Identification of Eilat virus and prevalence of infection among Culex pipiens L. populations, Morocco, 2016

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Highlights:

- Eilat virus is circulating in Morocco
- Eilat virus is present in *Culex pipiens* mosquito populations
- The detection of Eilat virus in larvae suggests its maintenance in the environment either through vertical transmission or through horizontal infection of larvae in breeding sites

Abstract:

Eilat virus (EILV) is described as one of the few alphaviruses restricted to insects. We report the record of a nearly-complete sequence of an alphavirus genome showing 95% identity with EILV during a metagenomic analysis performed on 1,488 unblood-fed females and 1,076 larvae of the mosquito *Culex pipiens* captured in Rabat (Morocco). Genetic distance and phylogenetic analyses placed the EILV-Morocco as a variant of EILV. The observed infection rates in both larvae and adults suggested an active circulation of the virus in Rabat and its maintenance in the environment either through vertical transmission or through horizontal infection of larvae in breeding sites. This is the first report of EILV out of Israel and in *Culex pipiens* populations.

Keywords:

Eilat virus, Alphavirus, *Culex pipiens*, Morocco

Introduction

Eilat virus (EILV) is a mosquito-borne alphavirus isolated from *Anopheles coustani* individuals in Israel in 1984 (Nasar et al., 2012). Contrary to most alphaviruses known to date, EILV is thought not to infect vertebrates (Nasar et al., 2012, Nasar et al., 2014). This restriction in host range has promoted the development of EILV recombinants as potential vaccines against other alphaviruses pathogenic to humans and livestock (Erasmus et al., 2018). Moreover, infection by *Chikungunya virus* is delayed in EILV-infected *Aedes aegypti*

mosquitoes, suggesting a possible role of EILV in limiting transmission of alphaviruses pathogenic to humans (Nasar et al., 2015). Despite these potential applications of EILV, little is known on its life cycle in natural settings, like its geographical and host ranges or its infection rates in the field, beyond the first description (Nasar et al., 2012).

Materials and methods

1- Mosquito sampling

We performed a mosquito survey in the region of Rabat (Morocco) from May to September 2016. Larvae and adult mosquitoes were collected at 3 localities: Vet school (IAV: 33,97479|-6,86957), Ounk Jmel (OJ: 34,005117|-6,772463) and Oulja (OL: 34,03333|-6,79999). Adults were collected using CO_2-baited traps set up for one night and larvae using classical larval dippers. All sites were sampled at the same dates, during 10 days per month. Larvae and adults were morphologically identified. Pools of approximately 30 dry adults were stored at -80°C, whereas pools of around 50 larvae were stored in 70% ethanol at -20°C.

2- Metagenomics approach

Each pool was resuspended in 500µl 1X PBS buffer with two ice-cold steel bearing balls (3-mm diameter) and grinded twice for 30 seconds at 30 Hz using a TissueLyser II (Qiagen). Aliquots from homogenized pools of a given stage (i.e. adult female, L3/L4 larva or L1/L2 larva) and site were pooled to generate nine samples. To remove non-encapsidaded nucleic acids, homogenates were digested with a nuclease cocktail consisting of 20 U of Exonuclease I (ThermoScientific), 5 U of RNase I (ThermoScientific) 25 U of benzonase (Merck Chemical) and 20 U of turbo DNase (Ambion) at 37°C for 60 minutes in 1 X DNase buffer (Ambion). Nucleic acids were then extracted using the Nucleospin RNA virus kit (Macherey Nagel) according to the manufacturer's protocol. In parallel to this extraction of RNA from

nuclease-treated homogenates, another aliquot of each homogenated pool was used to isolate total RNA using the Nucleospin RNA virus kit (Macherey Nagel).

cDNA was prepared in a reverse transcription reaction using the RevertAid First Strand cDNA synthesis kit (ThermoScientific) and using 5 µM of the 454-E-8N primer. This primer has been described previously (Victoria et al., 2009) and consist of a fixed region followed by a randomized octomer at the 3' end (CAT CAC ATA GGC GTC CGC TG NNNNNNNN). Double-stranded DNA was generated using the klenow fragment polymerase (ThermoScientific). A first PCR amplification was performed using the 454-E primer (Victoria et al., 2009) and the Phusion High-Fidelity DNA Polymerase kit (ThermoScientific) followed by a second PCR to ligate Illumina adapters (Kozich et al., 2013). Amplicons were purified using a magnetic bead capture (Agencourt). The size of PCR products was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies). Library concentration was estimated with the Library Quantification kit (Clontech Laboratories) according to the manufacturer's protocol. Libraries were then pooled in equimolar concentrations and sequenced in a MiSeq platform (Illumina; 250-bp reads in paired-end mode). After quality filtering and adaptor trimming with Cutadapt 1.6 (Martin, 2011), we de-novo assembled reads using Megahit version 1.0 (doi: 10.1093/bioinformatics/btv033) followed by an assembly of the resulting contigs with CAP3 (Huang, 1999). A homology search was done with the resulting contigs using Diamond (Buchfink et al., 2015) against the full GenBank protein database.

3- Phylogenetic analysis

The full-length sequences encoding both the nonstructural protein (nsP) and the structural protein (sP) of EILV-Morocco and 44 alphavirus sequences were aligned using the Clustal W program implemented in MEGA version 7 (Kumar et al., 2016). The intergenic regions (5'

and 3’ UTRs), the hypervariable domain of nsP3 and the N terminus of capsid sequences were excluded from the alignments, since these regions display considerable sequence divergence.

Phylogenetic trees were constructed using a maximum-likelihood method using the MEGA version 7.0 Software (Kumar et al., 2016). Confidence values for the tree clades were provided by bootstrap analysis of 1,000 datasets.

4- EILV prevalence

PCR primers were designed to detect EILV nucleic acids. The primer sequences were situated in a region in the structural polyprotein region conserved in the reference EILV sequence and the consensus sequence of EILV-Morocco. cDNA was synthesized from 10 µl of total RNA using the RevertAid First Strand cDNA synthesis kit (ThermoScientific) and random hexamer primer according to the manufacturer’s protocol. Then a SybrGreen qPCR assay coupled to melting curve analysis using the SensiFAST™ SYBR No-ROX kit (Bioline) was developed and performed on ABI 7500 Fast Real-Time PCR (Applied Biosystems). Amplification reaction mixture contained 2 µl of cDNA, 10 µl MasterMix 2x Sybr, 6.4 µl nuclease-free water, and 0.8 µl of primers F (5’-CCGCACCAATAACAACC-3’) and R (5’-CGATTTCCTGCCACCAC-3’) at 10 µM each. The cycling protocol started with a holding stage involving 2 min at 95°C, followed by 40 cycles of 95°C for 15 s and 58°C for 20 s. Melt curve was carried out over the range from 60°C to 95°C with a temperature increment of 0.1°C per second. Only cycle threshold [Ct] values below 36 and Tm of 85 ± 0.2°C were considered positives.

Results

A total of 1,488 unblood-fed females and 1,076 larvae of the mosquito *Culex pipiens* were collected at the three sites. Pool sizes ranged from 23 to 35 individuals for adults (49 pools),

from 53 to 64 for L1/L2 larval stades (11 pools) and from 27 to 35 for L3/L4 larval stades (14 pools) (Table 1).

We used a metagenomic approach to characterize the virome in the collected individuals. Nine libraries were generated, each library originating from RNA extractions from either larvae or adults from a given site. Among the virus-like sequences, we found a nearly-complete genome of an alphavirus (98.9% genome coverage) showing a 95% identity with the EILV genome collected in Israel in 1982 (accession number JX678730). EILV-like reads were not distributed homogeneously among libraries. Most of the EILV-like reads (99.999% out of 191,997 reads) were present in only two libraries, the libraries from adults from IAV and OJ sites. None of the other libraries provided a read number above an arbitrary detection threshold of ten reads.

We generated phylogenies based on nsP and sP ORFs to further define the taxonomy of this new alphavirus genome, tentatively named EILV-Morocco (Genbank accession number: MH921919). EILV-Morocco was classified into the same phylogroup with EILV and the two other alphaviruses with an arthropod-restricted host range, Taï Forest alphavirus (TALV) (Hermanns et al., 2017) and Mwinilunga alphavirus (MWAV) (Torii et al., 2018) (Fig.1). Genetic distance based on the full genome, using maximum likelihood methods between EILV-Morocco and EILV was 0.066 supporting that EILV-Morocco was a variant of EILV.

Given the absence of data on mosquito infection rates of EILV in nature, we analyzed the prevalence of EILV in the sampled mosquito populations. We developed a qRT-PCR that found 27 EILV-positive pools out of 49 pools of adults (55.1%) and 5 EILV-positive pools out of 25 pools of larvae (20.0%) (Table 1). Prevalence data from larvae could be biased due to the low RNA quality of larvae samples detected with capillary electrophoresis (Agilent 2100 Bioanalyzer; not shown) and should be interpreted cautiously. Quantitative analyses of infection rates are thus presented only for adult data.

Taking the three populations together, the EILV minimum infection rates was 1.81% (95% confidence interval (95% CI) = 1.14-2.50%) in adult females and the average true infection rate, calculated using a maximum likelihood method (Walter et al., 1980) implemented in the *binGroup* R package (Bilder et al., 2010) was 2.55% (95% CI = 1.72-3.71%). However, infection rates largely differed among the three sites, with a true infection rate of 4.05% in IAV and no positive pool detected in OL (Table 1).

Discussion

Our study described the first detection of EILV outside Israel, thus enlarging its known geographical range. In addition, this work is the first detection of EILV in wild populations of *Culex pipiens*, a vector of several arboviruses (Brugman et al., 2018, Amraoui et al., 2012). The observed infection rates imply an active circulation of the virus in the region of Rabat. Moreover, EILV detection in both larvae and adults may be indicative of vertical or interstadial transmissions. It would be interesting to determine whether EILV circulation plays a role in the efficiency of vector transmission of the arboviruses present in the region such as *West Nile virus* (El Rhaffouli et al., 2012). Further epidemiological and functional studies investigating the presence of the virus in other regions of Morocco as well as in other mosquito species are necessary.

Funding:

This study was partially funded by EU grant FP7-613996 Vmerge and is catalogued by the VMERGE Steering Committee as Vmerge025 (http://www.vmerge.eu). The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views of the European Commission.

Competing interests:

The authors declare that there are no conflicts of interest.

Fig.1. Phylogenetic analysis of Eilat-Morocco virus and representative alphaviruses. Phylogenetic tree were constructed using the maximum likelihood method in MEGA, version 7.0 software with 1000 bootstrap replicates based on multiple alignment of amino acid sequences of the non structural protein **(a)** and the structural protein **(b)**. Bootstrap values greater than 50 are shown near the branch nodes and the scale bar indicates the number of substitutions per site. The tree were rooted using the Salmon Pancreas disease virus sequence.

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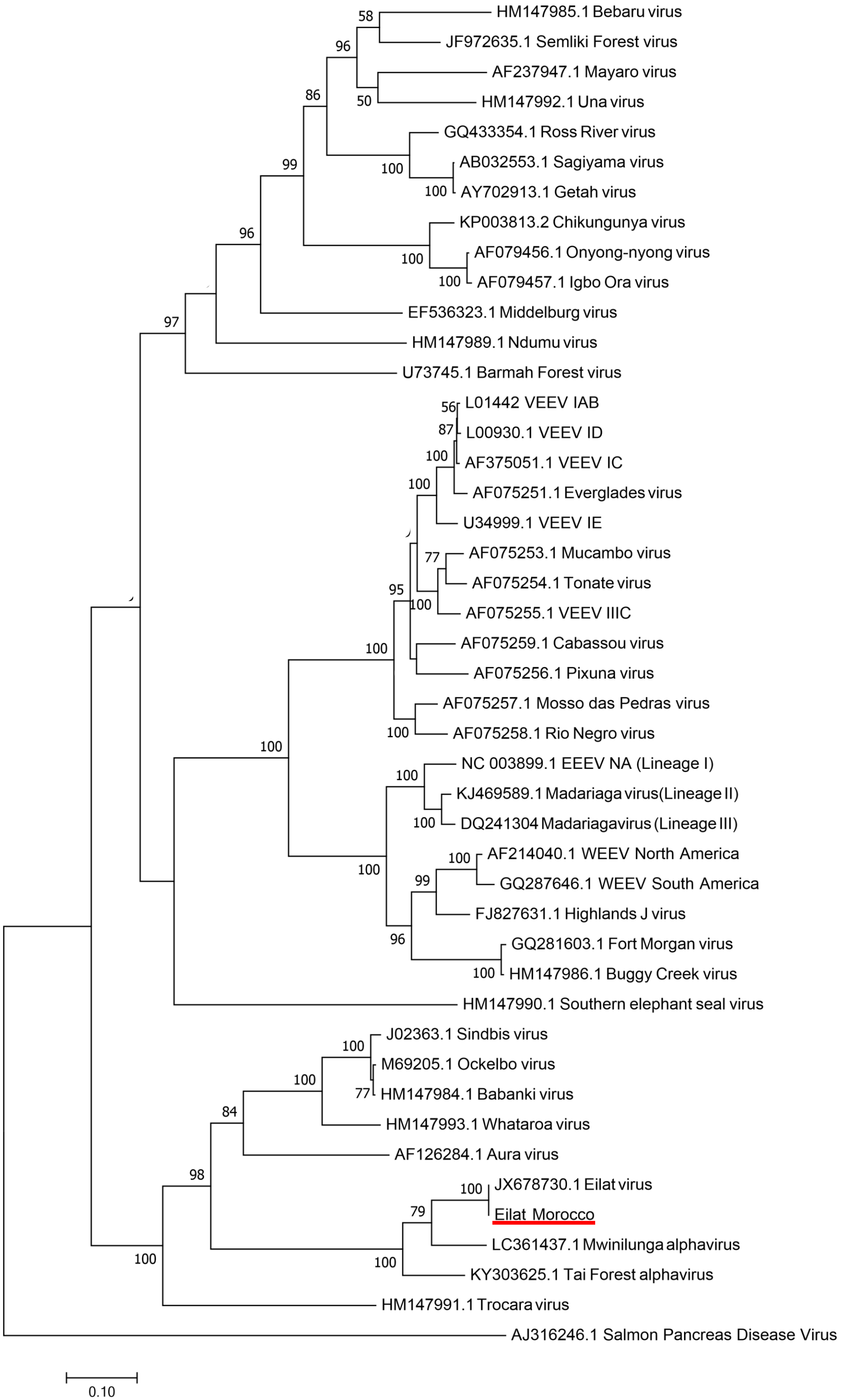
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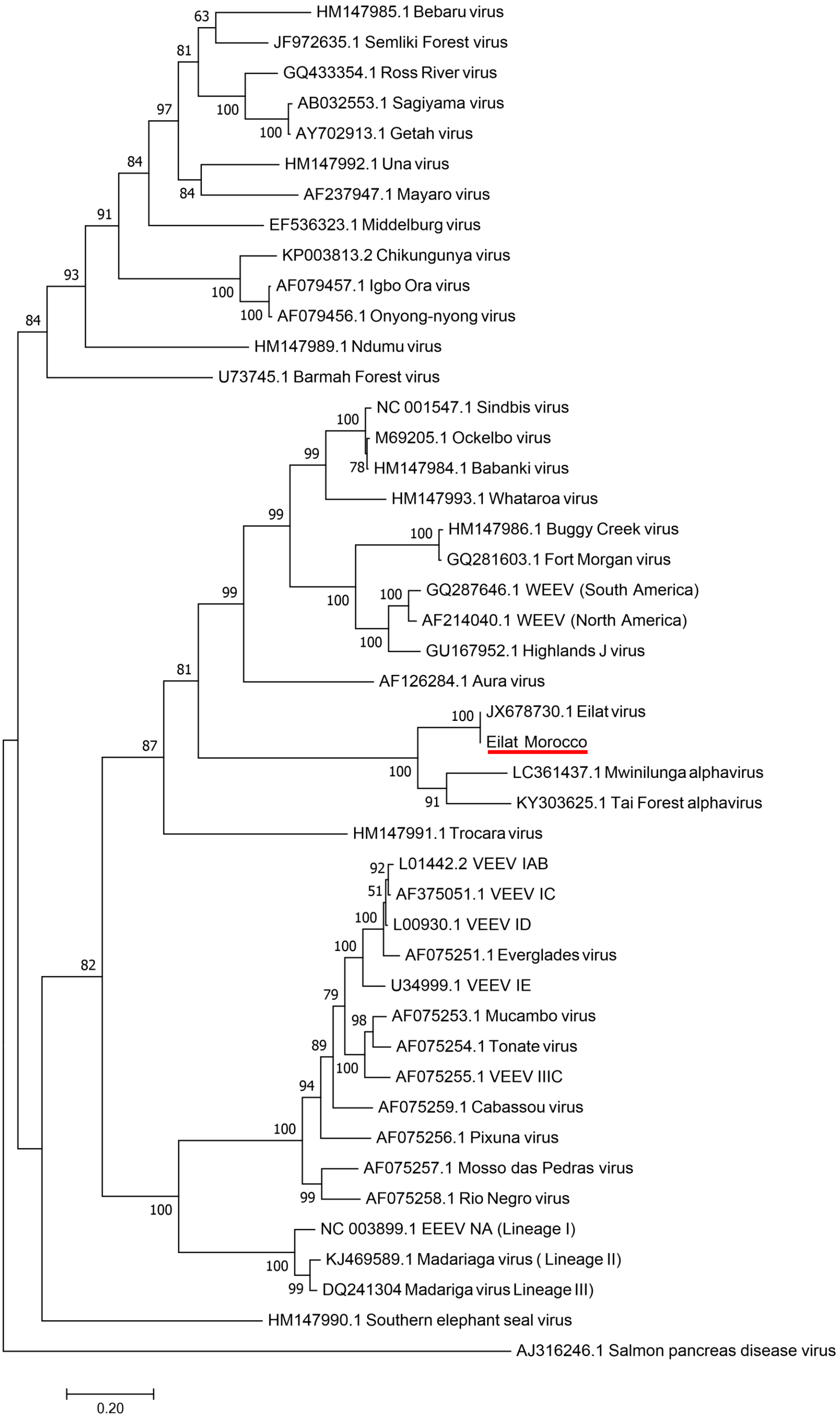
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AJ316246.1 Salmon pancreas disease virus
0.20

Table 1: Infection rates of Eilat-Morocco virus in adult *Culex pipiens* females collected in three sites of Rabat region (Morocco).

Site	Stages	No. pools	No. positive pools	Pool size	MIR [CI]	TIR [CI]
IAV	Adults	34	25	[20-34]	2.3969 [1.4687-3.3252]	4.0505 [2.6895-6.109]
OJ	Adults	7	2	[26-30]	0.9756 [0-2.3211]	1.0504 [0.1956-3.6087]
OL	Adults	8	0	[23-35]	0 [0-0]	0 [0-1.2956]
IAV	Larvae	5	3	[23-55]		
OJ	Larvae	14	1	[29-63]		
OL	Larvae	6	1	[27-64]		

IAV : Institut Agronomique et Vétérinaire Hassan II ; **OJ :** Ounk Jmel ; **OL :** Oulja

MIR: Minimum infection rate; **TIR:** True infection rate; **CI:** Confidence interval