LETTER TO THE EDITOR

TAXONOMIC REVISION OF THE FAMILY *CLOSTEROVIRIDAE* WITH SPECIAL REFERENCE TO THE GRAPEVINE LEAFROLL-ASSOCIATED MEMBERS OF THE GENUS *AMPELOVIRUS* AND THE PUTATIVE SPECIES UNASSIGNED TO THE FAMILY

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SUMMARY

New insights into the genetic structure and variability of grapevine leafroll-associated viruses (GLRaVs) gained through worldwide efforts in the last decade or so, and the production and use of new sets of serological reagents, have provided the solid foundation on which the present revision of the taxonomic structure of the family *Closteroviridae,* and the genus *Ampelovirus* in particular, is based. A comparative examination of the amino acid sequence divergence of three taxonomically relevant genes [RNA-dependent RNA polymerase (polymerase), heat shock protein 70 homologue (HSP70h) and coat protein (CP)] disclosed a difference among Grapevine leafroll-associated virus 4 (GLRaV-4), -5, -6 and -9 and a group of more recently described viruses (GLRaV-Pr, GLRaV-De and GLRaV-Car) below the 25% limit recently set by the International Committee on Taxonomy of Viruses (ICTV) as a discriminating criterion for the identification of species in the family *Closteroviridae.* This, plus the recognition that GLRaV-4, -5, -6 and -9 are serologically related, have similar biological and epidemiological traits, and that these viruses and GLRaV-Pr, GLRaV-De, GLRaV-Car have a genome with the same structure and size, supports the notion that they are all genetically divergent variants of a single species, GLRaV-4. The genus *Ampelovirus* is split into two subgroups designated I and II in recognition of the wide difference in the size and structure of the genome of the present members. Finally, the establishment of a fourth genus within the family *Closteroviridae,* comprising the unassigned putative species Grapevine leafroll-associated virus 7 (GLRaV-7), Little cherry virus 1 (LChV-1) and Cordyline virus 1 (CoV-1), is justified based on their molecular and biological characteristics that differ from those of members of the other three genera of the family.

Key words: plant viruses, closteroviruses, classification, taxonomy, *Velarivirus*.

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INTRODUCTION

Leafroll is a long known disease of European grapevines (*Vitis vinifera*) (for a historical review see Martelli and Boudon-Padieu, 2006), its symptoms varying with the cultivar, the infecting viruses and their combinations (Krake, 1993). American and Asian *Vitis* species are susceptible to infection but show no appar-

ent symptoms, except for a more or less pronounced decrease in vigour. Exceptions are *V. riparia* Gloire, *V. coignetiae* and *V. californica* which display leaf reddening when infected by some of the disease-associated viruses (Greif *et al.*, 1993; Saldarelli *et al.*, 2005; Klaassen *et al.*, 2011).

The viral nature of leafroll disease was inferred by the positive results of transmission trials made in Germany (Scheu, 1935) and confirmed 11 years later in California (Harmon and Snyder, 1946). The causal agent, however, had remained unknown until the late 1970s when Namba *et al.* (1979) found closterovirus-like particles in Japanese vines with leafroll symptoms, and associated this type of virus with the disease. The first partial characterization of two serologically different such viruses, referred to as "type I" and "type II", came a few years afterwards from Switzerland (Gugerli *et al.*, 1984). It was the beginning of a nomenclature based on the use of numerals to identify seemingly different viruses.

In the years that followed, the number of putatively new closterovirus species identified in vines with leafroll symptoms in Europe and the USA increased in such a disorderly way (Table 1), that a revision of their classification and nomenclature was required. The serological relationships of all leafroll-associated viruses reported in the literature were re-investigated to assess their taxonomic status, and their nomenclature was set in order (Boscia *et al.*, 1995). These authors, in accordance with the determination of the International Committee of Taxonomy of Viruses (ICTV) that Arabic rather than Roman numerals were to be used for virus names, renamed these viruses Grapevine leafroll-associated virus 1 to 5, and identified a sixth member of the group (GLRaV-6). The seventh (GLRaV-7) and eigth (GLRaV-8) member were reported later from Italy (Choueiri *et al.*, 1996) and the USA (Monis, 2000), respectively.

When the family *Closteroviridae* was revised in 2002, based on the molecular and epidemiological properties of its representatives as suggested by Karasev (2000), GLRaV-2 was assigned to the genus *Closterovirus,* comprising primarily aphid-transmitted viruses, GLRaV-1, -3, -4, -5, -6 and -8 were classified as approved or putative species in the genus *Ampelovirus,* comprising exclusively mealybug-transmitted viruses, whereas GLRaV-7 was given the status of unassigned putative species to the family (Martelli *et al.*, 2002). GLRaV-9, described

Table 1. Current classification and some properties of Grapevine leafroll-associated viruses (GLRaVs).

Virus	Genus	Coat protein (kDa)	Genome size (nts) (GenBank accession No.)	ORFs (No.)	Vectors	First record *fide* Boscia *et al.* (1995) and this paper
GLRaV-1	*Ampelovirus*	34	18,659 (JQ023131)	9	Mealybugs, soft scale isects	Gugerli *et al.* (1984)
GLRaV-2	*Closterovirus*	22	16,494 (AY88162)	8	Unknown	Zimmermann *et al.* (1990)
GLRaV-3	*Ampelovirus*	35	18,498 (EU259806)	12	Mealybugs, soft scale and scale insects	Zee *et al.* (1987)
GLRaV-4	*Ampelovirus*	35	13,830 (FJ467503)	6	Mealybugs	Hu *et al.* (1990)
GLRaV-5	*Ampelovirus*	35	13,384[a] (FR822696)	6	Mealybugs	Zimmermann *et al.* (1990); Walter and Zimmermann (1991)
GLRaV-6	*Ampelovirus*	35	13,807 (FJ467504)	6	Mealybugs	Gugerli and Ramel (1993); Gugerli *et al.* (1997)
GLRaV-7	Unassigned in the family	37	16,496 (HE588185)	10	Unknown	Choueiri *et al.* (1996)
GLRaV-8[b]	*Ampelovirus*	37	ND	ND	Unknown	Monis (2000)
GLRaV-9	*Ampelovirus*	35	12,588[a] (AY29781)	6	Mealybugs	Alkowni *et al.* (2004)
GLRaV-Pr	*Ampelovirus*	30	13,696 (AM182328)	6	Mealybugs	Maliogka *et al.* (2009);
GLRaV-Car	*Ampelovirus*	29	13,626 (FJ907331)	6	Unknown	Abou Ghanem-Sabanadzovic *et al.* (2010)

[a] Nearly complete sequence; [b] Cancelled from the 9th ICTV Report (Martelli *et al.*, 2011a); ND, not determined.

later by Alkowni *et al.* (2004), is currently retained as a putative ampelovirus species (Martelli *et al.*, 2011a).

From 2006 onwards, new ampelovirus isolates have been described (Saldarelli *et al.*, 2006; Maliogka *et al.*, 2008, 2009; Elbeaino *et al.*, 2009; Abou Ghanem-Sabanadzovic *et al.*, 2010) three of which have been extensively or totally sequenced and proposed as putative new species: Grapevine leafroll-associated virus Pr (GLRaV-Pr, sequence originally deposited in GenBank under the name of GLRaV-10), Grapevine leafroll-associated virus De (GLRaV-De, sequence originally deposited under the name GLRaV-11) (Maliogka *et al.*, 2008, 2009) and Grapevine leafroll associated-Carnelian virus (GLRaV-Car) (Abou Ghanem-Sabanadzovic *et al.,* 2010).

So, by 2011, the number of GLRaVs had grown to 12: one closterovirus (GLRaV-2), 10 ampeloviruses (GLRaV-1, -3, -4, -5, -6, -8, -9, GLRaV-Pr, GLRaV-De, GLRaV-Car) and one unassigned species (GLRaV-7). This is indeed a unique situation because, to the best of our knowledge, none of the known virus diseases of any woody crop has such a high number of viruses of the same type implicated in its aetiology.

Such an unusual scenario raises a number of questions, among which the following three seem to be most significant:

(i) Are all of the GLRaVs real viruses?
(ii) Should the existing ampeloviruses be retained as truly distinct species?
(iii) Is the current classification of GLRaVs correct?

In the following section an attempt is made to provide an aswer to the above queries based on the information that became available over the last decade or so.

Are all the GLRaVs real viruses?

The case of Grapevine leafroll-associated virus 8. The presence of a putative new ampelovirus denoted Grapevine leafroll-associated virus 8 (GLRaV-8), in a leafroll-affected vine [accession LR 102 (Monis and Bestwick, 1997)] was reported in 2000 from the USA (Monis, 2000). The identification was based on the isolation from the infected vine of a 37 kDa protein which was sequenced (GenBank accession No. AF233936) and used for raising monoclonal antibodies that did not react with antigens from GLRaV-1 to GLRaV-7 (Monis, 2000). The characterization of this virus did not progress further and, because the original virus source and the serological reagents are no longer available (J. Monis, personal communication), confirmatory work could not be carried out. However, the examination of two completely sequenced *Vitis vinifera* genomes has recently revealed that both contain the DNA counterpart of the putative GLRaV-8 RNA sequence (Bertsch *et al.,* 2009). The discovery that this sequence, rather than being of viral origin, is part of the grapevine genome, prompted the removal of GLRaV-8 from the membership of the genus *Ampelovirus* (Martelli *et al.*, 2011a).

So, by the end of 2011, the number of GLRaVs had dropped to 11, most of which (9) being unquestionably regarded as approved or putative members of the genus *Ampelovirus.*

Should the present ampeloviruses be retained as truly distinct species?

The membership of the genus *Ampelovirus* and, by and large, of the family *Closteroviridae,* has been determined by the discriminating criteria for the identification of virus species approved by the ICTV (Martelli *et al.* 2005) and enforced up to 2011:

(i) Particle size
(ii) Size of CP, as determined by deduced amino acid sequence data
(iii) Serological specificity using discriminatory monoclonal or polyclonal antibodies
(iv) Genome structure and organization (number and relative location of the ORFs)
(v) Amino acid sequence of relevant gene products (CP, CPm, HSP70h) differing by more than 10%
(vi) Vector species and specificity
(vii) Magnitude and specificity of natural and experimental host range
(vii) Cytopathological features (aspect of inclusion bodies and origin of cytoplasmic vesicles).

The fully or partially sequenced closteroviruses identified in leafroll-diseased vines were found to possess extensive variation in the size and structure of their genomes. Due to the ease of obtaining molecular data in comparison to biological data and controversial serological data caused by the inconsistent reactivity of some of the available reagents, the molecular parameters became the single most important criterion for the recognition of new species. When the narrow 10% boundary in the sequence identity of taxonomic relevant genes was broken, novel putative species were identified, thus favouring the growth of the population of leafroll-associated agents to the unprecedented current size.

The increase of the discriminating threshold from 10% to 25% for three taxonomically relevant genes (polymerase, HSP70h and CP) recently approved by the ICTV (Martelli *et al.*, 2011a) and the production of new sets of monoclonal antibodies to GLRaVs (Gugerli, 2009), now allows researchers to re-consider the taxonomic structure of the genus *Ampelovirus* and the validity of its grapevine-infecting members.

The case of *Grapevine leafroll-associated virus 1* and *3*. GLRaV-1 has a genome 18,659 nts in size, comprising 9 ORFs (10 genes) (GenBank accession No. JQ023131). The duplication of the hypervariable minor coat protein gene (CPm1 and CPm2) (Fig. 1A) (Little *et al*., 2001; Little, 2004), represents a unique trait of this virus. In trees constructed with the complete animo acid sequences of the phylogenetically relevant genes polymerase, HSP70h and CP, GLRaV-1 clusters with species of the genus *Ampelovirus,* next to *Grapevine leafroll-associated virus 3* (GLRaV-3) (Fig. 2), with which it is serologically very distantly related (Seddas *et al*., 2000). Divergence at the amino acid level of the CP sequence of 37 distinct viral isolates [AF195822, ABM05865, ACT79559, ACV52939 and Alabi *et al.* (2011)] ranges between 4 and 20%, whereas the divergence of the HSP70h gene sequence of 29 of the above isolates is the range of 10%, i.e. within the 25% boundary.

GLRaV-1 induces strong leafroll symptoms in naturally infected vines and in graft-inoculated indicators, and is transmitted aspecifically and with a semi-persistent modality by pseudococcid mealybugs of the genera *Heliococcus, Phenacoccus* and *Planococcus* and soft scale insects of the genera *Pulvinaria, Neopulinaria* and *Parthenolecanium* (Martelli and Boudon-Padieu, 2006; Tsai *et al*., 2010). Cytological modifications of infected grapevine cells consist of inclusion bodies made up of clusters of membranous vesicles with fibrillar content, originated from proliferation of the peripheral membrane of mitochondria, intermixed with loose aggregates of virus particles (Faoro *et al.,* 1991; Faoro, 1997).

GLRaV-3, the type species of the genus *Ampelovirus*, has a genome 18,498 nts in size comprising 12 ORFs (13 genes) (Fig. 1B). The divergence at the amino acid level of the CP sequence of 31 virus isolates from the USA (14 sequences), Brazil (6), South Africa (4), Portugal (3), People's Republic of China (3), Chile (2) and one each from Australia, Taiwan and Iran does not exceed 6%. The divergence of the HSP70h gene of eight distinct isolates is in the range of 3-4%, except for the 15% value of a New Zealand source (ABP49570). As to the polymerase, the divergence registered in nine different isolates is 5%, with a maximum of 12% for an Italian strain (ABC42904). None of these isolates breaks the 25% threshold of genetic variability.

Like GLRaV-1, GLRaV-3 is a strong leafroll inducer to naturally infected vines and graft-inoculated indicators, and is transmitted aspecifically and with a semi-persistent modality by pseudococcid mealybugs of the genera *Heliococcus, Phenacoccus, Planococcus* and *Pseudococcus*, soft scale insects of the genera *Pulvinaria, Neopulinaria Parthenolecanium, Coccus, Saissetia, Parasaissetia* and scale insects of the genus *Ceroplastes* (Martelli *et al*., 2011b). Cytopathological modifications are comparable to those induced by GLRaV-1, i.e. inclusion bodies

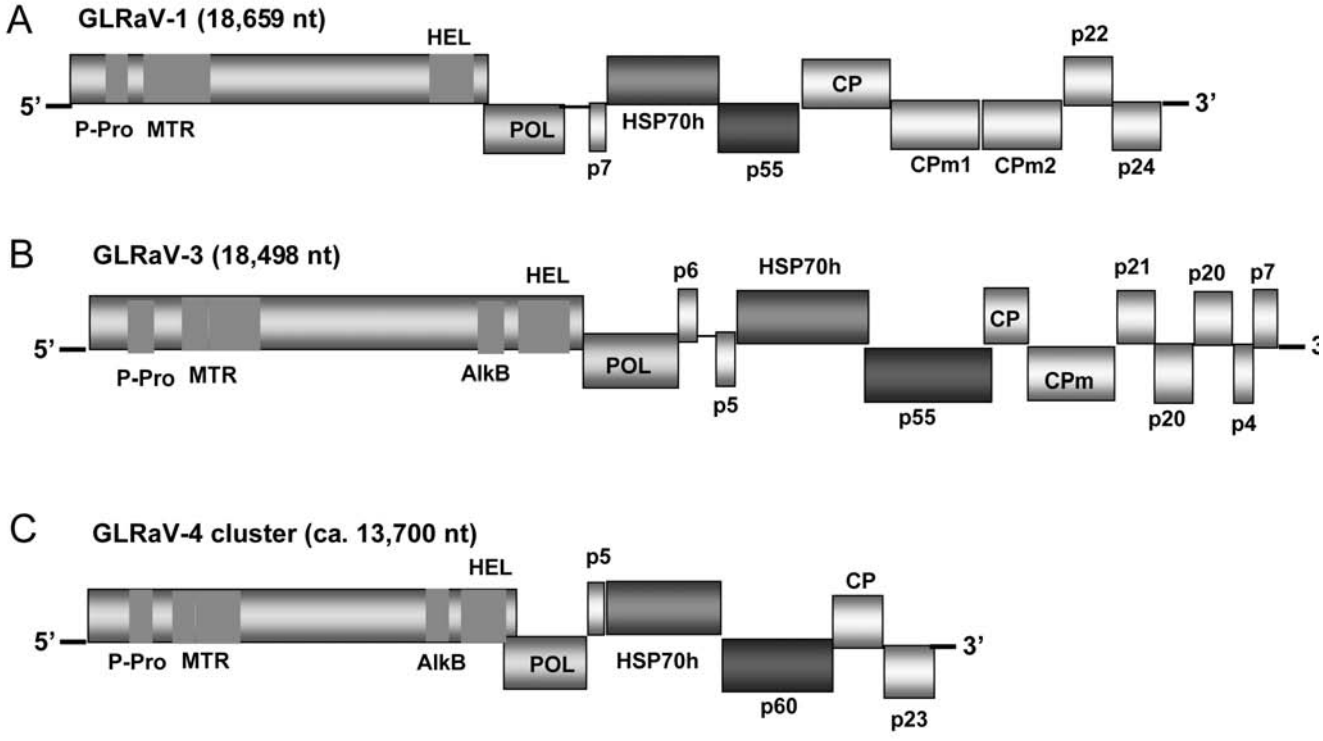

Fig. 1. Schematic representation of the genome structure of: (A) *Grapevine leafroll-associated virus* 1 (GLRaV-1) and (B) *Grapevine leafroll-associated virus* 3 (GLRaV-3), both members of Subgroup I of the genus *Ampelovirus*, and (C) Grapevine leafroll-associated virus 4 (GLRaV-4), the type representative of Subgroup II.

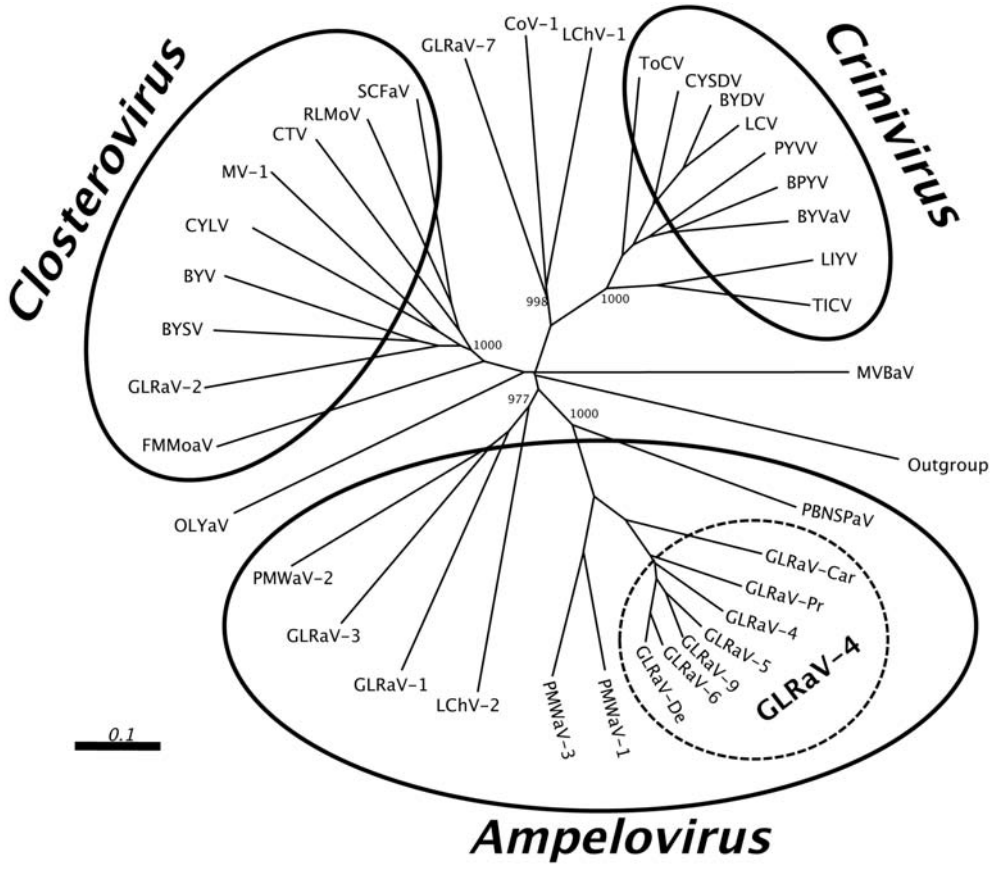

Fig. 2. Phylogenetic tree constructed with complete amino acid sequences of the HSP70h gene of members of family *Closteroviridae*. Distances are proportional to branch lengths. Bootstrap values are indicated at the main branch nodes. The bar represents 0.1 amino acid change per site. Viruses used in the tree, their abbreviations and accession numbers are: genus *Ampelovirus*: *Grapevine leafroll-associated virus 1* (GLRaV-1, AAF22740); *Grapevine leafroll-associated virus 3* (GLRaV-3, NP_813799); Grapevine leafroll-associated virus 4 (GLRaV-4, FJ467503); Grapevine leafroll-associated virus 5 (GLRaV-5, NC_016081); Grapevine leafroll-associated virus 6 (GLRaV-6, FJ467504); Grapevine leafroll-associated virus 9 (GLRaV-9, AAL63810); Grapevine leafroll-associated virus Car (GRLaV-Car; ACT67478); Grapevine leafroll-associated virus Pr (GLRaV-Pr, YP_002364305); Grapevine leafroll-associated virus De (GLRaV-De, AM494395); *Little cherry virus 2* (LChV-2; AF531505); *Pineapple mealybug wilt-associated virus 1* (PMWaV-1; AAL66711); *Pineapple mealybug wilt- associated virus 2* (PMWaV-2; AAG13941); *Pineapple mealybug wilt-associated virus 3* (PMWaV-3; ABD62350); *Plum bark necrosis stem pitting-associated virus* (PBNSPaV; YP_001552326). Genus *Closterovirus: Beet yellow stunt virus* (BYSV, AAC55662); *Beet yellows virus* (BYV, AAF14302), *Citrus tristeza virus* (CTV; NP_042864); *Carrot yellow leaf virus* (CYLV; YP_003075968); *Grapevine leafroll-associated virus 2* (GLRaV-2, AAC40858); *Mint virus 1* (MV-1, YP_224093); *Raspberry leaf mottle virus* (RLMoV, ABO15357), *Strawberry chlorotic fleck-associated virus* (SCFaV, ABI23185); Fig mild mottle-associated virus (FMMoaV, ACU57193). Genus *Crinivirus: Beet pseudoyellows virus* (BPYV, NP_940788); *Blackberry yellow vein-associated virus* (BYVaV, AAW67738); *Cucurbit yellow stunting disorder virus* (CYSDV, CAA11494); *Lettuce infectious yellows virus* (LIYV, NP_619695), *Lettuce chlorosis viru*s (LCV, ACQ82510); *Potato yellow vein virus* (PYVV, YP_054417); *Tomato infectious chlorosis virus* (TICV, ACN88745); *Tomato chlorosis virus* (ToCV, AAD01790); *Bean yellow disorder virus* (BYDV, ABY66965). Unassigned or unclassified viruses: Little cherry virus 1 (LChV-1, NP045004), Mint vein banding-associead virus (MVBaV, AAS57941); Olive leaf yellowing-associated virus (OLYaV, CAD29309); Grapevine leafroll-associated virus 7 (GLRaV-7, HE588185); Cordyline virus 1 (CoV-1; HM588723), Heat shock 70 protein from *Arabidopsis thaliana* (NP_187864) was used as outgroup.

made up of clusters of membranous vesicles originated from the proliferation of the peripheral membrane of mitochondria, intermixed with loose aggregates of virus particles (Faoro *et al.,* 1991; Faoro 1997).

Notwithstanding the similarity in the biological and epidemiological behaviour and the distant serological relatedness, GLRaV-1 and GLRaV-3 are recognized as distinct species (large difference in genome structure) and have an intraspecific molecular variability in the deduced amino acid sequence of polymerase and HSP70h, and CP genes that does not exceed 20%. These viruses, together with *Little cherry virus 2* (LChV-2) and *Pineapple mealybug wilt-associated virus 2* (PMWaV-2), form a coherent cluster in phylogenetic trees constructed with the sequence of HSP70h (Fig. 2) and the other two genes (data not shown). This cluster is comprised in a distinct branch of the tree, giving rise to an aggregate that, as suggested by Maliogka *et al.* (2008), constitutes a subgroup now denoted Subgroup I (Fig. 3).

The case of the Grapevine leafroll-associated virus 4 cluster. The identification of GLRaV-4, -5, -6 and -9 as distinct ampelovirus species was dictated primarily by the apparent lack of serological relationship among them (Boscia *et al.*, 1995; Alkowni *et al.*, 2004). However, when molecular data emerged (Good and Monis, 2001; Abou Ghanem-Sabanadzovic *et al.*, 2003, 2006; Dovas and Katis, 2003; Saldarelli *et al.*, 2006) it became evident that these four viruses formed a coherent phylogenetic cluster, that grew to seven members when the sequences of GLRaV-Pr, GLRaV-De, and GLRaV-Car became available (Maliogka *et al.*, 2008, 2009; Abou Ghanem-Sabanadzovic *et al.*, 2010) (Fig. 2). GLRaV-De has recently been identified as a variant of GLRaV-6 (Abou Ghanem-Sabanadzovic *et al.*, 2012).

Although it had been pointed out that these viruses could represent genetic variants of a single species (Martelli, 2009; Elbeaino *et al.*, 2009), no action for a taxonomic revision could be taken until the complete genomic sequences of GLRaV-4, -5 and -6 were obtained, as this represented the third obstacle standing on the way, the others being the above recalled 10% threshold and the apparent lack of serological relatedness. Since this task has now been accomplished (Abou Ghanem-Sabanadzovic *et al.*, 2012; Thompson *et al.*, 2012) and GLRaV-5, -6 and -9 proved to be serologically related to GLRaV-4 (Gugerli, 2009; Abou Ghanem-Sabanadzovic *et al.*, 2012), it becomes feasible to delineate, in accordance with these authors, a novel taxonomic scenario, whereby GLRaV-4 becomes a reference species comprising the formerly approved (GLRaV-5) and putative [GLRaV-6 (and its -De variant) and GLRaV-9] ampelovirus species and the unclassified GLRaV-Pr and GLRaV-Car (Fig. 3).

The foundation on which this revision rests is the recognition that:

(i) all these viruses have the smallest (*ca.* 13,700 nts) and the simplest [six ORFs (seven genes) and the lack of a recognizable CPm] genome within the family *Clos-*

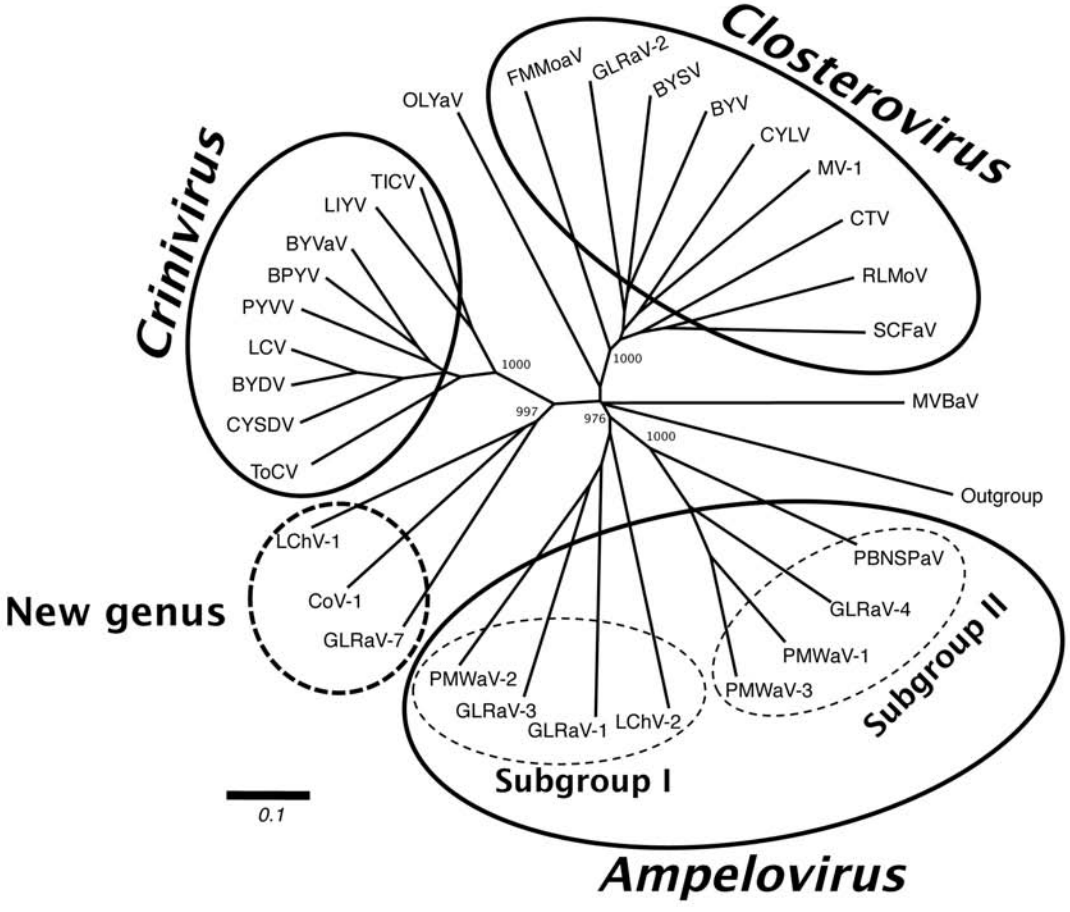

Fig. 3. Phylogenetic tree constructed with complete amino acid sequences of the HSP70h gene of members of family *Closteroviridae*. Distances are proportional to branch lengths. Bootstrap values are indicated at the main branch nodes. The bar represents 0.1 amino acid per site. Viruses used in the tree, their abbreviations and accession numbers are the same as in Fig. 2. The tree shows: (i) the suggested splitting of the genus *Ampelovirus* into two coherent subgroups including viral species with a large (in excess of 17,000 nts) and complex (9 to 12 ORFs) genome (Subgroup I) and with a smaller (aproximately 13,000-14,000 nts) and simpler (6 ORFs) genome (Subgroup II); (ii) the allocation of the three virus species (GLRaV-7, LChV-1 and CoV-1) included in the novel genus *Velarivirus,* in a branch of the tree next to that comprising members of the genus *Crinivirus*.

teroviridae (Fig. 1C), thus resembling very much the ancestral progenitor common to the family hypothesized by Dolja *et al.* (2006);

(ii) the molecular divergence at the amino acid level of the polymerase, HSP70h and CP genes of none of the viruses exceeds 25%, with the exception of the 33% value shown by the GLRaV-Car HSP70h (Tables 2, 3 and 4).

(iii) all viruses have a similar biological behaviour, i.e. association with a symptomatology milder than that elicited by GLRaV-1 and GLRaV-3 and transmissibility by pseudococcid mealybugs, though experimentally ascertained only for GLRaV-4, GLRaV-5, GLRaV-9, a Cypriot isolate of GLRaV-Pr (Sim *et al.*, 2003; Elbeaino *et al.*, 2009; Tsai *et al.*, 2010) and GLRaV-6 (E. Herrbach, personal communication)

As shown in Fig. 3, GLRaV-4 forms, together with *Plum bark necrosis stem pitting-associated virus* (PBNSPaV), *Pineapple mealybug wilt-associated virus 1* (PMWaV-1) and *Pineapple mealybug wilt-associated virus 3* (PMWaV-3), a phylogenetically coherent cluster of species comprised in a distinct clade denoted Subgroup II, which is significantly differentiated from Subgroup I (see also Maliogka *et al.*, 2008, 2009).

Is the current classification of GLRaVs correct?

The case of Grapevine leafroll-associated virus 7. GLRaV-7 was originally found in an unidentified apparently symptomless white-berried grapevine cultivar from Albania (accession AA42) which, however, induced leafroll symptoms onto grafted cv. Cabernet sauvignon indicators, thus justifying the name given to the virus (Choueiri *et al.*, 1996). The geographical distribution of GLRaV-7 is rather wide, as it comprises European (Albania, Armenia, Greece, Hungary, Italy, Switzerland), Near East (Egypt, Palestine, Turkey), North (USA) and South (Chile) American countries and China (Martelli, 2009).

Table 2. RNA-dependent RNA polymerase amino acid sequence identity (%) of members of the "GLRaV-4 cluster"

	GLRaV-9	GLRaV-5	GLRaV-6	GLRaV-4	GLRaV-Pr	GLRaV-Car	Mean divergence (%)
GLRaV-9	100	90	89	85	83	77	15
GLRaV-5	90	100	89	86	82	76	15
GLRaV-6	89	89	100	87	85	78	14
GLRaV-4	85	86	87	100	82	74	17
GLRaV-Pr	83	82	85	82	100	77	18
GLRaV-Car	77	76	78	74	77	100	24

Table 3. Heat shock protein 70 homologue amino acid sequence identity (%) of members of the "GLRaV-4 cluster"

	GLRaV-De	GLRaV-6	GLRaV-9	GLRaV-5	GLRaV-4	GLRaV-Pr	GLRaV-Car	Mean divergence (%)
GLRaV-De	100	92	85	86	81	79	67	18
GLRaV-6	92	100	84	85	78	78	66	20
GLRaV-9	85	84	100	90	82	80	67	19
GLRaV-5	86	85	90	100	82	80	68	18
GLRaV-4	81	78	82	82	100	78	67	22
GLRaV-Pr	79	78	80	80	78	100	69	23
GRLaV-Car	67	66	67	68	67	69	100	**33**

Table 4. Coat protein amino acid sequence identity (%) of members of the "GLRaV-4 cluster"

	GLRaV-De	GLRaV-6	GLRaV-5	GLRaV-9	GLRaV-4	GLRaV-Car	GLRaV-Pr	Mean divergence (%)
GLRaV-De	100	91	86	86	81	76	76	17
GLRaV-6	91	100	85	84	81	79	76	17
GLRaV-5	86	85	100	87	84	79	78	17
GLRaV-9	86	84	87	100	82	79	78	17
GLRaV-4	81	81	84	82	100	79	77	19
GLRaV-Car	76	79	79	79	79	100	77	22
GLRaV-Pr	76	76	78	78	77	77	100	23

GLRaV-7 has very flexuous filamentous particles with a most frequent length of 1500-1700 nm (with a predominance of 1500 nm long particles), exhibiting the cross banding and open structure of typical closterovirid virions. Its identification as a different species was largely based on the lack of reaction of an antiserum raised to its coat protein with any of the six grapevine-infecting closteroviruses known at that time (Choueiri *et al*., 1996). However, partial sequencing of the viral genome (Turturo *et al*., 2000) disclosed differences with members of the two genera of the family *Closteroviridae* with a monopartite genome (*Closterovirus* and *Ampelovirus*) suggesting GLRaV-7 be classified as an unassigned putatative species to the family, a position that it shares with Little cherry virus 1 (LChV-1) (Martelli *et al*., 2011a).

Contrary to all approved species of the genus *Ampelovirus*, GLRaV-7 and LChV-1 do not have a known vector. GLRaV-7, however, replicates in three different species of dodder (*Cuscuta reflexa*, *C. europea* and *C. campestris*) the first two of which could transmit the virus to *Tetragonia espansa* and *Nicotiana occidentalis,* respectively (Mikona and Jelkmann, 2010). This biological trait further differentiates GLRaV-7 from other agents of grapevine leafroll disease, an Australian source of which was transmitted by *C. campestris* from grape to grape but not to herbaceous hosts (Woodham and Krake, 1983).

Two GLRaV-7 isolates have been sequenced originating, respectively, from the Albanian accession AA42 (Mikona *et al*., 2009; Jelkmann *et al.,* 2012) and a Swiss selection of cv. Pinot Noir [accession FPS PN-23 (Al Rwahnih *et al.* (2012)].

The viral genome consists of 16,496 nucleotides (nt) arranged in 10 open reading frames (ORFs) (Fig. 4). As reported (Mikona *et al.,* 2009; Jelkmann *et al*., 2012; Al Rwahnih *et al.,* 2012), the genome encodes in the 5'→3' direction: (i) a polyprotein 267 kDa in size comprising the protease, methyltransferase, and helicase domains (ORF1a) and the 60 kDa RNA-dependent RNA polymerase (ORF 1b); (ii) a 8 kDa putative protein (ORF2) that overlaps ORF1b. This protein has predicted transmembrane helices and resembles small membrane proteins encoded by other closteroviruses, such as *Beet yellows virus* (BYV), where it is expressed by a subgenomic messenger RNA (Al Rwahnih *et al*., 2012); (iii) a 4 kDa hydrophobic protein with a putative transmembrane domain (ORF3); (iv) the 62 kDa HSP70h protein (ORF4); (v) a 10 kDa protein showing homology with the small-sized proteins (p4 to p10) coded for by RNA-2 of some criniviruses at the same genomic position (ORF5); (vi) a 61 kDa protein matching the comparable product, referred to as "p60", encoded by all members of the family *Closteroviridae* (ORF6); (vii) the coat protein (CP) 34 kDa in size (ORF7) and (viii) the minor coat protein (CPm) 69 kDa in size (ORF8). ORF9 and ORF10 putatively code for a 25 kDa and a 27 kDa protein, respectively, neither of which shares similarities with other viral proteins in the current database. It is to be noted that the AUG initiation codons of ORFs 2, 3 and 5 are not in an optimal context for expression (Lutcke *et al.,* 1987). The 5' and 3' untranslated regions (UTRs) are 47 and 283 nt in size, respectively, and have no apparent similarity with those of other closteroviruses. By analogy with other members of the family *Closteroviridae*, the genome expression strategy is thought to

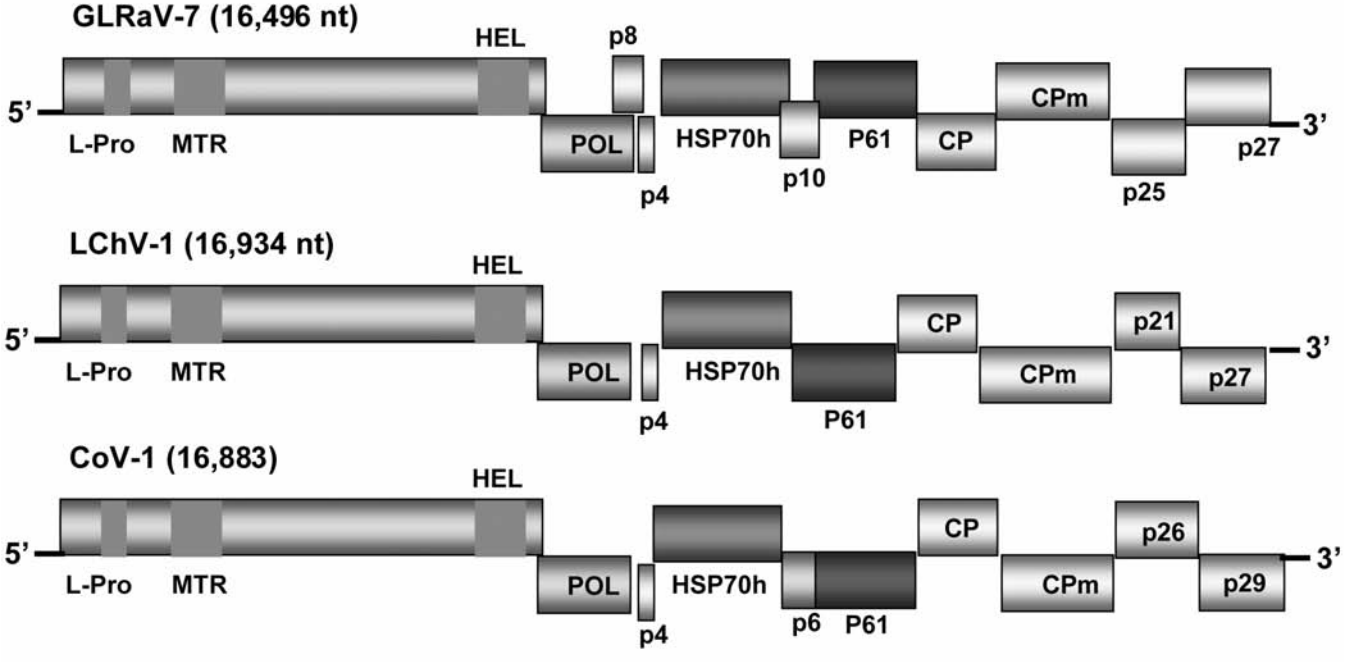

Fig. 4. Schematic representation of the genome structure of the members of the novel genus *Velarivirus*.

encompass direct translation and proteolytic processing of the polyprotein encoded by ORF1, a +1 ribosomal frameshift for the expression of the RdRp domain encoded by ORF1b and the expression of downstream ORFs by 3' co-terminal subgenomic RNAs (Martelli *et al*., 2011a).

The genome structure of GLRaV-7 resembles that of LChV-1 and of Cordyline virus 1 (CoV-1), a novel closterovirus-like virus infecting ti plants (*Cordyline fruticosa*) in Hawaii (Melzer *et al*., 2011), except for the apparent lack in LChV-1 of ORF2 and ORF5, and of ORF2 in CoV-1 (Fig. 4).

In phylogenetic trees constructed with the HSP70h sequences, GLRaV-7, LChV-1 and CoV-1 group together in a clade related to that comprising members of the genus *Crinivirus* (Fig. 2, 3). Furthermore, from a comparative analysis of three taxonomic relevant genes (polymerase, HSP70h and CP) of the three viruses (including both GLRaV-7 isolates) with comparable genes of members of the genera *Closterovirus*, *Ampelovirus* and *Crinivirus*, it appears that the highest identity at the amino acid level is shared with criniviruses (Table 5).

As discussed by Jelkmann *et al.* (2012), differences of GLRaV-7, LChV-1 and CoV-1 with members of the three extant genera of the family *Closteroviridae* reside in:

Ampeloviruses: (i) genome size and structure [number of genes intermediate between that of the largest (Subgroup I) and the smallest (Subgroup II) members of the genus]; (ii) biological traits, i.e. lack of a recognized vector, transmission through dodder to herbaceous hosts [ascertained for GLRaV-7 and LChV-1 (Jelkmann *et al.,* 2009; Mikona and Jelkmann, 2010)]; (iii) distant phylogenetic relationships (RdRp, HSP70h and CP protein identity at the amino acid level always lower than 30% for any gene).

Closteroviruses: (i) genome size and structure (lower number of genes, CPm preceding CP); (ii) lack of a recognized vector; (iii) distant phylogenetic relationships (RdRp, HSP70h and CP protein identity at the amino acid level always lower than 30% for any gene).

Criniviruses: Genome structure (monopartite versus bipartite/tripartite, diverse gene arrangement); (ii) lack of a recognized vector; (iii) phylogenetic relationships closer than that with closteroviruses and ampeloviruses but still distant (RdRp, HSP70h and CP protein identity at the amino acid level slightly exceeding 50% only for the polymerase gene).

Overall, these differences seemed to be relevant enough to support the suggestion that a fourth genus comprising GLRaV-7 and LChV-1 could be created within the family *Closteroviridae* (Martelli, 2011). The discovery of CoV-1 (Melzer *et al*., 2011), a third virus with molecular properties resembling those of GLRaV-7 and LChV-1, prompted the contemporary proposal for the establishment of a novel genus comprising GLRaV-7, LChV-1 and CoV-1 (Al Rwahnih *et al*., 2012; Jelkmann *et al.*, 2012), provisionally denoted *Velarivirus* (Al Rwahnih *et al*., 2012).

The validity of the name GLRaV-7 was questioned, based on the fact that the Swiss isolate of this virus does not induce symptoms in naturally infected cv. Pinot noir vines nor in graft-inoculated cv. Cabernet franc indicators (Al Rwahnih *et al*., 2012). Also the original Albanian source (AA42) was apparently symptomless, but induced leafroll symptoms on the indicators. By contrast, the Greek and Californian grapevine accessions analyzed for the presence of GLRaV-7 were reported to show "very mild or uncertain leafroll symptoms" (Avgelis and Boscia, 2001) or to be "symptomatic and asymptomatic" with no further details (Morales and Monis, 2007). Molecular re-examination of accession AA42 done at Bari and Dossenheim showed that no closteroviruses other than GLRaV-7 are present in this vine (P. Saldarelli and W. Jelkmann, unpublished information), and equally negative was an Illumina deep-sequencing run done at Bari (P. Saldarelli and A. Minafra, unpublished information). These findings support the notion that GLRaV-7 is a valid name worth retaining.

In conclusion, the critical re-examination of the taxonomic structure of the family *Closteroviridae* prompted by the outcome of the studies conducted over the last

Table 5. Amino acid identity (%) of complete sequences of three taxonomically relevant genes [RNA-dependent RNA polymerase (polymerase), heat shock protein 70 homologue (HSP70h), and coat protein (CP)] of GLRaV-7-Alb (numerator) and GLRaV-7-Swi (denominator) with the comparable genes of Little cherry virus 1 (LChV-1) and Cordyline virus 1 (CoV-1), and of all definitive members of the three current genera of the family *Closteroviridae.*

	Polymerase	HSP70h	CP
Little cherry virus 1 (LChV-1)	54/54	42/43	32/31
Cordyline virus 1 (CoV-1)	54/54	40/39	25/29
Crinivirus	47-53/48-49	34-40/37-39	15-21/18-22
Ampelovirus	23-29/25-28	21-27/25-26	8-14/10-14
Closterovirus	29-35/27-29	24-25/24-26	10-12/12-13

Table 6. Proposed new taxonomic configuration of the family *Closteroviridae*.

GENUS	VECTORS
Closterovirus	
Approved species	
Beet yellows virus (BYV)	Aphids
Beet yellow stunt virus (BYSV)	
Burdock yellows virus (BuYV)	
Carnation necrotic fleck (CNFV)	
Carrot yellow leaf virus (CYLV)	
Citrus tristeza virus (CTV)	
Grapevine leafroll-associated virus 2 (GLRaV-2)	
Mint virus 1 (MV-1)	
Wheat yellow leaf virus (WYLV)	
Putative species	
Alligator weed stunting virus (AWSV)	
Clover yellows virus (CYV)	
Dendrobium vein necrosis virus (DVNV)	
Festuca necrosis virus (FNV)	
Fig leaf mottle associated virus -1 (FLMaV-1)	
Fig mild mottle virus (FMMV)	
Raspberry leaf mottle virus (RLMV)	
Strawberry chlorotic fleck-associated virus (SCFaV)	
Ampelovirus	
Approved species	
Subgroup I	Mealybugs, soft scale and scale insect
Grapevine leafroll-associated virus 1 (GLRaV-1)	
Grapevine leafroll-associated virus 3 (GLRaV-3)	
Little cherry virus 2 (LChV-2)	
Pineapple mealybug wilt-associated virus 2 (PMWaV-2)	
Subgroup II	Mealybugs
Grapevine leafroll-associated virus 4 (GLRaV-4)	
Pineapple mealybug wilt-associated virus 1 (PMWaV-1)	
Pineapple mealybug wilt-associated virus 3 (PMWaV-3)	
Prunus bark necrosis stem pitting-associated virus (PBNSPaV)	
Crinivirus	
Approved species	
Abutilon yellows virus (AbYV)	Whiteflies
Bean yellow disorder virus (BYDV)	
Beet pseudoyellows virus (BPYV)	
Blackberry yellow vein-associated virus (BYVaV)	
Cucurbit yellow stunting disorder virus (CYSDV)	
Lettuce chlorosis virus (LCV)	
Lettuce infectious yellows virus (LIYV)	
Potato yellow vein virus (PYVV)	
Strawberry pallidosis-associated virus (SpaV)	
Sweet potato chlorotic stunt virus (SPCSV)	
Tomato chlorosis virus (ToCV)	
Tomato infectious chlorosis virus (TICV)	
Putative species	
Diodia vein chlorosis virus (DVCV)	
Cucurbit chlorotic yellow virus (CCYV)	
Velarivirus	
Cordyline virus 1 (CoV-1)	
Grapevine leafroll-associated virus 7 (GLRaV-7)	Unknown
Little cherry virus 1 (LChV-1)	
Approved unassigned species	
Mint vein banding virus (MVBaV)	Aphids
Putative unassigned species	
Olive leaf yellowing-associated virus (OLYaV)	Unknown

few years in a number of different laboratories from many countries, inspires a proposal which, as illustrated in Table 6, encompasses:

(i) the revision of the genus *Ampelovirus,* which is split into two subgroups accomodating, respectively, four species with a large (*ca.* 15,000 to over 18,000 nts) and complex (9 to 12 ORFs) genome (Subgroup I) and four species with a smaller (13,000-14,000 nts) and simpler (6 ORFs) genome (Subgroup II);
(ii) the drastic reduction in the number of members of Sugroup II due to the identification of GLRaV-5, -6, -9, -Pr, and -Car as molecular variants of GLRaV-4. These variants constitute phylogenetically distinct lineages (Maliogka *et al*., 2008) thus, to reflect former designations, they could be denoted GLRaV-4 strain 5; GLRaV-4 strain 6; GLRaV-4 strain 9; GLRaV-4 strain Pr and GLRaV-4 strain Car;
(iii) the creation of a fourth genus provisionally called *Velarivirus*, comprising two former unassigned viruses (GLRaV-7 and LChV-1) and the newly described CoV-1.

To become effective, the proposed taxonomic modifications must be examined and approved in a step-wise manner by the various bodies of the ICTV and then be ratified by the ICTV Plenum.

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