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Ambiviricota, a novel ribovirian phylum for viruses with viroid-like properties

Jens H. Kuhn,¹ Leticia Botella,² Marcos de la Peña,³ Eeva J. Vainio,⁴ Mart Krupovic,⁵ Benjamin D. Lee,^{6,7} Beatriz Navarro,⁸ Sead Sabanadzovic,^{9,10} Peter Simmonds,⁷ Massimo Turina^{11,12}

AUTHOR AFFILIATIONS See affiliation list on p. 8.

ABSTRACT Fungi harbor a vast diversity of mobile genetic elements (MGEs). Recently, novel fungal MGEs, tentatively referred to as ‘ambiviruses,’ were described. ‘Ambiviruses’ have single-stranded RNA genomes of about 4–5 kb in length that contain at least two open reading frames (ORFs) in non-overlapping ambisense orientation. Both ORFs are conserved among all currently known ‘ambiviruses,’ and one of them encodes a distinct viral RNA-directed RNA polymerase (RdRP), the hallmark gene of ribovirian kingdom *Orthornovirae*. However, ‘ambivirus’ genomes are circular and predicted to replicate via a rolling-circle mechanism. Their genomes are also predicted to form rod-like structures and contain ribozymes in various combinations in both sense and antisense orientations—features reminiscent of viroids, virusoids, ribozvirian kolmiovirids, and yet-unclassified MGEs (such as ‘epsilonviruses,’ ‘zetaviruses,’ and some ‘obelisks’). As a first step toward the formal classification of ‘ambiviruses,’ the International Committee on Taxonomy of Viruses (ICTV) recently approved the establishment of a novel ribovirian phylum, *Ambiviricota*, to accommodate an initial set of 20 members with well-annotated genome sequences.

KEYWORDS *Ambiviricota*, ambiviricot, ambivirus, Ascomycota, Basidiomycota, deltavirus, fungi, fungus, hairpin, hammerhead, ICTV, International Committee on Taxonomy of Viruses, phylum, *Riboviria*, ribozyme, twister, viroid, virus classification, virus nomenclature, virus taxonomy

The term ‘ambiviruses’ was first used for a group of unique mobile genetic elements (MGEs) with RNA genomes discovered in isolates of agaricomycete (Cerato-basidiaceae: *Ceratobasidium* sp. and Tulasnellaceae: *Tulasnella* sp.) and sordariomycete [Cryphonectriaceae: *Cryphonectria parasitica* (Murrill) M.E.Barr (1978)] fungi (1, 2). Soon after, several other ‘ambiviruses’ were characterized from mostly agaricomycetes [e.g., physalacriaceae *Armillaria* spp., bondarzewiaceae *Heterobasidion* spp., ceratobasidiaceae *Rhizoctonia* spp., and phanerochaetaceae *Phlebiopsis gigantea* (Fr.) Jülich (1978)] (3–5). These newly discovered MGEs were labeled “orphan-encoding sequences” because they contained open reading frames (ORFs) encoding products that, at the time of discovery, were not homologous to any of the protein sequences in the non-redundant protein database, as determined by Basic Local Alignment Search Tool (BLAST) comparison. These candidate viruses were missed in previous, highly comprehensive similarity-based virus-discovery searches in large metatranscriptomic datasets (6–8).

‘Ambiviruses’ from axenically cultured fungi have single-stranded RNA genomes that are 4.3–5.2 kb in length and encode at least two conserved proteins from non-overlapping ORFs in ambisense orientation (ORF-A and ORF-B) (Fig. 1). Some of these genomes have a third ORF that encodes proteins with sequences not conserved among the clade members. ORF-A encodes a highly divergent RNA-directed RNA polymerase (RdRP) containing typical A, B, and C motifs in the palm subdomain (9). ORF-B encodes a protein

Editor Felicia Goodrum, The University of Arizona, Tucson, Arizona, USA

Address correspondence to Jens H. Kuhn, kuhnjens@mail.nih.gov, or Massimo Turina, massimo.turina@ips.cnr.it.

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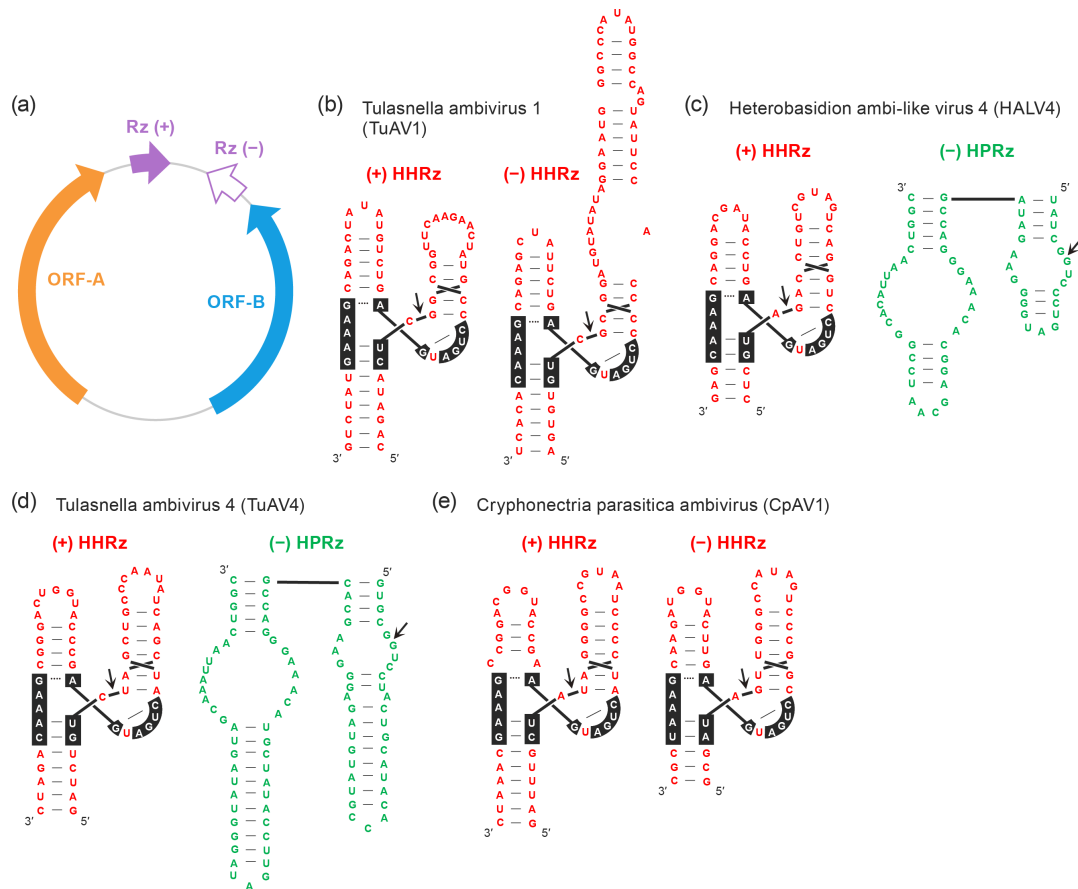


FIG 1 Genome organization and ribozymes of representative ‘ambiviruses.’ (a) Genome organization of ‘ambiviruses’ (sense: 5’→3’). (b–e) Secondary structures of the ribozymes contained in the plus (+) and minus (–) strands of representative ‘ambiviruses’ belonging to four distinct clades. Homologous ribozymes are shown with matching colors (red and green, respectively). ORF, open reading frame; Rz, ribozyme; HHRz, hammerhead ribozyme; HPRz, hairpin ribozyme. Arrows indicate self-cleavage sites.

conserved among ‘ambiviruses.’ The protein has no significant sequence or (predicted) structural similarity to functionally characterized proteins. Nevertheless, careful analysis of the ORF-B structure revealed the presence of two RNA recognition motifs (RRMs), with the second RRM resembling the palm domain of RdRPs and reverse transcriptases (RTs); however, neither RRM contains active site residues (M. Krupovic, personal observation). Thus, it is conceivable that the ORF-B-encoded protein is a cofactor for some aspects of genome replication.

Unlike most other described RNA viruses, ‘ambiviruses’ possess covalently closed circular RNA (cccRNA) genomes folding in a compact rod-like or branched secondary structure with the following ribozymes (Rzs) located at the 3’ termini of both ORFs: deltavirus ribozyme (DVRz), hammerhead ribozyme (HHRz), hairpin ribozyme (HPRz), and/or twister ribozyme (TWRz) (9, 10) (Fig. 1 and 2). Ribozyme activity and rolling-circle replication were experimentally confirmed *in vitro* and *in vivo* for three described ‘ambiviruses’: *Cryphonectria parasitica* ambivirus 1 (CpAV1), *Tulasnella ambivirus 1* (TuAV1), and *Tulasnella ambivirus 4* (TuAV4) (9). Together, these findings identified ‘ambiviruses’ as hybrids of viruses currently classified in realm *Riboviria*, kingdom *Orthornavirae* [which are defined as RNA viruses encoding RdRPs (11, 12)]; non-RdRP-encoding ribozyme-containing circular RNA viruses in realm *Ribozyviria*, family *Kolmioviridae* (hepatitis D virus 1 and relatives) (13); and, by extrapolation from those, viroids of the family *Avsunviroidae* (14), small circular single-stranded satellite RNAs (virusoids) (15), ‘epsilonviruses’ (7, 16), ‘zetaviruses’ (7), and ‘obelisks’ (17).

'AMBIVIRUS' DIVERSITY

Hundreds of 'ambivirus' genome sequences have been recently described (9, 18) (Fig. 2 and 3). For instance, a study of 46,500 fungal transcriptomes identified more than 2,500 'ambivirus' sequences that encode proteins with as little as 27% identity to previously described proteins; these 2,500 sequences likely represent genomes of at least 345 distinct 'ambiviruses' (18). Unsurprisingly, novel 'ambiviruses' continue to be discovered (19–22). Based on the organisms used for discovery, 'ambiviruses' infect fungi of at least four taxonomic divisions (Ascomycota, Basidiomycota, Chytridiomycota, and Glomeromycota). Of the discovered sequences, the biological host isolate has been experimentally validated and the viral genome confirmed by Sanger sequencing in basidiomycetes of *Armillaria* spp. (3, 21), *Heterobasidion* spp. (4, 23), and *Phlebiopsis gigantea* (5) and in two ascomycetes [*C. parasitica* and *Gibberella zeae* (Schwein.) Petch, (1936)] (formerly *Fusarium graminearum*) (2, 19). It is worth noting that these 'ambiviruses' are from fungi that are root associated except for *C. parasitica*, which is a tree trunk pathogen that is capable of a saprophytic lifestyle in soil. Given the enormous overall diversity of fungi, it is likely that the majority of unique 'ambiviruses' remain to be discovered.

ESTABLISHMENT OF PHYLUM AMBIVIRICOTA

To establish a framework for their initial formal classification, we analyzed the sequence dataset available at the beginning of 2023 to identify exemplar 'ambiviruses' with well-annotated genome sequences. The RdRPs encoded by ORF-A of the identified 23 viruses were analyzed for phylogeny (Fig. 4) and pairwise sequence similarity (Fig. 5). The 23 viruses formed four bootstrap-supported clades (Fig. 4 and 5). A preliminary species demarcation threshold of 90% pairwise sequence identity among translated ORF-A sequences supported their assignment to 20 distinct species.

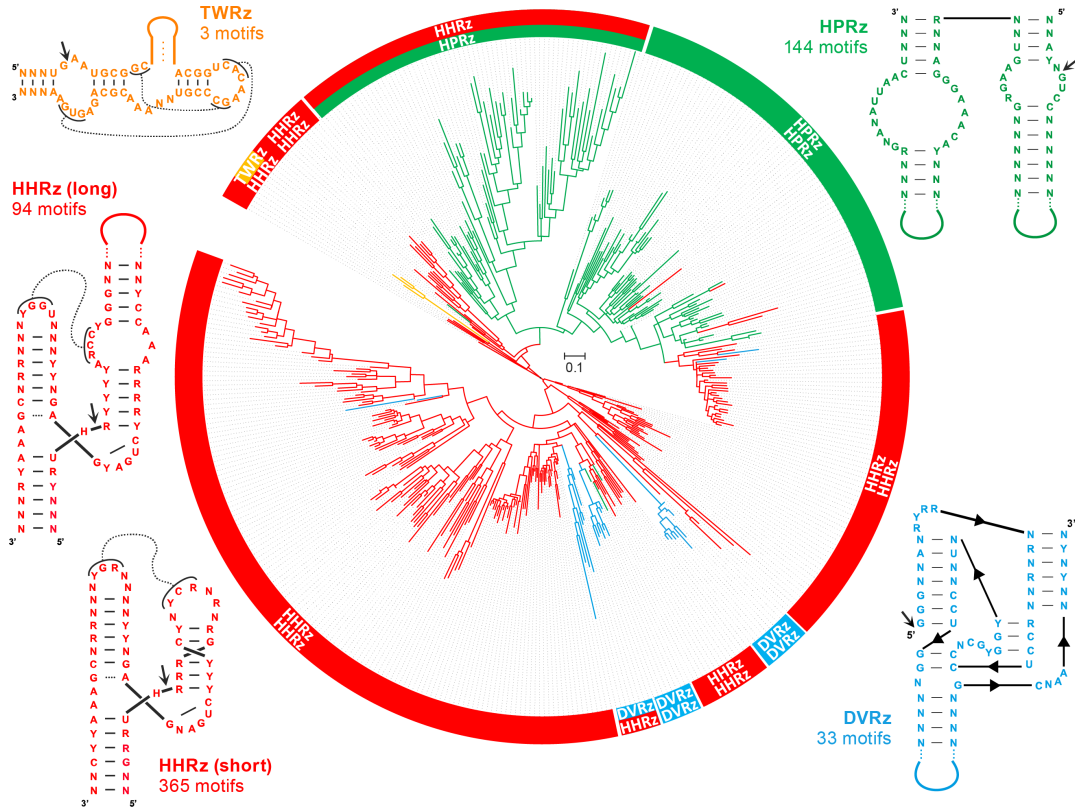


FIG 2 'Ambivirus' diversity. Maximum-likelihood phylogenetic tree of 'ambivirus' RNA-directed RNA polymerase (i.e., ORF-A-encoded protein) palimpsests of 439 distinct species-like operational taxonomic units. ORF, open reading frame; DVRz, deltavirus ribozyme; HHRz, hammerhead ribozyme; HPRz, hairpin ribozyme; TWRz, twister ribozyme. Sites of self-cleavage are indicated with arrows. Adapted from Fig. 2 in Forgia et al. 2023 (9).

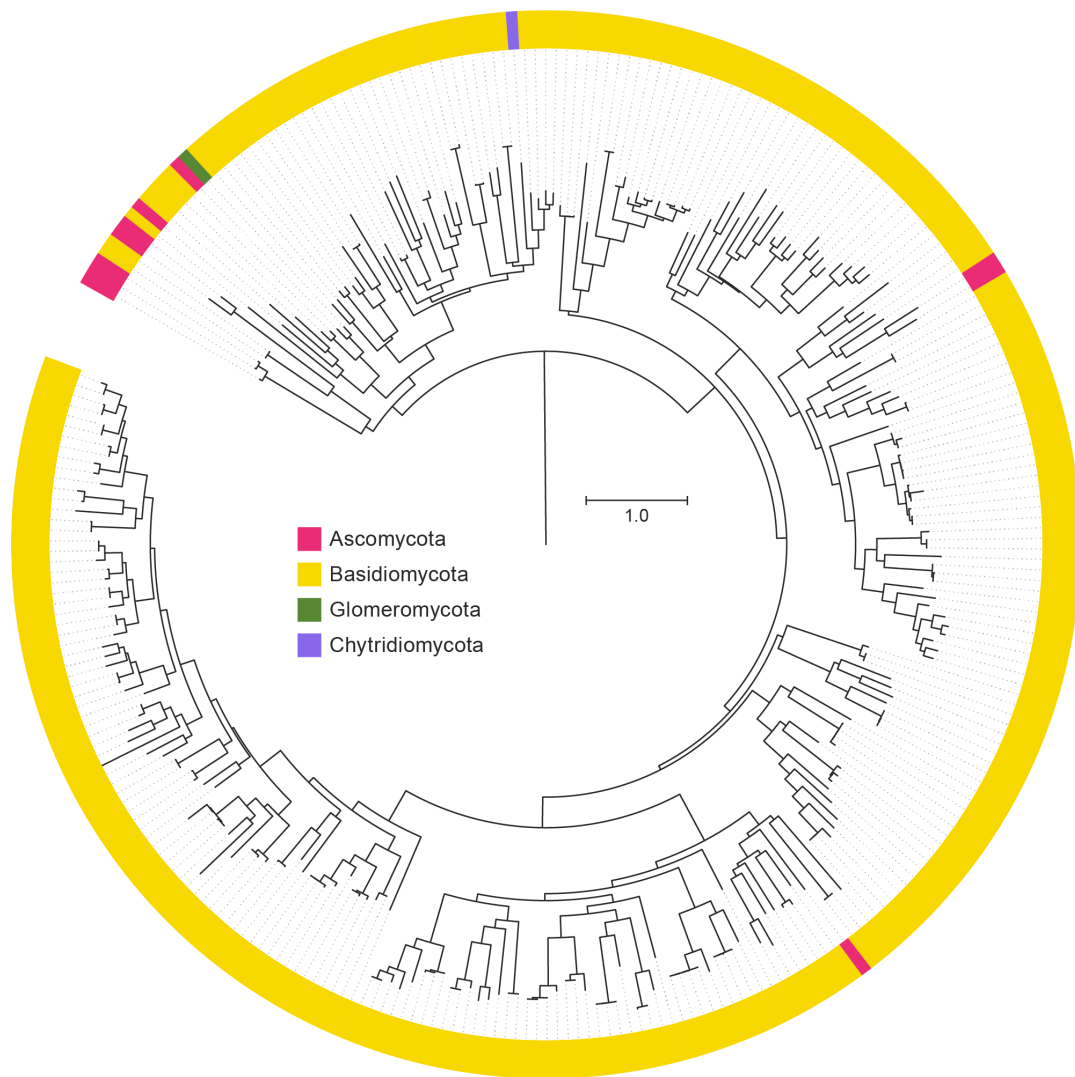


FIG 3 ‘Ambivirus’ diversity in fungal transcriptomes. Maximum-likelihood phylogenetic tree of ‘ambivirus’ RNA-directed RNA polymerases (i.e., ORF-A-encoded proteins), mid-point pseudo-rooted. Branches are colored according to the fungal division of the Sequence Read Archive (SRA) experiment from which the viral sequences were discovered. The scale bar is in average amino acid substitutions per site. Adapted from Fig. 3 in Chong and Lauber 2023 (18).

Based on these analyses, we proposed an initial taxonomic framework for the classification of these ‘ambiviruses’ to the International Committee on Taxonomy of Viruses (ICTV) in 2023 (29) that was approved and ratified in April 2024. The taxonomic framework will likely suffice to accommodate at least the already-known hundreds of unclassified ‘ambiviruses’ through the establishment of classes, orders, families, and genera. Due to the currently known diversity of ‘ambiviruses’ (Fig. 2 and 3), we conservatively proposed the establishment of four monogeneric families in a single order (*Crytulvirales*) and class (*Suforviricetes*) in a novel phylum *Ambiviricota* (Fig. 6).

Ambiviricots that form monophyletic clades in the RdRP phylogeny possess distinct types of ribozymes (Fig. 2), indicating that the two characters evolved independently of each other and are likely acquired/exchanged horizontally. The placement of ambiviricots within the global phylogenetic tree of RdRPs of members of the *Riboviria* remains ambiguous because of the substantial genetic divergence among them. However, comparisons based on predicted tertiary protein structures cluster ambiviricot RdRPs within *Orthornavirae* (30). Thus, due to the conservation of the RdRP, the hallmark protein of ribovirion orthornaviraens, being the only feature unifying ambiviricots, we proposed that phylum *Ambiviricota* be included in kingdom *Orthornavirae*.

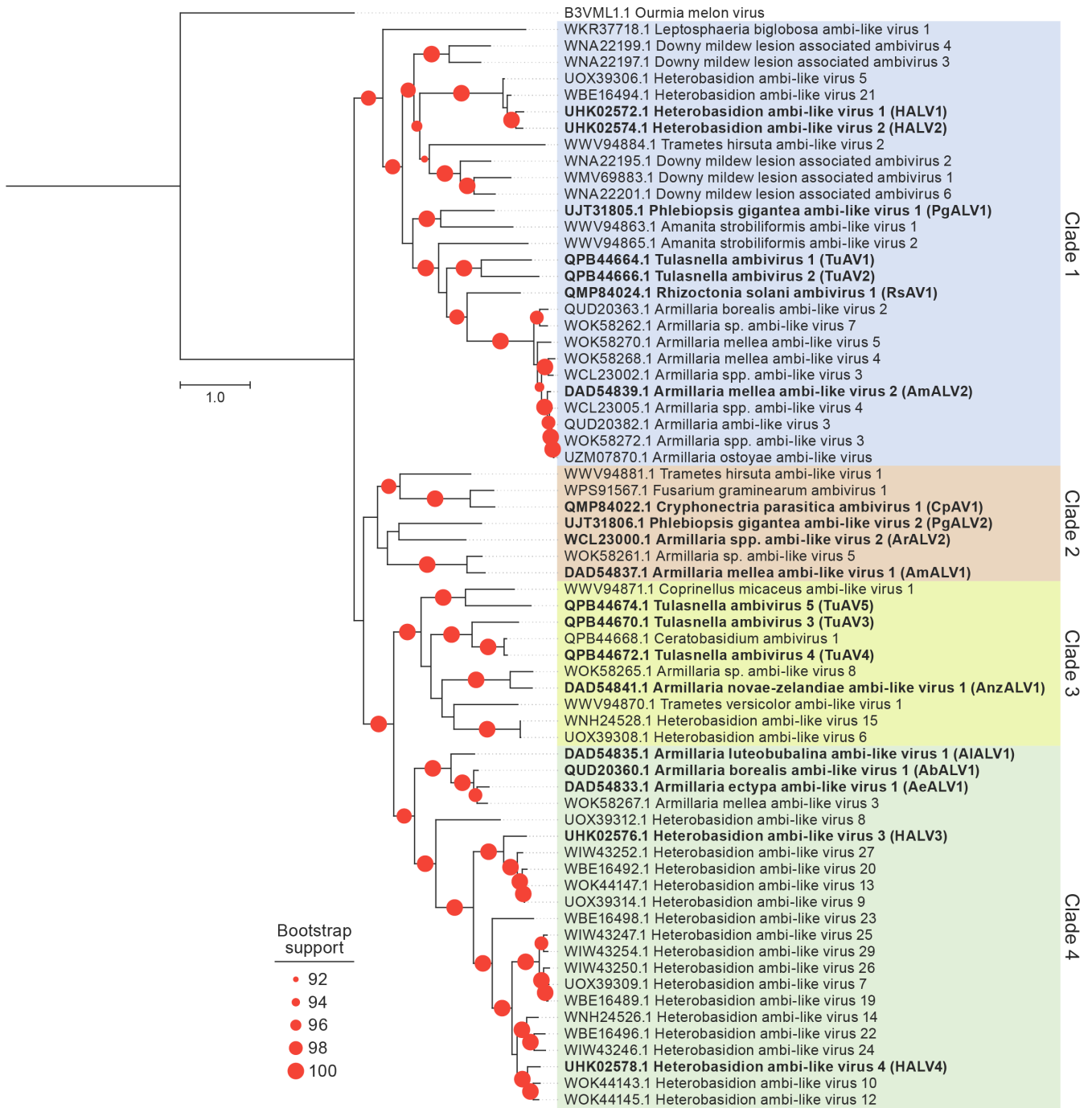


FIG 4 Phylogeny of ‘ambiviruses’ with well-annotated genome sequences. Phylogenetic tree based upon alignment of deduced amino acid sequences of well-annotated ‘ambivirus’ RNA-directed RNA polymerases (i.e., ORF-A-encoded proteins). Viruses written in bold font were used for the official taxonomic proposal to establish a phylum (see also Fig. 6). Alignment was performed with MAFFT v.7.525 (24, 25), whereas tree topology was calculated with the maximum-likelihood methodology implemented in IQ-TREE (26) using the ultrafast bootstrap method. The final version of the tree was drawn with Interactive Tree of Life (iTOL) v.6.9 (27). ORF, open reading frame.

OUTLOOK

The now-established *Ambiviricota* taxonomy will likely undergo rather drastic expansion in the immediate future. Beyond that, major questions will have to be addressed. As of now, *Ambiviricota* is a virus phylum. Per the ICTV’s International Code of Virus Classification and Nomenclature (ICVCN) Rule 3.3,

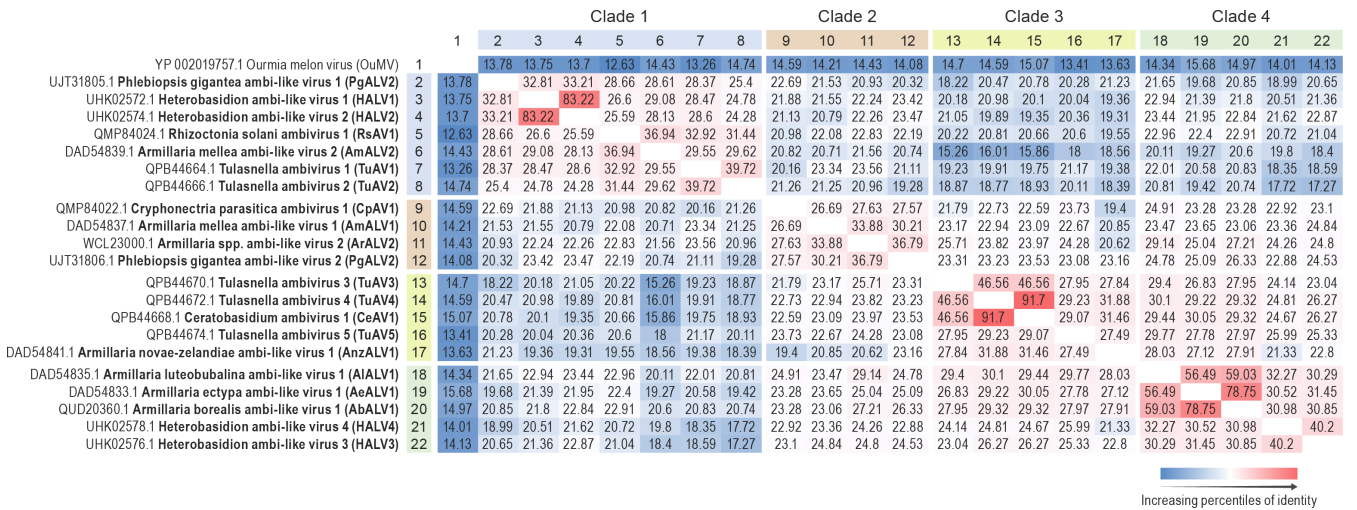


FIG 5 Percent (%) sequence identity of ‘ambiviruses’ (bold) with well-annotated genome sequences. Sequence identity matrix of RNA-directed RNA polymerase (i.e., ORF-A-encoded protein) sequences of all ‘ambiviruses’ used for the establishment of the initial taxonomic framework. Pairwise alignment was performed using Clustal Omega v1.2.4 (25, 28). Shades of color intensity from blue to red represent increasing percentiles of identity. ORF, open reading frame.

“[v]iruses *sensu stricto* are defined ... as a type of MGEs that encode at least one protein that is a major component of the virion encasing the nucleic acid of the respective MGE and therefore the gene encoding the major virion protein itself; or MGEs that are clearly demonstrable to be members of a line of evolutionary descent of such major virion protein-encoding entities ...” (31, 32).

Yet, it is currently unclear whether ambiviricot genomes are encapsidated, i.e., whether ORF-B encodes a protein akin to a capsid protein (CP). Given that many viral capsid proteins have been exapted from preexisting functionally diverse proteins (33), the possibility that the ORF-B-encoded protein functions in genome encapsidation cannot be ruled out but appears less likely than its involvement in genome replication. However, the apparent preferential distribution of ambiviricots in fungi is consistent with the capsid-less nature of many families of fungal viruses that are believed to have lost their capsid genes, switching to exclusively intracellular replication through fungal syncytia. Thus, whether ambiviricots should be considered viruses hinges on whether they have evolved from *bona fide* capsid-encoding viruses; yet, the evolutionary history of ambiviricots is currently unclear. However, all ambiviricots encode RdRPs, enabling their clustering with other RdRP-encoding viruses, all of which are classified within *Orthornavirae*. Indeed, no non-viral MGEs have been identified to encode palm-domain RdRPs thus far, supporting the placement of ambiviricots within a viral realm.

The taxonomic positioning of ambiviricots in *Orthornavirae* is associated with high uncertainty. Ambiviricots are highly reminiscent of non-RdRP-encoding ribozyme-containing circular RNA viruses (realm *Ribozoviria*: family *Kolmioviridae*) (13) and similar elements [such as viroids of the family *Avsunviroidae* (14), virusoids (15), ‘epsilonviruses’ (7, 16), ‘zetaviruses’ (7), and some ‘obelisks’ (17)], all of which have ribozyme-containing cccRNA genomes that replicate via the rolling-circle mechanism with the help of host-cell polymerases. Kolmiovirids are currently defined as negative-sense RNA viruses that exclusively have DVRzs and encode homologs of delta antigen (Dag). They infect animals and may require helper viruses for infection (13). Viroids of the family *Avsunviroidae* are defined as naked cccRNAs that contain ribozymes, do not encode proteins, and replicate autonomously in the absence of co-infection with a helper virus (14). Virusoids are basically viroids that are encapsidated with the help of co-infecting viruses (15). ‘Epsilonviruses’, ‘zetaviruses’, and ‘obelisks’ are at least superficially most similar to kolmiovirids in that they encode proteins and contain ribozymes, but their proteins bear little or no resemblance to Dag, and they often have ribozymes other than DVRzs

Family name	Genus name	Species name	Virus name (Abbreviation)
Dumbiviridae (clade 1)	Orthodumbivirus	<i>Orthodumbivirus armillariae</i>	Armillaria mellea ambi-like virus 2 (AmALV2)
		<i>Orthodumbivirus duatulasnellae</i>	Tulasnella ambivirus 2 (TuAV2)
		<i>Orthodumbivirus duoheterobasidii</i>	Heterobasidion ambi-like virus 2 (HALV2)
		<i>Orthodumbivirus phlebiopsis</i>	Phlebiopsis gigantea ambi-like virus 1 (PgALV1)
		<i>Orthodumbivirus rhizoctoniae</i>	Rhizoctonia solani ambivirus 1 (RsAV1)
		<i>Orthodumbivirus unatulasnellae</i>	Tulasnella ambivirus 1 (TuAV1)
Unambiviridae (clade 2)	Orthounambivirus	<i>Orthounambivirus cryphonectriae</i>	Cryphonectria parasitica ambivirus 1 (CpAV1)
		<i>Orthounambivirus duarmillariae</i>	Armillaria spp. ambi-like virus 2 (ArALV2)
		<i>Orthounambivirus phlebiopsis</i>	Phlebiopsis gigantea ambi-like virus 2 (PgALV2)
		<i>Orthounambivirus unarmillariae</i>	Armillaria mellea ambi-like virus 1 (AmALV1)
Quambiviridae (clade 3)	Orthoquambivirus	<i>Orthoquambivirus armillariae</i>	Armillaria novae-zelandiae ambi-like virus 1 (AnzALV1)
		<i>Orthoquambivirus duatulasnellae</i>	Tulasnella ambivirus 3 (TuAV3)
		<i>Orthoquambivirus tritulasnellae</i>	Tulasnella ambivirus 5 (TuAV5)
		<i>Orthoquambivirus unatulasnellae</i>	Tulasnella ambivirus 4 (TuAV4)
Trimbiviridae (clade 4)	Orthotrimbivirus	<i>Orthotrimbivirus duarmillariae</i>	Armillaria ectypa ambi-like virus 1 (AeALV1)
		<i>Orthotrimbivirus duoheterobasidii</i>	Heterobasidion ambi-like virus 3 (HALV3)
		<i>Orthotrimbivirus triarmillariae</i>	Armillaria borealis ambi-like virus 1 (AbALV1)
		<i>Orthotrimbivirus unarmillariae</i>	Armillaria luteobubalina ambi-like virus 1 (AlALV1)
		<i>Orthotrimbivirus unheterobasidii</i>	Heterobasidion ambi-like virus 4 (HALV4)

FIG 6 Current ambiviricote taxonomy (*Riboviria*: *Orthornavirae*: *Ambiviricota*: *Suforviricetes*: *Crytulvirales*). See Box 1 for etymology of established taxa.

(7, 16, 17). In the absence of any discernible evolutionary markers, ambiviricotes could be regarded to be in the same group as these three types of MGEs were it not for the encoded RdRP, the function of which in ambiviricotes is yet to be experimentally demonstrated. The biological characterization of ambiviricotes is only in its infancy, and infectious cDNA clones for in-depth studies have yet to be developed. However, in at least two cases (CpAV1 and *Fusarium graminearum* ambivirus 1), effects on fungal virulence and on mycotoxin production, respectively, were experimentally demonstrated (9, 19). Together, the growing knowledge about ambiviricotes and other cccRNA MGEs/viruses will likely spur discussions about whether their definitions ought to be amended and potentially lead to increased laboratory experimentation to shed light on their life cycles, evolution, and significance.

BOX 1. ETYMOLOGY OF ESTABLISHED TAXA IN FIG. 6

- *Ambiviricota*: after ‘ambivirus’ and phylum-specific suffix *-viricota*
- ambivirus: after Latin *ambi*, meaning “both” (ambiguous) and “around” (ambient), a reference to these mobile genetic elements (MGEs) having features of both orthornaviraens and ribozyme-encoding entities
- *armillariae*: after the host genus
- *cryphonectriae*: after the host genus
- *Crytulvirales*: after *Cryphonectria* and *Tulasnella*, the genera for fungi in which ‘ambiviruses’ were first discovered, and order-specific suffix *-virales*
- *duarmillariae*: after the host genus and Latin *duo*, meaning “two”
- *duatulasnellae*: after the host genus and Latin *duo*, meaning “two”
- *Dumbiviridae*: after Latin *duo*, meaning “two;” ‘ambivirus;’ and family-specific suffix *-viridae*
- *duoheterobasidii*: after the host genus and Latin *duo*, meaning “two”
- *Ortho-*: after Greek prefix *ortho*, meaning “straight”
- *phlebiopsis*: after the host genus
- *Quambiviridae*: after Latin *quattuor*, meaning “four;” ‘ambivirus;’ and family-specific suffix *-viridae*

- *rhizoctoniae*: after the host genus
- *Suforviricetes*: after *Sutela* and *Forgia*, the first authors of the first two papers describing 'ambiviruses' (1, 2); and class-specific suffix *-viricetes*
- *triarmillariae*: after the host genus and Latin *tria*, meaning "three"
- *Trimbiviridae*: after Latin *tria*, meaning "three;" 'ambivirus;' and family-specific suffix *-viridae*
- *tritulasnellae*: after the host genus and Latin *tria*, meaning "three"
- *Unambiviridae*: after Latin *unus*, meaning "one;" 'ambivirus;' and family-specific suffix *-viridae*
- *unarmillariae*: after the host genus and Latin *unus*, meaning "one"
- *unatulasnellae*: after the host genus and Latin *unus*, meaning "one"
- *unoheterobasidii*: after the host genus and Latin *unus*, meaning "one"

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AUTHOR AFFILIATIONS

¹Integrated Research Facility at Fort Detrick, Division of Clinical Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Fort Detrick, Frederick, Maryland, USA

²Department of Forest Protection and Wildlife Management, Faculty of Forestry and Wood Technology, Mendel University in Brno, Brno, Czech Republic

³Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Valencia, Spain

⁴Natural Resources Institute Finland (Luke), Helsinki, Finland

⁵Institut Pasteur, Université Paris Cité, Archaeal Virology Unit, Paris, France

⁶National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Maryland, USA

⁷Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

⁸Institute for Sustainable Plant Protection-CNR, Bari, Italy

⁹Department of Agricultural Science and Plant Protection, Mississippi State University, Mississippi State, Mississippi, USA

¹⁰Institute for Genomics, Biocomputing and Biotechnology, Mississippi State University, Mississippi State, Mississippi, USA

¹¹Institute for Sustainable Plant Protection-CNR, Torino, Italy

¹²Department of Plant Protection, School of Agriculture, The University of Jordan, Amman, Jordan

AUTHOR ORCID*s*

Jens H. Kuhn  <http://orcid.org/0000-0002-7800-6045>

Leticia Botella  <http://orcid.org/0000-0002-6613-5405>

Marcos de la Peña  <http://orcid.org/0000-0002-7949-8459>

Eeva J. Vainio  <http://orcid.org/0000-0002-6739-7968>

Mart Krupovic  <http://orcid.org/0000-0001-5486-0098>

Benjamin D. Lee  <http://orcid.org/0000-0002-7133-8397>

Beatriz Navarro  <http://orcid.org/0000-0001-8981-1018>

Sead Sabanadzovic  <http://orcid.org/0000-0002-2995-2633>

Peter Simmonds  <http://orcid.org/0000-0002-7964-4700>

Massimo Turina  <http://orcid.org/0000-0002-9659-9470>

AUTHOR CONTRIBUTIONS

Jens H. Kuhn, Conceptualization, Writing – original draft, Writing – review and editing | Leticia Botella, Writing – review and editing | Marcos de la Peña, Writing – review and editing | Eeva J. Vainio, Writing – review and editing | Mart Krupovic, Writing – review and editing | Benjamin D. Lee, Writing – review and editing | Beatriz Navarro, Visualization, Writing – review and editing | Sead Sabanadzovic, Visualization, Writing – review and editing | Peter Simmonds, Writing – review and editing | Massimo Turina, Conceptualization, Supervision, Visualization, Writing – review and editing

ETHICAL APPROVAL

This work did not include any work with humans or animals.

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