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Mycovirus Diversity and Evolution Revealed/Inferred from Recent Studies

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Abstract

High-throughput virome analyses with various fungi, from cultured or uncultured sources, have led to the discovery of diverse viruses with unique genome structures and even neo-lifestyles. Examples in the former category include splipalmiviruses and ambiviruses. Splipalmiviruses, related to yeast narnaviruses, have multiple positive-sense (+) single-stranded (ss) RNA genomic segments that separately encode the RNA-dependent RNA polymerase motifs, the hallmark of RNA viruses (members of the kingdom *Orthornavirae*). Ambiviruses appear to have an undivided ssRNA genome of 3∼5 kb with two large open reading frames (ORFs) separated by intergenic regions. Another narna-like virus group has two fully overlapping ORFs on both strands of a genomic segment that span more than 90% of the genome size. New virus lifestyles exhibited by mycoviruses include the yado-kari/yado-nushi nature characterized by the partnership between the (+)ssRNA yadokarivirus and an unrelated dsRNA virus (donor of the capsid for the former) and the hadaka nature of capsidless 10–11 segmented (+)ssRNA accessible by RNase in infected mycelial homogenates. Furthermore, dsRNA polymycoviruses with phylogenetic affinity to (+)ssRNA animal caliciviruses have been shown to be infectious as dsRNA–protein complexes or deproteinized naked dsRNA. Many previous phylogenetic gaps have been filled by recently discovered fungal and other viruses, which have

provided interesting evolutionary insights. Phylogenetic analyses and the discovery of natural and experimental cross-kingdom infections suggest that horizontal virus transfer may have occurred and continue to occur between fungi and other kingdoms.

INTRODUCTION

Viruses are as omnipresent in fungi as in other host organisms (172). Fungal viruses or mycoviruses attract considerable attention from a few perspectives. First, studies on mycoviruses have revealed the great diversity of viruses in terms of genome organization, lifestyles, and replication cycles. Second, if mycoviruses infect pathogenic fungi and reduce virulence, they have the potential to be used as virocontrol (biological control using viruses) agents. Next-generation sequencing (NGS) technologies have brought about an explosion of virus discoveries, considerably expanded the virosphere, and revolutionized our understanding of virus diversity and evolution (34, 140, 141). This is particularly true for the fungal virome. A large number of mycoviruses or mycoviral-like sequences have been reported since the late Professor S. A. Ghabrial and the corresponding author of this article described mycoviruses in Volume 47 of the *Annual Review of Phytopathology* (51). As shown via a PubMed (**https://pubmed.ncbi.nlm.nih.gov**) search using "mycovirus" as the keyword, the number of mycovirus-related papers published in the past two decades has rapidly and exponentially increased.

The fungal virosphere was believed to be heavily dominated by double-stranded (ds) RNA viruses a few decades ago. This turned out to be inaccurate and is thought to have resulted from a biased methodology (25). Many field-collected culturable fungal isolates were screened for the presence of dsRNA molecules, a hallmark of RNA mycovirus infection, which could be genomic RNA of dsRNA viruses or a replicative form dsRNA of single-stranded (ss) RNA viruses. This approach generally entails enrichment of the viral dsRNA fraction by cellulose affinity column chromatography with high cost-performance (97) and subsequent cDNA library construction followed by Sanger sequencing. However, this approach overlooks RNA viruses with titers of dsRNA below detection levels. It is also relevant that many negative-sense (−) ssRNA viruses and some positive-sense (+) ssRNA viruses do not accumulate dsRNA to high levels (8, 25, 45, 128, 135). However, recent deep RNA sequencing (RNA-seq) approaches using small RNA (36, 100), dsRNA, or ssRNA fractions (38, 92, 93) for cDNA library construction revealed a large number of (+)ssRNA viruses present in the fungal world. In addition, many (−)ssRNA viruses with undivided and divided genomes have been discovered (37, 72, 84, 89, 92, 93, 98, 164, 170). Since the discovery of the ssDNA virus in a phytopathogenic fungus (182), many similar ssDNA viruses have been detected in diverse organisms or environmental samples (20, 79, 83, 99, 146, 163). NGS of the total fungal ssRNA fraction, a possibly unbiased virus hunting method, has suggested that the global fungal virome is dominated by (+)ssRNA and dsRNA viruses, with a more limited representation of (−)ssRNA and ssDNA viruses. Neither pararetroviruses [reverse transcribing (RT) dsDNA viruses] nor true dsDNA viruses have yet been reported in fungi.

It should be noted that what we know about the fungal virus world (the mycovirosphere) is still based on sparse biased sampling of host fungi or related organisms (101). Examples of large-scale virome studies have been performed with culturable phytopathogenic ascomycetes and basidiomycetes, such as *Cryphonectria parasitica* (58), *Sclerotinia sclerotiorum* (178), *Heterobasidion* spp. (150, 160), *Fusarium* spp. (27, 82), *Botrytis cinerea* (55, 117, 131), *Rosellinia necatrix* (74), and *Magnaporthe oryzae* (115). Most of their fungal sources have been from Europe, the United States, and East Asia, after the reduction in the costs of NGS. Large-scale virus hunting studies with NGS

have been expanded biologically to endophytic, saprophytic, and mycorrhizal fungi (105, 145, 149, 157), as well as edible mushrooms (29), in geographically diverse regions of the world, including the Indian subcontinent (69), Africa (95), the Southern Hemisphere (99, 118), and the Arctic (133). Even marine fungi and oomycetes were targeted (14, 15, 103, 104). These studies have led to a better understanding of the great diversity and interesting evolution of fungal-related viruses.

In this review, we introduce the recently established taxonomy of mycoviruses, the diversity in virus lifestyles and genome organizations, and, lastly, the evolutionary histories of mycoviruses. To minimize the reiterations with previous review articles, the reader is encouraged to refer to several excellent review articles on fungal viruses and/or general mycovirus biology (48, 50, 57, 77, 151, 152, 178).

TAXONOMY OF MYCOVIRUSES

Over the past few decades, the diversity of viruses has massively expanded thanks to the advent of new technologies (34). This has led to a few major changes in virus taxonomy by the International Committee on Taxonomy of Viruses (ICTV). Those changes include the approval of virus species based solely on the entire coding sequences without their terminal noncoding sequences (168) that are generally important for virus replication. The ICTV Meta Data Resources (VMR) (version July 20, 2021; **https://talk.ictvonline.org/taxonomy/vmr/m/vmr-file-repository/13175**) now includes many viruses whose biological properties, such as host organisms or infectivity, are unknown. Since the proposal by Wolf et al. (176) that all RNA viruses, now known as members of the kingdom *Orthornavirae*, are divided into five branches corresponding to five phyla, the megataxonomy of the virus world by the ICTV has been established that includes the creation of higher taxa such as realms, kingdoms, phyla, and classes (143). Unrelated to the explosion of virus discoveries, the ICTV has adopted the binominal genus–species naming system for virus species, i.e., a genus name + a species epithet, by the ICTV, which could be (*a*) genus + Latin or Latinized epithet, (*b*) genus + alphanumeric epithet, or (*c*) genus + freeform (144). The descriptions of virus species and viruses in this review conform to the ICTV rules of orthography (183).

According to the latest ICTV taxonomy report and accepted list of all mycoviruses in the ICTV Master Species List 2020.v1 (**https://talk.ictvonline.org/**), there are 23 families containing a total of 206 mycovirus species (**Supplemental Table 1**). The majority of them have RNA genomes and are divided into nine families with (+)ssRNA genomes, two families with (−)ssRNA genomes, seven families with dsRNA genomes, one family with ssDNA genomes, and one family with RT-ssRNA genomes. In addition to these, the ICTV recently (March 2022) approved the creation of the (+)ssRNA viral families *Fusariviridae*, *Hadakaviridae*, and *Yadokariviridae*. How mycoviruses are currently classified into higher ranks of taxonomy, i.e., six phyla, *Negarnaviricota*, *Duplornaviricota*, *Kitrinoviricota*, *Pisuviricota*, *Lenarviricota*, and *Artverviricota*, is shown in **Figure 1**. High levels of similarities are detected in the genome architectures and organization between many fungal and plant RNA viruses. No bona fide dsDNA viruses, which are often found in animals and unicellular eukaryotes and prokaryotes, have yet been reported in fungi, as is the case with higher plants. In this regard, Dolja et al. (35) hypothesized that the presence of cell walls and plasmodesmata could serve as a barrier to infection by large dsDNA viruses. It should be noted that even small dsDNA viruses with a resemblance to polyomaviruses and papillomaviruses have not been discovered from plants. These small dsDNA viruses are detectable in vertebrates and are host specific and tissue specific. The same authors speculated that there is no route for horizontal virus transfer from vertebrates to plants (35), which would require an intimate ecological relationship. However, intimate ecological interactions occur between fungi and animals, which may not be as frequent as those between fungi and plants. There are septal pores for intercellular communication in the case of multicellular filamentous fungi, which are large enough even for cellular organelles to pass

Supplemental Material >

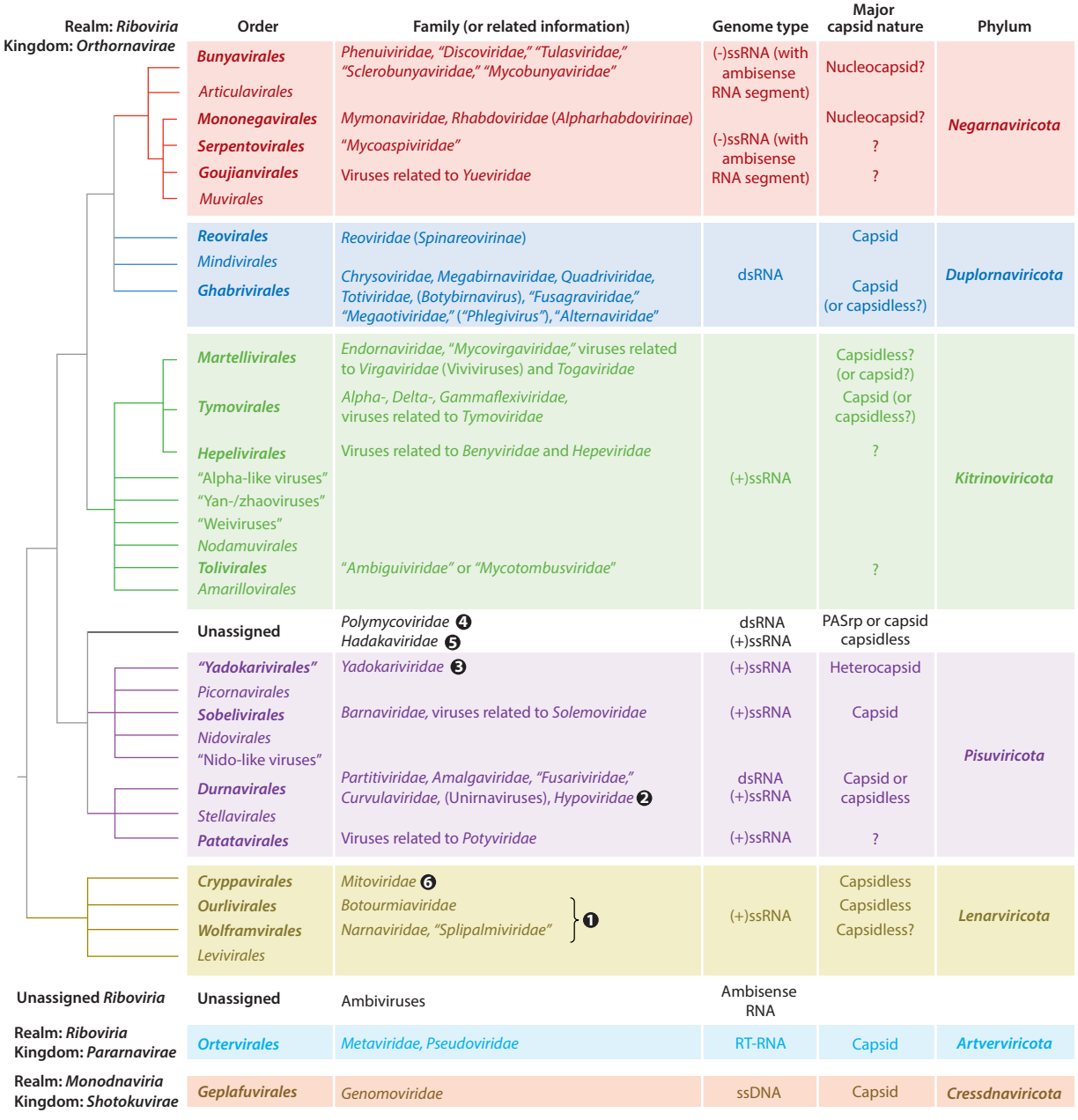

Figure 1

Cladogram of mycoviruses of the kingdom *Orthornavirae* (realm *Riboviria*) together with mycoviruses of kingdoms *Pararnavirae* (realm *Riboviria*) and *Shotokuvirae* (realm *Monodnaviria*). The proposed families (unassigned taxa) are shown in quotation marks. Genus or proposed genus is shown in parentheses. The orders that accommodate mycoviruses are indicated in bold. The encircled numbers (①–⑥) indicate different capsidless mycoviruses or other types of mycoviruses, except for polymycoviruses. "Alpha-like viruses," "Yan-/zhaoviruses," "Weiviruses," and "Nido-like viruses" in the Order column have yet to be classified officially. Descriptions in the Major Capsid Nature column correspond to the Family column. Abbreviations: dsRNA, double-stranded RNA; PASrp, proline-, alanine-, and serine-rich protein; (+), positive sense; (−), negative sense; RT-RNA, reverse transcribing RNA; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA.

through. Thus, dsDNA viruses could establish systemic infection within a colony once they enter fungal cells. There are many as-yet-unclassified mycoviruses, some of which are discussed below.

RECENTLY DISCOVERED MYCOVIRUSES WITH PECULIAR GENOME ORGANIZATION

In the past few years, a vast number of unusual viruses have been discovered in fungi and pseudofungi. These viruses appear to have peculiar genome architectures that were previously unknown. Below are some examples of such mycoviruses.

Yadokariviruses and Related Viruses

Yadokariviruses have been found in several fungi, including *Aspergillus* spp., and have at least two-segment (+)ssRNA genomes related to those of members of family *Virgaviridae* and other plant alpha-like viruses (family *Bromoviridae*) (22, 25, 30) (**Figure 2**). Examples include Plasmopara viticola lesion-associated yadokariviruses 1 to 4 (PvLaVVV1 to PvLaVVV4), Aspergillus flavus yadokarivirus 1 (AflVVV1), and Aspergillus fumigatus RNA virus 1 (AfuRV1). Yadokarivirus was proposed by an Italian research group after virga-virga (yadoka) to refer to two genomic segments of a yadokarivirus that are phylogenetically related to virgaviruses (22). Virga, meaning rod in Latin, was used in the family name *Virgaviridae*, which includes plant rod-shaped viruses such as tobacco mosaic virus (TMV, a tobamovirus) (2). A spherical particle form has been suggested for yadokariviruses (T. Massimo, personal communication). This group of viruses is generally reported to have two to three (+)ssRNA genomic segments but may have more segments depending on the viruses or viral strains (S. Honda, H. Kondo, N. Suzuki, unpublished data). The 5′-terminal sequence is conserved between the genomic segments of a yadokarivirus. The 3′-terminal sequence features vary depending on the particular yadokarivirus: Some yadokariviruses have a poly(A) tail, whereas others do not. A similar difference in the 3′-terminal sequence feature is observed among members of the family *Yadokariviridae* (7). The 3′-terminal sequences are conserved between the genomic segments of some yadokariviruses lacking a poly(A) tail. Yadokariviruses are unique in that their putative RNA methyltransferase (MT) domains are often encoded by two genomic segments separately (22, 25). A similar finding is that some other mycoviruses such as a (+)ssRNA hypovirus from *Rhizoctonia solani* have been reported to have two putative RNA helicase domains (1). The two putative MT domains (MT1 and MT2) are phylogenetically distinct; yadokarivirus MT1 clusters with those of virga-like viruses and is phylogenetically related to members of the family *Virgaviridae* as occurring for yadokarivirus RNA-dependent RNA polymerase (RdRP) domain regions, whereas MT2 is very distant from them and other related viruses (25). This suggests that MT2 domains may have been acquired from distant unknown viruses. Whether these two MT domains are essential for yadokarivirus replication and are functionally different remains elusive.

Ambiviruses and Other Mycoviruses with Ambisense Genome Nature

Another peculiar type of ssRNA viruses was first discovered in endomycorrhizal fungi (149). This group of viruses termed ambiviruses have also been detected in diverse phytopathogenic fungi such as *C. parasitica* (42), *R. solani* (42), *Armillaria* spp. (85), and *H. parviporum* (150). They have an enigmatic nonsegmented RNA genome of 4.5–5.0 kb with an ambisense coding nature, which possesses two open reading frames (ORFs) (A and B) on each strand, as shown in **Figure 2**. No functional motif was found in the two hypothetical proteins by a protein domain motif search. However, ORFA-encoded proteins contain the GDD motif (a hallmark of RdRP) and several key residues of other submotifs conserved in many RNA viruses, and ORFA-encoded proteins are thus assumed to be ambivirus RdRP (42, 149). ORFB-encoded proteins are homologous to

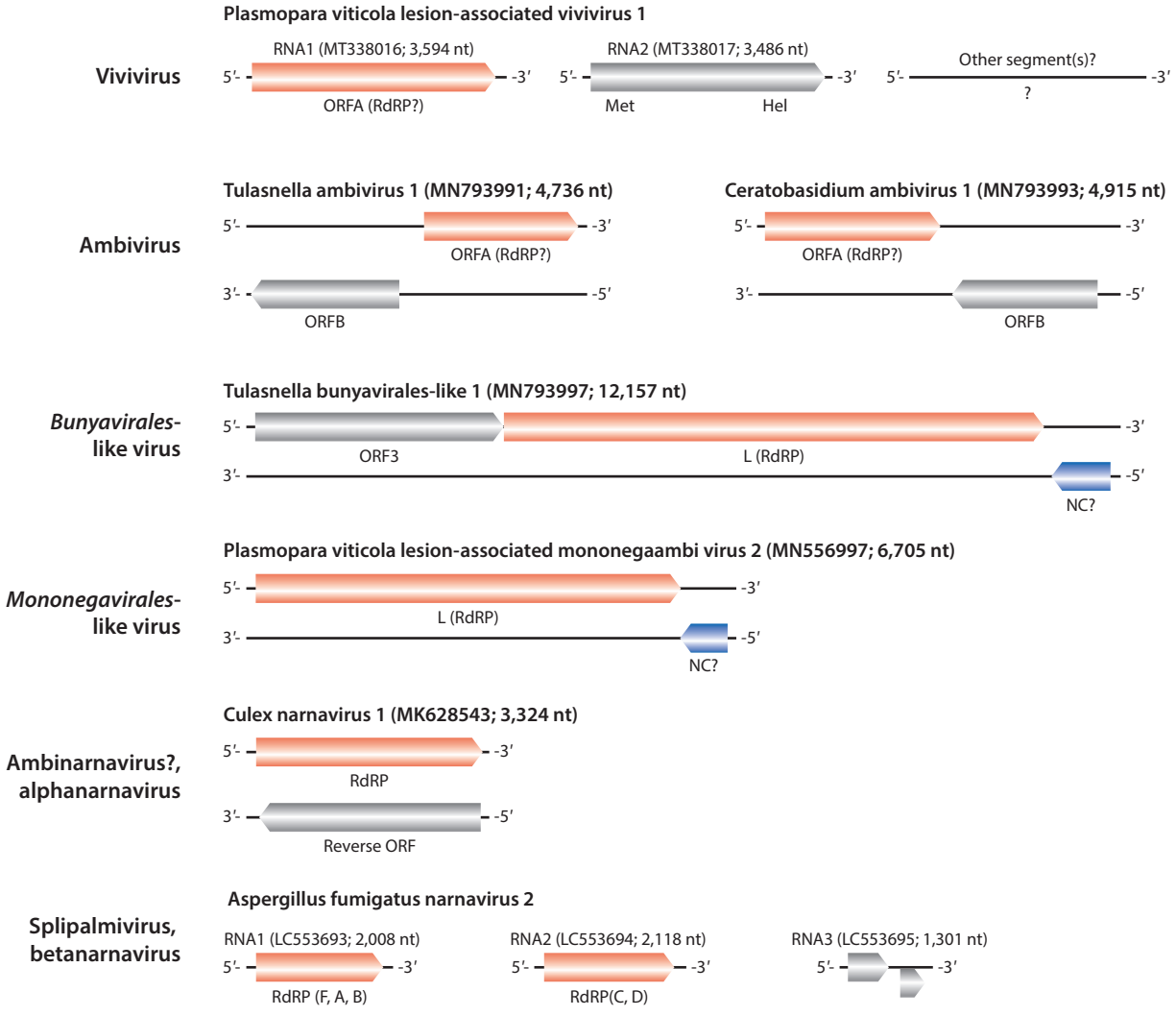

Figure 2

Peculiar genome organizations of recently discovered RNA mycoviruses. Schematic representation of the RNA genome organization with positive-strand or ambisense coding nature. Abbreviations: NC, nucleocapsid; ORF, open reading frame; RdRP, RNA-dependent RNA polymerase.

some ORFans (nonhost RNAs with no significant similarity with known protein sequences) of *Agaricus bisporus* (29). Interesting features of ambivirus are that (*a*), like members of *Bunyavirales* (see below), the two ORFs (A and B) are nonoverlapping and are juxtaposed in a head-to-head or tail-to-tail orientation and (*b*) the most abundant viral RNA form is a dimer ORFA-coding sense strand (149) (**Figure 2**). How the hypothetical proteins are expressed is elusive.

The ambisense coding nature has also been observed in some (−)ssRNA mycoviruses, such as members of the order *Bunyavirales*, including fungal phenuivirids (**Figure 2**; see also the section titled Evolutionary Considerations for Mycoviruses). The established animal and plant phenuivirids have multisegmented genomes, with one or more ambisense segments having two nonoverlapping ORFs and their intergenic regions often predicted to form stable stem-loop

structures. Transcripts are produced by the viral RdRP [large protein (L)] via a cap-snatch mechanism in which the 5′-capped end of host-derived mRNAs is snatched and then serves as a primer for synthesis of the subgenomic mRNAs from either the genome RNA or antigenome RNA strand. The transcription ends within or at the end of the noncoding intergenic region (123, 125). It should be noted that the stable stem-loop structures within ambisense viral segments may hamper sequencing analyses via NGS, leading to misassignment of a single ambisense segment as two fragments, as in the case of a plant-infecting phenuivirus (genus *Coguvirus*) (84, 187). However, additional different ambisense coding profiles have been discovered in fungal (−)ssRNA viruses. For example, some fungus-infecting putative members of the order *Mononegavirales* and *Bunyavirales* have an additional ORF(s) on the antigenomic RNA strand that would encode tentative nucleocapsid (N or NC) protein [known as major structural proteins for (−)ssRNA viruses] (22, 149) (**Figure 2**). Note that all plant and animal mononegaviruses and bunyaviruses encode N or NC genes on the genomic RNA strand (mononegaviruses) or the L-encoding segment (bunyaviruses). See below for other ambisense genomes discovered in (+)ssRNA narna-like viruses possessing an undivided genome with two almost entirely overlapping ORFs that occupy more than 90% of the genome length (22, 31, 33) (**Figure 2**).

Splipalmiviruses and Related Viruses

The fungal (+)ssRNA virus groups within the phylum *Lenarviricota* have been considerably expanded by the discoveries of splipalmiviruses and other related viruses, including mitoviruses and botourmiaviruses (**Figures 2** and **3**). Narnaviruses were historically coined for Saccharomyces cerevisiae 20S and 23S narnaviruses (ScNV20S and ScNV23S) that have a nonsegmented (+)ssRNA genome encoding only RdRP. Narna and narna-like viruses are phylogenetically divided into two groups tentatively termed alphanarnavirus and betanarnavirus (33) (**Figure 3**). The alphanarnavirus group accommodates authentic yeast narnaviruses (ScNV20S and ScNV23S), whereas the betanarnavirus group includes many other mycoviruses, among which are splipalmiviruses from the endomycorrhizal fungi (149) *S. sclerotiorum* (63), *B. cinerea* (131), *H. parviporum* (150), and *A. fumigatus* (25). The name splipalmivirus was recently given to a group of mycoviruses with the separate RdRP palm domains and phylogenetic affinity to narna and narna-like viruses by Turina and colleagues (149) (**Figure 2**). Polynarnavirus (63) and binarnavirus (131) were also proposed by different research groups. For the reasons mentioned by Sato et al. (135), the splipalmivirus was adopted as the appropriate name in this article. The most prominent characteristic of this group is that the divided RdRP domains, the hallmark of RNA viruses within the kingdom *Orthornavirae*, are separately encoded by two genomic segments (split RdRP domains with motifs F, A, and B and motifs C and D, respectively) (**Figure 2**). There are many multisegmented RNA viruses, but these viruses possess all the RdRP motifs in single proteins. No other members of the kingdom *Orthornavirae* have such a split RdRP except for a different type of narna-like virus, Aspergillus tennesseensis narnavirus 1 (AtenNV1) (26) (**Figure 3**). The genomic segment of splipalmiviruses, which encodes motifs C (GDD triad) and D, was identified earlier as representing narna-like viruses from leaves associated with grapevine powdery mildew and grapevine trunk pathogens (e.g., 22, 91). However, the other segments encoding RdRP motifs F, A, and B were not detected as genomic segments by these studies. Given that the presence of a narna-like virus (AtenNV1) was discovered with another type of divided RdRP (motifs F and A and motifs B, C, and D, respectively), the divided RdRP nature of these viruses might be distributed in viruses other than splipalmiviruses in the fungi.

There are a few important unanswered questions about splipalmiviruses. The two proteins encoded by the largest two segments are assumed to make up the functional RdRP complex.

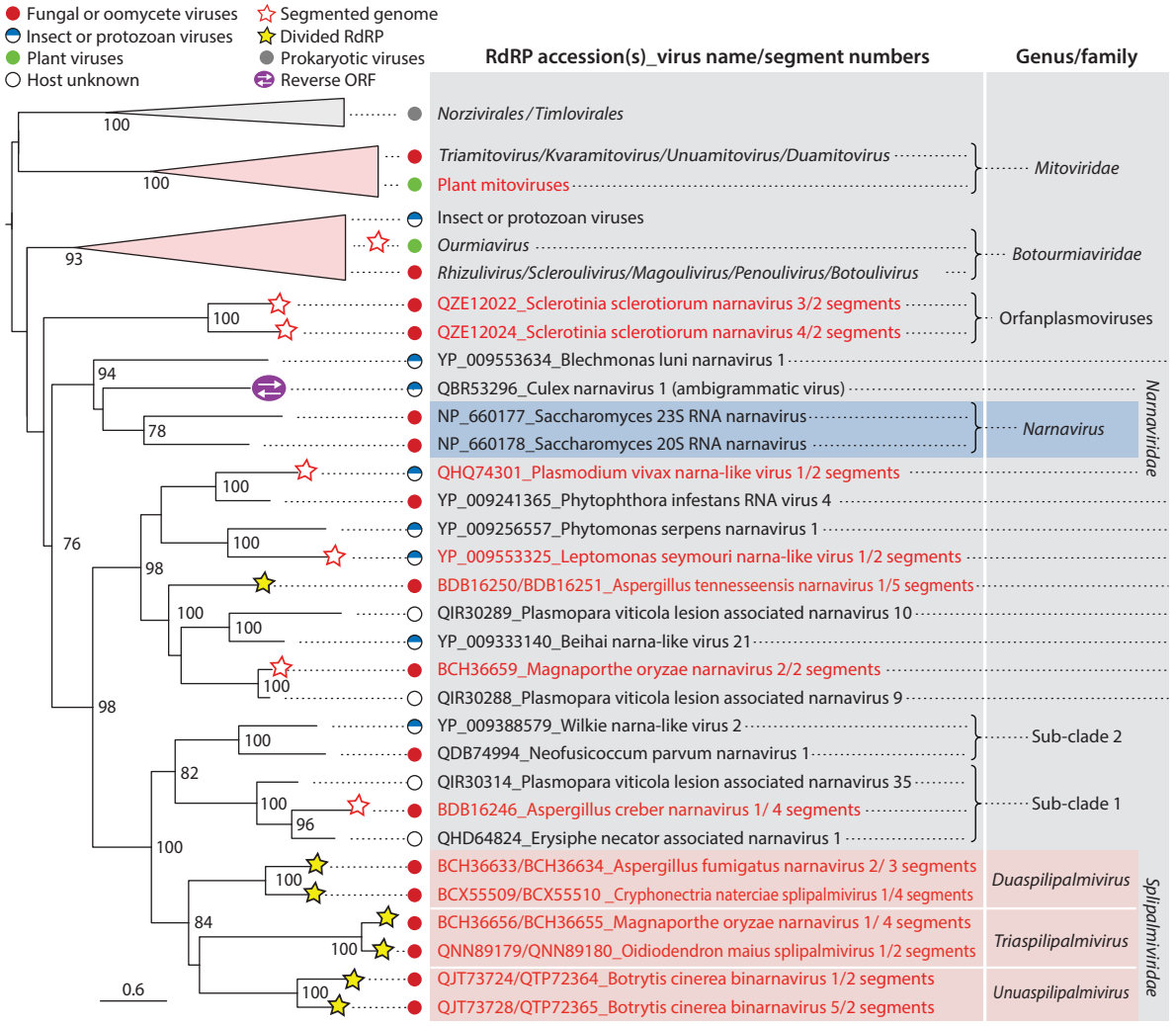

Figure 3

Phylogenetic relationships of viruses belonging to the phylum *Lenarviricota*. A multiple amino acid sequence alignment based on the RNA-dependent RNA polymerase (RdRP) sequences of selected viruses, including members of families *Mitoviridae*, *Narnaviridae*, and *Botourmiaviridae* and the proposed family *Splipalmiviridae* as well as other related unassigned taxa, was generated by MAFFT online version 7 (**https://mafft.cbrc.jp/alignment/server/**) (65). The resulting alignment was subjected to trimming of poorly aligned regions using trimAl version 1.3 (**http://phylemon.bioinfo.cipf.es**) (19) and then used to generate a maximum likelihood tree using PhyML 3.0 (**http://www.atgc-montpellier.fr/phyml/**) (53) with the best-fit model RtREV+G+I+F. The selected prokaryotic viruses of orders *Norzivirales* and *Timlovirales* were also included in this analysis and displayed in a collapsed state. The tree topology was obtained by the midpoint rooting method. Numbers at the nodes indicate bootstrap values of >70%. Abbreviation: ORF, open reading frame.

Homology-based modeling supports this idea (25). However, it is needed to show the physical interaction between the two split proteins and biochemical RdRP activity of the complex purified from infected mycelia or a reconstituted RdRP complex. Currently, there are no such RNA viruses with split RdRP motifs other than splipalmiviruses and AtenNV1. It is an open question as to whether there are other types of split RdRP RNA viruses or how splipalmiviruses exist

in infected cells. Concerning the second question, splipalmiviruses are probably capsidless like authentic yeast narnaviruses, which have been reported to exist as a ribonucleoprotein (viral RNA/RdRP) complex.

The other peculiar group includes narna-like viruses largely from insects and fungi that have an ambisense nature (22, 33). One strand encodes RdRP, whereas the other strand encodes a hypothetical protein showing no homology to known proteins except for counterparts of this group of viruses. Unlike the (−)ssRNA viruses or ambiviruses discussed above, the two ORFs of the viruses of this group overlap fully and correspond to approximately 90% of their entire genome size with the short terminal untranslated regions (**Figure 2**). This group of mycoviruses was designated as mycoambinarnavirus (22). The ambisense nature of some narnaviruses was previously noted by DeRisi et al. (31), who detected an additional large ORF, which spans nearly the full length of the reverse complement sequence of the virus genome. Independently, two new groups, Alphanarnavirus and Betanarnavirus, were proposed in the family *Narnaviridae* (33). The former includes nonsegmented narna-like viruses, including both the authentic (such as SsNV20S and SsNV23S) and ambisense types of the genomes, and the Betanarnavirus accommodates only non-ambisense narna-like viruses, including splipalmiviruses, with both nonsegmented and segmented genomes.

Multisegmented narna-like viruses with no ambisense nature, including splipalmiviruses, have also been reported from various organisms such as ascomycetes (63), arthropods (141), and protozoa (21) (**Figure 3**). Together with the above-discussed narna-like viruses, these add great diversity to the phylum *Lenarviricota*.

Other RNA Mycoviruses

In addition to the viruses listed above, there are many other groups of mycoviruses that have not yet been classified. For example, the family Ambiguiviridae (52) or Mycotombusviridae (189) was proposed to include Diaporthe RNA virus (DRV) (120) and other related viruses such as Magnaporthe oryzae RNA virus and soybean leaf-associated ssRNA virus 1 and 2 (18, 52, 88, 92, 93). As noted by Gilbert et al. (52), these viruses are phylogenetically related to tombusviruses (plant flavi-like viruses, phylum *Kitrinoviricota*, order *Tolivirales*) and commonly have a (+)ssRNA genome of approximately 3.0–4.0 kb with two ORFs encoding a hypothetical protein of unknown function and RdRP (**Figure 2**). The RdRP is assumed to be expressed as a fusion product with the 5′-proximal protein via readthrough of the amber termination codon UAG of the upstream ORF. Other features common to this group of viruses include the RdRP catalytic triplet GDN in place of GDD, which is possessed by most (+)ssRNA and dsRNA viruses, including tombusviruses. It is noteworthy that in vitro synthesized transcripts from the full-length cDNA of DRV were shown to be infectious to a fungal host *Diaporthe perjuncta* (96).

Reconstruction of Mycoviruses from Metagenomic Data

Sclerotinia sclerotiorum debilitation-associated DNA virus 1 (SsHADV1; phylum *Cressdnaviricota*, family *Genomoviridae*), also known as Sclerotinia gemycircular virus, is the first ssDNA mycovirus discovered to have a monopartite circular genome (182). The virus is transmitted by a mycophagous insect and can replicate in the vector (90) (see below). Recently, a tripartite fungal genomovirid from *F. graminearum* was also characterized (83). Most genomovirids were identified as metagenomes from a variety of environmental samples associated with animals, plants, and others and revealed largely monopartite ssDNA genomes (163). Among them, soybean leaf-associated gemycirculavirus 1 (SlaGemV1), which was discovered by leaf metagenomic analyses without known hosts, was reconstructed as an infectious entity (40, 92). SlaGemV1 can replicate

in not only filamentous ascomycetous fungi (*S. sclerotiorum*, *B. cinerea*, and *Monilinia fructicola*) but also insect (*Spodoptera frugiperda*)-cultured cells. This is reminiscent of an ssDNA virus (caribou feces–associated gemycirculavirus 1) from caribou feces metagenomic data whose reconstructed ssDNA genome was shown to infect an experimental plant host *Nicotiana benthamiana* (108). These studies are a milestone in that metagenomes are reconstructed as infectious entities and characterized biologically. To date, no such RNA mycovirus in metagenome analysis without known hosts has been reconstructed as an infectious unit.

Necessary Confirmation of Infectious Entity and Expression Strategy of Unusual Mycoviruses

Many metagenomic or metatranscriptomic virogenomic studies provide a platform for further virus characterization. The peculiar mycoviral genomes discussed above should be identified biologically as representing virus genomes. It is of great interest to investigate how many segments make up their genomes in a minimal form. To address these questions, the development of their reverse genetics will be helpful, as in the case of other well-studied mycoviruses. Furthermore, the segment number of reported viruses through metatranscriptomic analyses may have been underestimated, as NGS approaches are not so helpful in this regard. For segment number determination, in particular, the fragmented and primer-ligated dsRNA sequencing (FLDS) method (159) may be helpful, as demonstrated for many uncultured viruses based on the terminal sequence conservation among their segments (25, 158).

The unusual ambisense genome organization was observed in at least two groups: designated here as ambinarnaviruses and ambiviruses. The former group of viruses belongs to the phylum *Lenarviricota*, and the latter group of viruses is taxonomically distinct. Both groups are officially unclassified by the ICTV (see above). Both groups share common features in that one strand encodes the hallmark RdRP and the other strand encodes a protein of unknown function showing no homology to known proteins. An insect-infecting member of the first group, Culex narnavirus 1 (from *Culex tarsalis* CT cells), was shown, using reverse genetics, to require both proteins (126). The authentic yeast narnaviruses ScNV20S and ScNV23S are among the smallest and simplest viruses with a capsidless nature that encode only RdRP in their (+)ssRNA genome. These capsidless RNA viruses have been well-studied with biochemical and reverse genetics tools. The yeast narnaviruses and probably most narna-like viruses do not encode a capsid protein and exist as ribonucleoprotein complexes with the RdRP in the host cell cytoplasm. The yeast narnavirus RdRP binds the genomic RNA at a 1:1 stoichiometric molar ratio through interactions with both the 5′ and 3′ ends, which may help protect the viral RNA from degradation by host exonucleases (43, 44, 147, 175). This ribonucleoprotein complex form, along with the terminal structure, may protect the genomic RNA from degradation systems, including the 5′→3′ exonuclease (a proposed antiviral system with SKI1/XRN1 in the XRN family) (39). Little is known about the 5′ end structure of the narnaviral positive and negative strands that are important for their translation.

The RNA accumulation ratio of the two strands of an ambisense segment is an interesting area of investigation to gain insight into the expression level of two ambisense coding ORFs. The majority of ScNV20S viral RNA in the cell is (+)ssRNA molecules (45, 128). However, little is known regarding ambinarnaviruses. First, the ratio of the positive- versus negative-strand viral RNA accumulation in infected host cells must be determined. Turina's group suggested that the RNA accumulation ratio of the two strands varied among ambiviruses (149). How the reverse ORF on the negative-sense strand of ambiviruses or ambinarnaviruses is expressed is an open question. These unresolved problems are also relevant to the unique (−)ssRNA mycoviruses with ORFs on their positive-sense RNA, as exemplified above.

DIVERSITY IN MYCOVIRUS LIFESTYLE

The recent characterization of many peculiar mycoviruses has revealed considerable virus diversity and unusual genome structures, as discussed above, but also novel virus replication mechanisms and lifestyles. Yeast narnaviruses, along with prototype (+)ssRNA hypoviruses, were shown in the 1980s to present in a capsidless or naked form in the cytoplasm of infected cells. Recent research revealed several additional unique virus lifestyles of mycoviruses. There appear to be six different types of capsidless mycoviruses that have (+)ssRNA genomes, excepting polymycoviruses (family *Polymycoviridae*), which are classified as dsRNA viruses (**Figure 1**; see below). The first type (**Figure 1**, ❶) includes the aforementioned authentic narnaviruses, which are one of the simplest types of viruses (147, 175). Other mycoviruses in the phylum *Lenarviricota* such as fungal botourmiaviruses, may have a virus nature similar to that of yeast narnaviruses. The second type (**Figure 1**, ❷) comprises hypoviruses (picornavirus-like supergroup; phylum *Pisuviricota*) encapsulated in Golgi-derived vesicles, whose production is induced upon infection in filamentous fungi (153). This group includes (+)ssRNA endornaviruses (alpha-like supergroup; phylum *Kitrinoviricota*) whose replicative dsRNA form appears to be encased in host-derived cytoplasmic membranous vesicles (162). The third type (**Figure 1**, ❸) includes yadokariviruses (picornavirus-like supergroup; phylum *Pisuviricota*), which do not encode capsids but highjack the capsids of partner toti-like dsRNA viruses (phylum *Duplornaviricota*) (28, 59, 186). The fourth type (**Figure 1**, ❹) is dsRNA polymycoviruses (picornavirus-like supergroup; phylum *Pisuviricota*). They encode proline-, alanine-, and serine-rich protein (PASrp), but not capsid protein, and their genomic dsRNA is associated with their PASrp to form the ribonucleoprotein complex (64, 78). Recently discovered multisegmented (+)ssRNA viruses termed hadakaviruses (picornavirus-like supergroup) belong to the fifth type (**Figure 1**, ❺) and are phylogenetically related to polymycoviruses. Hadakaviruses likely exist as a naked form accessible by RNase A at least in mycelial homogenates (136). The last (sixth) type (**Figure 1**, ❻) accommodates mitochondrially replicating (+)ssRNA viruses (mitoviruses; phylum *Lenarviricota*). Their genome structure is similar to that of yeast narnaviruses, which encode only RdRP. Both their genomic RNA and replicative form dsRNA copurify with mitochondria (119, 177), and they use the mitochondrial genetic code and are thus assumed to be translated in mitochondria. However, little is known about how and where inside the mitochondria the mitovirus replicates and how its viral RdRP and RNA are associated. Below, we elaborate on the recently discovered neo-virus lifestyles of capsidless mycoviruses belonging to abovementioned groups ❸–❺.

Yado-Kari/Yado-Nushi Nature

The first such lifestyle was detected during a virus hunting study of many Japanese isolates of white root rot fungus *R. necatrix* (74, 179) and exhibited by a (+)ssRNA virus termed yado-kari virus 1 (YkV1) that is hosted or hetero-encapsidated by an unrelated dsRNA virus called yado-nushi virus 1 (YnV1) (**Figure 4**). YkV1 depends on YnV1 for viability; YnV1 as a full-fledged dsRNA virus can establish infection on its own. As described above, YkV1 is a member of the family *Yadokariviridae* in the phylum *Pisuviricota* and possesses a monosegmented (+)ssRNA genome encoding only a single polyprotein. The YkV1 polyprotein undergoes processing by the 2A-like peptide, which produces the N-terminal RdRP and the C-terminal protein of unknown function. Three strains of YnV1, which may be classified into a new family in the order *Ghabrivirales* (phylum *Duplornaviricota*), were detectable in the original *R. necatrix* strain W1032. Thus far, a related virus has been reported only from a Bangladeshi isolate of the phytopathogenic basidiomycete *Sclerotium rolfsii* (59). A series of studies have shown that YkV1 replicative form dsRNA is encapsidated by the capsid protein of YnV1 encoded by the 5′-proximal ORF of its two-ORF-type

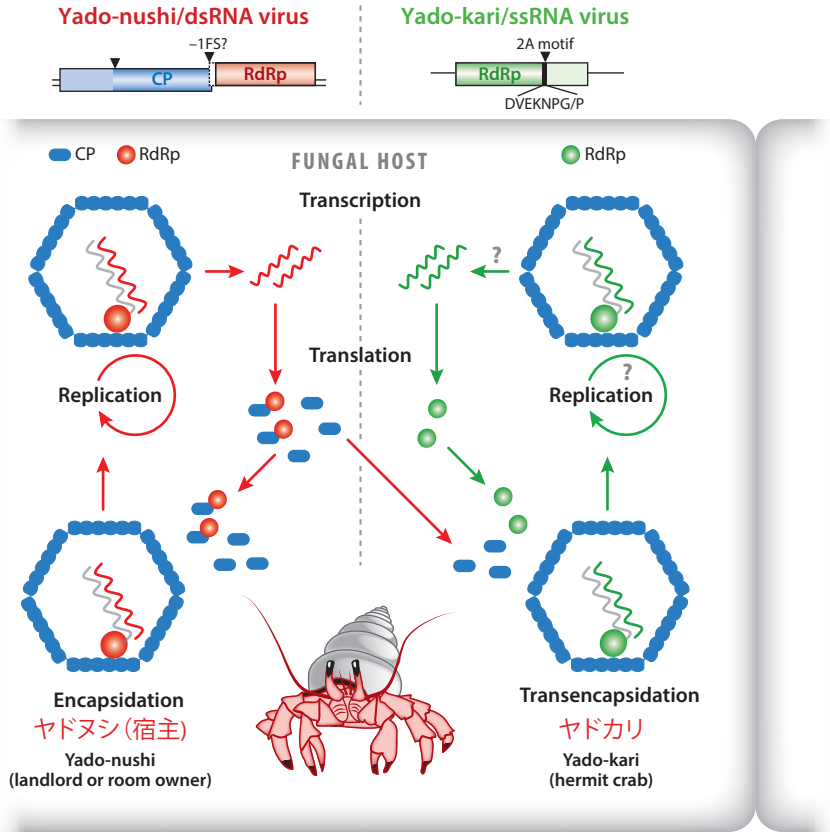

Figure 4

A model for a neo-virus lifestyle with the yado-kari/yado-nushi nature. Yado-kari virus 1 [YkV1, a calici-like (+)ssRNA virus] is hosted by the yado-nushi virus 1 (YnV1, a toti-like dsRNA virus) capsid. YkV1 replication and transcription likely occur in the heterocapsids as if YkV1 were a dsRNA virus, whereas YnV1 replication is enhanced by coinfecting YkV1 (186). Abbreviations: CP, capsid protein; RdRP, RNA-dependent RNA polymerase.

genome. The YnV1 capsid protein also encases YkV1 RdRP cleaved by the 2A-like peptide. Mutational analyses have clearly indicated that YkV1 RdRP liberated from the polyprotein and likely the C-terminal protein is also essential for YkV1 replication (28). Importantly, a portion of virus particles package only YkV1 RNA and RdRP, implying that the replication and transcription of YkV1 occur in heterocapsids; thus, YkV1 behaves as if it were a dsRNA virus (**Figure 4**). Interestingly, there are mutualistic interactions between YkV1 and YnV1: YkV1 highjacks the YnV1 capsid, whereas YnV1 replication is enhanced by coinfecting YkV1 (186).

The abovementioned viral neo-lifestyles pose intriguing questions that should be addressed in the future. The yado-kari/yado-nushi nature has been unambiguously demonstrated for the YkV1/YnV1 combination harbored in a Japanese strain of *R. necatrix*. There are ten ICTV-approved yadokarivirus species that are classified into two genera in the family *Yadokariviridae* and order *Yadokarivirales*. The partner dsRNA viruses of these exemplar yadokariviruses remain unidentified. However, we recently identified diverse dsRNA viruses within the order *Ghabrivirales*, which partner with other yadokariviruses, yado-kari virus 3 and 4 (YkV3 and YkV4), in the

Spanish strain of *R. necatrix* (Y. Sato, S. Hisano, C. J. López-Herrera, H. Kondo, N. Suzuki, unpublished results). From the previously reported coinfections of filamentous fungi by yadokariviruses and members of the order *Ghabrivirales*, their partnership is highly anticipated (59). Members of the order *Ghabrivirales* are considerably diverse, although their genomic organization is similar to only a few ORFs. This order currently has only four families approved by the ICTV (*Totiviridae*, *Chrysoviridae*, *Quadriviridae*, and *Megabirnaviridae*) but is expected to expand to more than 10 families (Y. Sato & N. Suzuki, unpublished results). The interfamily RdRP sequence identity is generally less than 30%. Each yadokarivirus appears to establish a species-specific partnership with a partner dsRNA virus, whereas yadokariviruses are collectively supported by diverse *Ghabrivirales* members. Additionally, a similar neo-virus lifestyle occurs in a plant that involves a (+)ssRNA tombus-like virus termed papaya meleira virus 2 (PMeV2, phylum *Kitrinoviricota*) and a member (papaya meleira virus) of the proposed family Fusagraviridae in the order *Ghabrivirales* (132). Interestingly, coinfection by PMeV2 with a yado-kari-like nature is implicated in the induction of the meleira disease in papaya characterized by severe sticky latex exudation. Thus, the yado-kari/yado-nushi nature may be prevalent in eukaryotic host organisms other than those found in the kingdom Fungi (59). How the partnership between yadokariviruses and partner dsRNA viruses is determined is of great interest.

As mentioned above, coinfection of *R. necatrix* by YkV1 enhances YnV1 accumulation. However, the impact of yadokarivirus coinfection on partner dsRNA viruses varies depending on their combination (Y. Sato & N. Suzuki, unpublished results).

Infectious Entities of Polymycoviruses with Colloidal Ribonucleoprotein or Naked dsRNA

The second peculiar virus neo-lifestyle was discovered in *A. fumigatus* infected by a tetrasegmented RNA virus. The virus was designated as Aspergillus fumigatus tetramycovirus 1 (AfuPmV1) and classified as a unique dsRNA virus in the family *Polymycoviridae* (64). Relatively large amounts of genomic RNA are detectable in infected mycelia associated with one of the virally encoded proteins, PASrp, of approximately 25 kDa. AfuPmV1 does not form typical rigid particles but rather exists as an RNA–protein complex in colloidal form. RdRP-based phylogenetic analyses show AfuPmV1 to be distantly related to animal caliciviruses (order *Picornavirales*) with (+)ssRNA viruses. PASrp can bind RNA and DNA in a sequence-nonspecific manner (134). AfuPmV1 putative RdRP has the presumed catalytic amino acid sequence GDNQ often found in RdRPs of (−)ssRNA viruses. Importantly the PASrp–dsRNA complex is infectious to protoplasts of the host fungus. This feature has been confirmed in other polymycoviruses. More surprisingly, AfuPmV1 is infectious as a purified naked dsRNA prepared by S1 nuclease and proteinase K treatment. Based on these attributes, AfuPmV1 is hypothesized to be an intermediate virus between (+)ssRNA and (−)ssRNA viruses and between capsidless and encapsidated RNA viruses. The polymycovirus infectious entities allowed the ICTV to classify them as dsRNA viruses.

There are other reported polymycoviruses from filamentous ascomycetes (e.g., 62, 78, 184). One of them was Colletotrichum camelliae filamentous virus 1 (CcFV1), which has been shown to form filamentous particles with a wide range of lengths (62). The filamentous particle was decorated by an antiserum against the viral protein PASrp, suggesting that this protein is a capsid protein. CcFV1 is infectious as a filamentous particle and as naked genomic dsRNA. CcFV1 is the only filamentous dsRNA virus reported thus far. Two distinct virus forms are reported for the two related polymycoviruses, CcFV1 and AfuPmV1. It is conceivable to anticipate that two homologous PASrps should lead to a similar virus morphology. Further morphological characterization of polymycoviruses is needed.

Hadaka Nature

Hadaka virus 1 (HadV1) strain 1NL was the first hadakavirus discovered in a phytopathogenic fungus, *Fusarium oxysporum*, from Pakistan (136). Subsequently, the second HadV1 strain 7n was isolated from another Pakistani *Fusarium nygamai* isolate (68). HadV1 1NL and 7n have 11 and 10 (+)ssRNA genomic segments, respectively, the largest segment number among (+)ssRNA viruses. Another hadakavirus was detected in an Ethiopian isolate of *Fusarium* spp. (S. Chiba, personal communication). Hadaka virus 1 was coined because of its capsidless nature, as Hadaka in Japanese means naked (136). These hadakaviruses show high phylogenetic affinity to polymycoviruses and encode three proteins homologous to the counterparts of polymycoviruses that include putative RdRP, putative MT, and hypothetical protein P2 (encoded by polymycovirus dsRNA2). The shared properties include the GDNQ catalytic motif in the RdRP core domain. However, notably, the hadakaviruses lack PASrp, a hallmark of polymycoviruses relatively tightly associated with their genomic dsRNA segments or a structural protein encoded by typical dsRNA viruses. The most interesting property associated with hadakaviruses is the hadaka nature. HadV1 replicative form dsRNA has been shown to be susceptible to RNase A in mycelial homogenates, which is distinct from the case of polymycoviruses and encapsidated dsRNA viruses (both are tolerant to RNase A in the homogenates). In this sense, capsidless (+)ssRNA hypoviruses are also different from hadakaviruses. The hypovirus replicative form dsRNA becomes susceptible to RNase in mycelial homogenates only after treatment with the nonionic detergent Triton X (Y. Sato, S. Hisano, N. Suzuki, unpublished data). This is reasonable considering that hypoviruses replicate in Golgi-derived membranous vesicles (54, 153). These observations allowed Sato et al. (136) to hypothesize that hadakavirus dsRNA exists in an RNase-accessible form, at least in mycelial homogenates.

To explore how hadakaviruses replicate and survive harsh host cellular environments is a future challenge. As noted above, the hadakaviruses are distinct from polymycoviruses or the capsidless hypoviruses. Along with the different RNase A susceptibility patterns, hadakaviruses show sedimentation profiles different from polymycovirus and hypoviruses. Hadakavirus replicative form dsRNAs are unable to be pelleted by ultracentrifugation and are accessible to nucleases without detergent treatment (136). In contrast, polymycoviruses and hypoviruses form RNA–protein complexes (colloidal forms) (64) or filamentous particles (62) and membranous vesicles, respectively, and both types of viruses are pelleted by ultracentrifugation. Polymycoviral PASrps are likely to bind nucleic acids in a sequence-nonspecific manner (134).

DIVERSITY IN THE IMPACT OF MYCOVIRUS INFECTIONS ON THEIR FUNGAL HOST AND MULTILAYER ECOSYSTEMS

Viruses Altering Host Fungal Lifestyles

It has long been known that most mycoviruses are associated with asymptomatic infections. This observation likely relies on the unbiased assay, i.e., dsRNA-based virus detection, and differs from pathogenic nature-centered plant and animal virus detection. Some fungal viruses convert the host fungus lifestyle from a virulent to a hypovirulent state. Examples include the prototype hypovirus, Cryphonectria hypovirus 1 (CHV1; *Hypoviridae*) (110, 127), SsHADV1 (a ssDNA genomovirus) (182), Rosellinia necatrix megabirnavirus 1 (a dsRNA virus; *Megabirnaviridae*) (24), Fusarium graminearum virus 1 (a hypo-like ssRNA virus; proposed family Fusariviridae), Heterobasidion partitivirus 13 (a dsRNA virus, *Alphapartitivirus*) (161), and many others, thus spanning a variety of virus groups infecting diverse fungi. In contrast, a limited number of mycoviruses are known to enhance virulence in their host fungi, such as an unidentified RNA virus infecting *Nectria radicicola* (3), polymycoviruses infecting the entomopathogenic fungus

Beauveria bassiana (78) and the human pathogenic fungus *Aspergillus fumigatus* (116), a quadrivirus (a toti-like quadripartite dsRNA virus; *Quadriviridae*) of *Leptosphaeria biglobosa* (139), and a chrysovirus (a multipartite dsRNA virus; *Chrysoviridae*) of *Alternaria alternata* (113). Readers are referred to recent reviews describing virus-induced hypovirulence and hypervirulence (e.g., 166), and virus-converting fungal lifestyles exemplified by SsHAD1-involved alterations (122, 185).

Cross-Kingdom Infections by Diverse Viruses with Potential Impacts on Multilayer Ecosystems

Plants, fungi, and animals, belonging to the three major eukaryotic kingdoms of multicellular organisms, Plantae, Fungi, and Animalia, have their own histories of evolution with different cellular environments and immune systems. Thus, it is assumed that there are barriers that prevent cross-kingdom infection and spread of single viruses. However, diverse eukaryotic viruses are predicted based largely on phylogeny to have been horizontally transferred among organisms across these three kingdoms (34, 176). Historically, rice dwarf virus (a dsRNA reovirus, phylum *Duplornaviricota*) was the first virus that has demonstrated the ability to infect both plants and insect vectors (46). Some other groups of viruses (such as plant-infecting rhabdoviruses, tospoviruses, and tenuiviruses, which cause serious diseases in important crops) are now known to replicate in and be transmitted by vector insects (such as hemipterans), shuttling between plants and animals (148). Recently, natural or experimental cross-kingdom infections by single viruses were reported between fungi and insects and, independently, between plants and fungi (6, 12, 40, 90, 106, 173) (**Figure 5**). These viruses crossing kingdoms are expected to be crucial players in ecosystem balance.

The first natural mycoviral cross-kingdom infection was demonstrated for the ssDNA virus SsHADV1, which can infect not only the fungal host *S. sclerotiorum* but also the vector fly *Lycoriella ingenua* (90) (**Figure 5**). Both naturally SsHADV1-infected fungal isolates and flies were readily detectable in stem rot–infested fields. *L. ingenua* can acquire SsHADV1 from infected mycelia on rapeseed leaves and transmit it vertically to offspring through eggs but also horizontally to uninfected fungal hosts. Interestingly, the virus appears to manipulate the host fungus to attract the vector insect via fungal secondary metabolites (volatiles), which serve as attractants to *L. ingenua*. This may not be so surprising because SsHADV1-like viruses (other genomovirids) are detectable from many insect-associated samples (80, 163). The second example of natural cross-kingdom infection is the infection of the notorious phytopathogenic basidiomycete, *Rhizoctonia solani*, by one of the best-studied plant viruses, cucumber mosaic virus (CMV; an alpha-like virus; phylum *Kitrinoviricota*) in the Inner Mongolia Province of China (6) (**Figure 5**). CMV strain Rs of fungal origin can be laterally transmitted under laboratory conditions from infected *R. solani* to plants and vice versa. CMV Rs shows slightly induced virulence in *R. solani*, whereas the well-explored CMV strain Fny of plant origin is symptomless in the fungus but induces mosaic in experimentally inoculated *N. benthamiana* by mechanical inoculation. The newly discovered CMV strain is different in genomic sequence (<2% divergence) from known reported subgroup Ia strains infecting plants but shares the tripartite genomic organization. *R. solani* can serve as a plant virus vector and acquire the virus from infected plants and transmit it to healthy ones. These two examples clearly show that mycoviruses could be transmissible by other host organisms serving as vectors from kingdoms other than Fungi.

The systemic replication of two well-studied plant viruses in fungi was shown in a compelling way by a few research groups. The first example is an alpha-like virus, tobacco mosaic virus (TMV; phylum *Kitrinoviricota*), which has been shown experimentally to be able to establish stable, i.e., for two months, infection of the phytopathogenic fungus *Colletotrichum acutatum* (94). The inoculation was carried out by mixing mycelia and purified TMV particles in liquid fungal culture.

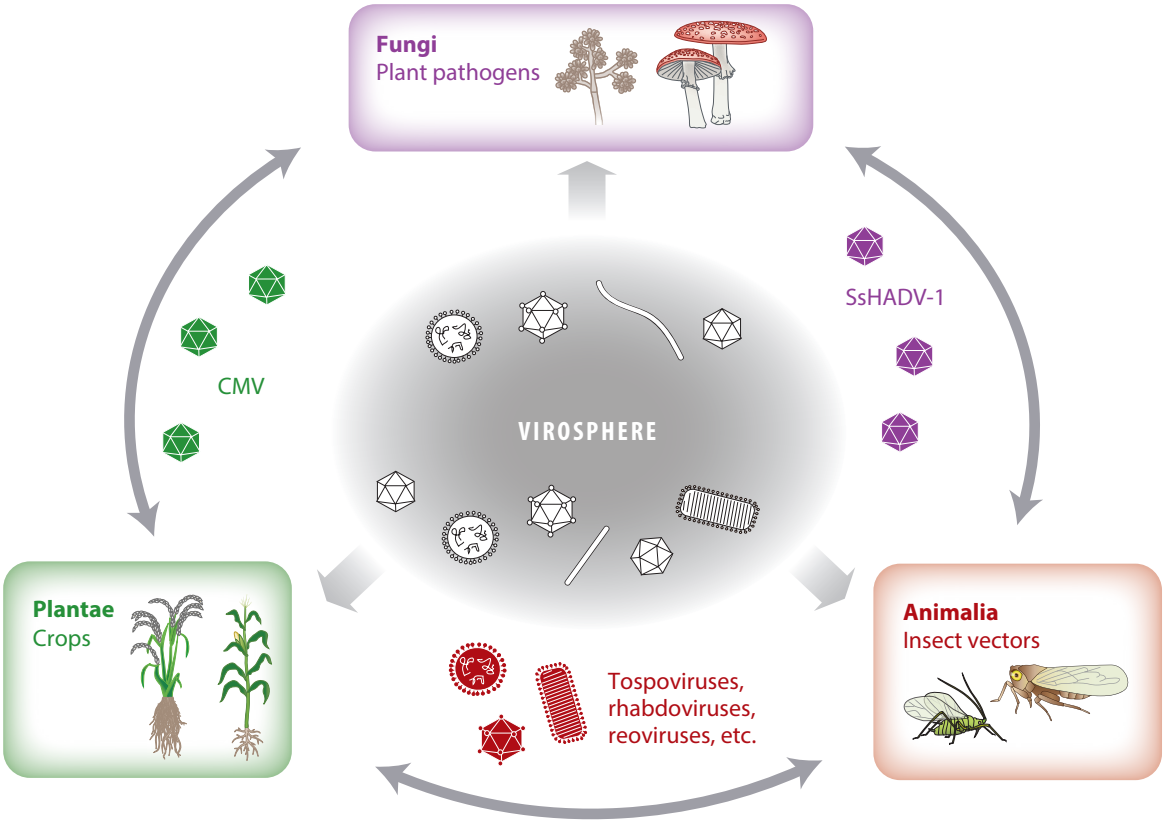

Figure 5

Trans-kingdom infections involving mycoviruses and fungal hosts. The figure illustrates natural cross-kingdom infections. Abbreviations: CMV, cucumber mosaic virus; SsHADV-1, Sclerotinia sclerotiorum debilitation-associated DNA virus 1.

How TMV enters the fungal cell remains largely unknown, as in the case of an ssDNA mycovirus (SsHADV1). TMV vector-based foreign protein expression and mediated virus-induced gene silencing was also confirmed in the fungus. RNA silencing (RNA interference) or quelling is well established in fungi whose genetic dissection was first pursued in a filamentous ascomycete *Neurospora crassa* (see 60). TMV is also able to infect *F. graminearum* by protoplast transfections as well, and its replication is augmented in RNA silencing–deficient mutants or by coinfecting a mycovirus (CHV1) that can suppress antiviral silencing activity (12). Another example of cross-kingdom infection is the bona fide fungal virus CHV1, the best-studied virus of filamentous fungi. CHV1 was shown to systemically infect a model plant, *Nicotiana tabacum*, only when the cell-to-cell movement protein (MP) of a plant virus origin is supplied transgenically or from replicating plant viruses such as TMV (12). Without plant viral MP, CHV1 replication can only be detectable in inoculated leaves. The tested plant viral MPs support the systemic spread of this capsidless mycovirus (CHV1), which is replicated in host-derived vesicles in fungi (153). In the presence of TMV, CHV1 can infect plants systemically and enhance the possibility of horizontal transfer of CHV1 to other fungi, including vegetatively incompatible ones through plants.

Viroids are the smallest infectious entity with noncoding circular RNAs that are pathogenic to plants. Recently, Liying Sun's group has shown experimentally that some viroids as the monomeric form of in vitro synthesized RNA replicate in a few tested filamentous fungi (173).

The tested viroids include hop stunt viroid (HSVd) and iresine 1 viroid (members of the family *Pospiviroidae*) and avocado sunblotch viroid (ASBVd; family *Avsunviroidae*), which replicate in the nucleus (pospiviroids) and chloroplast (avsunviroids) of their natural plant hosts, respectively. The above three viroids can establish stable infections in a few phytopathogenic fungi such as *C. parasitica*, *Valsa mali*, and *F. graminearum*. Interestingly, HSVd induces severe growth defects in *V. mali* but not in the other two fungi. HSVd and ASBVd underwent substitution mutation during successive subcultures of the infected RNA silencing–deficient mutants of *C. parasitica* and *F. graminearum*, respectively (174). Furthermore, HSVd can be horizontally bidirectionally transferred between *F. graminearum* and plants during infection, indicating the potential of fungi as a vector for plant-to-plant transmission of viroids and vice versa. This provides evidence that at least some viroids can be replicated in fungi. It is noteworthy that viroid-like small circular RNA has possibly been detected in fungi (41).

TMV, CHV1, and viroids, experimentally shown to cross host kingdoms, have yet to naturally infect plants, plants, and fungi, respectively. These cross-kingdom infections provide a surge in research interest and several intriguing questions. Examples include how single viruses adapt to the host environments of different host environments, how their replication site and host factors are different between the two kingdoms, how different kingdoms of host organisms incite defenses against single viruses, and how single viruses exert counter-defenses against different host defense responses. The cellular environments are different between fungi and members of the other kingdoms able to host the virus of interest. The abovementioned examples of cross-kingdom infections involve both encapsidated (CMV, TMV, SsHADV1) and capsidless ssRNA viruses (a hypovirus and CHV1) under certain conditions. CMV, TMV, and CHV1, particularly the former two, are well-studied in terms of replication. It is generally accepted that unnecessary genes in one of the alternate hosts are deleted or mutated via successive passage or maintenance in the one host, often leading to the revelation of functional roles of mutated genes. For example, maintaining rice dwarf virus (a plant dsRNA reovirus) exclusively in rice plants results in the generation of dysfunctional genomes, i.e., mutation of the S2 segment encoding the P2 outer capsid protein needed for receptor-mediated virus entry into insect host cells (121). A similar finding is in the genes for the G1 and G2 proteins of tomato spotted wilt virus [a plant (−)ssRNA bunyavirus], which undergo spontaneous mutation after long-term maintenance of the virus in host plants (reviewed in 49). Similar approaches should be helpful for elucidating the functional roles of viral genes in alternate hosts. In this regard, whether CMV and TMV retain the capsid protein and MP genes, which are essential for most plant viruses, is an interesting question, given the fact that many (+)ssRNA mycoviruses are capsidless and lack MP.

In fungi, as in insects and plants, RNA silencing is the primary antiviral defense in which host fungi perceive viruses and induce the RNA silencing pathway (111); there are noticeable differences in the RNA silencing pathway between the kingdoms. In *C. parasitica*, Dicer (Dicer-like protein) plays dual functional roles in not only post-transcriptional RNA silencing as the dsRNA-specific dicing enzyme but also transcriptional upregulation of several host genes together with the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex (4, 5, 138). Regulated host genes include RNA silencing of key genes such as dicer-like (*dcl*) and argonaute-like (*agl*; a putative slicer), as well as many other genes likely involved in the mitigation of symptom induction, thus forming a positive feedback loop. Whether such a regulation mechanism operates in other filamentous fungi needs to be explored. As counter-defense mechanisms, fungal viruses, like plant and animal viruses, encode RNA silencing suppressors (9, 137, 181) that appear to inhibit the upregulation of RNA silencing genes. No other modes of action of fungal virus suppressors of RNA silencing have yet been reported. RNA silencing in members of different kingdoms is under different regulation with different sets of key components, although small RNAs (typically 19–25 nucleotides

long) cross kingdoms using extracellular vesicles and work in both fungi and plants (17, 56). In this sense, short RNAs with a peak at 16 nucleotides derived from a plant mitovirus (a relative of fungal mitoviruses) are produced in the infected plant (107). This may suggest mitochondria-specific antiviral defense in plants.

EVOLUTIONARY CONSIDERATIONS FOR MYCOVIRUSES

Evolutional Aspects of dsRNA and (+)ssRNA Mycoviruses

Recent metagenomic and metatranscriptomic analyses have expanded our understanding of the virosphere in fungi (see above for details). These approaches allow us to understand the genetic divergence of mycoviruses and provide their evolutionary snapshots. Thus, some remarkable insights into mycoviral evolution are exemplified in this section [for dsRNA and (+)ssRNA viruses] and the following sections [for (−)ssRNA viruses].

Viral genome segmentation may have played an important role in genetics and evolutionary biology for RNA viruses (11, 112). The segmentation of a monopartite (+)ssRNA mycovirus (a deltaflexivirus in the family *Deltaflexiviridae*) has recently been proposed in Sclerotinia sclerotiorum deltaflexivirus 3 (SsDFV3) (98). A similar event could also be speculated for another (+)ssRNA mycovirus, a botrexvirus [Botryosphaeria dothidea botrexvirus 1 (BdBV1)], in the family *Alphaflexiviridae* (180). The genome segmentation of these two mycoviruses (SsDFV3 and BdBV1), both belonging to the same order, *Tymovirales* (the phylum *Kitrinoviricota*, also known as the alphavirus-like supergroup), possibly occurred independently from ancestral unsegmented flexiviruses during the course of evolution. Furthermore, botrexviruses (mycoviruses) show close sequence similarity to plant allexiviruses and likely have originated from ancestral plant viruses (148). However, no close relatives of deltaflexiviruses or gammaflexiviruses (members of a related mycoviral taxon) have been reported from plants. The benefits of genome segmentation are still poorly understood. However, these events will possibly provide genomic diversity through the exchange of the segments (reassortment) between related viruses, as previously described for animal and plant RNA viruses (73, 142).

Horizontal gene transfer (HGT) between virus and host or virus to virus has been recognized as an important driving force in the viral evolution (13, 81). In vertebrates and invertebrates, numerous endogenous nonretroviral RNA viral- or related viral-like elements [so-called EVEs (endogenous viral elements)] have been reported (61, 66). Similar viral-related footprints have also been discovered in the genomes of numerous plants and fungi (16, 23, 71, 72, 86). Some nonretroviral EVEs are widespread among the host genomes in particular kingdoms, such as bornaviruses in vertebrates (67), rhabdoviruses and nege-like viruses in invertebrates (insects) (70, 156), and partitiviruses and varicosaviruses (bipartite plant rhabdoviruses) in plants (23). However, the knowledge of the distribution and diversity of nonretroviral EVEs is still limited in fungal genomes. As a different direction of HGT, i.e., from virus to virus, the HGT events of cross-viral families have been proposed among diverse mycoviral-related dsRNA viral taxa with the S7 domain homologs (87). Similarly, the duplicated domains (putative viral helicase and MT) may have occurred in the genomes of SsDFV3, Aspergillus fumigatus RNA virus 1 (a vivivirus), and Rhizoctonia solani hypovirus 2 via HGT between (+)ssRNA viruses (1, 25, 98). Even HGT has also been predicted from a (+)ssRNA hypovirus to a dsRNA megabirnavirus (order *Ghabrivirales*) that has acquired a p29 papain-like protease domain (171).

The relatives of dsRNA and (+)ssRNA mycoviruses such as partitiviruses, totiviruses, chrysoviruses, and endornaviruses infect many plant species (129, 154). These plant RNA viruses are classified within the same genera (*Alphapartitivirus*, *Betapartitivirus*, *Totivirus*, *Alphachrysovirus*, and *Alphaendornavirus*), where related mycoviruses belong, and have persistent lifestyles in their host

plants: They lack the cell-to-cell MP and do not have an extracellular stage, and thus most likely transmit through the cell division (129). Based on the phylogenetic relationships, the abovementioned plant and fungal RNA viruses could be horizontally transferred between plants and fungi historically and probably currently (148). Among them, partitiviruses have left potential fossil records as mentioned above, and some integration events in members of the family Brassicaceae were roughly estimated at around 10–24 million years ago (23). This and some other examples have suggested the long-term coevolution between the partitiviruses and the host plants, whereas a similar coevolution between partitiviruses and fungal hosts has still not been uncovered. Under an experimental inoculation condition, two mycoviruses related to totiviruses and partitiviruses could replicate in plant cells (106). Thus, whether plant partitivirids could also infect fungal cells is still an open and interesting question.

Mycoviruses related to other plant alpha-like viruses in the family *Benyviridae* (order *Hepelivirales*, phylum *Kitrinoviricota*) and other RNA viruses in the *Potyviridae* (phylum *Pisuviricota*) have also been reported (169). Members of *Benyviridae* or bymoviruses in *Potyviridae* are transmitted by the zoospores of the plasmodiophorid protist (155). The ancestor of these viruses may have undergone trans-kingdom horizontal transfer between plants and protists on evolutionally timescales (35). The order *Martellivirales* (*Kitrinoviricota*) includes several important groups of plant viruses, such as families *Bromoviridae*, *Closteroviridae*, and *Virgaviridae*. Recent metagenomic studies have also demonstrated the presence of fungal-specific viruses (including viviviruses) related to virga- or other plant alpha-like viruses, which should form new virus taxa (families), respectively (22, 169). Interestingly, experimental inoculation approaches have demonstrated the infection of fungi by plant alpha-like viruses (CMV and TMV) (6, 94, 173). Inversely, a fungal RNA virus (CHV1) can replicate in plants, and TMV enables the systemic spread of this mycovirus in plants (12). Increasing evidence of cross-kingdom virus infections with many kinds of RNA viruses may allow us to understand the deeper evolutionary insights into the origin of RNA viruses (see also below).

The phylum *Lenarviricota* is composed of four classes, *Amabiliviricetes*, *Howeltoviricetes*, *Leviviricetes*, and *Miaviricetes*, and is rapidly expanding. The first and fourth classes accommodate mycoviruses in the families *Narnaviridae*, *Mitoviridae*, and *Botourmiaviridae*, and the other two classes include bacterial phages in the families *Atkinsviridae*, *Duinviridae*, *Fiersviridae*, *Solspiviridae*, *Bluneviridae*, and *Steizviridae*. Only this phylum and the phylum *Duplornaviricota* include prokaryote- and eukaryote-infecting members. The number of bacterial RNA viruses appears to be underestimated and the two phyla will be probably expanded. A vast number of members in the phylum *Lenarviricota* have been reported from eukaryotes, particularly fungi. The progenitors of narna-like fungal viruses are diverse and assumed to have originated from a bacterial phage (ancestral levivirus) because RdRP-based phylogenetic trees place levivirids at the base (107). The ancestral levivirus may have been brought with the α-proteobacterial progenitor of mitochondria during eukaryogenesis and then lost the capsid protein gene to evolve into a capsidless mitovirus. Mitochondrially replicating mitoviruses then might have moved their replication site into the cytosol to birth narna- and narna-like viruses, including botourmiaviruses (75, 176). During the coevolution of eukaryotes and narna-like viruses, mitoviruses have adjusted to their mitochondrial environments by including the mitochondrial genetic code such as the UGA encoding Trp in fungal mitochondria, whereas others (narnaviruses, botourmiaviruses, and many other relatives) seem to have adjusted to the cytoplasmic replication style. These viruses have also been isolated from or associated with plants and invertebrates in addition to fungi (107, 109, 141). Plant ourmiaviruses [a tri-segmented (+)ssRNA genome with nonenveloped bacilliform virions; family *Botourmiaviridae*] are assumed to have originated from fungal botourmiaviruses by acquiring the capsid protein and MP genes to adjust to the plant host environments (124). For other narna-like

viruses, as discussed in previous reviews (16, 165), horizontal transfer from both fungi to plants and plants to fungi could be possible during the course of evolution (109).

Evolutional Aspects of (−)ssRNA Mycoviruses

Recent studies have indicated that nonretroviral EVEs have been discovered in the genomes of fungi and provided evidence for the presence of extant (−)ssRNA viruses in filamentous fungi (71, 72). Subsequently, several studies have uncovered the presence of diverse mycoviruses with (−)ssRNA genomes, including mymonaviruses (order *Mononegavirales*) (89, 93, 170), phenui-like viruses, and bunya-like viruses (order *Bunyavirales*) (14, 15, 37, 93, 114). The metagenomic approach has also expanded the diversity of (−)ssRNA mycoviruses, including novel mycoviruses related to yueviruses (order *Goujianvirales*) and aspiviruses (order *Serpentovirales*), together with relatives of the abovementioned viruses with some enigmatic genomic structures (22, 63, 149, 169).

Many (−)ssRNA viruses encode glycoprotein gene(s), allowing virus cell entry into animal hosts, including vector insects for the case of plant viruses (32). In contrast, known (−)ssRNA mycoviruses do not have such genes, and thus they appear to lack a potential alternate host animal or an extracellular route for their infection of fungal hosts, similar to other mycoviruses except for the ssDNA virus SsHADV1. Interestingly, a mycovirus [Sclerotinia sclerotiorum rhabdovirus 1 (SsRhV1)] closely related to animal rhabdoviruses was reported first in fungi (98). SsRhV1 shares the genomic structure with the members of the subfamily *Alpharhabdovirinae*, including a putative glycoprotein, and likely forms a typical bullet-shaped virion. Viruses of *Alpharhabdovirinae* infect animals (vertebrates and/or invertebrates) but not plants (167). Therefore, the SsRhV1 infection may also occur through the extracellular route via unknown vectors, potentially including invertebrates.

Phenuiviruses and bunyaviruses infecting animals and plants are known to have bipartite, tripartite, or multipartite (−)ssRNA genomes (76), and recent studies have provided the first evidence for fungal phenuiviruses, i.e., Lentinula edodes negative-strand RNA virus 2 (LeNSRV2) and Entoleuca phenui-like virus 1 (EnPLV1), which have a segmented genome with a typical ambisense coding strategy (84, 164). These two mycoviruses share their genome structures with those of plant-infecting phenuiviruses (genus *Coguvirus*) (10, 102). Importantly, LeNSRV2 and EnPLV1 and their plant virus relatives (coguviruses and trisegment ruboviruses) encode the putative proteins similar to MPs (30K MP superfamily) (102, 130), suggesting the potential of trans-kingdom virus infection between plant and fungi as in the case of a plant RNA virus in the field (6). Aspi-like mycoviruses are related to ophioviruses (members *Aspiviridae*), which are known as plant pathogens, and some are transmitted by the zoospores of *Olpidium* spp. (47). Similar to the aforementioned scenario for beny-like and poty-like mycoviruses, evolutionary trans-kingdom virus infection may have occurred in the past (35).

This article does not touch on the evolution of fungal ssDNA genomoviruses, which still constitute a minor portion of the fungal virome. The reader is encouraged to refer to other elegant reviews on this issue (35, 188).

SUMMARY POINTS

1. Recent virus hunting studies with various groups of fungi, cultured or uncultured, led to the discoveries of many peculiar mycoviruses with unusual genome organizations and/or even with neo-lifestyles. It is readily anticipated that novel mycoviruses with unseen genome organizations will be discovered by searching fungi as-yet-unexplored as virus hosts.

2. Peculiar mycoviruses are exemplified by splipalmiviruses, ambiviruses, and ambinarnaviruses. Splipalmiviruses are narna-like viruses with a multisegmented (+)ssRNA genome that, unprecedently, encodes RNA-dependent RNA polymerase (RdRP) domains on separate segments. Ambiviruses have an undivided single-stranded RNA (ssRNA) genome with two open reading frames (ORFs) on both strands in a head-to-head or tail-to-tail orientation. Ambinarnaviruses encode RdRP with phylogenetic affinity to narnaviruses on the (+)ssRNA and would also encode a hypothetical protein on the other strand. Unlike those of ambiviruses, the two ORFs of ambinarnaviruses fully overlap.
3. Examples of the mycovirus neo-lifestyles include the yado-kari/yado-nushi nature, hadaka nature, and the lifestyle exhibited by polymycoviruses. Capsidless yadokariviruses divert the capsid of unrelated dsRNA viruses to *trans*-encapsidate their RNA and RdRP and, likely, use the same replication site. Hadakaviruses with a 10- or 11-segmented (+)ssRNA genome also have a capsidless nature that is distinct in RNase susceptibility and sedimentation profile from well-established capsidless hypoviruses or polymycoviruses. Polymycoviruses are phylogenetically close to hadakaviruses but encode proline-, alanine-, and serine-rich protein (PASrp) that binds their genomic double-stranded (dsRNA) and form complexes or filamentous particles. Polymycovirus genomic dsRNA, whether naked or associated with PASrp, is infectious.
4. The discovery of a great number of mycoviruses as well as other viruses has filled phylogenetic gaps and provided evolutionary insights. Horizontal virus transfer appears to (have) occur(ed) between different kingdoms of organisms, which is inferred from and suggested by phylogenetical analyses and the substantiation of cross-kingdom infections.

FUTURE ISSUES

1. Metagenomic and metatranscriptomic analyses revealed the genome structure of many mycoviruses. Are they biologically infectious as virus entities? If yes, what are the host range and the impacts on their host fungi?
2. It is difficult to determine the segment number of multipartite viruses that include newly detected mycoviruses through RNAseq approaches. How is fragmented and primer-ligated dsRNA sequencing (FLDS) or a similar method useful for this purpose?
3. Diverse mycoviruses with unique ambisense genomic segments have been discovered. How are the ORFs on each strand expressed? What are the terminal structures of the ambisense segments that may facilitate translation and replication? What is the ratio of the two strands of one genomic segment with ambisense nature?
4. How broadly do neo-lifestyles exhibited by some groups of mycoviruses prevail in other kingdoms of organisms? How do those with a capsidless or PASrp-associated nature (hadakaviruses and polymycoviruses) exist in infected fungal cells? How do these viruses evade fungal antiviral defense?
5. How is the yadokarivirus/dsRNA virus partnership determined? How is the accumulation ratio of yadokarivirus versus partner dsRNA virus determined?

6. How much does horizontal gene transfer between fungal viruses and viruses infecting organisms of other kingdoms and horizontal virus transfer contribute to shaping the fungal virome?

DISCLOSURE STATEMENT

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