Japanese TBEV was divided into three groups, among which the one consisting of Oshima 3-6, Oshima 5-10, Oshima A-1, Oshima C-1, Oshima I-1, Kik629/97, Miz416/97, Miz660/97, Oh696/97, and Oh701/97 corresponded to the previous group. TBEV was inferred to have been transmitted between Japan and Russia at least three times and to have been endemic to Japan for at least 700 years. These results were obtained by increasing the number of distinct sequences of Japanese TBEV in the analysis to thirteen.

In the present study, the rate of synonymous substitution for the E gene of TBEV was estimated to be 1.62×10^{-4} per site per year, which was similar to those ($[2.9–4.9] \times 10^{-4}$ per site per year) obtained in previous studies (Hayasaka et al., 1999; Jenkins et al., 2002; Hanada et al., 2004). These rates are smaller than those for other RNA viruses, such as human immunodeficiency virus type 1 and influenza A virus, which are of the order of 10^{-3} per site per year (Jenkins et al., 2002; Hanada et al., 2004). This is considered to be because TBEV mainly replicates in ticks, where the infectivity and virus titre are maintained at a low level, so that the rate of replication for TBEV is smaller than for other RNA viruses (Zanotto et al., 1995).

The time at interior node C was estimated to be 260–430 years ago in a previous study (Hayasaka et al., 1999). In addition, the divergence time of the Far Eastern and Siberian subtypes was estimated to be 1700–2100 years ago. These results have been obtained under the assumption of rate constancy among sites. Indeed, similar results were obtained under the same assumption in the present study; the time at C was estimated to be about 500 years ago, and the divergence time of the Far Eastern and Siberian subtypes about 2150 years ago. However, rate constancy among sites was rejected in the present study. When rate constancy among sites is assumed under the condition that this assumption is actually violated, the lengths of deep branches of the phylogenetic tree are usually underestimated (Nei and Kumar, 2000; Suzuki and Nei, 2002). Since the rate is estimated essentially by using the difference in the positions of exterior nodes, which are associated with shallow branches of the phylogenetic tree, it may not be greatly affected by a violation of the assumption. In fact, the rate was estimated to be 1.62×10^{-4} and 1.72×10^{-4} per site per year when the rate was assumed to be constant or to follow a Γ distribution among sites, respectively. However, since interior node C and the divergence of the Far Eastern and Siberian subtypes are associated with deep branches, the times obtained above may be underestimates. Indeed, when the rate was assumed to follow a Γ distribution among sites, the time at C was estimated to be about 550 years ago, and the divergence time of the Far Eastern and Siberian subtypes about 2800 years ago, which were older than previous estimates.

It should be noted that the variances for the estimates of evolutionary rate and divergence times obtained in the present study were relatively large. However, similar estimates were obtained under the assumptions of different models and alternative phylogenetic trees. In addition, the estimates obtained in the present study were largely consistent with those obtained in previous studies (Hayasaka et al., 1999; Jenkins et al., 2002; Hanada et al., 2004), as indicated above. These observations suggest that the estimates obtained in the present study are more or less reliable.

Although it is difficult to decide which of the five possible scenarios for the transmissions of TBEV between Japan and Russia is the most likely, they all appear to have taken place several hundred years ago. Since the Japanese archipelago separated from the Eurasian continent at least 20,000,000 years ago, it is likely that TBEV was transmitted between Japan and Russia across the sea. Since both Japan and Far East Russia are covered by the East Asian-Australian flyway of migratory birds, it is possible that migratory birds have transported infected ticks. In fact, *I. persulcatus* has been detected on migratory birds in Japan (Miyamoto et al., 1993).

In conclusion, Japanese TBEV belongs to the Far Eastern subtype, which is known to be highly pathogenic. Japanese TBEV was divided into three groups, and TBEV was inferred to have been transmitted between Japan and Russia at least three times, which were estimated to have occurred several hundred years ago. These results indicate that TBEV has not only been endemic but also transmitted multiple times to Japan.

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