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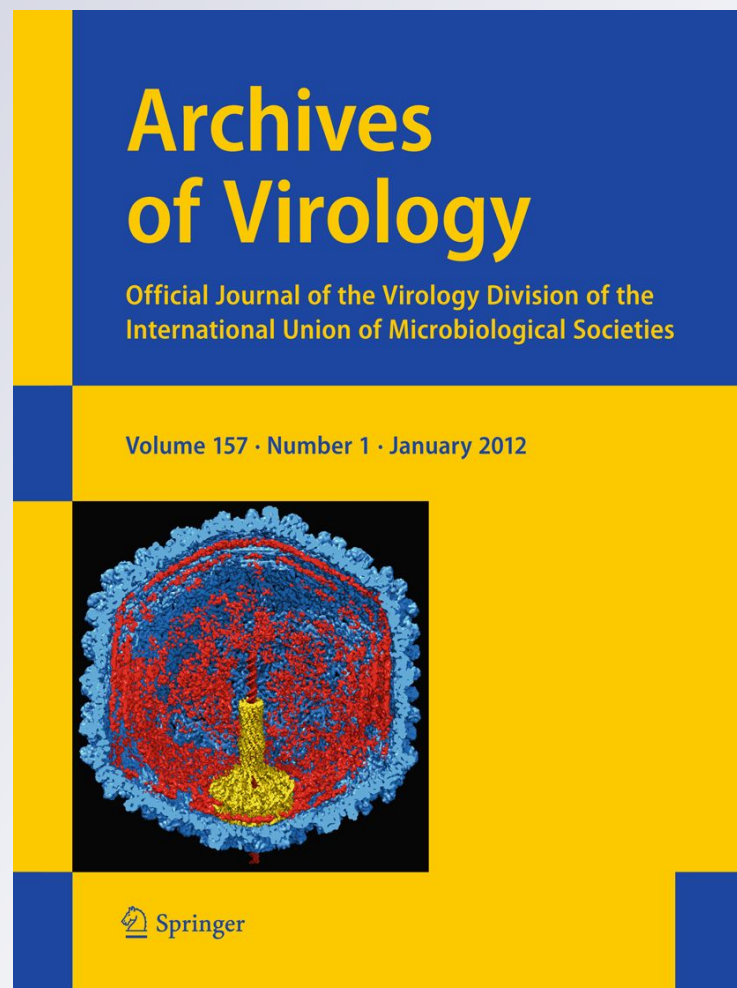
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## First record of a mosquito iridescent virus in *Culex pipiens* L. (Diptera: Culicidae)

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**Abstract** The mosquito iridescent viruses (MIVs) are large icosahedral DNA viruses that replicate and assemble in the cytoplasm of the host. Paracrystalline arrangements of virions that accumulate in the cytoplasm produce an iridescent color that is symptomatic of acute infections. In August 2010, we found larvae of *Culex pipiens* with these symptoms in suburban ditches around the city of La Plata, Argentina. Electron microscope studies, DNA sequencing, and phylogenetic analysis of the major capsid protein confirmed this as the first record of an MIV in *C. pipiens*.

**Keywords** Iridescent virus · Culicidae · *Culex pipiens* · Argentina

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Iridoviruses are pathogens of both vertebrates and invertebrates. Among invertebrates, common hosts are the aquatic stages of Diptera, particularly mosquitoes [10]. The viruses characterized from invertebrates have been assigned to two genera, both of which belong to the family *Iridoviridae*: the genus *Iridovirus*, which contains the majority of iridescent viruses and the genus *Chloriridovirus*, whose sole member is IV-3 from *Aedes taeniorhynchus* (Wiedemann) [2, 5]. The presence of an iridescent virus in mosquitoes was first reported in *Aedes taeniorhynchus* from Florida [5]. In subsequent years, these viruses have been detected in members of several other genera, such as *Ochlerotatus* Lynch Arribalzaga, *Psorophora* Robineau-Desvoidy and *Culiseta* Felt, but they have not been detected in *Anopheles* Meigen, and there are two reports of an iridescent virus infecting *Culex territans* Walker from Russia and Ukraine [10]. The mosquito iridescent viruses (MIVs) are large icosahedral viruses that replicate and assemble in the cytoplasm of the host, primarily in fat body cells. Paracrystalline arrangements of virions that accumulate in the cytoplasm produce an iridescent color when exposed to light on a dark background [1]. MIVs have been reported from the USA, Europe and Russia [10], while no data are available for the Neotropical Region.

*Culex pipiens* (Linnaeus) is a vector of pathogens that cause disease and an important pest worldwide. Immature stages develop in different habitats, including temporary and permanent pools. In Argentina, the most common breeding sites for this mosquito species are man-made drainage ditches in suburban areas of cities, from which a large number of mosquito adults emerge throughout the year [4].

In August 2010, during a survey for natural enemies of *C. pipiens*, we found fourth-instar larvae with infections

symptomatic of MIV. These larvae were collected from suburban ditches around the city of La Plata, Argentina (34°52'12.34"S, 57°52'8.59"W) that permanently contained water. The habitat was polluted with greasy substances and abundant organic matter from domiciliary drainage. Larvae were examined under a dissecting microscope using a black background. Of the 151 larvae collected, 16 (10.6%) showed turquoise iridescence similar to that described as typical for the family *Iridoviridae*.

Small pieces of infected larvae were prepared for electron microscopic studies. These were fixed in 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.3) for 3 h, postfixed in 1% osmium tetroxide for 2 h, dehydrated using increasing concentrations of ethanol into acetone and embedded in Epon-Araldite resin. Ultrathin sections (60 nm), stained with 2% uranyl acetate and lead citrate, were examined with an electron microscope at 75 kV [3]. The virus particle exhibited a hexagonal shape and had a dense central core surrounded by a lighter-staining outer shell (Fig. 1). The virus particles had an average diameter of 158 nm ( $n = 30$ ).

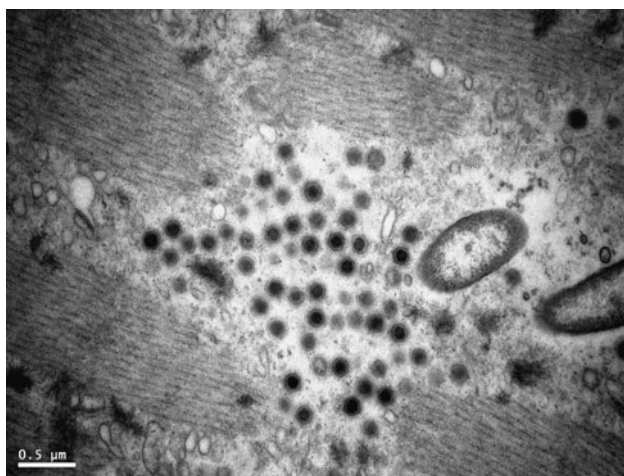
Two infected larvae were partially purified for DNA extraction. The larvae were homogenized in distilled water, filtered through cotton in a syringe, and centrifuged twice in order to remove cellular debris (1000 rpm for 10 min and 1500 rpm for 10 min). The supernatant was centrifuged at 15000 rpm for 30 min. Finally, the pellet was resuspended in 500  $\mu$ l of distilled water. One hundred microliters of this suspension containing MIV was digested with proteinase K (0.1 mg/ml) in lysis buffer plus SDS (10 mM Tris-HCl, pH 8.0; 200 mM NaCl; 1 mM EDTA, pH 8.0; 0.5% SDS). The mixture was incubated at 37 °C for 3 h and then centrifuged at 16,000g for 15 min. Viral DNA was purified by standard phenol/chloroform

extraction and ethanol precipitation [7]. The final pellet was dissolved in 20  $\mu$ l of double-distilled water.

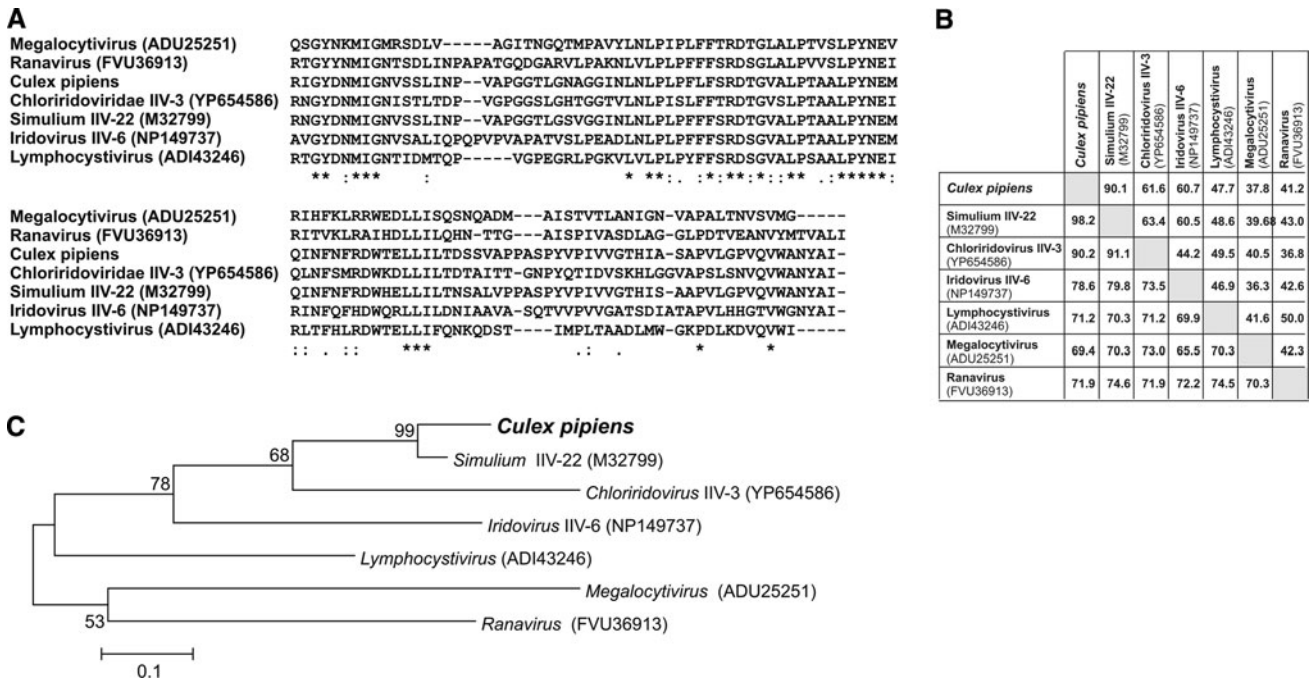
Using the MCP primers designed in the laboratory of JJ Becnel at CEMAVE (JJ Becnel, personal communication) and 1  $\mu$ l of MIV DNA (1/100 dilution) as template, a PCR amplification with Taq DNA polymerase (Invitrogen) was carried out under standard conditions (1X standard buffer, 0.25 U Taq DNA polymerase, 1.5 mM MgCl<sub>2</sub>, 1 mg/ml BSA, 0.2 mM each dNTP and 1  $\mu$ M each primer). The amplification profile used was as follows: one initial denaturing step of 1 min at 94 °C, 35 cycles of 10 s at 94 °C, 10 s at 56 °C and 20 s at 72 °C, followed by a final extension step of 3 min at 72 °C. The PCR samples were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining.

The expected fragment of  $\sim$ 300 bp was obtained. This fragment was cut out and recovered using silica matrix adsorption (GENECLEAN II Kit, BIO 101). The purified fragment was ligated into the pGEM-T vector (Promega) and electroporated into *E. coli* Top10 competent cells. After selection on LB agar plates containing 100  $\mu$ g/ml ampicillin and 20  $\mu$ g/ml X-gal, the recombinant clones were grown in liquid LB plus ampicillin. Plasmid DNA was purified using the alkaline lysis method [7] and sequenced at the Macrogen Center (Korea), employing T7 and SP6 universal primers. The *in silico*-translated protein sequence was compared to the corresponding sequence from the type member of each genus of the family *Iridoviridae* using multiple and pairwise alignments with ClustalX software [9] and phylogeny inference using MEGA4 software [8]. The partial sequence of the *Culex pipiens* MIV MCP protein clustered most closely with IIV-22 from *Simulium sp.* and members of the genus *Chloriridovirus*, especially IIV-3 from *Aedes taeniorhynchus* (Fig. 2). This result is in agreement with what has been reported by Delhon *et al.* [6]. The analyzed sequence is a small fragment of a well-conserved gene, and perhaps this is the reason that the consistency of three of the nodes is relatively low. In a paper describing IIV3, Delhon *et al.* [6] show a cladogram based on a concatamer of eleven conserved protein alignments. In this cladogram, invertebrate iridoviruses are clearly separated from the vertebrate iridoviruses. They also conducted a phylogenetic analysis of the complete MCP for which sequence data were available and found that IIV-3 and IIV-6 were clearly separated and that IIV-3 was more similar to a group of viruses including IIV-22, whose host is a black fly. It is clear in this analysis that the MIV from *Culex pipiens* is most similar to IIV-3, which is the type host for members of the genus *Chloriridovirus*.

Based on these results, we were able to confirm the presence of an iridescent virus infecting *C. pipiens* larvae based on symptoms, morphology, and molecular data. This report constitutes the first documentation of an iridescent



**Fig. 1 Transmission electron micrograph.** Ultrathin sections of infected *Culex pipiens* larvae showing particles of mosquito iridescent virus in areas of fat body near muscle



**Fig. 2 Sequence analysis.** **A.** Multiple alignment of the deduced protein sequence of *Culex pipiens* MIV MCP and the corresponding sequences from the type members of the different genera in the family *Iridoviridae*. **B.** Pairwise identity (upper right triangle) and homology (lower left triangle) between all analysed protein sequences. **C.** Evolutionary relationships of seven taxa, inferred using the minimum-evolution method. Node consistency (bootstrap test, 1000 replicates)

is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the following parameters: Model [amino: Poisson correction], Substitutions to include [All], Pattern among Lineages [Same (homogeneous)], Rates among Sites [Different (Gamma distributed)] and Gamma parameter [2.25]

virus in *C. pipiens* larvae, and this is also the first time that an iridescent virus has been described infecting mosquitoes in the Neotropical Region.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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