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Short communication

First genetic characterization of Usutu virus from *Culex pipiens* mosquitoes Serbia, 2014

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ABSTRACT

Since its first appearance in Europe, Usutu virus (USUV) diverged to several different genetic lineages. The virus was reported to date from multiple countries across Europe (Hungary, Italy, Switzerland, Spain, Germany, Czech Republic and Belgium). Considering the more frequently published impact of the virus on humans it is crucial to investigate locally circulating genetic variants and trace its evolution. We retrospectively analyzed mosquito samples from Serbia Vojvodina region, collected during 2014. In this study we report the results of the screening of 23,753 female mosquitoes (753 pools) for USUV-specific nucleic-acid. Out of the 753 pools sampled, the presence of USUV RNA was confirmed in 3 pools of *Culex pipiens* mosquitoes, collected in August. Based on their partial NS5 sequence, all strains were identical, therefore we adjusted one representative strain for complete genome sequencing. Based on phylogenetic analysis the Serbian USUV sequences were most closely related to the virus that emerged in Austria in 2001, in Hungary in 2005 and was circulating until 2015 in Hungary. This data presents a wider geographic distribution of this genetic variant and provides the first genetic data from this region.

USUV was first isolated in Africa in 1959 (Woodall, 1964), while the first evidence of the virus in Europe was noted in 1996 in Italy (Weissenböck et al., 2013). Since then, several European genetic lineages emerged and multiple independent introduction events were also suggested from Africa to Europe. Moreover, multiple dispersal events occurred between different European territories (Cadar et al., 2015; Cadar et al., 2017a, 2017b; Ziegler et al., 2016; Calzolari et al., 2017; Sieg et al., 2017). A recent publication described the exchange of USUV strains between Italy, Austria and Hungary; although until 2015 European lineage 1 genetic variant was described as the dominant locally circulating strain in Austria and Hungary (Bakonyi et al., 2017a). From Serbia, only serologic evidence of USUV is available, which permits any investigation on the possible origin of locally circulating genetic variants. USUV-specific antibodies were detected only in horse, wild bird and wild boar specimens in seroepidemiological studies conducted in the region so far (Lupulovic et al., 2011; Petrovic´ et al., 2013; Escribano-Romero et al., 2015). These studies denoted the Northern territory of the country (Vojvodina province) for the co-occurrence of WNV and USUV and well supported the need of surveillance studies in order to reveal genetic origins of these strains, however mosquito-related data is still scarce from the region. In this study we first reported molecular evidence for the presence of USUV in female mosquitoes collected in 2014, Vojvodina. We also provided evidence for a wider geographic distribution of the virus in Europe.

Female mosquitoes were collected as part of national mosquito control activities in Vojvodina province. A total of 23,753 adult female mosquitoes were collected from urbanized, human-inhabited areas and typical mosquito breeding sites within cities and small villages, from May to October 2014. Mosquitoes were trapped with CDC light traps baited with dry ice at 59 sampling sites belong to 9 municipalities. All collected mosquitoes were transported to the laboratory on dry ice and kept frozen at −80 °C until further processing. Each specimen was determined by species according to their taxonomic keys (Becker et al., 2003) using a stereomicroscope. Specimens were grouped by a maximum of 50 individuals per sampling event, species and collection site into pools and were processed as described previously (Kemenesi et al., 2014). These pools were subjected to USUV specific nested reverse transcription - polymerase chain reaction (RT-PCR), using degenerated

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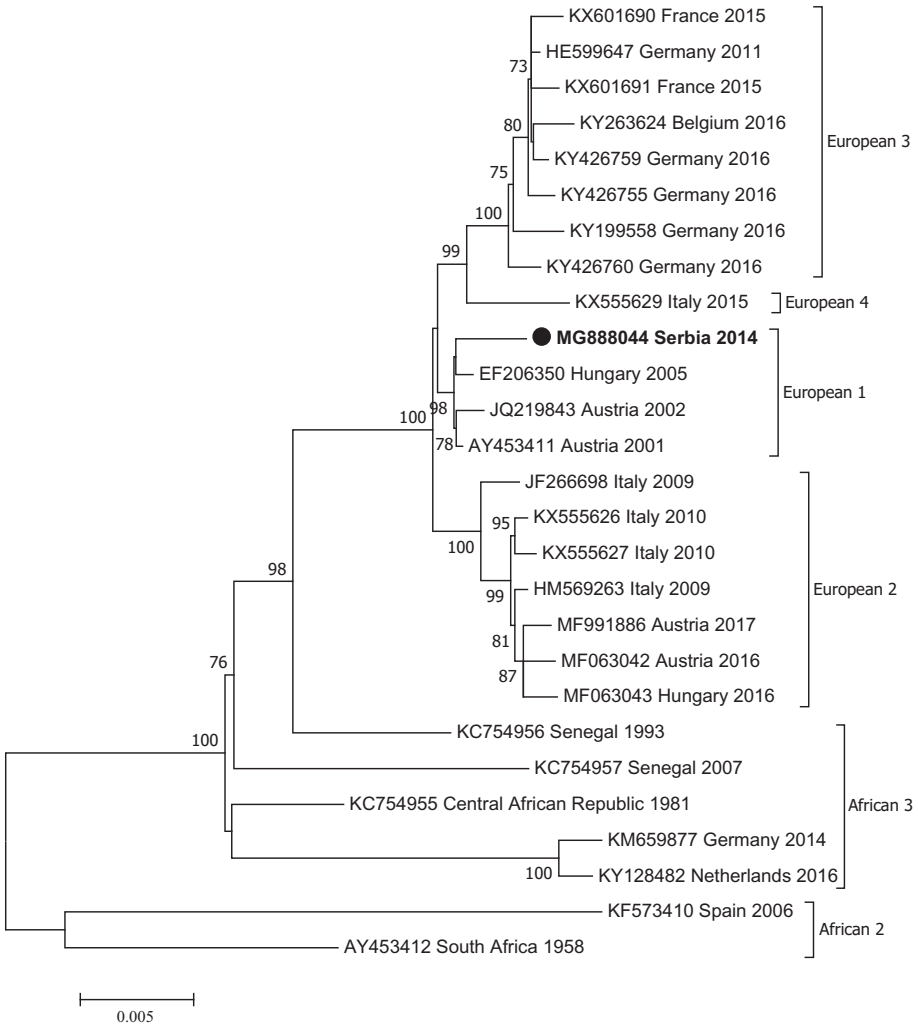

Fig. 1. The evolutionary history was inferred based on 10,302 nucleotide genome fragment by using the Maximum Likelihood method based on the Tamura-Nei model (+G). The best fit nucleotide substitution model was selected based on the Bayesian information criterion as implemented in the MEGA software. Scale bar indicates evolutionary distance, whilst branch support is indicated with bootstrap values. Group names are representing putative genetic lineages from Europe and Africa. Branch support values lower than 70 are not shown.

primers targeting the conserved NS5 gene of flaviviruses (Kuno et al., 1998). Amplicons originating from positive samples were purified with Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech, Taiwan) and bi-directionally sequenced with BigDye Terminator v1.1 Cycle Sequencing Kit according to the manufacturer's protocol on ABI Prism 310 DNA Sequencer platform (Applied Biosystems, USA). The complete genome of the Serbian USUV strain was amplified by RT-PCR method, resulting in four large genome segments. These large amplicons were subjected for nested PCR reactions, resulted in overlapping fragments of the genome. The amplicons were compiled into continuous sequence, using Geneious software (http://www.geneious.com) (Kearse et al., 2012). Phylogenetic analysis was implemented in MEGA 7 software based on 10,302 nucleotide genome fragments, containing the complete polyprotein coding sequence along with partial 3′ UTR, trimmed to the shortest sequence in the dataset.

The most abundant sampled species was *Culex pipiens* (n = 11,099, 47% of all mosquito specimens), followed by *Aedes vexans* (n = 10,005, 42% of all mosquito specimens). Dominant species of the investigated area were *Ochlerotatus caspius* (n = 1574, 7%), *Ochlerotatus sticticus* (n = 559, 2.4%) and *Coquillettidia richiardii* (n = 332, 1.4%) as well. All other species were represented with considerably lower number (n < 100, < 0.5% of all mosquito specimens). A total of 1437 female mosquitoes (68 pools) were collected in May, n = 4252 (158 pools) in June, n = 1512 (52 pools) in July, n = 13,683 (403 pools) in August, n = 54 (2 pools) in September and n = 2815 (70 pools) in October.

Out of the 753 pools sampled, the presence of USUV RNA was confirmed in 3 pools of *Culex pipiens* mosquitoes, collected in August from Titel (45°12′N, 20°18′E, 1 positive out of 38 pools) and Zrejanin (45°22′N, 20°23′E, 2 positives out of the 330 pools).

Cx. pipiens was frequently described as a primary vector of the virus across Europe (Ashraf et al., 2015; Fros et al., 2015; Nikolay, 2015; Cadar et al., 2017a). However, *Cx. pipiens* mosquitoes are originally considered with ornithophilic feeding behavior (Brugman et al., 2017), the two distinct biotypes (*Cx. p. pipiens* and *Cx. p. molestus*) show remarkable physiological and behavioral differences. While the *Culex p.* biotype *pipiens* rarely bite humans and seems to be strictly ornithophilic (i.e. bird biting host preference), females of the biotype *molestus* are mainly anthropophilic (Becker et al., 2012). At the same time, human host preference was identified in case of *pipiens* and *molestus* forms and their hybrids as well (Martínez-de la Puente et al., 2016). The adaptation of *Cx. pipiens* mosquitoes to human-altered environments led to their global distribution through dispersal via humans and, combined with their mixed feeding patterns on birds and mammals (including humans), predestine them as bridge vectors for pathogens transmitted between mammals and birds (Ashraf et al., 2015; Fros et al., 2015; Nikolay, 2015; Cadar et al., 2017a). Although we did not identify