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1 **Partial sequencing of recent Portuguese myxoma virus field isolates exhibits a high**  
2 **degree of genetic stability**

3

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26 **Keywords:** Myxoma virus, field strains, sequence analysis, co-evolution, European rabbit

1

2 **Summary**

3 To study genetic changes underlying myxoma virus evolution in its new host, the European  
4 rabbit (*Oryctolagus cuniculus*), we sequenced selected genomic regions of nine recent  
5 virulent field strains and a live attenuated vaccine strain (“MAV”, Germany). DNA was  
6 extracted from cell culture passaged myxoma virus. A total of 4863bp (approximately 3% of  
7 the genome) of ten regions spanning 12 genes of the myxoma viruses was sequenced and  
8 compared to the original virulent strain “Lausanne” and its attenuated field derivative strain  
9 “6918”. The field strains displayed a maximum of three (strains C43, C95) and a minimum of  
10 one (strains CD01, CD05) nucleotide substitutions. These were distributed through all  
11 analysed coding regions, except gene M022L (major envelope protein), where all strains were  
12 identical to “Lausanne” and “6918”. Two new single nucleotide insertions were observed in  
13 some of the field strains: within the intergenic region M014L/M015L and within gene  
14 M009L, where it leads to a frameshift. These insertions were located after homopolymeric  
15 regions. The vaccine strain displayed 37 nucleotide substitution, predominantly (95%) located  
16 in genes M022L and M036L. Interestingly, regions M009L and M014L/M015L of the  
17 vaccine were not amplified successfully, suggesting major genomic changes that could  
18 account for its attenuated phenotype. Our results support a high degree of genetic stability of  
19 myxoma virus over the past five decades. None of the analysed genome regions by its own  
20 seems sufficient for the genetic characterisation of field strains.

21

22 **Introduction**

23 Myxoma virus (MV) is a large double stranded-DNA virus of the genus Leporipoxvirus. In its  
24 natural hosts (*Sylvilagus brasiliensis* and *Sylvilagus bachmani*) it causes benign cutaneous  
25 fibromas. However, its introduction in the 1950s into a new host, the European rabbit

1 (*Oryctolagus cuniculus*), resulted in devastating epizootics with mortality rates above 99%.  
2 Interestingly, within a few years of the introduction of the virus into naïve European wild  
3 rabbit populations in Australia and Europe, a decrease from the initial nearly 100% mortality  
4 rates were observed. This process has been related to virus-host adaptation consisting of the  
5 evolution of attenuated viral strains and the natural selection of resistant rabbits (reviewed by  
6 Fenner & Ross, 1994). Extensive knowledge has been gained on subjects related to this co-  
7 evolution, such as the determination of virulence grades of circulating myxoma viruses (e.g.:  
8 Bárcena et al., 2000; Fenner & Ross, 1994; Marlier et al., 1999), the pathogenesis of myxoma  
9 virus infection of different clinical outcomes (Best & Kerr, 2000; Best et al., 2000), the  
10 rabbits' immune response (reviewed by Fenner & Ross, 1994), and the characterisation of  
11 virally encoded proteins able to modulate the host's immune response and thus clinical  
12 outcome of infection (e.g. Johnston & McFadden, 1994; Stanford et al., 2007; Willer et al.,  
13 1999; Zúñiga, 2002). Contrasting little is known about the genetic changes related to the  
14 evolution of myxoma virus in its new host (Kritas et al., 2008; Morales et al., 2008; Saint et  
15 al., 2001). The evolution of poxviruses has been studied based on whole-genome  
16 phylogenetic analyses rather than single gene phylogeny due to the occurrence of phenomena  
17 such as horizontal gene transfer (Bratke & McLysaght, 2008; Gubser et al., 2004; Hughes &  
18 Friedmann, 2004; Xing et al., 2006). To date, the complete genomes of only two myxoma  
19 viruses are available: the virulent strain "Lausanne", introduced in 1952 in Europe, and its  
20 attenuated field derivative "6918", obtained in 1995 in Spain (Bárcena et al., 2000; Cameron  
21 et al., 1999; Morales et al., 2008). The comparative analysis of both genomes has identified  
22 73 differences consisting of 67 base substitutions, 4 deletions and 2 insertions (Morales et al.,  
23 2008). Interestingly, events such as whole gene loss or gain were not observed.  
24 The understanding of genetic changes driving myxoma virus evolution in the European rabbit  
25 requires the genetic analysis of larger numbers of field strains. The large size of the viral

1 genome has hampered the availability of this kind of information, and partial gene sequences  
2 have shown little if any differences compared to the virulent strain Lausanne (Kritas et al.,  
3 2008; Saint et al., 2001). As gene order and content remained unchanged in the attenuated  
4 myxoma virus field strain “6918” (Morales et al., 2008), it may be sufficient to study virus  
5 evolution by analysing specific genomic regions. Our objectives were to study selected  
6 genomic regions of recent myxoma virus field isolates and to evaluate the suitability of these  
7 as molecular marker for virus evolution within the European rabbit.

8

## 9 **Material and Methods**

10

### 11 **1. Selection of viral genes**

12 The genome comparison between the virulent myxoma virus strain “Lausanne” (GenBank  
13 accession no. AF170726) and the attenuated field strain “9618” (GenBank accession no.  
14 AF552530), suggested that the observed disruptions in ORFs M135R, M148R, M009L,  
15 M036L may be related to loss of viral virulence (Morales et al., 2008). Thus, we included  
16 these genome regions in our analysis (Table 1). We also partially amplified gene M020L to  
17 confirm the insertion of the CTC codon for leucine at position 52 (Morales et al., 2008).  
18 Further five viral genome regions were selected, which include the following partial genes:  
19 M002L/R and M007L/R for being located in the terminal inverted repeats and encoding  
20 immunomodulatory proteins similar to TNF receptor and to rIFN gamma receptor,  
21 respectively (Johnston & McFadden, 1994; Stanford et al., 2007; Willer et al., 1999), M014L  
22 and M015L, which encode a kelch-like protein and the small subunit of ribonucleotide  
23 reductase, respectively; M022L encoding the major envelope protein, as this protein is  
24 exposed to the immune system of the host, and may reflect the effect of immune pressures;  
25 M137R and M138L because the corresponding sequence of the California myxoma virus

1 strain “MSD” (GenBank accession no. AF030894) was available for comparison and  
2 displayed major differences when compared with “Lausanne” (Jackson et al., 1999). The  
3 “MSD” strain naturally infects the brush rabbit (*Sylvilagus bachmani*) but causes fulminant  
4 disease in the European rabbit. A further locus, M061R, coding for the thymidine kinase gene,  
5 was selected for diagnostic purposes only (see below). The term “genomic region” was used  
6 to describe the amplified sequences, which correspond to either part of a single gene, or, in  
7 the case of M014L/M015L and M137R/M138R, to two adjacent genes and their respective  
8 intergenic noncoding region.

9

## 10 **2. Primers and sequencing**

11 The targeted genes and used primers are shown in Table 1. A hot start Taq polymerase  
12 (Hotstar Taq Polymerase, Qiagen) was used. The annealing conditions of the PCR reactions  
13 varied between 50 and 65°C. The PCR products were directly sequenced in both directions.  
14 The obtained sequences were edited, aligned and analysed using the software Bioedit (Hall et  
15 al., 1999). The obtained sequences were compared to sequences available in GenBank by  
16 performing BLAST searches. The deduced amino acid sequences were compared to the  
17 proteins of myxoma virus strain “Lausanne” and “6918”.

18

## 19 **3. Myxoma virus isolates**

20 Nine field strains were obtained from European rabbits displaying signs and/or lesions  
21 compatible with myxomatosis. They were collected between 2004 and 2007 in different  
22 locations in Portugal: four were obtained from wild rabbits in the southern province Algarve  
23 (C43, C95, C116, C152), three from wild rabbits in the north-eastern province Trás-os-  
24 Montes e Alto Douro (CB31, CB32, CB191), and two from an outbreak in domestic rabbits in  
25 the north-western province Douro Litoral (CD01, CD05). The vaccine strain “MAV” was

1 originally derived from a cell culture attenuated Californian myxoma MSD virus (Mckercher  
2 & Saito, 1964). It is currently used as seed for commercially available vaccines in Europe  
3 (Gorski et al., 1994). This vaccine strain was kindly made available by the company IDT  
4 Biologika GmbH, Dessau, Germany, through the Friedrich-Loeffler-Institut, Germany, and  
5 was used here as reference strain.

6 The diagnosis of myxomatosis was made by PCR targeting the thymidine kinase gene  
7 (M061R) using DNA extracts from eyelids (Table 1). In order to increment viral yield for the  
8 genetic characterisation, the viruses were passaged twice in RK-13 cells. Virus was harvested  
9 after submitting the infected cell culture twice to freeze-thaw cycles at -80°C. Cell-free  
10 supernatant was used for nucleic acid extraction using the QIAamp Viral RNA Mini Kit  
11 (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

12

#### 13 **4. Nucleotide sequence accession numbers**

14 The GenBank accession numbers of 16 sequences representing the observed nucleotide  
15 changes are FJ970492-FJ970507.

16

#### 17 **Results**

18 Ten genomic regions spanning 12 genes were selected for the partial genetic analysis of nine  
19 virulent myxoma virus field isolates obtained from seven wild and two domestic rabbits  
20 between 2004 and 2007 in Portugal, as well as from the German live attenuated vaccine strain  
21 "MAV". The sequence information on 4863bp (corresponding to 3% of the viral genome) was  
22 compared to that of strains "Lausanne" and "6918". We also confirmed the insertion of the  
23 CTC codon in gene M020L of all field strains as well as the vaccine strain (data not shown).  
24 The results are shown in Table 2. Within gene M002L/R (907bp) all field isolates displayed  
25 one single nucleotide substitution, albeit at three different locations. Six of the nine field



1 strains displayed a nonsynonymous substitution at position 1981, which was also observed in  
2 strain “6918”. The field strains CB191 and CD01 showed a synonymous substitution at  
3 position 1971. In field strain CD05, which originated from the same outbreak as CD01, a  
4 nonsynonymous nucleotide change at a different position (1975) was observed. The vaccine  
5 strain was identical to “Lausanne”. Within gene M007L/R (735bp), two nonsynonymous  
6 nucleotide substitutions were observed in field strain C43 and in the vaccine strain (positions  
7 8261 and 8494, respectively). Within gene M009L (477bp), five field strains (C95, C116,  
8 CB31, CB32, CB191) displayed a single nucleotide insertion (C) at position 12297 after a  
9 homopolymeric region of 5 cytosins, causing a frameshift after amino acid 280. The PCR  
10 amplification of the vaccine strain yielded two very weak bands of approximately 100 and  
11 400bp, inadequate for successful sequencing. Within the region spanning M014L/M015L  
12 (430bp) a single nucleotide insertion (A) was observed at position 16616 of the intergenic  
13 region of six field strains (C43, C95, C116, CB31, CB32, CB191) after a homopolymeric  
14 region of 10 adenosines. PCR amplification of this region of the vaccine strain was  
15 unsuccessful. At gene M022L (384bp) all field strains were identical to “Lausanne” and the  
16 vaccine strain displayed eight synonymous nucleotide substitutions. Within gene M036R  
17 (229bp), nonsynonymous nucleotide substitutions were observed at positions 37588 and  
18 37759, of the two field strains C95 and C152, respectively. The vaccine strain displayed 27  
19 nucleotide substitution, of which 5 were nonsynonymous. In gene M135R (229bp), the same  
20 nonsynonymous nucleotide substitution was observed at position 131772 in the field strains  
21 CB31 and CB32. The remaining, as well as the vaccine strain, were identical to “Lausanne”.  
22 Within the region spanning M137R/138L (958bp), a nonsynonymous nucleotide substitution  
23 was observed at position 133966 of field strain CB191, and a single nucleotide substitution at  
24 position 133846 in the intergenic non-coding region of strains CB31 and CB32. Within gene

1 M148R (436bp), nonsynonymous nucleotide substitutions were observed at position 142867  
2 of isolates C43, C95, C116, and at position 142804 of the vaccine strain.

3

#### 4 **Discussion**

5 Since the introduction of Myxoma virus into its new host population, the European rabbit, a  
6 decrease in disease mortality has been observed and myxomatosis has turned into a frequently  
7 cited example of virus-host adaptation, but genetic information on field strains is scarce  
8 (Kritas et al., 2008; Morales et al., 2008; Saint et al., 2001). In the present study only virulent  
9 virus strains were included, because field evidence of attenuated or atypical forms of disease  
10 is difficult to obtain, as these probably go undetected, e.g. we were unable to find evidence of  
11 myxoma virus infection in eyelid samples from 30 healthy wild rabbits hunted in southern  
12 Portugal (Pancas) and tested by PCR analysis of the TK gene as well as by three consecutive  
13 passages on RK-13 cells (data not shown). The analysed nucleotide sequences presented here  
14 corresponded to only approximately 3% of the complete viral genome, but they were selected  
15 based on the above-mentioned criteria and thus considered good candidates for detecting  
16 changes reflecting virus - host co-evolution. Interestingly, a maximum of only three (strains  
17 C43, C95) and a minimum of one nucleotide substitutions (strains CD01, CD05) were  
18 observed in each field strain. The attenuated strain “6918” only displayed four nucleotide  
19 substitutions within the analysed genome regions, one of which (position 1981 of gene  
20 M002L/R) was also found in six of the field strains, suggesting that this mutation is being  
21 fixed in some European wild rabbit populations. The major envelope gene (M022L) of  
22 myxoma is exposed to the rabbit’s immune system, and expected to be a good target for  
23 identifying viral variability. Surprisingly, the analysed gene sequences of the field isolates  
24 were identical to strain “Lausanne”, similar to that of a pathogenic myxoma virus isolated  
25 from an outbreak in vaccinated and non vaccinated commercial rabbits in Greece (Kritas et  
26 al., 2008). Within M022L of the vaccine strain, eight synonymous nucleotide substitutions

1 were found. No equivalent sequence information on the MSD strain was found in GenBank to  
2 understand whether these mutations are characteristic for this strain, or whether they may  
3 reflect pressures related to its attenuation process. Although our findings support the limited  
4 degree of genetic alteration found in recent myxoma virus field strains (Morales et al., 2008;  
5 Saint et al., 2001), the mutations observed in the selected genome regions allowed the  
6 individual identification and some grouping of the strains according to their geographic  
7 origin. For example, CB31 and CB32, which were collected from wild rabbits during the  
8 same occasion, are identical to each other but differ from CB191, which was sampled at a  
9 different geographic location, but also in the north of Portugal. Interestingly the strains  
10 obtained from domestic rabbits during an outbreak in a family farm (CD01, CD05) displayed  
11 mutations at different locations of gene M002L/R, but were otherwise identical to  
12 “Lausanne”.

13  
14 Within myxoma viruses, whole gene gain or loss does not seem to occur, but single or  
15 multiple base pair insertions and deletions which resulted in frameshift mutations have been  
16 described (Morales et al., 2008). We have found two homopolymeric sites, where a single  
17 nucleotide has been inserted in some of the virulent field strains: within gene M009L and in  
18 the noncoding region between M014L and M015L. Thus these single nucleotide insertions do  
19 not seem to affect virulence. However, major disruptions, at least in M009L, may indeed play  
20 an important role in attenuation, e.g. in strain “6918” (Morales et al., 2008). Although it has  
21 been argued that integrity of gene M009L may not be a critical virulence factor in myxoma  
22 virus, because of its disruption in the virulent Californian strain MSW (Labudovic et al.,  
23 2004) and its partial duplication in the attenuated Shope fibroma virus (Willer et al., 1999), it  
24 should be remembered, however, that these leporipoxviruses have evolved in different hosts,  
25 *Sylvilagus bachmani* and *S. floridanus*, respectively, whereas the natural host of myxoma

1 virus is *S. brasiliensis* (Fenner & Ross, 1994). They present a considerable degree of genetic  
2 heterogeneity (84-89%) and may not entirely be suitable for comparison with “Lausanne” and  
3 “6918”. Major changes are likely to be present in gene M009L of the vaccine strain,  
4 hampering PCR amplification and yielding two products of smaller sizes. The use of two  
5 other primer pairs overlapping the site of the deletion in “6918” (positions 11937-11947) was  
6 unsuccessful (not shown). The region spanning genes M014L/M015L of the vaccine strain  
7 could also not be amplified successfully, suggesting the occurrence of genomic changes  
8 requiring further investigations. Single indels in genes M135R and M148R were considered  
9 of putative relevance for the attenuation of “6918” (Morales et al., 2008). Our results support  
10 this hypothesis as these changes were not observed in the virulent field strains. However, they  
11 were neither found in the vaccine strain, indicating that they are not essential for attenuation.  
12 The disruption of another gene (M135R) was considered a potentially important determinant  
13 for the attenuation of the field strain “6918” (Morales et al., 2008). Again, this disruption does  
14 not seem essential for attenuation, as it did not occur in the vaccine strain. The sequence  
15 spanning genes m137R/m138L of the vaccine strain displayed 100% nucleotide identity with  
16 “Lausanne”. This is rather unexpected, as the sequence comparison with the “MSD” strain  
17 (Genbank accession no. AF030894), from which the vaccine strain was derived, displayed  
18 only 84% nucleotide identity to strain “Lausanne”. The vaccine strain has a long history of  
19 cell culture passaging, and it is thus very difficult to rule out possible cross contaminations  
20 with “Lausanne”-derived isolates or strains at any point.

21 Altogether, these results support those obtained with specific gene knockout viruses, in that  
22 various disruptions, on their own or in synergy, may affect virulence of myxoma viruses  
23 (Johnston & McFadden, 1994; Stanford et al., 2007; Willer et al., 1999). Further field strains  
24 should be analysed to assess the frequency of single base substitutions and indels as well as  
25 their importance in reflecting viral evolutionary processes, also considering that

1 misincorporations due to polymerase infidelity (*in vivo* and *in vitro*) may account for some of  
2 the observed differences between strains. Although focussing on only small parts of the viral  
3 genome, this study supports the relatively high degree of genetic stability of myxoma field  
4 strains over the past five decades (Saint et al., 2001, Morales et al., 2008). Based on our  
5 findings it is difficult to identify unique single gene markers of virus attenuation or evolution,  
6 indicating that analyses of larger portions of the genome are required.

7

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20

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**TABLE 1.** Selected genes and primers used for the genetic characterisation of myxoma virus field strains. The nucleotide positions refer to myxoma virus strain “Lausanne” (GenBank accession no. AF170726).

Gene (position)	Gene description	Forward primer (position)	Reverse primer (position)	Annealing temperature	Size PCR amplicon	Genomic Region: Positions of obtained sequence	Length of viral protein	Positions of deduced amino acids
M002L/R (1665-2645; 160109-159129)	Similar to TNF receptor	MT2F (1682-1702)	MT2R (2614-2634)	55°C	932bp	1706-2613; 160068-159161	326	12-313
M007L/R (7985-8776; 153789- 152998)	Similar to rIFN $\gamma$ receptor	MT7F (7990-8009)	MT7R (8748-8767)	55°C	758bp	8006-8741; 153768-153033	263	13-257
M009L (11601-13130)	Contains kelch motifs	M009F (11859-11878)	M009R (12383-12364)	55°C	525bp	11859-12336	509	263-424
M014L (15012-16565)	Kelch-like protein	NN1F= 16496F (16496-16515)	NN1R= 16931R (16912-16931)	65°C	436bp	16501-16931	517	1-21
M015L (16618-17586)	Ribonucleotide reductase, small subunit						322	220-322
M020L (19194-20531)	Serine/threonine protein kinase	M020F (20145-20164)	M020R (20458-20439)	55°C	314bp	20146-20413	445	38-125
M022L (22558-23673)	Major envelope protein	NN2F= 22913F (22913-22932)	NN2R= 23298R (23279-23298)	60°C	386bp	22914-23298	371	126-253
M036L (37209-39251)	Leucine zipper motif	M036F (37558-37577)	M036R (37845-37864)	55°C	307bp	37551-37858	680	464-564
M061R (57797-58333)	Thymidine kinase	TKU (57822-5783)	TK3 (58375-58393)	50°C	571bp	<b>Diagnostic PCR</b>		
M135R (131699-132235)	IL-1/IL-6 receptor-like	M135F (131647-131668)	M135R (131856-131876)	55°C	230bp	131642-131871	178	1-59
M137R (132908-133840)	Similar to vacA51R	A1F (133767-133787)	A1R (134405-134424)	60°C	658bp	133788-134746	310	295-310
M138L (133874-134746)	alpha 2,3-sialyltransferase	A2F (134340-134360)	1160R (134938-134957)	60°C	618bp		290	1-290
M148R (141626-143653)	Ankyrin motif; host range	M148F (142701-142720)	M148R (143119-143138)	55°C	438bp	142696-143132	675	360-504

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**TABLE 2.** Observed nucleotide polymorphisms and deduced amino acid variations in recent myxoma virus field strains. The nucleotide and amino acid positions refer to myxoma virus strain “Lausanne” (GenBank accession no. AF170726). The GenBank accession numbers of selected sequences (FJ970492-FJ970507) are indicated in bold italics below the respective polymorphic nucleotide positions.

Gene	Terminal region						Central region								Terminal region		
	M002L/R 907bp		M007L/R 735bp		M009L 477bp		M014L M015L 430bp	M022L 384bp		M036L 307bp		M135R 229bp		M137R/M138L 958bp		M148R 436bp	
	NT	AA	NT	AA	NT	AA	NT	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
<b>Strain 6918</b> (EU552530)	T1981 A2497 T2594	D222 F104 S18	A8064	F238	Deletion 11942- 11951	F/S after aa395, start Met 398	ID	ID	ID	Insertion n C37687	F/S after aa523, start Met 542	Insertion G131750	F/S after aa19, start Met 44	ID	ID	Deletion C142959	F/S after aa446, start Met 505
<b>C43</b>	T1981 FJ970494	D222	A8261 FJ970496	ID	ID	ID	ID <sup>)</sup> FJ970498	ID	ID	ID	ID	ID	ID	ID	ID	G142867 FJ970506	S416
<b>C95</b>	T1981	D222	ID	ID	Insertion C12297 FJ970497	F/S after aa280, start Met 303	ID <sup>)</sup>	ID	ID	T37588 FJ970500	M496	ID	ID	ID	ID	G142867	S416
<b>C116</b>	T1981	D222	ID	ID	Insertion C12297	F/S after aa280, start Met 303	ID <sup>)</sup>	ID	ID	ID	ID	ID	ID	ID	ID	G142867	S416
<b>C152</b>	T1981	D222	ID	ID	ID	ID	ID	ID	ID	T37759 FJ970501	M553	ID	ID	ID	ID	ID	ID
<b>CB31</b>	T1981	D222	ID	ID	Insertion C12297	F/S after aa280, start Met 303	ID <sup>)</sup>	ID	ID	ID	ID	T131772 FJ970503	C27	ID <sup>)</sup> FJ970504	ID	ID	ID
<b>CB32</b>	T1981	D222	ID	ID	Insertion C12297	F/S after aa280, start Met 303	ID <sup>)</sup>	ID	ID	ID	ID	T131772	C27	ID <sup>)</sup>	ID	ID	ID
<b>CB191</b>	T1971 FJ970492	ID	ID	ID	Insertion C12297	F/S after aa280, start Met 303	ID <sup>)</sup>	ID	ID	ID	ID	ID	ID	A133966 FJ970505	C261	ID	ID
<b>CD01</b>	T1971	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID
<b>CD05</b>	C1975 FJ970495	G224	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID	ID
<b>Vaccine strain</b> “MAV”	ID	ID	G8492 FJ970507	ID	No PCR amplicon (2 weak bands 100 + 400kb approx.)	-	No PCR amplicon	G23118 C23296 T23212 C23218 A23235 C23236 G23254 T23257 FJ970499	All ID	27 nt subst. FJ970502	L458 S491 S530 S532 S549 All other 22 ID	ID	ID	ID	ID	T142804 FJ970493	V395

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ID Identical to "Lausanne"

\*) Insertion (A) after nucleotide 16616 of a poly-A in the non-coding region.

§) Point mutation (C -> T) at position 133846 in the non-coding region.

F/S frameshift

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