First record of Aedes japonicus in Liguria region, Northwest Italy

Valeria Listorti*, Annalisa Accorsi, Maria Vittoria Riina, Simone Peletto, Pier Luigi Acutis, Valerio Carta, Alessio Ferrari, Cristiano Corona, Cristina Casalone, Carlo Ercolini, Lisa Guardone, Francesco Brunelli and Elisabetta Razzuoli

Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta.

Corresponding author at: Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta.
E-mail: valeria.listorti@izsto.it.

Summary
Aedes japonicus is an invasive Asian mosquito species, and to date it is widespread in many European countries. In Italy, it was first recorded in 2015 at the Austrian border and it then spread throughout the Northeast of the country. In 2019, it was also identified in Piedmont region, near the Swiss border. In the framework of the Italian program for prevention, surveillance, and response to Arboviruses, from June to November 2021, biweekly entomological surveillance was performed in the Liguria region (Northwest Italy). The collected mosquitoes were morphologically and genetically identified and molecularly analysed for the detection of West Nile and Usutu viruses. Six female mosquitoes, trapped on the 6th of July 2021 using a gravid trap in Albenga (Savona province), were morphologically identified as *Ae. japonicus* and the identification was genetically confirmed. The pool tested was negative for the presence of West Nile and Usutu viruses. The detection of *Ae. japonicus* was performed in a coastal area characterized by the presence of many floriculture activities. Considering the distance from the established *Ae. japonicus* mosquito populations in Italy and other European countries, this could represent an independent introduction in this country.

The invasive species *Aedes japonicus* (Theobald, 1901) is native to East Asia and the far East (Tanaka *et al.*, 1979, Kampen *et al.*, 2014). From the 90's it has spread in New Zealand (Laird *et al.*, 1994), the USA (Peyton *et al.*, 1999) and in Europe, where, after its first detection in the north of France in 2000 (Schaffner *et al.*, 2003), this species was observed in different European countries, such as Belgium (Versteirt *et al.*, 2009), Switzerland, Germany (Schaffner *et al.*, 2009), the Netherlands (Ibaňez-Justicia *et al.*, 2009), Austria, Slovenia and Hungary (Seidel *et al.*, 2016 a), Croatia (Klobucar *et al.*, 2019), and Spain (Eritjia *et al.*, 2019). It was also reported recently in Bosnia and Herzegovina and Serbia (Janssen *et al.*, 2020). This species was identified for the first time in Italy in 2015 at the Austrian border (Seidel *et al.*, 2016 b) and then spread in the Northeast of the country, colonizing two regions in few years (Montarsi *et al.*, 2016). In 2019, *Ae. Japonicus* was also isolated in Piedmont region (Northwest Italy), about 20 kilometres from the Swiss border, and in the two following years this species spread all over the region (Mosca *et al.*, 2022). The source of introduction of this species in European countries is not clear, a connection with the tyre trade importation and ground transport has been hypothesised (Medlock *et al.*, 2012).

Aedes japonicus is an experimentally proven vector for the transmission of flaviviruses such as Japanese Encephalitis virus (Huber *et al.*, 2014), Chikungunya, Dengue (Schaffner *et al.*, 2011) and Zika viruses (Jansen *et al.*, 2018); its vector competence for West Nile virus (WNV) is debated. A transmission

rate for WNV even higher than for *Culex pipiens* was observed in a study conducted in laboratory conditions (Turell *et al.*, 2001), while in another one *A. japonicus* seemed refractory to the infection with WNV (Huber et al., 2014). However, *Ae. japonicus* collected in the USA have been found infected with WNV (De Carlo *et al.*, 2020).

In Italy a national plan, named "Plan for prevention, surveillance, and response to Arboviruses", is enforced by the Ministry of Health (Italian Ministry of Health website).

Among the objectives of this plan are the surveillance of new invasive species, potential vectors of arboviruses, and the surveillance of WNV and Usutu virus (USUV) circulation in mosquitos. This plan provides for the identification on the national territory of sites for entomological and virological surveillance, in the period of maximum circulation of mosquitoes. Such sites are identified on the basis of the risk of WNV and Usutu viruses (USUV) circulation and of the risk of new invasive species introduction. The Liguria region is characterized by many important commercial and touristic ports, and also by various floriculture activities and markets, possible sources of introduction of invasive mosquito species. For these reasons, entomological surveillance is fundamental for their early detection and monitoring.

The aim of this work is to report the identification of *Ae. Japonicus* in Liguria in the framework of the above-mentioned surveillance plan.

A biweekly surveillance was performed in Liguria region (Northwest Italy) from June to November 2021. The traps were set up for both WNV-USUV surveillance and for invasive mosquitos monitoring. Adult mosquitoes were trapped using CO_2-lure-baited BG-sentinel traps or gravid traps, both types working overnight, in 20 sites.

After the collection, the mosquitoes were transported refrigerated to the entomological laboratory where they were morphologically identified using entomological keys (Severini et al., 2009, Becker *et al.*, 2010).

The ECDC guidelines (ECDC website) and specific entomological keys (Tsai-Yu *et al.*, 1946) were used for the identification of invasive species. In case of doubtful identification, single specimens were identified by molecular analysis: after homogenization in phosphatase-buffered saline (PBS), DNA was extracted using a commercial kit (ReliaPrep™ gDNA Tissue MiniPrep System – Promega, Madison, WI). Then, a portion of two different mitochondrial genes, cytochrome oxidase subunit I (*COI*) gene and nicotinamide adenine dinucleotide dehydrogenase subunit 4 (*ND4*) gene, were amplified by PCR following previously described methods (Dawnay *et al.*, 2007, Cameron *et al.*, 2010). The PCR products were sequenced on both strands using BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA) and each obtained consensus sequence was compared with sequences deposed in GenBank. A similarity ≥98.0% was considered as cut off for identification at the species level. Moreover, phylogenetic analyses were performed with MegAlign sequence alignment software (DNASTAR Inc., Lasergene package) to generate a phylogenetic tree with a bootstrap analysis to evaluate the statistical significance of the nodes. A value ≥70 corresponds to a statistical significance value ≥95% at the node.

Following the national surveillance plan, female specimens of the same mosquito species from each site were pooled to test for the presence of WNV and USUV. RNA was extracted using a commercial kit (Rneasy Mini Kit 250, QIAGEN, Valencia, CA) and analysed by a multiplex real-time reverse transcription-PCR for the simultaneous detection and differentiation of WNV Lineages 1 and 2 (Del Amo *et al.*, 2013), and by a real-time RT-PCR specific for USUV detection (Cavrini *et al.*, 2011).

Six female specimens of mosquito collected in Albenga, in the province of Savona (44.067169 N, 8.158698 E) using a gravid trap, on the 6th of July 2021, were individually morphologically identified as *Ae. japonicus* (Fig. 1).
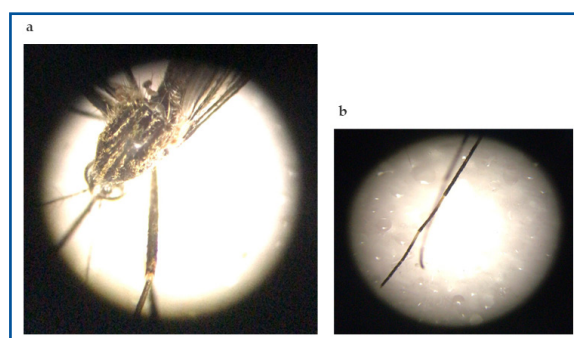

Figure 1. *a: Torax. Scutum dorsal side: stripes of golden scales, submedians long; b: Last segments of the third leg. Segments IV and V black.*

The genetic analyses confirmed the morphological identification. Regarding the *COI* gene, a 100% identity was found with the sequences of *Ae. japonicus* deposited in GenBank.

The same result was confirmed with the *ND4* gene analysis, where the similarity was 98.9%. Furthermore, the sequences showed the characteristic mutation in the position 181, which, according to Cameron et al. (Cameron *et al.*, 2010), represents a diagnostic site: T for *Ae. koreicus*, G for *Ae. japonicus*, A for the other *Aedes* species.

Representative sequences were submitted to GenBank with ON911330 accession number for the COI sequence and ON933815 for the ND4 sequence. Phylogenetic analyses confirmed the identification

showing clustering of the obtained sequences with other *Ae. japonicus* sequences from GenBank for both gene targets (Fig. 2). The pool tested was negative for WNV and USUV.
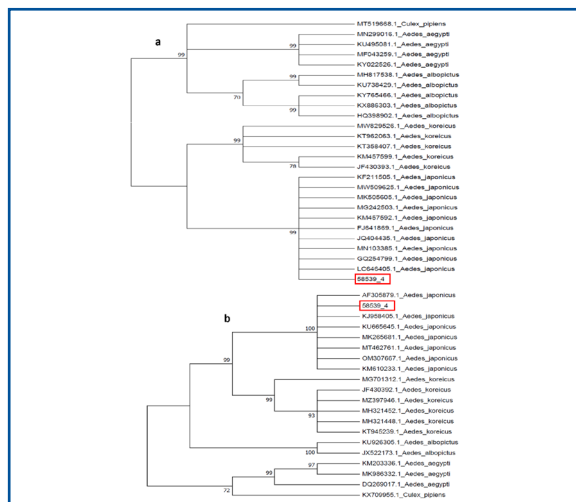

Figure 2. *Phylogenetic tree and bootstrap analysis for a) COI gene and b) ND4 gene.*

For the first time the invasive species *Ae. japonicus* was identified in Liguria region, Northwest of Italy, six years after its first detection in the region and in a site hundreds of kilometres away from its spreading area in the Northeast of Italy, and also from the other recent Italian finding in the Piedmont region. The described record could thus represent an independent introduction in a territory characterised by intensive floriculture activities, importing plants and flowers from many parts of the world. However, this hypothesis needs to be considered with caution as it was not possible to demonstrate the exact time and route of introduction. During the 2021 monitoring season, this was the only finding of *Ae. japonicus*. Also in other European countries, such as Germany, multiple independent introductions of this species have been observed in different areas and time (Schneider 2011, Zielke *et al.*, 2015, Reuss *et al.*, 2018). The southernmost detections reported for this species in Europe are in Spain, in the Asturian region (Eritjia *et al.*, 2019), and in Eastern countries such as Croatia, Bosnia and Herzegovina and Serbia (Janssen *et al.*, 2020). The site of the present detection is almost at the same latitude but it is characterised by different climate conditions.

The Liguria region has a Mediterranean climate, characterised by hot, dry summer and mild, wet winters. In particular, in the city of Albenga, the mean daily temperature ranges between 3-12°C during winter and between 19-29°C during summer. The optimal temperature for *Ae. japonicus* life-cycle has been experimentally investigated. An optimal female survival temperature at 25°C and minimal at 7°C have been proven. The larvae are capable to

hatch after exposure to 5°C water temperature and can survive at low temperatures for a long period. The hatching success decreases drastically at 0°C and -5°C (Reuss *et al.*, 2018). Thus, considering the winter temperature of the area, and the diapause capability of *Ae. japonicus* (Krupe *et al.*, 2021), it is possible to expect an overwintering. *Aedes japonicus* is considered a cold-tolerant species and in North America; it has been hypothesised that the expansion to southern latitudes is limited by temperatures regularly exceeding 30-35°C (Kaufman *et al.*, 2014). Moreover, experimentally the development to female adults has been observed at maximum 31°C (Reuss *et al.*, 2018). The low and high temperatures are limiting for *Ae. japonicus* and it is probably also for this reason that it is present only in some European countries. Moreover, Cunze et al. (Cunze *et al.*, 2016) modelled the possible future ecological niches for *Ae. albopictus* and *Ae. japonicus* in Europe, considering climate changes and environmental characteristics. The Mediterranean regions of Italy, characterised by a warmer climate, with summers that are becoming hotter and hotter over time, are not considered suitable for the presence of *Ae. japonicus*, that is less able to expand in warmer conditions compared to *Ae. albopictus* (Kaufman *et al.*, 2014).

In the Liguria region, other Aedes invasive mosquito species occurred, such as *Ae. albopictus*, widely present, and *Ae. koreicus* was detected for the first time in 2015, then it has become endemic in Genova province (Ballardini *et al.*, 2019). It would be interesting to observe the future distribution of *Ae. japonicus* in the Liguria region, which to date is one of the southernmost European sites of detection, and its distribution overlaps with other *Aedes* species. The province of Savona, where the pool was collected, is considered by the above-mentioned national plan on Arboviruses, to have a low level of risk for the circulation of WNV, because the virus has not been detected in the past five years. However, the circulation of the virus has been observed in the neighbouring province of Genova, an area with the same climate and environmental conditions. So, the future possible spreading of this species as potential vector could represent a risk also for the spread of arboviruses. Unfortunately, no control measures have been taken to eradicate this species. The entomological surveillance included in the national plan for prevention, surveillance, and response to Arboviruses allowed to detect *Ae. japonicus* in the Liguria region, in the northwest of Italy, which could represent an independent introduction in the Country. Data on the following monitoring activities will help in understanding if *Ae. japonicus* is becoming endemic in the area and if it is spreading to other sites.

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