Detection of mosquito-only flaviviruses in Europe

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The genus Flavivirus, family Flaviviridae, includes a number of important arthropod-transmitted human pathogens such as dengue viruses, West Nile virus, Japanese encephalitis virus and yellow fever virus. In addition, the genus includes flaviviruses without a known vertebrate reservoir, which have been detected only in insects, particularly in mosquitoes, such as cell fusing agent virus, Kamiti River virus, Culex flavivirus, Aedes flavivirus, Quang Binh virus, Nakiwogo virus and Calbertado virus. Reports of the detection of these viruses with no recognized pathogenic role in humans are increasing in mosquitoes collected around the world, particularly in those sampled in entomological surveys targeting pathogenic flaviviruses. The presence of six potential flaviviruses, detected from independent European arbovirus surveys undertaken in the Czech Republic, Italy,

Portugal, Spain and the UK between 2007 and 2010, is reported in this work. Whilst the Aedes flaviviruses, detected in Italy from Aedes albopictus mosquitoes, had already been isolated in Japan, the remaining five viruses have not been reported previously: one was detected in Italy, Portugal and Spain from Aedes mosquitoes (particularly from Aedes caspius), one in Portugal and Spain from Culex theileri mosquitoes, one in the Czech Republic and Italy from Aedes vexans, one in the Czech Republic from Aedes vexans and the last in the UK from Aedes cinereus. Phylogenetic analysis confirmed the close relationship of these putative viruses to other insect-only flaviviruses.

INTRODUCTION

Diseases caused by arthropod-borne viruses (arboviruses) are increasingly being reported from all over the world (Weaver & Reisen, 2010). Many pathogenic arboviruses are transmitted by mosquitoes and belong to the genus *Flavivirus*, such as dengue viruses, West Nile virus (WNV), yellow fever virus and Japanese encephalitis virus. The emergence of these viruses in recent years has led many countries to develop targeted surveillance of mosquito populations, which involves capturing of mosquitoes and detection of viral nucleic acid by molecular techniques. These surveys have led to reports of several sequences with close similarity to a group of flaviviruses associated exclusively with mosquitoes, named mosquito-only flaviviruses (MOFs).

This group includes cell fusing agent virus (CFAV), a virus isolated in 1975 from an *Aedes aegypti* cell line (Stollar & Thomas, 1975), and other viruses isolated directly from field-collected mosquitoes, including Kamiti River virus (KRV) isolated from *Aedes macintoshi* collected as larvae and pupae in Kenya (Crabtree *et al.*, 2003; Sang *et al.*, 2003), *Culex* flavivirus (CxFV) and *Aedes* flavivirus (AeFV) derived from *Culex* and *Aedes* mosquitoes in Japan (Hoshino *et al.*, 2007, 2009), Quang Binh virus from *Culex tritaeniorhynchus* sampled in Vietnam (Crabtree *et al.*, 2009), Nakiwogo virus from *Mansonia africana* caught in Uganda (Cook *et al.*, 2009) and Calbertado virus from *Culex tarsalis* sampled in Canada and Northern America (Bolling *et al.*, 2011; Pabbaraju *et al.*, 2009; Tyler *et al.*, 2011). A sequence detected in Thailand related to this virus group is present in GenBank (Wang Thong virus, accession no. AY457040). Further reports include one strain of CFAV isolated in Puerto Rico (Cook *et al.*, 2006), one detected in Mexico (Espinoza-Gómez *et al.*, 2011) and CxFV strains from different *Culex* spp. sampled in Guatemala (Morales-Betoulle *et al.*, 2008), Mexico (Farfan-Ale *et al.*, 2009), Uganda (Cook *et al.*, 2009), Iowa (Blitvich *et al.*, 2009), Chicago (Newman *et al.*, 2011), Trinidad and Texas (Kim *et al.*, 2009) and Colorado (Bolling *et al.*, 2011).

MOFs have unique characteristics, and this group of viruses could represent a primordial form of flaviviruses with replication restricted to mosquitoes and unable to infect vertebrate cells (Cammisa-Parks *et al.*, 1992; Cook & Holmes, 2006). This has been demonstrated by the exclusive isolation of these viruses from insect cell culture (Bolling *et al.*, 2011; Cook *et al.*, 2009; Crabtree *et al.*, 2003, 2009; Hoshino *et al.*, 2007, 2009; Kim *et al.*, 2009; Sang *et al.*, 2003). Furthermore, the numerous unsuccessful attempts to grow or to isolate these viruses in vertebrate cell cultures (Bolling *et al.*, 2011; Crabtree *et al.*, 2003, 2009; Hoshino *et al.*, 2007, 2009; Morales-Betoulle *et al.*, 2008; Sang *et al.*, 2003; Stollar & Thomas, 1975) suggest that MOFs are unable to replicate in vertebrates and do not represent a health risk for animals. The lack of a vertebrate host differentiates MOFs from other flaviviruses and raises the question of how these viruses persist in the environment. Laboratory studies have demonstrated the ability of KRV to infect *Aedes aegypti* mosquitoes via the oral route (Lutomiah *et al.*, 2007). Vertical transmission of different MOFs from adult mosquitoes to their offspring has also been reported (Bolling *et al.*, 2011; Cook *et al.*, 2006; Lutomiah *et al.*, 2007). In addition, MOF detection in mosquito males and immature stages has been observed (Bolling *et al.*, 2011; Crabtree *et al.*, 2003; Farfan-Ale *et al.*, 2009; Hoshino *et al.*, 2007, 2009; Sang *et al.*, 2003).

Much of the ecology of MOFs is still largely unknown, and the scarcity of knowledge on the life cycle and characteristics of these viruses highlights the need for further study. Moreover, the abundance of reports worldwide of MOFs highlights the ubiquity of these viruses in different mosquito species, and suggests that many of these viruses have yet to be discovered. In this study, we collated the findings of MOFs in five independent mosquito surveys conducted across Europe. Wetland locations were targeted in each survey due to high mosquito abundance in these areas. Using the sequence data derived from these studies, we derived a phylogeny for these viruses in the context of the genus *Flavivirus*.

RESULTS AND DISCUSSION

A total of 817 240 mosquitoes were pooled and tested in five independent surveys (Fig. 1). The most abundant species detected were *Culex pipiens* in Italy and Portugal, *Aedes caspius* in Spain, *Aedes vexans* in the Czech Republic and *Aedes cinereus* in the UK. Other species abundantly sampled were *Aedes caspius* and *Culex theileri* in Portugal, *Culex modestus*, *Culex theileri* and *Culex pipiens* in Spain, *Aedes caspius*, *Aedes vexans*, *Anopheles maculipennis* s.l. and *Aedes albopictus* in Italy, *Aedes rossicus* in the Czech Republic

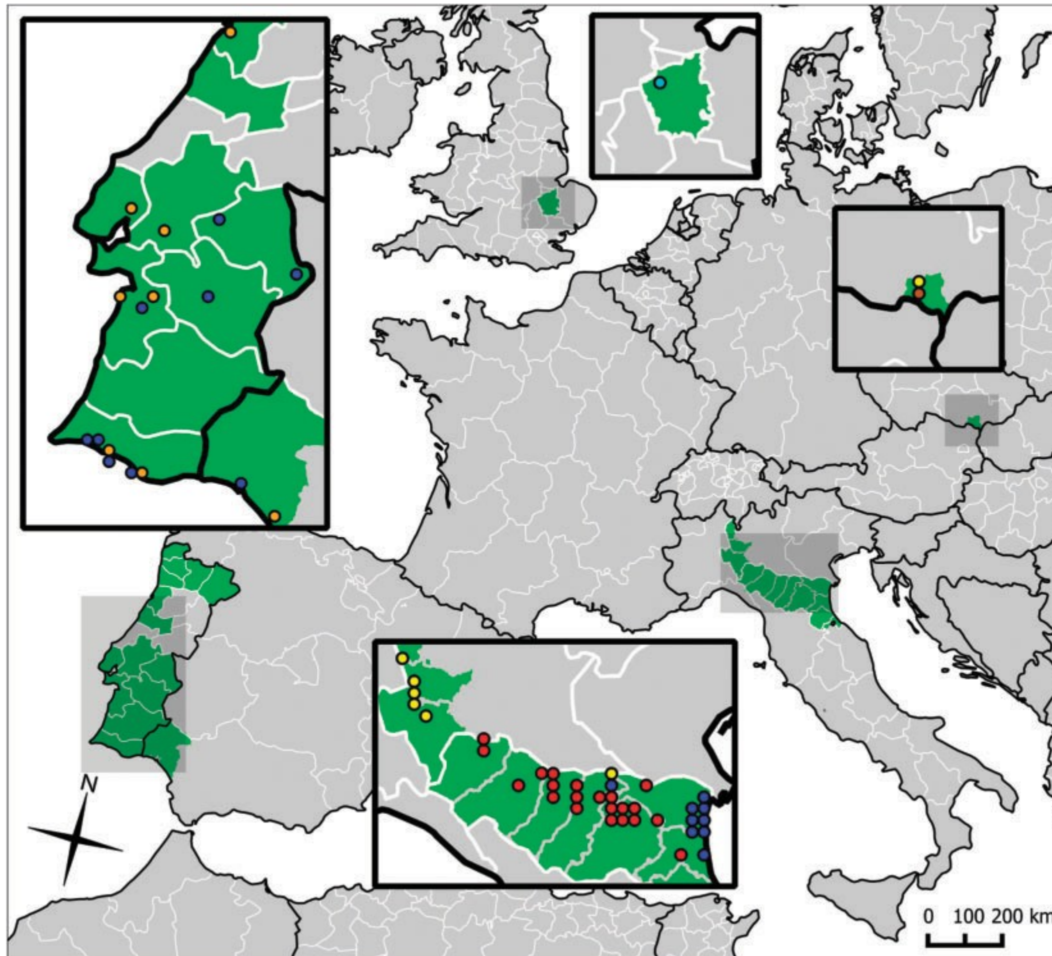

Fig. 1. Location of surveyed areas (in green) and of positive detections in the surveys at 10 km resolution. Red, *Aedes* flavivirus; azure, *Aedes cinereus* flavivirus; yellow, *Aedes vexans* flavivirus (Italy and Czech Republic); brown, *Aedes vexans* flavivirus (Czech Republic); orange, *Culex theileri* flavivirus; blue, *Ochlerotatus* flavivirus.

and *Coquellettidia richiardii* and *Culiseta annulata* in the UK (Table 1). Flavivirus-positive RT-PCR mosquito pools were reported in all countries (Fig. 1). The presence of the surveyed flaviviruses was detected in Italian mosquitoes, with 29 WNV-positive pools and 56 Usutu virus (USUV)-positive pools (Calzolari *et al.*, 2010a, b). Moreover, three groups of sequences similar to MOFs were detected in Italy. One was detected in 32 pools of *Aedes albopictus* mosquitoes and showed a high identity with the sequence of AeFV (GenBank accession no. AB488408) isolated in Japan in 2009; the other two virus sequences had lower identity with previously reported MOFs: one was detected in eight *Aedes caspius* pools and in one *Culex pipiens* pool, and the third sequence was detected in eight *Aedes vexans* pools. The sequence detections obtained in Italy from 2007 to 2009 have been reported previously (Calzolari *et al.*, 2010a, b). In Portugal, two groups of sequences similar to MOFs were detected, one in *Culex* and *Aedes* spp. (21 sequences), and the second in *Culex theileri* (11 sequences) mosquitoes, and were related to an insect flavivirus sequence detected in *Culex fuscocephala* in Thailand (Wang Thong virus, GenBank accession no. AY457040). In Spain, two sequence groups were also detected in the mosquitoes sampled, one from *Culex theileri* mosquitoes (four sequences) and one from *Aedes caspius* mosquitoes (three sequences). WNV and USUV were detected previously in *Culex perexiguus* mosquitoes in 2008 and 2009, respectively, in the same geographical area (south-west Spain) but at a different site (Vázquez *et al.*, 2011). In the Czech Republic, two sequence types were detected (with two and three sequences), all in *Aedes vexans* mosquito pools. Finally, all the sequences derived from flavivirus-positive *Aedes cinereus* mosquito pools from the UK were identical (Fig. 1, Table 2). A BLAST search performed in GenBank with the obtained amplicon sequences showed the highest identity rates with the MOF group (Fig. 2).

The described sequence groups showed a high degree of identity within each group (Table 3). In some cases, variation detected in these viral sequences (even taking into consideration the small size of the amplified NS5 sequence)

Table 1. Total number of specimens (%) and pools collected for each mosquito species in the surveys in each country

Mosquito species	Portugal 2007–2010		Spain 2007–2008		Italy 2007–2010		Czech Republic 2009		UK 2010	
	n (%)	Pools	n (%)	Pools	n (%)	Pools	n (%)	Pools	n (%)	Pools
Aedes albopictus					4 219 (0.6)	353				
*Aedes cantans**									20 (8.5)	4
*Aedes caspius**	6 625 (30.1)	166	23 927 (43.1)	1 005	65 922 (9.0)	1 086			10 (4.2)	2
Aedes cinereus					41 (<0.1)	15	150 (2.3)	3	96 (40.7)	20
*Aedes detritus**	462 (2.1)	16	830 (1.5)	155	79 (<0.1)	11				
*Aedes dorsalis**					13 (<0.1)	1				
*Aedes geniculatus**					674 (0.1)	28				
*Aedes punctor**					1 (<0.1)	1				
Aedes rossicus							800 (12.7)	16		
*Aedes sticticus**							500 (7.9)	10		
Aedes vexans					40 265 (5.5)	462	4 200 (66.7)	84		
Aedes spp.									5 (2.1)	1
Anopheles algeriensis	278 (1.3)	7	129 (0.2)	40						
Anopheles claviger	38 (0.2)	2							25 (10.6)	5
Anopheles maculipennis s.l.	101 (0.5)	15	167 (0.3)	79	4 510 (0.6)	122				
Anopheles plumbeus			1 (<0.1)	1		12				
Anopheles spp.	27 (0.1)	1				10				
Coquillettidia richiardii	13 (<0.1)	3	1 (<0.1)	1		4			45 (19.1)	5
Culex mimeticus	6 (<0.1)	1								
Culex modestus			9 695 (17.4)	381	2 471 (0.3)	90	350 (5.6)	7		
Culex perexiguus	507 (2.3)	38	2 330 (4.2)	237						
Culex pipiens	10 098 (45.9)	322	8 563 (15.4)	787	614 652 (83.8)	4 300	300 (4.8)	6		
Culex theileri	3 759 (17.1)	117	9 537 (17.2)	441						
Culex spp.			16 (<0.1)	5	15 (<0.1)	3				
Culiseta annulata	17 (<0.1)	5	119 (0.2)	64	4 (<0.1)	4			35 (14.8)	7
Culiseta longiareolata	42 (0.2)	13	242 (0.4)	87						
Culiseta spp.			0 (0.0)		3 (<0.1)	3				
Uranotaenia unguiculata	13 (<0.1)	1	5 (<0.1)	4						
Total	21 986	707	55 562	3 287	733 154	6 505	6 300	126	236	47

*According to Savage & Strickman (2004), *Ochlerotatus* taxon was considered to be a subgenus of the genus *Aedes*.

appeared to be very low; for example the 21 amplicons obtained from *Culex* and *Aedes* mosquitoes in Portugal were identical except for three polymorphisms in two sequences (PoMoFlavA95 and PoMoFlavR376, GenBank accession nos EU716416 and HQ676618, respectively), particularly considering that the positive mosquito pools belonged to two different genera and five species (*Culex pipiens*, *Culex theileri*, *Culex perexiguus*, *Aedes caspius* and *Aedes detritus*).

Unexpectedly, several groups of sequences from different countries showed high identity and grouped closely within the phylogenetic analysis (Fig. 2). In this analysis, the consensus sequences obtained from the different countries were compared with those available in GenBank, and representative flavivirus sequences were used. The new sequences grouped with previously described MOFs; they diverged from other known flaviviruses and were placed on six branches in the phylogenetic tree, suggesting the presence of different MOFs in Europe (Fig. 2). One sequence detected in Italy, Portugal and Spain, mainly from *Aedes* mosquitoes, was termed *Ochlerotatus* flavivirus (OcFV) in this study. A sequence detected in Portugal and Spain mainly from *Culex theileri* mosquitoes was termed *Culex theileri* flavivirus (CxthFV). A sequence detected in Italy from *Aedes albopictus* mosquitoes revealed high identity with the previously reported *Aedes* flavivirus. One sequence detected in the Czech Republic and in Italy from *Aedes vexans* mosquitoes was termed *Aedes vexans* flavivirus (AeveFV). A second sequence detected in the Czech Republic was derived from *Aedes vexans* mosquitoes. Finally, one sequence detected in the UK from *Aedes cinereus* mosquitoes showed little identity with other MOFs and formed its own branch within the phylogenetic analysis (Fig. 2, Table 3). As different protocols were utilized (Table 4), a portion of 155 bp of obtained sequences was aligned, producing a tree consistent with those obtained in other phylogenetic studies with longer sequences (Cook *et al.*, 2012); this tree showed

Table 2. Characteristics of the reported MOFs with the reference to the mosquito species, number, period, area and environment of detection

Virus	Mosquito species	No. detections (in GenBank)	Collection period	Collection years	Country	Collection area	Environment
Aedes flavivirus	*Aedes albopictus*	32 (29)*	June–October	2008, 2009, 2010	Italy	Pianura Padana	Floodplain
Ochlerotatus flavivirus	*Aedes caspius/detritus Culex pipiens/perexiguus/theileri*	33 (22)D	March–October	2007, 2008, 2009, 2010	Italy, Portugal, Spain	Lidi Ferraresi, Algarve, Alentejo, Andalusian	Inland and tidal wetland
Aedes vexans flavivirus	*Aedes vexans*	9 (9)d	July–November	2008, 2009	Czech Republic, Italy	Pianura Padana, South Moravia	Inland wetland
Czech *Aedes vexans* flavivirus	*Aedes vexans*	4 (4)§	August	2009	Czech Republic	South Moravia	Pond
Culex theileri flavivirus	*Culex theileri*	15 (9)||	March–October	2007, 2008, 2009, 2010	Portugal, Spain	Alentejo, Algarve, Centro, Ribatejo Andalusian	Inland and tidal wetland
Aedes cinereus flavivirus	*Aedes cinereus*	1¶	June–July	2010	UK	Cambridgeshire	Inland wetland

*GenBank accession numbers: Italy: GQ477004–GQ477012 and HQ441846–HQ441865.
DGenBank accession numbers: Italy: GQ476991–GQ476995 and HQ441842–HQ441845; Portugal: EU716415–EU716419, EU716421–EU716424 and HQ676618; Spain: HU4503, HU6404, HU6086.
dGenBank accession numbers: Italy: GQ476996–GQ477003; Czech Republic: JN802280.
§GenBank accession numbers: Czech Republic: JN802279 and JN802281–JN802283.
||GenBank accession numbers: Portugal: EU716420, HQ676619–HQ676621 and HQ676623; Spain: HU4212, HU4301, HU5659, HU5910.
¶Sequences available on request.

divergence between *Aedes*- and *Culex*-associated sequences (Fig. 2), although this was weakly supported by bootstrap values. This divergence has been reported by Cook *et al.* (2012).

Integration of flavivirus sequences into the mosquito genome has been reported (Crochu *et al.*, 2004; Katzourakis & Gifford 2010). To exclude the possibility that the detected sequences were the result of integration into the mosquito genome, the nature of the sequences obtained was investigated. In the Portuguese study, total RNA extracts from most of the positive mosquito samples were submitted to specific PCR amplification without reverse transcription. Specific amplification of total DNA extracts was also performed. No amplification was achieved using the same reaction conditions but omitting the reverse transcription step, or directly from total DNA extracts of mosquito macerates. In the Italian study, the flavivirus PCR was applied to total DNA extracted from two positive *Aedes albopictus* mosquito pools sampled in 2010 without any positive results.

In the Spanish study, samples prepared from positive pools were treated with RNase A before amplification and then were directly amplified without the previous reverse transcription step (Sánchez-Seco *et al.*, 2010). RNase treatment resulted in failure to amplify a flavivirus product, suggesting that the sequences obtained were most likely derived from RNA, probably of viral origin. In the Czech Republic samples, the same result was obtained even when the PCR was performed without reverse transcriptase, or despite RNase treatment, indicating the presence of the detected sequences in DNA form. All samples were subsequently subjected to DNase treatment, which resulted in the confirmation that, in at least one sample, the detected sequence was in RNA form only. These results did not indicate the presence of an integrated flaviviral sequence in a mosquito genome, as evidence of a reverse transcriptase mechanism was provided by CFAV infection in the C6/C36 cell line (Cook *et al.*, 2006, 2009), a surprising finding for an RNA virus that might replicate with a DNA intermediate.

For most of the PCR-positive mosquito pools, virus isolation in cell culture was attempted by inoculation of pooled or individual mosquito macerates in C6/36 cells and vertebrate cell lines (Table 4). Although, in this study, cell culture for several RT-PCR-positive mosquito homogenates was attempted, the isolation procedures were unsuccessful. However, one MOF, related to OcFV, detected in one mosquito pool collected in the southern region of Portugal in 2006, has previously been isolated in C6/36 cells (M. Niedrig, personal communication). The isolation of these viruses was also successful in Spain. Two detected MOFs were isolated in C6/36 cells from mosquitoes captured in 2002 and 2006. In both cases, the virus isolated developed a moderate cytopathic effect (CPE) and cell aggregation at 5–7 days post-infection (Vázquez *et al.*, 2012).

These findings demonstrated the widespread presence of different MOFs in Europe, related to other MOFs isolated

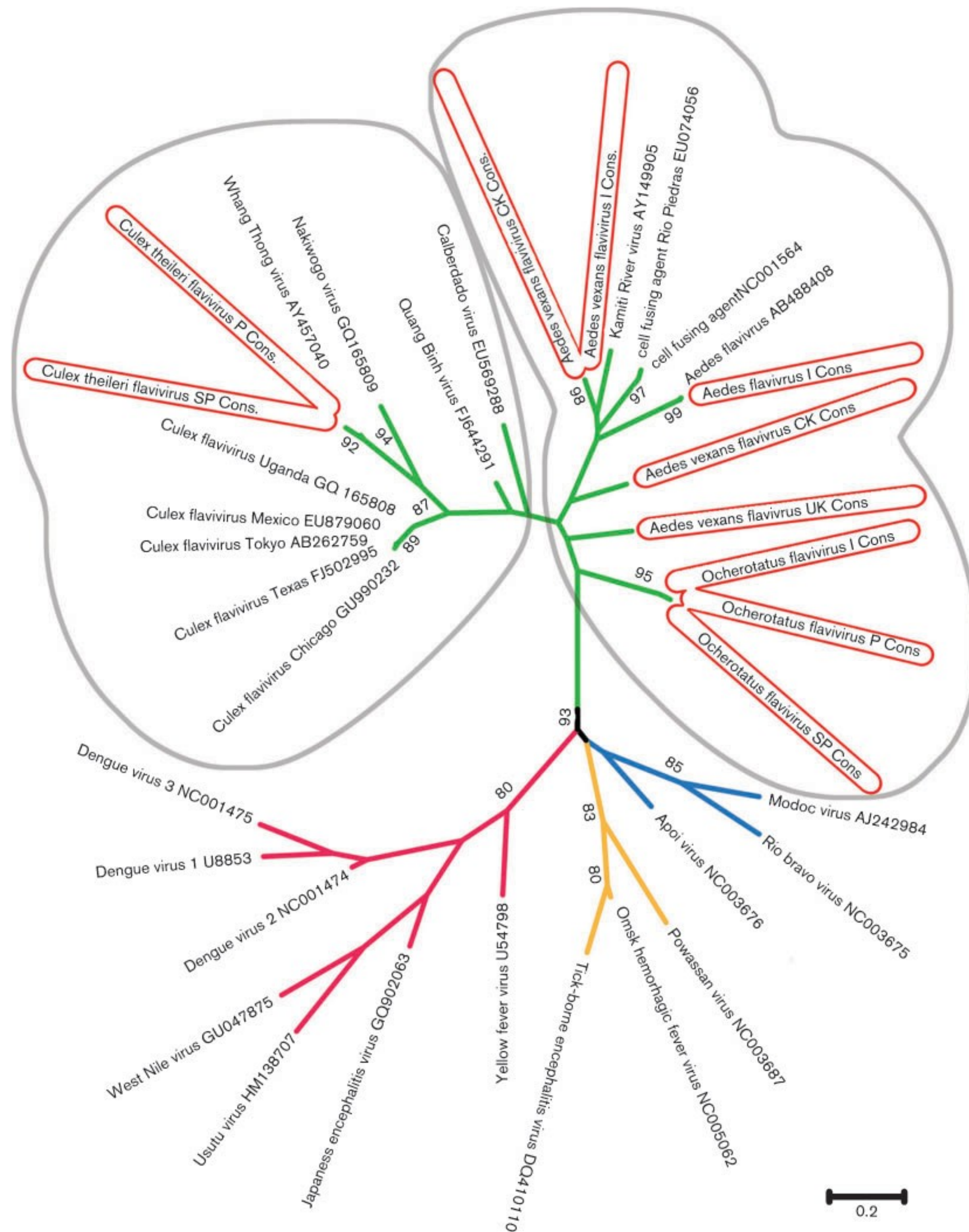

Fig. 2. Molecular phylogenetic analysis using the maximum-likelihood method (Kimura two-parameter model) of the MOF sequences reported in this work (part of the NS5 gene) and homologous GenBank sequences of other flaviviruses, with their respective GenBank accession numbers. The sequences from this work are circled in red; in grey are highlighted the Aedes- and Culex-associated MOF sequences, on the right and left, respectively. The tree with the highest log likelihood (−2920.7) is shown. An initial tree for the heuristic search was obtained automatically by the maximum-parsimony method. A discrete Γ distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter=1.1014)]. The rate variation model allowed some sites to be evolutionarily invariable (+I0, 23.8 % sites). The tree is drawn to scale, and bootstrap values (1000 replicate) of ≥80 % are shown. Bar, number of substitutions per site. The analysis involved 36 nt sequences. There were a total of 155 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Branches: green, mosquito-borne insect-only flaviviruses; blue, no-known vector; yellow, tick-borne flaviviruses; pink, mosquito-borne flaviviruses that can infect vertebrates. CK, Czech Republic; I, Italy; P, Portugal; SP, Spain; Cons., consensus sequence.

Table 3. Number of differences and percentage identity (bold) in sequences detected in each country (a) and between consensus sequences from the different surveys (b) (based on 155 bp)

The short sequences HQ441855, HQ441843, GQ477002 and GQ477003 were excluded from the analysis. I, Italy; P, Portugal; SP, Spain; CZ, Czech Republic; No. diff, number of differences in sequence; Mean diff., mean of differences for sequence.

(a)

Virus	Country	No. sequences	Identity (%)	No. diff.	Mean diff.
OcFV	I	8	100–96.8	0–5	1.86
	P	10	100–99.4	0–1	0.36
	SP	3	100–99.4	0–1	0.67
AeFV	I	32	100–98.1	0–3	0.83
AeveFV	I	6	100	0	–
	CZ	1	–	–	–
CxthFV	P	5	100–98.1	0–3	1
	SP	4	100–97.4	0–4	2
Czech AeveFV	CZ	3	99.4–97.4	1–4	2.67
Aedes cinereus flavivirus	UK	17	100	0	–

(b)

Virus		OcFV		AeFV		AeveFV		CxFV		Czech AeveFV	*A. cinereus* flavivirus
		I	P	SP	I	I	CZ	P	SP	CZ	UK
OcFV	I		96.8	97.4	63.2	66.5	66.5	66.5	66.5	73.5	74.2
	P	5		99.4	61.9	67.1	67.1	67.1	67.1	72.3	74.2
	SP	4	1		61.3	66.5	66.5	66.5	66.5	71.6	73.5
AeFV	I	57	59	60		78.1	78.1	69.7	69.7	72.3	73.5
AeveFV	I	52	51	52	34		100	67.7	67.7	72.3	72.9
	CZ	52	51	52	34	0		67.7	67.7	72.3	72.9
CxthFV	P	52	51	52	47	50	50		100	65.2	66.5
	SP	52	51	52	47	50	50	0		65.2	66.5
Czech AeveFV	CZ	40	40	41	41	42	42	52	52		75.5
A. cinereus flavivirus	UK	41	43	44	43	43	43	54	54	38	

worldwide. However, there are a number of difficulties in characterization of MOFs, which can be challenging, particularly the problem of their isolation in cell cultures: the MOF CPE can be weak (Hoshino *et al.*, 2009) or strain-dependent (Kim *et al.*, 2009), or only visible after a number of blind passages. In addition, the virus can go undetected by RT-PCR in cell-culture medium during early passage after inoculation (Bolling *et al.*, 2011).

The high sequence identity of the virus detected in Italian *Aedes albopictus* mosquitoes, previously detected in other studies in Italy (Calzolari *et al.*, 2010a; Roiz *et al.*, 2009), compared with the *Aedes* flavivirus isolated in Japan strongly suggested that these two viruses are closely related. Japan is the probable origin of this mosquito's recent expansion to North America and Europe (Hawley *et al.*, 1987; Rai, 1991); thus, it seems likely that the mosquitoes also brought AeFV with them. A further observation was that the mosquitoes from which other virus sequences were detected were collected in wetland ecosystems in the different countries (Table 2), although other environments were monitored in some surveys (Fig. 2). These observations could be explained by the presence of environmental factors favouring MOF persistence, or by a MOF influence on the bionomic features of infected mosquitoes, as has been described for carbon dioxide sensitivity induced in mosquitoes by different viruses (Shroyer & Rosen, 1983; Vazeille *et al.*, 1988); these influences could enhance mosquito adaptation to a particular ecosystem. Further experimental investigations will be required to support this hypothesis.

Table 4. Materials and protocols used in the different surveys

Method	Italy	Portugal	Czech Republic	Spain	UK
Traps	CDC modified traps (no light), CO_2 baited (self-produced)	CDC light traps, CO_2 baited	CDC light traps, CO_2 baited (BioQuip Products)	CDC light traps, CO_2 baited (self-produced)	Mosquito Magnet baited by CO_2, heat and Octenol attractant
Classification keys	Becker *et al.* (2010); Schaffner *et al.* (2001); Severini *et al.* (2009); Stojanovich & Scott (1997)	Ribeiro & Ramos (1999); Schaffner *et al.* (2001)	Becker *et al.* (2010); Kramář (1958)	Becker *et al.* (2010); Encinas Grandes (1982); Schaffner *et al.* (2001)	Schaffner *et al.* (2001); Snow (1990)
Maximum no. mosquitoes per pool	200 (Sutherland & Nasci, 2007)	50	50	50	10
Storage	−80 °C polypropylene cryotube	−80 °C polypropylene cryotube	−80 °C glass tube	−80 °C polypropylene cryotube	−80 °C polypropylene cryotube
Grinding	Copper-plated round balls and vortexing	Liquid nitrogen	Tissue Lyser II (Qiagen)	–	Scissors
Grinding medium	PBS	Minimal essential medium supplied with 10 % FBS, streptomycin (0.1 μg ml^{-1}) and fungizone (1 μg ml^{-1})	PBS	Minimal essential medium supplied with 10 % FBS	–
Centrifugation	4000 *g* for 3 min	12 000 *g* for 2 min	14 000 *g* for 10 min	10 500 *g* for 5 min	–
RNA extraction	Trizol LS Reagent (Invitrogen)	PureLink RNA Mini kit (Ambion)	Viral RNA Mini kit (Qiagen)	Viral RNA Mini kit (Qiagen)	MELT kit (Ambion) and Kingfisher 96 extraction robot (ThermoElectron)
DNA extraction	DNeasy kit (Qiagen)	–	–	–	–
Reverse transcription and PCR or one-step RT-PCR	Random hexamers (Roche Diagnostics) and SuperScript II	SuperScript One-Step RT-PCR (Invitrogen)	Titan One Tube RT-PCR kit (Roche)	One Step RT-PCR (Qiagen)	Random hexamers (Invitrogen) and reverse transcriptase (Promega)
PCR protocol	Scaramozzino *et al.* (2001)	Briese *et al.* (1999, 2002)	Scaramozzino *et al.* (2001)	Vázquez *et al.* (2012)	Johnson *et al.* (2010)
Gel band purification	Nucleospin Gel and PCR Clean-up (Machery-Nagel)	JET quick PCR Product Purification Spin kit (Genomed)	Illustra GFX PCR and Gel Band Purification kit (GE Healthcare)	Gel Extraction kit (Qiagen) or using QIAquick PCR Purification kit (Qiagen)	QIAquick gel extraction kit (Qiagen)
Cloning	–	–	Clone Jet PCR Cloning kit (Fermentas)	–	–
Sequencing	ABI Prism 3130 Genetic Analyzer (Applied Biosystems)	ABI Prism 3130 Genetic Analyzer (Applied Biosystems)	ABI Prism 3130xl Genetic Analyzer (Applied Biosystems)	ABI Prism 377 automated sequencer (Applied Biosystems)	ABI Prism 3100 Genetic Analyzer (Applied Biosystems)
Isolation	C6/C36 cells	C6/36 and Vero E6 cells	C6/C36 cells	C6/36, Vero or BHK-21 cells	–

The widespread distribution of MOFs suggests their potential as a tool to prevent the transmission of pathogenic flaviviruses due to superinfection phenomena, as suggested previously (Blitvich *et al.*, 2009; Crabtree *et al.*, 2009). Alternatively, the enhancement of WNV transmission in mosquitoes inoculated simultaneously with CxFV Izabal has been reported in Honduras (Kent *et al.*, 2010), indicating that the consequences of co-infection are not clear and require elucidation. Moreover, a positive association between CxFV and WNV was reported in field-collected mosquitoes (Newman *et al.*, 2011), and in the 2009 Italian survey (Calzolari *et al.*, 2010b), two *Aedes albopictus* pools sampled at the same site and in the same week were positive for AeFV and USUV, testifying to the persistence of both viruses in the environment.

Further studies are ongoing to achieve virus isolation of these flaviviruses through cell culture using mosquito cell lines to confirm the presence of viable viruses in these samples and to follow up their genetic characterization. This will involve evaluation of their potential to prevent or enhance the transmission of other pathogenic flaviviruses during co-infection.

The reported data suggest that MOFs have a broader geographical range in Europe than previously considered, with the probability that MOFs exist in natural mosquito populations throughout the world.

METHODS

Survey areas. In Italy, the survey area was the northern part of Emilia-Romagna, bounded on the north by the river Po and on the east by the Adriatic Sea, and a 91 000 ha Regional Natural Park sited in Lombardia region, 'Parco Lombardo della Valle del Ticino', that protects the Italian stretch of the Ticino River. All these areas were in Pianura Padana, the most important Italian plain, characterized by intensive agriculture and animal husbandry with scarce natural sites. All the monitored territories were densely populated and characterized by the abundant presence of villages and city and industrial areas (Fig. 1).

In Portugal, in 2007, only the Algarve region in the south was surveyed. From 2008, the survey area was enlarged to cover most of the country. A wide range of ecosystems was surveyed from rural and urban habitats, including some airports and sea ports. In the Algarve and north coastal areas, the study sites were coastal wetlands rich in avifauna and with a multitude of habitats characterized by marshlands, salt marshes, small islands, dunes and beaches. Fishery, aquaculture and salt works as well as farming areas with several hectares of rice fields and reed plantation were the most important human activities, but industrial complexes related to the fishing industry were also found (Fig. 1).

In the Czech Republic, the study was conducted at five sites in south-eastern Moravia in 2009. The localities included a lowland forest, the shore of a pond and a farmhouse. All these localities are characterized by high mosquito abundance. This region is characterized by a relatively warm and dry climate, and the surveyed area is endemic for several mosquito-borne human pathogens, including WNV (Hubálek *et al.*, 2010) (Fig. 1).

In Spain, mosquitoes were captured in the Guadalquivir marshes and adjoining wetlands, in the south-west of Spain and near to the Algarve in Portugal. Study areas were tidal marshes, freshwater marshes, coastal dunes and paddy fields in natural areas of the National Park of Doñana and Odiel Marshes Natural Park, with a high diversity and abundance of sedentary and migratory birds, particularly waterfowl, herons, waders and gulls (Fig. 1). In recent years, WNV has been circulating repeatedly in this area (Figuerola *et al.*, 2007).

In the UK, all mosquitoes analysed were collected at Woodwalton Fen, a wetland area in Cambridgeshire. Habitats included flooded grasslands and reed-bed swamp habitat, as well as wet woodland and ditch/groundwater-fed fen (Fig. 1).

Mosquito collections. Different traps baited with carbon dioxide were utilized for sampling mosquitoes (Table 4). All trap sites were georeferenced and traps worked at night from approximately 16 : 00 to 9 : 00.

Traps worked over different periods of the year in the different nations: from June to October in Italy and Portugal, from the beginning of April until the end of October 2009 in the Czech Republic, from March until the end of October in Spain and from April to October 2010 in the UK. Mosquitoes were identified to species level using morphological characteristics according to classification keys (Becker *et al.*, 2010; Encinas Grandes, 1982; Kramář, 1958; Ribeiro & Ramos, 1999; Schaffner *et al.*, 2001; Severini *et al.*, 2009; Snow, 1990; Stojanovich & Scott, 1997). The *Ochlerotatus* taxon was considered to be an *Aedes* subgenus (Savage & Strickman, 2004). Female mosquitoes were pooled according to date, location and species, with a maximum number of 10–200 individuals per pool.

The pooled mosquitoes were stored in tubes and frozen at 280 uC. Specimens were macerated by different methods: in liquid nitrogen or at room temperature, by mechanical methods, or by the addition of medium and metallic beads and shaking the samples (Table 4). The homogenate was clarified by centrifugation; finally, aliquots were collected from ground samples and submitted to biomolecular analysis.

Virus survey. RNA present in the aliquots was extracted using different commercial products according to the manufacturer's instructions (Table 4). Samples were analysed using different PCR protocols, targeted to an *NS5* gene fragment of flaviviruses (Briese *et al.*, 1999, 2002; Johnson *et al.*, 2010; Scaramozzino *et al.*, 2001; Vázquez *et al.*, 2012). PCR was performed on cDNA obtained by reverse transcription or by one-step PCR commercial kits according to the manufacturer's instructions (Table 4). Fragments obtained by *Flavivirus* genus PCR were sequenced by an automated fluorescence-based technique following the manufacturer's instructions. If necessary, a cloning passage was performed to obtain better-quality sequences.

The obtained sequences were used to perform basic local alignment searches (BLAST) in GenBank to confirm the specificity of the positive reaction and to estimate the degree of identity of the detected strains (Altschul *et al.*, 1997). The sequences obtained were aligned with available GenBank sequences and molecular phylogenetic analysis was performed by the maximum-likelihood method using the MEGA5 program (Tamura *et al.*, 2011). The Kimura two-parameter model was chosen among 24 different nucleotide substitution models for the lowest Bayesian information criterion value. The analysis involved 36 nt sequences, and a final dataset of 155 positions was utilized, eliminating all positions containing gaps and missing data (Fig. 2).

Virus isolation was attempted starting from the remaining part of the PCR-positive mosquito homogenates using the C6/36 cell line (Igarashi *et al.*, 1976) incubated at 28 uC, or adapted to 33 uC in Spain, and other vertebrate cell lines were incubated at 37 uC (Table 4).

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