Emergence of Two Different Genotypes of Bagaza Virus (BAGV) Affecting Red-Legged Partridges in Spain, in 2019 and 2021

Pilar Aguilera-Sepúlveda , Belén Gómez-Martín , Montserrat Agüero , Miguel Ángel Jiménez-Clavero * , Jovita Fernández-Pinero

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Article

Emergence of Two Different Genotypes of Bagaza Virus (BAGV) Affecting Red-Legged Partridges in Spain, in 2019 and 2021

Pilar Aguilera-Sepúlveda [1], Belén Gómez-Martín [2], Montserrat Agüero [2], Miguel Ángel Jiménez-Clavero [1,3,*] and Jovita Fernández-Pinero [1]

[1] Centro de Investigación en Sanidad Animal (CISA-INIA), CSIC, Valdeolmos, Spain; aguilera.pilar@inia.csic.es (P.A.-S.); majimenez@inia.csic.es (M.Á.J.-C.); fpinero@inia.csic.es (J. F.-P.)
[2] Laboratorio Central de Veterinaria (LCV), Ministry of Agriculture, Fisheries and Food (MAPA), Algete, Spain; mgmartin@mapa.es (B.G.-M.); maguerog@mapa.es (M.A.)
[3] CIBER of Epidemiology and Public Health (CIBERESP), Madrid, Spain; majimenez@inia.csic.es (M.Á.J.-C.)
* Correspondence: majimenez@inia.csic.es

Abstract: Bagaza virus (BAGV) is a flavivirus that affects avian species. In Europe, it was detected for the first time in Spain in 2010, exhibiting high genetic relatedness to Israel turkey meningoencephalomyelitis virus (ITMV) isolates from Israel. After a period of epidemiological silence, BAGV re-emerged causing important outbreaks in 2019 and 2021. This study aims to characterize the newly detected strains and to elucidate if these recent outbreaks were caused by single or different virus introductions into the country. Hence, Spanish BAGV isolates from 2019 (n=3) and 2021 (n=1) outbreaks, obtained from red-legged partridges in Cádiz, were sequenced and further characterized. The phylogenetic analyses showed that they belong to two different genotypes: BAGV Genotype 1 and 2. Isolates from 2019 belong to BAGV-Genotype 1, closely related to isolates from Senegal, where BAGV has been circulating since decades. In turn, the 2021 isolate belong to BAGV-Genotype 2, closely related to those detected in Spain in 2010. Additionally, the comparison of the viral polyproteins of several BAGV isolates from both genotypes support and confirm the phylogenetic findings. To conclude, BAGV has been introduced into Spain in at least three independent occasions, with alternating genetic clades, thus confirming that BAGV is able to occasionally reach southern Europe.

Keywords: Bagaza virus; emerging genotypes; phylogenetic analysis; molecular characterization; complete genome; *Alectoris rufa*; Spain

1. Introduction

Bagaza virus (*Orthoflavivirus bagazaense*, BAGV) is a flavivirus belonging to family *Flaviviridae*, genus *Orthoflavivirus*, close related to Israel turkey meningoencephalomyelitis virus (ITMV) and grouping in the Ntaya serocomplex. As other flaviviruses, it is mainly a mosquito-borne pathogen that affects birds (which are amplifying hosts), especially those belonging to Phasianidae family, such as turkeys, pheasants and partridges [1].

Bagaza virus was first isolated from *Culex* mosquitoes in Bagaza district, Central African Republic, in 1966 [2]. Since then, it has been detected in several African countries, India, Middle East and, more recently, in Europe [3–11]. Despite several countries have reported the detection of BAGV in mosquitos, the first isolation of this virus in a vertebrate host was in Spain in 2010 [9], associated with a high mortality outbreak in the south of the country that affected partridges and pheasants. More recently, in 2016-2017, BAGV has been noticed in Himalayan monal pheasants (*Lophophorus impejanus*) in South Africa [6], and in 2021 in a corn bunting and several red-legged partridges in Portugal [5]. On the other hand, the Israel turkey meningoencephalomyelitis virus (ITMV), detected

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in turkeys in Israel [12], is so close genetically to BAGV that both have been proposed as belonging to the same virus species [13].

Regarding pathogenesis, depending on the infected species, BAGV causes: apathy, weakness, unresponsiveness, impaired vision, severe hemolytic process and significant weight loss, among other disease signs [1,14–16]. Remarkably, BAGV can be transmitted rather efficiently by direct contact among red-legged partridges, at least under experimental conditions [1]. Mortalities of 30% in red-legged partridges [1] and 40% in grey partridges [14] have been observed upon BAGV infection, which implies an enormous impact both at socio-economic levels and in the abundance of the natural populations, as well as on the ecosystems of the Iberian Peninsula [17,18]. Although, based on serological detection in encephalitic patients from India during the acute phase of the infection, BAGV has been proposed as a zoonotic pathogen [8], it was found unable to infect mice in experimental conditions [1], which considerably stands against this claim.

The genome of BAGV consists of a linear, single stranded, positive sense RNA molecule of 10,900-11,000 nucleotides in length. This molecule encodes for a single polyprotein of 3427 amino acids, that is further processed into 3 structural proteins: capsid (C), pre-membrane (prM), and envelope (E), and seven non-structural proteins (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [18].

In Spain, BAGV was identified for the first time in September 2010, due to an unusual outbreak associated with high mortality rates in red-partridges (*Alectoris rufa*) and common pheasants (*Phasianus colchicus*) from Cádiz province (the southernmost province of the country) [9]. Circulation of the virus was confirmed serologically in the following seasons (2011-2012) [19]. After that, BAGV was not detected again in Spain until 2019 [10] and again in 2021, in mosquito [20] and in red-legged partridges. Moreover, BAGV has been reported for the first time in Portugal in September 2021, in a corn bunting (*Emberiza calandra*) and several red-legged partridges [5].

Bagaza virus is a neglected arbovirus of whom little is known apart from the information provided above. This study aimed at: 1) elucidating the origin of the recently re-emerged BAGV strains detected in Spain after nearly a decade of epidemiological silence, 2) establishing relationships with other circulating strains from different countries, 3) raising knowledge about the epidemiological situation and dispersal behavior of this pathogen. For this purpose, we have undertaken the molecular characterization and phylogenetic analysis of four Spanish BAGV isolates, obtained from outbreaks occurred in red-legged partridges in 2019 and 2021, in Cádiz, southern Spain.

2. Materials and Methods

2.1. Sample Collection and Preparation

Samples included in this study were collected from four affected red-legged partridges from two independent outbreaks. The first outbreak occurred in October 2019, in Vejer de la Frontera, a municipality located in Cádiz province; thus, samples from three red-legged partridges were collected. The second outbreak took place in August 2021, in Jerez de la Frontera, Cádiz province, 70 kilometers away from the 2019 outbreak (Figure 1); here, samples from another red-legged partridge were also collected. All samples analyzed in this study derive from brain tissues that were homogenized and subjected to initial PCR diagnostics [21] and then, to virus isolation in Vero and BSR cells as formerly described [22]. Viral isolates obtained in Vero cells were further analyzed by RT-PCR for BAGV [21]. Official diagnostic techniques were carried out at the Central Veterinary Laboratory, from Ministry of Agriculture (LCV, Algete, Spain). Then, the four isolates were stored at −80º C and sent to the Animal Health Research Center (CISA-INIA, CSIC, Valdeolmos, Spain) for further characterization.

2.2. Whole Genome Sequencing and Phylogenetic Analyses

Full genome sequencing was carried out by overlapping conventional RT-PCRs using primer sets previously described [9,13]. Additionally, extra primer sequences were specifically designed to

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cover the complete viral genomes (Supplementary Table S1). Amplified products were bi-directionally sequenced by Sanger's approach (kit Brilliant Dye Terminator Cycle Sequencing Kit version 3.1 Nimagen and Thermofisher Scientific - 3730 DNA Analyzer). Sequenced amplicons were further edited and assembled using SeqMan software (DNASTAR, Madison, WI, USA). Complete genome sequences obtained were submitted to GenBank.

Phylogenetic analyses included 24 BAGV and 6 ITMV previously published complete sequences, in addition to the 4 obtained Spanish BAGV of this study. To allow the incorporation of 4 additional African isolates to the study [6], further partial phylogenetic analysis targeting 1035 Nt of the NS5 coding region were carried out, including previously employed complete sequences. Tembusu virus (MN649267) was used in both analyses as outgroup. Multiple alignments were performed by ClustalW and phylogenetic trees were produced using the Maximum likelihood method (available in MEGA7 software) using GTR+G+I and TN93+I as optimal nucleotide substitution models for both 35 whole genome sequences analysis, and 39 partial genome analysis, respectively. Bootstrap analyses were inferred from 1000 replicates.

2.3. Polyprotein Analysis

Complete polyprotein sequences of the four isolates were obtained by EMBOSS Sixpack tool. Sequences alignment and further amino acidic homology studies were carried out by ClustalW. Initially, the four new Spanish sequences were compared with a representative of the first outbreak of BAGV in Spain (HQ644143), used as reference. Additionally, two sequences from Senegal 2014 (MF380434) and Portugal 2021 (LC730845) were included in the study. After that, a more complete analysis, including 17 polyprotein sequences from several countries and years, was performed.

3. Results

3.1. BAGV Isolates

All red-legged partridge's brain samples examined (n=4) were positive in virus isolation after 2 or 3 passages in Vero cells. BAGV isolates analyzed by real-time RT-PCR provided Ct values ranging from 11.6 to 12.5. The names assigned to the isolates were: BAGV_SPA/E/2019-01/RLP-b/3V (PP236854), BAGV_SPA/E/2019-02/RLP-b/3V (PP236853), BAGV_SPA/E/2019-03/RLP-b/3V (PP236852), and BAGV_SPA/E/2021-01/RLP-b/3V (PP236851) (Table 1).

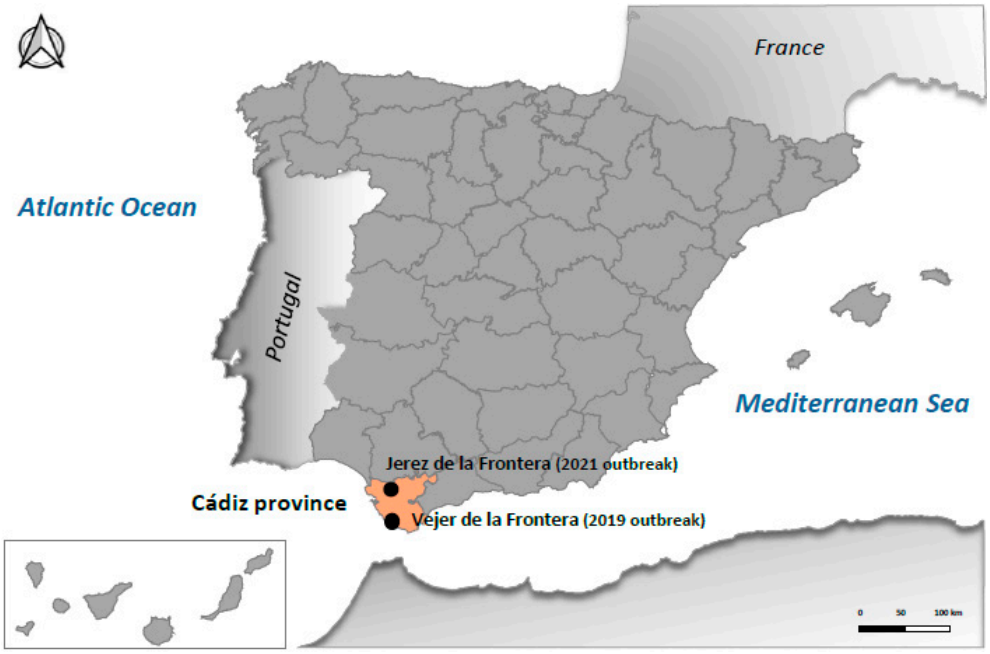

Figure 1. Representation of Spain highlighting Cádiz province and the municipalities where two BAGV outbreaks were reported in 2019 and 2021 in red-legged partridges.

Table 1. Information of the samples analyzed in this study. Results of the real-time RT-PCR, the virus isolation in cell culture, and GenBank accession no. are also presented.

Name of the isolate	Year	Location	Species	Tissue	RRT-PCR (Ct value)[1]	Viral isolation, cytopathic effect		GenBank Accesion nº
						BSR cells	Vero cells (nº of passage, p)	
BAGV_SPA/E/2019-01/RLP-b/3V	2019	Vejer de la Frontera (Cádiz, Spain)	Red-legged partridge	Brain	11.6	Weak	Positive (2p)	PP236854
BAGV_SPA/E/2019-02/RLP-b/3V	2019	Vejer de la Frontera (Cádiz, Spain)	Red-legged partridge	Brain	12.1	Weak	Positive (2p)	PP236853
BAGV_SPA/E/2019-03/RLP-b/3V	2019	Vejer de la Frontera (Cádiz, Spain)	Red-legged partridge	Brain	11.9	Weak	Positive (2p)	PP236852
BAGV_SPA/E/2021-01/RLP-b/3V	2021	Jerez de la Frontera (Cádiz, Spain)	Red-legged partridge	Brain	12.5	Neg.	Positive (3p)	PP236851

[1] Buitrago et al., 2012 [21].

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3.2. BAGV Sequences and Phylogenetic Analyses

Four full genome linear sequences of 10.973 (BAGV_SPA/E/2019-01/RLP-b/3V), 10.947 (BAGV_SPA/E/2019-02/RLP-b/3V and BAGV_SPA/E/2019-03/RLP-b/3V), and 10.920 (BAGV_SPA/E/2021-01/RLP-b/3V) nucleotides were obtained.

Phylogenetic relationships between these new isolates and other BAGV and ITMV isolates with full genome or partial (NS5) sequences available in GenBank were established. The phylogenetic analyses performed identified two genetic clusters or genotypes, here named BAGV-Genotype 1 and BAGV-Genotype 2 based on chronological order of detection. The first comprised the most ancient isolates detected (Israel, 1959-1995), as well as isolates from Senegal (1989-2014), India (1996), Ivory Coast (1998), which clustered together with the three Spanish isolates of 2019 analysed in this study. The second comprised more recent isolates from Israel (2010), Spain (2010), Zambia (2013), South Africa (2016, 2023), Namibia (2018) and Portugal (2021), which clustered together with the most recent BAGV identified in Spain (2021) here studied, as well as with another BAGV sequence from Spain, recently (2021) identified in *Anopheles atroparvus* mosquitoes, in Seville province, close to Cádiz [20]. Of note, both genotypes have been detected in a similar range of avian species (mainly phasianids) and mosquitoes (mainly from *Culex* genus) (Figure 2). When comparing the sequences included in each of the two genotypes, a mean nucleotide distance estimated in 0.06996 was found, which means a 7% of nucleotide divergence between the two defined genotypes. Partial phylogenetic analysis targeting 1035 Nt of the NS5 provided a similar tree topology, including the incomplete South African sequences (2017) within BAGV-Genotype 2 (Figure 3). However, the 2010 Israeli isolates did not clearly associate with any of the two main clusters, probably due to lower statistical support derived from the shorter length and lower variability of the partial NS5 sequence used in the analysis.

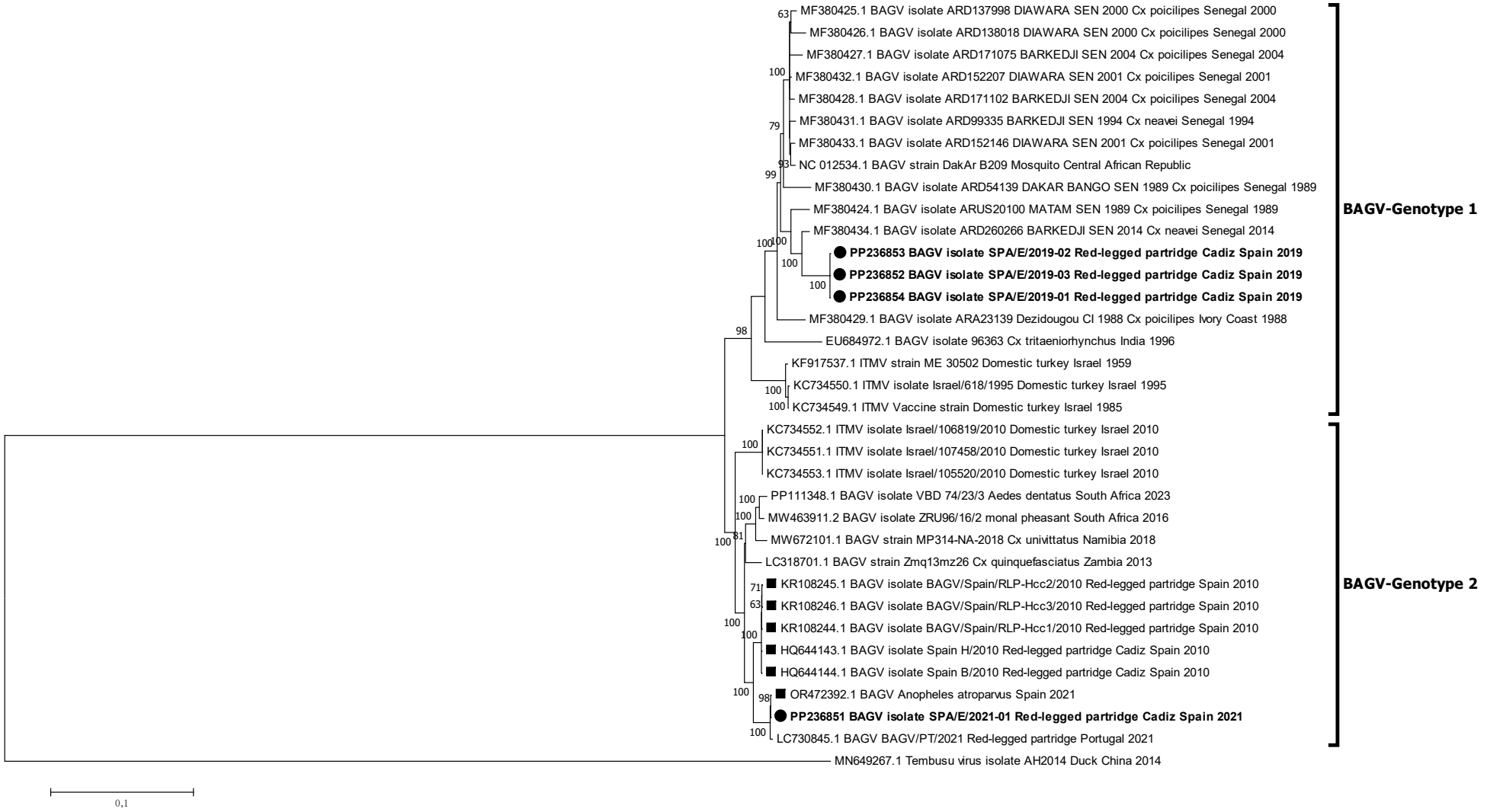

Figure 2. Phylogenetic analysis of 35 complete genome nucleotide sequences of BAGV/ITMV. BAGV genotypes 1 and 2 are indicated. Viral sequences are identified by GenBank accession number, name, species, country and year of isolation. Sequences emphasized in bold and with a circle were generated during this study. Other Spanish strains are marked with a square. Percentages of successful bootstrap replicates over 60% are indicated at tree nodes.

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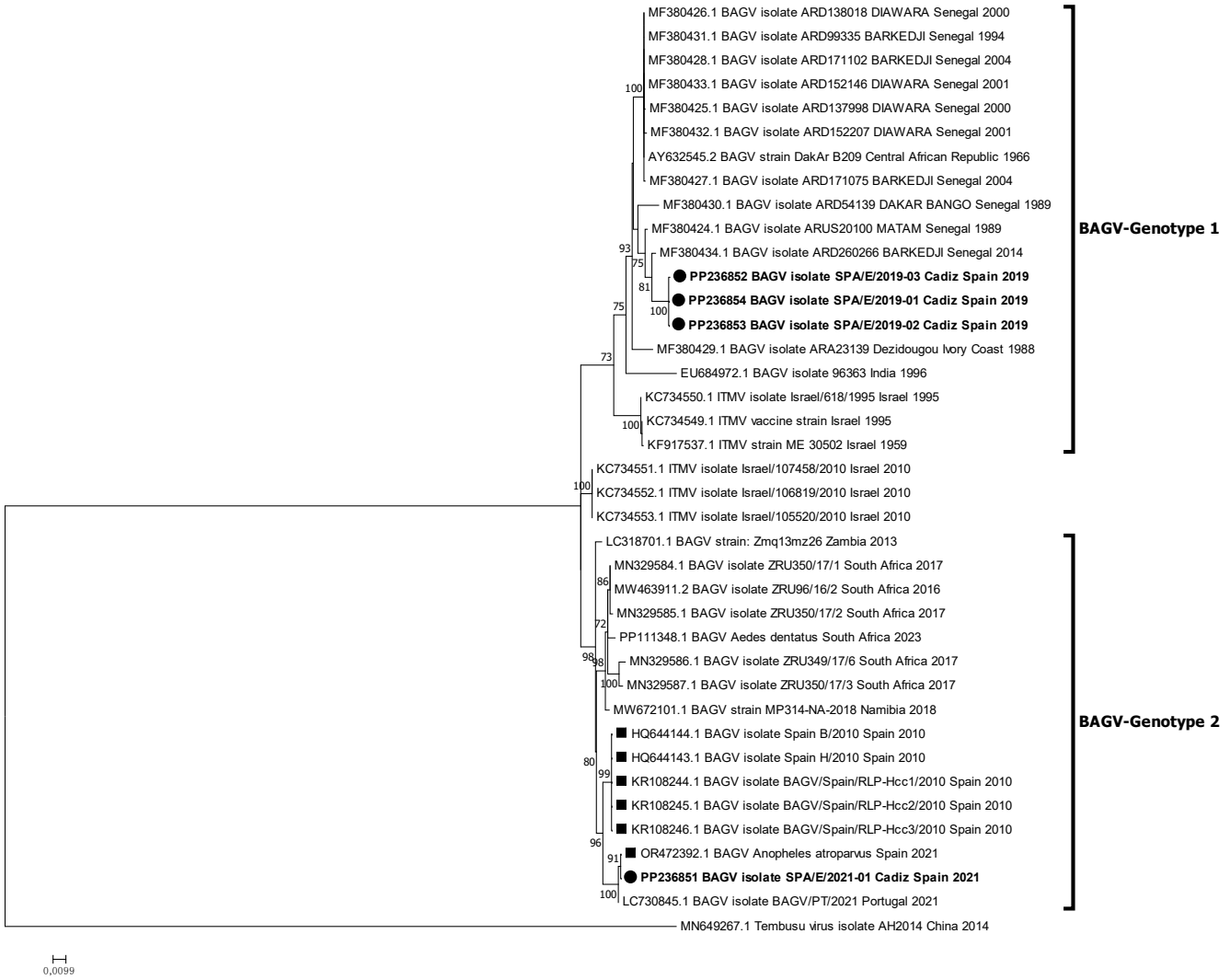

Figure 3. Phylogenetic analysis of 39 genome nucleotide sequences (1035 nt of NS5 region) of BAGV/ITMV. BAGV genotypes 1 and 2 are indicated. Viral sequences are identified by GenBank accession number, name, species, country and year of isolation. Sequences emphasized in bold and with a circle were generated during this study. Other Spanish strains are marked with a square. Percentages of successful bootstrap replicates over 60% are indicated at tree nodes.

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3.3. Analysis of the Viral Polyproteins

Each of the four full-length BAGV nucleotide sequences elucidated in this study yielded a polyprotein of 3427 amino acids, which were compared with those of their closest representatives from genotypes 1 and 2, that is, Senegal 2014 and Portugal 2021, respectively (the sequence OR472392 from *A. atroparvus* mosquitoes obtained in Spain, 2021 [20] was not included in this analysis as it is incomplete in GenBank). Indeed, a representative of the initial Spanish case of BAGV (2010) was also included. In total, seven BAGV polypeptides were compared (Table 2). Focusing on 2019 Spanish isolates, they are characterized by a specific amino acidic signature in six positions of the polyprotein, namely: positions 108, 109, 1332, 1836, 2215 and 3372 (marked in red in Table 2). More in detail, BAGV_SPA/E/2019-02/RLP-b/3V isolate is the only one in the study characterized by arginine (R) in position 164, instead of the glutamine (G) shown by the rest of the representatives. On the other hand, BAGV_SPA/E/2021-01/RLP-b/3V isolate has also a specific signature, particularly in the positions 83, 100, 896, 1051, 2283, 2358, 2435 and 3286 of the whole polyprotein, signature that is shared with the Portuguese isolate and differs from the initial Spanish BAGV-Genotype 2 representative (Spain 2010). When including more isolates from both clusters in the study (up to seventeen sequences), there are changes in seven amino acid positions that distinguished both BAGV genotypes (marked in red in Supplementary Table S2). The first substitution is located in glycoprotein E, position 564, while the rest of amino acidic differences are located in non-structural proteins, in positions 1010, 1039, 1511, 1834, 2262 and 2799 (Supplementary Table S2).

Table 2. Comparison of the amino acid substitutions between the complete genomes of the Spanish BAGV isolates. The first Spanish isolate from 2010 was used as the reference sequence. The closest representatives from BAGV-Genotypes 1 and 2 (Senegal 2014 and Portugal 2021, respectively) were included in the study for comparison. Black dot indicates the same amino acid as the reference sequence. Amino acids that are different from the reference sequence (Spain 2010) are highlighted in bold. Amino acids that characterize 2019 Spanish isolates from BAGV-Genotype 1 are marked in red.

		BAGV-GENOTYPE 2			BAGV-GENOTYPE 1			
PROTEIN	**Amino Acid Position**	**HQ644143.1 (Spain 2010) Red-legged partridge**	**PP236851 (Spain 2021) Red-legged partridge**	**LC730845.1 (Portugal 2021) Red-legged partridge**	**PP236854 (Spain 2019) Red-legged partridge**	**PP236853 (Spain 2019) Red-legged partridge**	**PP236852 (Spain 2019) Red-legged partridge**	**MF380434.1 (Senegal 2014) *Cx. poicilipes***
Flavi_capsid	53	A	.	.	T	T	T	T
	76	V	.	.	.	.	.	G
	83	K	R	R	.	.	.	
	92	M	.	.	.	.	.	.
	100	G	S	S	.	.	.	.
	108	T	.	.	I	I	I	.
	109	L	V	V	T	T	T	S
	113	I	.	.	V	V	V	V
	115	A	V	V	V	V	V	V
	116	V	.	.	A	A	A	A
Flavi_propepeptide	150	A	.	.	T	T	T	T
	164	G	.	.	.	R	.	.
	166	I	M	M	M	M	M	M
Flavi_glycoprot_E	564	T	.	.	A	A	A	A
Flavi_E_C	659	K	.	R	.	.	.	.
Flavi_E_stem	699	S	.	F	.	.	.	.
Flavi_NS1	838	E	.	D	.	.	.	.
	841	E	.	.	G	G	G	G
	842	K	.	.	R	R	R	R
	874	Q	.	.	.	.	.	P
	896	W	L	L	.	.	.	.
	909	G	.	.	.	.	.	E
	1010	I	.	.	V	V	V	V
	1051	K	R	R	.	.	.	.

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	1054	V	.	.	.	.	.	G
Flavi_NS2A	1174	V	M	M	M	M	M	M
	1202	L	.	M	.	.	.	.
DUF389 domain of unknown function	1305	R	.	.	K	K	K	K
	1307	V	.	.	I	I	I	I
	1319	V	.	.	I	I	I	I
	1332	I	.	.	V	V	V	.
	1511	K	.	.	R	R	R	R
Flavi_NS3 serine protease	1547	H	.	R	.	.	.	.
	1562	D	.	.	.	.	.	V
DEAD-like helicases superfamily	1758	V	.	.	I	I	I	I
	1834	M	.	.	V	V	V	V
	1836	V	.	.	L	L	L	.
Helicase superfamily c-terminal domain	1885	Q	.	P	.	.	.	.
	1887	N	.	Y	.	.	.	.
	2113	E	D	D	D	D	D	D
Flavi_NS4A	2215	V	.	.	A	A	A	.
	2262	V	.	.	I	I	I	I
	2265	T	.	.	A	A	A	A
Flavi_NS4B	2283	S	N	N	.	.	.	.
	2288	A	.	T	.	.	.	.
	2358	I	T	T	.	.	.	.
	2435	I	V	V	.		.	.
	2475	T	.	.	.	.	.	N
	2477	I	.	.	.	.	.	D
	2478	E	.	.	.	.	.	R
	2479	G	.	.	.	.	.	R
	2480	A	.	.	.	.	.	S
	2481	A	.	.	.	.	.	S
	2482	G	.	.	.	.	.	R
	2483	R	.	.	.	.	.	T
	2484	I	.	.	.	.	.	D
	2485	W	.	.	.	.	.	M
	2486	N	.	.	.	.	.	E
	2487	A	.	.	.	.	.	C
	2519	S	.	.	.	.	.	G
FtsJ FtsJ-like methyltransferase	2652	I	V	V	.	.	.	.
	2698	T	.	.	.	.	.	I
	2703	I	V	V	.	.	.	.
Flavi_NS5	2799	N	.	.	K	K	K	K
	2806	T	.	.	M	M	M	M
	2895	A	.	.	S	S	S	S
	3048	G	.	.	S	S	S	.
	3286	G	S	S	.	.	.	.
	3372	S	.	.	G	G	G	.

4. Discussion

Bagaza virus was first identified in Europe, concretely in southern Spain, in 2010, during an unusual high mortality event affecting red-legged partridges and common pheasants [9]. The next outbreak in Europe occurred 9 years after, in 2019, in red-legged partridges in the same region of Spain. In 2021, another outbreak occurred in the same Spanish province, affecting the same avian species, whereas in Portugal, BAGV was firstly detected in the same 2021 season, particularly in a corn bunting and several red-legged partridges [5] in an area that is close to the affected Spanish territory. Although this virus has been circulating in several African countries since at least the last three decades, it is striking why BAGV has been detected neither molecularly nor serologically in Spain during almost a decade since its first introduction, despite active and passive surveillance carried out in the territory. In this regard, the present study aimed to shed light into the epidemiological mechanisms by which this virus spreads between two continents by analysing and

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characterising the full genome sequences of four BAGV isolates from Spain. The phylogenetic analyses performed identify two genetic clusters, or genotypes, of BAGV, here named BAGV-Genotype 1 and BAGV- Genotype 2, integrating strains that have been circulating in different continents (Africa, Asia and Europe), in mosquito and avian populations.

On the one hand, BAGV- Genotype 1 comprises more ancient and longstanding variants, including the earliest isolates of ITMV from Israel (1958-1959), which were the responsible of an epizootic affecting turkeys in that country [23]. After that, the virus continued circulating in that territory, affecting turkey flocks, and becoming an emerging problem for the poultry industry in the country due to the high economic losses it caused. To cope with it, a live-attenuated vaccine was developed [24]. This genotype was also detected in mosquitoes during an outbreak of human encephalitis in 1996 in India, which, together with serological data, suggested a zoonotic potential for this virus [8], an aspect that still remains unclear. Besides, Genotype 1 representatives had been detected in *Culex* mosquito pools from West African countries, Senegal (1989-2014) and Ivory Coast (1988) [7], confirming a continued presence of this cluster in those regions. More recently, in 2019, this genotype emerged for the first time in Europe, concretely in Spain, in the same territory where the other genotype of BAGV (Genotype 2) was initially detected nine years before, in 2010, affecting red-legged partridges.

On the other hand, BAGV-Genotype 2 seems to have evolved more recently. Members of this cluster were first identified in 2010 in Israel and Spain [9,13], affecting phasianids (turkeys, pheasants and partridges). Later on, another strain from this cluster was reported in mosquitoes (*Culex quinquefasciatus*) in Zambia in 2013. Genotype 2 strains were later identified in two south-western African countries, particularly in *Cx. univittatus* mosquitos from Namibia (2018) [11] and in monal pheasants from South Africa (2016-2017) [6]. Another member of this genotype was also noticed in *Cx. perexiguus* from United Arab Emirates (2018) [3] (not included in the phylogenetic analyses due to the short length of this sequence). More recently, in 2021, strains of this genotype arose simultaneously in Spain, in red-legged partridges and mosquitoes [20], and in Portugal, affecting wild birds [5]. Lastly, in 2023, a Genotype 2 strain was identified again in South Africa in *Aedes dentatus* mosquitoes.

These analyses confirm that there are at least two main genotypes of BAGV actively circulating since decades in birds and mosquito populations in Africa and Asia, and more recently in Europe. More in detail, Portuguese authors propose four groups within the BAGV/ITMV monophyletic clusters, namely G1, G2, G3, and G4, separated by low intra genetic distances [18]. Following this proposal, BAGV-Genotype 1 would be divided into 2 groups: G1 gathering Israeli isolates from 1959 to 1995, while G2 would comprise Senegalese ones, as well as representatives from Central African Republic, India, Ivory Coast, and the Spanish isolates from 2019. On the other hand, BAGV-Genotype 2 would be divided also into 2 groups: G3 would gather Israeli isolates from 2010, while G4 would include isolates from the Iberian Peninsula, i.e. Spain, 2010 and 2021, and Portugal 2021, as well as African isolates from Namibia, Zambia, and South Africa.

These results reflect a remarkable dispersal capacity of BAGV through several territories and environments, as well as its ability to establish endemic cycles in different countries. Furthermore, they also support previous observations indicating that BAGV and ITMV likely belong to the same viral species [13,18]. Despite that, the International Commitee for Viral Taxonomy (ICTV) still classify them into two different viral species within the *Orthoflavivirus* genus (https://ictv.global/report/chapter/flaviviridae/flaviviridae/orthoflavivirus, ICTV 2023 release), a concept that is becoming obsolete as new sequence data become known. In addition, the study of the polyprotein supports the phylogenetic results, displaying each BAGV-Genotype a specific amino acidic signature in seven positions.

Focusing on the Spanish isolates, the emergence of BAGV in Spain in 2019 after almost a decade of silence was due to a new independent introduction of a BAGV-Genotype 1 variant from an African territory, probably related to Senegal. This connection between isolates from Senegal and Spain has also been observed in other flaviviruses such as West Nile virus [25,26], although this observation could be biased to the available sequences origin. Surprisingly, two years later, in 2021, a BAGV-

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Genotype 2 variant re-appeared in the territory, after eleven years undetected. This re-emergence of Genotype 2 was likely caused by a new introduction/s of BAGV in the Iberian Peninsula, as judged by the highly homologous amino acid pattern that the Spanish and Portuguese 2021 isolates share in the polyprotein, suggesting a common-close origin. Furthermore, this common pattern clearly differs from the original isolates from Spain (2010) as well as from other BAGV-Genotype 2 representatives. Moreover, Portuguese and Spanish 2021 isolates are not 100% homologous, neither at nucleotide nor at amino acid level, in fact, they differ in eight amino acid positions. Consequently, the almost simultaneous detection of BAGV-Genotype 2 in neighbouring Portuguese and Spanish territories might be due either to two separate introductions of the same viral strain, during the 2021 season, or by a single introduction that has been able to evolve and move near the border between these countries.

Hence, these findings allowed to reconstruct the main events related to the appearance of BAGV in the Iberian Peninsula. Firstly, they suggest that there have been, at least, three independent introductions of BAGV in Spain. The first one occurred in 2010, caused by a BAGV-Genotype 2. Then, after a silent period, a new cluster (Genotype 1) emerged in the same territory in 2019; finally, Genotype 2 re-emerged in 2021, affecting Spanish and Portuguese territories. Whether these variants are able to settle down and establish an enzootic cycle is still unknown, as more data are needed to confirm this hypothesis.

Regarding the impact of BAGV in animal health, this is an important pathogen not only for poultry farming (turkeys) but also for wild birds, and particularly for some species of game birds that are raised in farms for hunting purposes, such as pheasants and partridges, sustaining an economically important activity in some areas. For this reason, the presence of BAGV in red-legged partridges implies a great concern in nations such as France, Italy, Portugal and Spain, as this is a species of high economic-relevance in those countries, in fact, it is the only autochthone partridge species in Portugal [18]. Taking into account the results of this study and considering that phasianids are highly susceptible to BAGV infection, it is necessary to reinforce surveillance activities in the south of the Iberian Peninsula in order to provide an early warning and to apply the appropriate control measures to reduce the number of affected animals and the subsequent economic losses. Of note, BAGV has been detected in the Iberian Peninsula only when outbreaks occur in these avian species, and only one mosquito pool was found positive in 2021 [20], despite the intense flavivirus vector surveillance carried out in Southern Spain. This fact contrasts with the situation in Africa, where most BAGV data come from mosquito detections.

In summary, this study confirms the re-introduction of two BAGV variants in Spain, after a long period of epidemiological silence. As the red-legged partridge is highly susceptible to BAGV infection and disease, it would constitute a suitable target species for BAGV surveillance.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Sequences of primers specifically designed to complete the whole genome sequencing of the Spanish BAGV isolates displayed in this study; Table S2: Comparison of the amino acid substitutions between complete BAGV/ITMV isolates. The first Spanish isolate from 2010 was used as reference sequence. Spanish isolates (2019 and 2021) obtained in this study were included as well as their closest representatives from BAGV-Genotypes 1 and 2 (Senegal 2014 and Portugal 2021, respectively). Additionally, more representatives from several countries and years were incorporated in the study. Black dot indicates the same amino acid as the reference sequence. Amino acids that are different from the initial reference are highlighted in bold. Amino acid changes that distinguish BAGV-Genotype 1 and 2 are marked in red. Cell colours indicate the amino acid category: yellow = hydrophobic; green = polar; pink = acidic; blue = basic.

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