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First isolation of the Sindbis virus in mosquitoes from southwestern Spain reveals a new recent introduction from Africa

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ABSTRACT

Sindbis virus (SINV), is an *Alphavirus* of the family *Togaviridae*. This zoonotic arbovirus is transmitted by mosquitoes, primarily from the *Culex* genus, with bird species acting as amplifying vertebrate hosts. Occasionally it can also affect humans that are accidental hosts. SINV genotype I (SINV-I) has been isolated in mosquitoes and birds in South Africa and Northern Europe, producing fever outbreaks. In the last decades, there were several detections of SINV in Europe. In 2022, during the West Nile virus (WNV) mosquito surveillance program in Andalucía (Spain) implemented by the regional health administration, we detected the presence of both SINV and WNV in a *Culex perexiguus* pool, representing the first detection of SINV in Spain. After this finding, we screened 1149 mosquito pools to determine the status of SINV circulation in western Andalucía. We identified for the first time the presence of SINV in five different mosquito species. *Culex perexiguus* presented the highest infection rate by SINV. In addition, SINV was geographically widespread and distributed in four out of the five Andalucía's provinces studied, with Cadiz presenting the highest infection rate. All SINV genomes from South-western Spain characterised in this study belonged to SINV-I, previously detected in Europe and Africa. These isolated SINV-I strains presented low molecular variation among them and in the phylogenomic analyses they formed a monophyletic group that clustered with strains from Algeria and Kenya. These results suggest that, around 2017, a single new SINV introduction into the European continent occurred, probably from Northern (Algeria) or Central Africa.

1. Introduction

Sindbis virus (SINV), family *Togaviridae* and genus *Alphavirus*, was initially isolated from mosquitoes in Cairo, Egypt, in 1952 [1]. This zoonotic arbovirus primarily involves bird species as amplifying vertebrate hosts, *Culex* mosquitoes as vectors, and occasionally affects humans as accidental dead-end hosts [2]. Like other *Alphaviruses* such as Chikungunya, Ross River, or Mayaro viruses, SINV infection may be associated with symptoms like fever, exanthema, arthralgia, and myalgia. These symptoms can persist as long-lasting polyarthralgia/polyarthritis for several years in approximately 25 % of the patients [3].

SINV is a positive single-stranded RNA virus with a genome size of 11.7 kb [4]. Six genotypes (Genotypes I to VI) have been identified, each restricted to specific geographical regions. Genotype I (SINV-I) has been

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isolated in Europe, Africa, and the Middle East; SINV-II and SINV-VI in Australia; SINV-III was discovered in Southeast Asia; SINV-IV in Asia and the Middle East; and SINV-V (also known as Whataroa virus) in New Zealand [2].

SINV-I is the genotype associated with the reported fever outbreaks in South Africa, Northern Europe and Wester Russia [5,6]. The first cases of SINV infection in Northern Europe were observed in Sweden in 1967 [3]. Since the 1980s numerous Sindbis fever outbreaks have occurred in Russia (known as Karelian fever [6], and Northern Europe, (known as Pogosta and/or Ockelbo disease in Finland and Sweden respectively), with more than 3350 cases recorded in Finland and hundreds of cases in Sweeden [7,8]. In addition, serological evidence of SINV infection has also been found in humans [5] and birds [9] in other European countries [5]. In Spain, antibodies against *Alphavirus* in humans were studied using a hemagglutination inhibition assay. Although 4.1 % of the serum analysed showed a reaction against *alphavirus*, it was not possible to identify which virus caused the infection [10].

Geographic distribution of SINV-I has been linked with dispersal via migratory birds [2]. Phylogenetic analysis suggested a single introduction from Africa to Sweden, and from Sweden to Finland, Germany and Russia [11]. However, three independent introductions of SINV-I may have occurred in Central Europe, two from Northern Europe and one from Central Africa. These introductions led to a recombination of strains in Central Europe, forming two main groups. One group contains strains from Sweden, Finland, Norway, Germany, and Russia, while the other includes strains from Central African countries [11].

In Europe, SINV-I has been detected/isolated in various mosquito species, including *Culex torrentium*, *Culex pipiens* [1,5], *Culex modestus* [12], *Culex univittatus* [1], *Culex neavei*, *Culiseta morsitans*, and several *Aedes* species [5,8,13]. In addition, vector competence assays confirmed the capacity of *Cx. neavei*, *Cx. pipiens* and *Cx. torrentium* in the transmission of SINV-I [13,14].

Mosquito-based virus surveillance has been proposed as a reliable and cost-effective tool for the early detection of arbovirus outbreaks [15]. In 2022, SINV was serendipitously discovered in Western Andalucía (Spain) while analysing the WNV genome from a *Culex perexiguus* pool that had mosquitoes infected with WNV and SINV. This event represents the first detection of SINV in Spain. After this finding, we determined the status of SINV circulation in mosquitos in this area and characterised the genome sequences of SINV to analyse the genetic diversity and evolutionary relationships with the strains detected in other countries.

2. Material & methods

2.1. Mosquito sampling

Between May and October 2022, we trapped mosquitoes at 42 study sites in 5 different provinces (Cadiz, Cordoba, Huelva, Malaga and Seville) in Southwest Spain (Fig. 1). We placed CDC traps or BG traps for 24 h baited with 1 kg of dry ice. Mosquitoes were transported in dry ice to the laboratory and stored at −80 °C until morphological identification was carried out on a chill table, always maintaining the cold chain. Mosquito species identification was carried out following MoskeyTool

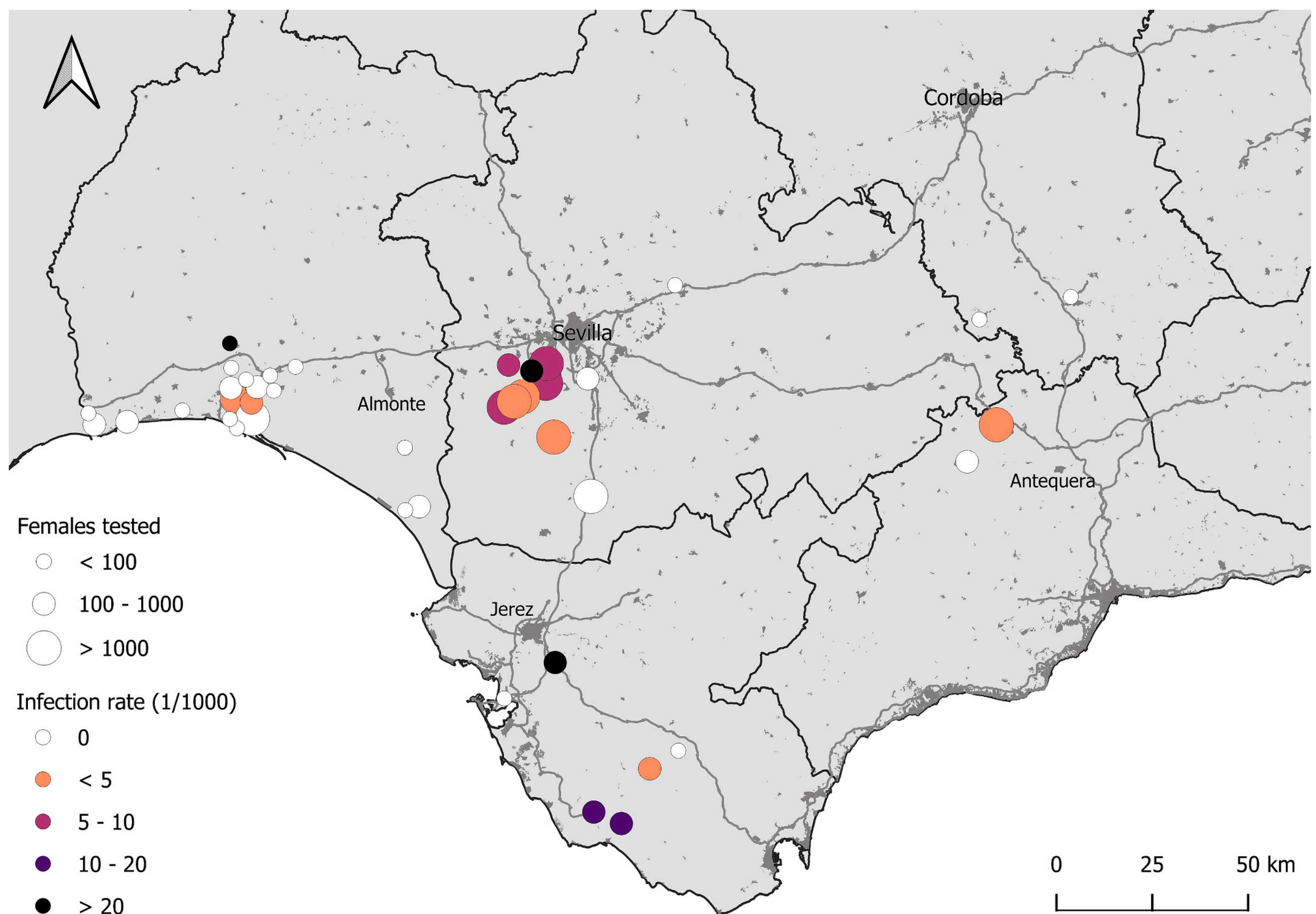

Fig. 1. Mosquito study sites. The size of the circle indicates the number of female mosquitoes analysed and the colour indicates SINV infection rates for each study sites.

key [16]. The identified mosquitoes were pooled by species, sex, date, and study site [pool ranged from 2 to 50] (Table 1).

2.2. SINV screening

Before RNA extraction female mosquito pools were homogenised in 500 μl MEM buffer (minimum essential buffer) supplemented with 10 % fetal bovine serum, 0.5 % penicillin and streptomycin, and 10 % L-Glutamine (Sigma-Aldrich, St. Louis, MO, USA) using a TissueLyser homogenizer (QIAGEN, Valencia, CA) for 2 min at 30 cycles/s. To clarify homogenates, they were centrifuged at 13,000 rpm for 5 min at 4C. Viral RNA was extracted using a Maxwell® extraction robot and the Viral Total Nucleic Acid Purification kit (Promega, Madison, WI, USA). We tested the presence of SINV using two sequential approaches. First, we used a real-time RT–QPCR that amplified a fragment of 134 bp of the non-structural proteins 1 (nsP1) region of SINV [17]. Samples with Ct > 40.0 were considered negative. The positive samples were further analysed using a generic alphavirus nested RT–PCR that amplified a fragment of 195 bp of the nsP4 region [18]. Virus identification was confirmed by sequencing the 195 bp fragments, and only successfully sequenced samples were considered positive for SINV.

2.3. Viral sequencing

Viral isolation was assayed in those samples with Ct value <30 of each study site (one or two samples from each study site), using African green monkey kidney cells (Vero) culture in T-25 flask. A volume of 100 μl of mosquitoes' homogenate was inoculated on Vero cells at 37 °C for

Table 1
Infection rates in the study sites analysed from the five provinces (95 % confident intervals are included).

Province	Study sites	Total mosquitoes	Range mosquitoes per pool	Pools analysed (N)	Positive pools (N)	Mean Ct-value	Infection rate	CILow 95 %	CIHigh 95 %
Cadiz	*Alcalá de los Gazules*	28	[1–7]	10	0	–	0	0	66
	Barbate	385	[2–50]	12	5	30	20	7	46
	Benalup Casas Viejas	802	[1–51]	35	1	35	1	0.7	6
	Jerez de la Frontera	338	[1–50]	13	6	39	32	12	69
	Puerto Real	21	[1–4]	11	0	–	0	0	87
	Vejer de la Frontera	187	[2–50]	8	2	30	13	2	41
Cordoba	*Lucena*	87	[2–41]	8	0	–	0	0	21
	Puente Genil	66	[1–24]	7	0	–	0	0	29
Huelva	*Álamos*	700	[1–50]	43	0	–	0	0	3
	Calatilla	1577	[1–50]	47	5	30	3	1	7
	Camping Giralda	109	[1–23]	19	0	–	0	0	18
	Casa de los Ingleses	25	[1–7]	6	0	–	0	0	74
	Celestino Mutis	1967	[1–50]	66	0	–	0	0	0.9
	Ciudad de los Niños	77	[1–15]	12	0	–	0	0	25
	Corrales	204	[1–50]	22	0	–	0	0	9
	Estación de aguas residuales de Huelva	515	[1–50]	24	1	36	2	1	9
	Estación de aguas residuales de Moguer	56	[1–7]	24	0	–	0	0	34
	El Rocio	2	[1]	2	0	–	0	0	617
	Gibraleon	24	[1–4]	13	1	24	43	3	174
	Golf El Rompido	74	[1–6]	23	0	–	0	0	26
	Granja Escuela	47	[1–7]	17	0	–	0	0	40
	Isla Canela	125	[3–50]	9	0	–	0	0	15
	Laguna de Santa Olalla	30	[1–7]	11	0	–	0	0	62
	Finca Las Herrumbes	59	[2–31]	6	0	–	0	0	32
	Palacia de Doñana	106	[1–22]	26	0	–	0	0	18
	Punta Umbría	13	[1–4]	6	0	–	0	0	137
	Ribera de Guadiana	61	[1–24]	15	0	–	0	0	31
	Silvasur	95	[1–49]	9	0	–	0	0	20
Malaga	*Campillos*	809	[1–51]	21	0	–	0	0	2
	Fuente de Piedra	1825	[1–50]	44	1	28	0.6	0.3	2
Seville	*Almensilla*	157	[9–50]	4	3	33	56	11	253
	Bollulos de la Mitación	237	[2–50]	8	2	30	10	2	30
	Los Palacios-Brazo del Este	6528	[1–51]	152	22	32	4	2	5
	Las Cabezas de San Juan	1618	[1–51]	59	0	–	0	0	1
	La Cañada de los Pájaros (La Puebla del Río)	1301	[1–50]	35	5	29	4	2	9
	Carmona	33	[1–9]	10	0	–	0	0	57
	La Dehesa de Abajo (La Puebla del Río)	2090	[2–50]	52	15	32	9	5	14
	Dos Hermanas	183	[1–24]	33	0	–	0	0	10
	Finca La Hampa (Coría del Río)	3326	[1–50]	82	26	33	10	6	14
	La Puebla del Río	3179	[1–50]	73	24	33	9	6	13
	Palomares del Río	1330	[1–50]	38	11	27	10	5	18
	Pinares de la Colina (La Puebla del Río)	1524	[1–50]	34	7	35	5	2	10

one hour, then, the medium was removed and fresh maintenance medium (MEM supplemented with 2 % FBS, 1 % penicillin-streptomycin, 1 % (200 mM) L-glutamine) was added. Cells were incubated at 37 °C and examined daily cytopathic effect (CPE). Whole genome sequencing was carried out on all the virus cultured that showed CPE. Viral RNA was extracted using the Quick-RNA Viral kit (Zymo, Irvine, CA, USA), quantified using QuantiFluor RNA System (Promega, Madison, WI, USA) and verified its integrity using Bioanalyzer 2100 and Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). Sample library preparation was performed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina® and NEBNext® Multiplex Oligos for Illumina®, Index Primers Set 3 (New England BioLabs Inc., Ipswich, MA, USA) and sequenced on an Illumina MiSeq v2 (300 Cycles).

To obtain a viral consensus genome, sequences were analysed following [19], using viralrecon pipeline v2.6.0 (https://github.com/nf-core/viralrecon) (accessed on 23rd November 2023) [20] written in Nextflow (https://www.nextflow.io/) in collaboration between the nf-core community (https://nf-co.re/) and the Bioinformatics Unit of the Institute of Health Carlos III (BU-ISCIII) (https://github.com/BU-ISCIII) (further details in Supplementary Material).

2.4. Phylogenetic and molecular clock analysis

Complete genomes obtained were compared with 48 complete SINV sequences available in Genbank (accessed on 25th January 2024) (Table S1). Multiple alignment was carried out with ClustalW test using MAFFT alignment [21]. GTR + G + I was chosen as the best fit model according to the Akaike Information Criterion (AIC) using jmoldeltest 2.1.10 [22] in CIPRES [23]. A maximum likelihood phylogeny was generated using this model and 1000 bootstraps in MEGA11 [24]. The genome SINV strain YN_22 (GenBank accession number: MH229928) was used as an outgroup. The pairwise-distances between sequences isolated from Spain were analysed using the Maximum Composite Likelihood model in Mega 11 [24].

To study the evolutionary history and divergence times of SINV-I we explored the temporal structure of the subset of sequences that belonged to SINV-I genotype. We carried out a root-to-tip regression analysis using TempEst [25]. The 53 sequences in the subset (Table S1) showed a temporal signal with a correlation coefficient of 0.78 and thus this subset was further analysed to estimate divergence time and evolutionary rates. We ran Bayesian Markov Chain Monte Carlo (MCMC) analyses using BEAST v.2.5 [26]. We tested 4 different combinations of demographic and molecular clock models: i) Strict clock and Coalescent exponential population model, ii) Strict clock and Coalescent Bayesian skyline model; iii) Relaxed log-normal clock and Coalescent exponential population model and iv) Relaxed log-normal clock and Coalescent Bayesian skyline model. We ran 60 million Bayesian MCMC generations sampling every 1000 generations. Convergence was assessed with Tracer v.1.7 [25]. We used Nested Sampling implemented in BEAST v.2.5 to select the best model [27]. Then, Maximum clade credibility (MCC) phylogenies were built using TreeAnnotator with 10 % burn-in. MCC trees were viewed and edited with the R package ggtree in R software v.4.3.1 (R Core Development Team, 2016). For each dataset we calculated posterior probabilities for each branch, nucleotide substitution rates and divergence times with error reported as the 95 % highest probability density (95 % HPD).

2.5. Statistical analysis

SINV infection rate for each study site, province and mosquito species was estimated by Maximum Likelihood (MLE), considering the number of mosquitoes in each pool. The infection rate is presented as the estimated number of mosquitos with positive SINV detection out of 1000 mosquitos, (i.e., MLE * 1000). The mean and 95 % confidence intervals were estimated with the function PoolPrev from the R package PoolTestR [28]. We carried out a Generalized Linear Model to analyse the differences in infection rates (dependent variable) between mosquito species and provinces with positive pools (independent variables) using the function PoolReg [28]. Statistical analyses were performed in R software v.4.3.1 (R Core Development Team, 2016).

3. Results

3.1. Mosquito trapping and SINV screening

A total of 31,920 female mosquitoes from six different species were collected in the study sites (Fig. 1). Among them, 1149 pools were analysed for the presence of SINV, and 11.92 % (137 pools) showed SINV amplification, with Ct values ranging from 16 to 40 (Ct value mean = 32), obtaining a total infection rate of 4.7 % (Table 1). All the positive samples by the RT-QPCR were confirmed by the generic alphavirus nested RT–PCR. The positive pools correspond to mosquitoes captured between June 23rd and October 21st, 2022. *Culex perexiguus* showed significantly higher SINV infection rates than *Ae. caspius* and *Cx. pipiens*. Meanwhile, *Cx. modestus* showed significantly higher SINV infection rates than *Cx. pipiens* (Table 2 and Tables S2 and S3). *Aedes detritus* was the only species where SINV was not detected.

The presence of SINV was confirmed in all the provinces except for Cordoba. Among the provinces, Seville showed a significantly lower SINV infection rate than Cadiz (Est = 0.70; Std. Error = 0.29; *P*-value = 0.02) (Table 2 and Table S3). The highest proportion of positive localities was found in Cadiz, with four out of the six localities studied having at least one positive pool (Fig. 1).

3.2. Virus isolation and phylogenetic analysis

SINV showed CPE at 3–4 days post-infection at passage 1 in Vero cells. We isolated virus from 19 cell culture, of which, we obtained 16 SINV complete genomes, all of them isolated from *Cx. perexiguus* captured at 14 study sites. Complete genome sequences were deposited in GenBank (accession numbers: PP879145 - PP879160; Table S4).

For the 16 SINV isolated, we sequenced 11,442 nucleotides (nt) corresponding to 3777 amino acids covering the four non-structural proteins regions nsP1 (1620 nt, 540aa), nsP2 (2421 nt, 807aa), nsP3 (1809 nt, 568aa) and nsP4 (1848 nt, 616aa), the coding region of the capsid C (798 nt, 264aa), the entire E3 protein (192 nt, 64aa), E2 protein (1269 nt, 423aa), 6 K protein (165 nt, 55aa), and the E1 protein (1320 nt, 440aa).

The maximum likelihood phylogeny based on the complete genomes showed that SINV isolates belonged to genotype I (Fig. 2). This genotype is divided into two main clades with well-supported values. All the genomes sequenced in Spain cluster together and are grouped in a clade

Table 2
Results of the multivariate model analysing the relationship between the infection rate estimate of SINV and the mosquito species and province (S.E. = standard error).

Variables	Category	Estimate	S.E.	Z	P
Mosquito species + Province	Intercept	−6.07	0.91	−6.69	<0.001
	Aedes caspius	0*			
	Culex laticintus	1.37	1.35	1.02	0.31
	Culex modestus	1.73	0.97	1.79	0.07
	Culex perexiguus	1.74	0.88	1.97	0.048
	Culex pipiens	−0.42	0.80	−0.52	0.60
	Cadiz	0*			
	Huelva	−0.57	0.70	−0.81	0.42
	Malaga	−1.39	1.21	−1.15	0.25
	Seville	−0.70	0.29	−2.41	0.016

*Cadiz is the category used as reference.
* *Aedes caspius* is the category used as reference.

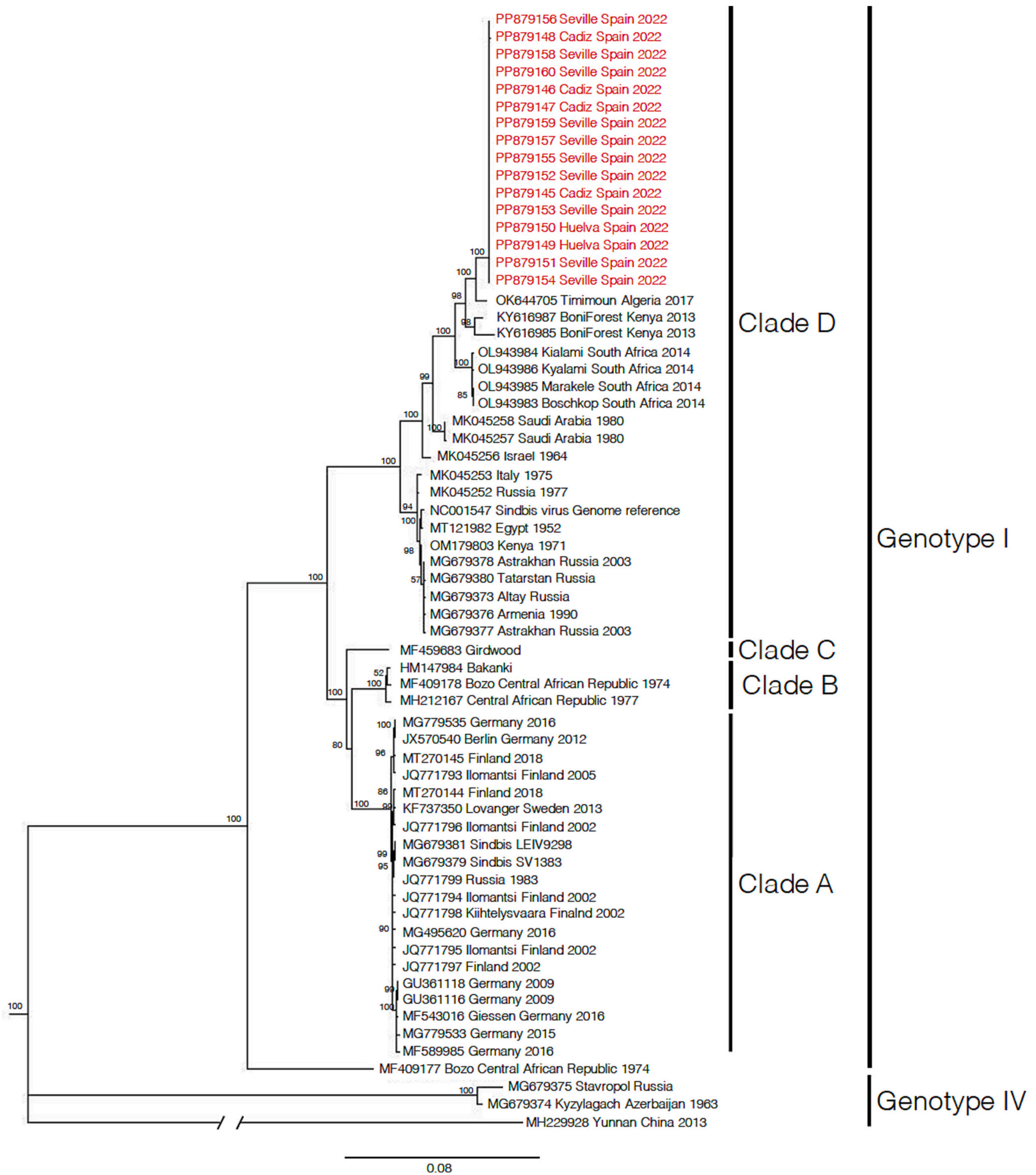

Fig. 2. Multiple likelihood phylogenetic tree of 64 complete genome sequences of SINV. Bootstrap values over 50 are given for 1000 replicates. Viral sequences are identified by GenBank accession number. Sequences in red were generated in this study. Clade information is based on Ling et al. [9]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that includes sequences from several African countries, the Middle East, and Southern Europe, being the sequences from viruses detected in Algeria in 2017 (3.8 % of identity, Table S5), in Kenya in 2013 and in South Africa in 2021 the more closely related to the Spanish ones (Fig. 2). Sequences from European countries with SINV-I outbreaks such as Finland or Sweeden are grouped in a different sister clade, which shares a common ancestor with sequences from Kenya. The evolutionary history of SINV-I was reconstructed using a strict clock with a Bayesian Skyline demographic model, which was the model with the best fit. The evolutionary rate of SINV-I was $6.64*10^{-5}$ substitutions/site/year (median = $6.61*10^{-5}$; 95 % HPD: $5.29*10^{-5}$-$8.01*10^{-5}$). The time to the most recent common ancestor (tMRCA) of the Spanish sequences dated back to 4.66 years (95 % HPD: 2.93–6.64 years) before 2022, indicating that SINV-I was introduced in the country around 2017

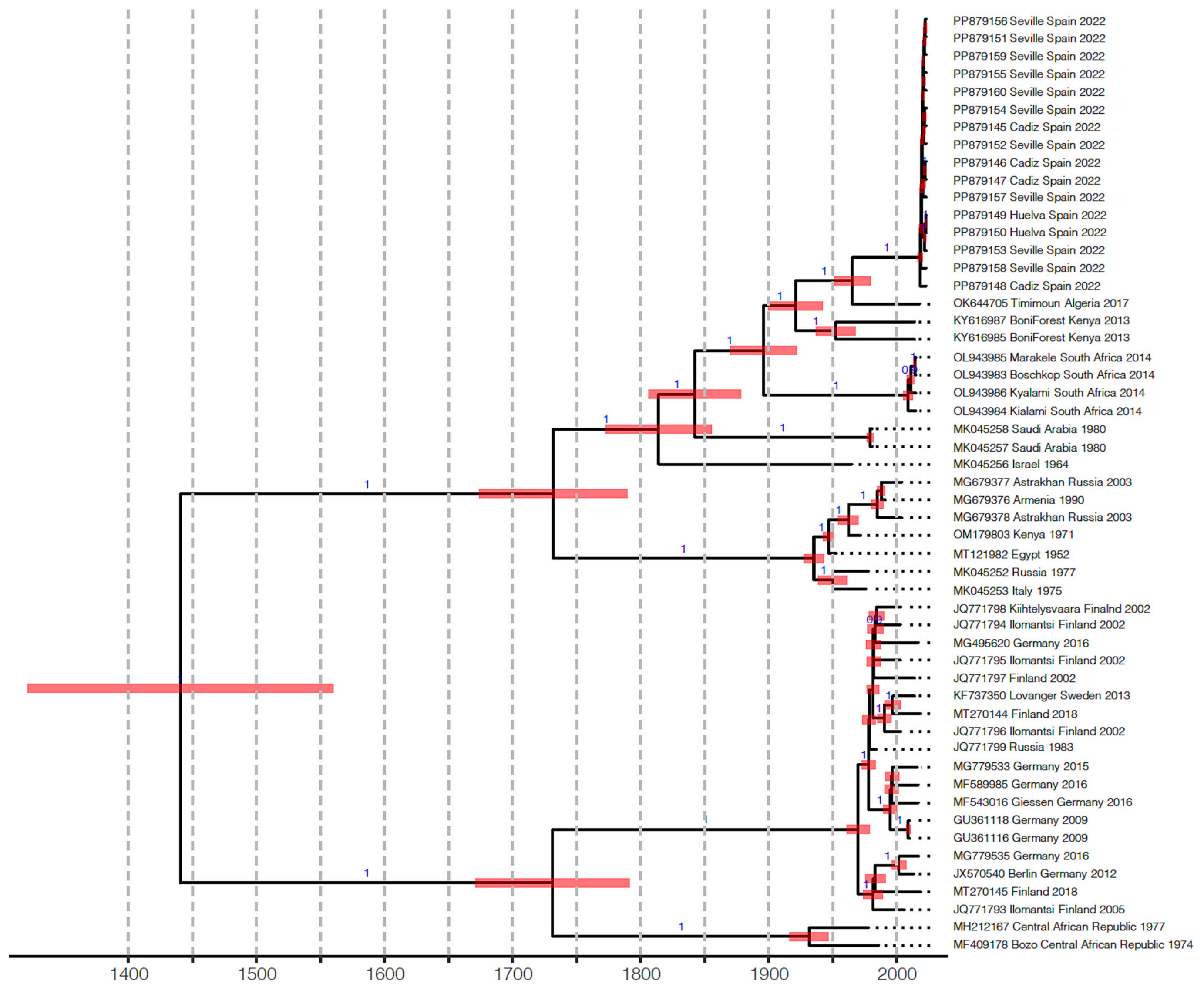

Fig. 3. Maximum clade creditability tree obtained using complete genomes of SINV-I strains (see table S1). The red bars represent divergence times, with the 95 % highest posterior density (HPD) intervals indicated by the highest probability density. The posterior probabilities (1–0.9) of the branches are shown in the tree. The timescale is located below the phylogenetic tree, and the dashed vertical lines mark the corresponding years. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3). Although the closest sample collected was collected in Algeria in 2017, the time to the most recent common ancestor between this sample and the Spanish ones was 57.74Y (95 % HPD: 44.87–72.71 years). These viruses diverged from the Kenyan and South African ones more than 100 years ago. This clade and the clades that were introduced in Northern Europe diverged around 500 years ago (Fig. 3).

3.3. Molecular characterisation

We compared the sequences obtained in this study with the reference genome SINV-I strain EgAR339 (GenBank accession number: NC001547). Our sequences presented differences at 443 nucleotides (nt) positions (3.87 %), with six nt inserted in nsP3. By molecular regions, nsP1 showed 46 differences, nsP2 showed 94 differences, nsP3 showed 84 differences, nsP4 showed 69 differences, capsid C showed 31 differences, E3 showed 6 differences, E2 showed 61 differences, 6 K protein showed 5 differences and E1 protein 47 differences. Compared to the genome from the strain from Algeria P29 (GenBank accession number: OK644705), we found 119 nucleotides differences (1.04 %). By molecular regions, nsP1 showed 12 differences, nsP2 showed 26 differences, nsP3 showed 27 differences, nsP4 showed 21 differences, E3 showed 1 difference, E2 showed 15 differences, 6 K protein showed 2 differences and E1 protein 15 differences. The capsid C regions of the strains from Spain did not show difference with the genome from Algeria.

We observed differences from the reference SINV genome in 55 amino acid (aa) positions (1.46 %) (Table 3). All the Spanish sequences showed an insertion of valine and aspartic acid at positions 448 in nsP3. Although the Spanish sequences shared identical aa changes at 37 sites, three of them showed an exclusive change in at least one amino acid position. In comparison with the Algeria strain sequence, there were changes in 18 amino acid positions (Table 3).

4. Discussion

In the current study, we report the first evidence of SINV circulation in mosquitoes in Southern Spain. In addition, sequencing whole genomes has confirmed that this is a new introduction into Europe, probably around 2017, with a different origin than those previously reported in other European regions.

The viruses detected in this study belong to the genotype I (SINV-I).

Table 3

Deduced amino acids in the 16 SINV-I strains from mosquitoes collected in 2022 in Southwestern Spain, showing the similarities and differences to the prototype SINV strain EgAR339 (GenBank Accession number NC_001547), and SINV-I P29 Argelia strain (GenBank accession number OK644705), in the complete genome.

	Amino acid positions with changes in genes																			
	NSP1								NSP2											
GenBank accesion number	106	145	217	268	401	404	441	496	108	276	330	438	467	468	471	631	634	652	657	667
SINV reference Strain (NC001547)	A	M	K	N	D	D	C	F	M	V	D	P	P	T	A	G	K	K	R	G
SINV Algeria strain (OK644705)	A	M	Q	K	E	E	S	Y	V	V	G	L	P	A	T	G	E	R	H	D
PP879145		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879146		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879147		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879148		T	K	K	E	E	S	Y	V		G	L	S	A	T	C	E	R	H	G
PP879149			K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879150			K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879151		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879152	T	T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879153		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879154			K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879155		T	K	K	E	E	S	Y	V	I	G	L		A	T	C	E	R	H	G
PP879156		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879157		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879158		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879159		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G
PP879160		T	K	K	E	E	S	Y	V		G	L		A	T	C	E	R	H	G

	NSP3															
GenBank accesion number	61	227	275	296	307	343	359	367	405	446	448*	459	470	472	476	479
SINV reference Strain (NC001547)	L	S	H	H	T	P	L	D	R	N		S	T	R	V	L
SINV Algeria strain (OK644705)	Q	C	H	Y	T	L	H	N	Q	I	insertionVD	S	M	C	E	P
PP879145	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879146	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879147	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879148	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879149	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879150	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879151	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879152	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879153	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879154	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879155	Q	C	Y	Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879156	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879157	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879158	Q	C		Y	I	L	H	N	R	I	insertionVD	P	M	C	E	P
PP879159	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P
PP879160	Q	C		Y		L	H	N	R	I	insertionVD		M	C	E	P

	NSP4						C	E2					E1			
GenBank accesion number	42	111	244	278	569	576	80	3	23	70	172	263	73	211	237	278
SINV reference Strain (NC001547)	H	L	E	Y	G	T	P	I	V	K	R	P	H	K	S	I
SINV Algeria strain (OK644705)	Y	S	Q	H	D	A										
PP879145	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879146	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879147	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879148	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879149	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879150	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879151	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879152	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879153	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879154	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879155	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879156	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879157	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879158	Y	S	E	H	D	A	S	T	E	E	G	S	Q	N	A	V
PP879159	Y	S	E	H	D	A		T	E	E	G	S		N	A	V
PP879160	Y	S	E	H	D	A		T	E	E	G	S		N	A	V

No changes were found in the gene E3 nor 6 k.

* Between amino acids 448 and 449 of the NSP3, the samples analysed showed an insertion of two amino acids (Valine "V" and Aspartic acid "D").

Despite the presence of SINV-I in other European countries, the strains isolated in Spain are in a different clade and more related to sequences from Northern-Central African countries, such as Argelia or Kenya indicating a possible African origin for Spanish SINV. The molecular clock analyses indicated that all the Spanish sequences derive from a single, and recent introduction, probably around 2017, but the divergence times with the African sequences ranged from around 50 to 100 years. Thus, before this new introduction in South Spain, there has been viral circulation and spread of this clade across Africa. The clade where the Spanish samples cluster also includes samples from the Middle East, but the time to the most recent common ancestor estimated for these strains is 200 years. Furthermore, the sister clade that includes samples from South/Central Europe, Asia and Africa diverged from the Spanish clade around 300 years ago supporting a long history of circulation and introduction of SINV-I virus between Africa, Asia and Europe. This recent introduction in Southwest Spain is also confirmed by the low rate of amino acid changes observed among the sequences obtained in our study (see Table S5). Previous studies investigating the evolutionary history and spread of SINV-I in Europe indicated that the origin of the virus in Northern Europe was a single introduction into Sweden that later spread to Finland and Germany [11]. Ling et al. [11] proposed that three introductions had occurred in Europe, two from Northern Europe, and one from Central Africa. Our results add one new recent introduction from Africa. The intercontinental spread of bird-hosted viruses, such as WNV, is frequently linked to migratory bird movements [29]. In this sense, the study area in Southwest Spain is one of the main stopovers in the migratory pathway northward, supporting the hypothesis that the virus was probably introduced to Europe by migratory birds from Africa.

The presence of other mosquito-borne viruses with similar ecology and life cycles to SINV (i.e., WNV and Usutu virus), has been previously detected in *Cx. perexiguus* and *Cx. pipiens* in Southwestern Spain [30]. Notably, a significant WNV outbreak occurred in 2020 [31]. During this outbreak, there was a WNV infection rate estimate of 1 % in *Cx. perexiguus* in the study site 'Dehesa de Abajo' [30]. The SINV-I infection rate estimated in *Cx. perexiguus* in 2022 in the same area is 9 %, which indicates a very intense circulation of SINV-I in Southwestern Spain in 2022 (Table 1). We detected SINV-I in five of the six mosquito species analysed. Four of them belong to the genus *Culex*, and *Cx. perexiguus*, showed the highest infection rate of SINV-I. This virus has been previously detected and/or isolated in *Cx. perexiguus* in Algeria [32]. The feeding behaviour of these species is mainly ornithophilic, but occasionally they also feed on mammals, including humans. This behaviour allows the amplification of the virus between birds and facilitates the spillover to humans [33]. In Southwestern Spain, *Cx. perexiguus* is well-known for its important role in the cycle of other mosquito-borne viruses such as WNV or Usutu virus [30]. This species is mainly linked to the amplification of the viruses between birds because the analysis of its feeding patterns shows a clear predominance of bloodmeals from different avian species [33]. Thus, we propose that *Cx. perexiguus* would also have a significant role in the enzootic transmission of SINV. Currently, there are no studies that analyse the vector competence of *Cx. perexiguus* for SINV. Experimental quantification of its vector competence is needed to determine the role of this mosquito species in SINV transmission.

Additionally, other mosquito species of the genus *Culex* that feed both on birds and humans, might act as bridge vectors, increasing the likelihood of spillover to humans. This might be the case of *Cx pipiens*. Its ecology is characterised by a high abundance in urban areas, a diverse feeding behaviour that includes a wide range of bird species and a strong attraction to humans, making it a potential bridge vector for enzootic viruses [30,34].

Another potential bridge vector could be *Cx. modestus*, a competent vector for WNV and USUV feeds on birds and mammals [35]. Although *Cx. modestus* is one of the less abundant mosquito species in southwestern Spain [36], we found high infection rates, suggesting its role as a SINV-I vector. In fact, SINV-I has also been detected in *Cx. modestus* in

the Czech Republic [12].

SINV-I has been previously detected in *Ae. caspius* in Israel [37], as well as in other *Aedes* species in Sweden as *Aedes cinereus* [7]. However, the capacity of SINV transmission by *Aedes* mosquitos must be confirmed by vector competence assays. Vector competence assays of these mosquito species for transmission of SINV-I would provide valuable information about the potential role these species could play in the transmission dynamics of this mosquito-borne virus.

The high infection rate of SINV-I in mosquitoes observed in 2022 suggests that some cases of human infections could be occurring in these areas. Serological studies on residents in these localities are necessary to clarify its incidence in humans. In addition to SINV-I negative impact on human health, imported Chikungunya cases are frequently reported in Spain in travellers coming from endemic areas [38]. Chikungunya disease and Sindbis fever, show similar symptomatology, and important levels of antibodies cross-reactions between SINV infections and other *alphavirus* have been previously demonstrated [39]. Consequently, differential diagnosis against SINV should be considered to confirm Chikungunya diagnosis based on serological analyses if human infections are confirmed.

5. Conclusion

We identify, for the first time, SINV in mosquitoes from Spain showing that *Cx. perexiguus* may be an important vector of this virus, although its vectorial competence should be confirmed in laboratory assays. The Spanish SINV isolates, which belong to Genotype I, are likely to have been recently introduced in Spain by migratory birds from Northern-Central Africa. Furthermore, studies are required to assess the potential endemicity of SINV in Spain as well as to understand its future spread and impact on public health. Given the high circulation in 2022, we propose that it could be necessary to consider its inclusion in the differential diagnosis of arboviral diseases by the Spanish National Health System.

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CRediT authorship contribution statement

Rafael Gutiérrez-López: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **María José Ruiz-López:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Juan Ledesma:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Sergio Magallanes:** Writing – review & editing, Methodology. **Cristina Nieto:** Methodology. **Santiago Ruiz:** Data curation. **Carolina Sanchez-Peña:** Writing – review & editing. **Ulises Ameyugo:** Writing – review & editing. **Juan Camacho:** Methodology. **Sarai Varona:** Writing – review & editing, Methodology, Formal analysis. **Isabel Cuesta:** Writing – review & editing, Investigation, Formal analysis. **Isabel Jado-García:** Writing – review & editing. **María Paz Sanchez-Seco:** Writing – review & editing, Validation, Resources. **Jordi Figuerola:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Ana Vázquez:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

We declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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