ENVELOPE GENE SEQUENCE VARIATION AMONG JAPANESE ENCEPHALITIS VIRUSES ISOLATED IN KOREA

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Summary. – The nucleotide (nt) sequences of the envelope (E) gene of 4 Japanese encephalitis virus (JEV) isolates from Korea (K82PO1, K87P39, K91P55 and K94PO5) were determined and the deduced amino acid (aa) sequences were compared within themselves and with the published sequences of 16 other JEV strains originating from other parts of Asia. Homologies of 87.2 – 95.6% at the nt level and 95.8 – 98.0% at the aa level among the Korean JEV isolates were found. aa positions 89, 129, 220, 225, 327, 366, 456 and 477 characterized the Korean isolates. According to the phylogenetic analysis based on the E gene nt sequence, the Korean isolates formed distinct subgroup consisting of at least 2 genetic types.

Key words: Japanese encephalitis virus; Korean isolates; envelope gene sequence; variation

JEV is a mosquito-borne flavivirus that causes an arboviral disease of major public health importance in Asia including Korea. More than 35,000 cases with 10,000 deaths are reported annually from this continent (Ni and Barrett, 1995).

Identifying the genotypic and phenotypic differences in JEV isolates from different time periods and geographic sites produces an important information for choosing vaccine candidate strains, studying virus evolution, and reviewing the efficacy of monovalent inactivated vaccines (Kobayashi *et al.,* 1984; Chen *et al.,* 1990, 1992; Wills *et al.,* 1992). The use of monoclonal antibodies (MoAbs) has revealed additional variation in JEV antigenicity and distinct immunotypes of JEV have been detected (Kobayashi *et al.,* 1984; Wills *et al.,* 1992). On the other hand, data on the partial sequence of precursor membrane protein M (preM) gene were used to divide 44 JEV strains into four genotypes (Chen *et al.,* 1990, 1992). However, the preM gene sequences did not indicate sufficiently enough corresponding serological differences (Ni *et al.,* 1994).

Important biological activities of JEV, namely haemagglutination, virus neutralization, virion assembly, membrane fusion and virus binding to cellular receptors are associated with its 53 K E protein (Gritsun *et al.,* 1995). Recently, the nt and deduced aa sequences of the E gene of tick- and mosquito-borne flaviviruses were used for construction of their phylogenetic trees and classification (Marin *et al.,* 1995). Also Ni and Barrett (1995) showed a variation and phylogenetic tree of 13 JEV isolates , originating from different geographical locations, based on the structural protein gene of these viruses. Korean JEV isolates showed different optimal pH of haemagglutination and MoAb crossreaction with the Nakayama-NIH strain which has been used to produce inactivated vaccine in Korea (Cho *et al.,* 1994).

In this study, four JEV isolates from a pool of *Culex tritaeniorhynchus* collected at Wando and Young Kwang provinces in Korea were studied. Their complete E protein genes were cloned and sequenced with the aim to analyse the genetic variation of JEV isolates from one geographical area. These sequences were compared also with published E gene sequences of 16 JEV strains which were isolated in different Asia regions with the aim to characterize the intratypic and inertypic variation of the four Korean strains at molecular level.

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Abbreviations: aa = amino acid; DDT = dithiothreitol; E = envelope; JEV = Japanese encephalitis virus; MoAb = monoclonal antibody; nt = nucleotide; PCR = polymerase chain reaction; preM = precursor membrane protein M; SDS = sodium dodecyl sulphate

Table 1. Isolation history JEV strains compared in this study

Strain	Year	Location	Source	Sequence source
K94PO5	1994	Korea, Wando	*C.tritaeniorhynchus*	This paper(U34929)*
K91P55	1991	Korea, Wando	*C.tritaeniorhynchus*	This paper(U34929)*
K87P39	1987	Korea, Wando	*C.tritaeniorhynchus*	This paper(U34929)*
K82PO1	1982	Korea, Young Kwang	*C.tritaeniorhynchus*	This paper(U34929)*
Nakayama	1935	Japan, Tokyo	Human brain	McAda *et al.*, 1987
JaOArS982	1982	Japan, Osaka	Mosquito pool	Sumiyoshi *et al.*, 1987
SA-14	1954	China, Xian	*Culex pipiens*	Nitayaphan *et al.*, 1990
Kamiyama-1	1966	Fukuoka	Human brain	Hasegawa *et al.*, 1992
Beijing-1	1949	China, Beijing	Human brain	Hasegawa *et al.*, 1992
691004	1969	Sri Lanka	Human brain	Hasegawa *et al.*, 1992
Muar	1952	Singapore	Human brain	Hasegawa *et al.*, 1992
DH 20	1985	Nepal, Dharan	Human brain	U03690*
KPP034-35CT	1982	Thailand, Kampanghet	Mosquito pool	U03690*
P3	1949	China, Beijing	Mosquito pool	U03690*
8256	1982	China, Taiwan	Mosquito pool	U03690*
826309	1982	India, Goa	Human brain	U03690*
Saigon	1962	Vietnam, Saigon	NK	U03690*
Indonesia	NK	Indonesia	Mosquito pool	U03690*
p20778	NF	NF	NF	Z34095*
733913	NF	NF	NF	Z34095*

*GenBank accession number. NK = not known; NF = not found.

Viruses. The 4 Korean and 16 other isolates of JEV are described in Table 1. The Korean isolates used in this study underwent less than 5 passages in suckling mouse brains.

cDNA synthesis. Viral RNA was extracted from mouse brain suspensions (10% w/v) with sodium dodecyl sulphate (SDS) and phenol-chloroform. To produce cDNA, total RNA was reverse transcribed using random hexamer primers (660 ng/μl, BRL), reverse transcriptase (200 U, SuperScript™II), 10 mmol/l dNTP mixture and 100 mmol/l dithiothreitol (DTT) in a 20 μl volume. Samples were incubated at 42°C for 30 mins and at 95°C for 5 mins.

Polymerase chain reaction (PCR). In order to analyze the sequence of the E gene region we synthesized and purified 4 DNA primer pairs. The following primers complementary to the JEV Nakayama strain genomic sequence were used (McAda *et al.*, 1987). Of four sense primers,

primer 1 (nt 625-644), 5'TACTTCACCATCCTCCTGCT3';
primer 2 (nt 997-1016), 5'GGAAGCATTGACACATGTGC3';
primer 3 (nt 1430-1449), 5'GTTGTTGCTCTTGGGTCACA3';
primer 4 (nt 1765-1784, 5'ACTCAAAGGTGCTAGTCGAG3'.

Of four antisense primers,

primer 5 (nt 1110-1129), 5'AGTGGTGGTTCCATGCACAA3';
primer 6 (nt 1519-1538), 5'GGTGGCCTGATGTTAACTTC3';
primer 7 (nt 1892-1911), 5'CTTTGCCCAGCGTGCTTCCA3';
primer 8 (nt 2162-2181), 5'ATACCTATCCACCCAGGCTT3'.

Four overlapping regions (nt 625-1129, 997-1538, 1430-1911, 1767-2181) were amplified in 22 cycles (94°C for 40 secs, 58°C for 1 min, and 72°C for 1 min). The PCR products were electrophoresed in 1.5% agarose gels.

Cloning and sequencing. The PCR products were cloned into PCR™II vector (Invitrogen). Ligated products were used to transform competent *E. coli* INVαF' (Invitrogen) cells. For sequencing, the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical) was used according to the manufacturer's directions. Sequences were determined from at least two clones of each independent PCR product. Sequence data were analyzed by using the DNASIS and PROSIS programmes. Phylogenetic analyses were performed using the PAUP 3.1.1 programme.

The nt and aa sequence homologies of the E gene of the JEV strains under study are shown in Table 2. According to these data the variation of E gene among the compared JEV strains was not related to either geographical location or isolation year. Also, viruses isolated from humans or from mosquito vector did not share E gene sequence homology properties due to interaction with their host. These results correspond to findings reported by other workers (Chen *et al.*, 1990; Wills *et al.*, 1992; Ni and Barrett, 1995). Slight interannual variation was seen in the sequences of the Korean strains (maximum homology of 95.6% (nt) and 98.0% (aa)).

The receptor-binding fusion sequence aa 98-111 (Mandl *et al.*, 1989), the predicted T-helper cell recognition sites in the region of aa 426-457, and the potential N-glycosylation sites in the region of aa 154-156 (Gritsun *et al.*, 1995) did not show any variation in the most JEV strains with a few exceptions (Fig. 1). There were no changes in aa 138, 176, 270 and 333 (Ni *et al.*, 1994) related to the attenuation among the four Korean isolates. These results support those showing a similarity of the virulence of the Korean strains to that of the Nakayama strain (Cho *et al.*, 1994).

Table 2. E protein nucleotide and amino acid sequence homology (identity, %) among 20 JEV strains

	K94PO5	K91P55	K87P39	K82PO1	Nakayama	Beijing 1	JaOArS982	691004	Kamiyama 1	SA-14
K94P0	–	98.0	95.8	97.2	97.2	96.8	97.4	96.0	97.2	97.2
K91P55	94.3	–	96.8	98.0	97.8	97.4	97.6	96.6	97.4	97.2
K87P39	87.2	91.9	–	96.8	97.4	97.4	97.2	96.4	97.0	96.8
K82PO1	90.1	94.7	95.6	–	98.2	97.8	98.4	97.0	98.2	98.0
Nakayama	87.6	91.5	95.6	94.3	–	99.2	99.4	98.8	99.2	99.0
Beijing 1	87.6	91.5	95.6	94.1	97.9	–	99.0	98.0	98.8	98.6
JaOArS982	88.1	91.7	97.0	95.3	97.0	97.1	–	98.2	99.8	99.2
691004	87.1	91.0	95.2	93.9	99.3	97.4	96.6	–	98.0	97.8
Kamiyama 1	87.8	91.7	96.0	94.8	97.9	97.0	97.6	97.3	–	99.0
SA-14	88.1	91.9	97.2	95.5	97.2	97.1	98.7	96.7	97.6	–
733913	87.7	91.6	96.2	94.7	96.7	96.4	98.0	96.3	96.6	98.0
8256	87.9	91.8	96.6	95.1	96.8	96.8	98.1	96.4	97.0	98.3
826309	87.6	91.1	95.6	94.3	96.3	96.1	97.4	95.8	96.6	97.4
DH20	87.6	91.2	96.6	95.0	97.4	96.7	98.0	96.9	96.8	98.5
Indonesia	87.8	91.7	96.7	95.0	97.1	97.0	99.3	96.6	97.1	98.4
KPPO34	88.6	90.0	95.3	93.7	95.5	95.2	96.4	95.0	95.4	97.5
p20778	88.2	91.5	96.2	94.8	97.7	97.0	97.8	97.2	97.3	97.7
Saigon	86.7	90.4	94.7	93.3	97.7	96.3	96.2	97.2	96.4	96.4
P3	87.4	91.0	96.0	94.2	96.8	96.5	97.6	96.2	96.8	97.8
Muar	77.3	78.9	79.5	79.9	80.5	80.4	80.8	80.7	80.8	80.8
	733913	8256	826309	DH20	Indonesia	KPPO34	p20778	Saigon	P3	Muar
K94P0	96.8	97.4	96.6	97.0	97.2	97.0	97.2	95.6	96.0	89.1
K91P55	97.4	97.4	96.8	97.2	97.4	97.0	97.4	95.6	96.6	89.5
K87P39	97.0	96.8	96.6	96.4	96.8	96.4	97.0	94.9	96.0	88.3
K82PO1	97.8	98.0	97.6	97.8	98.0	97.8	98.2	96.2	97.0	89.1
Nakayama	99.2	99.0	98.6	99.2	99.2	98.6	99.2	97.6	98.2	90.3
Beijing 1	98.8	98.6	98.2	98.4	98.6	98.2	98.8	96.8	97.8	89.7
JaOArS982	99.0	99.2	98.8	99.0	99.2	98.8	99.4	97.4	98.0	90.7
691004	98.2	97.8	97.4	98.0	98.0	97.4	98.0	96.6	97.0	89.9
Kamiyama 1	98.8	99.0	98.6	98.8	99.0	98.6	99.2	97.2	97.8	90.5
SA-14	98.6	99.2	98.4	99.0	98.8	99.2	99.0	97.4	97.6	89.9
733913	–	98.6	98.2	98.4	98.6	98.2	98.8	96.8	97.8	90.1
8256	97.4	–	98.4	99.4	99.6	98.8	99.0	97.8	98.0	90.1
826309	97.3	96.7	–	98.2	98.4	98.0	98.6	96.6	97.2	89.5
DH20	97.2	98.5	96.5	–	99.6	98.6	98.8	97.6	97.8	90.1
Indonesia	97.6	99.5	96.9	98.7	–	98.4	99.0	97.6	98.0	90.3
KPPO34	95.9	96.1	95.3	96.5	96.2	–	98.6	97.0	97.2	89.9
p20778	97.1	97.1	96.4	97.4	97.3	96.2	–	97.2	97.8	88.7
Saigon	95.5	96.9	95.2	96.8	96.9	94.6	96.2	–	95.8	88.7
P3	96.8	97.4	96.2	97.2	97.6	95.8	96.8	95.6	–	88.9
Muar	80.6	80.4	80.6	80.3	80.6	81.7	81.0	79.9	80.2	–

All values were calculated by using the PAUP 3.1.1. programme and based on the shorter of the two sequences in the pairwise comparison. Values above dashes (identity trace) are % of identical amino acids and those below dashes are % of identical nucleotides.

According to Ni and Barrett (1995), four variable E protein aa, namely aa 51, 209, 244 and 366, were found. In this study, two other variable aa (aa 227 and 327) were identified. Sequence variation in aa 89, 129, 220, 225, 327, 366, 456 and 477 was characteristic for the Korean strains (Fig. 1). It is not yet confirmed that these aa alterations are related to serological differences as compared with Nakayama strain. However, mutations in E gene sequence affecting the virulence and neutralization have been reported by many researchers (Ni *et al.,* 1994; Gritsun *et al.,* 1995). Therefore, the E gene variation may affect the serological behaviour of JEV.

The phylogenetic tree based on the E gene of 20 JEV strains has two main branches comprising the Muar strain on one branch and all other strains on the other branch divided into subgroups. The formation of two distinct sublineages among the 4 Korean isolates suggests that there are 2 genetic types of JEV existing in Korea and that the strain divergence can occur even in a limited geographic region (Fig. 2). These

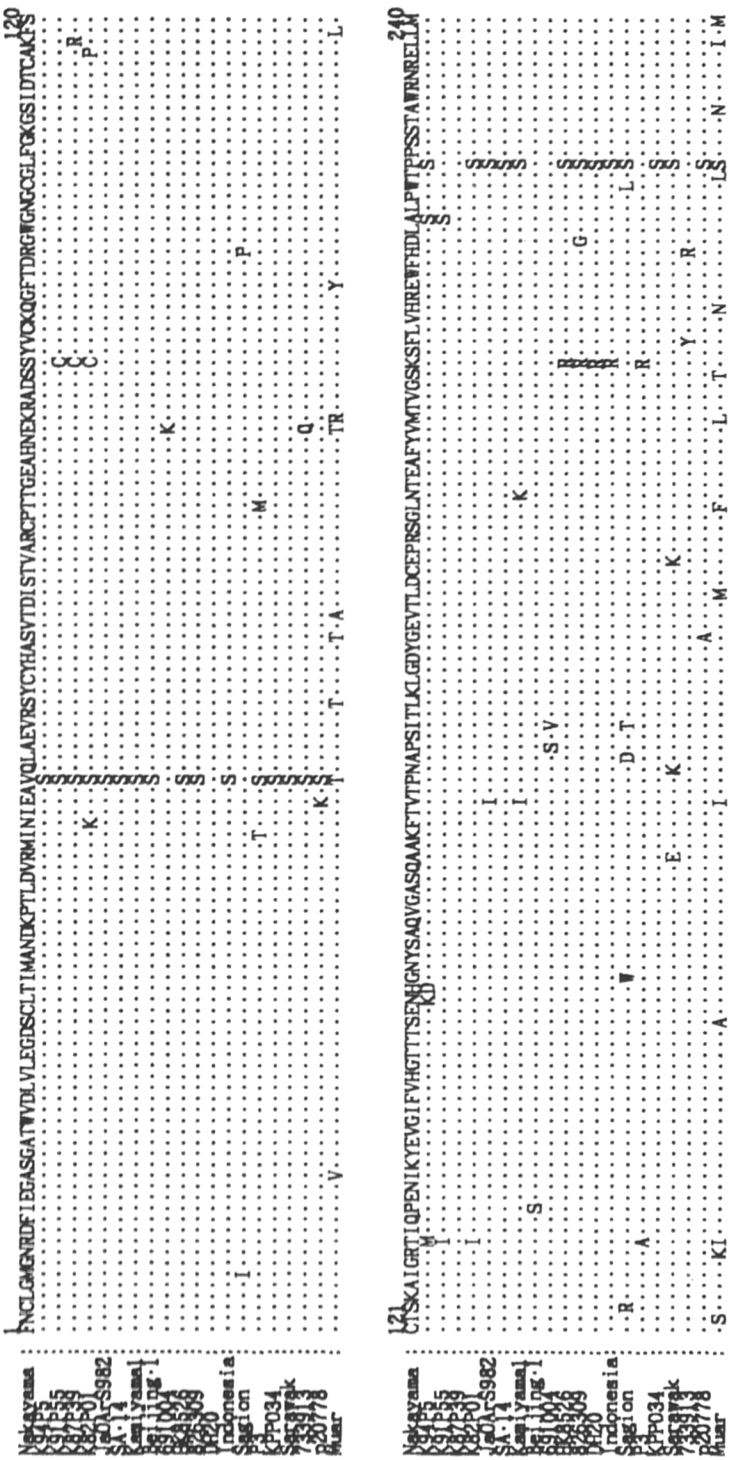

Fig. 1

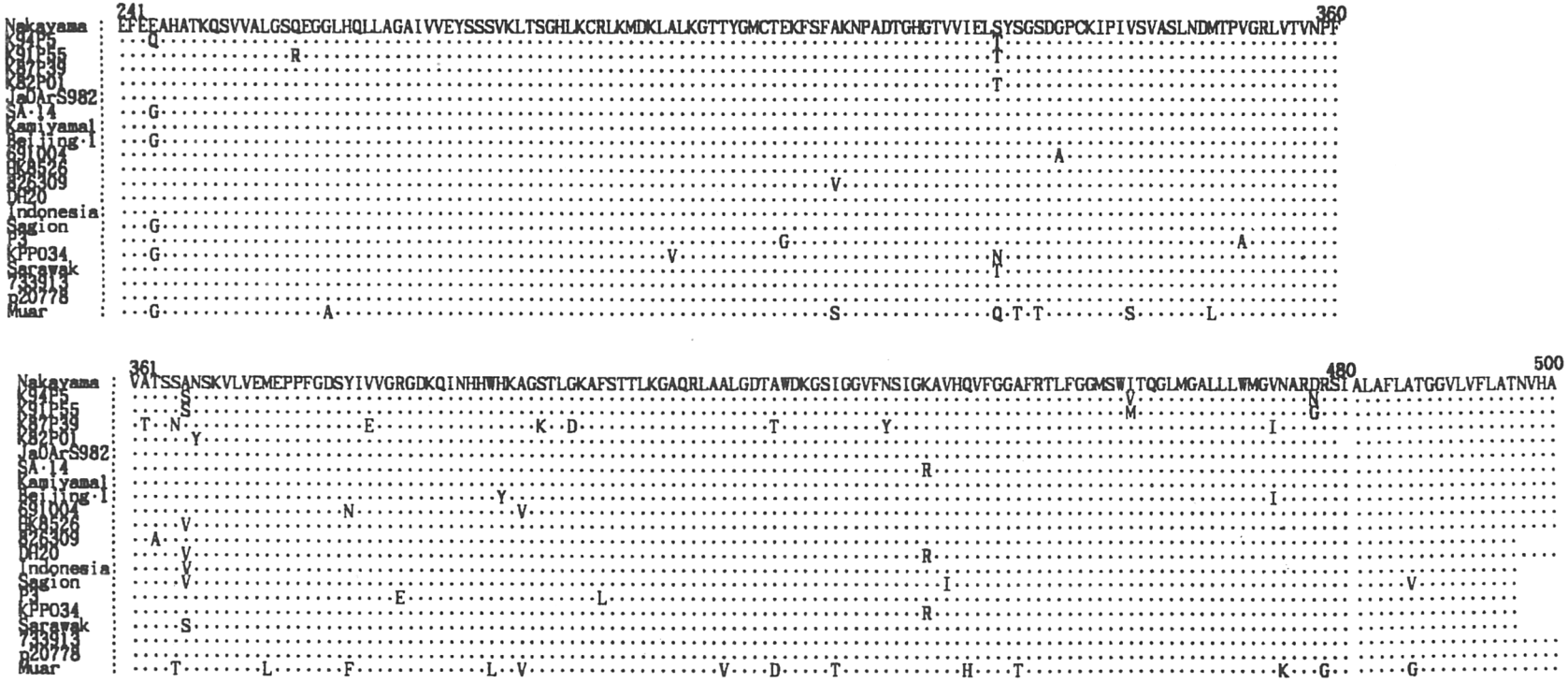

Fig. 1

Alignment of E protein amino acid sequences of 20 JEV strains

Amino acid positions are numbered within the E protein. Dots indicate identities.

molecular data are consistent with those of the serological studies (cross-reactions in haemagglutination and with Mo-Abs) reported previously (Cho *et al.*, 1994).

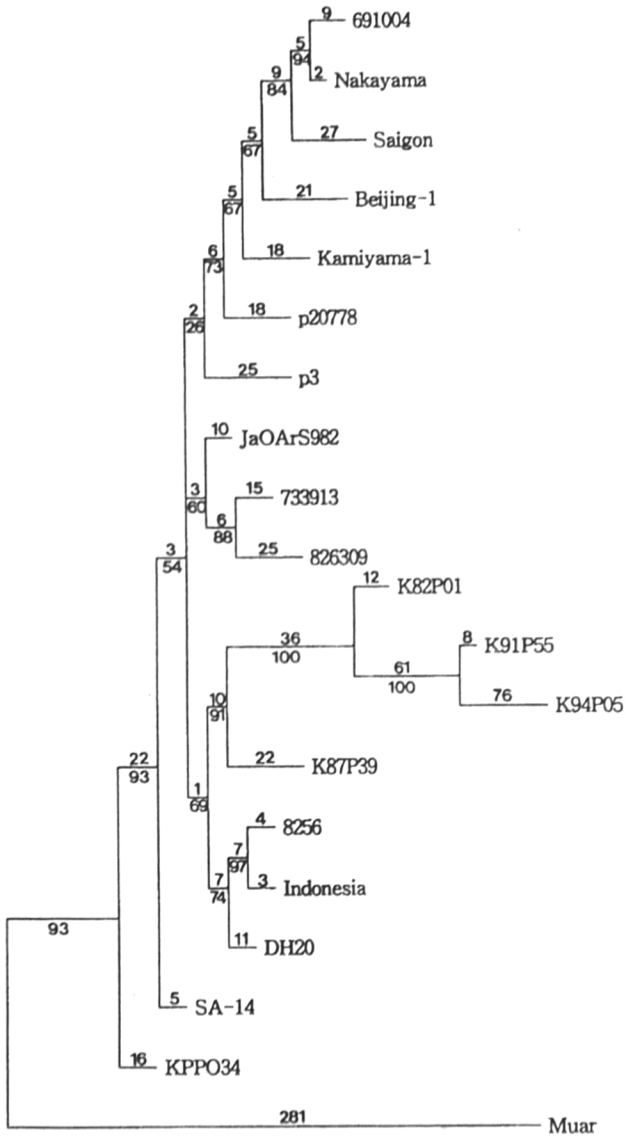

Fig. 2
Phylogenetic tree of 20 JEV strains isolated in Asia according to the E gene sequence
Phylogenetic analyses were made on the basis of nucleotide sequences by using the PAUP 3.1.1 programme. One thousand bootstrap replications were performed and confidence limits are listed as % below each branch. Horizontal lengths of branches and the numbers above each branch are proportional to nucleotide sequence differences.

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