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Broadening the *Chrysoviridae* horizons: a new geographical area and potential arthropod vector for the Xanthi chryso-like virus

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Abstract

Mosquitoes are widespread arthropods that serve as vectors for a broad spectrum of viruses, many of which pose a substantial threat to humans. Conversely, some viruses may seemingly present with beneficial implications for the health of man whilst impinging on other domains of life, such as the fungi-infecting mycoviruses. This study is only the second one so far to describe the exceptionally scarcely identified Xanthi chryso-like virus (XCLV), member of the mycopathogenic *Chrysoviridae* family, in a new geographical area and a novel potential arthropod vector, the *Culex pipiens* mosquito. Interestingly, the XCLV was initially fortuitously detected by West Nile virus-specific primers directed at a conserved part of the *NS5* gene, possibly indicative of a genetic resemblance and shared ancestry reflected in the *NS5* evolutionary heritage. Detection and characterization of the virus and insect alike was done via PCR and Sanger sequencing. This investigation draws attention to our knowledge of viral pervasiveness – be it topographical or vectorial – and how humble it truly is. Additionally, we would propose that more attention be given to arthropod-borne viral pathogens that might prove advantageous to human health.

Keywords

Mosquito, arthropod-borne, vector, RNA virus, mycovirus, *Chrysoviridae*, sequencing, Serbia

Introduction

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Mosquitoes are ubiquitous insects that may harbor a large variety of RNA viruses, with a significant impact on human health. Often, they carry a rich viral community which may facilitate genetic interactions as recombination and reassortment, possibly giving rise to new and emerging agents of disease [1].

With the increasing use of next generation sequencing, a whole panoply of mosquito viromes may be scrutinized in short periods of time. These boast an assortment of viral families pertaining to vertebrates, invertebrates, plants, fungi, protozoa [2]; furthermore, novel virus sequences and undescribed species are also practically a given result of sequencing [2-4].

Notwithstanding the extensive array of viruses carried by hematophagous arthropods, the most attention is given to human pathogens. Nevertheless, these insects serve as reservoirs for numerous viruses of various other species (e.g. plant and fungal viruses), hence, tentative beneficial effects of diverse mosquito viromes to human public health may be considered

Viruses infecting fungi have been described in many fungal species, including human fungal pathogens [5-8], and may tentatively prove favorable to humans in that they interfere with fungal physiology. Chrysoviruses are among recognized mycopathogens associated with mosquitoes.

Chrysoviridae are small, double-stranded multipartite RNA viruses, pathogens of ascomycetous or basidiomycetous fungi, plants and possibly insects [9]. Viruses associated with this family have been known to negatively influence fungi infecting humans, plants and have even been suggested as an agent for biological control [10-13]. Amongst the more thoroughly studied related pathogens, the Xanthi chryso-like virus (XCLV) is localized in a niche of unclassified chrysoviral agents [14].

In this study we report on a fortuitous discovery of this so far exceptionally rarely detected virus by means of PCR detection, using primers directed at a conserved part of the West Nile virus (WNV) *NS5* gene. Moreover, XCLV has been identified in a novel arthropod, the *Culex pipiens* mosquito, broadening the spectrum of potential XCLV reservoirs. Having in mind the extensive interaction between insects, humans, plants and ubiquitous fungi, this finding highlights a possible role of mosquitoes as vectors of fungus-infecting viruses, with so far insufficiently elucidated implications on both human and plant health.

Materials & Methods

Mosquito collection and RNA extraction

The analyzed mosquitoes were collected within the scope of routine WNV environmental screening program, regularly implemented by the Institute for Biocides and Medical Ecology, Belgrade, Serbia (IBME). Insect sampling was done in the north of Serbia (Figure 1) during the period 2018 to 2020, which covered the time of the largest epidemic of WNV infections in Southeast Europe in humans up to that point.

Field sample collection was performed using specialized BG Sentinel® (Type 1 and Type 2) mosquito traps, which contain dry ice (CO_2 in solid state, in the form of pellets) as an attractant, placed in

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a well-insulated container (volume 3L). Upon setting the trap CO_2 is released, which attracts female mosquitoes. During trap recollection, live mosquito specimens were separated from dead ones *in situ*. Living specimens were stored in a portable refrigerator for sample transport and momentarily frozen on dry ice (-80 ˚C). Once frozen,the mosquitoes were counted, sorted and transferred into corresponding labeled (date, location, city) sample tubes, upon a preliminary identification at the genus level. These were stored on dry ice and taken back to the IBME, where the mosquitoes were pooled in groups of approximately 50 individuals and subsequently homogenized prior to RNA extraction. A total of 200 mosquito pools further analyzed for the presence of WNV via Real-Time PCR, according to commercial PCR test protocol (Sacace Biotehnologies, Como, Italy) were found pathogen RNA-positive. Extracted RNA was transported on dry ice to the Virology Laboratory of Instutute of Microbiology and Immunology, University of Belgrade Faculty of Medicine for further in-depth molecular characterization.

Genome detection, identification and phylogenetic analysis

Final confirmation of insect species was done by PCR detection of 16S RNA (one step RT-PCR Takara Bio Inc., Shiga, Japan), using universal 16S primers listed in Table 1 [15].

The protocol of WNV detection and characterization included nested PCR amplification with Sanger sequencing of amplified products. In short, mosquito pool RNA aliquots were subjected to a one-step reverse transcription PCR protocol (Takara Bio Inc., Shiga, Japan), followed by nested PCR with primers that amplified a conserved part of the WNV *NS5* gene region coding for a nonstructural protein with RNA-directed 5'-3' RNA polymerase activity. The *NS5* gene-specific primers [16] are listed in Table 1, along with XCLV-specific primers for nested PCR which amplified ~750 bp of the *P3* putative protease gene. These primers were designed within this study, based on the 4 XCLV sequences existing in public databases [17]. Detected PCR products were thereafter Sanger-sequenced on a Genetic analyzer 3730 (Applied Biosystems, Waltham, MA, USA). The obtained sequence data were assessed utilizing the Sequencing Analysis Software v5.4 (Applied Biosystems, Waltham, MA, USA).

Basic sequence identification was performed by the BLAST tool for comparison to the viral sequences in the NCBI database [18].

In order to reconstruct phylogeny of XCLV, all sequence data in the NCBI database accepted by July 2022 were included in the study. Considering the fact that prior to this study only four XCLV sequences have been available in the NCBI database, we also downloaded all Chryso-like virus (CLV) sequences which correspond to partial amplified *P3* putative protease gene of XCLV. Sequence alignment was done by MAFFT 7 software (https://mafft.cbrc.jp/alignment/server/) and manually inspected. The resulting alignment consisted of 15 CLV sequences 800 nucleotides (nt) in length. Prior to further phylogenetic analysis alignment was screened for recombination using algorithms as implemented in the Recombination

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Detection Program 4 (RDP4) [19]. The jModeltest 0.1.1 software [20] selected General Time Reversibile (GTR) distance model as the best fitting substitution model using all 88 proposed models. The MEGA 6 software package [21] was used to conduct phylogenetic analysis by maximum likelihood (ML) method with 1000 bootstrap replicates.

Results

Among a total of 200 studied pools, an unexpected finding of XCLV was made in four samples (submitted to the NCBI GenBank and IN-DEPTH Project (https://indepth.rs/) sequence databases). This was further confirmed by using XCLV-specific primers encompassing a 736 nt region of the *P3* putative protease gene and substantiated by subsequent bidirectional Sanger sequencing.

BLAST analysis of the obtained nucleotide sequences indicated the highest similarity to XCLV (Accession no. MW520404) and Soufli chryso-like virus (Accession no. MW520403) with 96.38% and 89.20%, respectively. Likewise, the highest similarity based on the protein level was observed with P3 putative protease of XCLV (Accession no. QRD99897) and P3 putative protease of Soufli chryso-like virus (Accession no. QRD99896) with 96.99% and 95.31%, respectively.

The studied 730 bp CLV dataset sequences comprised 266 amino acids of *P3* putative protease gene. Potential recombination events were not detected in the studied alignment. Average nucleotide distance of all analyzed nucleotide sequences was 0.51 (0.0-0.95, SD=0.35), with amino acid divergence of 0.13%. Comparative analysis of XCLV sequences from Serbia revealed a nucleotide distance of 0.02 (Figure 1).

The general topology of the phylogenetic tree for the studied dataset showed two separate clades, both with high bootstrap. The first clade consisted of seven Hubei chryso-like virus sequences, of which five are from Australia and two from USA, collected in 2015 and 2017, respectively. The second tree clade included four XCLV sequences from Serbia together with XCLV and Soufli chryso-like virus from Greece, collected in 2020 and 2018, respectively. The tree was rooted with two Hubei chryso-like virus sequences from Australia.

In XCLV-positive samples the identification of mosquito species was further achieved on a genetic level, as described above. Sanger sequencing followed by BLAST alignment confirmed that all samples contained *Culex pipiens* mosquitoes.

A topographical distribution of mosquito collection sites, with indication of the locations where XCLV was identified is shown in Figure 2. Of note, other mosquito-borne viruses, like Usutu virus and WNV, have also been found in the north of Serbia (Novi Sad, Titel). Interestingly, every XCLV sequence was detected in *Culex* spp. pools gathered solely during 2020, but not prior to this year.

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Discussion

Mosquitoes are cosmopolitan insects inhabiting a wide spectrum of biomes. As vectors of infectious agents, these hematophagous arthropods are a cause for great health concern. The investigation of mosquito viromes can yield a flood of data, with the eye of public health oftentimes largely drawn to the zoonotic exponent of this sequencing information deluge. We draw attention to a group of non-human pathogens, namely the *Chrysoviridae* mycovirus family, that could tentatively present with beneficial implications for the health of man.

This study used classical PCR and Sanger sequencing in order to investigate possible presence of West Nile virus in a population of mosquitoes in Serbia, Belgrade. We made a serendipitous detection of the uncommon XCLV, so far described in a single work by Konstantinidis and coworkers [17]. Furthermore, herein we uncover *Culex pipiens* as a new arthropod vector for this chrysovirus, which is as yet an unreported find. The XCLV has been so far scantily mentioned and the full extent of its distribution, be it geographical or vectorial, is yet to be elucidated. The addition of new XCLV sequences will aid in mapping the presence of this virus.

Since the first definitive description of mycoviruses some 60 years ago [22], it has been recognized that viruses are ubiquitous in all major groups of filamentous fungi [23]. *Chrysoviridae* could be involved in impairing phenotypes of the rice blast fungus *Magnaporthe oryzae* [10], the most destructive pathogen of rice worldwide [13]. *Magnaporthe oryzae* chrysovirus 1 strains A (McCV1-A) and B (McCV1-B) were the first reported mycoviruses causing reduced pigmentation, changed colony morphology, and weakened growth – all signs of hypo virulence in the host fungus [13]. This may be a trait that could be exploited to impart a useful effect on industrial plant life.

Interestingly, chrysoviruses were found to impinge on human fungal pathogens as well, such as *Cryptococcus neoformans* [12]. Product of the McCV1-A was even suggested as a protein candidate for a pharmaceutical agent against *C. neoformans* disease [12]. The Alternaria alternata chrysovirus 1 (AaCV1) modulates the pathogenicity of its namesake fungal host, *A. alternata,* as well [13]. Alternaria is known to cause opportunistic infections in immunocompromised humans [24], so the impaired growth caused by the AaCV1 [13] might prove advantageous against the disease. The Aspergillus fumigatus chrysovirus (AfuCV) has also been identified in the eponymous *Aspergillus fumigatus* fungus [25]. Mycoviruses have also been suggested as epigenetic factors inducing alterations in the pathogenicity of plant-infecting fungi [13]. In recent research by Ejmal et al. [11] the authors observed the *Aspergillus thermomutatus chrysovirus* 1 to exert a reduction in the number of conidia from a clinical isolate of the titular fungal pathogen at 20˚C, suggesting the possible use of the virus as a biological control agent.

The genus *Culex* comprises more than 760 taxa, some of which are the most significant vectors of human diseases. Environmental changes influence their geography, heralding changes in their habitat [26].

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Vis-à-vis their topographical expansion lies an as yet unexplored potential for the spread of infectious agents conceivably obliging to humans, such as the present chrysoviruses. Moreover, these viruses are not bound to a singular arthropod genus – the XCLV apparently shares genetic similarity to Soufi chryso-like virus, previously described in the *Uranotaenia unguiculata* mosquito [17]. Our findings of the XCLV in the *Culex* arthropod might hence mean that this is not the only mosquito carrying a chrysovirus within its virome, hinting at a larger distribution of these mycoviruses throughout insect reservoirs and geographic areas alike. The width of the topographical dispersal itself could have significant implications for the value of chrysoviruses to humans.

In recent times plagued by zoonotic epidemics, continuous monitoring of mosquito-associated viral communities can give health systems an edge over advancing disease. Apart for the search for pathogens of concern, it would be advisable to screen for beneficial microorganisms that could tentatively safeguard human health and prove valuable in industry. Additionally, sequencing might prove as the method of choice for pathogen detection, as it is obvious from our research that techniques such as Real-Time PCR and nested PCR can fail in discerning mixed-virus infections.

Finally, biologic interventions with beneficial mycoviruses – an endeavor of opposite approach, but similar outcome to that of *Wolbachia*-based insect population suppression [27] – could hence be undertaken. The exploits posited as biological control agents could possibly be extrapolated to managing human and animal fungal diseases, as mycoviruses have a high specificity to fungal pathogens [11]. This is hitherto a sparsely charted territory, but an attractive one to explore nonetheless.

Our work broadens the arthropod-associated horizons for viral circulation ever so slightly. We stress, however, the added importance of screening insects for the presence of possibly useful microorganisms. The geographical spread of viruses does not, and most certainly will not cease to astound, and should be duly given an important place in molecular investigation. The topographical divergence among viromes in mosquitoes [28] calls for site-to-site sampling and NGS approach in order to more precisely map viral presence in specific areas, expanding our understanding of these at times most useful pathogens.

References

1. Sall AA, Zanotto PM, Sene OK, Zeller HG, Digoutte JP, Thiongane Y, Bouloy M. Genetic reassortment of Rift Valley fever virus in nature. J Virol. 1999 Oct;73(10):8196-200. doi: 10.1128/JVI.73.10.8196-8200.1999.

2. Atoni E, Wang Y, Karungu S, Waruhiu C, Zohaib A, Obanda V, Agwanda B, Mutua M, Xia H, Yuan Z. Metagenomic Virome Analysis of Culex Mosquitoes from Kenya and China. Viruses. 2018 Jan 12;10(1):30. doi: 10.3390/v10010030.

3, Thannesberger J, Rascovan N, Eisenmann A, Klymiuk I, Zittra C, Fuehrer HP, Scantlebury-Manning T, Gittens-St Hilaire M, Austin S, Landis RC, Steininger C. Highly Sensitive Virome Characterization of *Aedes aegypti* and *Culex pipiens* Complex from Central Europe and the Caribbean Reveals Potential for Interspecies Viral Transmission. Pathogens. 2020 Aug 21;9(9):686. doi: 10.3390/pathogens9090686.

4. Stanojević, M., Li, K., Stamenković, G., Ilić, B., Paunović, M., Pešić, B., Maslovara, I. Đ., Šiljić, M., Ćirković, V., & Zhang, Y. (2020). Depicting the RNA Virome of Hematophagous Arthropods from Belgrade, Serbia. Viruses, 12(9), 975. https://doi.org/10.3390/v12090975.

5. Bao X, Roossinck MJ. Multiplexed interactions: viruses of endophytic fungi. Adv Virus Res. 2013;86:37-58. doi: 10.1016/B978-0-12-394315-6.00002-7.

6. Ghabrial SA, Dunn SE, Li H, Xie J, Baker TS. Viruses of Helminthosporium (Cochlioblus) victoriae. Adv Virus Res. 2013;86:289-325. doi: 10.1016/B978-0-12-394315-6.00011-8.

7. Dawe AL, Nuss DL. Hypovirus molecular biology: from Koch's postulates to host self-recognition genes that restrict virus transmission. Adv Virus Res. 2013;86:109-47. doi: 10.1016/B978-0-12-394315-6.00005-2.

8. Hillman BI, Cai G. The family narnaviridae: simplest of RNA viruses. Adv Virus Res. 2013;86:149-76. doi: 10.1016/B978-0-12-394315-6.00006-4.

9. International Committee on Taxonomy of Viruses. *Chrysoviridae*. Available from: https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsrna-viruses/w/chrysoviridae [Accessed on 23 Feb 2022]

10. Urayama S, Sakoda H, Takai R, Katoh Y, Minh Le T, Fukuhara T, Arie T, Teraoka T, Moriyama H. A dsRNA mycovirus, Magnaporthe oryzae chrysovirus 1-B, suppresses vegetative growth and development of the rice blast fungus. Virology. 2014 Jan 5;448:265-73. doi: 10.1016/j.virol.2013.10.022.

11. Ejmal MA, Holland DJ, MacDiarmid RM, Pearson MN. A novel chrysovirus from a clinical isolate of Aspergillus thermomutatus affects sporulation. PLoS One. 2018 Dec 20;13(12):e0209443. doi: 10.1371/journal.pone.0209443.

12. Urayama S, Fukuhara T, Moriyama H, Toh-E A, Kawamoto S. Heterologous expression of a gene of Magnaporthe oryzae chrysovirus 1 strain A disrupts growth of the human pathogenic fungus Cryptococcus neoformans. Microbiol Immunol. 2014 May;58(5):294-302. doi: 10.1111/1348-0421.12148.

13. Moriyama H, Urayama SI, Higashiura T, Le TM, Komatsu K. Chrysoviruses in Magnaporthe oryzae. Viruses. 2018 Dec 8;10(12):697. doi: 10.3390/v10120697.

14. National Center for Biotechnology Information. *Taxonomy browser*. Available from: https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=249310.

15. Veith M, Kosuch J, Vences M. Climatic oscillations triggered post-Messinian speciation of Western Palearctic brown frogs (Amphibia, Ranidae). Mol Phylogenet Evol. 2003 Feb;26(2):310-27. doi: 10.1016/s1055-7903(02)00324-x.

16. Vázquez A, Sánchez-Seco MP, Palacios G, Molero F, Reyes N, Ruiz S, Aranda C, Marqués E, Escosa R, Moreno J, Figuerola J, Tenorio A. Novel flaviviruses detected in different species of mosquitoes in Spain. Vector Borne Zoonotic Dis. 2012 Mar;12(3):223-9. doi: 10.1089/vbz.2011.0687.

17. Konstantinidis K, Dovrolis N, Kouvela A, Kassela K, Rosa Freitas MG, Nearchou A, de Courcy Williams M, Veletza S, Karakasiliotis I. Defining virus-carrier networks that shape the composition of the mosquito core virome of a local ecosystem. Virus Evol. 2022 Apr 16;8(1):veac036. doi: 10.1093/ve/veac036.

18. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990 Oct 5;215(3):403-10. doi: 10.1016/S0022-2836(05)80360-2.

19. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B. RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol. 2015 May 26;1(1):vev003. doi: 10.1093/ve/vev003.

20. Posada D. jModelTest: phylogenetic model averaging. Mol Biol Evol. 2008 Jul 25(7):1253-6.

21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013 Dec 30(12):2725-9.

22. Hollings M. Viruses associated with a die-back disease of cultivated mushrooms. Nature. 1962;196: 962-965.

23. Kim JM, Jung JE, Park JA, Park SM, Cha BJ, Kim DH. Biological function of a novel chrysovirus, CnV1-BS122, in the Korean Cryphonectria nitschkei BS122 strain. J Biosci Bioeng. 2013 Jan;115(1):1-3. doi: 10.1016/j.jbiosc.2012.08.007.

24. Fernandes C, Mota M, Barros L, Dias MI, Ferreira ICFR, Piedade AP, Casadevall A, Gonçalves T. Pyomelanin Synthesis in Alternaria alternata Inhibits DHN-Melanin Synthesis and Decreases Cell Wall Chitin Content and Thickness. Front Microbiol. 2021 Aug 27;12:691433. doi: 10.3389/fmicb.2021.691433.

25. Jamal A, Bignell EM, Coutts RH. Complete nucleotide sequences of four dsRNAs associated with a new chrysovirus infecting Aspergillus fumigatus. Virus Res. 2010 Oct;153(1):64-70. doi: 10.1016/j.virusres.2010.07.008.

26. 21. Sadeghi M, Altan E, Deng X, Barker CM, Fang Y, Coffey LL, Delwart E. Virome of > 12 thousand Culex mosquitoes from throughout California. Virology. 2018 Oct;523:74-88. doi: 10.1016/j.virol.2018.07.029.

27. Wang GH, Gamez S, Raban RR, Marshall JM, Alphey L, Li M, Rasgon JL, Akbari OS. Combating mosquito-borne diseases using genetic control technologies. Nat Commun. 2021 Jul 19;12(1):4388. doi: 10.1038/s41467-021-24654-z.

28. Shi M, Neville P, Nicholson J, Eden JS, Imrie A, Holmes EC. High-Resolution Metatranscriptomics Reveals the Ecological Dynamics of Mosquito-Associated RNA Viruses in Western Australia. J Virol. 2017 Aug 10;91(17):e00680-17. doi: 10.1128/JVI.00680-17.

Author contributions

Conceptualization – M.J. and V.Ć. Methodology, V.Ć., M.Š., A.L.; Writing – original draft preparation, M.J. and V.Ć.; Writing—review and editing, M.J., V.Ć., M.S., G.S., A.L. and M.Š.; All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

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Informed consent statement

Not applicable.

Data Availability Statement

All sequenced data are submitted to the NCBI GenBank database and the IN-DEPTH project website (indepth.rs).

Conflict of Interest

The authors declare no conflict of interest.

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Figures and Tables with Captions

Table 1. Universal primers for the 16S mitochondrial ribosome subunit used in confirmation of mosquito species along with virus-specific primers.

Target	Primer name	Primer sequence (5'-3')	Annealing temparature (˚C)	Primer position in genome (nt)	Reference
16S rRNA	16 Sar	CGCCTGTTTATCAAAAACAT	60	837-1416	13
	16 SBR OiR	CCGGTCTGAACTCAGATCACGT			
WNV *NS5* gene	1NS5F	GCATCTAYAWCAYNATGGG	50	9035-10146	14
	1NS5R	CCANACNYNRTTCCANAC			
	2NS5F	GCNATNTGGTWYATGTGG	50	9120-10122	
	2NS5R	TRTCTTCNGTNGTCATCC			
XCLV	Xanthi_out_F	TGCGGTGTGACAT	52	2108-2904	Designed in this study *
	Xanthi_out_R	AATATTACCAGCTT			
	Xanthi_inn_F	TTACTTGTGCAGGTACT	52	2141-2876	
	Xanthi_inn_R	GGGCAGATCTAATTCCA			

WNV – West Nile virus; XCLV – Xanthi chryso-like virus.

* The primer position on the 3rd segment of the XCLV genome designed according to the sequence MW520404 from the NCBI database.

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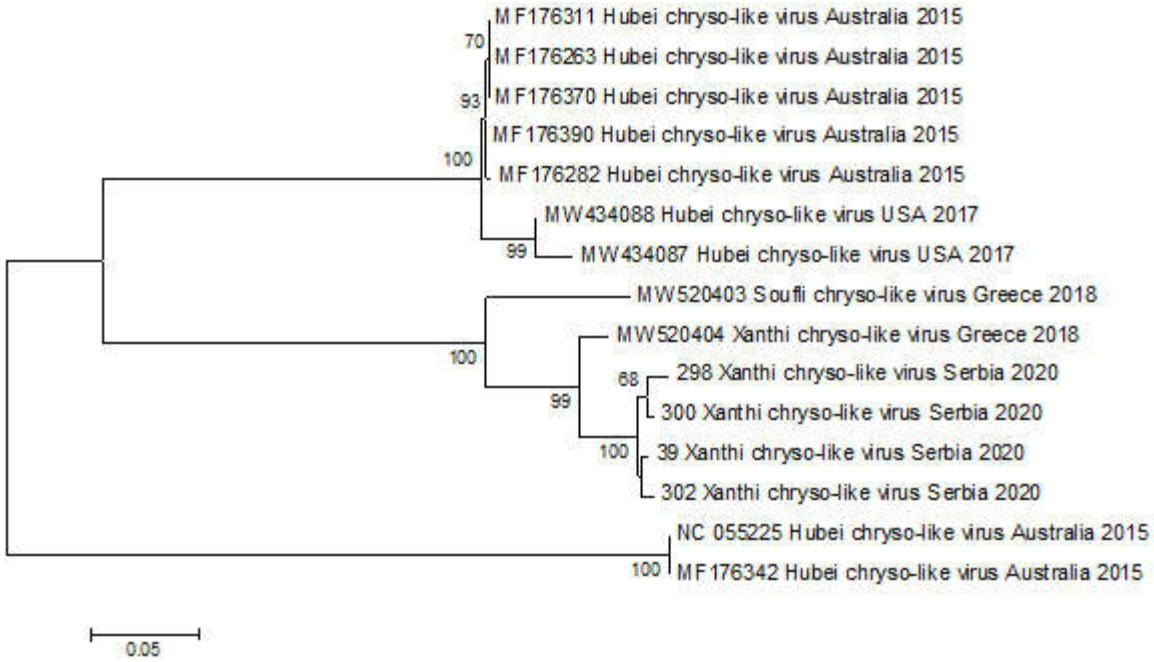

Figure 1. Maximum likelihood phylogenetic tree generated by MEGA 6 software, based on 800nt of 15 chryso-like viruses. Bootstrap values >65% are shown in the tree nodes. Sequences indicated by GenBank Accession number, name of the virus, country and year of isolation.

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Figure 2. Map of Serbia with pins indicating mosquito-sampling sites discussed in this work. Red pins signify locations where XCLV was detected.