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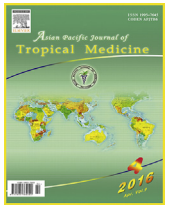

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Phylogeny of Murray Valley encephalitis virus in Australia and Papua New Guinea

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ABSTRACT

Objective: To study the genetic diversity of Murray Valley encephalitis virus (MVEV) in Australia and Papua New Guinea.

Methods: MVEV envelope gene sequences were aligned using Clustal X and manual editing was performed with Bioedit. ModelTest v. 3.7 was used to select the simplest evolutionary model that adequately fitted the sequence data. Maximum likelihood analysis was performed using PhyML. The phylogenetic signal of the dataset was investigated by the likelihood mapping analysis. The Bayesian phylogenetic tree was built using BEAST.

Results: The phylogenetic trees showed two main clades. The clade Ⅰ including eight strains isolated from West Australia. The clade Ⅱ was characterized by at least four epidemic entries, three of which localized in Northern West Australia and one in Papua New Guinea. The estimated mean evolutionary rate value of the MVEV envelope gene was 0.407×10^{-3} substitution/site/year (95% HPD: 0.623×10^{-4}–0.780×10^{-3}). Population dynamics defines a relative constant population until the year 2000, when a reduction occurred, probably due to a bottleneck.

Conclusions: This study has been useful in supporting the probable connection between climate changes and viral evolution also by the vector point of view; multidisciplinary monitoring studies are important to prevent new viral epidemics inside and outside new endemic areas.

1. Introduction

Murray Valley encephalitis virus (MVEV) is a zoonotic *Flavivirus*, member of the Japanese encephalitis serocomplex, which is actually endemic in Northern West Australia and Papua New Guinea (PNG). MVEV primarily exists in a transmission cycle between *Culex annulirostris* and birds. It is considered the causal agent of Murray Valley encephalitis (previously known as Australian encephalitis) and humans are generally considered to be dead-end hosts [1]. The evolution of MVEV in Australia and PNG has proceeded independently and the MVEV strains in circulating Australian are not systematically re-seeded from endemic regions of PNG. Major outbreaks of MVEV occurred in Australia in 1951, 1956 and 1974, with the virus first being isolated during the 1951 outbreak. MVEV may have caused

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earlier outbreaks of Australian 'X' disease in 1917–1918, 1922 and 1925 [2]. In the most recent outbreak of MVEV in 1974, 58 cases of encephalitis were identified [3], indicating the significance of this disease despite the infrequency of epidemics.

The majority of infections with MVEV are asymptomatic or cause a non-specific febrile illness usually accompanied by headache, myalgia and occasionally rash [6]. However, approximately 1:150 to 1:1 000 infections with MVEV, clinical encephalitis results [4]. After the incubation period of up to four weeks, clinical cases usually present with fever (commonly accompanied by convulsions in children), headache, malaise, and altered mental status, which may be followed by progressive neurological deterioration, parkinsonian tremor, cranial nerve palsies, peripheral neuropathy, coma, flac-cid paralysis, and death [4].

Since the mid-1970s a surveillance program for MVEV has been in place in West Australia. Surveillance using sentinel chicken flocks is a good system to monitor the spread of infection. In addition, mosquito collection trips are undertaken annually during the latter part of the wet season. Opportunistic mosquito collections are conducted in other regions of Northern West Australia, generally following the detection of MVEV seroconversions in sentinel chickens. Prevention and control of MVEV are still a challenge because include difficulties in controlling mosquito numbers during periods of extensive flooding, and a lack of other prophylactic or treatment measures for MVEV. Mosquitoes are processed for virus isolation to determine infection rates in mosquitoes and to investigate which mosquito species are involved in MVEV transmission [4]. The aim of this study was to define the phylogenic analysis of MVEV in Australia and Papua New Guinea, under a time-scale and a population dynamics, considering the recent GenBank entries not previously subjected to phylogenetic studies [5].

2. Materials and methods

The dataset consisted in 45 sequences of envelope (*E*) and polyprotein gene, downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and isolated in different regions of Northern West (NW) Australia, except for the two strains isolated in PNG, and selected on the basis of the following criteria: (1) all the available sequences (2) already published in peer-reviewed journals and with (3) known sampling date and location.

All MVEV *E* gene sequences were aligned using Clustal X and manual editing was performed with Bioedit to a final alignment length of 462 bp [6] after removing gaps. ModelTest version 3.7 was used to select the simplest evolutionary model that adequately fitted the sequence data [6]. The phylogenetic signal of the dataset was investigated by using of the likelihood mapping analysis of 10 000 random quartets by using TreePuzzle as already described [7]. Groups of four randomly chosen sequences (quartets) were evaluated using Maximum likelihood. For each quartet, the three possible unrooted trees were reconstructed under the selected substitution model. If more than 30% of the dots fall into the center of the triangle, the data are considered unreliable for the purposes of phylogenetic inference. The best-fitting nucleotide substitution model was tested with a hierarchical likelihood ratiotest implemented in Modeltest software v. 3.7 [6]. The statistical robustness and reliability of the branching order within the phylogenetic tree was confirmed by the approximate likelihood-ratio test approach; all calculations were performed using PhyML [8]. Statistical support for specific clades and clusters was assessed by bootstrap analysis considering bootstrap values > 70%. The dated tree, was estimated by using a Bayesian MCMC approach (Beast v. 1.7.4, http://beast.bio.ed.ac.uk/) [9] implementing a HKY using both a strict and an uncorrelated log-normal relaxed clock model.

As coalescent priors, we compared three parametric demographic models of population growth (constant size, exponential, expansional) and a Bayesian skyline plot (BSP, a non-parametric piecewise-constant model). The Bayes factor (BF) analysis showed that the relaxed clock fitted the data significantly better than the strict clock (2 lnBF = 8 018 for relaxed clock). Under the relaxed clock the BF analysis showed that the exponential growth model was better than the other models (2 lnBF > 7). Chains were conducted for at least 50×10^6 generations, and sampled every 5 000 steps. Convergence was assessed on the basis of the effective sampling size after a 10% burn-in using Tracer software v. 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Only parameter estimates with effective sampling size's of >200 were accepted. Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals and the best fitting models were selected by a BF (using marginal likelihoods) implemented in Beast [9]. The evolutionary distances among different groups were calculated with MEGA6 software [10] using p-distance model. It was performed two different type of evolutionary distance: the first between clade Ⅰ and clade Ⅱ; the second between the subclades A1, A2 and B. The demographic history was also analyzed on the MVEV *E* gene sequences by performing the Bayesian skyline Plot to give an interpretation of the phylodynamic feature of the dataset.

3. Results

The phylogenetic noise of the dataset is shown in likelihood mapping (Figure 1). The percentage of dots falling in the central area of the triangles was 5.6%, and as the dataset showed no more than 30% of noise, it contained sufficient phylogenetic signal.

The phylogenetic tree performed with PhyML 3.0, shows two clades highly significant under a geographic selection (Figure 2).

The clade Ⅰ including eight strains isolated from West Australia. The clade Ⅱ was characterized by at least four epidemic entries, three of which localized in NW Australia and one in Papua New Guinea. The genetic distance between the two clades (0.137 $P = 0.013$) is a good suggestion for the hypothesis of two distinct epidemic entries. The Bayesian phylogenetic tree performed using a Bayesian MCMC approach is show in Figure 3.

The estimated mean evolutionary rate value of the MVEV *E* gene was 0.407×10^{-3} substitution/site/year (95% HPD: 0.623×10^{-4}–0.780×10^{-3}). The root of the tree dated back to the year 1759. Two main clade were identified, clade Ⅰ posterior probability (*PP*) = 0.99 including the strains from Western Australia and dated back to the year 1948 (95% HPD: 1875–1995) and clade Ⅱ which included the strains from Papua New Guinea and the Australian coasts (*PP* = 0.63) which dated to 1826 (95% HPD: 1643–1980).

Inside clade Ⅰ, subclade Ⅰa (*PP* = 0.81) dated to the year 1966 (95% HPD: 1917–1997) and subclade Ⅰb (*PP* = 0.96) to the year

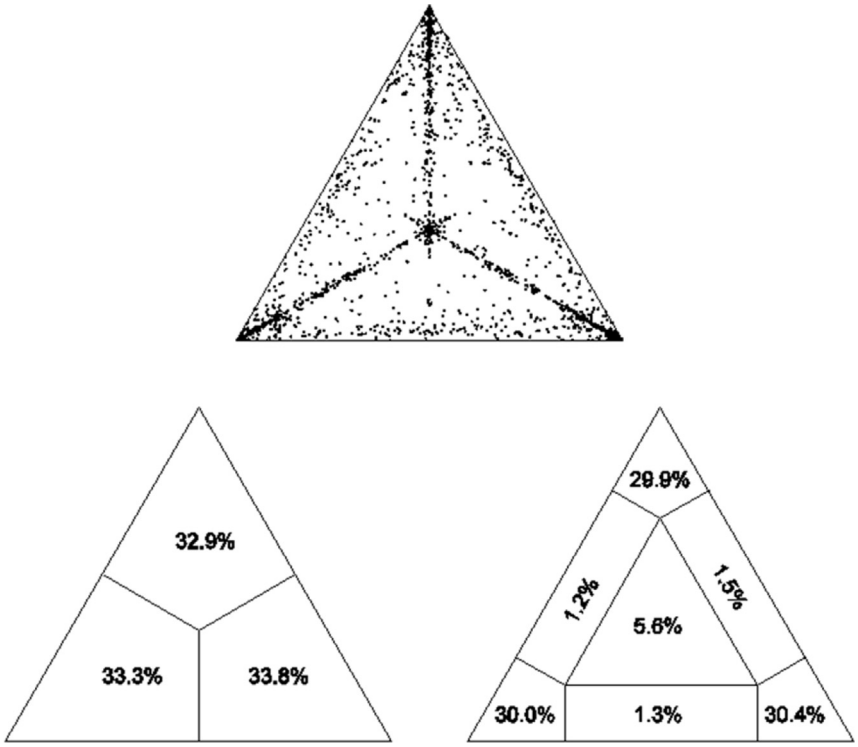

Figure 1. Likelihood mapping of MVEV *E* gene dataset.
The dots inside the triangles represents the likelihood of the possible unrooted topologies for each quartet. Numbers indicate the percentage of dots in the centre of the triangle corresponding to phylogenetic noise (star-like trees).

1980 (1978–1999). Inside clade Ⅱ, a major subclade (Ⅱ a), which dated back to the year 1939 (95% HPD: 1860–1989), can be found. Subclade Ⅱa was characterized by different epidemic entries registered in different time intervals. One epidemic entry was in 1980 (95% HPD: 1938–2006) and was determined by two strains isolated from Papua New Guinea (labeled as PNG65298 and PNG69198) that grouped together, separately respect to the other sequences. Another entry was in 1952 (95% HPD: 1889–1990) and included the majority of the strains of subclade Ⅱa.

Specifically, inside the clade which dated back to 1969 (95% HPD: 1928–1990) a major clade (A) and a minor cluster (B) can be described. Inside clade A, two different clusters (A1 and A2) were evident. Cluster A1 (*PP* = 0.98) seems to have a spreading region around the West Cape York in the NE Australia, and is datable approximately in the year 1992 (95% HPD: 1981–2000). Cluster A2 (*PP* = 0.63) dated to 1996 (95% HPD: 1983–2005) included strains localized in the New South Wales region (SE Australia) and represents a separate and more recent entry of

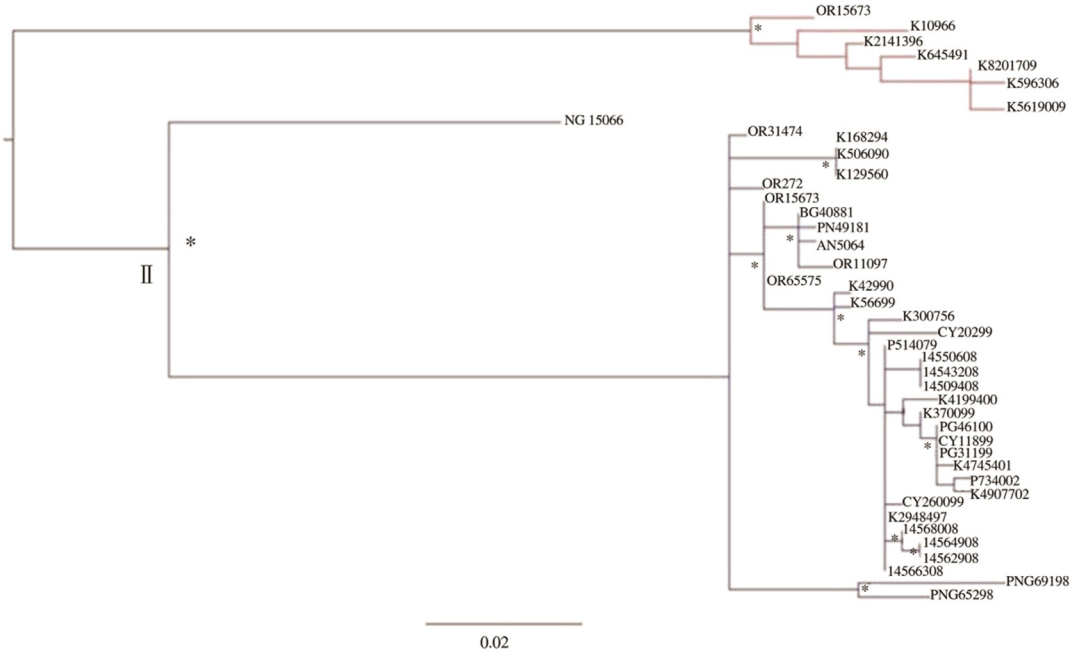

Figure 2. Maximum likelihood tree of the 46 MVEV *E* gene sequences.
*: significant statistical support for the clade subtending that branch (bootstrap support > 70%). The scale bar indicates 2% of nucleotide divergence.

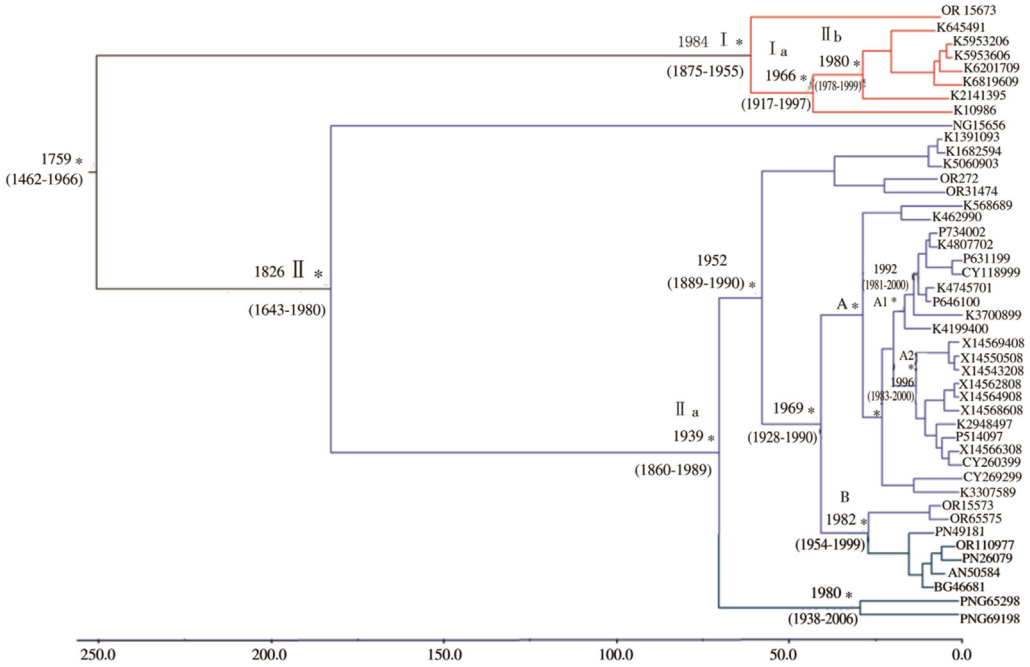

Figure 3. Bayesian phylogenetic tree of the MVEV *E* gene sequences.
In different colors: clade Ⅰ and Ⅱ; subclades A1, A2 and B. Time scale covers 250 years in the past beginning from year 2009 (last date of isolation strain in the dataset). The time of the most recent common ancestor, with the credibility interval based on 95% HPD, was reported in years. Scale years is reported at the bottom of the figure.

MVEV. Cluster B (*PP* = 0.78) dated back to the year 1982 (95% HPD: 1954–1999) and included strains from NW Australian region. The genetic distances among these groups are shown in Table 1. The mean genetic distance showed that subclade A1 and A2 are closer than the other subclades (0.010 *vs*. 0.023 and 0.021).

The BSP representing the estimates changes in effective population size through time is reported in Figure 4. The analysis of the BSP defined a relative constant population grow until the year 2000, when a strong reduction probably occurred due to a bottleneck.

4. Discussion

This phylogenetic study provides different suggestions about the diffusion and the biological history of MVEV in Australia and PNG. However previous studies highlighted the geographical distribution of this virus in Australia and PNG, the additionally evidences of the time-scale analysis define a relative constant and well-localized presence of MVEV in these regions. The assumption that a prevalent distribution of the principal vector (*Culex annulirostris*) is defined by two strong conditions such as relative ecosystem and density population of humans in wetlands. These two conditions suggested that this virus have maintained a constant fitness and numerosity of its population for a lot of decades.

As already described [5] the hypothesis of wide-spreading in Australia of MVEV is probably due to two factors: a first contribution of viremic migratory water-birds [11,12] and a second contribution of wind-borne infected mosquitoes [13,14]. But since reduced density of waterbirds might explain the fewer MVEV case numbers in South-eastern Australia in 2011 compared to 1974, in spite of there being similar climatic conditions [15].

This is the first study that estimated the mean evolutionary rate of MVEV *E* gene sequences and that reported the time-scaled phylogeny and phylodynamics of this virus. We estimated a mean evolutionary rate of MVEV *E* gene of 0.407×10^{-3} substitution/site/year (95% HPD: 0.623×10^{-4}–0.780×10^{-3}). Our time-scaled phylogeny reconstruction showed two main clades, labeled Ⅰ and Ⅱ, which dated back respectively to the year 1948 (95% HPD: 1875–1995) and 1826 (95% HPD: 1643–1980) indicating two distinct epidemic entries of this virus.

Moreover the phylodynamics analysis performed in the present study showed a relative constant population growth until the year 2000, followed by a reduction, probably due to climatic changes and decreasing circulation of the vectors. Climatic changes can gradually modify the ecosystem of Culex and waterbirds [16]. In the endemic regions of NW Australia the presence of MVEV population is only partially influenced by variation in rain-falling: in wetlands the intensity of rain-falling could have a detrimental effect to *Culex* larvae, decreasing the vector fitness; in arid grasslands an initial increasing of *Aedes* can leads to a more extensive viral diffusion.

The North to South diffusion of MVEV could probably increase from stronger tropical cyclone in northern WA activity. However, it is likely that some areas will have increases in

Table 1
Estimates of evolutionary divergence over sequence pairs between subclades groups.

	Subclade A1	Subclade A2	Subclade B
Subclade A1			
Subclade A2	0.010 (P = 0.003)		
Subclade B	0.023 (P = 0.006)	0.021 (P = 0.006)	

The number of base differences per site from averaging over all sequence pairs between suclades A1, A2, and B are shown. Standard error estimates were obtained by a bootstrap procedure (1 000 replicates). The analysis involves 30 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions with less than 95% site coverage were eliminated.

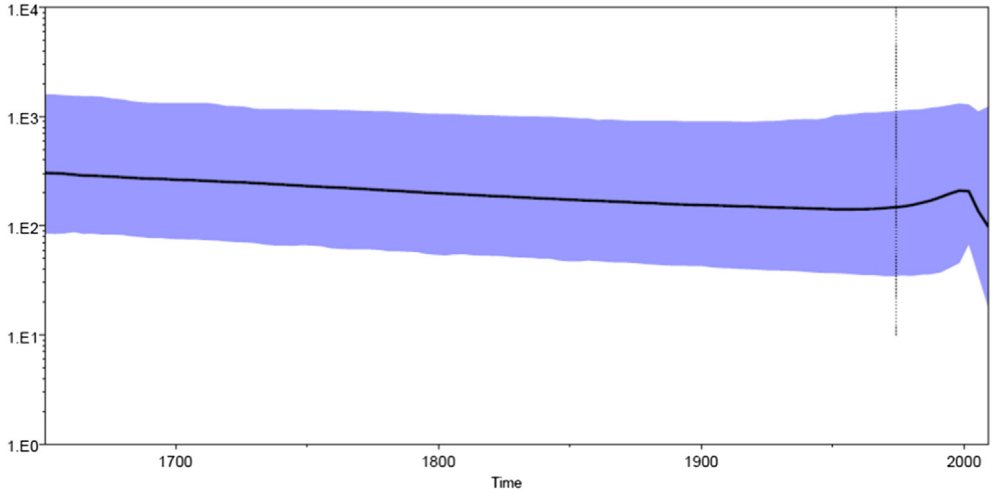

Figure 4. BSP of the MVEV *E* gene.
The effective number of infections is reported on the Y-axis. The colored area corresponds to the credibility interval based on 95% HPD.

arbovirus activity and human infection with predicted climate change, but risk of increased transmission will vary with locality, vector, host and human factors.

In conclusion, our sophisticated phylogenetic methods have been useful in supporting the probable connection between climate changes and viral evolution also by the vector point of view. Moreover, considering climate changes and emerging importance of MVEV (as a JEV family member), it will be necessary to investigate and monitor this kind of outbreaks in order to prevent new viral epidemics inside and outside endemic areas.

Conflict of interest statement

We declare that we have no conflict of interest.

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