Family *Flexiviridae*: A Case Study in Virion and Genome Plasticity

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Abstract

The plant virus family *Flexiviridae* includes the definitive genera *Potexvirus*, *Mandarivirus*, *Allexivirus*, *Carlavirus*, *Foveavirus*, *Capillovirus*, *Vitivirus*, *Trichovirus*, the putative genus *Citrivirus*, and some unassigned species. Its establishment was based on similarities in virion morphology, common features in genome type and organization, and strong phylogenetic relationships between replicational and structural proteins. In this review, we provide a brief account of the main biological and molecular properties of the members of the family, with special emphasis on the relationships within and among the genera. In phylogenetic analyses the potexvirus-like replicases were more closely related to tymoviruses than to carlaviruses. We postulate a common evolutionary ancestor for the family *Tymoviridae* and the two distinct evolutionary clusters of the *Flexiviridae*, i.e., a plant virus with a polyadenylated genome, filamentous virions, and a triple gene block of movement proteins. Subsequent recombination and gene loss would then have generated a very diverse group of plant and fungal viruses.

INTRODUCTION

Historically, all organisms including viruses were classified based primarily on their morphology and reproductive cycle. Later, the rise of molecular biology and comparative genomics revolutionized understanding of virus taxonomy and evolution, and revealed unexpected relationships between apparently dissimilar viruses, as well as surprising genetic differences between viruses that look alike (3, 43). The recently established family of plant-infecting positive-strand RNA viruses, *Flexiviridae* (2), highlights both of these seemingly opposing tendencies. As the family name immediately suggests, its members share the flexuous morphology of their elongated helical virions. A similar morphology is found in two other plant virus families, *Potyviridae* (115) and *Closteroviridae* (31, 78). Earlier phylogenetic analysis demonstrated that capsid proteins forming filamentous virions of plant viruses are related to each other and likely originate from a single ancestral protein (29). However, whole genome comparisons showed that the virions of the three families harbor strikingly distinct genomes of the alphavirus-like (*Closteroviridae* and *Flexiviridae*) and picornavirus-like (*Potyviridae*) superfamilies (60).

The principal basis for the establishment of the family *Flexiviridae* was the strong phylogenetic relationships between the viral replicational and structural proteins and common features of genome design. All members of the family share monopartite, 3′-polyadenylated genomes that encode closely related methyltransferase, RNA helicase, and RNA polymerase domains of the viral replicase. In contrast, one or two proteases, and AlkB-homology domains are found in replicases of some, but not all, family members. There is also a remarkable diversity in the number and nature of the 3′-proximal viral genes that are expressed via formation of subgenomic mRNAs. These genes encode viral movement proteins that belong to either the "p30-like" superfamily, or to the triple gene block movement complex (69, 91), the nucleic acid-binding proteins, and several unique proteins found in a limited number of family members. These genetic differences translate into a variety of host ranges, pathogenicity levels, and overall epidemiology exhibited by the viruses within the family *Flexiviridae*.

The currently recognized family genera are *Allexivirus*, *Capillovirus*, *Carlavirus*, *Foveavirus*, *Mandarivirus*, *Potexvirus*, *Trichovirus*, and *Vitivirus*. An additional genus, *Citrivirus* (121), with *Citrus leaf blotch virus* (CLBV) as the type species, is under scrutiny for approval by the International Committee on Taxonomy of Viruses. The unassigned family members include *Banana mild mosaic virus* (BanMMV), *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV), *Potato virus* T (PVT), and *Sugarcane striate mosaic-associated virus* (SCSMaV) (2). The recently described Banana virus X (BanVX) is another putative member of the family that does not seem to fit in any of the extant genera (113), whereas African oil palm ringspot virus (AOPRV) (88) and Asian prunus virus-1 (APruV-1), -2 (APruV-2), and -3 (APruV-3) (75) are additional putative members of the genus *Foveavirus*.

In this review, we provide a brief account of the main biological and molecular properties of the *Flexiviridae* with a special emphasis on the relationships within and among the component genera. Using detailed phylogenetic analysis in relation to the recent progress in understanding the basic pathways of virus-host coevolution, we attempt to develop a coherent and parsimonious scenario for the origin and evolution of this viral family.

BIOLOGICAL PROPERTIES

Host Range and Pathogenicity

In natural and agricultural settings, flexiviruses infect a wide variety of wild and cultivated hosts including herbaceous

and woody dicotyledonous and, less often, monocotyledonous plant species. Most of the viruses are pathogenic to agricultural crops, their infections generating mild to extremely severe diseases. Most members of the genera *Capillovirus*, *Citrivirus*, *Foveavirus*, *Mandarivirus*, *Trichovirus*, and *Vitivirus* and the unassigned species CGRMV and CNRMV are agents of woody host diseases, whereas the genera *Potexvirus*, *Carlavirus*, and *Allexivirus* and the remaining unassigned species in the family tend to infect herbaceous plants.

Several capilloviruses, foveaviruses, and vitiviruses share the capacity to elicit modifications of the host xylem known as stem pitting or grooving, depending on whether the woody cylinder is marked by localized, shallow surface indentations (pits), or by long narrow depressions (grooves). An emblematic example of this type of disorder is the rugose wood complex of grapevine (76) in the etiology of which the vitiviruses *Grapevine virus A* (GVA) and *Grapevine virus B* (GVB), and the foveavirus *Grapevine rupestris stem pitting-associated virus* (GRSPaV) are involved, as the putative agents of Kober stem grooving (22, 41) corky bark (11), and Rupestris stem pitting (83), respectively. Another vitivirus, *Grapevine virus D* (GVD), is associated with an especially serious condition of certain grapevine cultivars known as corky rugose wood (10) (**Figure 1**).

Plants infected with GVA, GVB, and GRSPaV often harbor a population of viral sequence variants (45, 46, 83, 107), some of which endowed with differential pathogenicity. Thus, the comparative analysis of the 3′-terminal regions of a number of GVA isolates allowed their separation into three molecular groups that share sequence identity within groups above 90% and below this value between the groups. Only isolates of group III induced mild symptoms in the experimental host *Nicotiana benthamiana* (45). These isolates appeared not to induce in grapevine cv Shiraz a serious disorder known in South Africa as Shiraz disease (SD), whereas a variant of group I dominated in SD-affected vines (46). By contrast, one of the three sequenced GRSPaV variants was shown to infect *Vitis rupestris* without inducing symptoms (84).

Apple stem grooving virus (ASGV), a capillovirus, and *Apple stem pitting virus* (ASPV), a foveavirus, are elicitors of wood abnormalities (Plate 1) in some apple and pear cultivars, especially from Far Eastern countries, and may cause crippling graft incompatibility such as the Japanese apple topworking disease (27, 55). The citrus strain (tatter leaf)

GVA: *Grapevine virus A*

GVB: *Grapevine virus B*

GVD: *Grapevine virus D*

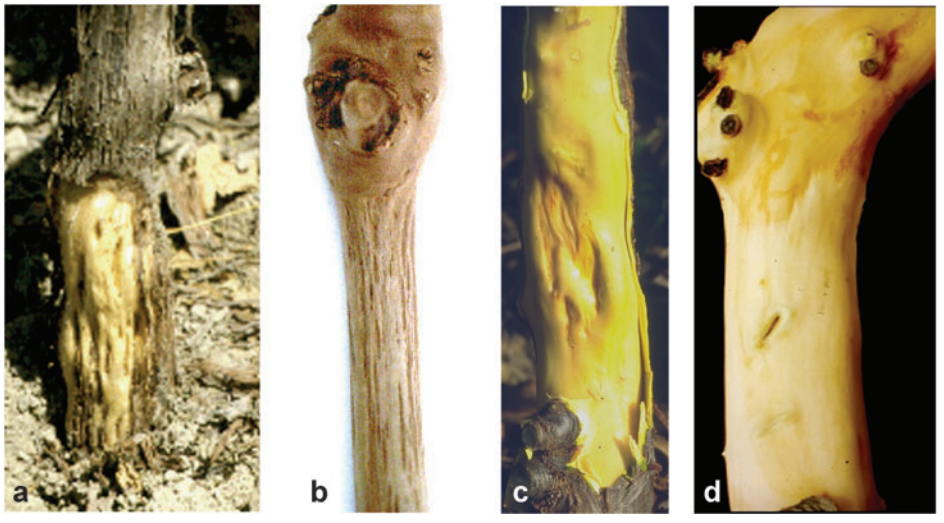


Figure 1

(*a*) Field-grown vine affected by rugose wood; (*b*) stem grooving induced on Kober 5BB by *Grapevine virus A*; (*c*). stem pitting induced on Virginia Crab by *Apple stem grooving virus*; (*d*) mild stem pitting induced on *Malus platicarpa* by *Apple stem pitting virus*. (*c* and *d* courtesy of Dr. L Giunchedi)

PVX: *Potato virus X*
PVY: *Potato virus Y*
PVS: *Potato virus S*

of ASPV is strongly pathogenic to grafted trifoliate rootstocks, which react with bud union crease and stem grooving (101). As its name suggests, the tentative capillovirus Nandina stem pitting virus (NSPV) is associated with a stem pitting in *Nandina domestica* (heavenly bamboo) (4), and the unassigned CGRMV and CNRMV with a wood pitting of cherry (27, 126).

Apricot latent virus (ApLV), a foveavirus inducing symptomless infections in most apricot cultivars (94), possesses two molecular variants pathogenic to peach, in which they cause foliar diseases called peach asteroid mosaic and peach sooty ringspot, respectively (27, 42). Similar symptoms of chlorotic leaf spotting and fruit deformation are associated with peach infection by Asian prunus viruses (75), while AOPRV is held responsible for a lethal disease of African oil palms (88). None of the putative flexiviruses infecting banana (BanMMV and BanVX) seems to be a serious pathogen (40, 113).

Whereas infection by CLBV generates bud union crease in plants grafted on trifoliate rootstocks (39), *Indian citrus ringspot virus* (ICRSV), the only known member of the genus *Mandarivirus*, induces foliar yellow ringspots and a rapid decline of affected trees (18).

Trichoviruses are mainly pathogens of stone fruit trees (apricot, cherry, almond, peach, and plum) and of grapevine (*Grapevine berry inner necrosis virus*, GINV). In these hosts, trichoviruses elicit a variety of symptoms, ranging from delayed bud break, stunted growth, mottling and deformation of the leaves (Peach mosaic virus, PcMV; Cherry mottle leaf virus, ChMLV), to severe damage of fruits, i.e., false plum pox, plum bark split, fruit necrosis of cherry and apricot (*Apple chlorotic leafspot virus*, ACLSV), and necrosis of grape berries (GINV) (27, 67, 111).

Viruses of the genera *Allexivirus*, *Carlavirus*, and *Potexvirus* are predominantly pathogens of herbaceous plants. The genus *Potexvirus* comprises species with important economic impact. In an inquiry coordinated by Milne (86), the potexvirus *Potato virus X* (PVX) was among the top 10 most damaging viruses in seven of nine wide geographical areas including North America, South America, Europe, India, China, Africa, and Australia/New Zealand. However, although infection of solanaceous crops such as potato, tomato, pepper, and tobacco by PVX alone could result in diseases of some consequence, effects were much worse when this virus was in mixture with others [*Potato virus Y* (PVY), in particular]. In the same inquiry, the carlavirus *Potato virus S* (PVS) was reported as a pathogen of concern only in South America, in line with the notion that carlavirus infections are frequently symptomless. Symptoms, when shown, are usually of the mosaic type, much the same as with potexviruses (33, 100, 123). The host ranges of allexiviruses are limited to *Allium* species, in which they are symptomless or induce mild diseases (6).

There is only limited information on the molecular determinants of flexivirus pathogenicity. Two examples include the 25-kDa protein encoded by ORF2 of PVX and the 10-kDa protein encoded by ORF5 of GVA, both of which are involved in symptom development and suppression of RNA silencing (14, 20, 37, 50, 127).

Epidemiology

With a few exceptions, members of the family *Flexiviridae* are transmissible from natural to experimental hosts by mechanical inoculation. Viruses that invade and multiply in parenchymatous tissues, especially members of the genera *Potexvirus*, *Carlavirus*, *Allexivirus*, and *Trichovirus*, are more readily transmissible in this way than vitiviruses, which are phloem-restricted in the natural host, or foveaviruses, which are transmitted with difficulty (ASPV and ApLV) or not at all (GRSPaV).

In vegetatively propagated hosts, virus dissemination over long distances takes place primarily through the distribution of infected

propagative materials. Thus, members of the family that do not have a vector rely exclusively on graft-transmission for their survival and dissemination. All flexiviruses infecting woody hosts, regardless of the genus, are efficiently spread by nursery productions, which are largely responsible for the worldwide deterioration of the sanitary status of fruit tree crops. Infected bulbs, tubers, offshoots, and the like are similarly responsible for the dispersal of potex-, carla-, and allexiviruses (48). An exception is *Lilac chlorotic leafspot virus* (LiCLSV), which, although being transmitted in a semipersistent manner by aphids (15, 16), is currently classified as a capillovirus (2). However, the taxonomic allocation of LiCLSV is under scrutiny and removal from the list of approved species is being considered.

Although seeds represent important natural routes for virus dissemination, seed transmission is hardly relevant to the economy of the family *Flexiviridae*. Examples are few, represented by a capillovirus (the citrus strain of ASGV), three potexviruses [PVX, *Clover yellow mosaic virus* (ClYMV), and *White clover mosaic virus* (WClMV)], and four carlaviruses [*Hop mosaic virus* (HpMV), *Pea streak virus* (PeSV), *Red clover vein mosaic virus* (RCVMV), and some isolates of *Cowpea mild mottle virus* (CPMMV)]. Transmission rates of individual viruses are generally low and do not exceed 10% (81, 87).

Flexivirus infection is introduced at a site by mechanical contact or from the activity of vectors. The first of these modes of transmission is, by and large, typical of potexviruses, which do not have vectors, are very stable, extremely infectious, and multiply to high titers in their hosts. Contact between an infected and a healthy plant is thought to be sufficient for propagating the infection (100). Some carlaviruses (e.g., PVS) behave in a similar way (59), although their natural transmission occurs by aphids in a nonpersistent manner (123). An exception is CPMMV, which is the only known carlavirus transmitted by the whitefly *Bemisia tabaci* (17). The molecular mechanism underlying aphid transmission of carlaviruses has not been elucidated.

Most members of the genera *Allexivirus* and at least three of the genus *Trichovirus* are mite-borne; the vectors in all cases are eriophyd mites (97). *Aceria tulipae* is the alleged vector of the allexiviruses *Garlic virus C* (GarV-C), *Garlic virus D* (GarV-D), and *Shallot virus X* (ShVX) and the tentative species Garlic mite-borne latent virus (GarMbLV), Shallot mite-borne latent virus (ShMbLV), and Onion mite-borne latent virus (OmbLV) (116, 117).

Among trichoviruses, PcMV is transmitted by *Eriophyes insidiosus* (58), ChMLV by *Eriophyes inequalis* (97), and GINV by *Colomerus vitis* (64). The mode of transmission of these viruses is still undetermined. Notwithstanding the lack of definitive experimental evidence, Oldfield & Proeseler (97) suggested that transmission of eriophyd-borne viruses of *Allium* species and woody perennials is of the persistent type.

Vitiviruses are the only members of the family that are vectored by mealybugs. GVA was the first RNA virus shown to be transmissible by *Pseudococcus longispinus*, a pseudococcid mealybug (102) and, ten years later, transmission of GVB by *Planococcus ficus* was obtained (11). Although GVA and GVB remain the only mealybug-transmissible vitiviruses, the number of vectors has grown to six different species in the genera *Planococcus*, *Pseudococcus*, and *Heliococcus* and one species in the soft scale insect genus *Neopulvinaria* (80). As experimentally determined with *Ps. longispinus*, transmission of GVA is semipersistent (66).

Relations with Cells and Tissues

Modifications induced in host cells by flexiviruses have been studied either in the natural hosts for potexviruses, carlaviruses, and allexiviruses, or in experimentally infected herbaceous indicators for capilloviruses, foveaviruses, trichoviruses, and vitiviruses (23, 34, 70, 77, 96).

In general, flexivirus infections do not give rise to cytopathic structures (e.g., inclusion bodies) specific enough to be diagnostic at the genus level (77). Rather, it seems that cytoplasmic and, sometimes, nuclear accumulations of virions in various forms are the most frequent, if not the only intracellular trait common to all members of the family. Differences, however, exist in the outward appearance of these accumulations. Thus, potex-, viti-, and capillovirus aggregates have a flexuous appearance, whereas those of carla-, tricho-, and foveaviruses are rather stiff (34, 70, 96). The size of the aggregates can vary, with PVX accumulations being always massive (70, 77). Large, bundle-like viral aggregates are also seen in cells infected by vitiviruses and carlaviruses, whereas accumulations of allexi-, capillo-, and trichoviruses are relatively small (34, 96)

Species-specific cytopathic structures are elicited by some potex- and carlaviruses. Examples include (*i*) PVX-induced "laminated inclusions"; (*ii*) amorphous inclusions made up of excess coat protein in the cells infected by ClYMV; (*iii*) striking crystalline cytoplasmic and nuclear inclusions associated with Argentine plantago virus and Boussingaultia mosaic virus (70). As to carlaviruses, the whitefly-transmitted CPMMV was reported to elicit unusual brush-like particle aggregates in the infected cells (70).

The cytopathological features common to vitiviruses are more extensive and consist of (*i*) virion aggregates in the shape of bundles, whorls, banded bodies, or stacked layers; (*ii*) cell wall thickenings; (*iii*) proliferation and accumulation of endomembranes; (*iv*) vesicular evaginations of the tonoplast protruding into the vacuole (34, 80). Movement proteins of GVA and GVB were identified by gold immunolabelling in association with cell walls and plasmodesmata (105).

VIRION STRUCTURE

Particles of flexiviruses are formed by a single type of capsid protein subunit with a mol. wt. ranging from 21 kDa in GVD to 41 kDa in CLBV, and encapsidate a single RNA molecule of ~6 to ~9 kb (**Table 1**) constituting ~5% of the particle weight (2). Studies of the two other families of plant-infecting filamentous viruses, closteroviruses (99, 106) and potyviruses (112), revealed that their virions possess terminal tail-like structures formed by additional viral proteins. Because these proteins were also implicated in virus transport, the tails were proposed to represent specialized movement devices (28). The ability of PVX RNA and virions to associate with the viral 25-kDa movement protein in vitro (57) suggests that the virions of flexiviruses may also possess the tail-like appendages required for virus transport in infected plants.

Filamentous virions of flexiviruses have modal lengths ranging from ~470 nm in potexviruses to ~1000 nm in citriviruses, a

Table 1 Properties of the family *Flexiviridae* genera

Genus	Virion length (nm)	Pitch of the helix (nm)	Genome size (kb)	CP (kDa)	ORFs	MP type	Rep (kDa)
Potexvirus	470–580	3.3–3.6	5.9–7.0	22–27	5	TGB	146–191
Mandarivirus	650	~3.4	7.5	36	6	TGB	187
Allexivirus	~800	~3.2	8.1–8.8	26–28	6	TGB	175–194
Carlavirus	610–700	~3.4	7.4–8.5	31–40	6	TGB	226–238
Foveavirus	723–800+	3.6–4.0	8.7–9.3	28–45	5 or 6	TGB	244–247
Capillovirus	640–700	3.4–3.8	6.5–7.4	25–27	2 or 3	30 K	214–241
Vitivirus	725–785	3.4–3.6	7.4–7.6	21–23	5	30 K	195–196
Trichovirus	640–760	3.8	7.5–8.1	21–22	3 or 4	30 K	216–217
Citrivirus	960	UD	8.7	41	3	30 K	227

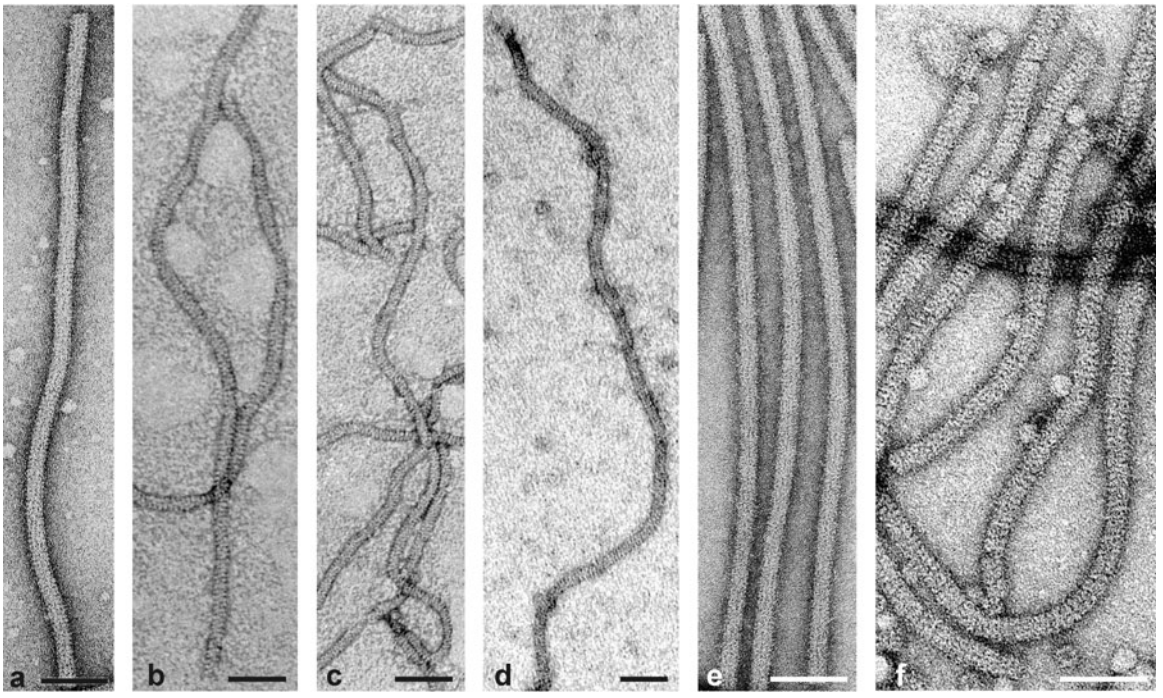


Figure 2

Virus particles of some genera of the family *Flexiviridae*. (*a*) *Potato virus X* (*Potexvirus*). (*b*) *Grapevine virus B* (*Vitivirus*). (*c*) *Apple chlorotic leaf spot virus* (*Trichovirus*). (*d*) *Apple stem pitting virus* (*Foveavirus*). (*e*) *Carnation latent virus* (*Carlavirus*). (*f*) *Indian citrus ringspot virus* (*Mandarivirus*). Bars = 50 nm. (*a*, *e*, *f* courtesy of Dr. R.G. Milne; *b*, *c*, courtesy of Dr. M.A. Castellano; *d*, courtesy of Dr. A. De Stradis.)

diameter of 12–14 nm (**Table 1**) and, when discernible, show an axial canal of ~1.5 nm in diameter. There are 8.9 subunits per turn of the helix in PVX virions (98, 108, 114). Depending on the genus, flexiviruses exhibit varying degrees of flexibility, with potexviruses and carlaviruses being the least flexuous. In fact, the latter are semirigid and show a tendency to curve to one side (85). Citri- and capilloviruses have an intermediate flexibility, whereas virions of all remaining genera are extremely flexuous, with an open structure readily visible in the electron microscope (**Figure 2**).

The surface of carlavirus and potexvirus particles is marked by intersecting sets of distinct grooves (85), one of which runs along the particle length and the other follows the viral helix (98). The particles of all family members exhibit distinct cross-banding. This feature is outstanding in the species of the very flexuous genera and is less evident in potexviruses and carlaviruses (85). Cross-banding spacing corresponds to the pitch of the primary helix (12, 114). Carlavirus and potexvirus particles are marked also by near-longitudinal lines along their length that correspond to rows of subunits lying on secondary helices (85, 114).

GENOME ORGANIZATION AND EXPRESSION

Genome organization of the typical *Flexiviridae* family members is summarized in **Figure 3**. Although the number of ORFs (open reading frames) varies between genera and sometimes between species, the first and largest ORF is always a replicase gene.

ORF: open reading frame

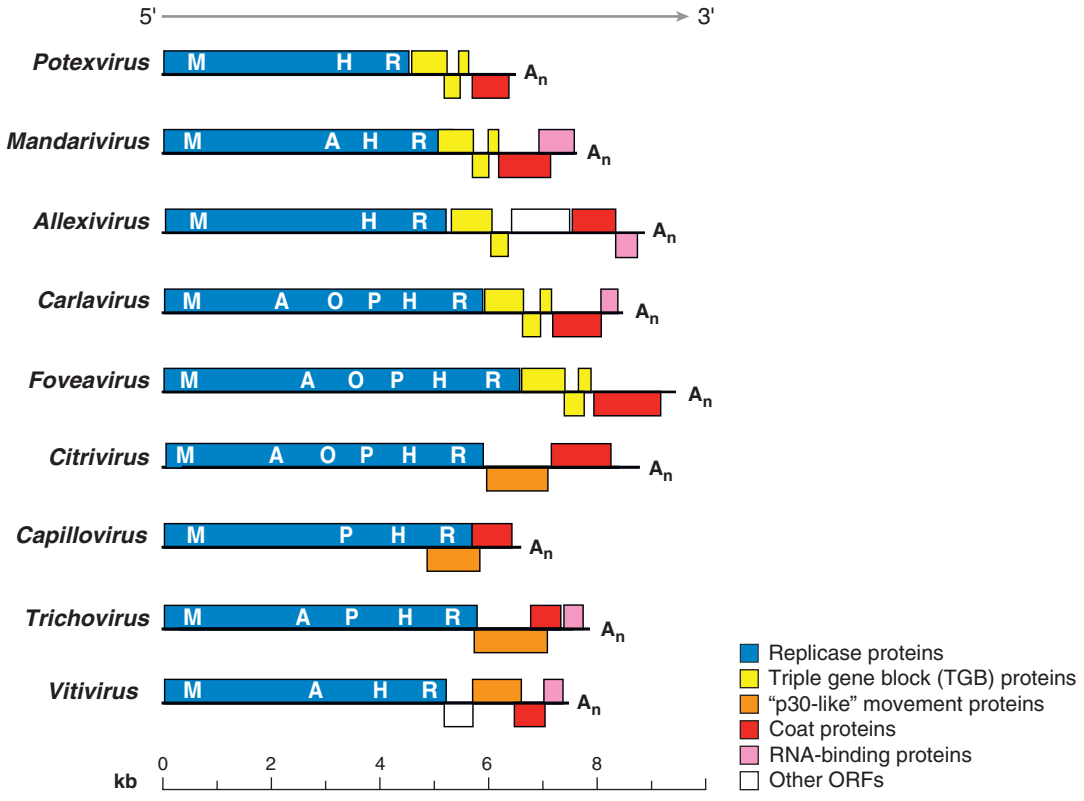


Figure 3

Diagram showing genome organization for the genera of the family *Flexiviridae*. Blocks represent predicted open reading frames (ORFs). The methyltransferase (M), AlkB (A), OTu-like peptidase (O), papain-like protease (P), RNA helicase (H), and RNA-dependent RNA polymerase (R) domains of the replicase are shown. RNA-binding proteins are present in some, but not all, members of the genus *Trichovirus*.

This is likely the only ORF translated directly from the capped genomic RNA, although it was suggested for GVA that a shorter-than-genomic RNA could also be involved in replicase expression (36). The remaining ORFs are expressed from 3′-coterminal subgenomic (sg) mRNAs, some of which could be bicistronic. At least in some potexviruses, sgRNAs are packaged (7).

Replicase ORF

Although the size of the replicase protein varies between the family members from ~150 to ~250 kDa, this protein invariably contains a set of functional domains whose amino acid sequences and order are conserved in all viruses of the alphavirus-like superfamily of the positive-strand RNA viruses (60). A methyltransferase type 1 domain (Met; **Figure 3**) is located near the N terminus of the replicase (104). This domain functions in capping the viral RNAs and in the case of *Bamboo mosaic virus* (BaMV, genus *Potexvirus*) was shown to exhibit both GTP methyltransferase and guanylyltransferase activities (53). A catalytic RNA-dependent RNA polymerase domain (RdRp; **Figure 3**) with a characteristic core motif S/TGx$_3$Tx$_3$NS/Tx$_{22}$GDD is present near the C terminus of the replicase. It was shown that the BaMV RdRp is capable of recognizing the 3′-terminal

sg: subgenomic

RdRp: RNA-dependent RNA polymerase domain

pseudoknot structure to initiate minus-strand RNA synthesis (21). An RNA helicase domain of superfamily 1 (Hel; **Figure 3**) is localized upstream from the RdRp domain (44). Similar to Met and RdRp, Hel is indispensable for viral RNA replication, as demonstrated for PVX (26). Besides its presumed function in unwinding RNA, BaMV Hel was also implicated in cap formation (71).

In addition to the Met-Hel-RdRp module, replicases of the animal viruses of the alphavirus-like superfamily universally possess a papain-like cysteine protease domain (P-Pro) that processes the replicase polyprotein (60). The distribution of P-Pro among plant alphavirus-like viruses is more limited. In the family *Flexiviridae*, P-Pro is located upstream from Hel in carla-, capillo-, fovea-, citri-, and trichoviruses, and in BanMMV, CGRMV, CNRMV and SCSMaV, as was first demonstrated by (68) for *Blueberry scorch virus* (BlScV), genus *Carlavirus* (**Figure 3**). In BlScV, the autocatalytic processing by P-Pro appears to be required for RNA replication. In potexviruses and other genera lacking P-Pro, the replicase polyprotein functions in a nonprocessed form (7).

Subsequently, a second proteinase domain located immediately upstream from the papain-like protease was recognized in a subset of flexiviruses (13, 74). This domain belongs to a new superfamily of the proteases distantly related to papain and typified by the Ovarian Tumor (OTU) gene involved in oocyte morphogenesis in *Drosophila*. In addition to eukaryotic organisms, OTU proteases are found in several pathogens including diverse DNA, negative-strand RNA, and positive-strand RNA viruses where they were suggested to play a significant role in virus-host interactions, possibly involving the ubiquitin-dependent pathway (74). Our search of the PFAM database confirmed the presence of OTU domains in most carla-, fovea-, and fovea-like viruses (J.K., unpublished data). The exact function of this predicted OTU protease in the infection cycles of the flexiviruses presents an interesting problem for future research.

Another unexpected recent finding was the identification of the AlkB-like domain in the replicase polyproteins of several flexiviruses and closteroviruses (5, 13). This domain is located downstream from the Met, similar to OTU, and its function in virus reproduction is currently unknown. Although the AlkB domain was identified in most of the *Flexiviridae* genera, it exhibits nontrivial distribution among the viruses within genera (see below).

OTU: Ovarian Tumor

TGB: triple gene block

ORFs Encoding Movement Proteins

Downstream of the replicase ORF, flexiviruses encode one or more proteins involved in viral cell-to-cell movement. Five of the family genera, *Allexivirus*, *Carlavirus*, *Foveavirus*, *Mandarivirus*, and *Potexvirus*, as well as BanMMV, CGRMV, CNRMV, and SCSMaV, possess a set of three partially overlapping ORFs known as a triple gene block (TGB) (89). Most likely, these ORFs are expressed from two species of the 3′-coterminal sgRNAs (90, 118). A TGB module is also found in members of the genera *Benyvirus*, *Hordeivirus*, *Pecluvirus*, and *Pomovirus*, alphavirus-like plant viruses with rod-shaped virions. However, the first and third proteins of the TGB of these viruses are considerably larger than the corresponding proteins in the family *Flexiviridae* (91). Functions of the TGB proteins were recently reviewed and are not discussed in detail here (see 91, 119). In brief, a ~25-kDa TGBp1 has ATPase, RNA-binding and RNA-helicase activities, and is believed to increase the size exclusion limit of plasmodesmata. The smaller proteins TGBp2 and TGBp3 have, respectively, two and one transmembrane domains, and localize to endomembranes and cell walls. The three proteins are believed to act in concert to enable cell-to-cell movement of the viral genomes. Although the exact mechanism by which the movement occurs remains unknown, it was found that TGBp1 of PVX possesses RNA silencing suppressor activity (122). Moreover, it was shown that this activity

TMV: *Tobacco mosaic virus*

CP: capsid protein

is essential, but not sufficient, for PVX movement (8).

The *Flexiviridae* genera *Capillovirus*, *Citrivirus*, *Trichovirus* and *Vitivirus* have a single movement protein that is a member of the 'p30-like' superfamily typified by the 30-kDa movement protein of *Tobacco mosaic virus* (TMV) (69, 82, 92). The p30-like movement proteins occur in a large number of diverse plant viruses with RNA and DNA genomes. Mutational study confirmed the role of the p30-like protein in the cell-to-cell movement of GVA (38, 105). It was also found recently that the 50-kDa movement protein of ACLSV suppresses systemic, but not local, RNA silencing (125). Therefore, the functional profile of this protein overlaps that of the unrelated PVX movement protein, TGBp1, emphasizing the role of silencing suppression in virus transport and mechanistic convergence among the two classes of the flexiviral movement proteins.

3′-Proximal ORFs Encoding Capsid and Other Proteins

The ORF coding for the capsid protein (CP) is either the 3′-most or penultimate ORF in the flexiviral genomes (**Figure 3**). Typically, CP is expressed from an abundant, functionally monocistronic sgRNA (30, 38). In the genus *Capillovirus*, the CP ORF is contiguous with the replicase ORF, but analysis of the RNA patterns suggested that the CP may be expressed from a sgRNA (73).

In the genera *Allexivirus*, *Carlavirus*, *Mandarivirus* and *Vitivirus*, the 3′-most ORFs encode RNA-binding proteins with a zinc ribbon motif (20, 37, 47). Similar proteins are found in some, but not all, viruses in the genus *Trichovirus* (54). It was recently shown that the nucleic acid-binding protein of GVA is able to suppress RNA silencing (127), suggesting that this function may be conserved in other related proteins of flexiviruses.

Viruses in the genera *Allexivirus* and *Vitivirus* possess additional ORFs encoding unique proteins of unknown functions (**Figure 3**). Among these, the 42-kDa product of the allexivirus ORF4 was implicated in virion assembly (120), while the 19-kDa product of the vitivirus ORF2 was suggested to aid virus transmission by the mealybugs (38).

EVOLUTIONARY HISTORY

The availability of entire genome sequences for a large number of viruses and their host organisms has enabled large-scale phylogenetic analyses and provided unprecedented opportunities for data-informed reconstructions of viral origins and evolution in relation to evolution of cellular life forms (24, 35, 61). It is now widely accepted that the positive-strand RNA viruses share a universally conserved RdRp and represent an ancient, monophyletic, evolutionary lineage (3, 56, 60). The absence of viral RdRp homologs among cellular organisms was recently interpreted to support its direct origin from a precellular RNA-protein world (62). Furthermore, it was argued that the stark contrast between the scarcity of the prokaryote-infecting positive-strand RNA viruses and the enormous abundance of such viruses in eukaryotes suggests that the diverse lineages of the extant positive-strand RNA viruses have evolved at the time of eukaryogenesis (62). This notion finds increasing support in the discovery of marine positive-strand RNA viruses that infect evolutionary distant unicellular eukaryotes (25, 65, 93, 110). Indeed, the fact that these marine viruses belong to picornavirus-like and flavivirus-like superfamilies indicates that these superfamilies have evolved in early eukaryotes. Much less plausible is an alternative scenario that would call for massive introduction of viruses that infect land plants and animals into marine plankton communities. Alphavirus-like viruses of unicellular eukaryotes are yet to be found. Because the deep evolutionary roots of the alphavirus-like superfamily cannot thus be revealed, we focused on the later phase in evolution that yielded current genomic diversity within the family *Flexiviridae*. The evolutionary scenario

for flexiviruses was deduced using extensive phylogenetic analysis of the viral replicases, CPs, and movement proteins.

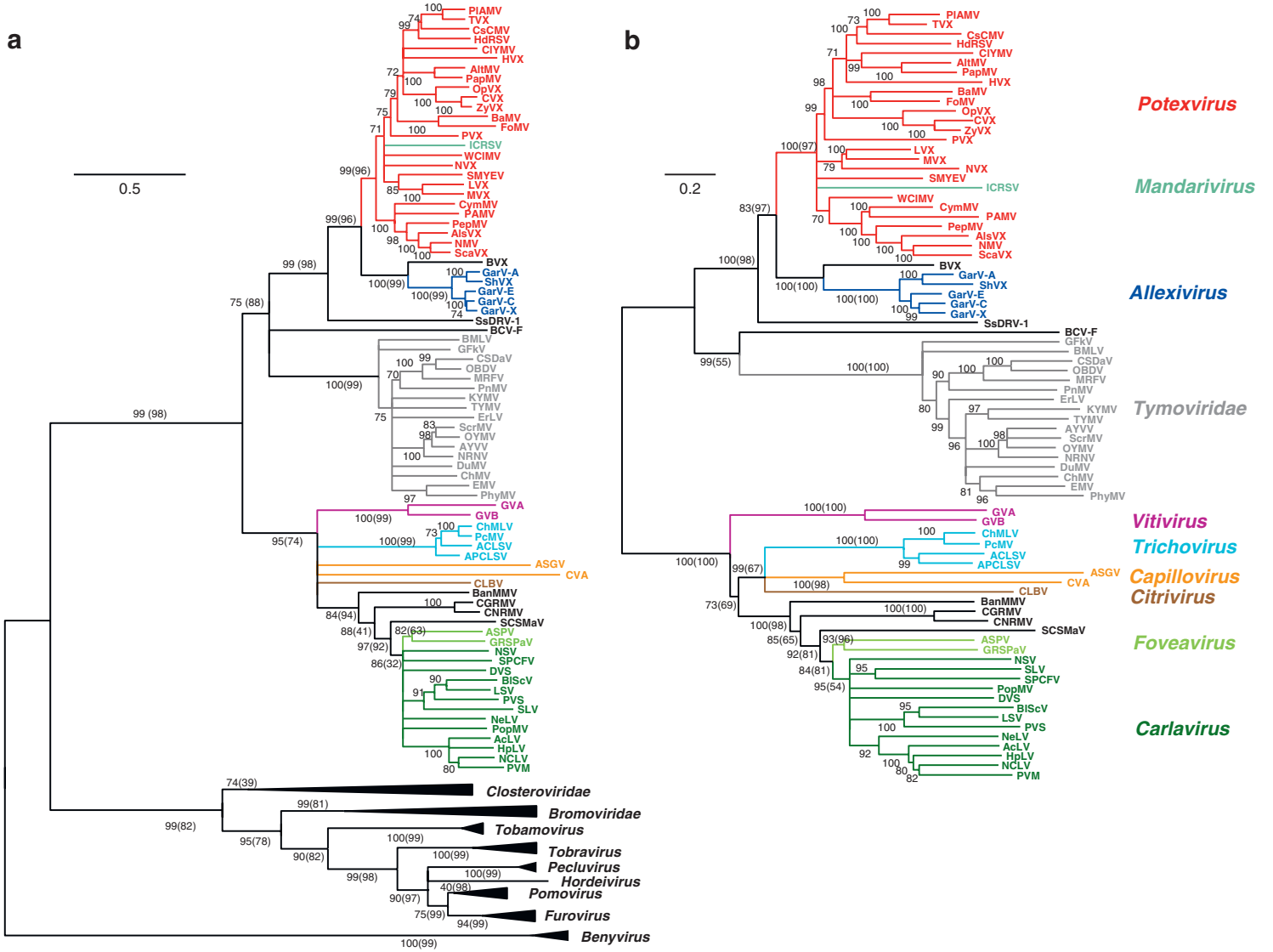
Phylogenetic Analyses of the Viral Proteins

The replicase. Because RdRp is the only protein universally encoded in the nondefective positive-strand RNA viruses, its phylogeny is the principal determinant of the evolutionary framework in this vast virus class. In addition, the Met and Hel domains are conserved throughout the alphavirus-like superfamily (60), therefore facilitating further refinement of the viral phylogenies. In agreement with the original finding (103), our expanded analysis of the three replication-related domains revealed that within the alphavirus-like superfamily, families *Flexiviridae* and *Tymoviridae* are most closely related to each other to the exclusion of other plant virus families such as *Closteroviridae* and *Bromoviridae* and unassigned genera such as *Tobamovirus* and *Benyvirus* (**Figure 4*a***). Moreover, our analysis shows that the *Flexiviridae* can be divided into two clusters, one containing potex-, allexi-, and mandariviruses (collectively called here potexvirus-like viruses), and the other containing carla-, viti-, capillo-, fovea-, and trichoviruses (carlavirus-like viruses). Surprisingly, the replicases of the potexvirus-like viruses are more closely related to those of the *Tymoviridae* than to the carlavirus-like replicases (**Figure 4*b***).

In addition to plant flexi- and tymoviruses, this evolutionary lineage of the viral replicases includes three fungal viruses, Botrytis cinerea virus F (BCV-F), Botrytis virus X (BVX), and Sclerotinia sclerotiorum debilitation-associated RNA virus (SsDRV); two flexuous filamentous viruses; and a capsid-less virus, respectively (51, 52, 124). Conspicuously, our analysis of the complete replicase sequences (**Figure 4*b***), as well as separate Hel domains (not shown), suggested that the filamentous BCV-F is related more closely to the *Tymoviridae* than to any of the *Flexiviridae* clusters. This conclusion is also supported by the similarity of the papain-like proteinases found in BCV-F and tymoviruses that both belong to the C21 PFAM family of endopeptidases (results of HMMER search of PFAM database performed in this study), unlike proteases of carlavirus-like viruses that are of C23 or C34 PFAM families (potexvirus-like viruses lack proteases altogether). In contrast, BVX shows highest similarity to allexiviruses, whereas SsDRV is a peripheral member of the potexvirus-like cluster (**Figure 4**). Such remarkable evolutionary affinities between the fungal and plant RNA viruses are not without a precedent: The capsid-less viruses of the family *Hypoviridae* are the evolutionary kin of the plant family *Potyviridae* (95). Collectively, these observations suggest the amazing propensity of the filamentous plant viruses to cross the host kingdom barriers and spawn their evolutionary descendants to plant-pathogenic fungi. This direction of transfer seems to be supported by the higher diversity of relevant plant viruses and affinity of their fungal cousins to particular plant virus lineages.

The capsid proteins. As noted above, CPs of the flexiviruses share the conserved structural core and evolutionary origin with CPs of two other families of filamentous plant viruses, *Closteroviridae* and *Potyviridae* (19, 29). A phylogenetic tree of the flexiviral core CP sequences reveals two clusters with good bootstrap support (**Figure 5*a***). The first and largest cluster contains potex-, allexi-, mandari-, carla-, fovea-, and citriviruses, as well as the filamentous fungal virus BVX. The second cluster includes capillo-, tricho-, and vitiviruses along with another fungal virus, BCV-F. However, even though both the replicase tree (**Figure 4**) and the CP tree (**Figure 5*a***) contain two major clusters each, the sets of the genera that fall into these clusters are distinct.

The movement proteins. As discussed above, flexiviruses possess two unrelated types



of movement proteins that belong to either the TGB complex or the p30-like superfamily. In accord with previous work (reviewed in 91), our phylogenetic analysis shows that flexivirus TGB proteins represent a compact cluster distinct from their evolutionary counterparts in the rod-shaped plant viruses of the alphavirus-like superfamily (**Figure 5*b***). Therefore, the monophyletic origin of the flexiviral TGB proteins seems to be well supported.

The p30-like movement proteins of plant viruses are generally quite variable (92). Due to this and the availability of fewer sequences, support for the monophyletic relationship of the flexiviral p30-like proteins is not very robust beyond the genus level, yet sufficient to claim their common origin (data not shown; see also 82).

AN EVOLUTIONARY SCENARIO FOR THE *FLEXIVIRIDAE/ TYMOVIRIDAE* LINEAGE

We used comparisons of the phylogenetic trees for viral proteins and the genome designs of the extant viruses to reconstruct a hypothetical common ancestor and to develop the most parsimonious scenario for the diversification of the family *Flexiviridae*. The former task presents an immediate challenge due to the undisputable clustering of the potexvirus-like replicases with those of tymoviruses to the exclusion of carlavirus-like replicases (**Figure 4**). It appears that the most sensible way of solving this paradox is recognizing that the family *Tymoviridae* and two related, but distinct, evolutionary clusters comprising the family *Flexiviridae* shared a common evolutionary ancestor.

In an attempt to reconstruct an ancestor of the *Flexi-/Tymoviridae* lineage, we postulated that it was a plant virus encoding a minimal set of proteins required for RNA replication, encapsidation, and virus movement from cell to cell. Because all flexi- and tymoviruses possess closely related Met, Hel, and Pol domains, these domains must have been present in the ancestor's replicase (**Figure 6**). The presence of a Met domain implies that the ancestor's genome was 5′-capped. Because the genomes of all but one extant genus in this lineage are polyadenylated, the common ancestor was likely to possess a poly(A) tail that was replaced by a tRNA-like structure in the ancestor of the genus *Tymovirus* (32).

It also appears likely that the ancestral replicase harbored a P-Pro domain. Indeed, this domain is present in all tymoviruses and flexiviruses with carlavirus-like replicase with a single exception of the genus *Vitivirus* (**Figure 4**). Moreover, related P-Pro is universally conserved in the animal viruses of the alphavirus-like superfamily. The simplest explanation of such a distribution pattern is that P-Pro was present in a common ancestor of

Figure 4

Phylogenetic trees of replicase amino acid sequences. Genera within the *Flexiviridae* are indicated in different colors. (*a*) Tree created from the fused alignments of methyl-transferase, helicase, and polymerase conserved core domains of plant infecting "Alpha"-like viruses. Deletion of the variable regions between the conserved domains was required to obtain a robust alignment; (*b*) tree created from the alignment of the entire replicase sequence of flexiviruses, tymoviruses, and related viruses. Alignments and phylogenetic analysis were performed using the Mega 3.1 package (63). Presented trees were created by neighbor joining using the JTT matrix and pair-wise gap deletion. Numbers on branches indicate percentage of bootstrap support out of 1000 bootstrap replications. Branches with less than 70% bootstrap support were collapsed. Similar phylogenies were obtained using the Maximum Parsimony method, and the corresponding bootstrap values are indicated between brackets in branches above the genus level. Scales indicate JTT amino acid distances. Distantly related genera and families that formed well-supported monophyletic clades were collapsed into a triangle, the size of which corresponds to the variation found within the clade. Accession numbers of sequences used for constructing the trees are in **Table 2**.

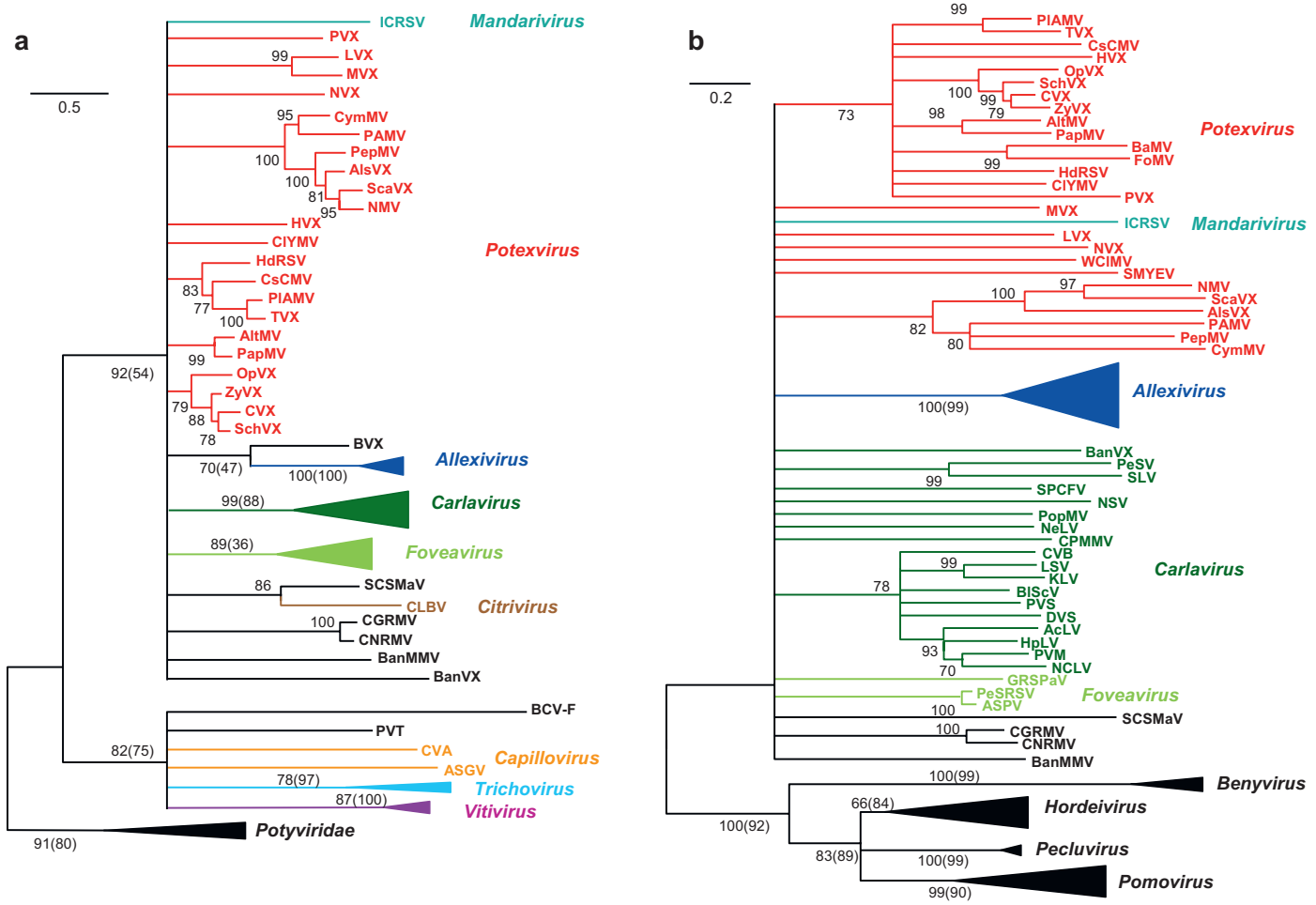


Figure 5

Phylogenetic analyses of the flexivirus protein sequences: (*a*) capsid protein; (*b*) triple gene block protein 1 helicase domain. Colors and methods are as in **Figure 4**. Accession numbers of sequences used for constructing the trees are in **Table 2**.

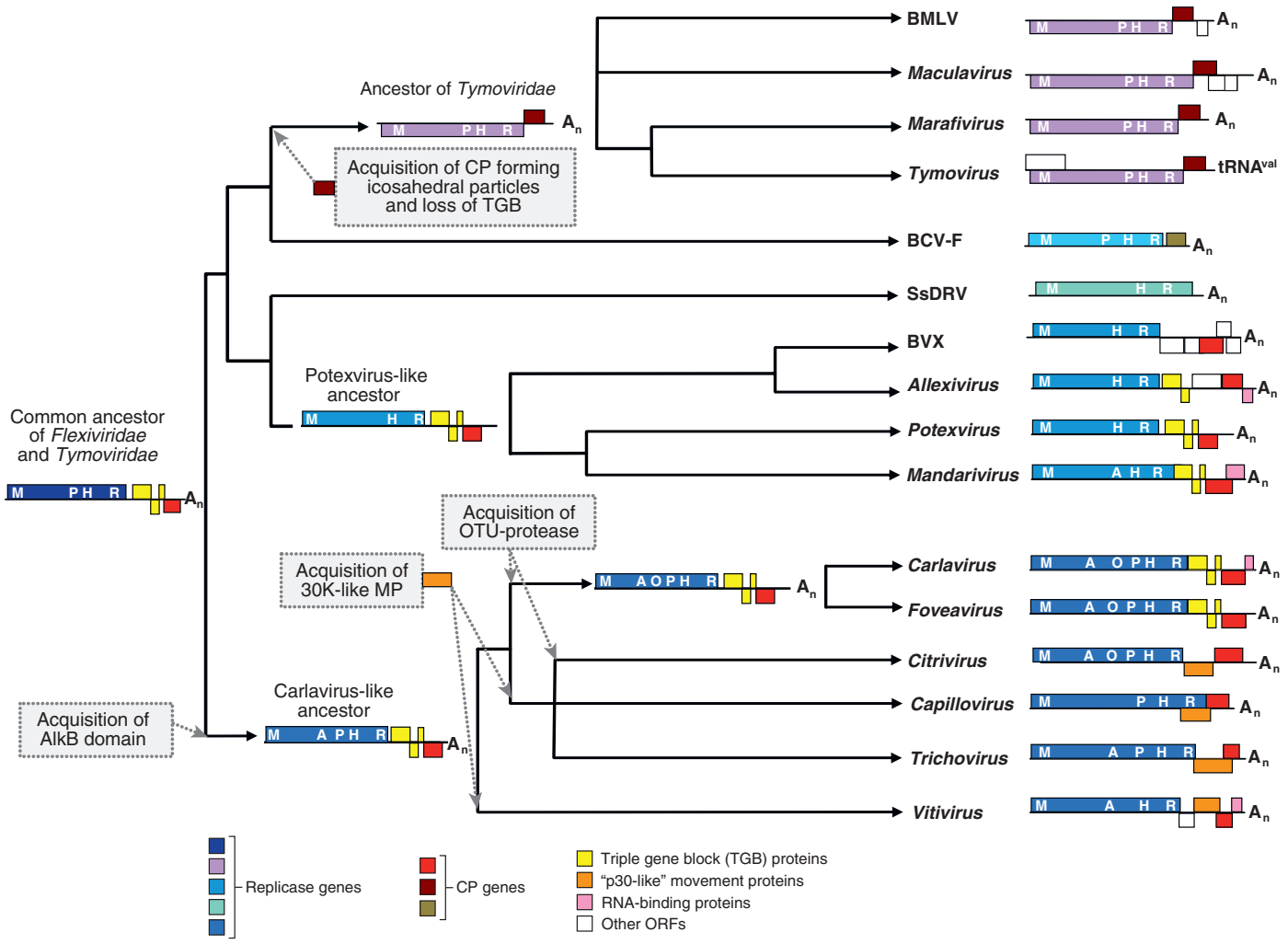


Figure 6

Evolutionary scenario for the families *Flexiviridae* and *Tymoviridae*. Boxes represent open reading frames. Replicase gene domains are as in **Figure 3**. M, methyltransferase; A, AlkB; O, OTu-like peptidase; P, papain-like protease; H, RNA helicase; R, RNA-dependent RNA polymerase.

the entire alphavirus-like superfamily (60), as well as in the common ancestor of the flexi-/tymoviral lineage with independent loss of P-Pro in the potexvirus-like viruses and vitiviruses.

Since two of the three major clusters within the *Flexi-/Tymoviridae* lineage possess filamentous capsids, it is reasonable to assume that the common ancestor of this lineage was also a filamentous virus. This notion is supported by the fact that two fungal viruses with evolutionary affinities to either tymoviruses (BCV-F) or potexvirus-like viruses (BVX) also have filamentous particles. Under this scenario, the last common ancestor of the family *Tymoviridae* acquired an unrelated CP, forming icosahedral particles after divergence from the BCV-F ancestor (**Figure 6**). The alternative, implying that the common ancestor had an icosahedral particle, would require CP replacement on three independent occasions involving ancestors of the potexvirus-like and carlavirus-like viruses, as well as BVF, which seems less likely.

It is a much harder proposition to define the ancestral type of a movement protein for the *Flexi-/Tymoviridae* lineage. Some viruses within the family *Tymoviridae* possess a unique type of movement protein with no homologs outside the family, whereas in others there is no recognizable movement protein at all (79). As described above, the flexiviruses employ either TGB-type or p30-like movement proteins. However, TGB proteins are present in both potexvirus-like and carlavirus-like clusters of flexiviruses, whereas p30-like proteins are present only in the latter cluster. Furthermore, TGBp1 of the flexiviruses are significantly smaller than those found outside this family. This observation may suggest that flexiviral TGBp1 is closer to an ancestral form of this protein, while accretion of additional domains in TGBp1 of rod-shaped viruses occurred later in evolution.

Taken together, these considerations suggest that the common ancestor of the *Flexi-/Tymoviridae* lineage possessed a TGB movement complex that has been lost in tymoviruses concomitant with the CP replacement. We further propose that after the separation of two flexiviral evolutionary clusters, the potexvirus-like viruses retained TGB, whereas p30-like movement proteins have replaced TGB proteins in a subset of the carlavirus-like genera (**Figure 6**).

Phylogenetic analysis of the flexiviral CPs reveals strong correlation of the CP evolution with the type of movement protein present in a viral genome (**Figure 5*a***). The larger of the two major CP lineages includes viruses that possess TGB, while the smaller includes capillo-, tricho-, and vitiviruses, all of which have p30-like proteins. This phylogenetic pattern is indicative of coevolution of CPs and movement proteins, possibly due to a coupling of the encapsidation and movement functions in the viral life cycle. The only exception from such protein coevolution in flexiviruses is CLBV, which possesses a p30-like protein in combination with a CP that clusters with those of TGB-harboring viruses. This anomaly can be explained by a relatively recent recombination event that replaced one of the components of the usual movement protein-CP couple.

The distribution pattern of the AlkB domain within replicases of numerous positive-strand RNA viruses represents an interesting evolutionary puzzle. So far, this domain was found in all but one (*Capillovirus*) genera of the *Flexiviridae* (**Figure 7**) and in none of the *Tymoviridae* genera (**Figure 6**). Conspicuously, AlkB is present in some, but not all, viruses that belong to the same genus. For instance, among *Potexvirus* members, *Papaya mosaic virus* (PapMV) and ClYMV possess AlkB, whereas PVX and WClMV do not. Likewise, in the genus *Carlavirus*, AlkB is found in BlScV and *Poplar mosaic virus* (PopMV), but not in *Potato virus M* (PVM) or PVS. There are, however, only a handful of known AlkB-containing viruses outside the family *Flexiviridae* (**Figure 7**). These include *Grapevine leafroll-associated virus-3* (GLRaV-3) and Little cherry virus-1 (LChV-1) (family *Closteroviridae*), Blackberry virus Y (BVY) (family

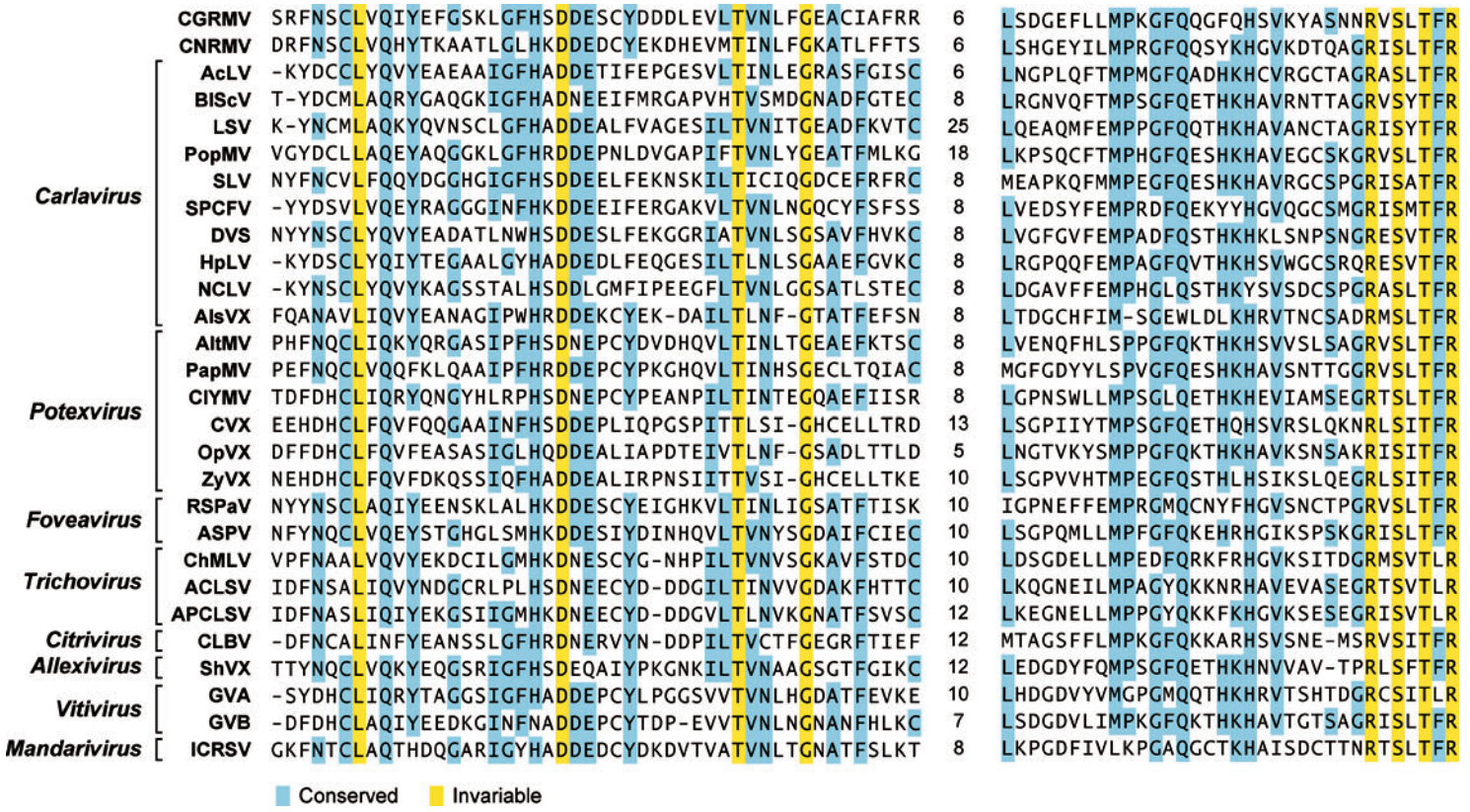


Figure 7

Alignment of AlkB domains from flexiviruses. Conserved amino-acids of the AlkB domain are highlighted in blue, invariable residues in yellow.

Table 2 Abbreviations, names, taxonomic position and sequence accession numbers of the viruses reported in this article. Only names of definitive virus species are italicized.

Abbreviations	Virus names	Genus	Family	Acc. No
ACLSV	*Apple chlorotic leaf spot virus*	*Trichovirus*	*Flexiviridae*	m58152
AcLV	*Aconitum latent virus*	*Carlavirus*	*Flexiviridae*	ab051848
AlsVX	*Alstroemeria virus X*	*Potexvirus*	*Flexiviridae*	ab206396
AltMV	*Alternanthera mosaic virus*	*Potexvirus*	*Flexiviridae*	ay863024
AOPRV	African oil palm ringspot virus	*Foveavirus*	*Flexiviridae*	af468004
APCLSV	*Apricot pseudo-chlorotic leaf spot virus*	*Trichovirus*	*Flexiviridae*	ay713379
ApLV	*Apricot latent virus*	*Foveavirus*	*Flexiviridae*	af057035
ApruV-1	Asian prunus virus 1	*Foveavirus*	*Flexiviridae*	dq205236
ApruV-2	Asian prunus virus 2	*Foveavirus*	*Flexiviridae*	dq205237
ApruV-3	Asian prunus virus 3	*Foveavirus*	*Flexiviridae*	dq205238
ASGV	*Apple stem grooving virus*	*Capillovirus*	*Flexiviridae*	d14995
ASPV	*Apple stem pitting virus*	*Foveavirus*	*Flexiviridae*	d21829
AYVV	*Anagyris vein yellowing virus*	*Tymovirus*	*Tymoviridae*	ay751780
BaMV	*Bamboo mosaic virus*	*Potexvirus*	*Flexiviridae*	d26017
BanMMV	*Banana mild mosaic virus*	Unassigned	*Flexiviridae*	af314662
BanVX	Banana virus X	Unassigned	*Flexiviridae*	ay710267
BCV-F	Botrytis cinerea virus F	Unassigned ssRNA+ virus		af238884
BVY	Blackberry virus Y	Unassigned	*Potyviridae*	ay994084
BRpNV	Black raspberry necrosis virus	*Sadwavirus*	Unassigned	Not available
BlScV	*Blueberry scorch virus*	*Carlavirus*	*Flexiviridae*	l25658
BMLV	Bombyx mori Macula-like latent virus	*Maculavirus*	*Tymoviridae*	ab186123
BVX	Botrytis virus X	Unassigned ssRNA+ virus	*Flexiviridae*	ay055762
CGRMV	Cherry green ring mottle virus	Unassigned	*Flexiviridae*	af017780
ChMLV	Cherry mottle leaf virus	*Trichovirus*	*Flexiviridae*	af170028
ChMV	*Chayote mosaic virus*	*Tymovirus*	*Tymoviridae*	af195000
CLBV	*Citrus leaf blotch virus*	*Citrivirus*	*Flexiviridae*	aj318061
CLV	*Carnation latent virus*	*Carlavirus*	*Flexiviridae*	aj010697
ClYMV	*Clover yellow mosaic virus*	*Potexvirus*	*Flexiviridae*	d29630
CNRMV	Cherry necrotic rusty mottle virus	Unassigned	*Flexiviridae*	af237816
CoLV	*Cole latent virus*	*Carlavirus*	*Flexiviridae*	ay340584
CPMMV	*Cowpea mild mottle virus*	*Carlavirus*	*Flexiviridae*	af024629
CsCMV	*Cassava common mosaic virus*	*Potexvirus*	*Flexiviridae*	u23414
CSDaV	Citrus sudden death-associated virus	*Marafivirus*	*Tymoviridae*	ay884005
CTV	*Citrus tristeza virus*	*Closterovirus*	*Closteroviridae*	ay170468
CVA	*Cherry virus A*	*Capillovirus*	*Flexiviridae*	x82547
CVB	Chrysanthemum virus B	*Carlavirus*	*Flexiviridae*	s60150
CVX	*Cactus virus X*	*Potexvirus*	*Flexiviridae*	af308158
CymMV	*Cymbidium mosaic virus*	*Potexvirus*	*Flexiviridae*	u62963
DuMV	*Dulcamara mottle virus*	*Tymovirus*	*Tymoviridae*	ay789137
DVS	Daphne virus S	*Carlavirus*	*Flexiviridae*	aj620300

(*Continued*)

Table 2 (*Continued*)

Abbreviations	Virus names	Genus	Family	Acc. No
EMV	*Eggplant mosaic virus*	*Tymovirus*	*Tymoviridae*	j04374
ErLV	*Erysimum latent virus*	*Tymovirus*	*Tymoviridae*	af098523
FoMV	*Foxtail mosaic virus*	*Potexvirus*	*Flexiviridae*	m62730
GarV-A	*Garlic virus A*	*Allexivirus*	*Flexiviridae*	ab010300
GarV-B	*Garlic virus B*	*Allexivirus*	*Flexiviridae*	ab010301
GarV-C	*Garlic virus C*	*Allexivirus*	*Flexiviridae*	ab010302
GarV-D	*Garlic virus D*	*Allexivirus*	*Flexiviridae*	ab010303
GarV-E	*Garlic virus E*	*Allexivirus*	*Flexiviridae*	aj292230
GarV-X	*Garlic virus X*	*Allexivirus*	*Flexiviridae*	aj292229
GarMbLV	Garlic mite-borne latent virus	*Allexivirus*	*Flexiviridae*	Not available
GCLV	*Garlic common latent virus*	*Carlavirus*	*Flexiviridae*	x81138
GFkV	*Grapevine fleck virus*	*Maculavirus*	*Tymoviridae*	aj309022
GINV	*Grapevine berry inner necrosis virus*	*Trichovirus*	*Flexiviridae*	d88448
GLRaV-3	*Grapevine leafroll associated virus-3*	*Ampelovirus*	*Closteroviridae*	af037268
GRSPaV	*Grapevine rupestris stem pitting-associated virus*	*Foveavirus*	*Flexiviridae*	af026278
GVA	*Grapevine virus A*	*Vitivirus*	*Flexiviridae*	x75433
GVB	*Grapevine virus B*	*Vitivirus*	*Flexiviridae*	x75448
GVD	*Grapevine virus D*	*Vitivirus*	*Flexiviridae*	y07764
HdRSV	*Hydrangea ringspot virus*	*Potexvirus*	*Flexiviridae*	ay707100
HpLV	*Hop latent virus*	*Carlavirus*	*Flexiviridae*	ab032469
HpMV	*Hop mosaic virus*	*Carlavirus*	*Flexiviridae*	ab051109
HVX	*Hosta virus X*	*Potexvirus*	*Flexiviridae*	aj620114
ICRSV	*Indian citrus ringspot virus*	*Mandarivirus*	*Flexiviridae*	af406744
KLV	*Kalanchoe latent virus*	*Carlavirus*	*Flexiviridae*	aj293570
KYMV	*Kennedya yellow mosaic virus*	*Tymovirus*	*Tymovirus*	d00637
LiCLSV	*Lilac chlorotic leafspot virus*	*Capillovirus*	*Flexiviridae*	Not available
LSV	*Lily symptomless virus*	*Carlavirus*	*Flexiviridae*	aj516059
LVX	*Lily virus X*	*Potexvirus*	*Flexiviridae*	aj633822
LChV-1	Little cherry virus 1	Unassigned	*Closteroviridae*	y10237
MRFV	*Maize rayado fino virus*	*Marafivirus*	*Tymoviridae*	af265566
MVX	Mint virus X	*Potexvirus*	*Flexiviridae*	ay789138
NCLV	*Narcissus common latent virus*	*Carlavirus*	*Flexiviridae*	am158439
NeLV	*Nerine latent virus*	*Carlavirus*	*Flexiviridae*	dq098905
NMV	*Narcissus mosaic virus*	*Potexvirus*	*Flexiviridae*	d13747
NRNV	Nemesia ring necrosis virus	*Tymovirus*	*Tymoviridae*	ay751778
NSV	Narcissus symptomless virus	*Carlavirus*	*Flexiviridae*	am182569
NSPV	Nandina stem pitting virus	*Capillovirus*	*Flexiviridae*	Not available
NVX	*Nerine virus X*	*Potexvirus*	*Flexiviridae*	ab219105
OBDV	*Oat blue dwarf virus*	*Marafivirus*	*Tymoviridae*	u87832
OmbLV	Onion mite-borne latent virus	*Allexivirus*	*Flexiviridae*	Not available
OpVX	Opuntia virus X	*Potexvirus*	*Flexiviridae*	ay366209
OYMV	*Ononis yellow mosaic virus*	*Tymovirus*	*Tymoviridae*	j04375
PAMV	*Potato aucuba mosaic virus*	*Potexvirus*	*Flexiviridae*	s73580

(*Continued*)

Table 2 (*Continued*)

Abbreviations	Virus names	Genus	Family	Acc. No
PapMV	*Papaya mosaic virus*	*Potexvirus*	*Flexiviridae*	d13957
PcMV	Peach mosaic virus	*Trichovirus*	*Flexiviridae*	dq117579
PepMV	*Pepino mosaic virus*	*Potexvirus*	*Flexiviridae*	af484251
PeSRSV	Peach sooty ringspot virus	*Foveavirus*	*Flexiviridae*	af318062
PeSV	*Pea streak virus*	*Carlavirus*	*Flexiviridae*	af354652
PhyMV	*Physalis mottle virus*	*Tymovirus*	*Tymoviridae*	y16104
PlAMV	*Plantago asiatica mosaic virus*	*Potexvirus*	*Flexiviridae*	z21647
PnMV	*Poinsettia mosaic virus*	Unassigned	*Tymoviridae*	aj271595
PopMV	*Poplar mosaic virus*	*Carlavirus*	*Flexiviridae*	x65102
PVM	*Potato virus M*	*Carlavirus*	*Flexiviridae*	d14449
PVS	*Potato virus S*	*Carlavirus*	*Flexiviridae*	aj863509
PVT	Potato virus T	Unassigned	*Flexiviridae*	d10172
PVX	*Potato virus X*	*Potexvirus*	*Flexiviridae*	x05198
PVY	*Potato virus Y*	*Potyvirus*	*Potyviridae*	x12456
RCVMV	*Red clover vein mosaic virus*	*Carlavirus*	*Flexiviridae*	Not available
ScaVX	*Scallion virus X*	*Potexvirus*	*Flexiviridae*	aj316085
SchVX	Schlumbergera virus X	*Potexvirus*	*Flexiviridae*	ay366207
ScrMV	*Scrophularia mottle virus*	*Tymovirus*	*Tymoviridae*	ay751777
SCSMaV	Sugarcane striate mosaic-associated virus	Unassigned	*Flexiviridae*	af315308
ShMbLV	Shallot mite-borne latent virus	*Allexivirus*	*Flexiviridae*	Not available
ShVX	*Shallot virus X*	*Allexivirus*	*Flexiviridae*	m97264
SLV	*Shallot latent virus*	*Carlavirus*	*Flexiviridae*	aj292226
SMYEV	*Strawberry mild yellow edge virus*	*Potexvirus*	*Flexiviridae*	d12517
SPCFV	*Sweet potato chlorotic fleck virus*	*Carlavirus*	*Flexiviridae*	ay461421
SsDRV	Sclerotinia sclerotiorum debilitation-associated RNA virus	Unassigned ssRNA+ virus		ay147260
TMV	*Tobacco mosaic virus*	*Tobamovirus*	Unassigned	v01408
TVX	*Tulip virus X*	*Potexvirus*	*Flexiviridae*	ab066288
TYMV	*Turnip yellow mosaic virus*	*Tymovirus*	*Tymoviridae*	x07441
WClMV	*White clover mosaic virus*	*Potexvirus*	*Flexiviridae*	x06728
ZyVX	*Zygocactus virus* X	*Potexvirus*	*Flexiviridae*	ay366208

Potyviridae) (109), and Black raspberry necrosis virus (BRpNV) (genus *Sadwavirus*) (49).

Close inspection of the list of AlkB-containing viruses (**Figure 7**) allows two generalizations: (*i*) almost exclusively, the AlkB domain is found in replicases of the three families of filamentous plant viruses; (*ii*) most, if not all, AlkB-containing viruses infect woody or perennial plants. The latter tendency may be attributed to the functional advantages provided by AlkB for the long-term survival of viruses within the single infected plant. Indeed, the cellular AlkB was implicated in repair of RNA methylation damage (1), an activity that would provide an obvious benefit of higher genetic stability for viral RNA genomes in woody or perennial plants.

A patchy distribution of AlkB among viral genomes makes it difficult to fathom its exact evolutionary history that would necessarily require multiple events of acquisition and/or loss within different virus taxa.

Because AlkB is most frequently present in the viruses of carlavirus-like cluster of *Flexiviridae*, we marginally favor its inclusion into a common ancestor of the viruses in this cluster (**Figure 6**). Since AlkB is widely conserved in cellular organisms from bacteria to humans, but found only in a minority of plant-infecting viruses, it was most likely acquired into virus genomes via recombination with the corresponding host mRNA. The following evolution of AlkB has likely included its repeated loss in certain carlavirus-like viruses and acquisition by some potexvirus-like viruses via recombination of the viruses coinfecting the same host plant. It also does not seem unreasonable to suggest that *Flexiviridae* donated AlkB to a few AlkB-containing viruses outside this family.

A similar line of reasoning applies to the mosaic distribution of the OTU-like protease among the subset of carlavirus-like viruses. Under the most parsimonous scenario, the OTU domain was acquired by the common ancestor of the carla- and fovea-viruses and donated to citrivirus via horizontal gene transfer (**Figure 6**).

Another protein of uncertain evolutionary history is the Zn-ribbon-containing, RNA-binding protein present in five flexiviral genera (**Figure 3**). These proteins are so variable in sequence that alignments have proven unsuitable for robust phylogenetic reconstruction (data not shown). An apparently random distribution of these five genera in both evolutionary clusters of the flexiviruses (**Figure 6**) is compatible with two scenarios. According to the first, the RNA-binding protein was present in the common ancestor and was lost on multiple occasions. According to a second scenario, this protein was acquired by one of the genera (e.g., *Carlavirus*), and then distributed to other flexiviruses through horizontal gene transfer. It appears that flexiviruses may have donated a related RNA-binding protein to a single virus within the genus *Closterovirus*, *Citrus tristeza virus* (CTV), where it functions as a suppressor of RNA silencing (20, 72).

CONCLUSIONS AND TAXONOMIC IMPLICATIONS

The most prominent aspect of the evolutionary scenario devised here and summarized in **Figure 6** is the unusual fluidity of the flexiviral genomes compared to those of the related rod-shaped or icosahedral RNA viruses of the alphavirus-like superfamily. This fluidity could be explained by the relative ease with which the filamentous virions can accommodate the increase or reduction in the size of the viral genome. Indeed, the only other family of plant viruses that exhibits comparable genomic variability is an evolutionarily distant family of filamentous alphavirus-like viruses, *Closteroviridae* (31).

There is no region of flexiviral genomes that is left untouched by genetic variation. Even the most conservative genomic core that encodes the RNA replicase exhibits multiple events of acquisition or loss of such domains as P-Pro, OTU-like protease, or AlkB. Another major determinant of the viral life style, the virion architecture that is sometimes defined as nothing less than "the viral self" (9), underwent a dramatic transformation at the descent of the *Tymoviridae* family from a common ancestor with potexvirus-like viruses. The levity with which flexiviruses switch the modes of their cell-to-cell movement between TGB and p30-like proteins is also amusing. This levity seems to be even more extreme in the *Tymoviridae* lineage where some viruses possess exotic movement proteins or no apparent movement protein at all, as in the genus *Marafivirus*. The genes for nucleic acid-binding proteins that wander among flexiviral genomes complete the picture of genetic unrest that seems to reign within the *Flexi-/Tymoviridae* evolutionary lineage.

Do flexiviruses benefit from their genetic plasticity? It seems that the answer is yes if one considers the ability of flexiviruses to find novel ecological niches, e.g., by evolving into fungal viruses and sometimes changing the capsid architecture or even leaving it behind, as is the case for SsDRV. Another aspect of

flexiviruses that stands out is their propensity for infecting woody plants. A rapidly growing list of flexiviruses found in trees, shrubs, and vines, from poplars to blueberries to hops (**Table 2**) provides a testimony to this notion.

In addition to revealing the vagaries of virus evolution, our analysis illustrates the difficulty of classifying viruses that unceasingly evolve via exchange of genetic modules. If taxonomy is to reflect the likely evolutionary pathways, it should be based on phylogenetic analysis of the only character shared by all positive-strand RNA viruses, which is the RdRp. We generally followed this line of thought even though we expanded our analysis by inclusion of the Met and Hel domains conserved in the alphavirus-like superfamily (**Figure 4**). A radical taxonomic interpretation of this analysis would call for separation of the existing family *Flexiviridae* into two families corresponding to potexvirus-like and carlavirus-like lineages. A more conformist alternative to this interpretation would take into account additional evolutionary considerations. One of these is a shared origin and morphology of the capsid. Another consideration is that the genomes of flexiviruses are peppered with such genetic modules as TGB and genes encoding a nucleic acid-binding protein or an AlkB domain. Importantly, none of these modules is found in the otherwise closely related family *Tymoviridae*, and, except for TGB and 30 k, these proteins are seldom seen outside flexiviruses. Taken together, our phylogenetic reconstructions seem to favor retaining the family *Flexiviridae*, with the possible establishment of two subfamilies, to account for the bifurcated evolutionary history of the viral replicases.

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Errata

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