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Molecular epidemiology of Aleutian mink disease virus causing outbreaks in mink farms from Southwestern Europe: a retrospective study from 2012 to 2019

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ABSTRACT

Background: Aleutian mink disease virus (AMDV) causes major economic losses in fur-bearing animal production. The control of most AMDV outbreaks is complex due to the difficulties of establishing the source of infection based only on the available on-farm epidemiological data. In this sense, phylogenetic analysis of the strains present in a farm may help elucidate the origin of the infection and improve the control and biosecurity measures.
Objectives: This study had the following aims: characterize the AMDV strains from most outbreaks produced at Spanish farms between 2012–2019 at the molecular level, and assess the utility of the combined use of molecular and epidemiological data to track the possible routes of infection.
Methods: Thirty-seven strains from 17 farms were partially sequenced for the NS1 and VP2 genes and analyzed phylogenetically with other strains described worldwide.
Results: Spanish AMDV strains are clustered in four major clades that generally show a good geographical correlation, confirming that most had been established in Spain a long time ago. The combined study of phylogenetic results and epidemiological information of each farm suggests that most of the AMDV outbreaks since 2012 had been produced by within-farm reservoirs, while a few of them may have been due to the introduction of the virus through international trade.
Conclusions: The combination of phylogenetic inference, together with epidemiological data, helps assess the possible origin of AMDV infections in mink farms and improving the control and prevention of this disease.

Keywords: AMDV; American mink; disease outbreaks; phylogeny

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INTRODUCTION

Aleutian disease (AD) is probably the main infectious cause of economic losses in global mink farming [1]. Its aetiological agent is the Aleutian mink disease virus (AMDV), which is taxonomically classified as *Carnivore amdoparvovirus 1* [2]. The development of AD depends on the virulence of the AMDV strain and the genetics of the hosts, affecting the Aleutian genotype mink [3]. Virulent strains produce high mortality rates in Aleutian mink. Typically, the infection causes progressive immune complex-associated syndrome, leading to glomerulonephritis and arteritis [4]. In newborn kits, the infection produces acute interstitial pneumonia that is usually fatal [5]. In contrast, most AMDV infections in non-Aleutian mink are asymptomatic and even non-persistent, even though some animals can show typical disease [4,6]. In addition, an AMDV infection has been described in other mustelids as well as in other mammals [7-9].

AMDV is a non-enveloped virus with a single-stranded DNA encoding three non-structural proteins (NS1–NS3) and two capsid proteins (VP1 and VP2) [2]. NS1 is essential for viral replication and has a high degree of genetic variability, making it suitable for epidemiological studies [1,10,11]. VP2 is the main capsid protein, and its coding sequence contains a characteristic hypervariable region [12,13]. Both genes have been used widely to assess the differences between strains worldwide. Overall, these studies reported between 3 and 7 different clusters of AMDV strains, usually grouped according to their geographical origin but not according to their pathogenicity [1,11,14-17]. Several studies reported high diversity in some countries/regions and even the coexistence of different strains in a single farm, suggesting that international and local trade may play an important role in the distribution of AMDV [10,15,16,18].

Regarding AMDV infections in Spanish mink farms, since the introduction of counter-inmunoelectrophoresis (CIEP) testing, the percentage of positive farms has decreased from 100% in 1980 to approximately 25% in 2019. This reduction has been attributed mainly to test-and-removal/stamping-out strategies and the closure of many farms. On the other hand, the eradication of the disease is challenging because of the extreme persistence of the virus in the environment, which can favor farm reinfection [19,20]. Furthermore, the trade of breeding stock/equipment and the circulation of people between farms (workers, visitors, technical staff) can be involved in spreading the virus [21]. Accordingly, the application of molecular epidemiology has proved useful for tracking possible sources of AMDV infections [11,22]. This has encouraged many producing countries to characterize their AMDV strains molecularly, and study their relationship with strains from different regions and countries [14,15,23,24]. On the other hand, available data on Spanish AMDV strains are extremely scarce, and they do not represent the whole Spanish mink sector. Therefore, this study characterized the AMDV strains from most infected Spanish farms between 2012–2019 by partial NS1 and VP2 sequencing and phylogenetic analysis, including the contemporary sequences previously described worldwide. In addition, the phylogenetic inference was combined with epidemiological information of the farms for establishing the possible routes of infection and improving the specific preventive measures in each case.

MATERIALS AND METHODS

Sample selection
Between 2012–2019, 17 mink farms (14 from Spain, two from NW Portugal and one from SW France) were identified as AMDV-infected by CIEP testing within the framework of the annual

control program. Farms from Portugal and France were included owing to their historical and current relationship with the Spanish mink sector and their proximity to the Spanish border. In this retrospective study, all available AMDV biological and environmental positive samples from these farms were identified in the authors' specimen bank. In addition, relevant epidemiological information about AMDV infections between 2012 and 2019 was collected (date of infection/outbreaks, introduction of breeding stock animals, commercial and working relationships with other farms). All these samples were previously confirmed by quantitative polymerase chain reaction using previously described protocols [25]. Overall, 2 types of samples were selected for this study: spleen samples from necropsies of CIEP-positive animals, and swab samples from different farm elements in contact with CIEP-positive animals. Where possible, at least 2 positive samples from the same farm and year were selected. Therefore, 37 samples were selected for molecular characterization at the NS1 and VP2 genes (**Table 1**).

NS1 and VP2 amplification and sequencing

Two pairs of previously described primers were used to amplify the NS1 and VP2 partial sequences: AMDV2/AMDV3 for NS1 [10] and VP2 for/VP2rev for VP2 [12]. The PCR reactions were performed in a final volume of 25 µL containing a final concentration of 2.5 U of Taq DNA Polymerase, 1 × reaction buffer, 2 mM of $MgCl_2$, 0.2 mM of dNTP's (all reagents supplied by NZYTech Lda., Lisbon, Portugal), 0.5 µM of each primer, and 5 µL of sample DNA. A touch-down PCR thermal protocol was used for both targets: hot start (95℃ for 60 sec), 10 touch-down cycles (95℃ for 20 sec/55℃ for 30 sec/68℃ for 60 sec) reducing the annealing temperature by 0.5℃ in each cycle, 28 cycles (95℃ for 20 sec/50℃ for 30 sec/68℃ for 60 sec), and a final extension (68℃ for 5 min) [15]. The reactions were run in a Bio-Rad T100 thermocycler (Bio-Rad Laboratories Inc., USA).

The PCR products were visualized on a 1% agarose gel in 1 × TAE buffer stained with RedSafeTM (iNtRON Biotechnology Inc., USA). Subsequently, the PCR products were purified and sequenced in a 3730xl DNA analyzer (Applied Biosystems, USA). The chromatograms obtained from both the forward and reverse strands were assembled and proofread manually using ChromasPro 2.1.4 (Technelysium, Australia). All consensus sequences were deposited in the GenBank NCBI database under accession numbers MN652195-MN652268.

Phylogenetic analysis

The sequences were aligned using the ClustalW algorithm, generating a sequence identity matrix for the NS1 and VP2 sequences in BioEdit 7.2.5 [26]. Identical sequences from the same year and farm were eliminated for phylogenetic analyses to simplify the trees. Five phylogenetic trees were constructed. First, the consensus sequences obtained in this study were analyzed in three datasets: NS1, VP2, and concatenated NS1+VP2 sequences. Second, the sequences available in GenBank from other mink-producing countries and previously described Spanish AMDV sequences (from farmed and wild animals) were selected. These sequences were analyzed together with the sequences of this study in two global datasets for NS1 and VP2. A list of the GenBank sequences used for the analysis of each target is available (**Supplementary Tables 1** and **2**). The best-fitting nucleotide substitution model was selected using the Akaike Information Criterion with free software jModelTest v.2.1.10 [27,28]. Phylogenetic trees were constructed using MrBayes 3.2.7 software [29] using the Bayesian approach with Markov Chain Monte Carlo sampling (10,000,000 generations sampling every 1,000 steps). The trees were visualized and edited in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The Gray Fox amdovirus sequences for NS1 and VP2 (GenBank Accession No. JN202450) were included in the analysis as an outgroup to infer the ancestral relationships.

Table 1. List of the qPCR-positive samples employed in the study, including the sample type, year of collection, country of origin (region), and type of infection

Farm code	Sample code	Sample type	Collection date	Origin: country (region)	Type of infection*
540	540.1/2014	Nest box	2014	Spain (center)	Endemic infection until 2014 (cleared by stamping-out)
542	542.1/2017	Cage	2017	Spain (center)	Endemic infection (closed in 2016)
	542.2/2017	Slaughter box	2017		
545	545.1/2015	Spleen	2015	Spain (center)	Endemic infection until 2012 (cleared by stamping-out) Recurrent sporadic outbreaks since 2015
610	610.1/2013	Cage	2013	Spain (NW Galicia)	Epizootic outbreak in 2012 (endemic infection since then)
	610.2/2016	Catching gloves	2016		
612	612.1/2014	Cage	2014	Spain (NW Galicia)	Epizootic outbreak in 2014 (endemic infection since then)
	612.2/2016	Slaughter box	2016		
616	616.1/2016	Spleen	2016	Spain (NW Galicia)	Sporadic outbreak in 2016 (cleared by test-and-removal)
619	619.1/2013	Cage	2013	Spain (NW Galicia)	Epizootic outbreak in 2013 (endemic infection since then)
	619.2/2016	Catching gloves	2016		
622	622.1/2014	Slaughter box	2014	Spain (NW Galicia)	Epizootic outbreak in 2014 (endemic infection since then)
	622.2/2014	Catching gloves	2014		
	622.3/2016	Catching gloves	2016		
	622.4/2016	Cage	2016		
	622.5/2016	Slaughter box	2016		
	622.6/2018	Catching gloves	2018		
	622.7/2018	Nest box	2018		
633	633.1/2018	Slaughter box	2018	Spain (NW Galicia)	Epizootic outbreak in 2018 (cleared by stamping-out)
	633.2/2018	Cage	2018		
661	661.1/2013	Spleen	2013	Spain (NW Galicia)	Epizootic outbreak in 2012 (endemic infection since then)
	661.2/2013	Spleen	2013		
	661.3/2013	Cage	2013		
	661.4/2016	Slaughter box	2016		
672	672.1/2019	Spleen	2019	Spain (NW Galicia)	Sporadic outbreak in 2018 (currently under test-and-removal)
	672.2/2019	Spleen	2019		
936	936.1/2015	Spleen	2015	Spain (SW Galicia)	Sporadic outbreaks in 2015 and 2018 (cleared by test-and-removal)
	936.2/2018	Cage	2018		
	936.3/2018	Nest box	2018		
985	985.1/2015	Spleen	2015	Spain (SW Galicia)	Sporadic outbreak in 2015 (cleared by test-and-removal)
6109	6109.1/2014	Slaughter box	2014	Spain (NW Galicia)	Epizootic outbreak in 2014 (closed in 2015)
162	162.1/2018	Catching gloves	2018	Portugal (NW border)	Epizootic outbreak in 2014 Endemic infection since 2015 (closed in 2018)
	162.2/2018	Cage	2018		
1130	1130.1/2018	Catching gloves	2018	Portugal (NW border)	Epizootic outbreak in 2014 Endemic infection since 2015 (closed in 2018)
	1130.2/2018	Cage	2018		
382	382.1/2014	Nest box	2014	France (SW)	Epizootic outbreak in 2013 (cleared by stamping-out in 2015)
	382.2/2015	Slaughter box	2015		

ELISA, enzyme-linked immunosorbent assay.
*Endemic infection: farms with a high prevalence for several years, which usually select tolerant animals using the Iodine Agglutination Test or ELISA; Sporadic outbreak: cases with a low within-farm spread of the infection (prevalence <2%), cleared mainly by test-and-removal strategies; Epizootic outbreak: cases with an explosive spread of the infection within the whole farm in a few months (high prevalence) that were cleared by stamping-out or became endemic infections.

RESULTS

All samples (n=37) were amplified and sequenced for both NS1 and VP2 genes. After manual proofreading and alignment, all sequences were trimmed to the same length (328 bp for NS1; 628 bp for VP2). The NS1 sequences showed 83.2–100% identity to each other, whereas the VP2 sequences had 91–100% identity (**Supplementary Tables 3** and **4**). In cases where 2 or more samples from the same farm were analyzed, the obtained sequences were virtually identical for both genes independent of the year of sampling (99.5–100% identity), with farm 936 as the only exception, which showed different sequences for 2015 and 2018 (92.6% for NS1; 96.9% for VP2).

Regarding the phylogenetic analysis of the three Spanish datasets, the General Time Reversible model with gamma-distributed rate variation across sites (GTR+G) was found to

be the best-fit substitution model. The three phylogenetic trees are shown in **Figs. 1**, **2**, and **3** (NS1, VP2, and concatenated NS1+VP2, respectively). The nomenclature of the clades used in the NS1 tree was conserved in the other trees to facilitate comprehension.

Based on the NS1 phylogenetic tree, the AMDV sequences from the Spanish farms were divided into three clades (**Fig. 1**). Clade I was divided into three subclades with a certain geographical relationship. Subclade Ia was gathered from three farms in the northwest of Galicia (NW Spain), as well as the sequences from the French farm (382). Subclade Ib was clustered from three farms, one from SW Galicia and the two farms from NW Portugal. Subclade Ic only presented one recent sequence from a farm in the southwest of Galicia (farm 936), which had previously been infected in 2015 with another strain from subclade Ib. Clade II was the smallest, with three farms in the center of Spain and the other in SW Galicia. Clade III comprised of six farms from NW Galicia and was divided in subclade IIIa, with five farms very close to each other with an identical NS1 sequence, and IIIb with a single farm separated geographically from farms of subclade IIIa.

The VP2 phylogenetic tree (**Fig. 2**) differed slightly from the NS1 tree, forming four main clades. VP2 clade I included the same sequences as NS1 subclade Ia. Clade II was the smallest again, but only comprising the three farms in the center of Spain (same as NS1 subclade IIa). Clade III was identical to NS1 clade III. Finally, the new VP2 clade IV gathered the sequences of NS1 subclades Ib, Ic, and IIb, thereby representing the four farms in SW Galicia and NW Portugal.

The NS1+VP2 concatenated tree was also divided into four clades (**Fig. 3**) as the VP2 phylogenetic tree, with only some differences in terminal branching. Interestingly, both

Fig. 1. Bayesian phylogenetic tree based on the NS1 sequences of AMDV obtained in this study. Posterior probabilities (%) are shown next to the nodes. Sequences are called the "Farm ID. Sample number_Year of collection," and each clade is represented with a different color. Gray Fox amdovirus (sequence from GenBank Accession No. JN202450) was included in the analysis as an outgroup.
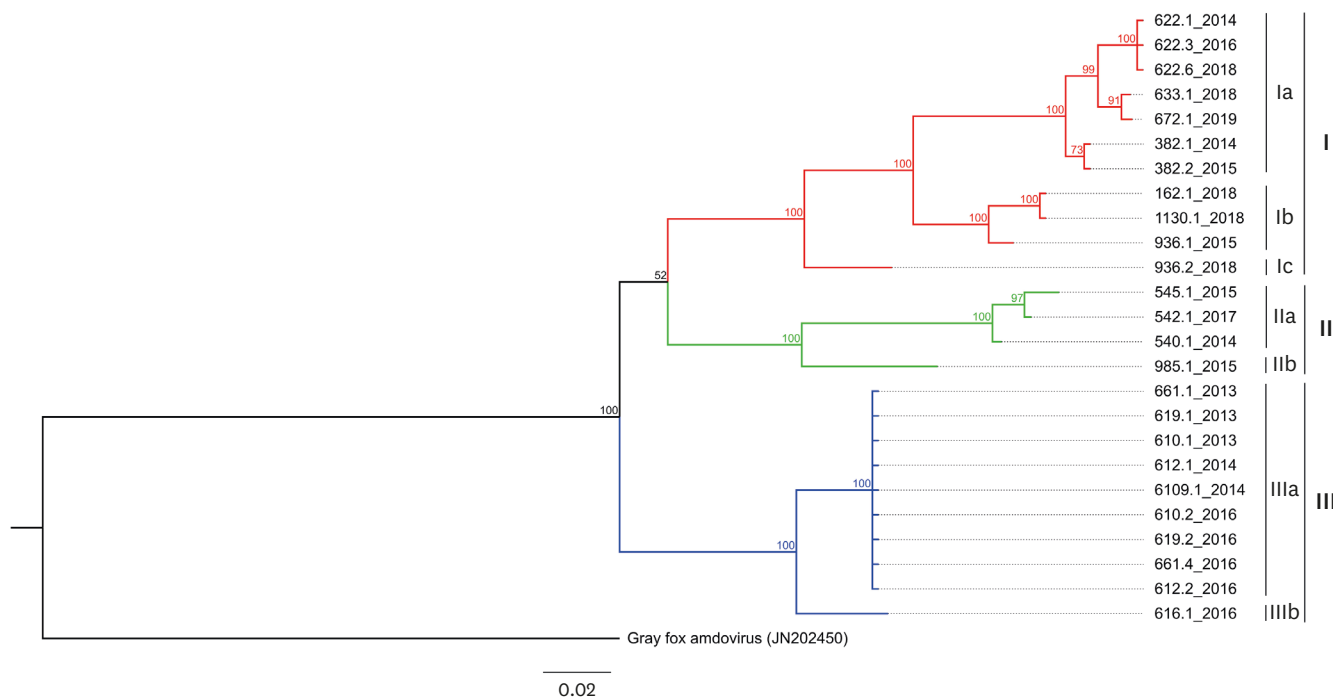AMDV, Aleutian mink disease virus.
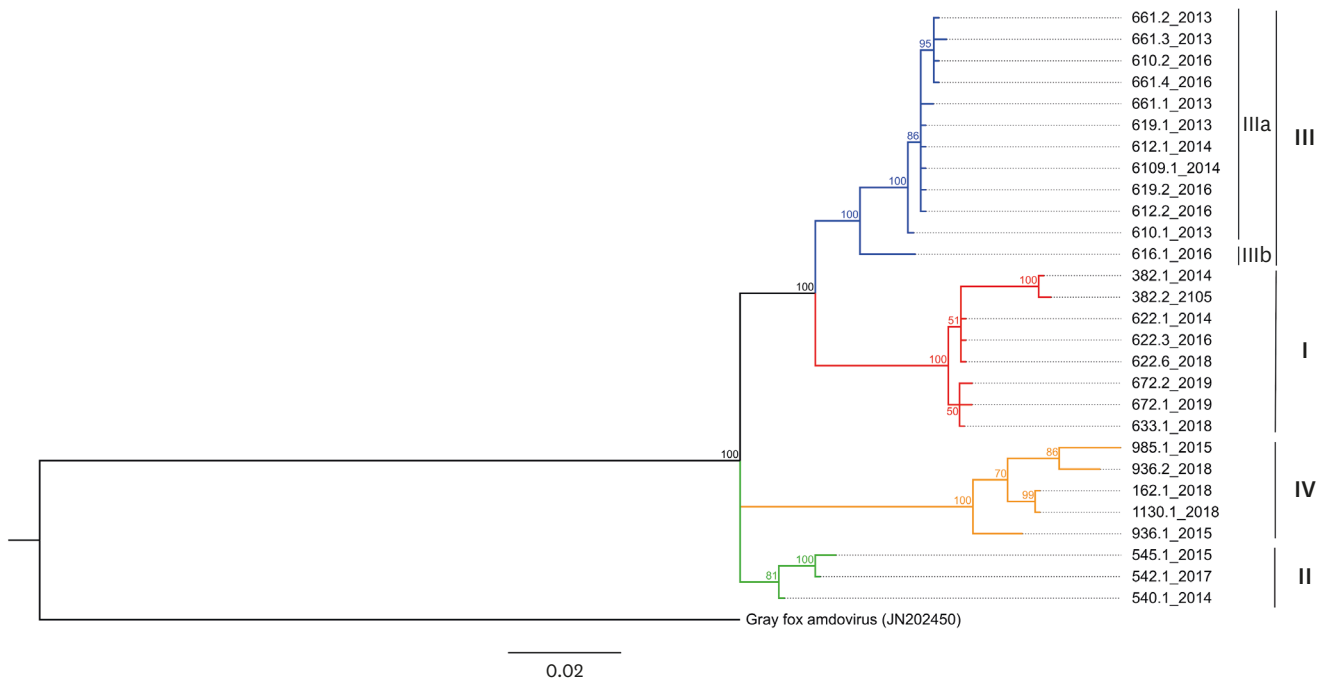
Fig. 2. Bayesian phylogenetic tree based on the VP2 sequences of AMDV obtained in this study. Posterior probabilities (%) are shown next to the nodes. Sequences are called "Farm ID.Sample number_Year of collection," and each clade is represented with a different color. Gray Fox amdovirus (sequence from GenBank Accession No. JN202450) was included in the analysis as an outgroup.
AMDV, Aleutian mink disease virus.

Fig. 3. Bayesian phylogenetic tree based on the concatenated NS1 and VP2 sequences of AMDV obtained in this study. Posterior probabilities (%) are shown next to the nodes. Sequences are named as "Farm ID.Sample number_Year of collection," and each clade is represented with a different color. Gray Fox amdovirus (sequence from GenBank Accession No. JN202450) was included in the analysis as an outgroup.
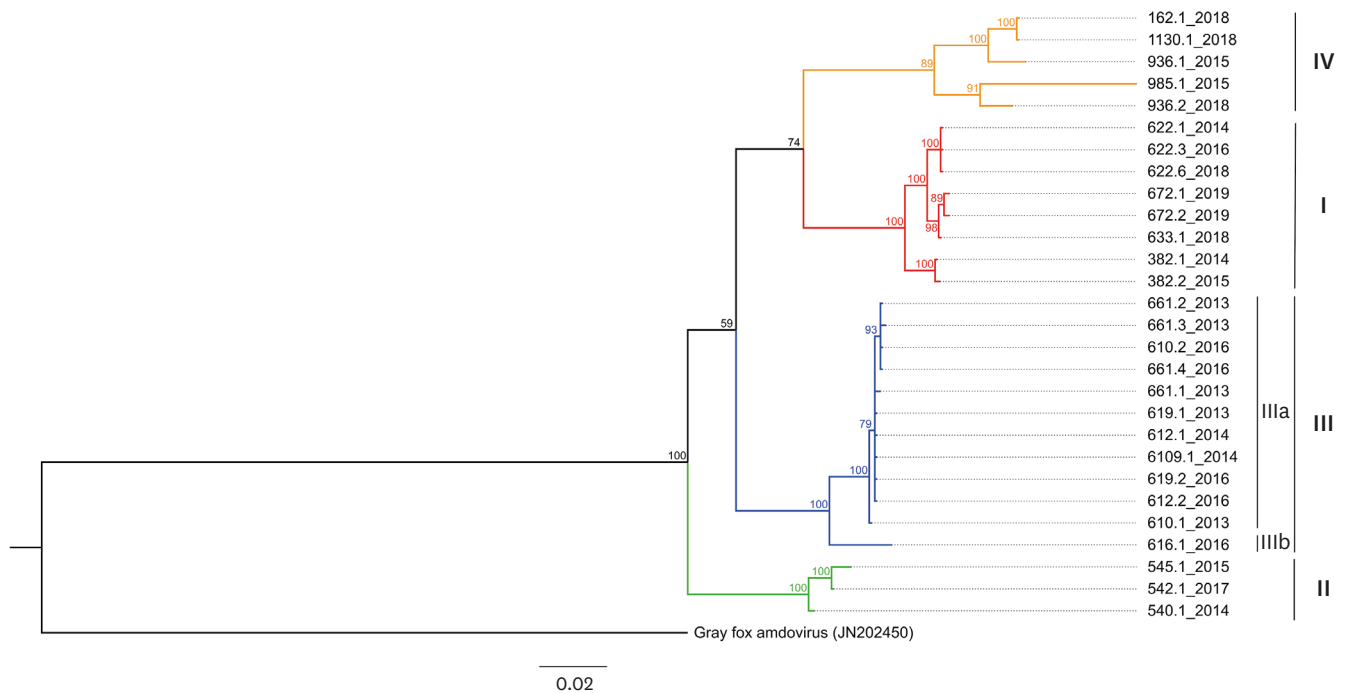AMDV, Aleutian mink disease virus.

trees showed high concordance with the geographical distribution of the farms, particularly for clades II, III, and IV (**Fig. 4A**). Therefore, the sequences from clades III and IV were only detected in NW Spain and NW Portugal. On the other hand, there was a clear geographical division between both clades because clade III sequences only appeared in farms from NW Galicia (**Fig. 4B**). In contrast, the clade IV sequences were detected exclusively in SW Galicia, forming a spatial cluster with the Portuguese farms (**Fig. 4C**). Similarly, the clade II strains were only described in the center of Spain. Finally, the clade I sequences also exhibited geographical clustering in NW Galicia (**Fig. 4B**), except for those from the French farm.

Regarding the phylogenetic analysis of the two global datasets of the NS1 and VP2 sequences, the best substitution model in both cases was the GTR with gamma-distributed rate variation across the sites and a proportion of invariable sites (GTR+G+I). The two trees are presented as Supplementary Files 3 and 4 because of their large size.

The global NS1 phylogenetic tree (**Supplementary Fig. 1**) illustrates the distribution of the Spanish strains regarding those published previously. The sequences from clade III of this
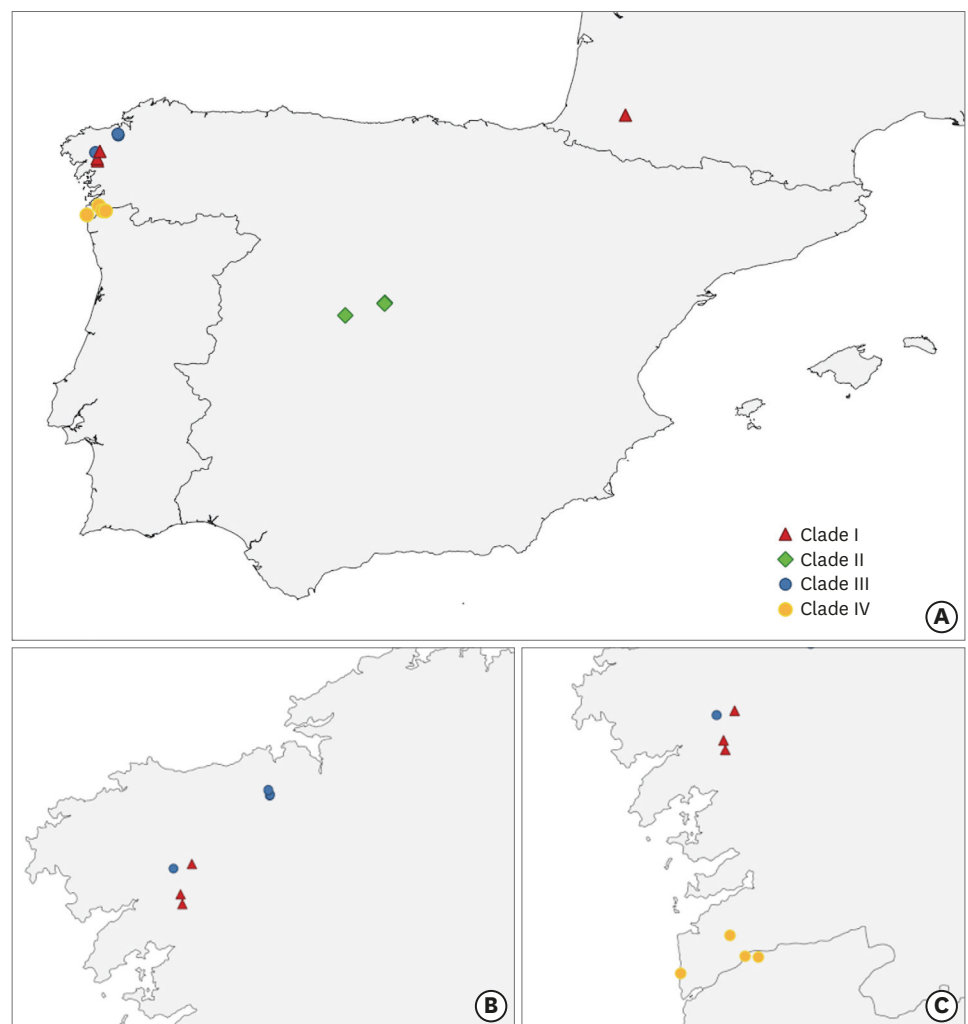

Fig. 4. Geographical location of the farms included in the study, with different symbols and colors for each of the clades identified in the phylogenetic analysis of concatenated fragments (NS1 and VP2).

study were grouped with the only three Spanish sequences previously described [23], without a strong relationship with the strains from other countries. The clade II sequences clustered mainly with the strains from Poland, Italy, Greece, and Sweden. On the other hand, clade I appeared fragmented. The subclade Ia sequences clustered with sequences from Poland, The Netherlands, and Greece, whereas the sequences of subclades Ib and Ic were grouped in a large cluster with strains from all over the world.

The global phylogenetic tree for the VP2 sequences (**Supplementary Fig. 2**) showed that the sequences of the Spanish clades II and III were quite conserved, and did not have a close relationship with those reported in other countries. In contrast, the clade I and IV sequences were distributed among other worldwide sequences. In the case of clade I, the Spanish sequences mostly clustered together with sequences from Poland, Finland, Russia, and Belarus, whereas the clade IV sequences were grouped mainly with the strains from Finland and Denmark.

DISCUSSION

The Spanish mink sector is currently characterized as having a low percentage of AMDV-infected farms, due mainly to the application of a voluntary control program in most Spanish farms developed by the Galician Association of Mink Breeders. This program included annual CIEP-testing of the whole breeding stock, high standards of biosecurity, eradication strategies (test-and-removal/stamping-out), and exhaustive cleaning and disinfection protocols for AMDV outbreaks. In addition, the Spanish mink sector was the first to introduce a method for testing for the presence of AMDV in the farm environment to improve the control of AD [25,30]. Therefore, molecular characterization of the Spanish strains is necessary to elucidate any epidemiological association between them and to help complete the molecular knowledge of the AMDV strains from a global point of view. Therefore, this work presumably gathers all the AMDV strains implied in Spanish outbreaks since 2012.

In general, the phylogeny of Spanish strains showed a clear geographical relationship between the different clades, as described by other authors [1,11,17], particularly for NS1+VP2 concatenated analysis. The phylogenetic tree obtained from the NS1 target alone allowed differentiation of the main clusters. On the other hand, it did not show the same resolution as the concatenated one because some sequences from farms with no geographical, commercial or historical relationship clustered together, such as farm 985 in clade II (**Fig. 1**). This has been mentioned previously [11,15,22], indicating that an analysis of the longer fragments or even the whole genome can build better, higher-resolution phylogenetic relationships.

Overall, the farms of each clade identified in the concatenated tree not only present a geographical relationship but also share some epidemiological features (**Table 2**). The sequences from clade I belong to three farms in NW Galicia and one in SW France. Farm 622 suffered an epizootic outbreak in 2014, which spread rapidly within the farm, possibly boosted by the fact that part of the breeding stock was Aleutian mink. Since then, this farm only bred non-Aleutian genotypes, performing an intensive selection of AMDV-tolerant breeding stock and routine cleaning and disinfection procedures. This exhaustive control program could explain why all sequences detected from this farm in the period 2014–2018 were 100% identical because, as suggested previously, the genetic stability may be the result of a bottleneck imposed by the selection pressure [1]. This farm introduced animals and farm

Table 2. Clades and subclades obtained in the phylogenetic analysis of the concatenated fragments (NS1 and VP2), farms in each clade/subclade, and observations about the farms of each clade/subclade

Clade	Subclade	Included farms	Observations
I		622	Near Santiago de Compostela except for farm 382 (Southwest of France). All these outbreaks are recent infections. 633 and 672 belong to the same owner.
		633	
		672	
		382	
II		540	Central area of Spain (Madrid and Ávila provinces). Old and recent infections.
		542	
		545	
III	IIIa	661	Cluster of farms in the "Monte Xalo" area with a maximum distance of 2.5 km between them (infection started in 2012 and still active).
		610	
		612	
		619	
		6109	
	IIIb	616	50 km away from the farms of subclade IIIa, but historically and currently related to them.
IV		162	Southwest of Galicia and adjacent border area of Portugal. 162 and 1130 belong to the same owner.
		1130	
		985	
		936 (outbreaks of 2015 and 2018)	

supplies from other European countries in 2014. Therefore, one possible explanation for the introduction of AMDV would be international trade, a route that has also been suggested by other authors [15,16,24]. This hypothesis is supported by global phylogenetic analysis, where the sequences from this farm were related more to other contemporary sequences described in Poland, The Netherlands, and Greece (**Supplementary Figs. 1** and **2**) than to other Spanish sequences. Similar to farm 622, farm 633 experienced an epizootic outbreak that was detected at the beginning of 2018 in a barn housing Aleutian mink. In this case, however, the infection was cleared after a stamping-out strategy and thorough cleaning and disinfection. The introduction route of AMDV in this farm is unclear, even though two hypotheses have been proposed. On one hand, the infection could have its origin in farm 622 because of its proximity, but the homology between both strains is not extremely high (**Supplementary Tables 3** and **4**). On the other hand, farm 633 purchased farm equipment from other European countries in 2017. Hence, AMDV might have been introduced through international trade, as suggested by the global phylogenetic analysis (**Supplementary Figs. 1** and **2**).

In contrast, farm 672 suffered a sporadic outbreak in 2018, which was restricted to a batch of animals moved from farm 633 immediately before the latter's outbreak was detected. This is probably because there were no Aleutian genotypes on this farm; the infection is currently under control by test-and-removal. Therefore, together with the high identity between the sequences of both farms (≈100%), the introduction of AMDV-infected animals is the most likely explanation for the infection of farm 672. Regarding farm 382, although the detected sequences are also related to other European sequences, the lack of epidemiological data did not indicate the origin of the infection.

Regarding clade II, all sequences belong to three farms in the center of Spain, which have remained endemically infected for decades. From an epidemiological and historical point of view, these farms were related to the founding farms established in this region in the 1950s [31], and they remained practically isolated from the rest of the Spanish sector. Hence, these strains may represent the old ones from this area. This could explain why these sequences appear to be the most conserved ones in this study, even though similar NS1 sequences have recently been described in Poland [23] (**Supplementary Fig. 1**). Considering the high similarity

between the strains of this clade and the extreme resistance of AMDV to environmental conditions, the occasional appearance of outbreaks in farm 545 might be related to the maintenance of within-farm environmental reservoirs of these old strains [19,25].

Similarly, the sequences from clade III also showed a high degree of intraclade identity, particularly for those of subclade IIIa (**Table 2**). The time sequence of the outbreaks indicates that the epizootic outbreak of farm 661 in 2012 was possibly the first, which then spread among neighboring farms because of their location and interrelation. Because these strains appear to be quite conserved (**Supplementary Figs. 1** and **2**), the initial outbreak in farm 661 may have been caused by the existence of AMDV environmental reservoirs, considering that this farm had suffered several sporadic outbreaks from 2000 to 2008. On the other hand, farm 616 (subclade IIIb) represents a particular case because it only presented a sporadic outbreak in 2016 with a strain related to subclade IIIa. Therefore, this strain might be an ancestor of subclade IIIa, representing the old strains that existed in this region since the establishment of these farms. On the other hand, the lack of samples preceding 2013 only allows a supposition.

Finally, clade IV was the most heterogeneous cluster in this study, but all sequences belonged to four farms in the same geographical area (**Fig. 4B**). Most farms from this region were founded in the 1960s. Therefore, stable populations of wild American mink have been present in this area since the 1980s due to farm escapees [32,33], and have recently shown a high AMDV seroprevalence (47%) [34]. Therefore, the differences found among these sequences could be related to the variation or recombination of old strains in this region, as previously suggested [35]. This may explain why the NS1 and VP2 sequences from farm 985 grouped differently in the phylogenetic trees, or why the strains obtained from farm 936 in 2015 and 2018 presented moderate homology despite their phylogenetic relation. On the other hand, the sequences of this clade also appear to be related to recent sequences described in other countries (**Supplementary Figs. 1** and **2**), so the introduction of the AMDV from a foreign source cannot be ruled out.

One limitation of this study was the lack of samples from Spanish wild fauna, which impeded an analysis of their possible role in the establishment of new infections. Unfortunately, only one VP2 sequence has been described [7], showing an scarce relationship with the sequences of this study (90.9–96.5% identity) (**Supplementary Fig. 2**). Therefore, further studies, including AMDV strains of wild mink and other susceptible species, will be necessary. Nevertheless, this study drafted a molecular map of the Spanish AMDV strains and is the first description of Portuguese and French sequences. This will help enhance the global knowledge of the molecular diversity of this virus. These results indicate a moderate molecular diversity of AMDV in Spain. They suggest that most strains were established in this country a long time ago and subsequently spread between farms within the same geographical area. Nevertheless, a few of them are presumably of recent introduction. The use of the NS1+VP2 concatenated genes showed a better correlation with the epidemiological data collected at the farms than using a single target. In this sense, the results show a robust geographical relationship for each of the four identified clusters. This indicates that the exhaustive control program developed by the breeders' associations together with the publication of the AD status of the farms, commercial restrictions, and biosecurity measures have made it possible to limit AMDV infections to a few located areas. Therefore, the use of phylogenetic inference combined with epidemiological information, has helped improve understanding of the routes of infection and their prevention. Overall, this method will be a helpful tool for the control of this disease.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIALS

Supplementary Table 1
List of previously described NS1 sequences (obtained from NCBI GenBank database) used for the global phylogenetic analyses

Click here to view

Supplementary Table 2
List of previously described VP2 sequences (obtained from NCBI GenBank database) used for the global phylogenetic analyses

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Supplementary Table 3
Identity matrix for the NS1 sequences obtained in this study

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Supplementary Table 4
Identity matrix for the VP2 sequences obtained in this study

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Supplementary Fig. 1
Global Bayesian phylogenetic tree based on the NS1 sequences obtained in this study and the NS1 sequences described worldwide (n = 255). Posterior probabilities (%) are shown next to the nodes. Sequences from this study are named as "Farm ID.Sample number_Year of collection", and each clade is represented with a different colour.

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Supplementary Fig. 2
Global Bayesian phylogenetic tree based on the VP2 sequences obtained in this study and the VP2 sequences described worldwide (n = 78). Posterior probabilities (%) are shown next to the nodes. Sequences from this study are named as "Farm ID.Sample number_Year of collection", and each clade is represented with a different colour.

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