Genetic variability and phylogenetic analysis of *Lagovirus europaeus* strains GI.1 (RHDV) and GI.2 (RHDV2) based on the RNA-dependent RNA polymerase (RdRp) coding gene

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Lagovirus europaeus GI.1 (RHDV-rabbit haemorrhagic disease virus) and GI.2 (RHDV2-rabbit haemorrhagic disease virus 2), family *Caliciviridae*, genus *Lagovirus*, are etiological factors of the rabbit haemorrhagic disease (RHD). This small RNA virus is a great model for tracking the variability and evolution of RNA viruses, because it uses an RNA-dependent RNA polymerase (RdRp) to replicate its own genetic material. This polymerase determines the fidelity and the rates of replication and mutation of the virus, conditioning its adaptation to the environment and even to a new host, and thus influencing evolution of the virus. The aim of this study was to determine the genetic variability and phylogenetic relationships of 105 *Lagovirus europaeus* strains with different genotypes based on the RdRp gene. The strains came from around the world in the years of 1987–2017. The aforementioned group of 105 strains included 14 strains whose RdRp sequences were obtained and analysed in this study, and the rest were retrieved from GenBank: 74 strains classified as genotype GI.1 (RHDV), 14 as GI.2 (RHDV2), 2 strains of *Lagovirus europaeus* not assigned to any genotype, and a MRCV strain, the sequences of which were collected from GenBank. Among the 14 strains whose RdRp sequences were obtained in this study, the highest variability was presented in the Austrian 237 strain from 2004. The genetic distance between the Austrian 237 strain and the remaining thirteen analysed strains ranged from 0.117 to 0.123 (from 11.7% to 12.3% nucleotide substitutions). The lowest variability, however, was recorded for Hungarian, Czech and Austrian strains. On the phylogenetic tree, the 14 analysed strains were allocated into GI.1c (G2), GI.1d (G3-G5) and GI.1a (RHDVa). Analysis of the genetic variability of the 105 strains of *Lagovirus europaeus* indicated a growing genetic distance between the strains, both in time and location. Phylogenetic analysis showed a division of the strains into seven groups, dictated by the chronology, geographical location and evolutionary events in the history of the virus, such as mutations and recombinations.

Key words: RdRp, *Lagovirus europaeus*, RHDV, RHDV2, rabbit, variability, phylogenetic analysis

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INTRODUCTION

Lagovirus europaeus genotype GI.1 (RHDV) and GI.2 (RHDV2/b) are etiologic factors of Rabbit Haemorrhagic Disease (RHD), belonging to the *Caliciviridae* family, *Lagovirus* genus. The disease was recorded for the first time in 1984 in China, in the Jiangsu Province, in rabbits imported from Germany (Liu *et al.*, 1984). In less than a year, the disease had spread across China, causing rabbit deaths on a massive scale (Liu *et al.*, 1984). In Europe, the disease was first reported in Italy in 1986; with further occurrences recorded in Czechoslovakia in 1987, in Germany in 1988, in France and Spain in 1989, and at the turn of 1988 in Poland (Hukowska-Szematowicz *et al.*, 2013). The RHDV strains isolated in the earliest period of RHDV occurrence in Europe (1987–1989) were characterised with high homology and low genetic variability among one another, and are referred to as the reference strains, and include the Czech V351 strain from 1987, as well as strains from 1989: German FRG, Spanish AST89, French SD, and Italian BS89. In 1996, the first case of RHDV was recorded (Capucci *et al.*, 1998), which was different from classic RHDV in the aspect of antigenicity, genetic variability and phylogenetic analysis of the gene encoding structural protein VP60, referred to as RHDVa (Capucci *et al.*, 1998; Kerr *et al.*, 2009; Kinnear & Linde, 2010; Fitzner & Niedbalski, 2017). It was also evidenced that RHDVa strains are characterised with higher virulence and mortality rates in rabbits (Capucci *et al.*, 1998). Continuous monitoring points out that in some areas, RHDVa seems to replace RHDV, as is currently being observed in Australia (Mahar *et al.*, 2018b). Phylogenetic surveys have classified the RHDV strains into six genogroups (G1-G6) (Le Gall-Recule *et al.*, 2003), while recently, they have been allocated into four variants of *Lagovirus europaeus* GI.1 (RHDV): GI.1a (previously G6/RHDVa), GI.1b (previously G1), GI.1c (previously G2), GI.1d (previously G3-G5), according to a newly proposed nomenclature (Le Pendu *et al.*, 2017).

A breakthrough in RHDV evolution involved the occurrence in 2010 of a new genotype in France (Le Gall-Recule & Zwingelstein, 2011) which, within the framework of the newly proposed nomenclature, was referred to as *Lagovirus europaeus*-GI.2 (RHDV2/b) (Le Pendu *et al.*, 2017). RHDV2 has spread to other countries: Italy (Le Gall-Recule *et al.*, 2013), Spain (Dalton *et al.*, 2012), Portugal (Abrantes *et al.*, 2013), United Kingdom (Westcott *et al.*, 2014), Scotland (Baily *et al.*, 2014), the Azores

(Duarte *et al.*, 2015), Australia (Hall *et al.*, 2015), Macaronesia (Lopes *et al.*, 2018), Poland (Fitzner & Niedbalski, 2018) and Tunisia (Rahali *et al.*, 2019). Continuous monitoring suggests that in many areas it replaces the older locally circulating strains, such as GI.1b (G1), G1.1c (G2), GI.1d (G3-G5), and GI.1a (RHDVa) (Le Gall-Recule *et al.*, 2013; Calvete *et al.*, 2014; Dalton *et al.*, 2014; Lopes *et al.*, 2015a; Mahar *et al.*, 2018a; Rouco *et al.*, 2018). RHDV2 shows greater genetic variability than between RHDVa and classic RHDV, with a similarity of nucleotide sequence of the VP60 gene between wild-type RHDV2 and RHDV of approximately 82% (Dalton *et al.*, 2012; Le Gall-Recule *et al.*, 2013). Studies on RHDV pathogenesis caused by RHDV2 have revealed that the disease lasts longer, with the virus having lower virulence and causing lower mortality than recorded after infection with RHDV. Furthermore, RHDV2 can cause infection not only in young rabbits, including those aged 11 days (Dalton *et al.*, 2012), which used to be considered resistant to the old RHDV genotypes; but also in vaccinated rabbits (Le Gall-Recule *et al.*, 2013), which has also been confirmed in Poland (Fitzner & Niedbalski, 2018). It was also evidenced that RHDV2 causes diseases in other lagomorph species, including the Sardinian Cape hare (*Lepus capensis mediterraneus*) (Puggioni *et al.*, 2013), Corsican (Italian) hare (*Lepus corsicanus*) (Camarda *et al.*, 2014), Iberian hare (*Lepus granatensis*) (Lopes *et al.*, 2014) and European hare (*Lepus europaeus*) (Hall *et al.*, 2017), and recently even in the mountain hare (*Lepus timidus*) (Neimanis *et al.*, 2018) and hares in England (Bell *et al.*, 2019), unlike RHDV and RHDVa, which are believed to be characteristic for the European rabbit (*Oryctolagus cuniculus*). The latest case of infection is the sixth *Lepus* species to be infected with RHDV2, which suggests that the susceptibility to RHDV2 can be widespread among the *Lepus* species (Neimanis *et al.*, 2018; Bell *et al.*, 2019). Holms and Grenfell (Holms & Grenfell, 2009) explain that the occurrence of new variants of the virus in the wild is the result of four factors: (1) adaptive evolution of the host's genes that engage in the most thorough interaction with the host's immune response, and react at the "host-pathogen" level, (2) interaction among the locally circulating viruses, (3) possible outbreak dynamics in time and space, and (4) disease spread control methods. Hypothetically, it can be assumed that in the case of RHDV, we are dealing with all four factors.

Since the isolation of RHDV more than 30 years ago, the genetic variability and evolution of RHDV have shown a specificity in the RdRp enzyme used by the virus to replicate its own genetic material and to express its genome. The enzyme has no corrective ability to repair the many errors generated during the replication process (Chen *et al.*, 2003). RdRp determines the fidelity, as well as the speed of replication and mutation, and conditions its adaptation to the environment and even to a new host (Chen *et al.*, 2003), thus affecting the evolution of the virus (Smertina *et al.*, 2019). It is also for the above reasons that RdRp may prove to be a more appropriate molecular target to track variability and phylogeny than the capsid protein (Koonim, 1991), as for example, the new RHDV2 genotypes reveal an increasing variability in their genome, which is due to recombination at the edge of structural and non-structural fragments, including RdRp (Lopes *et al.*, 2018). To date, there has been no research carried out on the variability and phylogeny of RHDV (including RHDV, RHDVa, and RHDV2) based on RdRp. With the emergence of new RHDVa strains in Australia (Mahar *et al.*, 2018b) and RHDV2 in the Macronesian Islands (Lopes *et al.*, 2018) and Poland

(Fitzner & Niedbalski, 2018), and due to the fact that RHDV2 has extended its infectious spectrum to hares, it seems crucial to monitor the variability of this virus for epidemiological reasons.

This study aimed at assessing genetic variability and performing a phylogenetic analysis of a total of 105 strains of *Lagovirus europaeus,* with RHDV and RHDV2 genotypes originating from around the world in the period of 1987–2017, pursuant to the nucleotide sequence of the RdRp coding gene. The aforementioned group included 14 strains whose RdRp sequences were obtained and analysed in this study (deposited by the author in GenBank), and other strains whose RdRp sequences were retrieved from GenBank: 74 strains classified as genotype GI.1 (RHDV), 14 as GI.2 (RHDV2), 2 strains of *Lagovirus europaeus* not assigned to any genotype, and a MRCV strain, the sequences of which were collected from GenBank.

MATERIALS AND METHODS

***Lagovirus europaeus* GI.1 (RHDV) strains.** The research included 14 strains of *Lagovirus europaeus*, which according to the newly proposed classification and nomenclature system for *Lagoviruses*, are classified in group GI.1 (RHDV) (Table 1). All the strains originated from research centres in Europe, namely the Central Veterinary Institute, Institute of Debrecen, Hungary; the Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, Czech Republic; Friedrich-Loeffler Institute, Institut für Virusdiagnostik, Greifswald, Germany. RHDV strains used for the genetic studies were prepared in the form of a suspension in glycerol.

Isolation of viral RNA. Total RNA from fourteen RHDV strains were isolated from the suspension in glycerol using a High Pure Viral RNA Kit (Roche Diagnostic, Switzerland) according to the manufacturer's recommendations. RNA obtained in this manner was directly used for a reverse transcription reaction, or stored at −80ºC for further analysis.

Reverse transcription (RT) reaction – cDNA synthesis, PCR reaction and primers. A complementary strand of the nucleic acid (cDNA) of the gene encoding RdRp from 14 RHDV strains was obtained in the reverse transcription reaction on a matrix of viral RNA using the Reverse Transcriptase enzyme (RevertAid H Minus M-MuLV Reverse Transcriptase) (Fermentas, Lithuania). The reaction was performed using a reverse transcription kit – First Stand cDNA Synthesis Kit (Fermentas, Lithuania) with the following components: Random Hexamer primer at a concentration of 100 µM, dNTPs at10 mM, RevertAid H Minus M-MuLV Reverse Transcriptase-200U/µl, 5xReaction Buffer, RiboLock RNase inhibitor and water for molecular biology. For the reaction, RNA of the relevant strains (24V,118V, 94VM, V411, 1600VM, 1042, V562, V560, 894V, 948V, 1447V, Wika, 72V, 237) (Table 1) was used. Before the reaction mixture was prepared, RNA of the 14 analysed RHDV strains was heated for 5 minutes at 65°C, and then stored on ice until the mixture was prepared. The RT reaction was performed in a total volume of 20 µl and included: 1.0 µl of Random Hexamer primer, 6.0 µl of water for molecular biology, 4.0 µl RT buffer, 1.0 µl RiboLock Rnase inhibitor, 2.0 µl dNTPs, 1.0 µl RevertAid H Minus M-MuLV Reverse Transcriptase, and 5.0 µl RNA of the relevant strain (Table 1). The RT reaction was conducted in a T-gradient Thermocycler (Biometria, Germany) using the following temperature-

Table 1. *Lagovirus europaeus* – GI. 1 (RHDV) strains evaluated in this study and subjected to analysis of genetic variability and phylogenetic analysis.

No.	GI.1 (RHDV) strains Country of origin/Year of identification	Host	GenBank accession number
1.	24V_Hungary1989	*Oryctolagus cuniculus*	BankIt2247194/MN218422
2.	118V_Hungary1989	*Oryctolagus cuniculus*	BankIt2247194/MN218423
3.	94VM_Hungary1989	*Oryctolagus cuniculus*	BankIt2247194/MN218424
4.	V411_Austria1989	*Oryctolagus cuniculus*	BankIt2247194/MN218425
5.	1600VM_Hungary1990	*Oryctolagus cuniculus*	BankIt2247194/MN218426
6.	1042_Hungary1992	*Oryctolagus cuniculus*	BankIt2247194/MN218427
7.	V562_CzechR1992	*Oryctolagus cuniculus*	BankIt2247194/MN218428
8.	V560_CzechR1993	*Oryctolagus cuniculus*	BankIt2247194/MN218429
9.	894V_Hungary1994	*Oryctolagus cuniculus*	BankIt2247194/MN218430
10.	948V_Hungary1994	*Oryctolagus cuniculus*	BankIt2247194/MN218431
11.	1447V_Hungary1996	*Oryctolagus cuniculus*	BankIt2247194/MN218432
12.	Wika_Germany1996	*Oryctolagus cuniculus*	BankIt2247194/MN218433
13.	72V_Hungary2003	*Oryctolagus cuniculus*	BankIt2247194/MN218434
14.	237_Austria2004	*Oryctolagus cuniculus*	BankIt2247194/MN218435

Due to the long strain names in the text, only the first part of the strain name was given. The sequences have been reported to GenBank and accepted, received accession numbers and will be made available in a public database at the time of publication.

time profile: 25°C – 5 minutes, 42°C – 60 minutes, 70°C – 5 minutes and 4°C – 1 minute. cDNA samples were stored at 2–8°C until further analyses. The PCR reaction was prepared in a 25 µl mixture containing: 2.0 µl of the primer pair (1.0 µl each primer: RHDV _RTFor and RHDV _RTRev) (Sigma Aldrich, Germany), 1.0 µl dNTPs (Fermentas, Lithuania), 2.5 µl Taq Plus buffer (Genoplast, Poland), 0.5 µl Taq plus DNA polymerase (Genoplast, Poland), 18.0 µl of water for molecular biology (Eppendorf, Germany), and 1.0 µl cDNA of the relevant RHDV strain (Table 1) added as the last component in the reaction mixture. The PCR reaction was performed in a Mastercycler proS Thermocycler (Eppendorf, Germany), applying the following amplification conditions: preliminary denaturation – 94°C – 3 min, and then 35 cycles comprised consecutively of: denaturation (94°C – 30 s), primer annealing (63°C – 1 min), chain elongation (72°C – 2 min); final elongation (72°C – 8 min), and cooling of the reaction mixture to 4°C. Reaction products were stored at 4°C until further analyses. Primers were designed on the basis of comparison of the nucleotide sequence encoding RdRp of the FRG RHDV strain (M67473) with the sequence of RHDV homologues. For amplification of the RdRp coding sequences (positioned in the genome at 3750-5350), the following primers were used: forward RHDV_RTFor 5'cactggcaagytgttggggtttc3', reverse RHDV_RTRev 5'gttccgggaactgatgctgtgg3'. A gene of length 1600 bp was amplified**.** Primer synthesis was performed by Sigma-Aldrich (Germany).

Electrophoresis of PCR products, preparative amplification and preparation of the RHDV genes for sequencing. For the purpose of visualisation of the PCR products, electrophoresis was performed in 2% agarose gels (Prona, USA) dyed with ethidium bromide (Fermentas, Lithuania). Molecular weight marker GeneRuler 200 bp (Fermentas, Lithuania) was used for evaluation of the size of the products. Interpretation of the electrophoretic analysis results was completed using a UV visualisation kit (UVP, Germany). After visualisation

of the PCR reaction products, a preparative PCR reaction was performed to obtain the appropriate amount of products for sequencing. Conditions and the course of the reaction were identical to the conditions described above. After visualisation of the PCR products on a gel, they were isolated (from the gel) using the Gel-Out kit (A&A Biotechnology, Poland), according to the manufacturer's protocol. The PCR products were then sent to Genomed, Poland, for sequencing.

Comparative analysis of nucleotide sequences of the gene encoding RdRp, creation of the distance matrices and the phylogenetic tree. The obtained 1284 nucleotide-long sequences of the gene encoding RdRp from the 14 RHDV strains were deposited in GenBank (Table 1). Comparative analysis of these nucleotide sequences involved a total of 105 *Lagovirus europaeus* strains (Tables 1 and 2), as generated by the MEGA 5.0 software, permitting genetic variability to be determined by generation of a distance matrix. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al.*, 2004) with the MEGA 5.0 software (Kumar *et al.*, 2018). Variability within the nucleotide and amino acid sequences of RdRp was determined against a reference sequence of the V351 strain (U54983). Supplementary Table S1 and Table S2 (at https://ojs.ptbioch.edu.pl/index.php/abp/) show this variability also with respect to other reference strains from 1989: FRG (M67473), AST89 (Z49271), SD (Z295514), BS89 (X87607). The distance matrix shows the genetic distances for all sequence pairs within the range of 0.000 to 1.000. Genetic distance (manifesting genetic variability) between the sequences of a pair of strains corresponds to the number of nucleotide substitutions (expressed as a percentage) which occurred in such sequences since their divergence from the output sequence. For a clear presentation of the test results, genetic variability is expressed as percentage. Distance matrix tables (Supplementary Material – Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/) show the values of nucleotide substitutions, which correspond to the percentage of genetic variation.

Table 2. *Lagovirus europaeus* strains obtained from GenBank and used for the genetic variability and phylogenetic analyses.

No.	*Lagovirus europaeus* genotype	Strain, origin and year of identification	Host	GenBank accession number
1.		V-351_CzechR1987	*Oryctolagus cuniculus*	U54983
2.		KGM_Poland 1988	*Oryctolagus cuniculus*	KP144790
3.		PD_Poland1989	*Oryctolagus cuniculus*	KP144789
4.		FRG_Germany1989	*Oryctolagus cuniculus*	M67473
5.		BS89_Italy1989	*Oryctolagus cuniculus*	X87607
6.		SD_France1989	*Oryctolagus cuniculus*	Z29514
7.		AST89_Spain1989	*Oryctolagus cuniculus*	Z49271
8.		Eisenhuttenstadt_Germny1989	*Oryctolagus cuniculus*	EF558578
9.		Mexico89_Mexico1989	*Oryctolagus cuniculus*	AF295785
10.		Italy90_Italy1990	*Oryctolagus cuniculus*	EU003579
11.		Korea90_Korea1990	*Oryctolagus cuniculus*	EU003580
12.		Ascot_UnitedKingdom1992	Rabbit	EF558575
13.		Meiningen_Germany1993	*Oryctolagus cuniculus*	EF558577
14.		Jena_Germany1993	*Oryctolagus cuniculus*	EF558576
15.		MAŁ_Poland1994	*Oryctolagus cuniculus*	KU882093
16.		BLA_Poland1994	*Oryctolagus cuniculus*	KP144792
17.		CB137_Pt_PortugalAlpiarca1995	*Oryctolagus cuniculus algirus*	JX886002
18.		Saudia Arabia_SaudiaArabia1996	*Oryctolagus cuniculus*	DQ189078
19.		Frankfurt5_Germany1996	Rabbit	EF558573
20.		Frankfurt12_Germany1996	Rabbit	EF558572
21.		Hartmannsdorf_Germany1996	Rabbit	EF558586
22.		Triptis_Germany1996	Rabbit	EF558583
23.	GI.1 (RHDV)	JXCHA97_China1997	Rabbit	DQ205345
24.		GSK_Poland1998	*Oryctolagus cuniculus*	KU882092
25.		Lagovirus europaeus_France2000	*Oryctolagus cuniculus*	MH190418.1
26.		Daschwald_Germany2000	Rabbit	EF558582
27.		Erfurt_Germany2000	Rabbit	EF558581
28.		Iowa_USA2000	Rabbit	AF258618
29.		ZD0_Poland2000	*Oryctolagus cuniculus*	KU882095
30.		Bahrain_Bahrain2001	Rabbit	DQ189077
31.		NY01_USA2001	Rabbit	EU003581
32.		UT01_USA2001	Rabbit	EU003582
33.		Rossi_Germany2002	Rabbit	EF558584
34.		Whnrh_China2002	Rabbit	DQ280493
35.		Hokkaido_Japan2002	Rabbit	AB300693
36.		OPO_Poland2004	*Oryctolagus cuniculus*	KU882094
37.		CD_China2004	Rabbit	AY523410
38.		GRZ2004_Poland2004	*Oryctolagus cuniculus*	KP144791
39.		L145_Poland2004	*Oryctolagus cuniculus*	KY679902
40.		KRY_Poland2004	*Oryctolagus cuniculus*	KY319033
41.		W147/05_Poland2005	*Oryctolagus cuniculus*	KY319035
42.		IN05_USA2005	Rabbit	EU003578
43.		HYD_China2005	Rabbit	JF412629
44.		CB194Pt_PortugalChaves2006	*Oryctolagus cuniculus algirus*	JX886001
45.		AUS/NSW/M9/2007_Australia2007	*Oryctolagus cuniculus*	KX357678

46.		AUS/NSW/M2/2007_Australia2007	*Oryctolagus cuniculus*	KX357677
47.		Sch07_China2007	Rabbit	KY171748
48.		NZ61_NewZealand2008	Rabbit	EF558580
49.		NZ54_NewZealand2008	Rabbit	EF558579
50.		AUS/SA/ORA383/2008_Australia2008	*Oryctolagus cuniculus*	KX357695
51.		AUS/ACT/PI-1/2009_Australia2009	*Oryctolagus cuniculus*	KX357672
52.		AUS/ACT/GUN1-52/2009_Australia2009	*Oryctolagus cuniculus*	KX357668
53.		AUS/ACT/GUN1-37/2009_Australia2009	*Oryctolagus cuniculus*	KX357667
54.		AUS/ACT/AIN-2/2009	*Oryctolagus cuniculus*	KX357654
55.		AUS/ACT/AIN-1/2009	*Oryctolagus cuniculus*	KX357653
56.		NJ2009	Rabbit	HM623309
57.		AUS/ACT/MtPt-4/2010	*Oryctolagus cuniculus*	KX357671
58.		AUS/ACT/MtPt-2/2010	*Oryctolagus cuniculus*	KX357670
59.		STR2012	*Oryctolagus cuniculus*	KF677011
60.		STR2-2013	*Oryctolagus cuniculus*	KY679904
61.		SKO2013	*Oryctolagus cuniculus*	KY319034
62.		GLE2013	*Oryctolagus cuniculus*	KY319032
63.		RED1-2013	*Oryctolagus cuniculus*	KY679903
64.		AUS/NSW/BER1/2013/12_Australia2013	Domestic rabbit	KY628307
65.		AUS/NSW/BER2/2013/12_Australia2013	Domestic Rabbits	KY628309
66.		AUS/NSW/BER3/2014/01_Australia2014	Domestic Rabbits	KY628310
67.		AUS/NSW/BlueGums1/2014/03_Australia2014	Wild rabbit	KT006732.1
68.		AUS/NSW/OUR1/2014/06_Australia2014	Wild rabbit	KY628318
69.		AUS/NSW/OUR2/2014/06_Australia2014	Wild rabbit	KY628319
70.		STR2014_Poland2014	*Oryctolagus cuniculus*	KY679905
71.		BIE2015_Poland2015	*Oryctolagus cuniculus*	KY319031
72.		AUS/NSW/WAL1/2015/01_Australia2015	Domestic Rabbits	KY628320
73.		HB_China2016	Rabbit	KY437668
74.		CBMad17-1_PortugalMadeiraFunchal2017	*Oryctolagus cuniculus*	MF407655
75.		RHDV-N11_SpainNavarra2011	*Oryctolagus cuniculus*	KM878681
76.		Zar11-11_Spain,2011	*Oryctolagus cuniculus*	KP129398
77.		CBVal16_ PortugalValpacos2012	*Oryctolagus cuniculus*	KM979445
78.		Rij06-12_Spain2012	*Oryctolagus cuniculus*	KP129395
79.		Seg08-12_Spain2012	*Oryctolagus cuniculus*	KP129396
80.		Tar06-12_Spain,2012	*Oryctolagus cuniculus*	KP129397
81.		Zar06-12_Spain,2012	*Oryctolagus cuniculus*	KP129399
82.	GI.2 (RHDV2)	16PLM1_SpainCanaryIslandsLa Palma2016	*Oryctolagus cuniculus*	MF407653
83.		CBMad17-2_ PortugalMadeiraPaulSerra2017	*Oryctolagus cuniculus*	MF407656
84.		CBMad173_PortugalMadeira PaulSerra2017	*Oryctolagus cuniculus*	MF407657
85.		PSM2_PortugalMadeiraPortoSanto2016	*Oryctolagus cuniculus*	MF407654
86.		CBPico17-1_PortugalAzoresPico2017	*Oryctolagus cuniculus*	MF407651
87.		CBPico17-2_ PortugalAzoresPico2017	*Oryctolagus cuniculus*	MF407652
88.		CBAlgarve14-3/2014_PortugalAlgarve2014	Rabbit	KM115715.2
89.	GI. (RCV)	MRCV	Rabbit	GQ166866
90.	Strains not assigned to any genotype	P19_PortugalPorto1994	*Oryctolagus cuniculus*	KY765610
91.		P95_PortugalToresNovas1996	*Lepus granatensis*	KJ943791

Due to the long strain names in the text, only the first part of the strain name was given.

The evolutionary history was inferred using the Maximum Likelihood method and General Time Reversible model (Nei & Kumar, 2000), the most statistically appropriate method for the selected data. A discrete Gamma distribution was used to model the evolutionary rate differences among sites. Evolutionary analyses were conducted with MEGA 5.0 (Kumar *et al.*, 2018). Reliability of phylogenetic trees were assessed using the bootstrap method (%). Distance matrices were also developed (using the method stipulated above) for particular groups of the strains generated in the phylogenetic tree in order to determine the intra-group variability. In the last phase, the nucleotide sequences of the 105 RHDV strains were translated to amino acid sequences, and a distance matrix was generated to determine their variability using the MEGA 5.0 software (Kumar *et al.*, 2018).

RESULTS

Genetic variability of *Lagovirus europaeus* strains

Nucleotide sequences of genes coding RdRp, obtained in this study from 14 *Lagovirus europaeus* strains, with the GI.1 genotype (RHDV), (Table 1), had a length of 1284 nucleotides and covered positions 3955-5238 in the viral genome, while the full RdRp coding sequence has a length of 1548 nucleotides and occupies positions 3756-5301. Genetic variation of the 14 analysed strains within RdRp indicated occurrence of many polymorphic sites with local mutations in the form of transitions (C↔T, G↔A) and transversions (G↔T, A↔T, C↔G). Transitions were more frequent, comprising over 90% of mutations, involving frequent C↔T changes (over 50%) versus G↔A. Among the 10% of transversions, all mutations occurred with a comparable frequency.

Among the 14 analysed strains (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/), the highest variability within the RdRp gene was presented in the Austrian 237 strain from 2004, which showed a genetic distance of 0.124 from the reference V351 strain from 1987 (12.4% nucleotide substitutions, Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The genetic distance between the Austrian 237 strain and the remaining thirteen strains ranged from 0.117 to 0.123 (from 11.7% to 12.3% nucleotide substitutions). A lower genetic variability was observed for the Hungarian strains from 1994 and 1996 – 1447V (8%), and 894V, 948V (7.6%); and the German Wika strains from 1996 (6.9%). The lowest variability, however, was recorded for: Hungarian strains from 1990, 1989, 1992, 2003 – 1600VM (1.1%), 24V, 118V, 94VM, 1042, 72V (1.3%); Austrian from 1989 – V411 (1.2%), and Czech from 1992 and 1993 – V562 (1.3%), V560 (1.6%). Supplementary Table S2 (at https://ojs.ptbioch.edu.pl/index.php/abp/)presents a matrix for the comparison of the predicted 428 RdRP amino acid sequences in the 105 analysed *Lagovirus europaeus* strains. The RdRp amino acid sequence obtained in this study was at positions 1316-1743, which constituted over 80% of the full RdRp amino acid sequence. Among the 14 strains analysed here, the highest genetic variability in the amino acid sequence *versus* the V351 strain, was observed for Wika (2.4%) and the 237 strain (1.9%). In the case of strains 24V89, 118V, 94VM, V411, V562, 1600VM, and 72V, variability was at 0.9%. For other strains from the 1990s, however, the variability was as follows: 1447V (1.7%), 1042 (1.4%), V560 (1.2%), 894V (1.7%), 948V (1.7%).

Analysis of the 105 *Lagovirus europaeus* strains genetic variability (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/) on the basis of the RdRp gene revealed that the strains have an increasing genetic distance, observed both in the time scale and in the geographic scale, with the highest genetic variability in the area of RdRp gene recorded for five strains: three from Australia- AUS/NSW/BER-2, AUS/NSW/BER-3, AUS/NSW/WAL-1 from 2013, 2014, 2015, one from Portugal - CBMad17-1 from 2017, and one MRCV strain. The above strains from Australia and Portugal showed a genetic distance from 36.2% to 37.8% *versus* the V351 sequence, amounting to, respectively: 36.2% for AUS/NSW/BER-2 and AUS/NSW/WAL-1, 36.3% for AUS/NSW/BER-3, 37.8% for CBMad17-1. Equally high variability in this group was recorded for MRCV: 35.4% (*vs.* V351), 34.7% (*vs.* FRG), 37.2% (*vs.* AST89), 37.9% (*vs.* AST89), and 36.9% (*vs.* BS89). Among the 105 analysed strains (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/), the highest variability (based on the RdRp amino acid sequence) was recorded in five strains, including three from Australia, one from Portugal (Madera), and the MRCV strain (12–13.0%). Assessed against the V351 sequence, the results were as follows: 12.0% for AUS/NSW/BER-2 and AUS/NSW/WAL-1; 12.5% for AUS/NSW/BER-3/2014 and 13.0% for CBMad17-1. Equally high variability was recorded for MRCV: 12.3% (*vs.* V351), 11.6% (*vs.* FRG), 11.8% (*vs.* AST89 and SD) and 12.0% (*vs.* BS89).

The high variability, not previously reported, within the RdRp gene was obtained for eight *Lagovirus europaeus* strains with the GI.2 genotype (RHDV2), isolated in 2011, 2012 and 2016, originating from Portugal and Spain (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The above strains revealed a genetic distance of 15.8% for Rij06-12, 15.9% for RHDV-N11; 16.1% for CBVal16; 16.1% for Tar06-12; 16.3% for Zar06-12; 16.3% for Zar11-11; 16.4% for Seg08-12, and 16.5% for 16PLM1. A lower genetic variability (11.1%-12.9%) of the GI.2 genotype (RHDV2) (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/) was reported for strains from Portuguese islands in 2016–2017 – 12.1% for CBMad17-3 and PSM2, 12% for CBMad17-2, 12.9% for CBPico17-2 and CBPico17-1; and one strain of CBAlgarve14-3 from 2014 from mainland Portugal – 11.1%. On the basis of the RdRp amino acid sequence (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/), the RHDV2 strains revealed a genetic distance *versus* V351 ranging from 2.8% to 3.1%: 2.8% for Rij06-12, Zar06-12, Zar11-11 and 3.1% for RHDV-N11, CBVal16, Tar06-12, Seg08-12, 16PLM1. For other Portuguese strains in this genotype, the variability ranged from 1.7% to 2.6% (1.7% for CBMad17-2 and CBAlgarve14-3/2014, 1.9% for CBMad17-3 and PSM2 and 2.6% for CBPico17-2 and CBPico17-1).

Genetic variability for two Portuguese strains, P19 from 1994 and P95 from 1996, which so far have not been assigned to any genotype, were 14.7% and 14.8%, respectively (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The lowest genetic variability among the Portuguese strains was for CB137_Pt-8.5% and CB194_Pt-10.5% (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The P95 and P19 strains showed 3.1% variability in the RdRp amino acid sequence, similarly to some RHDV2 strains. The lowest genetic variability among the Portuguese strains was observed for CB137_Pt-1.4% and CB194_Pt-2.6% (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/).

A genetic variability of 11.8–13.6% within RdRp was obtained for 28 strains from around the world from the period of 1996–2014, and classified as GI.1a (G6/RH-DVa) (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). The genetic distance *versus* the V351 sequence was as follows: 11.8% nucleotide substitutions for the Hartmannsdorf strain, 12.1% for Erfurt and Rossi, 12.2% for IN05, 12.3% for UT01, 12.4% for Hokkaido and GRZ2004, 12.5% for Triptis and Red1-2013, 12.6% for JXCha97, as well as W147_05 and NY01, 12.7% for Daschwald, 12.8% for Iowa, 12.9% for L145, as well as KRY and NJ2009, 13.0% for CD and HYD, as well as for Sch07, 13.1% for GLE2013 and STR-2-2013, 13.2% for WHNRH, 13.4% for SKO and STR2014, and 13.4% for BIE2015, 13.6% for STR2012, and 12.4% for the Austrian 237 strain analysed here. Based on the RdRp amino acid sequence, the genetic distance of the above 28 strains with respect to the V-351 sequence ranged from 2.1% to 2.6% (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/).

A lower genetic variability within RdRp was recorded for strains isolated in the 1980s, 1990s, and after 2000 (Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). Genetic distance for strains from the 1980s (including the strains analysed in this study) *versus* V351 ranged from 0.9% to 7.6%, and was as follows: 0.9% for FRG, 1.0% for KGM, 1.1% for PD, 1.2% for V411, 1.3% for 24V89, 118V, and 94VM, 2.8% for Mexico89, 6.5% for BS89, 7.0% for Eisenhuttenstadt, 7.2% for AST89 and 7.6% for SD. Strains from the 1980s showed a 0.9–1.2% variability in the RdRp amino acid sequence (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/).

Genetic distance for strains from the 1990s (including strains analysed in this study) *versus* V351 ranged from 1.0% to 8.0%, and was as follows: 1.0% for MAL, 1.1% for 1600VM, 1.3% for 1042 and V562, 1.5% for Italy90, 1.6% for V560, 2.6% for Korea-90, 4.5% for SaudiArabia, 6.9% for Wika, 6.6% for Meiningen and BLA, 6.8% for Frankfurt5 and Frankfurt12, 6.9% for Ascot, 7.6% for 894V, 948V and Jena, 7.8% for GSK, and 8.0% for 1447V. Genetic distance for strains from the 1990s versus V351, determined on the basis of the RdRp amino acid sequences, and including strains analysed in this study, ranged from 0.9% to 2.4% (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/).

Genetic distance for strains isolated after 2000 (including 14 Australian strains) *versus* V351 ranged from 1.3% to 8.4%, and was as follows: 1.3% for 72V, 2.2% for HB, 2.5% for NZ54 and NZ61, 3.4% for AUS/SA/ORA383, 3.4% for AUS/ACT/PI-1, 4.3% for AUS/NSW/M2, 4.5% for AUS/ACT/MtPt-4 and AUS/NSW/M9, 4.7% for AUS/ACT/MtPt-2, 4.8% for AUS/ACT/AIN-2 and AUS/ACT/AIN-1, 5.0% for AUS/ACT/GUN1-37 and AUS/ACT/GUN1-52, 6.0% for AUS/NSW/BlueGums1 and AUS/NSW/BER-1, 6.7% for OPO, 6.8% for AUS/NSW/OUR-1 and AUS/NSW/OUR-2, 7.8% for Lagovirus europaeus_France2000, 8.1% for ZD0, 8.4% for Bahrain. Variability in the group of the above 14 Australian strains from the period of 2007–2014 ranged from 3.4% to 6.8%. Genetic distance for strains isolated after 2000, based on the RdRp amino acid sequences, ranged from 0.9% to 1.9%. The Australian strains *versus* V351 revealed a distance of 1.4%-1.9% (Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/).

Phylogenetic analysis of the *Lagovirus europaeus* strains

The image of phylogenetic interdependencies (Fig. 1) among 105 *Lagovirus europaeus* strains, build on the basis of RdRp, has revealed a clear division of these strains into 7 groups, where the 14 strains analysed in this study were allocated into GI.1c (G2), GI.1d (G3-G5), and GI.1a (RHDVa) groups. Distance matrices generated for groups of *Lagovirus europaeus* strains (Table S3–S9 at https://ojs.ptbioch.edu.pl/index.php/abp/), isolated pursuant to the topology of the phylogenetic tree (Fig. 1), revealed that variability in RdRp within the groups (intra-group variability) is much lower than variability obtained for all 105 *Lagovirus europaeus* strains. Lower variability was caused by much lower numbers of nucleotide substitutions in such groups.

Group GI.1b (G1) included 11 strains from the period 1989–2017, namely the last 28 years, and these were the strains isolated in the first period of the disease occurrence in Europe, as well as contemporary strains from continental Portugal and the Portuguese islands. Evolutionary relations in this group clearly point to a chronological distance that has divided the strains into two subgroups. The first has been formed by RHDV from 1989-SD, AST89, Eisenhuettenstadt, with CB137_Pt from 1995 and CB194_Pt from 2006. While the latter by Portuguese strains from the period of 2014-2017: PSM2, CBMad17-3, CBMad17-2, CBPico17-2, CBPico17-1, CBAlgarve14-3. The distance matrix generated to determine intragroup genetic variability (Table S3 at https://ojs.ptbioch.edu.pl/index.php/abp/) revealed variability ranging between 0.2–9.7%. The shortest genetic distance in this group was recorded, mutually, for strains CBMad17-3 from 2017 and PSM2 from 2016 (0% nucleotide substitutions), and then for CBPico 17-1 and CBPico17-2 from 2017, and for CBMad 17-2 and CBMad 17-3 from 2017 (in both cases 0.2% nucleotide substitutions), as well as AST89 and Eisenhuettenstadt (0.9%). Portuguese strain CB137Pt showed a genetic distance to strains in its subgroup between 3.1–3.5%, and to other Portuguese strains between 6.8–9.7%.

Group GI.1c (G2) was comprised of 35 strains (including nine strains analysed in this study) from the period of 1987–2014, originating from around the world, including Australia. Evolutionary relations in this group divided the strains into three subgroups. The first subgroup included European strains phylogenetically deriving from the Czech V351 strain from 1987, which included nine strains analysed in this study, as well as the following strains: Italy 90, PD, KGM, MAL, FRG. The second subgroup included strains from New Zealand and Australia. The third subgroup included HB, Korea-90, Mexico89, and SaudiArabia (Fig. 1). Intragroup variability (Table S4 at https://ojs.ptbioch.edu.pl/index.php/abp/) ranged from 0% to 8.8%. The shortest genetic distance in this group was recorded for strains NZ54 and NZ61 (0%), 72V and 94VM (0%), V351 and FRG (0.9%). The most genetically distanced from other strains in this group were the four Australian strains from 2013 and 2014, which revealed a genetic distance of 5.7–8.8%.

Group GI.1d (G3-G5) was comprised of 16 strains (including four strains analysed in the course of this study) from the period of 1989–2004, predominantly originating from Europe, and one from Asia. Evolutionary relations in this group divided the strains into two subgroups. Subgroup one included Wika, Frankfurt15, Frankfurt12, BLA, and OPO. Subgroup two: BS89, Ascot, Meiningen, Lagovirus europaeus, Bahrain, Jena,
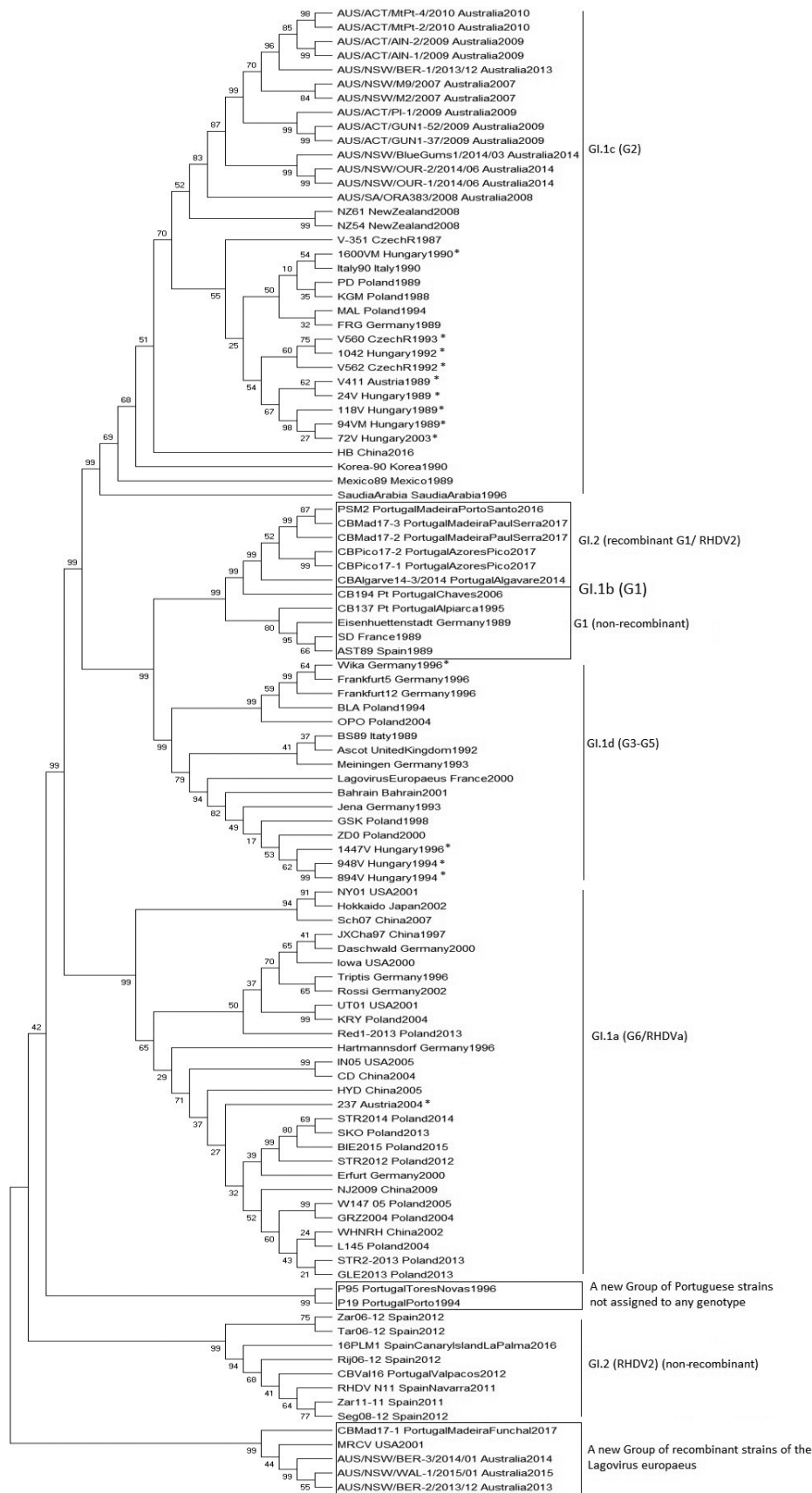
Figure 1. Phylogenetic tree of 105 strain of *Lagovirus europaeus* build on the basis of the RdRp coding gene nucleotide sequence.
The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. Evolutionary analyses were conducted with MEGA 5.0. Reliability of the phylogenetic trees was assessed using the bootstrap method (%).

GSK, ZD0, 1447, 948V, 894V. Intragroup variability (Table S5 at https://ojs.ptbioch.edu.pl/index.php/abp/) totalled 0–5.0%. The shortest genetic distance in the group was recorded for strains 894V and 984V (0%), Frankfurt5 and Frankfurt12 (0.2%), as well as Wika, Frankfurt5 and Frankfurt12 (0.2%), BLA and OPO (0.5%). The most genetically distanced were the French *Lagovirus europaeus* and Bahrain. *Lagovirus europaeus* recorded a large genetic distance *versus* BS89 (3.5%), Ascot (3.6%), Meiningen (3.7%), BLA (3.9%), Wika (4.5%), Frankfurt12 and Frankfurt 5 (4.4%). Similarly, Bahrain recorded a genetic distance *versus* BS89 (3.9%), Ascot (4.0%), Meiningen (4.0%), BLA (4.4%), Wika (5.0%), Frankfurt12 and Frankfurt 5 (4.9%).

Group GI.1a (G6/RHDVa) was comprised of 28 strains (including 1 strain analysed in this study) from the period of 1996–2015, originating from around the world. The strains in this phylogenetic group were divided into three subgroups. Subgroup one was comprised of: NY01, Hokkaido, Sch07; subgroup two: JXCha97, Daschwald, Iowa, Triptis, Rossi, UT01, KRY, RED1-2013, and subgroup three: Austrian 237, Hartmannsdorf, IN05, CD, HYD, STR2014, SKO, BIE2015, STR2012, Erfurt, NJ2009, W14705, GRZ2004, WHNRH, L145, STR2-2013, GLE2013. The location of the 237 strain in this particular group testifies to it being a GI.1a variant. Intragroup variability totalled 0.2–6.1% (Table S6 at https://ojs.ptbioch.edu.pl/index.php/abp/). The shortest genetic distance in this group was recorded for strains STR2014 and SKO (0.2%), BIE2015 and STR2014 (0.3%), SKO and STR2012 (0.5%), STR2014 and STR2012 (0.6%), Rossi and Triptis (0.9%). The most genetically distanced in this group was strain Sch07, recording a significant genetic distance from most strains: Hokkaido (3.0%), NY01 (3.7%), JXCha97 (3.8%), Triptis (4.0%), Rossi (4.3%), Iowa (4.3%), Daschwald (4.6%), Red1-2013 (5.1%), Erfurt (5.4%), UT01 (5.4%), 237 (5.5%), L145 (5.5%), KRY (5.5%), STR2-2013 (5.7%), BIE2015 (5.7%), Hartmannsdorf (5.7%), GRZ2004 (5.8%), W147_05 (5.8%), NJ2009 (5.8%), IN05 (5.9%), SKO (5.9%), WHNRH (6.0%), CD (6.0%), HYD (6.0%), STR2012 (6.0%), STR2014 (6.0%), GLE2013 (6.1%).

The image of phylogenetic relations based on RdRp clearly pointed to a new monophyletic group situated between GI.1a-1d and RHDV2, with a high statistical support (bootstrap value 99%). It was created by two strains of the Portuguese origin: P95_PortugalToresNovas1996 (host: *Lepus granatensis*) and P19_PortugalPorto1994 (host: *Oryctolagus cuniculus*), which so far have not been assigned to any genotype. The strains (Table S7 at https://ojs.ptbioch.edu.pl/index.php/abp/) revealed a genetic distance from one another of 0.017/1.7% nucleotide substitutions.

Group GI.2 (RHDV2) was formed by eight strains from the period of 2011–2016, originating from Spain and Portugal. The strains were divided into two subgroups: one from 2012: Zar06-12, Tar06-12, and the other from 2011-2016: 16PLM1, Rij0612, CBVal16, RHDVN11, Zar11-11, Seg08-12. Intragroup variability (Table S8 at https://ojs.ptbioch.edu.pl/index.php/abp/) ranged from 0.5 to 4.1%. The shortest genetic distance in this group was recorded for RHDV_N11 *versus* Zar11-11 (0.5%), Seg08-12 (0.7%), and Rij06-12 (0.9%), and mutually between CBVal16 and Rij06-12 (0.9%). The most distanced in the group was Tar06-12 from 2012, which showed a significant genetic distance *versus* Rij06-12 (3.1%), CBVal16 (3.2%), and 16PLM1 (4.1%), as well as Zar06-12 and 16PLM1 (3.6%).

The picture of phylogenetic dependencies based on the RdRp gene revealed a new group of recombinant strains of *Lagovirus europaeus* (Fig. 1), with high bootstrap support values (99%). Phylogenetic dependences in this group suggest that the Portuguese and Australian strains are associated with MRCV (chosen as the reference strain in this analysis) and the Portuguese strain is additionally associated with RHDV2. Intragroup variability (Table S9 at https://ojs.ptbioch.edu.pl/index.php/abp/) totalled 0.5–17.5% (exclusive of MRCV) and 0.5–18.3% (inclusive of MRCV). The shortest genetic distance was recorded, mutually, for Australian strains: AUS/NSW/BER-2 *versus* AUS/NSW/WAL (0.2%), AUS/NSW/BER-2 *versus* AUS/NSW/BER-3 (0.5%), as well as AUS/NSW/WAL-1 *vs.* AUS/NSW/BER-3 (0.7%). Portuguese CBMad17-1, in turn, revealed a very large genetic distance *versus* Australian strains AUS/NSW/WAL-1 (17.3%) and *versus* AUS/NSW/BER-2 and AUS/NSW/BER-3 (17.5%). It must be noted that CBMad17-1 was found to be the most genetically distanced from MRCV (18.3%), which was a greater distance than recorded in respect to the remaining strains, namely: AUS/NSW/BER-2 (16.2%), AUS/NSW/BER-3 (16.4%) and AUS/NSW/WAL-1 (16.4%).

DISCUSSION

Genetic variability of viruses depends on the frequency of mutations taking place in the genome, and external factors of the environment where the pathogens are present (Belshaw *et al.*, 2008). The core natural factor that conditions a mutation comes from errors in imprecise replication of nucleic acids by RdRp. This polymerase is incapable of repairing errors occurring during synthesis of descendant RNA strands (Steinhauer *et al.*, 1992). The scale of errors is illustrated by an example where one error occurs per one replication cycle for RNA viruses as compared to one error per 300 cycles for DNA viruses (Smertina *et al.*, 2019). Genetic variability can also result from other mechanisms, such as homological recombination between closely related RNA viruses. The pace of recombination seems to vary and be specific to each virus species, as some recombine very frequently, while others seem to be limited, showing little proof of recombination (Forrester *et al.*, 2008). The emergence of a new genotype or variant in the case of RNA viruses most frequently involves mutations within the RdRp gene or within the capsid genome, particularly the subunit encoding surface epitopes, with the new genotype being more or less virulent or more stable in the environment (Capucci *et al.*, 1998; Belshaw *et al.*, 2008; Bigoraj *et al.*, 2011). Studies on genetic variability of RHDV, among others conducted on the basis of the VP60-coding gene, revealed both – mutations and recombinations (Abrantes *et al.*, 2008; Forrester *et al.*, 2008; Hukowska-Szematowicz *et al.*, 2013; Lopes *et al.*, 2015b; Mahar *et al.*, 2016; Hu *et al.*, 2017; Lopes *et al.*, 2018). It is suggested that the rabbit plague outbreak recorded in China in 1984 was caused by recombination of the genetic material originating from the Angora rabbits imported from Germany. This variability type was also identified for three RHDV strains in the RdRp gene (Forrester *et al.*, 2008). According to some authors, such RHDV variability type as recombination will be increasingly frequent in viral evolution because the recombinants can be characterised with higher pathogenicity and different tropism than the input strains (Abrantes *et al.*, 2008; Forrester *et al.*, 2008; Lopes *et al.*, 2015b), and the phenomenon begins to play an im-

portant role in generating biological diversity of RHDV2 (Lopes *et al.*, 2015b).

Analysis of genetic variability in 105 *Lagovirus europaeus* strains, based on the RdRp gene, indicated that these strains feature an increasing genetic distance, observed both in the time scale and in geographic scale, while RHDV variability occurs both, through mutation and recombination, which is a consequence of employing the existing genetic diversity to develop new genome combinations (Lopes *et al.*, 2017). The results of the analyses point out that the highest mutation potential in the studied RdRp gene (15.8–16.5%) was recorded for the RHDV2 genotype in Portugal and Spain. The results indicate that these are non-recombinant RHDV2 strains. Smertina and others (Smertina *et al.*, 2019) speculated that RHDV2 in Australia could have acquired a relative fast polymerase, which may explain its higher virulence, and apparent evolutionary success. During 18 months from arrival, RHDV2 had largely replaced the endemic RHDV strains in Australia (Mahar *et al.*, 2018a). Therefore, replication speed may mark viral efficiency because viruses with a higher replication speed can produce more copies of its genome, resulting in a greater number of variants, even if RdRp error rate remains the same (Smertina *et al.*, 2019). Lower mutation potential within the area of the RdRp gene was observed for strains classified as GI.1a (G6) (11.8–13.6%), GI.1c (G2) (1.0–6.8%) and GI.1d (G3-G5) (6.5–8.4%). The mutation potential, however, of the Australian strains in GI.1c (G2) from the period of 2007-2014 totalled 3.4–6.8%, which points to the fact that the mutation coefficient in RdRp had increased in the Australian population of the virus. A very interesting situation was found for some strains (PSM2, CBMad17-3, CBMad17-2, CBPico17-2, CBPico17-1, CBAlgarve14-3/2014) identified in 2014, 2016 and 2017 in GI.1b (G1), which showed variability at 11.1–12.9%, similar to GI.1a (G6/RHDVa) (11.8–13.6%), but phylogenetically were not found in this group. This suggests that these are G1/RHDV2 recombinants, which is in line with the results of previous studies (Mahar *et al.*, 2016; Lopes *et al.*, 2018; Silverio *et al.*, 2018). Genetic variations of 7.0–10.5% of the remaining strains (CB194Pt, CB147 Pt, Eisenhuettenstadt, SD, AST89) in this group, supported by the results of studies by other authors (Abrantes *et al.*, 2012; Lopes *et al.*, 2017), indicate that these are the G1-non-recombinant strains, both within the studied polymerase gene and in the VP60 and VP10 genes (Lopes *et al.*, 2017). Genetic variability of 14.7% and 14.8% in the RdRp gene was observed in the case of Portuguese strains P95 and P19, which may have been the source of a new variant of polymerase which did not stabilize in the environment. On the other hand, very high genetic variability, which may indicate recombination of genetic material between rabbit viruses, was observed for CBMad-17-1 (37.8%), AUS/NSW/BER-3 (36.3%) and AUS/NSW/BER-2 and AUS/NSW/WAL-1 strains (36.2%).

The highest variability within the amino acid sequence of RdRp was recorded for recombinant strains (12.0–13.0%) and MRCV (12.3%). High variability was observed for RHDV2 genotype (2.8–3.1%) and recombinants P19 and P95 (3.1% for both strains), and then in group GI.1a (RHDVa) (2.1–2.6%), in GI.1b (G1) for strains from continental Portugal and Portuguese islands (1.4–2.6%), and for strains from the 1980s (0.9–1.2%), the 1990s (0.9–2.4%), and after 2000 (0.9–1.9%), including Australian strains (1.4% to 1.9%). However, discussions in this area seem to be problematic, given that no similar studies have been conducted.

The matrix of phylogenetic relations (Fig. 1) among the 105 *Lagovirus europaeus* strains on the basis of RdRp gene sequence has revealed a clear division of the strains into 7 groups. The allocation to the groups depended on chronology, geographic origin, and evolutionary events in the viral history, such as recombination. The location of some strains on the phylogenetic tree, supported by their variability results, testifies to them being recombinants, forming phylogenetically new groups, while recombination seems to be an increasingly frequent event in the viral evolution (Lopes *et al.*, 2018). Such a situation applies to two strains of the Portuguese origin: P95 and P19. On the phylogenetic tree, the strains are positioned between GI.1a-1d and RHDV2 and have not been assigned to any genotype. Their location on the phylogenetic tree indicates that this is a new group of recombinant strains which "for a moment" appeared in Portugal. Phylogenetic analysis by Lopes and others (Lopes *et al.*, 2017) showed that in the region of the genome encoding structural proteins VP60 and VP10, the strains were similar to pathogenic strains from the GI.1b group (G1), but in the region of the genome that encodes non-structural proteins they had created a new group which was 13% divergent from known strains. According to Lopes and others (Lopes *et al.*, 2017), there are no further reports of this strain surviving in the environment. The absence of information could be due to limited sampling (Lopes *et al*; 2017). Another important issue is the fact that the P95 strain was isolated from an Iberian hare (*Lepus granatensis*), which points to breaching the species barrier, although for some unknown reason the infection was not sustained in the environment (Lopes *et al.*, 2017). Perhaps the occurrence of this strain line can be explained with the survival strategy of the virus through speciation in one of two directions, namely: change of the infected host species or through a change within the same host species. The former strategy which probably occurred in the case of P95 required many changes and was a major adaptation challenge, hence the virus did not survive. One must also account for the fact that perhaps, in the case of the two strains, there was a similar situation as that was observed in the noroviruses (Mahar *et al.*, 2013), where recombination resulted in a new RdRp variant with a higher mutation potential (14.8% and 14.7% nucleotide substitutions, respectively, for P95 and P19). This was supposed to imply a higher genetic variability of RHDV and improved general robustness of the viral population against the selection pressure but, for unknown reasons, this mechanism has failed (Mahar *et al.*, 2013).

The GI.1b (G1) group contains strains that are similar in two ways. On one hand, the groups were created by chronology, as it contains the "classical" strains of RHDV from 1989 and strains CB137 from 1995 and CB194 from 2006, which probably are evolutionarily related to classical strains (Abrantes *et al.*, 2012). On the other hand, this group also includes the G1/RHDV2 recombinant strains currently circulating in the Portuguese islands and replacing the existing G1 strains in the wild rabbit population (Lopes *et al.*, 2015a). It should be noted that the CBAlgarve14-3 strain, in a phylogenetic analysis based on the VP60 gene sequence (Lopes *et al.*, 2015a; Mahar *et al.*, 2016), showed positioning in the RHDV2 genotype, i.e. behaved differently than in our study. This fact underlines the importance of research not only on genes encoding structural proteins such as VP60, but also of non-structural proteins such as RdRp, in order to determine the designation of strains to genotypes. In group GI.1c (G2), strains were characterised

with both, a chronological structure: from the period of 1987–1996, and a geographic structure: strains from Australia from the period of 2007–2014, and from New Zealand. Evolutionary relations of Australian and New Zealand strains point to them as being descendants of a European ancestor, the Czech V351 strain, which was artificially released in the mid-1990s in Australia and New Zealand to control the population of the artificially introduced European Rabbit (Cooke & Fenner, 2002; Eden *et al.*, 2015). Furthermore, evolutionary relations in this group point out that all of the remaining strains forming this group derive from the Czech V351 strain. Group GI.1d (G3-G5) was predominantly formed by European strains from the period of 1989–2004, with some of them focusing around the Italian Bs89 strain from 1989. It must be pointed out that this group also included strains which, in prior studies (Forrester *et al.*, 2008), were found to have undergone recombination in the RdRp-coding region, including: Frankfurt5, Frankfurt12, and Wika, but this event did not result in a durable new RdRp variant. Among the strains analysed, 28 originating from the period of 1996–2015 had formed a monophyletic group GI.1a (G6/RHDVa), statistically very well supported, with a variability of 11.8–13.6%, with intragroup variability totalling 0.2–6.1%.

Phylogenetic relations deduced from the RdRp gene had revealed a new phylogenetic group, gathering original non-recombined RHDV2 strains from the period of 2011–2016 circulating in Spain, continental Portugal and the Canary Islands (Lopes *et al.*, 2018; Silverio *et al.*, 2018). The last group in the phylogenetic tree was formed by four strains from Australia, one from Portugal, and by MRCV. Phylogenetic relations in this group suggest that currently the population of *Lagovirus* is mixed between Europe and Australia, as also reported by other authors (Mahar *et al.*, 2016). Phylogenetic relations in this group and genetic variability suggest that the Portuguese CBMad17-1 strain from 2017 is a recombinant that arose between rabbit caliciviruses and RHDV2, although the occurrence of the strain in Madera still remains a mystery. Positioning of Australian strains AUS/NSW/BER-3, AUS/NSW/BER-2, and AUS/NSW/WAL-1 on the phylogenetic tree points to their phylogenetic relations with rabbit calciviruses, and suggests they are recombinants of rabbit caliciviruses and RHDV or rabbit caliciviruses and RHDV or RHDVa, because Australian RHDV strains have been gradually replaced since 2013 with the GI.1a variant (Mahar *et al.*, 2018a).

CONCLUSIONS

The extent and significance of genetic variability through RNA mutation and recombination has not yet been thoroughly investigated, and the study presented here approximates this issue to some extent, which is important in the context of viral evolution, epidemiology and pathogenesis. So far, the main direction of genetic variation and evolution of RHDV which has been present worldwide for over 30 years, has led from RHDV, through RHDVa, to RHDV2. It seems that the recorded variability within the RdRp gene for the analysed lagoviruses, in particular RHDV2, is of major importance for its expansion and survival in the environment.

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