行政院國家科學委員會補助專題研究計畫成果報告

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※　台灣地區日本腦炎病毒感染生態學與不同宿主的　※
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計畫類別：■個別型計畫　□整合型計畫
計畫編號：NSC89－2314－B－002－418－Y
執行期間：2000 年 8 月 1 日 至 2001 年 7 月 31 日

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執行單位：台灣大學公共衛生研究院流行病研究所

中 華 民 國 91 年 2 月 6 日

中文摘要

日本腦炎是一經由蚊子媒介的傳染病，會引起嚴重的神經症狀及後遺症，在亞洲地區是重要的公共衛生課題。根據之前的研究，以 prM 的分子演化樹可將亞洲地區的日本腦炎病毒分成四個基因型。而在台灣地區，根據詹等的研究 (2000) 以部分的 C/prM 的基因序列共 280 個鹼基所做的分析，台灣地區的日本腦炎病毒可分為三個聚落 (cluster)。但是在分子演化上，僅僅以 280 個鹼基進行演化樹的分析，其結果有可能無法提供足夠甚或錯誤的訊息。因此本研究嘗試以較長的基因序列對台灣地區日本腦炎病毒株進行分子演化的分析。由於外套膜蛋白 (Envelope, E) 是主要引起中和抗體以及病毒與細胞受器結合的部位，也與病毒的毒力有很大的關連，因此本研究選取了這段基因作為研究的目標；另外，日本腦炎病毒的 3' 端非譯區 (3' untranslated region, 3'UTR) 到目前為止也尚未有對其進行分子演化研究的報告，因此本研究也將其納入分析。研究使用的病毒株包括有來自蚊子、豬以及人腦的分離株。在外套膜基因，總共對 30 株的病毒進行分析；在 3' 的非譯區則對 32 株的病毒進行分析。所使用的序列則是自行定序的或是從 GenBank 收集而來。

分析的結果顯示，外套膜的分子演化樹可將台灣地區的日本腦炎病毒株分成三個聚落，第一個聚落包含了台灣北部與中部彰化地區的分離株；第二個聚落則是台中地區以及台灣南部的病毒株。而第三個聚落則包括了 1965 年之前的人腦分離株及台灣的疫苗株 (中山與北京株)。而 3' 端非譯區的分子演化樹與外套膜基因分析的結果相似，均可將台灣的病毒株分成三個聚落。在詹 (2000) 的研究中，彰化的蚊子分離株 CH1392，演化上是與第三個聚落較為相近，但在本研究發現，無論是外套膜或是 3' 端的非轉譯區的演化樹，CH1392 均是南部地區的病毒株在同一聚落。而 1997 年的小琉球白腹叢蚊分離株 T1P1，在演化上則是與台灣南部的病毒相近，表示這株病毒應該是從台灣本島被引進小琉球的。而在外套膜基因序列的相似性上，病毒株之間在核酸序列的相似性介於 93.6% 到 100%，而在胺基酸的相似度則是介於 93.7% 到 100% 之

胜太 (Internal membrane fusion peptide) 、受器結合模組 (Receptor binding motif) 以及抗原決定位 (epitopes)，除了少數幾個病毒株外，在大部分的病毒株都相當的守舊(Conserved)，並沒有太大的變異。而在所有的病毒株之中，1998 年的人腦分離株 CJN，在外套膜的胺基酸序列上，包括了在抗原決定位的胺基酸序列上，有最多的變異，但是否就是這些變異造成其中樞神經毒性則有待進一步的研究。經由分子演化樹的結果也顯示，除了第三個聚落是全部由人腦分離的病毒株所組成以外，第一個與第二個群落都包括有蚊子、豬和人腦的分離株，由此結果看來台灣地區的日本腦炎病毒並無一個特別的分支(lineage) 是與人類的病例有關的。在 3' 端非譯區的核酸序列方面，大部分的變異及缺損 (deletion) 多集中在 5' 端的 70 個核酸序列內，而在核酸序列位置 10642 到 10654 則是另一個變異較多的區域。3' 端最末端的 100 個核酸則是相當的守舊，沒什麼太大的變異。此外，在 3' 非譯區的分子演化樹上，有四株非台灣地區的病毒株 （韓國、澳洲、印度）也納入進行分析，結果卻顯示兩株同樣是由印度分離的病毒並不會聚集在一起形成同一個聚落，而是與台灣的病毒聚集在一起。

Abstract

Japanese encephalitis (JE) still has public health threat in Asia because of its high mortality, serious neurological sequelae and approximately 35,000 cases and 10,000 deaths reported. annually. Taiwan is an endemic area of JEV and the virus is quite active even after mass immunization since 1968. Therefore, this study attempted to isolate JEV from mosquitoes in pig farm and used RT-PCR, sequence alignement and phylogenetic analysis of E and 3' untranslated region (3'UTR) of JEV to understand the evolution of this virus JEV in Taiwan compared to other geographic areas and various hosts to provide enough solid information for viral pathogenesis, molecular epidemiology, and vaccine development.

Two JEV strains from the field-caught mosquitoes around pig farms in Tainan during July to Aug, 2000 were isolated (TN207 and TN208). Total 30 JEV strains were used and two newly isolated JEV strains (CJN and T1P1) were also added. We found that phylogenetic results of both E and 3' UTR regions showed that Taiwanese JEV strains were divided into 3 clusters. The first cluster included the viruses circulated in northern and central Taiwan and the second cluster was composed of those strains originated from southern Taiwan. The third cluster involved mainly human brain isolates such as Ling (1965), TL, and Beijing (1949). The E region of 30 JEV strains revealed the high identity 93.6 -100% and 93.7-100% at the levels of nucleotide and amino acid sequences, respectively. No host-specific substitution was observed but the internal membrane fusion peptide (E101-108) and the receptor binding motif (RGD) (E387-389) were highly conserved among all the strains. In addition to those viruses in third cluster, there were other human brain isolates positioned in first and second clusters. This result suggested there was no a special lineage of virus which might be responsible for

of E protein were very conserved among strains. In the sequence analysis of 3' UTR, our results showed that most variations and deletions were located within the first 70 nucleotides of 5' portion. The terminal 100 nucleotides of 3' portion were highly conserved. In the phylogeny of 3' UTR, although several strains originated from areas outside Taiwan were used in our study, no grouping pattern associated with geographic distribution and hosts were observed.

Chapter 1 Introduction

Japanese encephalitis (JE), an important public health problem in Asia. Approximately 35,000 cases and 10,000 deaths are reported annually (Tsai et al., 1999). Most infections of Japanese encephalitis virus (JEV) are subclinical but its case fatality rate is the highest among arboviral encephalitis, even up to 30%. JEV is transmitted by mosquitoes and principally replicates in pigs and mosquitoes.

JEV is an enveloped, positive sense, single-stranded RNA virus, belongs to genus Flavivirus, family *Flaviviridae* (Chambers et al., 1990; Kuno et al., 1998). Morphologically, JEV is spherical, the diameter is approximately 40-50 nm. Nucleocapsid is composed of a capsid (C) protein and viral RNA, enclosed in a lipid membrane derived from cellular membrane. JEV genome size is 10,976 kb, encodes an uninterrupted open reading frame (ORF), flanked by 95 and 585 base untranslated regions (UTR) at the 5' and 3' ends (Rice, 1996; Sumiyoshi et al., 1987). The order of encoded proteins is 5'-C-prM-E-NS1-NS2A-NS2b-NS3-NS4A-NS4B-NS5-3'. E protein is associated with the attachment of virus to receptors, fusion with membrane, and neutralization epitope (Chambers et al., 1990).

Molecular phylogeny of JEV could separate JEV strains into 4 genotypes. Taiwan is an endemic area of JEV and viruses fell into the genotype 3 (Chen et al., 1990; Williams et al., 2000). On the other hand, according to Jan et al. (2000), the phylogeny of partial C/prM gene could classify Taiwanese JEV strains into three clusters. Cluster 1 principally contained strains circulate in northern Taiwan and cluster 2 was consisted of strains from southern Taiwan. Cluster 3 had two human brain isolate and two mosquito

isolates.

3' UTR of flavivirus has been suggested to be related to viral replication and RNA synthesis (Chambers et al., 1990; Rice, 1996). Phylogenetic analysis of 3' UTR has been performed in several flavivirus, i.e. dengue virus (Shurtleff et al., 2001), tick-borne encephalitis virus (Wallner et al., 1995), and yellow fever virus (Wang et al., 1996). However, there has not been any studies about the phylogeny of 3' UTR of JEV until now.

In this study, we isolated two new JEV strains from the field and then subjected them to the phylogenetic analysis. We performed phylogenetic analyses of E gene and 3' UTR of JEV strains from Taiwan and other areas. By the phylogeny of these two regions, we can understand the evolutionary relationship among these viruses and to know is there any association between virus and other parameters such as hosts, time of isolation and geographic distribution.

Chapter 3 Materials and Methods

A. Isolation of JEV from the Field

Study Site and Field Caught Mosquitoes

In mid-July and mid-August, 2000, mosquitoes were trapped by the mosquito lamp in a pig farm in Liou Chia Hsiang (六甲鄉), Tainan County (台南縣). Mosquitoes were trapped in the evening. The mosquito lamp were hold for about 3 hours from 6:00 pm to 9:00 pm then mosquitoes were collected by a net and stored in 4℃ in the refrigerator and transported to our laboratory as soon as possible. Mosquitoes will then be stored at -70℃ once they arrive at lab of infectious disease epidemiology at NTU until use.

Cells

C6/36 cells derived from *Ades albopictus* and BHK-21 cells were used in this study (both cell lines were kindly provided from Dr. Duane Gubler at the Vector-borne infectious disease laboratory at the Centers for Disease Control, Fort Collins, Colorado, USA.). C6/36 were grown in 28℃ incubator and maintained in Dulbeco's minimal essential medium (DMEM) (Gibco BRL, USA) and Mitsuhasi and Maramorosch insect medium (MM) (Sigma, USA) (both medium were 1:1 mixed) with 10% fetal calf serum (FCS) (Atlantic, USA) supplemented with streptomycin, penicillin G and amphotericin B (Atlantic, USA). BHK-21 cells were grown in 37℃ incubator and cultured in minimal essential medium (MEM) (Gibco BRL, USA) with 10% FCS containing the same antibiotics as above.

Virus Isolation

Procedures for virus isolation were depicted as the flow chart in Fig 3.1. Mosquitoes were pooled together on the ice. Each pool was consisted of 5-15 mosquitoes. The pools of mosquitoes were added with either 5 ml of cold PBS containing 5% FCS or 5 ml of cold DMEM and MM (1:1 mixed) with 5% FCS containing antibiotics. Mosquitoes with medium were homogenated on ice bath by sterilized mortars and pestles. The homogenates were then transferred to 15 ml centrifuge tube and centrifuged at 1,500 rpm for 10 minutes. After centrifugation, the supernatant were filtered through 0.2 μM filter (Millipore, USA). About 100 ul of the filtered supernatant samples was added to each confluent monolayer of mosquitoes C6/36 cells grown in 12-well plates after removing the media. The plate were put in 28℃ incubator for 1.5 hours with periodic agitation per 15 minutes and then added with mosquito media as the above for incubation at 28°C for 7 days. All the cultured medium were collected and centrifuged at 1,500 rpm for 10 minutes to remove the cell debris. The harvested medium were aliquoted to several vials and stored at -70℃ for further research. The centrifuged media were inoculated on BHK cells and screened by plaque assay to search for any virus initially

Plaque Assay

Because JEV can cause CPE and plaque of BHK cells, plaque assay was chosen to screen the existence of virus in our samples initially. BHK-21 cells grown in 12 well plates (Coring, USA) were used for plaque assay. 2×10^5 cells were seed in each well and incubated for 2 days. When the monolayer of cells were formed, the medium was removed. The 100 μl of serial dilutions of samples was added in each well of BHK cells. Subsequently, cells were incubated in 37℃ for 1.5 hour with periodic agitation per 15 minutes. After the adsorption, MEM medium containing 1% methylcellulose (Sigma,

USA) supplemented with 2% FCS and antibiotics and antimycotics was added (2 ml/well). Plates were placed in 37℃ incubator for 3 days. Cells were observed microscopically every day for CPE. After 3 days, the methylcellulose was flushed out by PBS. Plaques were visualized by staining with 1% crystal violet in 20% methanol overnight. The numbers of plaques per ml were represented as plaque forming units (PFU)/ml.

Indirect Immunoflourecence Assay (IFA)

C6/36 cells were scraped down, and then dropped on a 12 well Teflon-coated glass slide. Air dried cells were fixed by cold (-20℃) acetone for 10 minutes. Ten μl of 1: 100 diluted mouse anti-JEV IgG (mouse anti-JEV IgG, Nakayama strain, TropBio, USA) was added. The slide was placed at 37℃ incubator for 30 minutes. After incubation, the slide was washed with PBS for 10 minutes. Subsequently, 10 μl of 1:50 diluted FITC-conjugated goat anti-mouse IgG (Sigma, USA) was added on the cell and incubated at 37℃ for 30 minutes. Slide was washed with PBS for 10 minutes and added with glycerol. The result was examined by a fluorecent microscope.

RNA Extraction and Reverse Transcriptase –Polymerase Chain Reaction

Viral RNA was extracted by QIAmp viral R Viral RNA was extracted by QIAmp viral RNA extraction kit (Qiagen, Germany). Procedures were accorded to the manufacturer's recommendation. Extracted RNA was stored at -70℃ until use. Primer pairs, PE1 (sense primer, 5'-AGT-TAA-CAT-CAG-GCC-ACC-TGA-3', nucleotide position 183-1833) and PE2 (anti-sense primer, 5'-GTT-CCA-TCT-CGA-CCA-GCA-C-3', nucleotide position 2085-2103) (Chung et al., 1996) were used to detect the JEV RNA. SuperScript II reverse transcriptase (RT) (Gibco BRL, USA) was used to convert RNA

into cDNA. RNA and anti-sense primer were heated at 80℃ to denature possible secondary structures. Subsequently, RT buffer, 10mM dNTP, 0.1 M DTT, Rnase inhibitor (Gibco BRL, USA), senese-primer and 200 units of RT were added. The mix was incubated at 42℃ for reverse transcription. Converted cDNA was stored at -20℃ for further use. PCR was performed by Platinum *Taq* DNA polymerase (Gibco BRL, USA). The temperature for denature, annealing and extension is 94℃, 55℃ and 72℃ respectively. The DNA was amplified in 35 cycles. For positive samples, the whole E gene was also amplified. Six primer pairs spanning the whole E region were used. Sequences of these primers were listed in table 3.1 (provided by Dr WJ Chen and Dr SS Chiou, Dept of Parasitology, Chang Gung University). The PCR products were electrophresised by 2 % agarose gel (SeaKem LE Agarose, FMC, USA) and visualized by staining of ethidium bromide.

Sequencing of PCR Products

PCR products were electropheresised by 2 % agarose gel and the band of DNA was excised. DNA was extracted by DNA extraction kit (Viogene, Taiwan). BigDye Sequencing kit (ABI, USA) and ABI 377 autosequencer (ABI, USA) were used to determine sequences.

Multiple Alignment of Sequences

The sequence of E gene of the newly isolated JEV was determined. A phylogenetic analysis of the E gene was performed to elucidate the relationship of this new virus with other Taiwanese local strains. The list of JEV strains used in this study and their year of isolation, host, location and GenBank accession number is shown in table3.2. Twenty-one local strains and six reference strains (Nakayama, Beijing-1, P3, SA14,

JaOAr01, JaGAr982) were included in the study. Based on the study of Williams et al., (2000), the sequence of a Indonesia JEV strain, JKT5441 (U70406), was used as the outgroup here.

Nucleotide sequences were aligned by Clustal X program (Thompson et al., 1997). The parameters were using the default settings. Gap-opening penalty was 10 and gap-extension penalty is 0.2. Results of alignment were inspected visually and adjusted manually in the environment of BioEdit (Hall, 1999) if needed. Percentages of identity among sequences were calculated by BioEdit. Results of alignment were visualized, processed and presented by GenDoc program (Nicholas et al., 1997).

Phylogentic Analysis of JEV Using E Gene

Phylogenetic trees were constructed by neighbor-joining (NJ) (Saitou et al., 1987), maximum parsimony (MP), and maximum likelihood (ML) (Felsenstein, 1981) methods. NJ tree was constructed by MEGA version 2.0 (Kumar et al. 2001). The multiple alignment of E gene was subjected to the program and bootstrapped (Felsenstein, 1985) for 1000 times. Nucleotide distances were estimated using Kimura-two-parameter (K2P) model (Kimura, 1980) with transition/transversion ratio of 2.0.

MP and ML trees were constructed by PHYLIP package (Felsenstein, 1993). The multiple sequence alignment was subjected to the SEQBOOT program for bootstrap analysis of 1000 replicates. The generated dataset was then imported into DNAPARS program for constructing MP trees. The CONSENSE program was used to build a majority consensus bootstrapped tree. ML tree was build by DNAML program with

transition/transversion ratio of 2.0. Results produced by PHYLIP package were visualized by TreeView program (Page, 1996).

B. Phylogenetic Analysis of 3' Untranslated Region

Virus and Cells

Taiwanese JEV strains studied in this study were propagated in C6/36 mosquito cells or BHK cells as in the section A of this chapter. The Nakayama strain was propagated in mouse brain, BHK, PS, and C6/36 cells. The year of isolations, hosts, and locations of strains used in this study were listed in table 3.3.

RNA Extraction and RT-PCR

Viral RNA was extracted by QIAmp viral RNA extraction kit (Qiagen, Germany). Procedures were following to the manufacturer's recommendation. Extracted RNA was stored at -70℃ until use. SuperScript one step RT-PCR system (Gibco BRL, USA) was used to perform RT-PCR. Two pairs of primers were used to amplify the 3' UTR region. Sequences of primers were listed in table 3.4. The cDNA was synthesized at 50℃ for 45 minutes, then the synthesized cDNA was subsequently subjected to PCR in the same container. PCR conditions were 35 cycles of denaturing at 94℃ for 1 minutes, annealing at 45℃ for 1 minutes, and extension at 72℃ for 1 minutes. PCR products were elecphoresized by 2% agarose gel and excised and purified. The extracted PCR products were directly sequenced by BigDye Sequencing kit (ABI, USA) and ABI 377 autosequencer (ABI, USA).

Phylogenetic Analysis

Sequences of 3' UTR of JEV were aligned by Clustal X program. The parameters were using the default settings: gap-opening penalty is 10 and gap-extension penalty is 0.2. The result of multiple alignment was examined visually and adjusted manually using BioEdit program.

Procedures for construction of phylogenetic trees were as described in section A of this chapter. MEGA version 2 was used to construct NJ tree and PHYLIP package for MP and ML trees. For NJ and ML methods, transition/transversion ratio was set as 2.0 and K2P substitution model was employed. Sequence of Kunjin virus 3' UTR (L24512) was used as the outgroup to root the tree.

Chapter 4 Results

A. Isolation of JEV from the Field

Isolation of JEV From Mosquitoes

From July to August, 2000, mosquitoes were collected from a rural area in Tainan County. 14 pools of mosquitoes collected in July were homogenated and for virus isolation. Only one pool out of fourteen pools was positive in plaque assay. RNA extracted from this samples was subjected to RT-PCR and the result was positive (Fig 4.1). In addition to positive plaque assay and RT-PCR data, the C6/36 cells inoculated with this sample was re-tested by IFA and the result was also positive (Fig 4.2A). This strain was designated as TN207 because it was isolated in Tainan County in the year of 2000 (207:July (7), 2000). On the other hand, mosquitoes collected in August were grouped into 15 pools for virus isolation. One out of 15 pools was positive in plaque assay. RNA extracted from this sample was tested by RT-PCR and the result was positive (Fig 4.1). Result of IFA of this sample was also positive (Fig 4.2B). This virus strain was designated as TN208 (Tainan, August (8), 2000). Both JEV strains were isolated from pools consisted of alive mosquitoes without blood in their belly. However, because the species of these mosquitoes were not identified right away, origins of these two virus strains were pending. These two newly isolated strains were subsequently used in the following phylogenetic analysis.

Multiple Alignment of Sequences of E Gene

The sequence of E gene of TN207 was determined. We also collected sequences of E gene of Taiwanese JEV strains from GenBank for analysis. Strains with whole sequence of E were chosen because longer sequences may provide more information

and more reliable results of phylogenetic analysis (Graur et al., 1999). Nucleotide and deduced amino acid sequences of E gene were aligned using the algorithm of progressive alignment. Results of multiple alignment are shown in Fig 4.3 (nucleotide) and Fig 4.4 (amino acid). The percentage of identity of nucleotide sequences and amino acid among strains are presented in table 4.1 and table 4.2.

On average, nucleotide sequences of E gene among Taiwanese strains were highly homologous. The overall percentage of identity was higher than 93%, ranging from 93.6% to 100%. The highest identity of 100% was observed in two strains isolated from Taichung (NT109 in 1984 and NT113 in1985) and in two Changhua isolates (CH2195 in1994 and CH1949 in 1992). The lowest identity of 93.6% was observed between a human brain isolate from Taiwan and a mosquito isolate in China, CJN and P3 strains, with 96 nucleotide differences, 69 substitutions are transition and 27 are transversion. Substitutions of nucleotide are random within E gene and there is no heterogeneity of substitution rate over sites.

At the level of amino acid of E region, there are also high homologies among virus strains. The overall identity of amino acid sequences is over 93%, ranging from 100% to 93.7%. The highest similarity is 100% which was found quite frequently in table 4.2. The lowest identity of 93.7% was observed between CJN and p3 strains. The putative receptor binding motif, Arg-Gly-Asp (RGD, E387-389), is highly conservative among JEV strains except the p3 strain. In p3 strain, the non-polar glycine (G) at residue 388 changed into polar glutamic acid (E). The internal membrane fusion peptide, residue 101-108, is also conservative among all these JEV strains. However, there are no host-specific amino acid substitutions being observed among strains isolated from

mosquito, pigs, and human.

In conclusion, the overall nucleotide and amino acid sequences of E gene of Taiwanese and six reference JEV strains are highly homologous. CJN strain, a human brain isolate in 1998, is the most diverse compared one with other strains at both nucleotide and amino acid level.

Phylogenetic Analysis of E Gene

Twenty three Taiwan local strains isolated from different hosts and years and six reference strains were used to construct the phylogeny of E gene. Another 3 JEV strains isolated in recent four years were also subjected to the analysis. Trees build by neighbor-joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) are presented in Fig 4.5, Fig 4.6, and Fig 4.7.

Topologies of these trees are almost identical. All trees consistently classify JEV strains into three clusters. Bootstrapping for 1000 times was performed during the process of construction of trees by NJ and MP methods. Results of bootstrapping are relatively low at some branches. For example, bootstrap values of the branch separating viruses into two major groups (1 and 2) are 65% in NJ tree (Fig 4.5) and 23% (229 out of 1000 bootstraps) in the MP tree (Fig 4.6). However, the same topology and pattern of grouping can be observed in the phylogenetic tree build by maximum likelihood. Because identical topology was obtained by three different methods and programs, these results should be acceptable.

According to the phylogenetic trees, the cluster 1 contains strains isolated from

northern and central Taiwan including CH1949, CH2195, CJN, ML117, Ch392, CN80, CH109, T263 and S67. However, a southern Taiwan isolate, CC27, is also included in this cluster. Cluster 2 principally includes strains isolated from central and southern Taiwan including NT109, NT113, CC94, NT80, CH1392, T1P1 and TN207. Interestingly, T1P1, a strain isolated from Liu-Chiu islet located offshore the Pingtung county, is phylogenetically closest to TN207 which is a Tainan county isolate in 2000. Cluster 3 contains four early JEV human brain isolates, they are Nakayama, Ling, Beijing and TL, respectively. However, p3 strain, a China early isolate, forms a independent branch distinct from other three clusters. Although 4 early strains isolated from human brain form the cluster 3, there are no patterns associated with the time and hosts of isolation among virus strains in clusters 1 and 2.

B. Phylogenetic Analysis of 3' Untranslated Region

RT-PCR of 3' UTR

Results of RT-PCR are shown in figure 4.10. Sequences of 3' UTR of eleven local strains were amplified in present study. PCR products are visualized after the electrophoresis. The size of all PCR products are identical and no obvious deletion are observed (Fig 4.8).

Multiple Alignment of Sequences of 3' UTR

Sequences of 3' UTR of 11 JEV strains were determined and compared with sequences of other JEV strains obtained from different geographic areas for phylogenetic analysis. Nucleotide sequences of 3' UTR of total 29 JEV strains ranging from position 10392 to 10969 (numbering according to Sumiyoshi et al., 1987) were

aligned (Fig 4.9). The result showed an extensive homology of 3' UTR among JEV strains. Seventy nucleotides within the 5' terminal portion following the stop codon was found to be more variable than other regions. Deletions of 25, 15 and 13 nucleotides were found in Ling, FU and K94P05 strains, respectively. A small variable region was also found within nucleotide position 10642 to 10654.

Phylogenetic Analysis of 3' UTR

Phylogenetic trees of 3' UTR were constructed by NJ (Fig 4.10), MP (Fig 4.11), ML (Fig 4.12) methods are presented.

All phylogenetic trees buiid by different methods have the similar topology although the bootstrap values of MP tree are low. Taiwanese strains are clustered into one cluster. Two Japanese strains (JaOArS982 and JaGAr), three Chinese strains (SA-14, SA14-2-8 and SA14-2-2) and one Indian strain (GP78) were also included in this group. An early Chinese isolate, p3, forms a distinct branch by itself. Three early isolates from different geographic areas, Beijing (China), Ling (Taiwan) and Vellore (India) strains formed another group. However, the position of Nakayama strain is not consistent in phylogenetic trees constructed by different methods. The Australian strain, FU, and the Korean strain, K94P05, were not grouped with other strains and formed two distinct lineagess. Two Indian strains, Vellore and GP78 did not fell into the same group but each clustered with other Asian strains instead. It was found that the tree topology of 3' UTR of JEV doesn't correspond to a variety of parameters, such as the year of isolation, geographic origins of isolation or hosts of isolation.

Chapter 5 Discussion

In present study, we performed phylogenetic analysis of Taiwanese JEV strains using E gene and 3' UTR to reveal the evolutionary relationship among local strains. The high homology of nucleotide and amino acid sequence of E gene was observed. According to the phylogeny of E gene, Taiwanese JEV strains can be divided into three genotypes corresponding to the geographic distribution of isolation.

Our study examined the degree of homology of E gene among local and reference JEV strains. It was found that the homology ranging from 93.6% to 100% at nucleotide level and 93.7% to 100% at amino acid level. It is unusual that an RNA virus has so high similarity of nucleotide and amino acid sequence. The RNA virus has the error-prone RNA polymerase resulting in the high mutation rate thus forms a swarm of much variants during the process of infection which called quasispecies (Domingo et al., 1997; Eigen, 1993). Although JEV is RNA virus, the E gene is very stable even when strains isolated from different geographic areas at interval of several decades. For example, the amino acid sequence of E region of CC94, a Taiwanese strain isolated in 1984, is completely identical to a China strain SA14 isolated in 1954 (table 4.2). Yang and colleagues (2000) calculated the ratio of nonsynonymous substitution rate (d_N) and synonymous substitution rate (d_S) of E gene of JEV to understand the selection pressure on this region. They identified no positive selection pressure but discovered a strong purifying selection pressure operating on the E gene. Their result suggested that when a mutation resulting in a change of amino acid then this altered phenotype will not be fixed and may be deleterious to the individual organism. The E protein has the most important biological functions associated with viral entry and receptor binding

(Chambers et al., 1990; Rice, 1996). To maintain these functions, too many mutations will not be allowed during the evolution of JEV and this may lead to the high homology of E gene. The constraint proposed by these functions may be the possible reason for the conservation of E protein.

In this study, we used E gene of whole length comprising 1500 nucleotides to construct the phylogeny of Taiwanese JEV strains. The phylogeny of E gene separated viruses into three clusters. The first cluster contained strains principally isolated from northern Taiwan and the second cluster included strains isolated from southern Taiwan. Early isolates Ling and TL with two vaccine strains Nakayama and Beijing strains formed the third group. Our result is similar to the phylogeny of C/prM genes by Jan et al. (2000). However, the previous work by Jan and colleagues used only 280 nucleotides of the region of the junction between C and prM gene to build the phylogenetic tree. It has been proposed and cautioned that short sequences (<300 nt) used in the phylogenetic analysis of flavivirus tend to produce unreliable information (Kuno et al., 1998; Westaway et al., 1997). Therefore, our study of the phylogenetic analysis using different tree constructing methods and programs produced consistent results so the phylogeny of whole E gene should be more reliable and provide more accurate information.

Three newly isolated strains, i.e. CJN, T1P1 and TN207, were included in these analyses. CJN strain was isolated in Taoyuan and grouped with other northern Taiwan strains. TN207 and T1P1 were isolated in southern Taiwan and clustered with other strains forming the second group. T1P1 was isolated from the Liu-Chiu islet offshore Pingtung county. The result of phylogenetic analysis suggested that T1P1 strain might

originate from a virus strain circulating in southern Taiwan but how did this virus move from the Taiwan island to Liu-Chiu islet is still a mystery.

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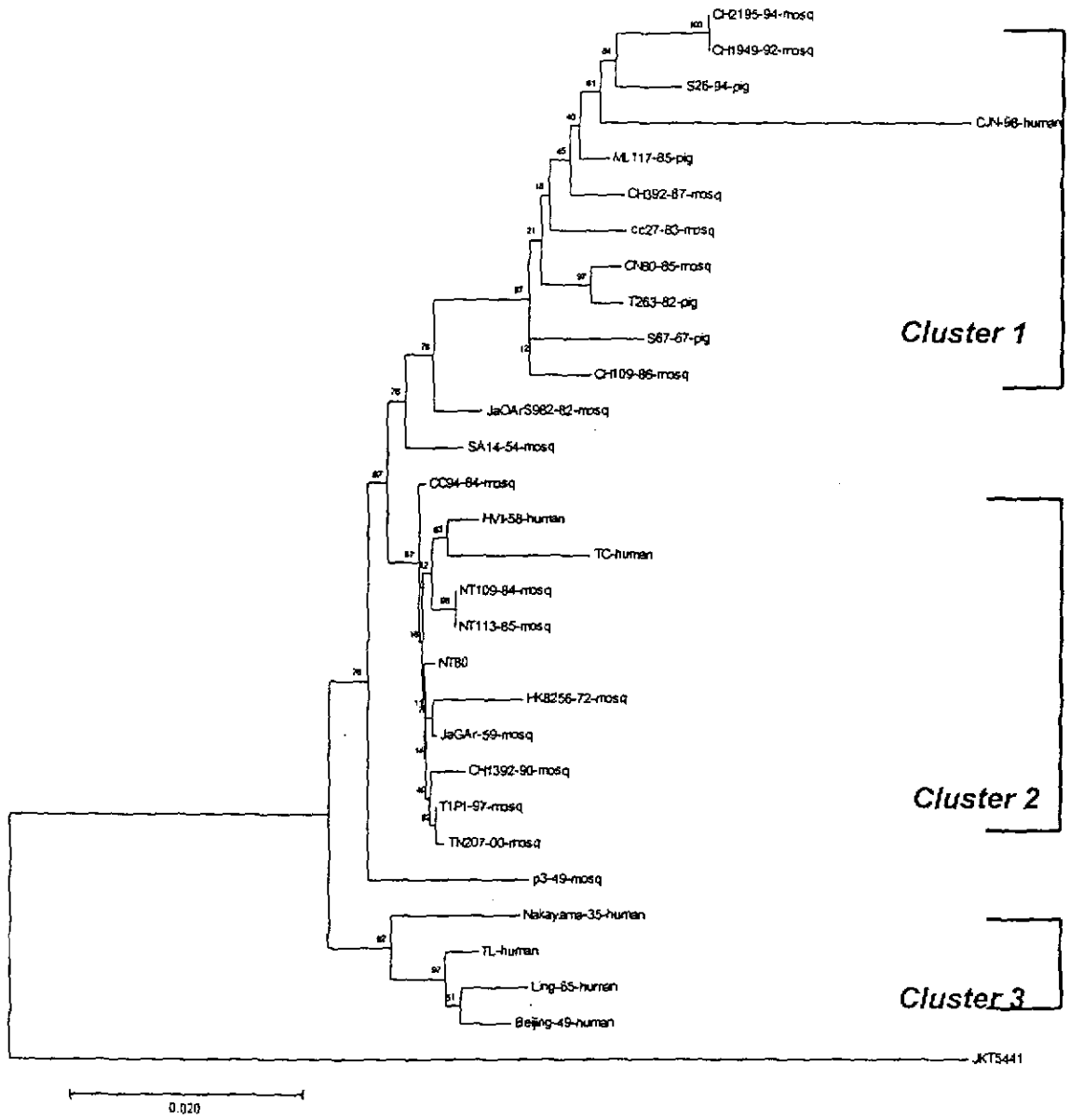

Fig 4.5

Phylogenetic tree of E gene constructed by neighbor-joining method; bootstrapped for 1000 times. Bootstrap values are presented above branches of the tree. Names of virus strains are followed by the year of isolation and host. Mosq: mosquito.

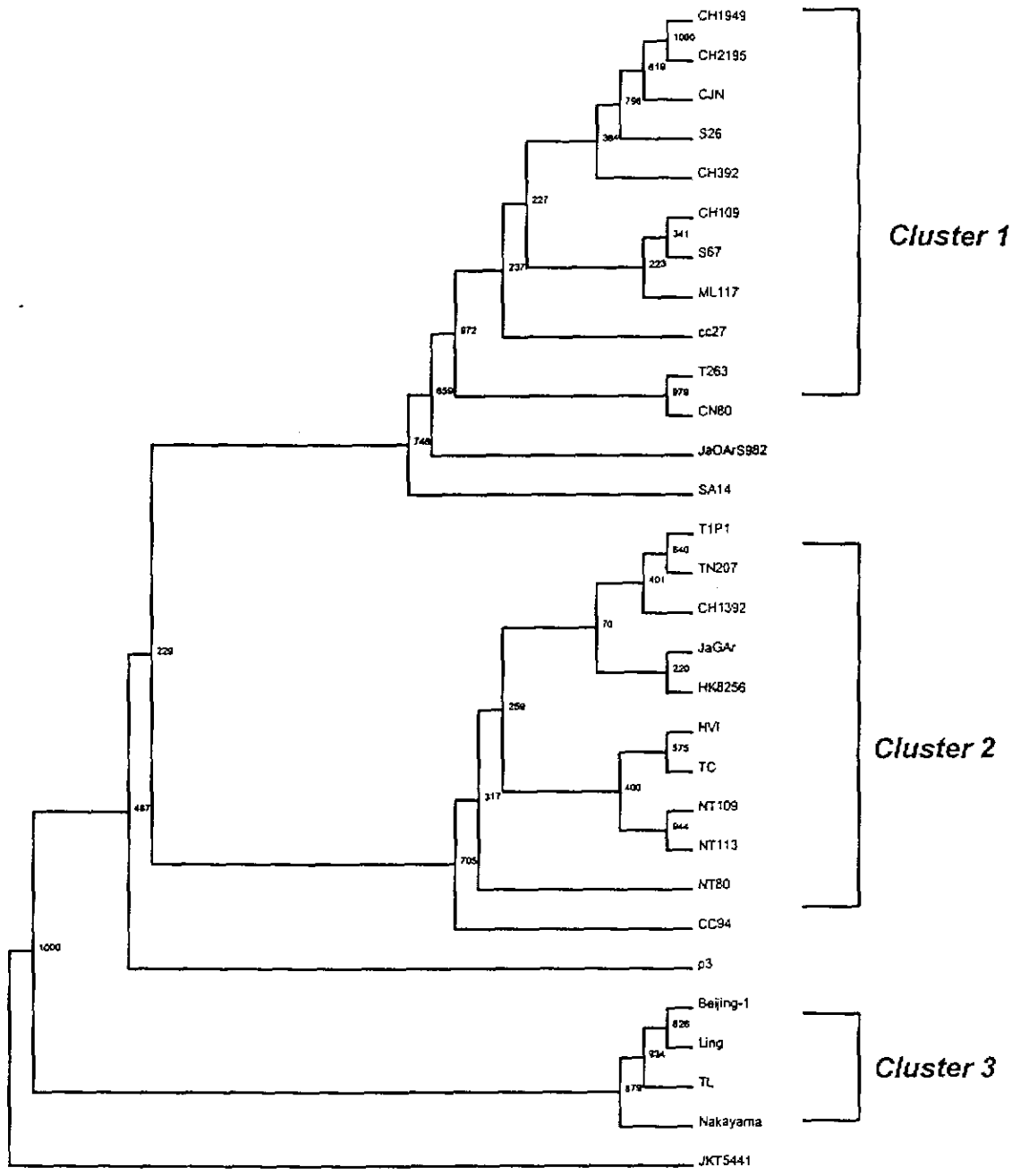

Fig 4.6

Phylogenetic tree of E gene constructed using maximum parsimony method by DNAPARS program. Bootstrap values are labeled above branches of the tree.

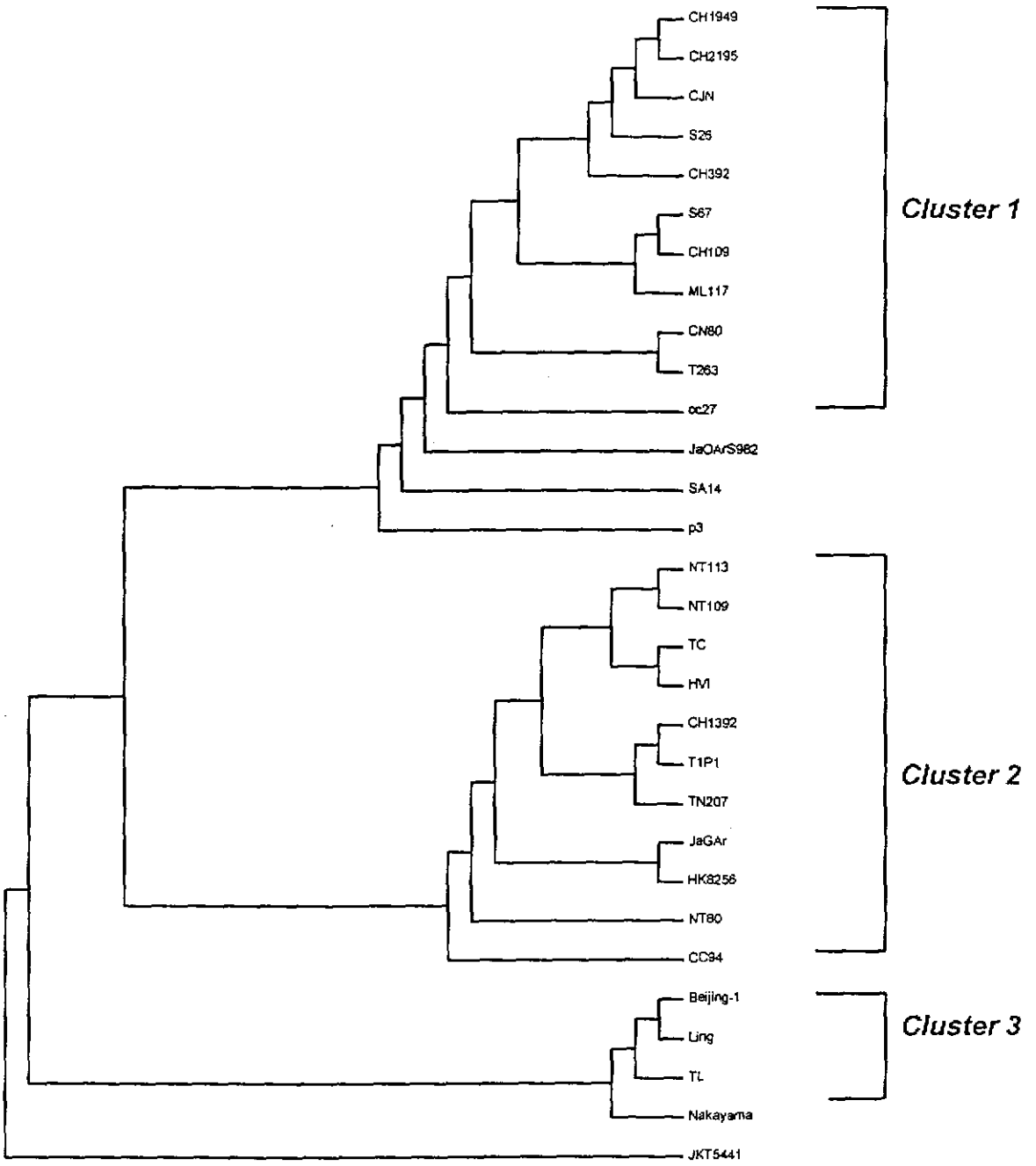

Fig 4.7

Phylogenetic tree of E gene constructed using maximum likelihood method by DNAML program in PHYLIP package.

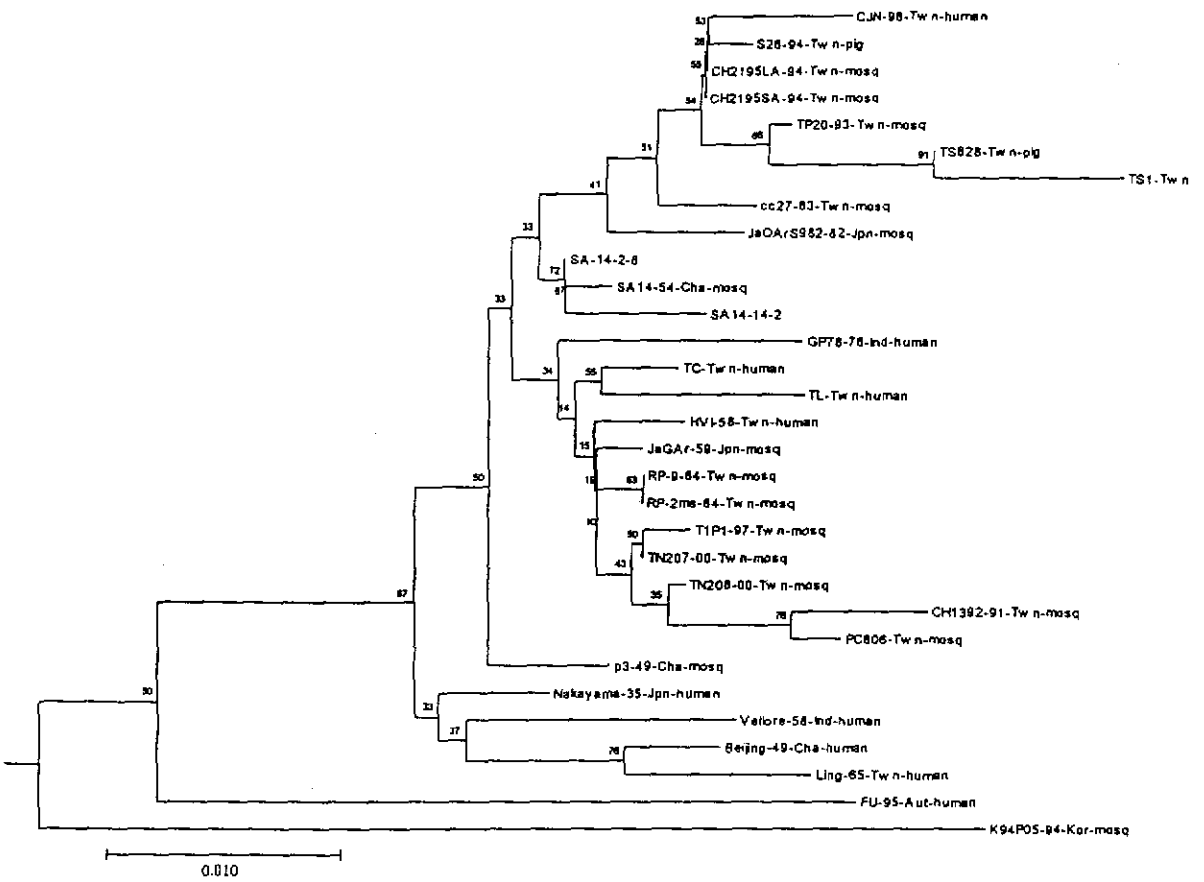

Fig 4.10

Phylogenetic tree of 3' UTR of JEV using neighbor-joining method by MEGA v2 program. Bootstrapped for 1000 times and rooted by Kunjin virus (not shown). Strain names are followed by year of isolation, location of isolation and host of isolation. Tw: Taiwan; Jpn: Japan; Cha: China; Ind: India; Aut: Australia; Mosq: mosquito.

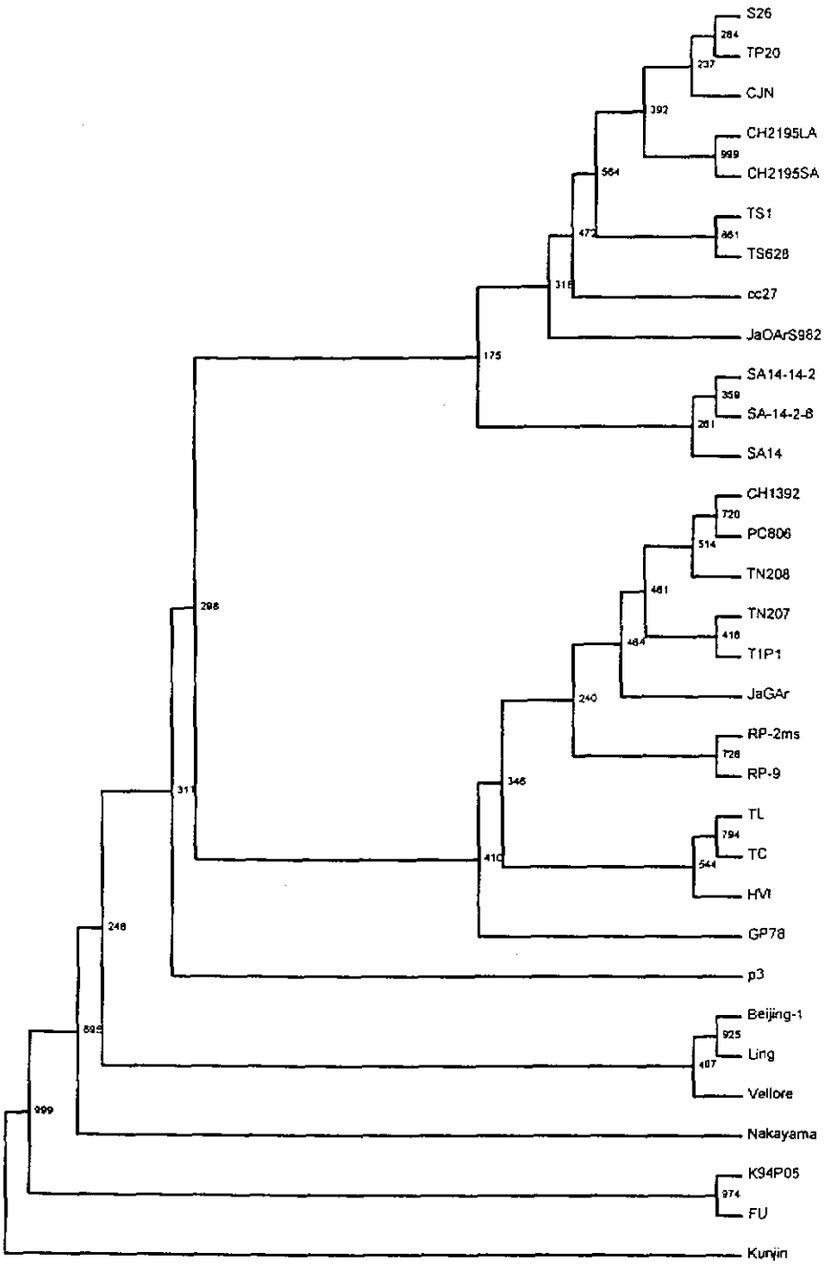

Fig 4.11

Phylogenetic tree of 3'UTR of JEV using maximum parsimony method by DNAPARS. Bootstrapped for 1000 times.

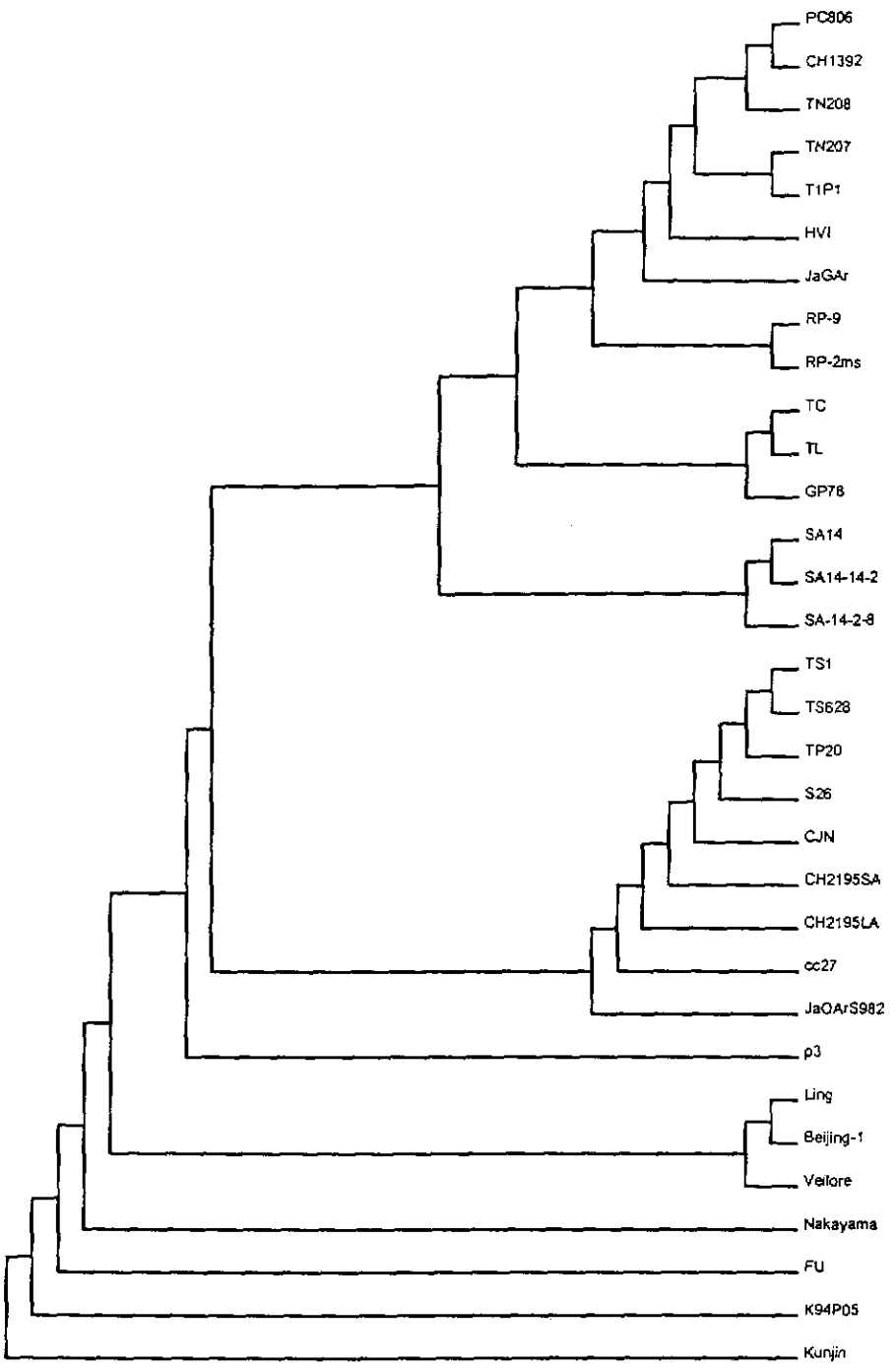

Fig 4.12

Phylogenetic tree of 3' UTR using maximum likelihood method by DNAML.