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LETTER TO THE EDITOR

Molecular traces of a putative novel insect flavivirus from *Anopheles hyrcanus* mosquito species in Hungary

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The genus *Flavivirus* includes large number of single stranded RNA viruses, many of which have been recognized as pathogens of significant importance to human health including dengue virus, yellow fever virus and West Nile virus (*1, 2, 3, 4*). The majority of flaviviruses is classified as arthropod-borne viruses that are transmitted horizontally between vertebrate host and arthropod-vectors (mosquito or tick) while other group of viruses within this genus is also maintained in the nature by horizontal transmission and infects vertebrate host but their vectors are still unknown (*5, 6, 3*). In the last decade a small group of flaviviruses had been recognized, that appears to replicate only in mosquitoes but lacks the ability to replicate in vertebrate host. These flaviviruses show global distribution and had been categorized as insect-specific flaviviruses (ISFVs) (*5, 6, 2*). This group can be divided into two separate phylogenetic groups. The first group (classical ISFVs) consists of flaviviruses that are phylogenetically distinct from all other known flaviviruses such as cell fusing agent virus (CFAV) and Kamiti River virus (KRV). While the second group (dual-host affiliated ISFVs) consists of viruses that are phylogenetically linked to the arthropod borne and not-known vector flaviviruses such as Chaoyang virus (CHAOV) and Donggang virus (DONV) (*6*). Although their incidental role in the nature is barely understood, many recent studies focusing on determining the presumable role of ISFVs show that some ISFVs have a tendency to enhance or suppress the replication of flaviviruses associated with human diseases (*2, 6, 8*).

In this study *Anopheles hyrcanus* mosquito samples were collected in multiple locations from Hungary and were tested for the presence of flavivirus-related sequences. Consensus flavivirus-specific PCR was used to determine a partial NS5 sequence. Phylogenetic analyses suggest the presence of a putative novel ISFV among Hungarian *An. hyrcanus* mosquitoes.

Mosquitoes were collected with EVS CO_2 Mosquito Trap baited with dry ice and white light from mosquito breeding areas near the city of Pécs and Debrecen in Hungary, from May to September 2013. Mosquito identification, sample preparation and PCR screening were fulfilled as previously described (*9, 10*). Briefly, after identification of mosquito species according to taxonomic keys (*11*), specimens were grouped by species, collection site and date and pools were created consisting of maximum 50 individuals per each pool. Following mosquito homogenization in PBS and centrifugation, homogenates were subjected to RNA extraction and tested by nested reverse transcription-PCR assays. To exclude the amplification of possibly integrated flaviviral

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Abbreviations: ISFVs = insect specific flaviviruses; cISFVs = classical ISFVs; AnFV = anopheles flavivirus

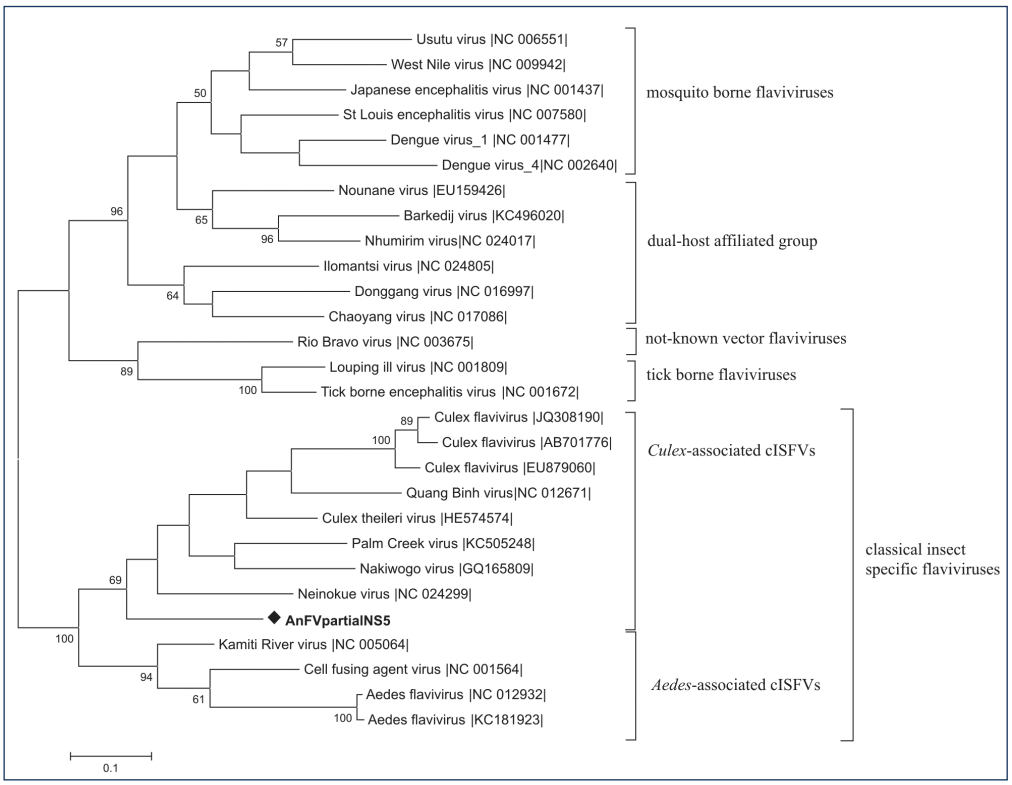

Fig. 1

Phylogenetic tree of the novel AnFVpartialNS5 strain and other members of the genus *Flavivirus* for the partial NS5 and full genome nucleotide dataset

Phylogenetic tree was constructed from the nucleic acid sequence alignments using the maximum likelihood method based on the General Time Reversible model (GTR+G+I) of the program MEGA v6.0 software. The number of bootstrap replications was 1000.

sequences, which were previously described in *Aedes albopictus* mosquitoes (*12*), DNase digestion of the mosquito pool supernatant (*13*) was performed before viral nucleic acid extraction. PCR amplicons were directly sequenced bidirectionally (BigDye Terminator v1.1 cycle sequencing kit) in ABI Prism 310 DNA Sequencer instrument (Applied Biosystems). Basic sequence manipulation and verification were performed using GeneDoc v2.7 software. Nucleotide sequences were aligned by ClustalX v2.0 software, and a phylogenetic tree was constructed from the nucleic acid sequence alignments using the maximum likelihood method based on the General Time Reversible model (GTR+G+I) of the program MEGA v6.0 software. The number of bootstrap replications was 1000.

Altogether 283 female *An. hyrcanus* mosquitoes were collected and combined in 8 pools. Flavivirus-related sequences were detected in 1 and 2 pools collected in Pécs and Kisvárda respectively. Nucleotide BLAST homology revealed a putative novel ISFV, which showed the highest nucleotide identity of 76% to Nakiwogo virus (*1*). In order to evaluate the taxonomic status of our sequence we aligned the nucleotide sequences of the most frequent representatives of arthropod-borne (tick and mosquito) flaviviruses, nearly all representatives of insect specific flaviviruses from both classical (cISFVs) and dual-host affiliated ISFVs and one representative from not-known vector (NKV) flaviviruses. Examined sequences showed the formation of the same main phylogenetic clusters as described previously. Classical ISFVs generated distinct phylogenetic group from all other known flaviviruses and separated into two main clades composed of cISFVs which are usually associated with *Aedes* spp. mosquitoes or *Culex* spp. mosquitoes (Fig. 1) (*6, 1, 14*). Our putative novel ISFV sequence branched within the cluster of cISFVs. Besides, it showed homology with *Culex*-associated insect-specific flaviviruses and formed a distinct branch within this cluster indicating that anopheles flavivirus (AnFV) is a possible new member of the *Culex*-associated insect-specific flaviviruses (Fig. 1). *Anopheles hyrcanus* is known for its potential role as *Plasmodium* sp. and *Dirofilaria* sp. vector (*15, 16*). Furthermore, the presence of Tahyna virus, was also described in this mosquito species (*17*). However its possible capability for hosting flaviviruses is described in this study

for the first time. Other species within the *Anopheles* genus were described previously in association with flaviviruses, precisely with Quang Binh and Culex flaviviruses, which were reported in *Anopheles sinensis* mosquitoes (*2*).

Although we described a potential novel member within the group of ISFVs, relatively short fragment which was analyzed is not sufficient to make long-term or even final conclusions. Further experiments and field screening of *An. hyrcanus* are needed to clarify the presence and exact position of this tentatively novel member within *Flavivirus* genus.

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