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Mitovirus UGA(Trp) codon usage parallels that of host mitochondria

Max L. Nibert*

Department of Microbiology & Immunobiology, Harvard Medical School, Boston, MA 02115, USA

Abstract

Mitoviruses replicate in mitochondria of their host fungi. They have small RNA genomes that encompass a single ORF encoding the viral RdRp. Since UGA codons encode Trp in fungal mitochondria, the RdRp ORF of a typical mitovirus includes multiple UGA codons. In some mitoviruses, however, the ORF has no such codons, suggesting that these particular viruses may be under selective pressure to exclude them. In this report, new evidence is presented that host fungi whose mitoviruses have no or few UGA codons are distinctive in also having no or few UGA codons in their core mitochondrial genes. Thus, the relative exclusion of such codons in a subset of mitoviruses appears to reflect most fundamentally that UGA(Trp) is a rare mitochondrial codon in their particular hosts. The fact that UGA(Trp) is a rare mitochondrial codon in many fungi appears not to have been widely discussed to date.

Keywords

fungus; mitochondrion; mitovirus; mycovirus; Narnaviridae; rare codon; RNA virus; UGA

INTRODUCTION

Genus *Mitovirus*, in family *Narnaviridae*, comprises fungal viruses with small RNA genomes, most or all of which replicate in their hosts' mitochondria (Cole et al., 2000; Hillman and Cai, 2013; Hong et al., 1998, 1999; Polashock and Hillman, 1994; Rogers et al., 1987; Wu et al., 2016). Only five mitovirus species from two host fungi, the chestnut blight fungus *Cryphonectria parasitica* and the Dutch elm disease fungus *Ophiostoma novo-ulmi*, have been ratified to date: *Cryphonectria mitovirus 1*, *Ophiostoma mitovirus 3a*, *Ophiostoma mitovirus 4*, *Ophiostoma mitovirus 5*, and *Ophiostoma mitovirus 6* (Buck et al., 2005; Hong et al., 1998, 1999; Polashock and Hillman, 1994). However, there are now at least 99 annotated accessions in the Nucleotide database (NR/NT) at GenBank that appear to encompass the complete protein-coding sequences of these and other mitoviruses, from at least 29 different fungal host species in phylum Ascomycota, subphylum Pezizomycotina;

*Corresponding author. +1-617-432-4829. mnibert@hms.harvard.edu.

phylum Basidiomycota, subphylum Agaricomycotina or Pucciniomycotina; and phylum Mucoromycota, subphylum Glomeromycotina (fungal taxonomy per Hibbett et al. (2007), updated by Spatafora et al. (2016)) (Table S1). The genome sequence lengths across this collection range from 2.1 to 4.4 kb (median, 2.6 kb).

The genome sequence of each of these viruses encompasses a single long ORF encoding a deduced protein that spans between 650 and 1140 aa (median, 726 aa) and includes conserved motifs indicative of a viral RdRp. This protein is presumably required for both plus- and minus-strand RNA synthesis of each virus. Phylogenetic analyses of the deduced RdRp sequences (Fig. 1) suggest that they indeed represent a monophyletic clade of viruses that encompasses current genus *Mitovirus* and is distinguishable from the other current genus (*Narnavirus*) in family *Narnaviridae*, to which only two ratified species (*Saccharomyces 20S RNA narnavirus* and *Saccharomyces 23S RNA narnavirus*) have been assigned to date (Buck et al., 2005; Esteban et al., 1992; Hillman and Cai, 2013). The RdRps of mito- and narnaviruses have been reported to share some limited sequence similarities with those of RNA bacteriophages such as Qbeta in family *Leviviridae* (Esteban et al., 1992; Polashock and Hillman, 1994). This has led to the further suggestion that these viruses might have evolved from levivirus-like ancestors, possibly associated with bacterial endosymbionts including those from which mitochondria evolved (Bruenn et al. 2015; Hillman and Cai, 2013; Hong et al. 1998; Shackelton and Holmes, 2008).

For translation of the RdRp, mitovirus mRNAs must rely on the same genetic code as the endogenous mitochondrial mRNAs of their respective hosts. Notably, the mitochondrial genetic code of fungi (translation table 3 or 4), like that of many other eukaryotes, includes two Trp codons: not only UGG as in the standard nuclear/cytosolic code (translation table 1) but also UGA, which serves as a stop codon in the standard code. UAA and UAG are hence the only two stop codons in the endogenous mitochondrial mRNAs of most fungi, and UGA(Trp) codons are commonly found within the ORFs of those mRNAs (see evidence in Table S2 as introduced below). Similarly, the RdRp ORFs of most of the mitoviruses in Table S1 include a substantial number of UGA codons. Indeed, in the RdRp ORFs across this full collection of viruses, the median numbers of Trp codons per virus are 10 UGA vs. 4 UGG (median percentages, 67% UGA vs. 33% UGG). As indicated in previous reports, this finding strongly supports the conclusion that these viruses replicate in fungal mitochondria, because the many UGA codons within their RdRp ORFs would specify stops if used for mRNA translation in the cytosol.

It is important to note, however, that there are exceptions to the general conclusion that UGA codons are common among the mitoviruses in Table S1. In particular, two viruses from *Rhizophagus* species (phylum Mucoromycota, subphylum Glomeromycotina) (Kitahara et al., 2014), ten viruses from *Rhizoctonia* species (phylum Basidiomycota, subphylum Agaricomycotina) (Abdoulaye et al., 2017; Bartholomäus et al., 2016; Das et al., 2016; Lakshman et al., 1998; Marzano et al., 2016; Zhang et al., 2015), and one virus from *Macrophomina phaseolina* (phylum Ascomycota, subphylum Pezizomycotina) (Marzano et al., 2016) contain no UGA codons. Additionally, three other viruses from *Rhizoctonia solani* (Marzano et al., 2016) and one each from *Agaricus bisporus* and *Clitocybe odora* (phylum Basidiomycota, subphylum Agaricomycotina) (Heinze, 2012) contain only a low percentage

of UGA codons (4–21%) vs. UGG codons (79–96%). These exceptions are the focus of this report.

Several previous authors have noted that any such mitoviruses with no UGA codons might express their RdRps and replicate their genomes not only in mitochondria but also or instead in the cytosol of their respective hosts (Das et al., 2016; Hillman and Cai, 2013; Hong et al., 1999; Kitahara et al., 2014; Lakshman et al., 1998; Zhang et al., 2015). Moreover, such capacity for cytosolic replication might have provided the selective pressure for this subset of mitoviruses to lack UGA codons. In this report, however, evidence is presented that runs counter to that argument. Of particular note is evidence that UGA(Trp) is a rare (unpreferred, infrequently used) codon in the endogenous core mitochondrial genes of fungi from families *Ceratobasidiaceae*, *Agaricaceae*, *Tricholomataceae*, and *Glomeraceae*, to which *Rhizoctonia* species, *Agaricus bisporus*, *Clitocybe odora*, and *Rhizophagus* species respectively belong. Thus, the presence of no or few UGA codons in the mitoviruses from these fungi appears to be explained more fundamentally by the fact that the molecular machinery for mRNA translation in these particular hosts' mitochondria simply disfavors such codons, i.e., UGA(Trp) is a rare mitochondrial codon in these hosts.

RESULTS

In Fig. 1, some of the mitoviruses possessing no UGA codons are phylogenetically juxtaposed to ones possessing many more of these codons. For example, Thanatephorus cucumeris mitovirus (*T. cucumeris* is a teleomorph of *Rhizoctonia solani*) contains no UGA codons but is flanked in the tree by Botrytis cinerea mitovirus 3 and Fusarium poae mitovirus 3, which respectively contain 11 and 16 of these codons (representing 85% and 94% of their Trp codons) (highlighted by a vertical bar at right in Fig. 1). From a simple perspective, it seems unlikely that viruses sharing such close phylogenetic relatedness would exhibit the fundamental difference in site(s) of intracellular replication that has been suggested by several previous authors: some viruses replicating only in mitochondria (those with UGA codons), other viruses replicating in the cytosol instead of or in addition to mitochondria (those with no UGA codons). Considering this simple perspective, alternative explanations were explored for why only a small subset of the mitoviruses in Table S1 has no or few UGA codons.

One reasonable alternative would seem to be that UGA(Trp) may be a rare mitochondrial codon in particular fungi, just as various nuclear/cytosolic codons are rare in different organisms (Komar, 2016). A recent article by Hegedusova et al. (2014) has indeed provided evidence in this regard for a number of fungal species in phylum Basidiomycota. For the current report, that analysis was expanded by surveying all fungal taxa, at the level of taxonomic order and below, for which both complete/annotated mitochondrial genome sequences and associated mitovirus sequences have been reported to NR/NT at GenBank. For three of the fungal host species in Table S1 (*Buergenerula spartinae*, *Helicobasidium mompa*, and *Macrophomina phaseolina*), no complete mitochondrial genome sequences have been reported to NR/NT, even for other species in the same taxonomic orders of fungi to which these species belong, and thus these three mitovirus hosts could not be included in the current analysis. For the numerous other mitovirus hosts in Table S1, however, protein-

coding sequences of the 11–14 mitochondrial core genes *atp6*, *atp8*, *atp9*, *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, and *nad6* (Hegedusova et al., 2014; Losada et al., 2014) were identified within the complete mitochondrial genome sequences of relevant fungi. The protein-coding sequences from these mitochondrial core genes were then concatenated and examined for their codon usage frequencies, in order to ascertain whether UGA(Trp) is a rare mitochondrial codon in any of these fungi.

The summary of results from this analysis in Table S2 reveals that 14 different taxonomic families or orders include fungal species that have had both their complete/annotated mitochondrial genome sequences and their associated mitovirus sequences reported to NR/NT. For the fungal species from four of these families (*Ceratobasidiaceae*, *Agaricaceae*, *Tricholomataceae*, and *Glomeraceae*), their associated mitoviruses have low percentages of UGA codons (0–21%, median 0%) vs. UGG codons (79–100%, median 100%), and their endogenous core mitochondrial genes strikingly also have low percentages of UGA codons (0–4%) vs. UGG codons (96–100%). Reciprocally, for the fungal species from the other ten families or orders (*Cryphonectriaceae*, *Ophiostomataceae*, *Nectriaceae*, *Ceratocystidaceae*, *Sclerotiniaceae*, *Helotiaceae*, *Pleosporinae*, *Pezizales*, *Pucciniales*, and *Bondarzewiaceae*), their associated mitoviruses have higher percentages of UGA codons (31–100%, median 77%) vs. UGG codons (0–69%, median 23%), and their endogenous core mitochondrial genes strikingly also have higher percentages of UGA codons (69–100%) vs. UGG codons (0–31%). These findings are graphically summarized in Fig. 2. Thus, UGA(Trp) is a rare codon only in the mitochondria of host fungi whose associated mitoviruses also have no or few UGA codons. This noteworthy correlation suggests that a small subset of mitoviruses possesses no or few UGA codons most fundamentally because the molecular machinery for mRNA translation in their hosts' mitochondria disfavors those codons.

DISCUSSION

The fact that only a small subset of mitoviruses (13 of 99 in Table S1) has no UGA codons is intriguing, because it suggests that these particular viruses may have been under selective pressure to exclude these codons. One source of such selective pressure could be the associated benefit for each of these viruses to have the capacity to express its full-length RdRp, and perhaps then also to replicate its genome, in the fungal host cytosol. UGA codons in the RdRp ORFs of these viruses would have thereby been excluded because they would specify translational stops in the cytosol, preventing synthesis of the full-length RdRp there. This seems to be a compelling argument, particularly in the absence of any other such argument to explain the small subset of mitoviruses that have no UGA codons. In this report, however, a different source of selective pressure has been identified. The evidence here suggests instead that the small subset of mitoviruses that have few or no UGA codons do so because UGA(Trp) is a rare (unpreferred, infrequently used) codon in their particular hosts' mitochondria. According to this new argument, the disfavoring of UGA(Trp) codons by the molecular machinery for mRNA translation in these hosts' mitochondria has provided the fundamental selective pressure to maintain the low number of UGA codons in the associated mitoviruses, regardless of any consequent potential for cytosolic replication if all UGA codons are excluded.

Might this new explanation have any broader significance for understanding prior and ongoing evolution of mitoviruses? If a mitovirus with no or few UGA codons, from a fungal host in which UGA(Trp) is a rare mitochondrial codon, were by some means horizontally transmitted into a new fungal host in which UGA(Trp) is a more common mitochondrial codon, then that virus would become newly free to accumulate those codons. Mitochondrial fusion after hyphal anastomosis (Polashock et al., 1997) would be a conceivable mechanism for such horizontal transmission. Reciprocally, if a mitovirus with a higher number of UGA codons, from a fungal host in which UGA(Trp) is a more common mitochondrial codon, were by some means horizontally transmitted into a new fungal host in which UGA(Trp) is a rare mitochondrial codon, then that virus would become newly subject to selective pressure to reduce its number of these codons. Successful horizontal transmission of mitoviruses back and forth between these two different types of fungal hosts, defined by their different Trp codon biases, would thus seem to remain possible in at least some instances, with the number of UGA codons newly reached after each such transmission being determined by the selective balance between virus and host.

Deeper evolutionary implications might also be inferred, but with less certainty. One possibility is that the last common ancestor to extant mitoviruses might have had no or few UGA codons and that the molecular machinery for mRNA translation in mitochondria of subsequent hosts was then the fundamental determinant of whether the evolving mitoviruses were either free to accumulate UGA codons or not. *Rhizophagus* species, from order Glomerales in subphylum Glomeromyocytina, phylum Mucoromycota, are the earliest-branching fungi (James et al., 2006) from which mitoviruses have been reported to date (Kitahara et al., 2014), and notably UGA(Trp) is a rare mitochondrial codon in these fungi as well as in their associated mitoviruses (Tables S1 and S2). UGA is a rare mitochondrial codon also in the other basal fungi for which complete/annotated mitochondrial genome sequences, though no mitoviruses, have been reported to date, specifically fungi from subphyla Glomeromyocytina, Mortierellomycotina, and Mucoromycotina in phylum Mucoromycota; subphylum Kickxellmycotina in phylum Zoopagomycota; phylum Blastocladiomycota; and phylum Chytridiomycota (Table S2). Thus, there appears to have been an extended period during the evolutionary radiation of basal fungi in which the last common ancestor to extant mitoviruses, with no or few UGA codons, might have infected one of these basal fungi.

The phylogenetic tree suggests at least three distinguishable clades of viruses within current genus *Mitovirus*, labeled I–III in Fig. 1. Clades I and II largely correspond with those distinguished by Hillman and Cai (2013), with clade III now also distinguishable. Viruses with no or few UGA codons are found in all three clades, though they make up a small fraction of Clade I, a larger fraction of Clade II, and most of Clade III. In addition, viruses derived from both ascomycetes and basidiomycetes are found in Clades I II, and III, and ones also from glomeromycetes only in Clade III to date. Some lower branch support values (60–66%) near the mitovirus root in this and other trees make interpretations tentative. However, the fact that the earliest-branching clade of mitoviruses in Fig. 1, Clade III, includes viruses derived from the earliest-branching clade of fungi reported to carry mitoviruses to date, glomeromycetes, is consistent with the suggestion above that the last common ancestor to extant mitoviruses might have infected a basal fungus.

From a simple genetic or mechanistic perspective, why might UGA(Trp) be a rare mitochondrial codon in some fungi, but not in others? All of the ascomycetes in Table S2 have at least one tRNA-Trp with anticodon UCA encoded in the mitochondrial genome, allowing preferential translation of codon UGA and thereby explaining why UGA is not a rare mitochondrial codon in these fungi. In contrast, all of the basal fungi in Table S2, up to and including the species in subphylum Glomeromycotina, phylum Mucoromycota, appear to have a single tRNA-Trp encoded in the mitochondrial genome, with anticodon CCA allowing preferential translation of codon UGG and thus consistent with the fact that UGA is a rare mitochondrial codon in these fungi. The basidiomycetes in Table S2, on the other hand, are mixed in these regards, as Hegedusova et al. (2014) have also noted for certain basidiomycetes. Three species in Table S2—*Phakopsora meibomiae*, *Phakopsora pachyrhizi*, and *Heterobasidion irregulare*—have at least one tRNA-Trp with anticodon UCA encoded in the mitochondrial genome, allowing preferential translation of codon UGA and thereby explaining why UGA is not a rare mitochondrial codon in these three basidiomycetes. However, three other species in Table S2—*Rhizoctonia solani*, *Agaricus bisporus*, and *Tricholoma matsutake*—have a single tRNA-Trp encoded in the mitochondrial genome, with anticodon CCA allowing preferential translation of codon UGG and thus consistent with the fact that UGA is a rare mitochondrial codon in these three basidiomycetes.

This new explanation for why some mitoviruses lack UGA codons does not directly imply that these viruses have failed to exploit the consequent potential for RdRp translation, and perhaps then also genome replication, in the cytosol of their respective hosts. Those outcomes of a mitovirus having no UGA codons remain logical possibilities, at least as based on the immediate facts. On the other hand, one can argue that yet-to-be identified host factors are likely to be involved in mitovirus replication and are also likely to be localized to either mitochondria or the cytosol, not both. Published evidence for presence of Thanatephorus cucumeris mitovirus (TcMV) RNA in the cytosol as well as in mitochondria (Jian et al., 1997; Lakshman et al., 1998) is noteworthy, but perhaps not definitive because controls for proper separation of the different subcellular fractions were not analyzed in parallel. Published evidence for TcMV transfer from donor to recipient fungal strain by hyphal anastomosis without apparent co-transfer of donor mitochondria (Charlton and Cubeta, 2007; Jian et al., 1997) is also noteworthy, but perhaps again not definitive because the co-transfer of donor mitochondria allowing TcMV transmission by fusion with recipient mitochondria (Polashock et al., 1997) seems not to have been wholly ruled out. Whether TcMV or any other of the viruses with no UGA codons can indeed replicate in the cytosol of their respective hosts is clearly a subject that warrants further direct study.

MATERIALS AND METHODS

Multiple sequence alignments were performed using MAFFT 7.3 (L-INS-i) as implemented with defaults at http://mafft.cbrc.jp/alignment/server/. Phylogenetic analyses were performed using PhyML 3.0 (Guindon et al., 2010) as implemented at https://www.hiv.lanl.gov/content/sequence/PHYML/interface.html with the following parameters differing from the defaults: Sequence type/model, Amino acids/JTT, LG, rtREV, or WAG (each yielded very similar results); Gamma shape parameter, estimated from data; Proportion of invariant sites, estimated from data; Starting tree(s) optimization, Tree topology and Branch length; Tree

improvement, Best of NNI and SPR; Branch support, Approximate Likelihood Ratio Test (aLRT), SH-like supports. Codon frequencies in single or concatenated ORFs were calculated using the program Codon Usage in the Sequence Manipulation Suite as implemented at http://www.bioinformatics.org/sms2/codon_usage.html. Mitochondrial core genes and spliced mRNA sequences were identified via annotations in the GenBank accession for each complete mitochondrial genome sequence. Mitochondrially encoded tRNAs were also identified via annotations in each GenBank accession, but then confirmed using the program ARAGORN (Laslett and Canback, 2004) as implemented at http://mbio-serv2.mbioekol.lu.se/ARAGORN/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mitoviruses replicate in fungal mitochondria, where both UGG and UGA encode Trp
- Most mitoviruses contain a substantial number of UGA(Trp) codons, but some do not
- The latter derive from fungal hosts in which UGA(Trp) is a rare mitochondrial codon

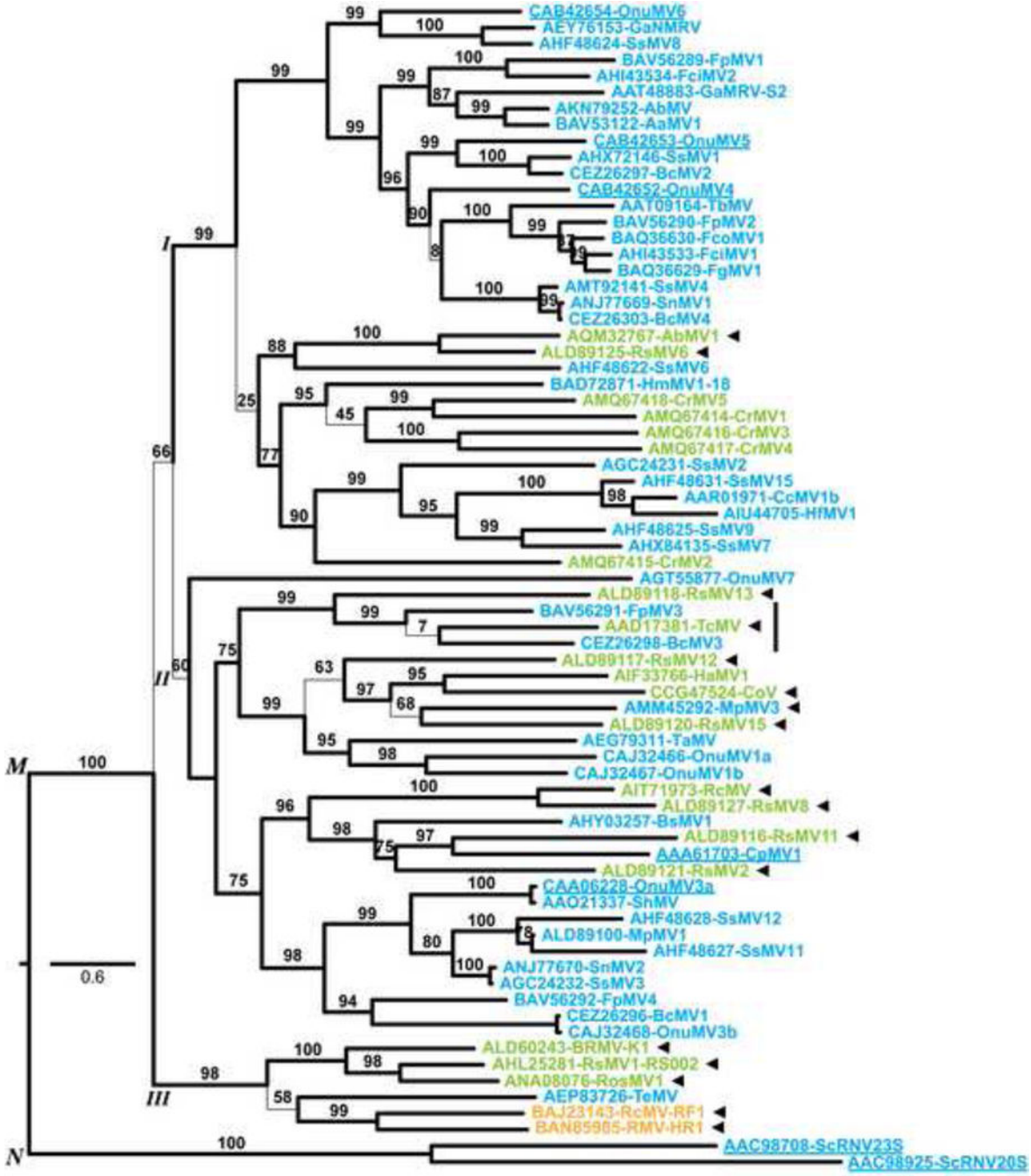

Fig. 1.

Phylogenetic tree, genus *Mitovirus*. This maximum-likelihood phylogram for the deduced RdRp aa sequences of these viruses was generated as described in Materials and Methods (alignment program, MAFFT; PhyML substitution model, rtREV; estimated gamma shape parameter, 1.222; estimated proportion of invariant sites, 0.010; midpoint rooting). Thin lines, branches with <70% support. *M*, genus *Mitovirus* root; *N*, genus *Narnavirus* root; *I–III*, three apparent clades within genus *Mitovirus*. The same three clades have been identified by Xu et al. (2015), but numbered differently. Viruses representing ICTV-ratified species are

underlined (five in genus *Mitovirus*; two in genus *Narnavirus*). ◄, mitoviruses with no or few UGA codons (0–21%) vs. UGG codons (79–100%). Hosts of the represented viruses: cyan, ascomycetes; green, basidiomycetes; orange, glomeromycetes. Some viruses in Table S1 were not included in this analysis because their RdRp sequences are very similar (>70% aa identity) to one of the other viruses from the same host species. The GenBank accession numbers listed in this figure are for the RdRp sequences. See Table S1 for full virus names.

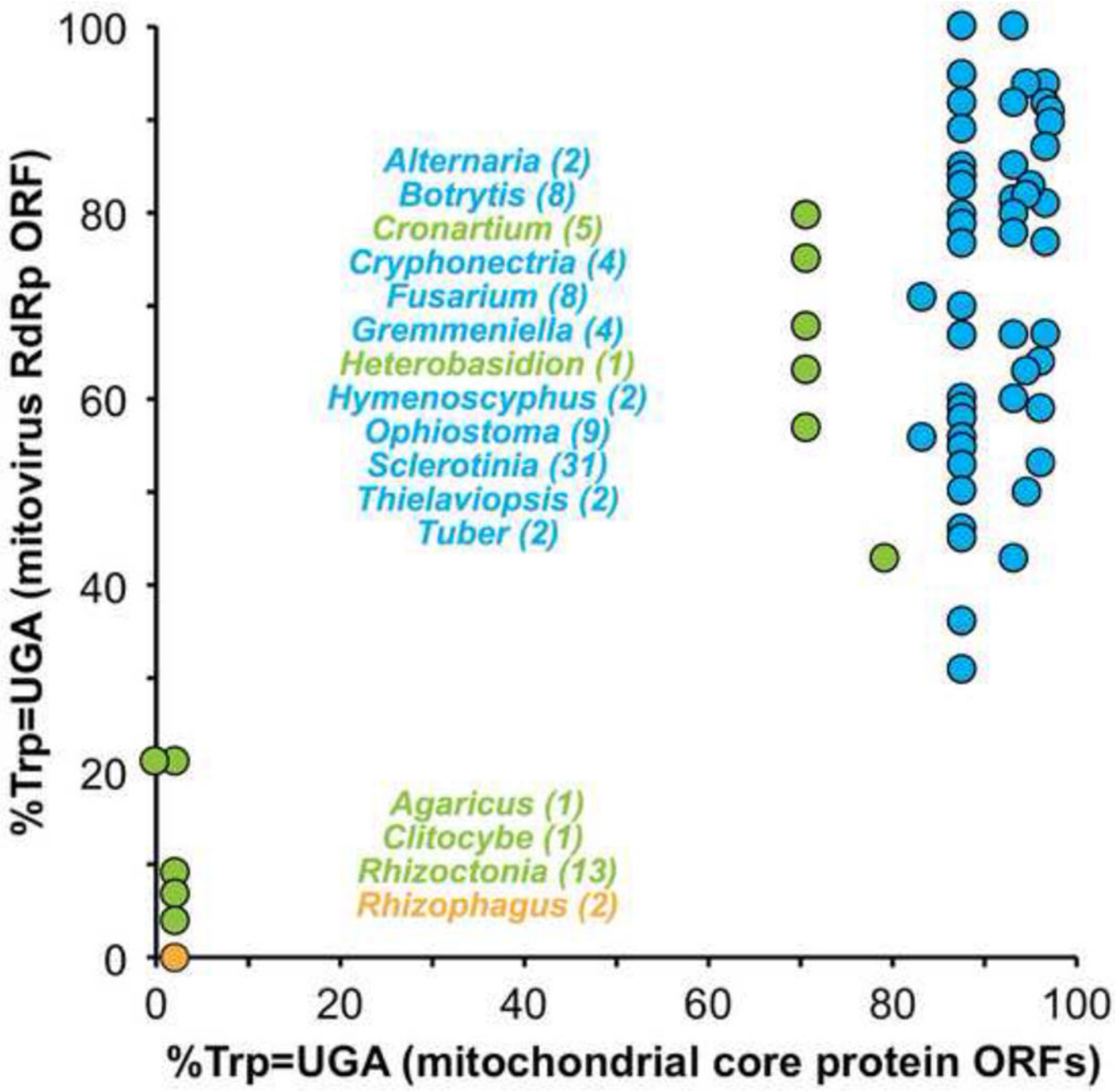

Fig. 2.

Scatter plot of the percentage of Trp codons that are UGA (vs. UGG) in the RdRp ORF of each mitovirus from Table S1 (*y* axis) and in the mitochondrial core protein ORFs of its respective fungal host (*x* axis). Mitochondrial core proteins are defined in the text. Only the four total viruses from *Buergenerula spartinae*, *Macrophomina phaseolina*, and *Helicobasidium mompa* in Table S1 are not included here, because the complete mitochondrial genome sequences of those fungal species (or other fungal species in the same order or family) have not yet been reported to NR/NT in GenBank. The %UGA value used for the mitochondrial core protein ORFs of each host is the mean of the value range for each

respective order or family of fungi in Table S2. Mitovirus host genera represented in the graph are listed alphabetically beside the respective two clusters of points; the number of mitoviruses from each genus, as represented in Table S1, is also indicated. Color-coding of hosts is the same as in Fig. 1. Several points in the graph are obscured because they overlap, especially for *Rhizoctonia* and *Rhizophagus* mitoviruses with values $y = 0$, $x = 2$.