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Detection of Myxoma Virus DNA in Ticks from Lagomorph Species in Spain Suggests Their Possible Role as Competent Vector in Viral Transmission

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ABSTRACT: Myxoma virus (MYXV) causes morbidity and mortality in European wild rabbits (*Oryctolagus cuniculus*) worldwide, and recently in Iberian hares (*Lepus granatensis*) in Spain. We aimed to assess the presence of MYXV-specific DNA in ixodid ticks collected from both hosts. A total of 417 ticks harvested from 30 wild lagomorphs, including wild rabbits and Iberian hares were collected from southern Spain. Enzyme-linked immunosorbent assay and PCR-sequencing were used to detect virus exposure and presence, respectively. Antibodies to MYXV were detected in 68% (17/25) of wild rabbits and in 67% (2/3) of Iberian hares. We detected MYXV DNA in 50.7% of pools of two different tick species (nymphs and adults of *Rhipicephalus pusillus*, and nymphs of *Hyalomma lusitanicum*) parasitizing rabbits and hares. The obtained partial sequence of the viral major envelope protein gene showed a mutation (G383A) within the MYXV_gp026 locus between the rabbit strain and Iberian hare strain (recently isolated in tissues of infected hares from Spain). However, in our study, the viral DNA presence was detected for the first time using tick DNA as the PCR-template, but the possible role of ticks as vectors of MYXV still needs to be elucidated.

Key words: Ixodid ticks, *Lepus granatensis*, mutation, myxoma virus, *Oryctolagus cuniculus*.

Myxoma virus (MYXV) is a member of the genus *Leporipoxvirus* (family Poxviridae) that causes subclinical infection or localized cutaneous fibromas in its native host, South American rabbits of the genus *Sylvilagus*. However, it can occasionally cause lethal virus disease of wild and domestic European rabbits (*Oryctolagus cuniculus*; Fenner and Chapple 1965). Even though species of hare are considered to be resistant to MYXV infection, sporadic cases of myxomatosis have been reported in the European brown hare (*Lepus europaeus*) and mountain hare (*Lepus timidus*; Kerr et al. 2015). In addition, high mortality rates have recently been described in Iberian hare (*Lepus granatensis*) populations in Spain (García-Bocanegra et al. 2019, in press).

Myxoma virus was introduced in France in 1952 and spread rapidly, causing high mortality rates in European rabbits throughout Europe (Fenner and Chapple 1965). The virus was detected for the first time in Iberian hare populations in Spain in 2018, where it caused mortality rates higher than 50% (García-Bocanegra et al. 2019). The current molecular analysis on the complete ha-MYXV virus genome (ha-MYXV-Tol strain showed more than 110 mutations with respect to the MYXV Lausanne reference genome, supporting a new MYXV isolate from Iberian hares (Águeda-Pinto et al. 2019; Dalton et al. 2019). This new MYXV encoded three disruptive genes (M009L, M036L, and M152R; Águeda-Pinto et al. 2019) and a novel 2.8 kb insertion (Ins-H1 sequence) related to MYXV genes and open reading frames M060R, M061R, M064R, and M065R (Dalton et al. 2019). The latter are hypothetically related to jumping species barriers (Dalton et al. 2019) and more effectively defeat the defences in the new host (Águeda-Pinto et al. 2019). In the Iberian Peninsula, myxomatosis is an endemic disease that causes near-extinction changes in wild rabbit populations associated with epizootic outbreaks (Calvete et al. 2002). Recent

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epidemiological data from most European Union countries indicates that mosquitoes (Culicidae) and black flies (Simulidae) are directly involved in the viral dissemination. However, Kerr et al. (2015) stated that flea transmission of myxomatosis can occur throughout the year, whereas mosquitoes and other flying vectors such as midges and black flies are more seasonal. The role in transmission of MYXV by other Arthropoda species such as ticks remains unclear. Our aim was to investigate the presence of MYXV DNA isolated from ixodid ticks collected from European wild rabbits and Iberian hares in Spain.

A total of 27 European wild rabbits legally hunted during summer 2017 in Cadiz (12 hunting estates) and Seville (one hunting estate) provinces from southern Spain, and three Iberian hares hunted in one hunting estate in Badajoz province (western Spain) during summer 2018 were studied. Myxomatosis-like clinical signs and lesions, when present, were recorded for each animal. The presence of antibodies to MYXV in serum was also determined using a commercial indirect enzyme-linked immunosorbent assay (INgezim Mixomatosis R.17.MIX.K1®, Ingenasa, Madrid, Spain) following the manufacturer's instructions. The reported sensitivity and specificity rates of this enzyme-linked immunosorbent assay are 98% and 99%, respectively. Feeding ticks were harvested from the sampled lagomorphs, transported alive to the laboratory under refrigeration and grouped for each host (pool compositions are given in Table 1) according to developmental stage, sex, and species (Gil-Collado et al. 1979) and were kept at –80 C until DNA extraction with DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany). The MYXV major envelope protein gene (*m022L*) was amplified by PCR (Farsang et al. 2003). DNA from attenuated virus (POXLAP Strain León-162, Ovejero Laboratory, León, Spain) was employed as a positive control. Finally, nine ExoSAP-it (Affimetrix Inc., Santa Clara, California, USA) purified PCR products from eight sampling locations were sequenced in both directions with the BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Darmstadt, Germany) using an ABI 3130 genetic analyzer (Applied Biosystems). Seventy-seven nucleotide sequences were aligned to conduct evolutionary analyses in MEGA7 (Kumar et al. 2016). Nine sequences (n=9; but two from one hare) from this study were the field strain, but one sequence was from an attenuated commercial vaccine (strain León-162), for a total of 10 sequences. The remaining sequences were downloaded from GenBank as follows: 27 from Australian wild rabbits (*Oryctolagus cuniculus*), 27 from European wild rabbits, and one from Brazilian rabbits (*Sylvilagus brasiliensis*). Also, two more sequence from wild Spanish hares were included, for a total of four of the hare viruses. Moreover, four sequences from vaccine strain and another six sequences that did not fit in the previous groups, were also incorporated. The inferred tree was performed using the Maximum Likelihood method under the Kimura 2-parameter + G model (Kimura 1980).

Differences between the outputs of the PCR analysis on tick pools and the detection of clinical signs of MYXV infection in lagomorphs were assessed using a Pearson's chi-square test. The same statistical test was used to study the association between detection of DNA in ticks and detection of antibodies in animals. Tick PCR output at the animal level was considered positive when at least one of its tick pools displayed a positive result. In all tests, significance was set at $P<0.05$.

Antibodies to MYXV were detected in 68% of wild rabbits and in 67% (2/3) of the Iberian hares analyzed. Clinical signs and lesions compatible with MYXV infection were observed in 57% (17/30) of the animals. Using PCR, we analyzed DNA from 67 tick pools from rabbits (*Rhipicephalus pusillus*: 20 pools of adult males, 19 pools of adult females, and 12 pools of nymphs; *Hyalomma lusitanicum*: 16 pools of nymphs), and two individual ticks (one male and one female *R. pusillus*) from hares were analyzed by PCR (Table 1). We detected MYXV DNA in 50.7% (35/69) of tick pools. Both ticks collected from one hare were also positive for MYXV infection. We detected

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TABLE 1. Results of myxoma virus detection in European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*), and in ticks infecting them in Southern Spain. Summary of serology results achieved on lagomorphs and PCR results obtained on tick pools. Bold type indicates ticks with myxoma virus DNA, from which the sequences have been analysed. Three ticks were randomly selected if there were between three and eight ticks of the same species and same reproductive stage; four ticks were randomly selected if there were between nine and 10, and five ticks were randomly selected if there were more than 10 ticks.[a]

Hosts					Infecting ticks							
					Rhipicephalus pusillus						*Hyalomma lusitanicum*	
					Adults				Nymphs		Nymphs	
Specimen	Species	Sampling site[b]	Serology[c]	Signs and lesions[d]	Male (*n*)	Male (pools/ticks)	Female (*n*)	Female (pools/ticks)	*n*	Pools/ticks	*n*	Pools/ticks
1	**Rabbit**	1	POS	Yes	1	NEG/1	0	—	0	—	**12**	**POS/5**
2	Rabbit	1	POS	No	1	NEG/1	2	NEG/2	0	—	12	NEG/5
3	**Rabbit**	3	POS	Yes	0	—	**1**	**POS/1**	0	—	22	NEG/5
4	**Rabbit**	2	POS	Yes	2	NEG/2	0	—	0	—	**15**	**POS/5**
5	Rabbit	4	NEG	No	1	NEG/1	0	—	0	—	21	NEG/5
6	**Rabbit**	5	POS	Yes	3	POS/3	**3**	**POS/3**	0	—	5	POS/3
7	Rabbit	5	NEG	No	1	POS/1	4	NEG/3	0	—	13	POS/5
8	Rabbit	5	POS	Yes	6	NEG/3	1	POS/1	0	—	0	—
9	Rabbit	6	POS	No	2	NEG/2	1	NEG/1	0	—	0	—
10	Rabbit	6	NEG	No	1	NEG/1	2	NEG/2	0	—	0	—
11	Rabbit	7	NEG	No	0	—	0	—	0	—	1	NEG/1
12	Rabbit	8	POS	No	0	—	5	NEG/3	11	NEG/5	6	NEG/3
13	Rabbit	8	POS	Yes	1	NEG/1	6	POS/3	5	NEG/3	0	—
14	Rabbit	8	POS	Yes	1	POS/1	6	POS/3	76	POS/5	2	POS/2
15	**Rabbit**	9	POS	Yes	14	POS/5	**7**	**POS/3**	6	POS/3	3	POS/3
16	Rabbit	11	NEG	No	2	NEG/2	1	NEG/1	1	NEG/1	26	POS/5
17	Rabbit	3	NEG	Yes	0	—	0	—	2	NEG/2	13	NEG/5
18	Rabbit	5	POS	No	2	NEG/2	1	NEG/1	2	NEG/2	1	NEG/1
19	Rabbit	5	POS	No	0	—	0	—	8	NEG/3	0	—
20	**Rabbit**	10	POS	No	0	—	0	—	0	—	**19**	**POS/5**
21	Rabbit	10	POS	No	0	—	0	—	1	NEG/1	6	NEG/3
22	Rabbit	12	NEG	No	3	NEG/3	4	NEG/3	6	POS/3	0	—
23	**Rabbit**	13	POS	Yes	1	POS/1	**6**	**POS/3**	2	POS/2	0	—
24	Rabbit	13	—	Yes	4	POS/3	1	POS/1	0	—	0	—
25	Rabbit	13	NEG	Yes	2	POS/2	6	POS/3	0	—	0	—
26	Rabbit	13	POS	Yes	4	POS/3	4	POS/3	0	—	0	—
27	Rabbit	13	—	Yes	1	POS/1	3	POS/3	1	POS/1	0	—
28	**Hare**	14	POS	Yes	**1**	**POS/1**	**1**	**POS/1**	0	—	0	—
29	Hare	14	POS	Yes	0	—	0	—	0	—	0	—
30	Hare	14	NEG	Yes	0	—	0	—	0	—	0	—

[a] Pools/ticks = ratio of analyzed pools/number of ticks within the pool; POS = positive; NEG = negative; — = no data.

[b] Sampling sites (1 to 12) are located in Cadiz province (1 = 36°26′39″N 5°46′33″W, 2 = 36°25′38″N 5°45′33″W, 3 = 36°37′20″N 5°53′35″W, 4 = 36°33′34″N 5°49′07″W, 5 = 36°34′43″N 5°57′11″W, 6 =36°33′50″N 5°47′06″W, 7 = 36°34′40″N 5°51′44″W, 8 = 36°31′23″N 5°56′58″W, 9 = 36°22′17″N 5°47′26″W, 10 = 36°32′34″N 5°49′07″W, 11 = 36°35′02″N 5°49′56″W, 12 = 36°41′43″N 5°46′06″W); sampling site 13 (37°23′39″N 5°52′36″W) is in Seville province, and sampling site 14 (39°21′49″N 7°21′51″W) is in Badajoz province).

[c] Results of serology performed by enzyme-linked immunosorbent assay.

[d] Observed myxomatosis-like clinical signs and lesions.

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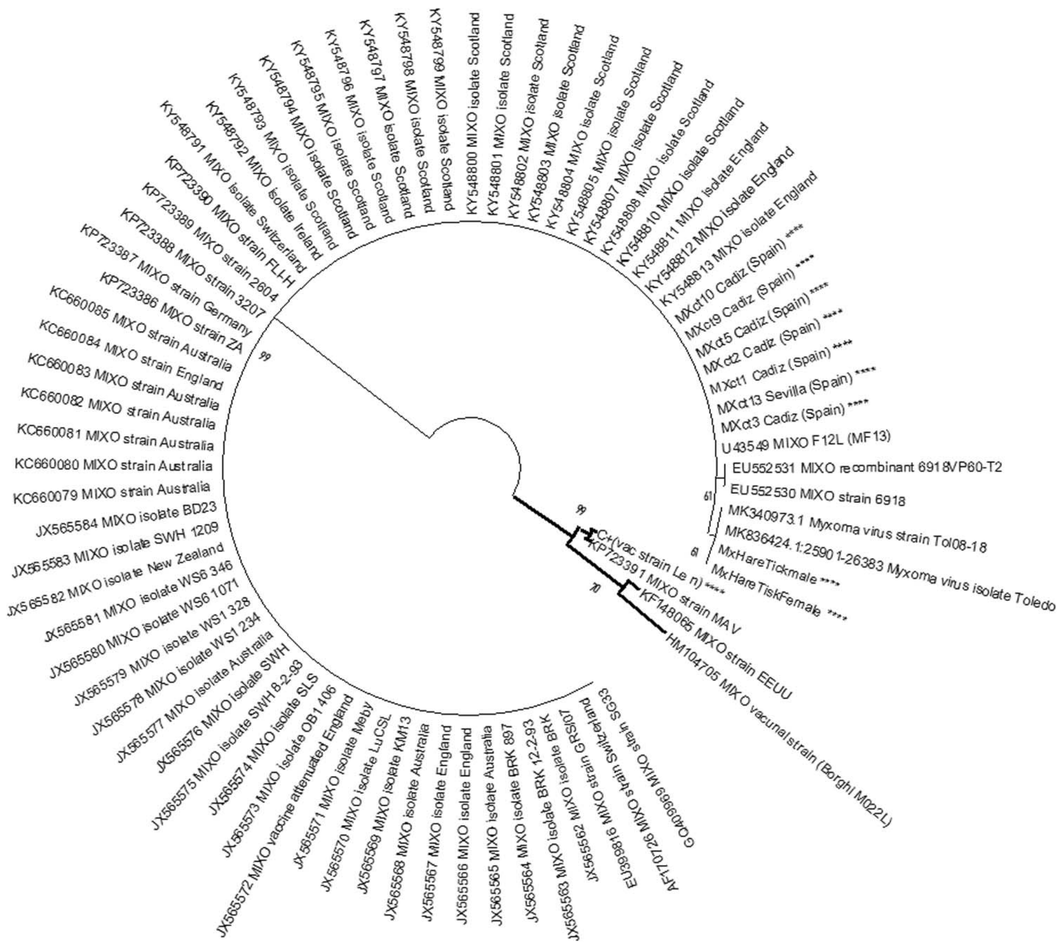

FIGURE 1. Molecular phylogenetic comparison of myxoma viruses from European wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) by Maximum Likelihood method. The tree with the highest log likelihood (–915.2677) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method and a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach. A discrete Gamma distribution was used to model evolutionary rate among sites (five categories [+G, parameter = 0.1466]). The analysis involved 77 nucleotide sequences. There was a total of 483 positions in the final dataset. Sequences from this study are shown using asterisks (****). The branches of each of the two main clades are shown with different thickness from the central point.

MYXV DNA in ticks in the three provinces investigated and in 11 of the 14 hunting estates sampled. The MYXV sequences from ticks (n=7, from seven different hunting states) from rabbits had homologue sequences (MK340923-MK340929), but were different (mutation G383A within the MYXV_gp026 locus) from sequences (n=2) amplified and detected in Iberian hare ticks (MK340920 and MK340922). These last two sequences were identical to each other and to those from Águeda-Pinto et al. (2019) and Dalton et al. (2019). The phylogenetic tree (Fig. 1) revealed two strongly divergent clades (99% bootstrap level and located in opposite directions of the circular tree), with a wide majority of sequences from natural infections falling exclusively in the major one, having a worldwide distribution and including all Iberian rabbits. The sequence from control (POXLAP Strain León-162, MK340921) was different from any sequence within the mayor

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TABLE 2. Summary of amino acid substitutions (Nonsynonymous sites) of the major envelope protein (gene *m022L*) of myxoma virus using all eight protein sequences from GenBank data for myxoma virus strains with the length obtained in this study of host lagomorphs in Spain.

Accession no. (strain)	*n* sequences in GenBank	Amino acid no.[a]						Host/virulence
		60 (triplet)	74 (triplet)	78 (triplet)	111 (triplet)	128 (triplet)	129 (triplet)	
AF170726 (Lausane strain)	112	T (ACC)	A (GCA)	V (GTC)	R (CGG)	R (CGG)	C (TGT)	European rabbit/most pathogenic
MK340973 (Tol08-18 strain)	4	T (ACC)	A (GCA)	V (GTC)	R (CGG)	Q (CAG)	C (TGT)	Hare/pathogenic
EU552531 (Recombinant_virus_6918VP60-T2)	2	T (ACC)	A (GCA)	V (GTC)	W (TGG)	R (CGG)	C (TGT)	European rabbit/non pathogenic
MK388096 (Aust/NSW/Euchareena (3)/02-2013)	1	T (ACC)	A (GCA)	V (GTC)	R (CGG)	R (CGG)	C (TGT)	European rabbit/unknown
KP723391 (strain_MAV)	3	S (AGC)	V (GTA)	I (ATC)	R (CGG)	R (CGG)	C (TGT)	European rabbit/attenuated vaccine
KF148065 (strain_California/San_Francisco_1950)	1	S (AGC)	V (GTA)	I (ATC)	R (CGG)	S (AGC)	C (TGT)	European rabbit/high virulent
HM104705 (strain_Borghi)	1	S (AGC)	A (GCA)	V (GTC)	R (CGG)	S (AGC)	C (TGT)	European rabbit/atenuated vaccine
AY136665.1	1	—	—	—	—	R (CGG)	G (GGT)	European rabbit/clinical case

[a] — = no data.

clade. The MYXV sequences from ticks taken from the Iberian hare showed 100% identity to the hare-MYXV previously isolated in this species (MK836424.1 and MK340973.1) and described earlier (Águeda-Pinto et al. 2019; Dalton et al. 2019). Interestingly, hare-MYXV from ticks collected from Iberian hare showed a single nucleotide polymorphism (C to T) at the 26,111-genome position (Dalton et al. 2019), causing a nonsynonymous substitution (R to Q) at the 128 amino acid position (Table 2). This mutation could be associated with a particular epidemiological significance such as the promotion of adaptive changes (Dalton et al. 2010). Indeed, in England, hares became clinically ill by the same viral agent infecting rabbits (Barlow et al. 2014). It is accepted that the major envelope gene of myxoma (*m022L*) is exposed to the rabbit's immune system (Muller et al. 2010), providing a good gene target for identifying viral variability. In addition, genomic changes due to big gene deletions or a single nucleotide insertion or deletion that modifies open reading frames, might attenuate phenotypes of the MYXV, but not so in the case of nonsynonymous (missense) mutations (Muller et al. 2010). Other authors point out that MYXVs might adapt to a new host due to single-point mutations (Dalton et al. 2010).

Information about the role of ticks as possible vectors is still scarce. Pioneer experimental studies reported MYXV transmission based on the direct inoculation of macerated *Rhipicephalus sanguineus* tick tissues into rabbits (Blanc et al. 1961). In our study, we detected MYXV-specific DNA at a high frequency, in both sexes, and in both developmental stages (adults and nymphs) of two different species of ticks feeding on rabbits and hare. However, the vector competence of the tick species identified in our study has not been experimentally assessed; therefore, a classic vector role cannot be proposed nor discarded.

The detection of MYXV DNA in ticks might simply be the result of the digestion of a blood meal from a viremic lagomorph. The association between the detection of the virus in ticks and the presence of clinical signs in the

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26 animals (χ^2_{1df}=11.87, $P<0.005$) could corroborate this finding, but also, that could indicate virus transmission from the tick to the rabbit in recent infections. Likewise, the lack of correlation in the chi-square test between MYXV DNA in ticks and MYXV antibodies in 26 lagomorphs (χ^2_{1df}=0.65, P=0.420) does not support tick to lagomorph transmission, or vice versa; this finding could indicate previous exposure to the virus. Antibody detection in animals with acute infections (less than 3 wk) is limited. Because we detected virus DNA in ticks from animals with negative serology, this suggests that these ticks could have been feeding on a recently infected host, leading us to think of a possible transstadial transmission. New avenues on research are opened because the present results demonstrated the presence of viral DNA in ticks; this suggests the need for further in vivo experiments. We identified three limitations of this research: not testing tissues of lagomorphs for MYXV DNA, the limited number of animals sampled, and the absence of methods used to detect the whole virus in ticks, such as virus isolation in cell culture or negative staining electron microscopy.

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LITERATURE CITED

Águeda-Pinto A, de Matos AL, Abrantes M, Kraberger S, Risalde MA, Gortázar C, McFadden G, Varsani A, Esteves PJ. 2019. Genetic characterization of a recombinant myxoma virus leap into the Iberian hare (*Lepus granatensis*). *Viruses* 11:530.

Barlow A, Lawrence K, Everest D, Dastjerdi A, Finnegan C, Steinbach F. 2014. Confirmation of myxomatosis in a European brown hare in Great Britain. *Vet Rec* 175:75–76.

Blanc G, Ascione L. 1961. Quelques expériences sur le rôle possible de transmetteuse et réservoir de virus de la tique *Rhipîcephalus sanguineus Latr*. dans la myxomatose. *Bull Soc Path Exot* 54:935–939.

Calvete C, Estrada R, Villafuerte R, Osácar JJ, Lucientes J. 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. *Vet Rec* 150:776–782.

Dalton KP, Martín JM, Nicieza I, Podadera A, de Llano D, Casais R, Gimenez S, Badiola I, Agüero M, Duran M, et al. 2019. Myxoma virus jumps species to the Iberian hare. *Transbound Emerg Dis* 66:2218–2226.

Dalton KP, Nicieza I, Baragão A, Alonso JM, Parra F. 2010. Molecular characterisation of virulence graded field isolates of myxoma virus. *Virol J* 7:49.

Farsang A, Makranszki L, Dobos-Kovács M, Virág G, Fábián K, Barna T, Kulcsár G, Kucsera L, Vetési F. 2003. Occurrence of atypical myxomatosis in Central Europe: Clinical and virological examinations. *Acta Vet Hung* 51:493–501.

Fenner F, Chapple PJ. 1965. Evolutionary changes in myxoma virus in Britain. An examination of 222 naturally occurring strains obtained from 80 counties during the period October–November 1962. *J Hyg-Camb* 63:175–185.

García-Bocanegra I, Camacho-Sillero L, Caballero-Gómez J, Agüero M, Gómez-Guillamón F, Manuel Ruiz-Casas J, Manuel Díaz-Cao J, García E, José Ruano M, de la Haza R. In press. Monitoring of emerging myxoma virus epidemics in Iberian hares (*Lepus granatensis*) in Spain, 2018–2020. *Transbound Emerg Dis.* doi: 10.1111/tbed.13781.

García-Bocanegra I, Camacho-Sillero L, Risalde MA, Dalton KP, Caballero-Gómez J, Agüero M, Zorrilla I, Gómez-Guillamón F. 2019. First outbreak of myxomatosis in Iberian hares (*Lepus granatensis*). *Transbound Emerg Dis* 66:2204–2208.

Gil-Collado J, Guillén-Llera JL, Zapatero-Ramos LM. 1979. Claves para la identificación de los Ixodoidea españoles (adultos). *Rev Iber Parasitol* 39:107–118.

Kerr P, Liu J, Cattadori I, Ghedin E, Read AF, Holmes EC. 2015. Myxoma virus and the Leporipoxviruses: An evolutionary paradigm. *Viruses* 7:1020–1061.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33:1870–1874.

Muller A, Silva E, Abrantes J, Esteves PJ, Ferreira PG, Carvalheira JC, Nowotny N, Thompson G. 2010. Partial sequencing of recent Portuguese myxoma virus field isolates exhibits a high degree of genetic stability. *Vet Microbiol* 140:161–166.

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