UDC 576.858

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MOLECULAR CHARACTERIZATION OF AN IRIDOVIRUS ISOLATED FROM MOSQUITO *AEDES FLAVESCENS*

В роботі представлені результати вивчення молекулярних властивостей іридовіруса комара Aedes flavescens методами електронної мікроскопії, електрофорезу білків, рестрикційного аналізу вірусної ДНК та ПЛР. Молекулярна вага головного капсидного білку іридовірусу A. flavescens, розмір вірусної ДНК та специфічна ПЛР-ампліфікація свідчать, що даний ізолят належить до родини Iridoviridae. Натомість за білковим та рестрикційним профілями іридовірус комара A. flavescens відрізняється від іншого іридовіруса комара Aedes taeniorhynchus, типового представника роду Chloriridovirus.

An iridovirus, isolated from mosquito Aedes flavescens in Kiev region, was investigated by the methods of electron microscopy, analysis of viral protein in gradient SDS polyacrylamide gel electrophoresis, restriction of viral DNA and amplification of a segment of viral DNA in polymerase chain reaction. All approaches yielded results consistent with the suggestion that mosquito iridovirus Ae. flavescens was a member of the family Iridoviridae. Moreover RFLP analysis of viral DNA, number of virus proteins and molecular weight of major capsid protein distinguished iridovirus Ae. flavescens from another mosquito iridovirus Ae. taeniorhynchus, the type species of the genus Chloriridovirus.

Introduction. Iridoviruses are large DNA viruses that replicate in the cytoplasm of infected cells. Iridovirus genomes are circularly permuted and terminally redundant, and range in size from 105 to 212 kbp. The family *Iridoviridae* is currently subdivided into five genera: *Chloriridovirus, Iridovirus, Lymphocystivirus, Megalocytivirus*, and *Ranavirus*. Iridoviruses have been found to infect invertebrates and poikilothermic vertebrates, including amphibians, reptiles, and fish [1].

Invertebrate iridescent viruses (IIVs) are icosahedral particles of approximately 120–200nm in diameter that infect invertebrates, mostly insects, in damp or aquatic habitats. These viruses cause two types of disease: one patent and the other covert (inapparent). An abundance of virus particles in the cells of patently infected insects causes them to develop an obvious iridescent color that typically ranges from violet, blue, green, or orange [2].

Iridoviruses that infect mosquitoes and midges belong to the genus *Chloriridovirus.* The taxonomic situation is even more unsatisfactory for the genus *Chloriridovirus* , which, despite numerous records of infections in many species of mosquitoes and midges of medical and veterinary importance, consists of a single species, *Invertebrate iridescent virus 3* (IIV-3) from the saltmarsh mosquito, *Ochlerotatus* (*Aedes*) *taeniorhynchus*. That is why the newly isolated mosquito iridovirus *Ae. flavescens* was investigated with the purpose to understand its relatedness within family *Iridoviridae.*

Materials and methods. Virus propagation. An iridovirus, isolated from mosquito *Ae. flavescens* in natural reservoirs of the Kyiv region*,* was propagated in multihost wax-moth larvae *Galleria mellonella.* The wax-moth larvaes were infected by intraperetonal injection and incubated at temperature 20-22 ^{0}C. In 14 days after injection the mosquito iridovirus was purified from wax-moth larvae *G. mellonella*.

Virus purification. The mosquito iridovirus *Ae. flavescens* was purified from the infected wax-moth larvae *G. mellonella* by the differential centrifugation method. Briefly, after homogenization of infected larvae *G. mellonella*, cell debris was separated by centrifugation at 3000 × *g* for 5 min at 10°C. The pellet was discarded and the supernatant centrifuged in ultracentrifuge Beckman L5-50B in a rotor SW-40 for 40 min at 70500 × *g* at 4°C. Characteristic blue pellet confirmed the presence of mosquito iridovirus virions. The virus pellet was suspended in TNE (50 mM Tris-HCl, 150 mM NaCl, 1 mM disodium ethylene diaminetetracetic acid [EDTA], pH 7.5) and centrifuged at 1100 × *g* for 5 min at 10°C. The suspension was layered onto a 10 to 50% (w/w) linear sucrose gradient in TNE. After centrifugation at 70500 × *g* for 40 min at 4°C, 1 visible band near the bottom of the tube were collected, diluted in fresh TNE and centrifuged at 70500 × *g* for 40 min at 4°C. The virus pellet was placed into TNE at a final protein concentration of 1,7 mg ml^{-1}.

Electron-microscopy. For electron-microscopy investigation the viral suspension was stained with 2% uranyl acetate and observed in microscopy EM-125 (SEO).

DNA extraction. Viral genomic DNA was extracted using a 25:24:1 phenol:chloroform:isoamyl alcohol (IAA) mixture, followed by ethanol precipitation [3]. Briefly, purified virus was incubated with Proteinase K in the presence of lysis buffer for 3 h at 56°C and then treated with IAA. A 0.1 volume of 3 M sodium acetate was added to the aqueous phase following IAA extraction followed by 2.5 volumes of cold 95% ethanol. The mixture was incubated overnight at –20°C, followed by centrifugation at 12 500 × *g* to precipitate the DNA. The supernatant was removed and the pellet was washed with cold 70% ethanol and then air-dried. TE buffer was added and the DNA pellet was incubated for 5 min at 37°C to dissolve the DNA. DNA concentrations were determined by spectrophotometer (APEL PD-303 UV) and the samples were stored at –20°C.

Analysis of virion polypeptides. Purified virus (1,7 mg protein ml^{-1}) in TNE was mixed 1:1 with 2× sample application buffer, heated to 100°C for 5 min. Virion polypeptides were separated by SDS-PAGE in 5-20% gradient gel according to the system of Laemmli [4]. Molecular weight standards (Fermentas) were included in gel. After electrophoresis, the gels were stained with Coomassie blue R-250 and the approximate molecular weight of the virion polypeptides was estimated by their mobility relative to the molecular weight standards.

Restriction fragment length polymorphism (RFLP). For RFLP analysis, 1 to 2 μg of viral DNA from purified preparations of iridovius *Ae. flavescens* was incubated with 10 U of *Eco*RI, *Bam*HI, *Xba*I, *Hind*III, *Hpa*II and *Msp*I endonuclease (Fermentas) for 16 h at 37°C. DNA fragments were separated by electrophoresis on 0.5% agarose gels and observed after staining with 1% ethidium bromide.

Primer selection and oligonucleotide synthesis. We designed oligonucleotide primers to amplify DNA of iridovirus *Ae. flavescens*. Using nucleotide sequences of all iridoviruses a set of oligonucleotide primers was designed for ribonucleoside reductase small subunit (RNRS) gene [5]. Selected oligonucleotides were tested for possible secondary structure and self-complementarity using Vector NTI 11 software (INVITROGEN). The forward primer (MIV_RNRS_F) sequence was 5' – CTC CAC GAC CAG AGT GCT AAA G – 3' and the reverse primer (MIV_RNRS_R) sequence was 5' – TGC TAC GAC AAG TGG GAT ACG C – 3'. Oligonucleotides were synthesized commercially (Metabion). The predicted location of the 350 base-pair (bp) amplified product within the ribonucleoside reductase small subunit (RNRS) gene was based on the

sequence of Invertebrate iridescent viruses 3 (IIV-3; GenBank Accession No. DQ643392) [6].

Polymerase chain reaction (PCR) assay. The reagents used for PCR amplification were from Fermentas. Each 25 µl reaction mixture (prepared on ice) contained the sample DNA, 2,5 µl of 10× PCR buffer, 2 mM $MgCl_2$, 2.5 µl of dNTP mixture, 10 pmol of each primer, 0.75 U of *Taq* polymerase and distilled water up to total volume. A MasterCycler (Eppendorf) was used for the PCR as follows, Cycle 1: 94°C for 2 min; Cycles 2 to 30: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min; Cycle 31: 72°C for 5 min. Control received the PCR mixture containing no DNA template (reagent control). After the PCR, products were transferred to a 1.0% agarose gel, electrophoresed, and DNA was visualized by ethidium bromide staining.

Results and Discussion. In this study, we described the isolation and characterization of a potentially novel iridescent virus from mosquito *Ae. flavescens* . Our data from electron microscopy reveal that iridescent virus *Ae. flavescens* exhibits the major morphological characteristics of an iridovirus. A sixfold symmetry of virus particles can be observed in negatively stained material. With sizes of 200 ± 5 nm, the particles exhibit dimensions ranging between the reported typical sizes of large (genus *Chloriridovirus*: 180–200 nm) invertebrate iridoviruses [1].

The ultrastructural investigations of viral particles show an electron-dense core surrounded by an internal lipid bilayer and an outer electron-dense shell. This is in accordance with structural data of other members of the family *Iridoviridae* [7]. The nucleoprotein core of iridoviruses is surrounded by an internal lipid membrane and a protein shell forming the icosahedral particle.

Gradient SDS-PAGE of purified virions revealed the presence of 12 polypeptides ranging from 14,5 to 122 kDa. The molecular weight of a major capsid protein (MCP) was a 48 kDa. Interestingly that number of proteins of another mosquito iridovirus *Ae. taeniorhynchus* is 9 ranging from 15,5 to 98 kDa and molecular weight of MCP is approximately 55 kDa (Tab. 1). These data have the characteristics of those of iridoviruses [1].

Table 1. **The molecular weight of proteins of mosquito iridoviruses isolated from *Ae. flavescens* and *Ae. Taeniorhynchus***

№	Molecular weight of proteins, Da	
	Aedes flavescens iridescent virus	*Aedes taeniorhynchus iridescent virus*
1	14 500	15 500
2	18 000	19 000
3	34 000	30 000
4	48 000	37 000
5	53 000	54 000
6	57 500	68 500
7	60 000	84 000
8	63 000	94 000
9	80 500	98 000
10	108 500	
11	114 000	
12	122 000	

Restriction enzyme analysis of isolated iridovirus *Ae. flavescens* DNA with the endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, *Xba*I, *Msp*I, and *Hpa*II has revealed that the restriction fragment patterns of iridovirus *Ae. flavescens* differ from those obtained from experiments with iridovirus of mosquito *Ae. taeniorhynchus* (Tab. 2) and iridoviruses of lower vertebrates, such as Rana esculenta iridovirus (REIR) or *Frog virus 3* (FV-3). The genome of FV-3 does not show cleavage sites for *Eco*RI, but it exhibites numerous cleavage sites for *Bam*HI (Ahne *et al.,* 1998) [8]. In contrast to invertebrate iridoviruses, the genome of vertebrate iridoviruses has been shown to be methylated by a virus-specific methyltransferase. The methylation of viral DNA at cytosine residues is a characteristic stage of the replication cycle of these viruses, protecting newly synthesized virus DNA from digestion by vertebrate cytoplasmic endonucleases. Methylated viral DNA can be distinguished from unmethylated forms by digestion with the endonucleases *Msp*I and *Hpa*II. These restriction enzymes have the same cleavage site (CCGG). However, whereas *Msp*I is able to cleave viral DNA in the presence of methylcytosine, *Hpa*II is not. DNA of Iridovirus *Ae. flavescens* is degraded by both endonucleases. Thus, it must be unmethylated and exhibits the expected characteristics of invertebrate iridovirus DNA.

Table 2. **Comparative restriction fragment length polymorphisms of the viral DNA from iridoviruses *Ae. flavescens* and *Ae. taeniorhynchus* ***

Restriction endonuclease	*Aedes taeniorhynchus iridescent virus*		*Aedes flavescens iridescent virus*	
	Number of fragments	Fragment length, kbp	Number of fragments	Fragment length, kbp
*Bam*HI	24	0,323-23,9	11	5,3-24,1
*Eco*RI	11	0,238-55,753	23	2,1-24,3
*Hind*III	45	0,08-23,969	25	1,3-13,0
*Xba*I	19	0,021-31,765	18	3,1-24,3
*Hpa*II	560	0,004-6,272	19	1,5-11,9
*Msp*I	560	0,004-6,272	19	1,5-11,9

* – RFLP analysis of *Aedes taeniorhynchus iridescent virus* was made by usage the Vector NTI11 software

Using nucleotide sequences of all iridoviruses a set of oligonucleotide primers was designed for RNRS gene. After 30 cycles of amplification of mosquito iridovirus *Ae. flavescens* genomic DNA, PCR products of about 350 bp were visible on agarose gels stained with ethidium bromide.

Knell *et al.* (2006) and Tang *et al.* (2007) have recently reported the isolation of an iridescent viruses from mopane warm *Imbrasia* (*Gonimbrasia*) *belina* and sergestid shrimp *Acetes erythraeus* [9, 10]. We have described an invertebrate DNA virus from *Ae. flavescens,* termed *Aedes flavescens* iridescent virus (AfIV), which can be

classified as a member of the genus *Chloriridovirus.* The impact of this virus in the natural environment is unknown. Although, it is not known whether or not this iridovirus can infect any other mosquito species, many members of the *Iridoviridae*, such as IIV-6 and ranaviruses, are known to have large host ranges.

Interest in invertebrate iridoviruses has been limited by the perception that they have little potential as biological control agents against insect pests. However, there is now growing awareness of the potential impact of sublethal IIV disease on the dynamics of insect populations, including insect vectors of medical importance worldwide. Studies with insects indicate that sublethal iridovirus infections can also seriously impact host fitness through reduction in reproductive capacity, body size, and longevity [7]. The mosquito iridovirus *Ae. flavescens* can be promising biological control agent against insect pests, as it causes covert disease and reduces mosquito stocks.

Therefore, future experiments are required to establish whether AfIV is a new species within the genus *Iridovirus* or just an isolate of a previously known virus species. In addition, it would be worthwhile to investigate the susceptibility of AfIV to other species of *Aedes* and to explore the prevalence of these viruses in wild stocks.

Conclusion. Electron microscopy and molecular approaches yielded results consistent with the suggestion that mosquito iridovirus *Ae. flavescens* was a member of the family *Iridoviridae*. Moreover RFLP analysis of viral DNA, number of virus proteins and molecular weight of MCP distinguished iridovirus *Ae. flavescens* from another mosquito iridovirus *Ae. taeniorhynchus*, the type species of the genus *Chloriridovirus*. Presently, a large numberof mosquito iridoviruses, that have been reported in literature, are insufficiently studied and consequently they are not classified. Our observation may help to understand the relatedness of mosquito iridovirus *Ae. flavescens* within family *Iridoviridae.*

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Надійшла до редколегії 25.11.10

UDC 578.85/.86

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DETECTION AND IDENTIFICATION OF VIRUSES AFFECTING IRISES IN LITHUANIA AND UKRAINE

Робота виконана згідно зі спільною Литовсько-Українською програмою співробітниками кафедри вірусології Київського національного університету імені Т. Шевченка та лабораторії вірусів рослин інституту ботаніки в рамках проекту "Моніторинг складу вірусних патогенів декоративно-квіткових культур в Україні та Литві". Мета даної роботи – дослідження вірусних захворювань півників (Iris L.) ботанічних садів Литви та України; ідентифікацію збудників проводили за допомогою класичних та новітніх, молекулярно-біологічних, методів. Вірус помірної мозаїки півників (IMMV) був виявлений в усіх досліджених зразках як Литви так і України. Методами рослин індикаторів, електронної мікроскопії, імуноферментного аналізу, в модифікації DAS, та зворотно-транскрипційної полімеразної ланцюгової реакції (RT-PLR) з деяких литовських зразків були ізольовані та ідентифіковані віруси тютюнової мозаїки (TMV), кільцевої плямистості тютюну (TRSV) та кільцевої плямистості томатів (ToRSV).

According to the joint Lithuanian–Ukrainian program the research workers of Department of Virology T. Shevchenko Kyiv National University and Plant Virus Laboratory of Nature Research Center Institute of Botany are fulfilling research project "Monitoring of diversity of virus pathogens infecting ornamental plants in Ukraine and Lithuania". The aim of this work was to investigate viral diseases affecting irises (Iris L.) grown in Botanical gardens in Lithuania and Ukraine; to identify their agents applying the classical and modern, molecular biology methods. Iris mild mosaic virus (IMMV) was detected in all investigated Lithuanian and Ukrainian samples; Tobacco mosaic (TMV), Tobacco ringspot (TRSV), and Tomato ringspot (ToRSV) viruses have been isolated and identified in some investigated Lithuanian samples by the methods of test–plants, electron microscopy, double–antibody sandwich enzyme–linked immunosorbent assay (DAS–ELISA), and reverse transcription polymerase chain reaction (RT–PCR).

Introduction. Irises have been used for centuries both as ornamentals and as a source of perfumes and medicine. This wide-ranging genus of more than 200 species is native to the temperate regions of the Northern hemisphere. There are many hybrids. Irises are divided into main two groups: rhizomatous and bulbous.

Spread of viruses in irises remains a serious problem. Until now 15 viruses affecting irises have been described in literature: *Iris mild mosaic virus*, *Narcissus latent virus*, *Iris severe mosaic virus*, *Bean yellow mosaic virus*, *Tomato spotted wilt, Impatiens necrotic spot virus*, *Turnip yellow mosaic virus* [1], *Tobacco ringspot virus, Tobacco mosaic virus, Tobacco rattle virus*, *Iris fulva mosaic virus*, *Cucumber mosaic virus, Broad bean wilt virus*, *Lilac chlorotic leafspot virus*, *Iris germanica virus*.

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Materials and methods. Plant material was collected in Botanical Garden of Vilnius University, Experimental Station of Field Floriculture and collection of iris breader O. Griniuviene in Lithuania; Botanical garden of Kiyv T. Shevchenko university, and N. N. Griško Kyiv National Botanical Garden in Ukraine.

Viruses have been identified by the methods of test–plants [2], electron microscopy (EM), double–antibody sandwich enzyme–linked immunosorbent assay (DAS–ELISA) and reverse transcription polymerase chain reaction (RT–PCR) [3, 4, 5, 6].