

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY NOTE 1

Proteomic analysis of Tupanvirus particles

Proteomic analysis of Tupanvirus soda lake particles revealed 127 proteins, nearly half ($32/127 = 25.1\%$) of which are unknown and eight of which are encoded by ORFans ($11/87 = 12.6\%$). Besides the expected major structural components [major capsid protein (L679) and core protein (L614)], transcription components constitute the largest functional category associated to the viral particle (13 gene products, including subunits of DNA-dependent RNA polymerase, RNA helicases, and mRNA capping enzyme). Moreover, other functional categories were detected in Tupanvirus particles, including protein/lipid modification (14), oxidative pathways (6), DNA topology and repair (3), particle structure (4), and others (87). 59 and 25 out of 127 TPV virion-associated proteins are shared with mimivirus (APMV) and Cafeteria roenbergensis virus respectively, out spanning all functional categories analyzed (Supplementary Data 2).

18S rRNA intronic sequence within Mimiviridae and Tupanvirus

The study of 18S rRNA sequences in tupanviruses showed that both copies are located in an intergenic region (Fig. 6; Supplementary Fig. 6A,B). The genomic environment analysis revealed the presence of many hypothetical proteins and ORFans next to the two copies and a tRNA island, composed of seven tRNAs, 15 kbp away from the core sequence of copy 1 (Supplementary Fig. 6A,B). Best hit analysis showed that the first 100 sequences, for both copies, belong mainly to the intronic regions of 18S sequences from different organisms such as fungi, amoebas and algae (Supplementary Fig. 6C,D). The presence of the 18S rRNA intronic sequence was also observed in members of lineages A, B and C of the *Mimiviridae* family (Fig. 6) Lineages A and B presented just

one copy which is located in an intronic region next to a self-splicing group I intron endonuclease. Lineage C presented two copies of the 18S rRNA sequence: copy 1 was disposed in a similar pattern of the copies presented in both tupanviruses (in intergenic regions) and copy 2 showed a most similar pattern to the unique copies of lineages B and C (in an intronic region also next to a self-splicing group I intron endonuclease) (Fig. 6 A-E). Phylogenetic analyses suggested that 18S rRNA copies 1 and 2 of mimiviruses lineage C had separate and different origins. However, Tupanvirus 18S rRNA copy 1 and 2 seem to be related to mimiviruses lineage A and B (single copy) and fungi mitochondrial 18S rRNA intronic region (Fig. 6F). We also observed the presence of three or four copies of the 18S-like sequences in some *Chlorella virus*, which were all phylogenetically inter-related (Fig. 6F). Analyses involving FISH and qPCR of the intronic 18S ribosomal region in tupanvirus soda lake demonstrated that, although these 18S rRNA copies are located in intergenic regions, they are highly expressed during the entire infection, especially in intermediate and later phases (6 and 12 hours post infection) (Supplementary Fig. 8).

Profile of infectiveness of Tupanvirus

Tupanvirus was first isolated in both *Acanthamoeba castellanii* and *Vermamoeba vermiformis*, suggesting a broader host-range compared to other previously described amoebal giant viruses. In light of this, we tested the infectiveness of the new isolate on a large panel of protozoa. Four distinct infectiveness profiles were observed (Supplementary Table 1). Cytopathic effect (CPE), increase of viral titer, and genome replication were observed in *A. castellanii*, *A. polyphaga*, *A. sp E4*, *A. griffini*, *V. vermiformis*, *Dysctiostelium discoideum*, and *Willartia magna*, characterizing a productive cycle wherein these hosts were permissive to Tupanvirus. An abortive cycle

was observed in *Acanthamoeba sp michelline* and *A. royreba*, wherein CPE and Tupanvirus genome replication were observed, but there was no particle formation since the viral titer did not increase. *Trichomonas tenax* were completely refractory to Tupanvirus, since CPE, genome replication and increase of viral titer were not observed. The fourth profile was observed in RAW247, THP-1 cells and in *Tetrahymena hyperangularis*, a ravenous free-living protist, wherein Tupanvirus was able to induce a cytopathic effect, but neither an increase of viral titer nor genome replication were observed, thus we concluded that TPV was toxic to the susceptible cell - an unprecedented profile among amoebal giant viruses.

Tupanvirus modulates a non-host organism

Tetrahymena sp was susceptible to Tupanvirus, which caused several effects (Fig. 7G; Fig. 8); however it could not replicate within the protist. In light of this, we hypothesized that the reduction of physiological activity of a (non-host) predator could increase the virus' fitness. We put it to the test by performing *in vitro* simulations, wherein *Acanthamoeba castellanii* (AC) and *Tetrahymena* sp cells were put together and infected at M.O.I. of 10 (Tupanvirus or APMV), and observed over 12 days. Input of fresh medium and permissive host (AC) was done at days four and eight post infection. After the input, Tupanvirus reached higher titers while APMV was extinguished, since the latter was not able to modulate the predator organism, unlike Tupanvirus (Fig. 8N). When we induced a previous ribosomal RNA shutdown in *Tetrahymena* with geneticin, APMV was able to survive until the last day of observation, similar to tupanvirus. This indicates that viral particles' toxicity confers an advantage in a habitat of intense competition where giant viruses are abundant (e.g. water environment), favoring the encounter between the virus and a permissive host.

Altogether, this data suggest that viral particles can act as active “non-alive” players favoring viral progeny maintenance, in a distinct way of their canonical role of transmitting genetic information. Considering that Tupanvirus is sister group of mimivirus, such modulation mechanism could be an ancient inheritance from an ancestor of *Mimivirus* or *Mimiviridae*. This new mechanism demonstrates that selective pressures over virion content go beyond the metastability properties in early steps of host infection, and can act as unexpected players to protect the rest of the viral progeny against predation.

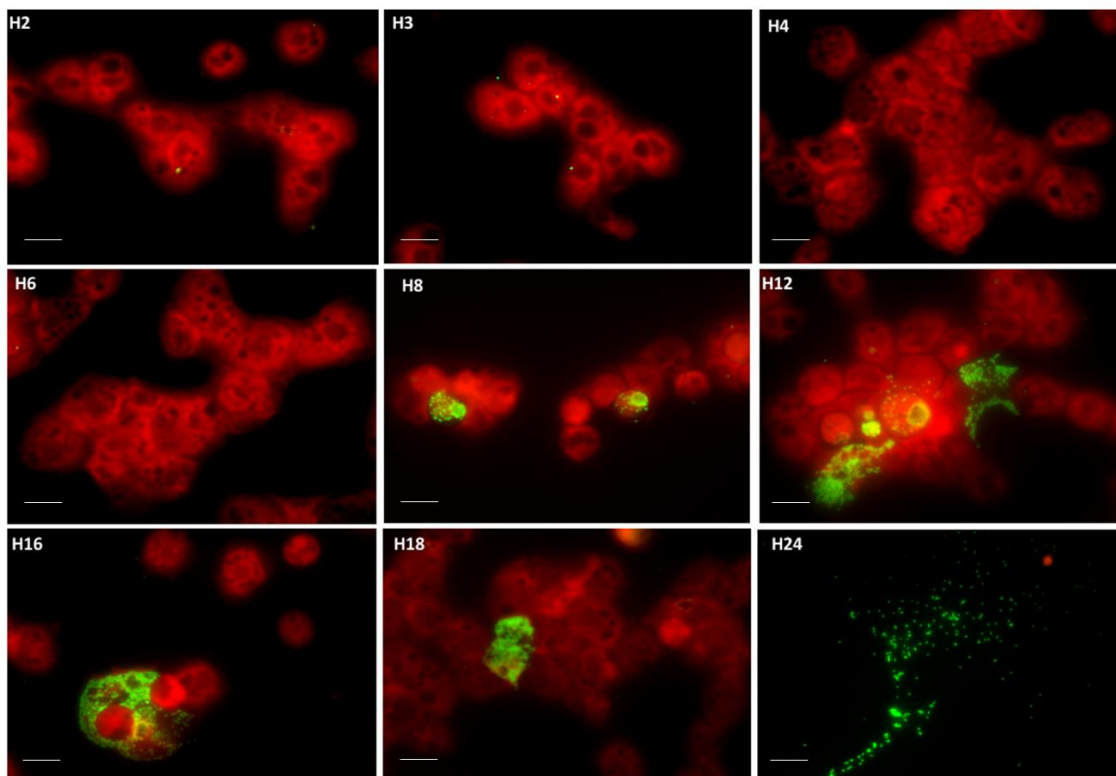
rRNA shutdown does not seem to be related to ribophagy

The cytotoxic phenotype caused by Tupanvirus in host (*A. castellanii*) and non-host cells (*Tetrahymena*) is a circumstance never previously described and seems to be related to the shutting down of host ribosomal amounts (Fig. 7C; Fig. 8B,C,D,K,L). In a first moment, we believed that the autophagy might be related to Tupanvirus infection. In order to corroborate or not this hypothesis, we developed other several experiments involving the use of acidification inhibitors (chloroquine and bafilomycin A), the observation of acidification of infected cells, as well as the silencing of the gene that encodes an autophagy-related protein, Atg8-2. The treatment of amoebas with chloroquine (a lysosomotropic agent that accumulates inside the acidic parts of the cell, including endosomes and lysosomes, preventing acidification and, consequently, the fusion of endosomes and lysosomes) or bafilomycin A (a vacuolar H⁺ ATPase inhibitor that acts in the late phase of autophagy) did not prevent the occurrence of the ribosomal shutdown caused by tupanvirus infection, suggesting that this phenomenon is not dependent of the autophagosomes formation, a hallmark of ribophagy (Fig. 7C,D; Supplementary Fig. 9). In order to confirm this first experiment, we also opted to do the

silencing (siRNA) of Atg8-2, that is one of the ubiquitin-like proteins required for autophagosome formation, representing thus a cell marker related to ribophagy. Curiously, the gene silencing also does not prevent viral induced ribosomal shutdown in *Acanthamoeba castellanii*, corroborating the results observed with the inhibitors treatment (Fig. 7C,D). We also checked the pH of cells infected by tupanvirus or mimivirus, in presence or absence of bafilomycin A. Tupanvirus, in contrast to mimivirus, induces a strong acidification of whole amoebal cytoplasm at the time of 9 hours after infection (Fig. 7B; Supplementay Fig.9A,B). In mimivirus-infected amoebas and non-infected amoebas, it was possible visualize only localized acidic vacuoles, but in a scale quite smaller than that observed to tupanvirus infected cells (Fig 7B; Supplementay Fig.9A,B). Interestingly, the intense acidification caused by tupanvirus is not affected by the bafilomycin A treatment, suggesting that this decrease in pH could be induced by an another acidification mechanisms not related to H⁺ ATPase pump (Fig. 7B; Supplementay Fig.9A,B). Although tupanvirus induces a remarkable acidification of host cells concomitantly with the ribosomal RNA shutdown, the biological meaning of such acidification and its possible relation to ribosomal shutdown remain to be investigated. As demonstrated in main data, Tupanvirus (inactivated or not) also causes degradation of host (*Acanthamoeba castellanii*) and non-host (*Tetrahymena*) nucleolus/nucleus (Fig. 7F,G), which may contribute to rRNA shutdown, since the nucleolus is important to ribosomal biogenesis. Taken together, these results indicate that this cytotoxic phenotype caused by tupanvirus is a mechanism not-related to the canonical ribophagy/autophagy process.

SUPPLEMENTARY FIGURES

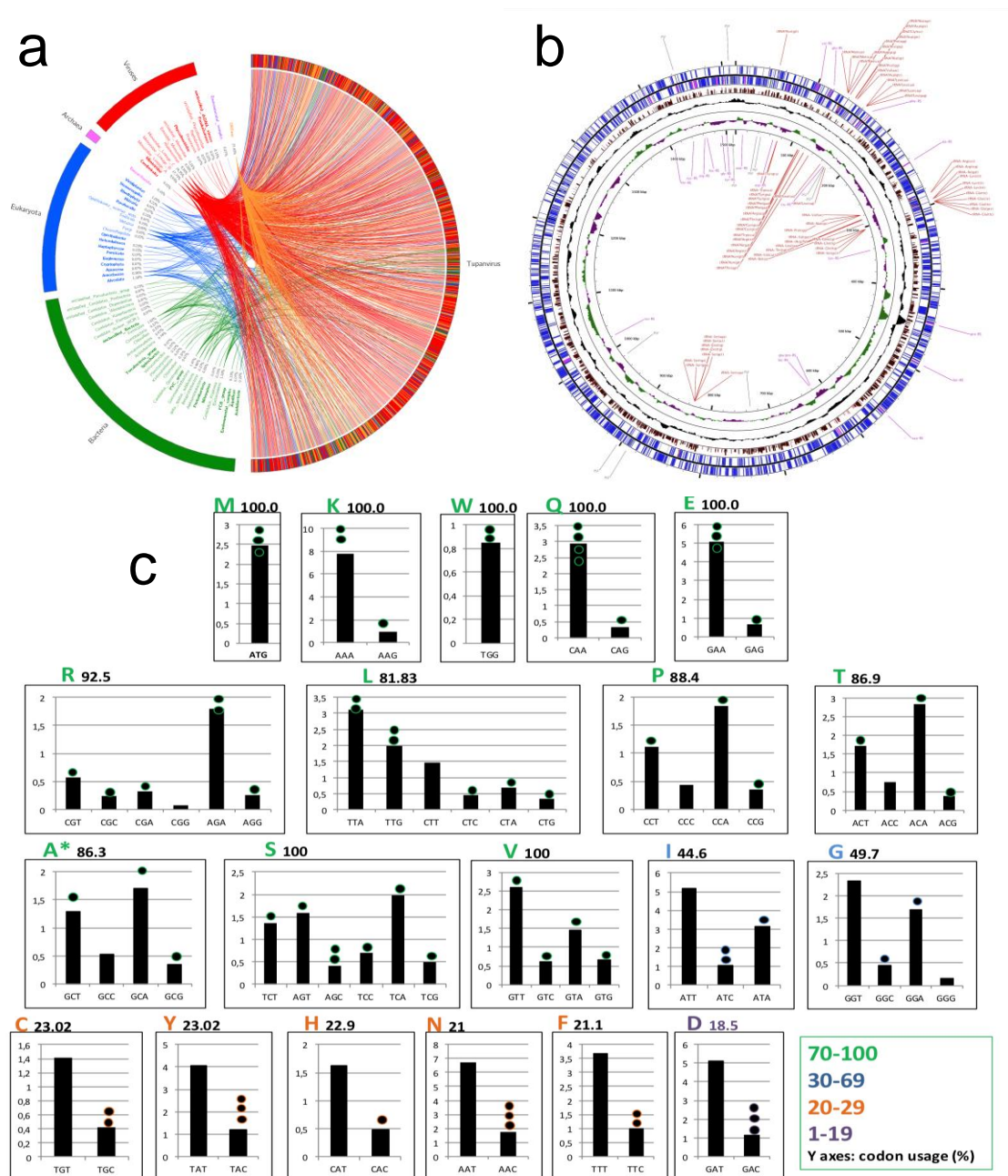
Supplementary Figure 1: Tupanvirus soda lake cycle in *A. castellanii* observed by immunofluorescence. Cells were infected at a multiplicity of infection of 1 and observed at different time points post-infection. In green, viral particles detected by anti-tupan particle antibody produced in mouse; in red, amoeba cytoskeleton. H: hours post-infection. Scale bar, 10 μ m.



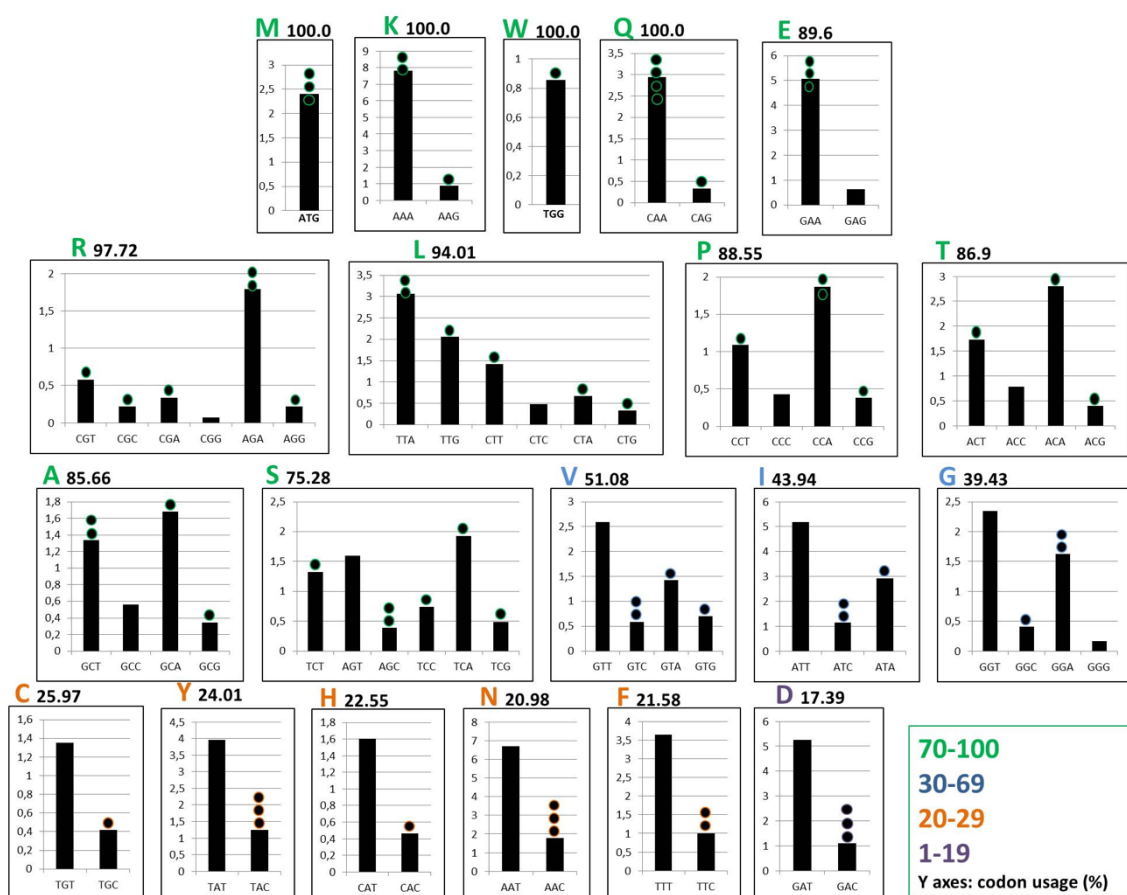
Supplementary Figure 2: Tupanvirus deep ocean genomic information. (a)

Rhizome; (b) circular representation of the genome highlighting the translation-related factors; and (c) analysis of Tupanvirus deep ocean 70 tRNAs distribution among amino acid (aa) categories, isoacceptors and their relation to viral aa usage. Bars represent the percentage of use of a given codon (isoacceptor) related to a given aa. Dots above bars represent codons in which Tupanvirus deep ocean presents one or more related tRNAs.

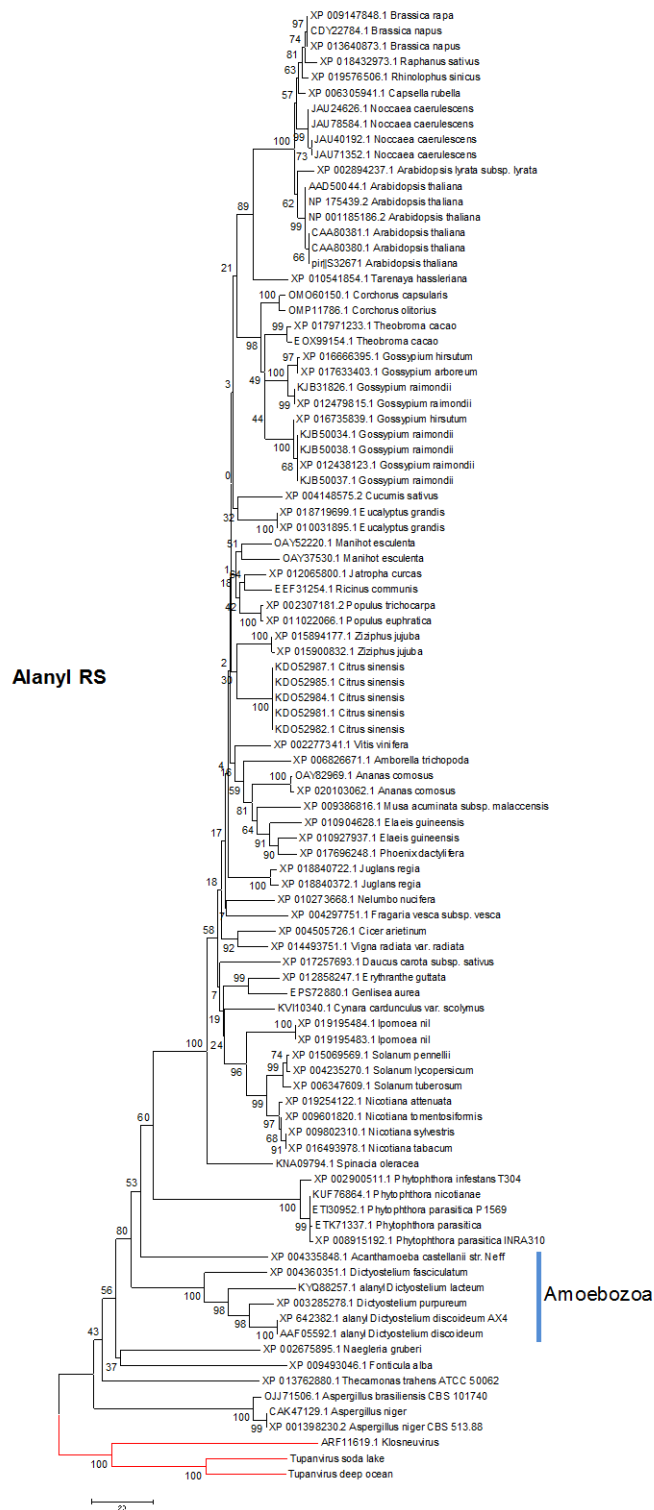
*Ambiguous tRNA – also predicted for pyrrolysine.

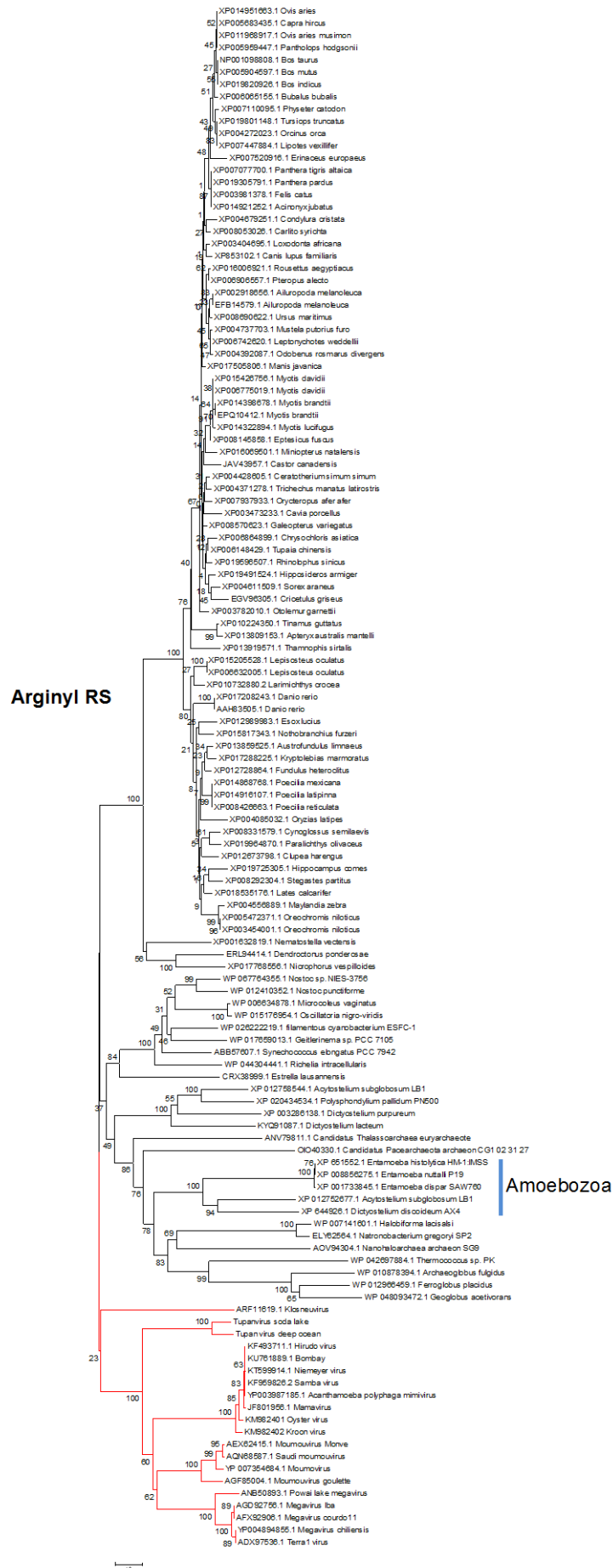


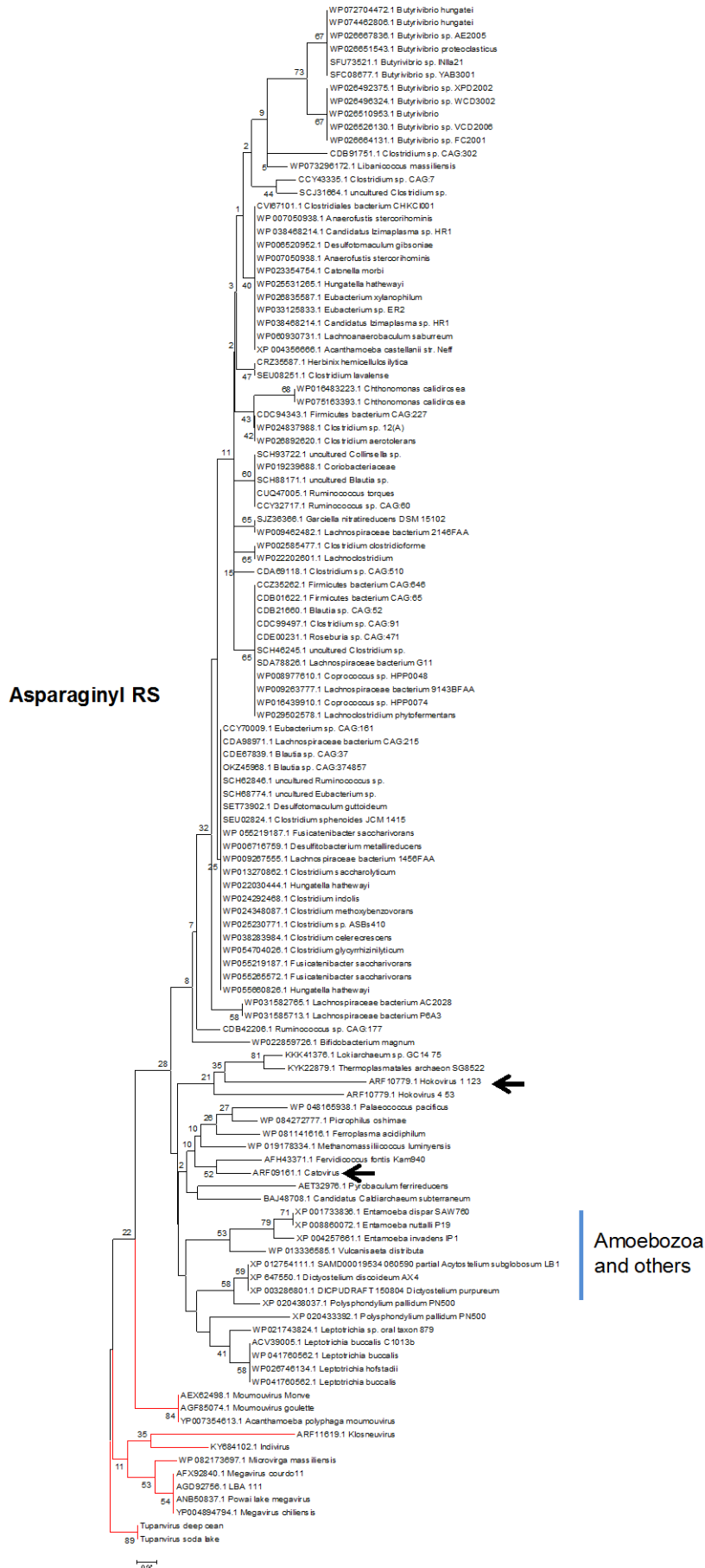
Supplementary Figure 3: Analysis of 67 tRNA distribution in tupanvirus soda lake among amino acid (aa) categories, isoacceptors and their relation to viral aa usage. Bars represent the percentage of use of a given codon (isoacceptor) related to a given aa. Dots above bars represent codons in which Tupanvirus presents one or more related tRNAs. Numbers above the squares represent the percentage of codon occurrence covered by tupanvirus tRNA, considering each aa. These numbers determined the colours, according to the legend at the bottom right corner of the figure.



**Supplementary Figure 4: Maximum likelihood phylogenetic trees of the 20
Tupanvirus aminoacyl tRNA synthetases (aaRS).**

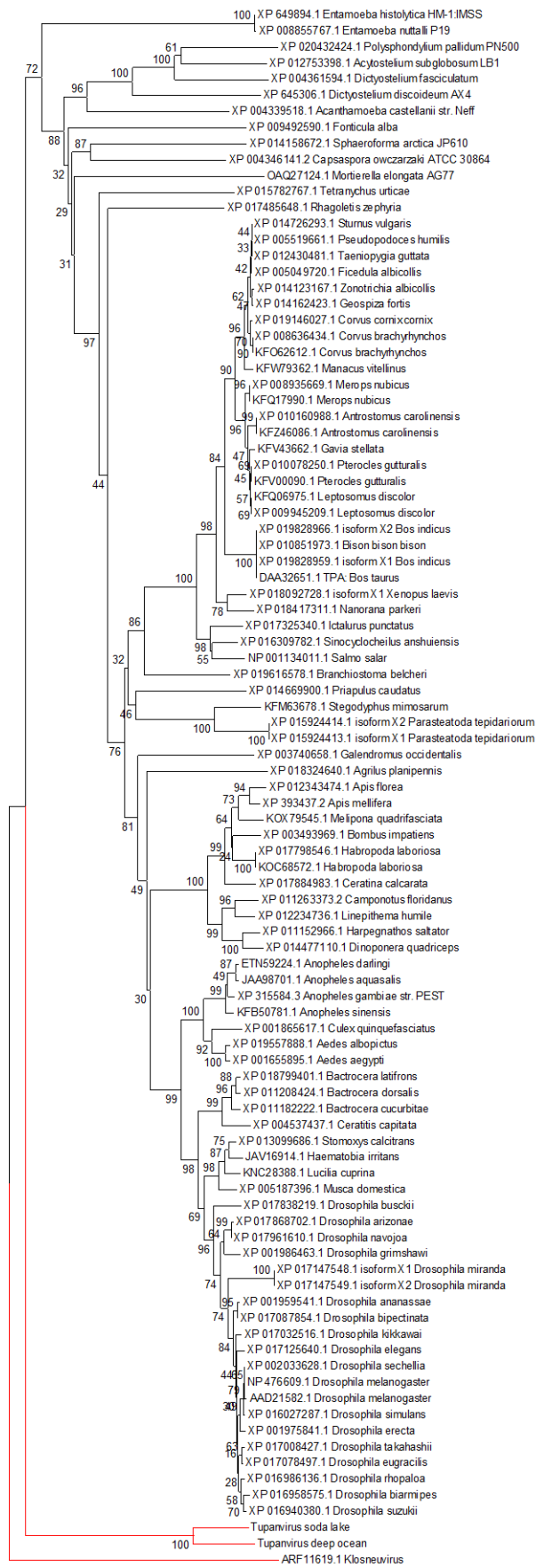


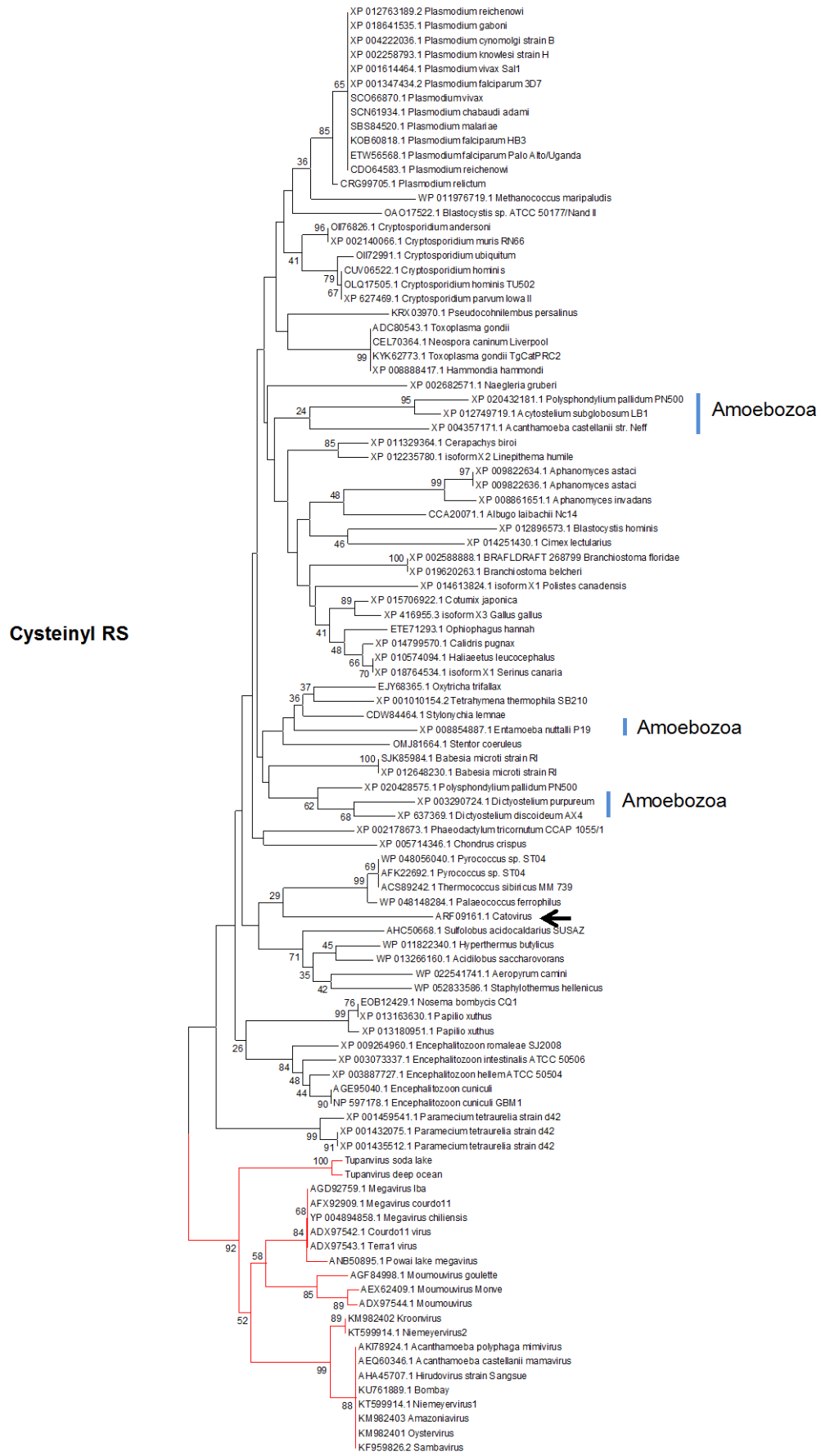


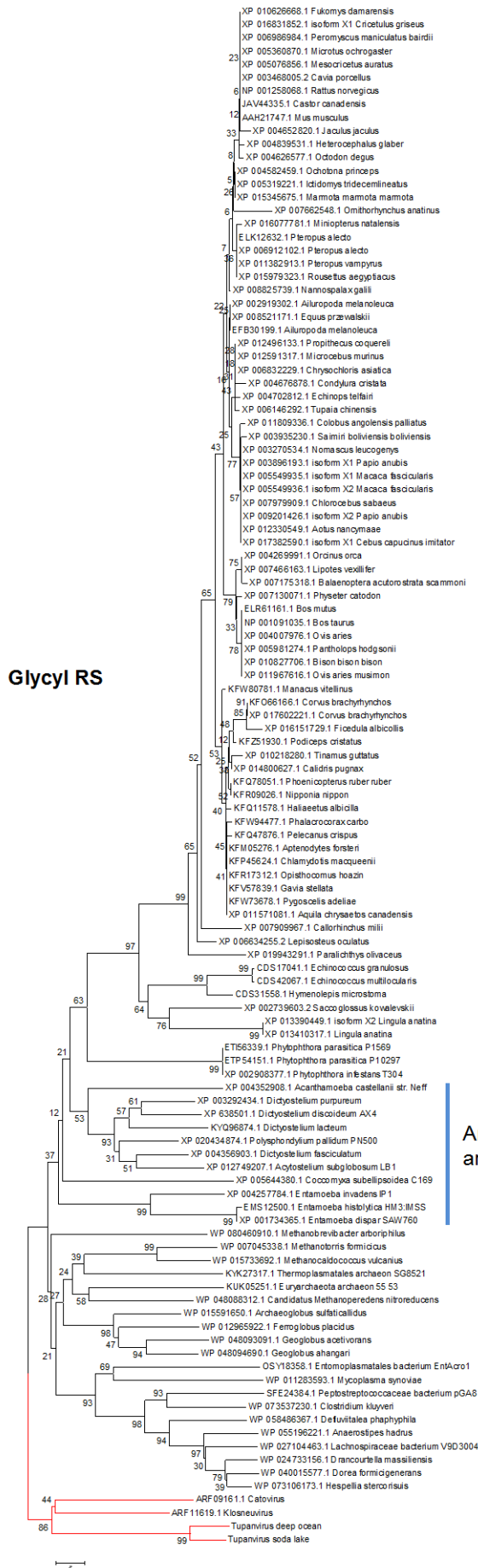


Amoebozoa

Aspartyl RS

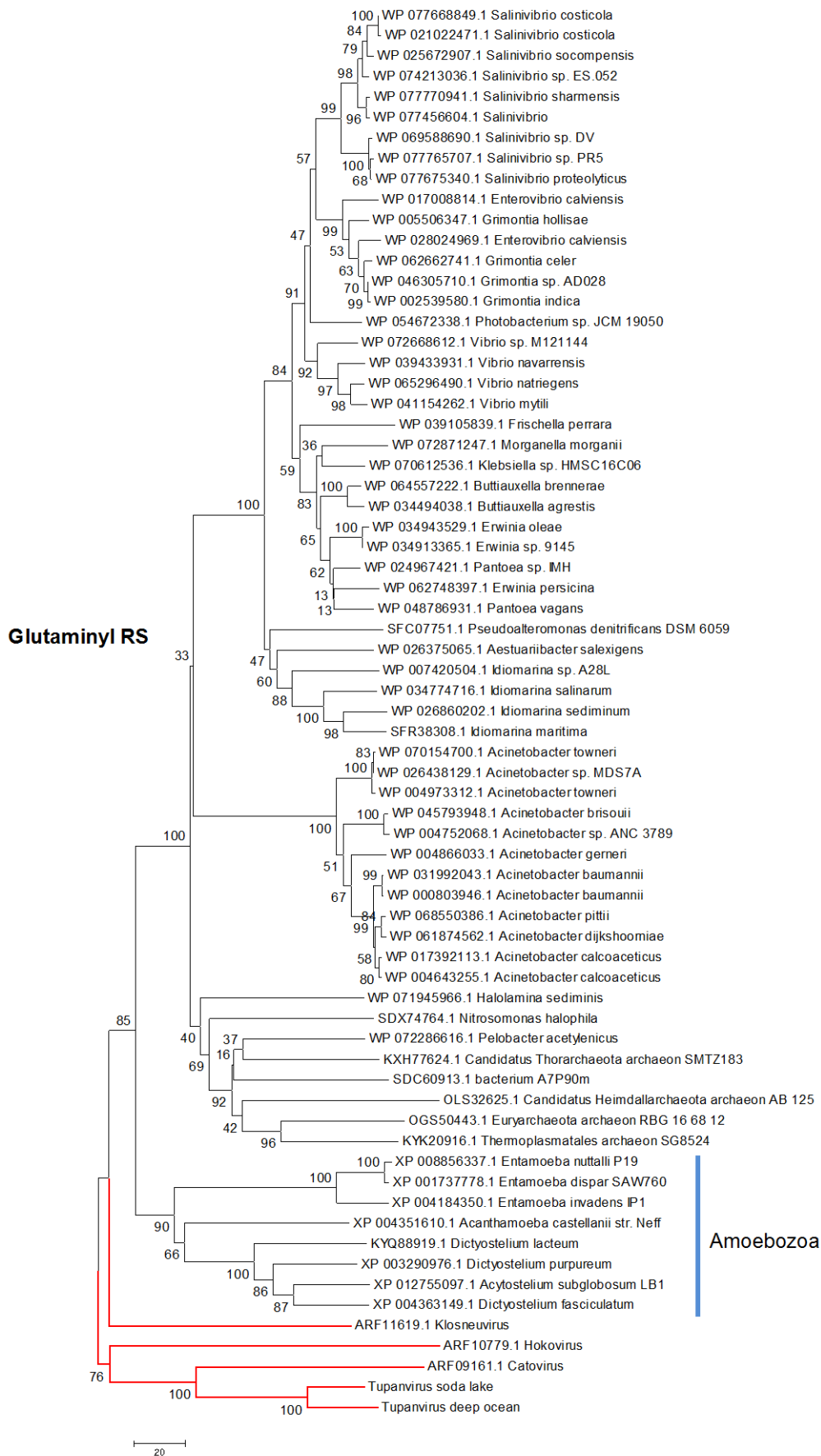






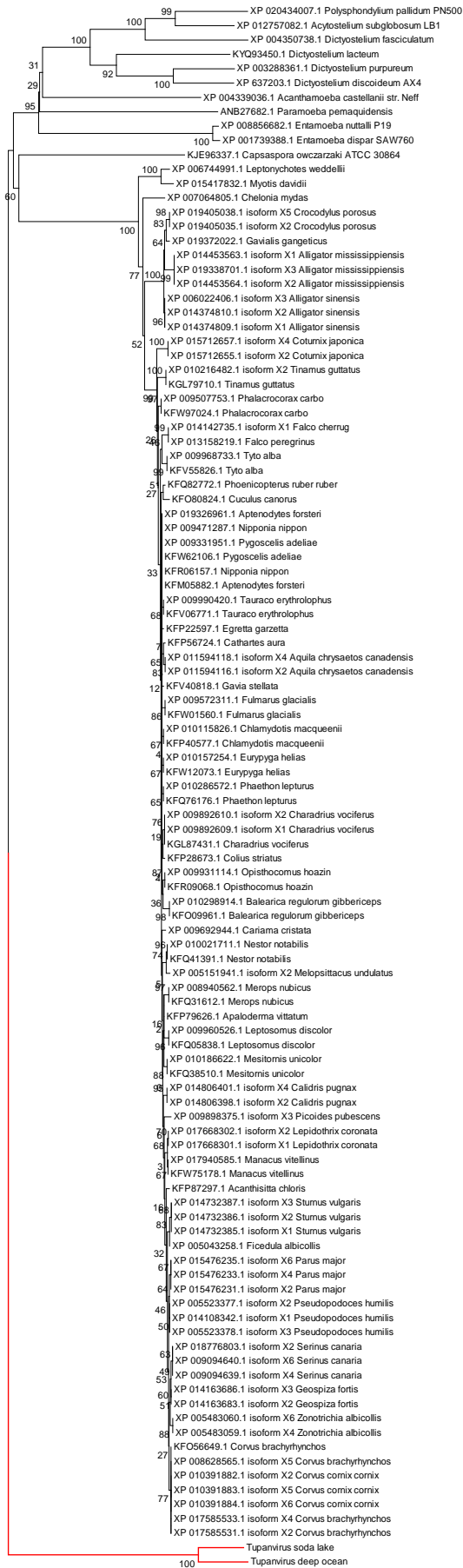
Glycyl RS

Amoebozoa and others

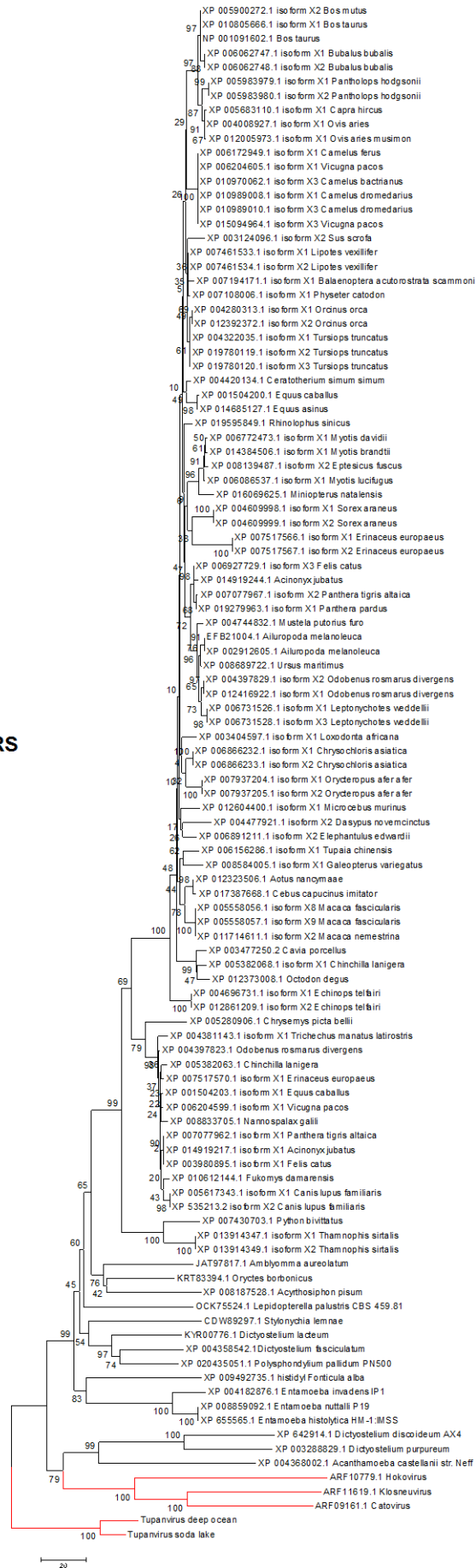


Amoebozoa

Glutamate-Prolyl RS

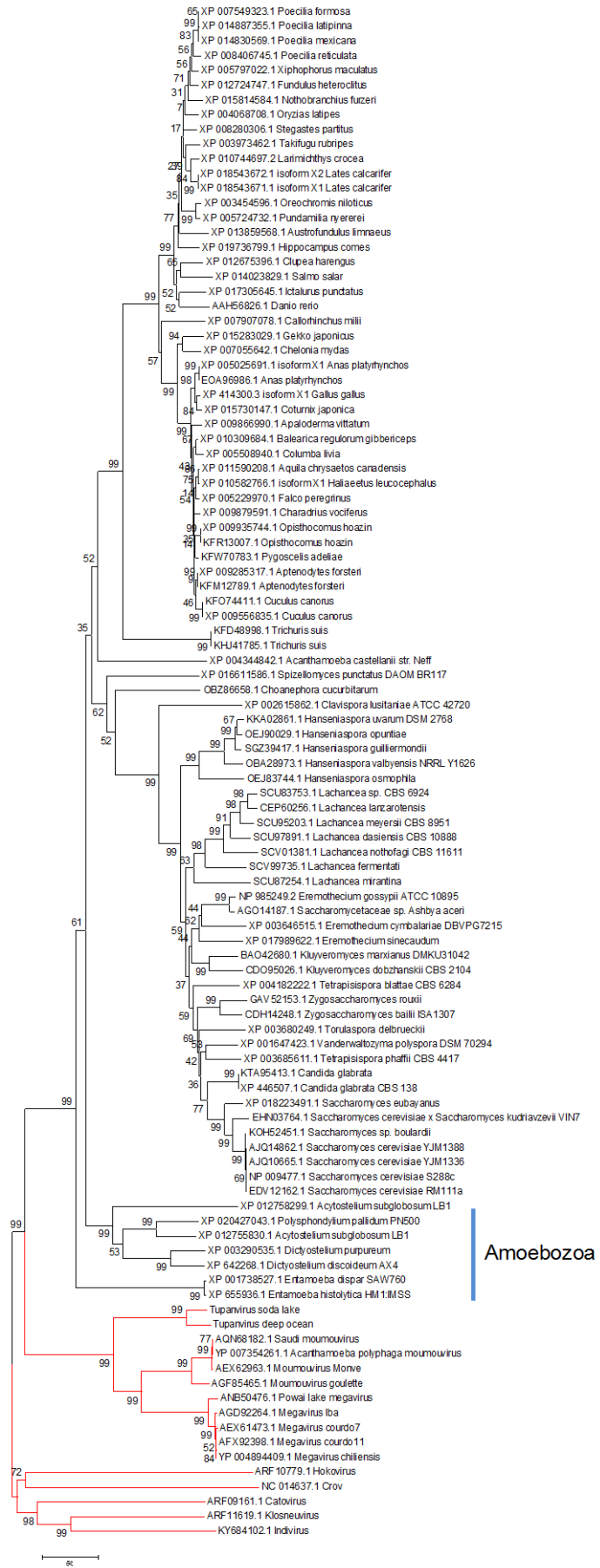


Histidyl RS



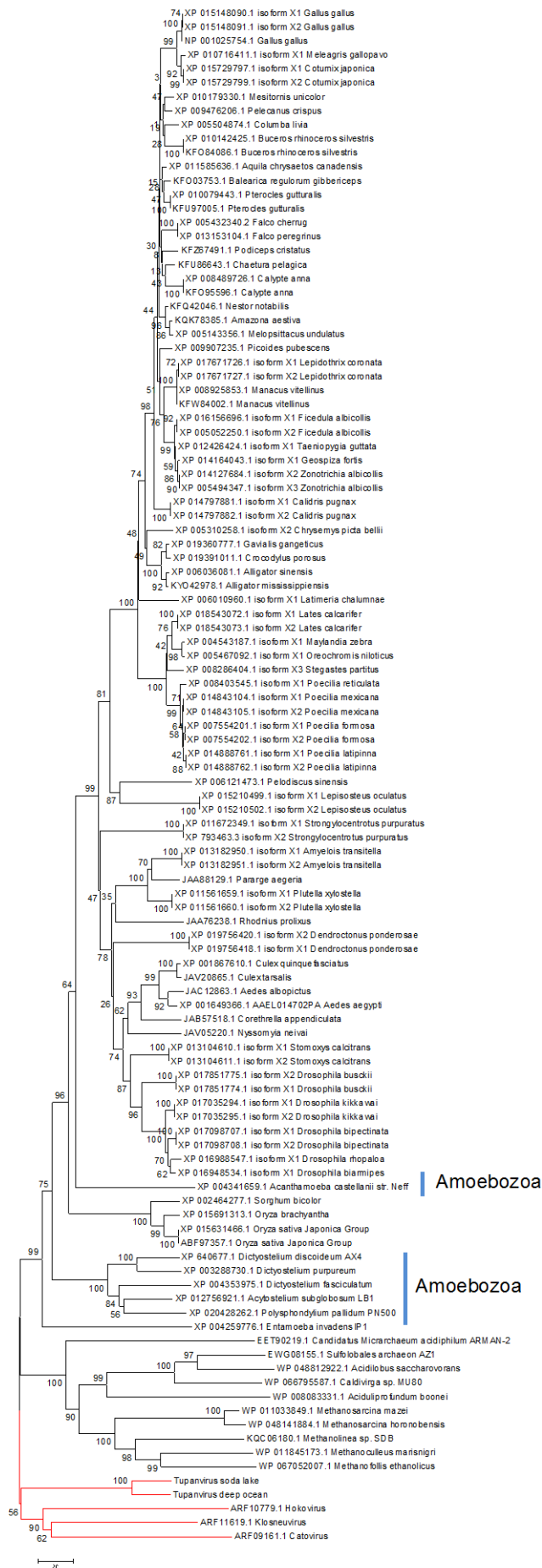
Amoebozoa and others

Isoleucyl RS



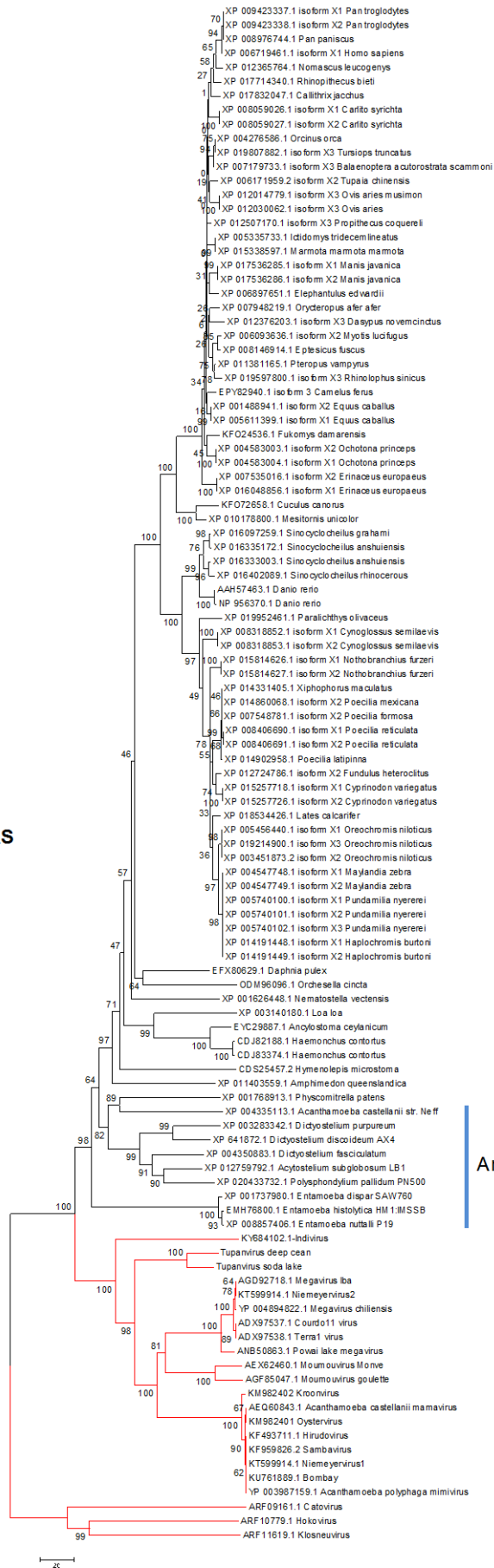
Amoebozoa

Lysyl RS

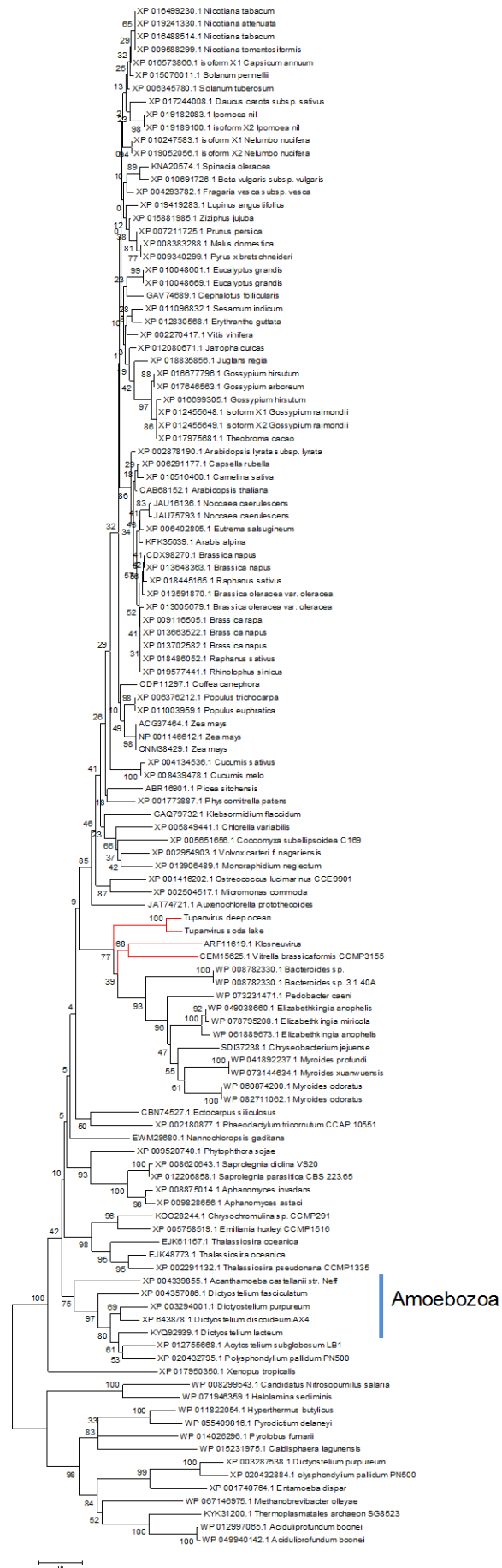


Methionyl RS

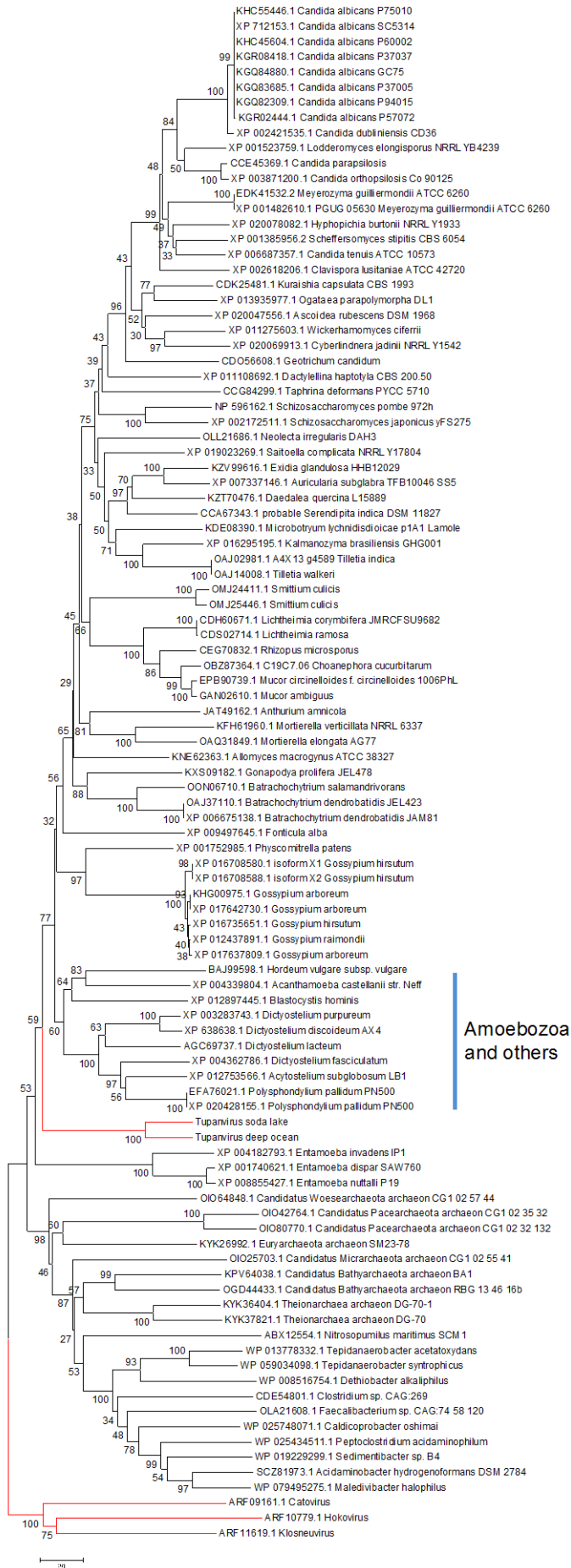
Amoebozoa



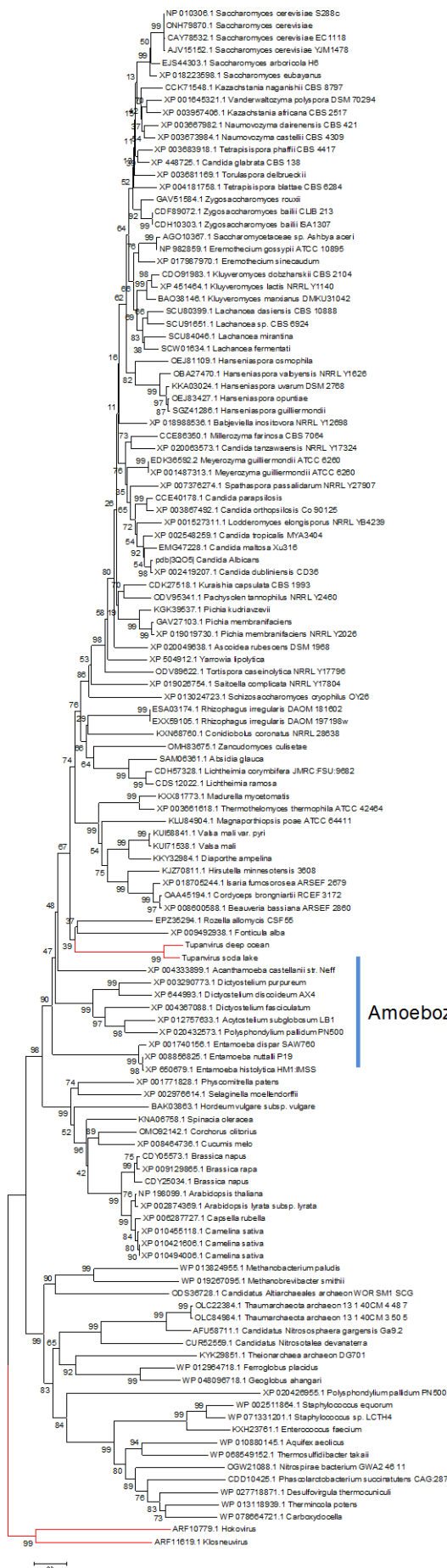
Phenylalanyl RS



Prolyl RS

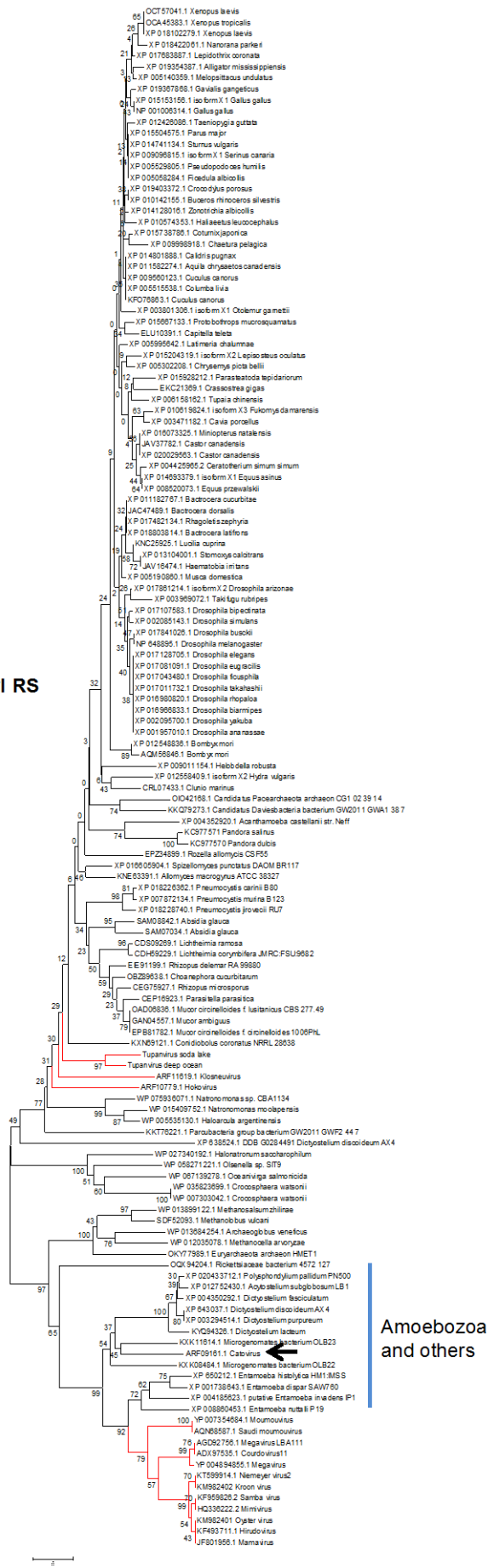


Seryl RS



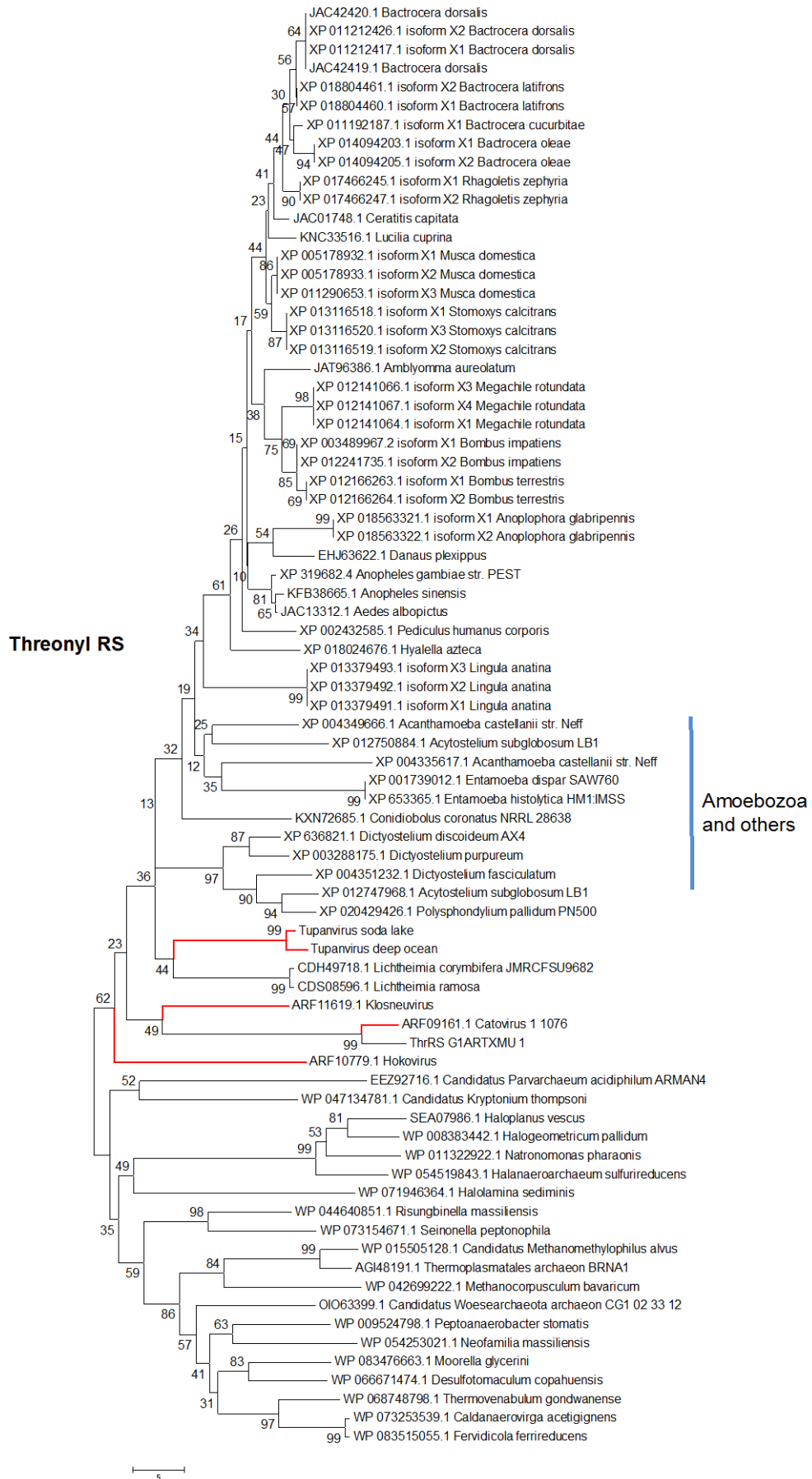
Amoebozoa

Tyrosyl RS



Amoebozoa and others

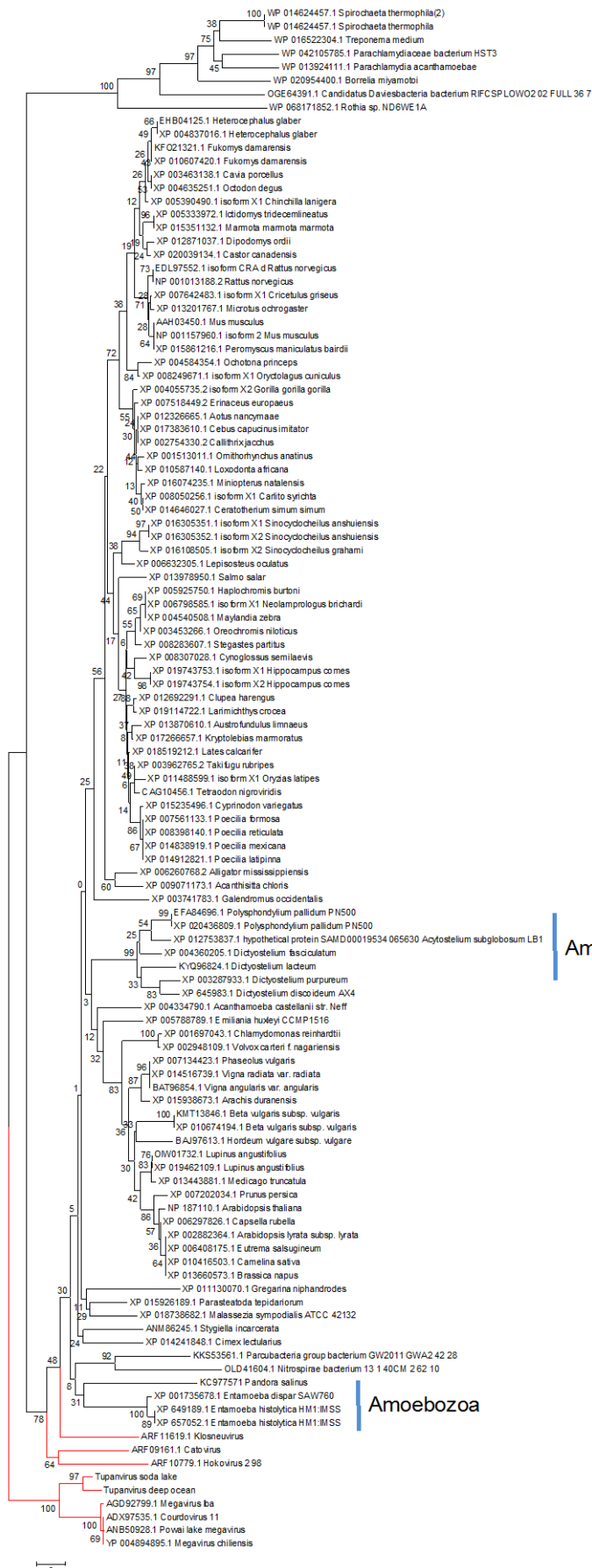




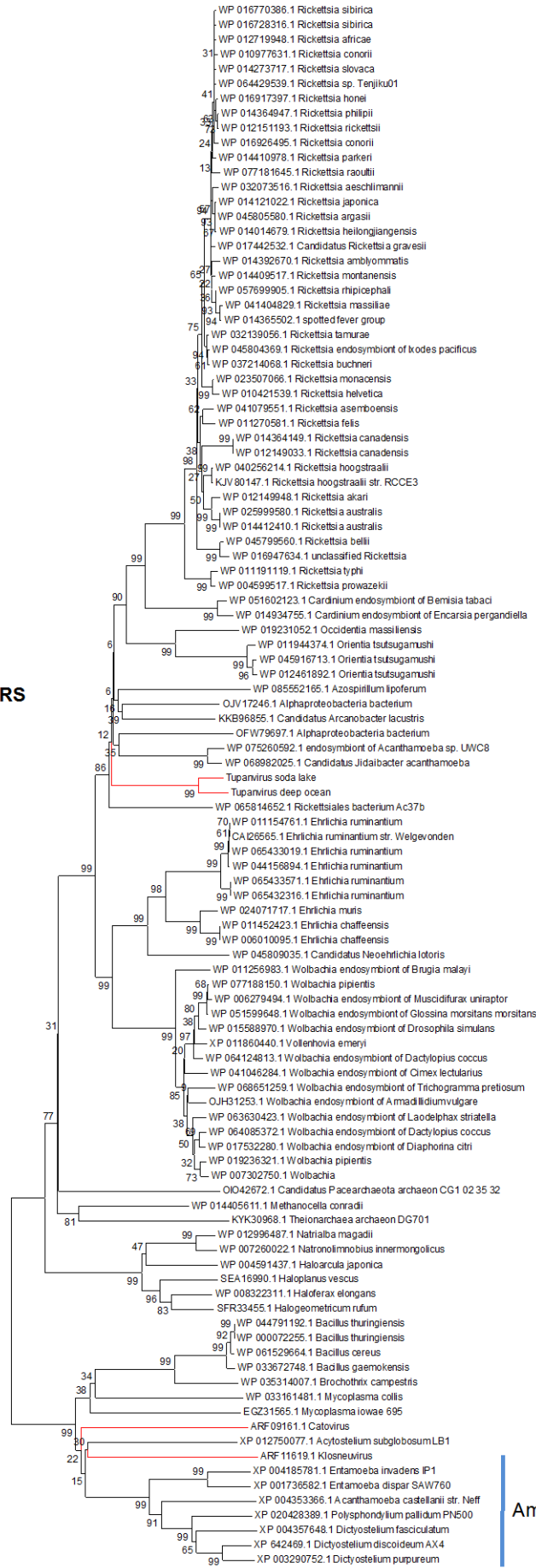
Tryptophanyl RS

Amoebozoa

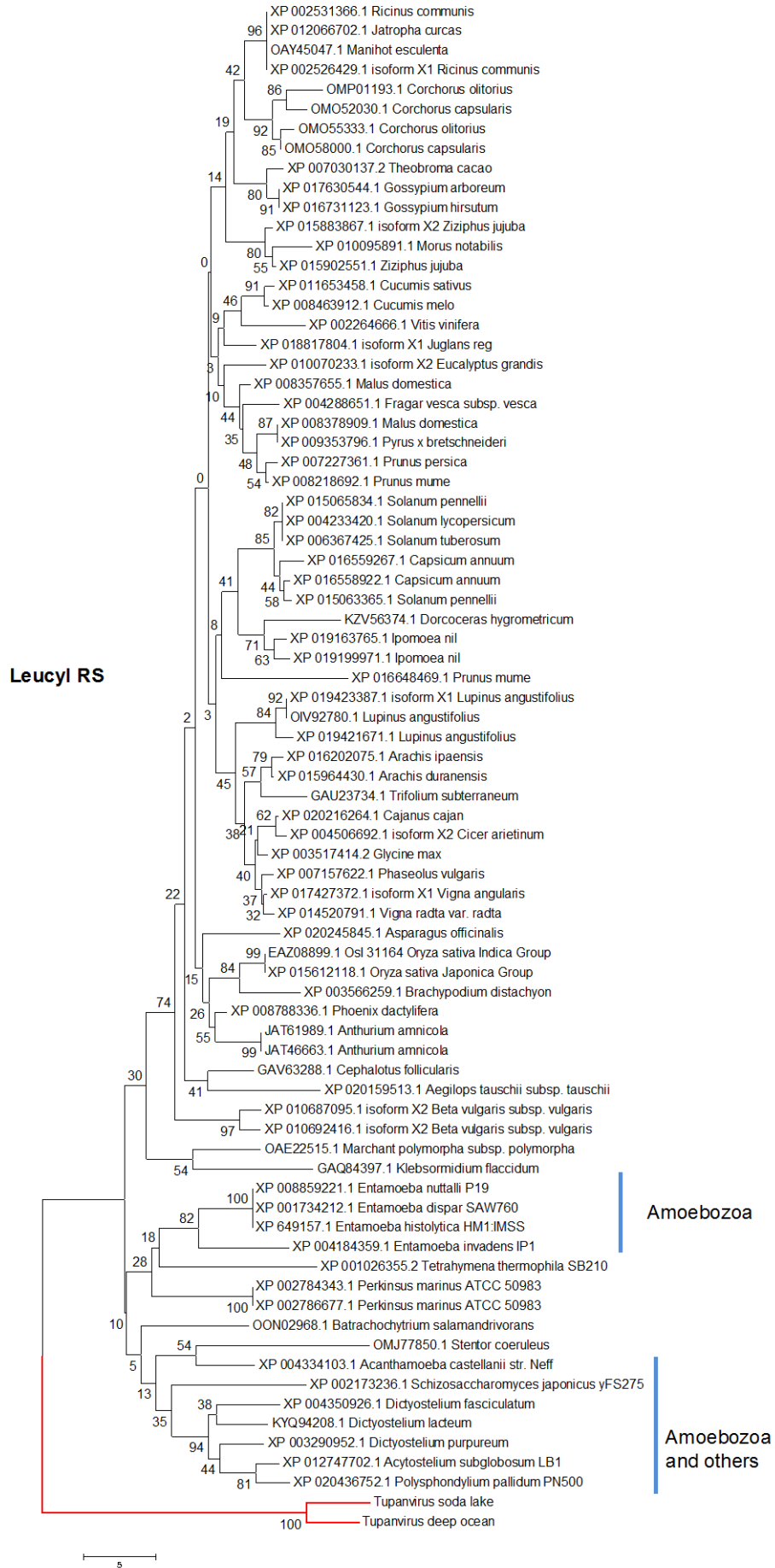
Amoebozoa



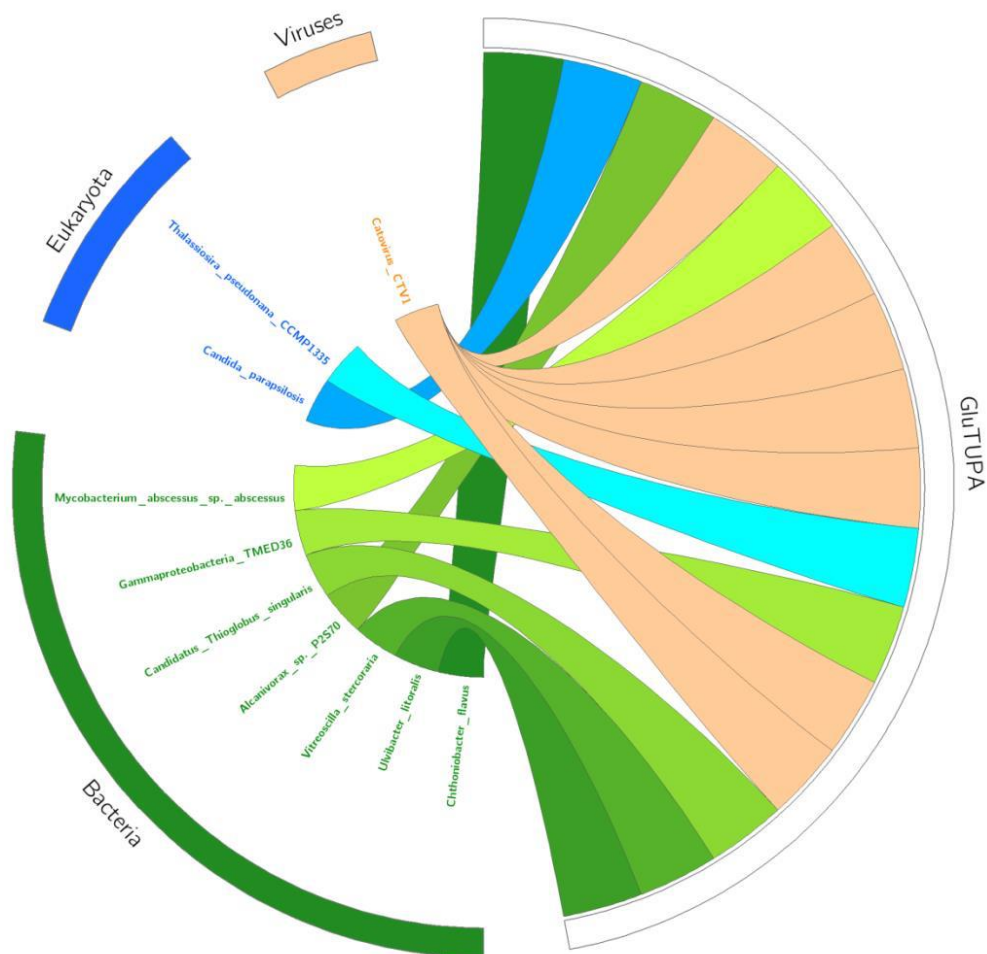
Valyl RS



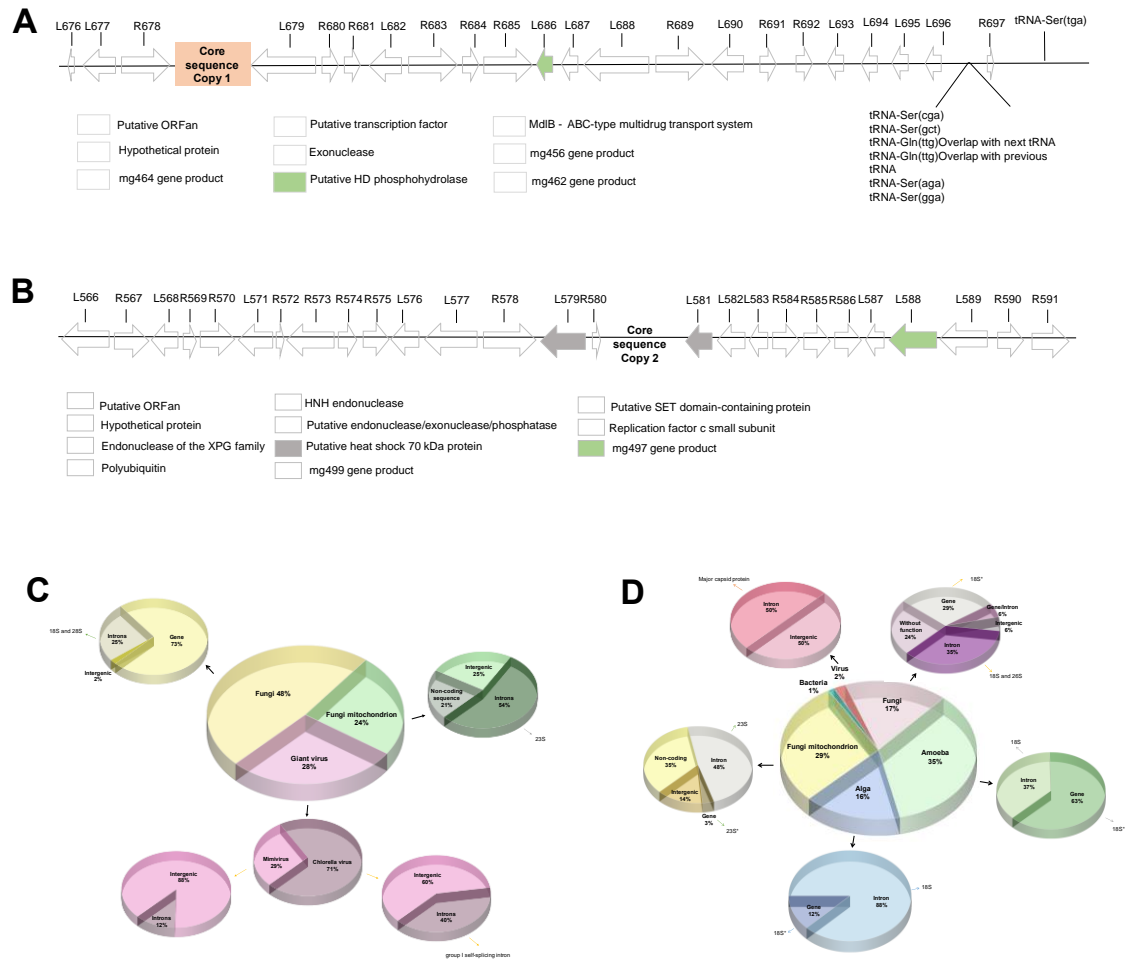
Amoebozoa



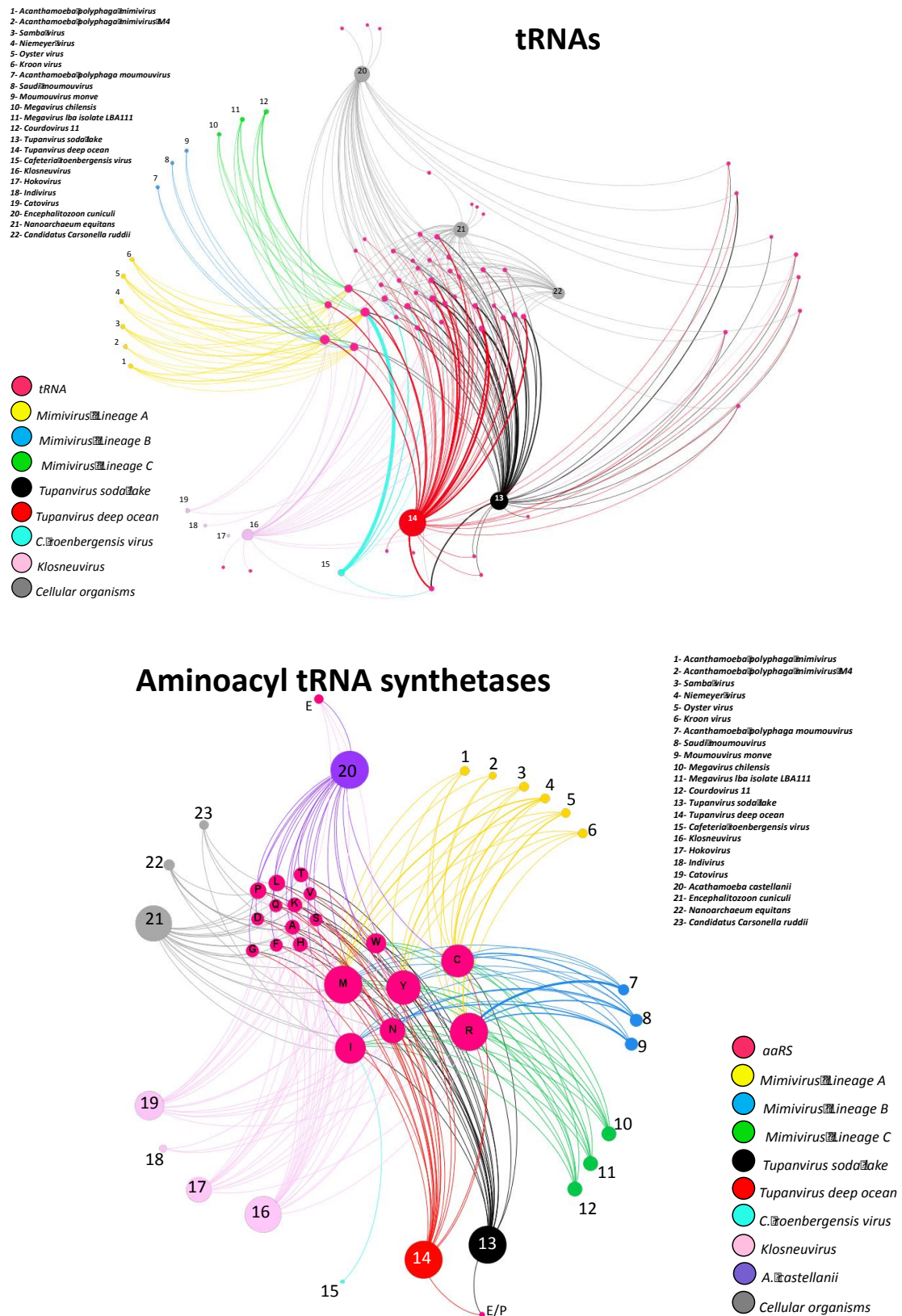
Supplementary Figure 5: The rhizome of the glutaminyl-tRNA synthetase of Tupanvirus soda lake. The sequence of the glutaminyl-tRNA synthetase was split into short fragments and blasted against the NR database. Best hit was selected and integrated in a circular gene data image. Taxonomic origins are colored in blue for eukaryote, green for bacteria and orange for viruses.



Supplementary Figure 6: Genomic environment and best-hit analyses of Tupanvirus soda lake 18S rRNA intronic regions. Genomic environment of copy 1 (a) and copy 2 (b). The 100 best hits for copies 1 (c) and 2 (d).

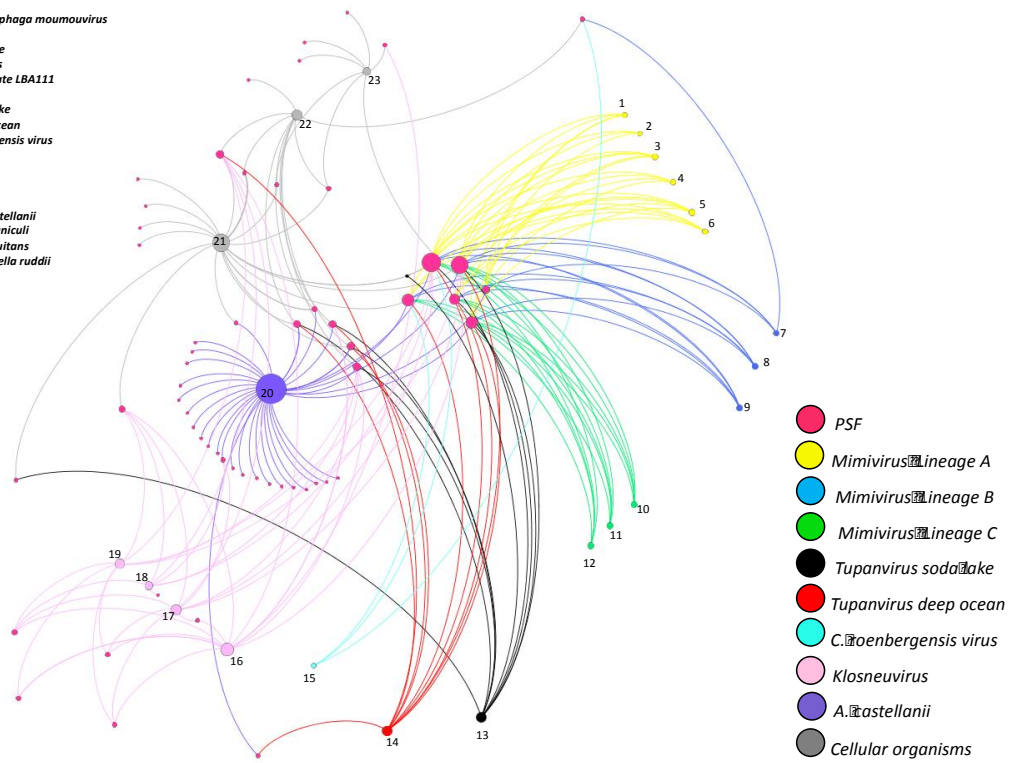


Supplementary Figure 7: Networks of translation-related category of genes of tupanviruses, Klosneuviruses, other mimiviruses and cell organisms.

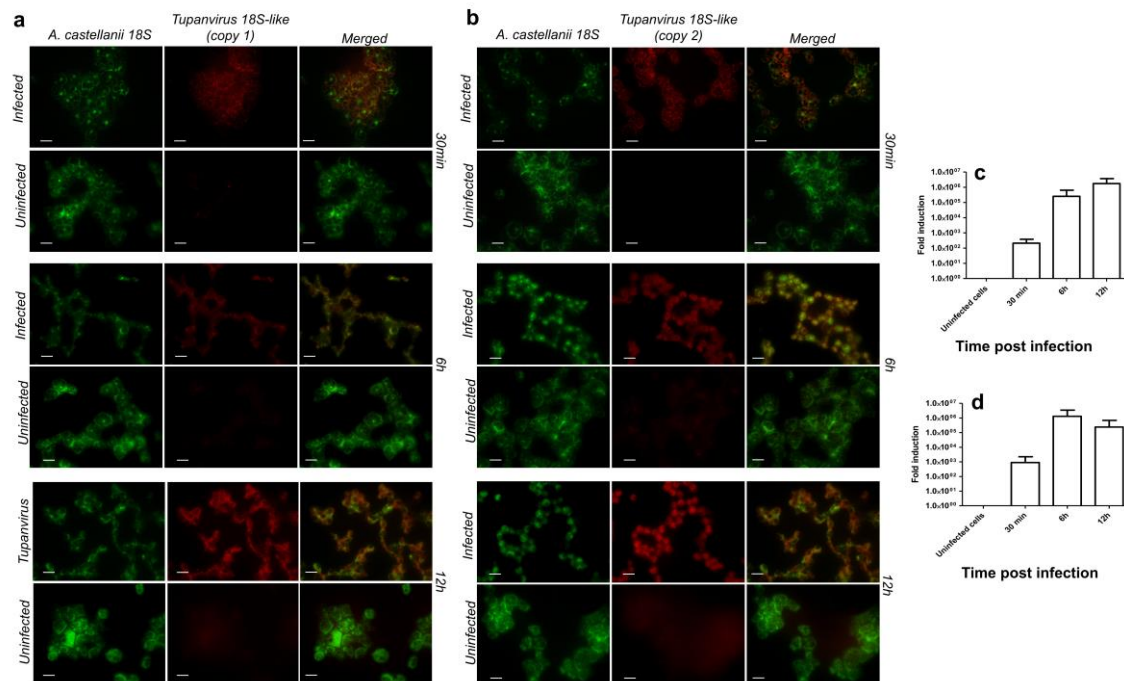


Protein Synthesis Factors

- 1- *Acanthamoeba polyphaga mimivirus*
- 2- *Acanthamoeba polyphaga mimivirus M4*
- 3- *Samba virus*
- 4- *Niemeyer virus*
- 5- *Oyster virus*
- 6- *Kroon virus*
- 7- *Acanthamoeba polyphaga moutou virus*
- 8- *Saudi moutou virus*
- 9- *Moutou virus manve*
- 10- *Megavirus chilensis*
- 11- *Megavirus Iba isolate LBA111*
- 12- *Courdivirus 11*
- 13- *Tupanvirus soda lake*
- 14- *Tupanvirus deep ocean*
- 15- *Cafeteria roenbergensis virus*
- 16- *Klosneuvirus*
- 17- *Hokovirus*
- 18- *Indivirus*
- 19- *Catovirus*
- 20- *Acanthamoeba castellanii*
- 21- *Encephalitozoon cuniculi*
- 22- *Nanoarchaeum equitans*
- 23- *Candidatus Carsonella ruddii*



Supplementary Figure 8: Analysis of Tupanvirus 18S rRNA intronic region (copies 1 or 2) expression in infected *Acanthamoeba castellanii* cells at 30 minutes and at 6 and 12 hours post-infection. Fluorescence in situ hybridization (FISH) (a and b) and qPCR (c – copy 1 and d – copy 2). In red, viral 18S rRNA intronic copies; in green *Acanthamoeba* 18S rRNA region (a and b). FISH analysis related to 24 hours post-infection; copy 1 is also shown in Figure 7a. Scale bar, 10 μ m. Error bars (c and d), standard deviation. The experiments were performed 3 times independently, with two replicates each.



Supplementary Figure 9: Acidification of *Acanthamoeba castellanii* cytoplasm after

Tupanvirus soda lake and mimivirus infection (9 h.p.i.). (a) Tupanvirus causes

strong acidification of amoebal cytoplasm even in the presence of bafilomycin A. (b)

Quantification of the acidification demonstrated in (a). (c-f) Even in the presence of

chloroquine or bafilomycin A, the rRNA shutdown induced by Tupanvirus is not

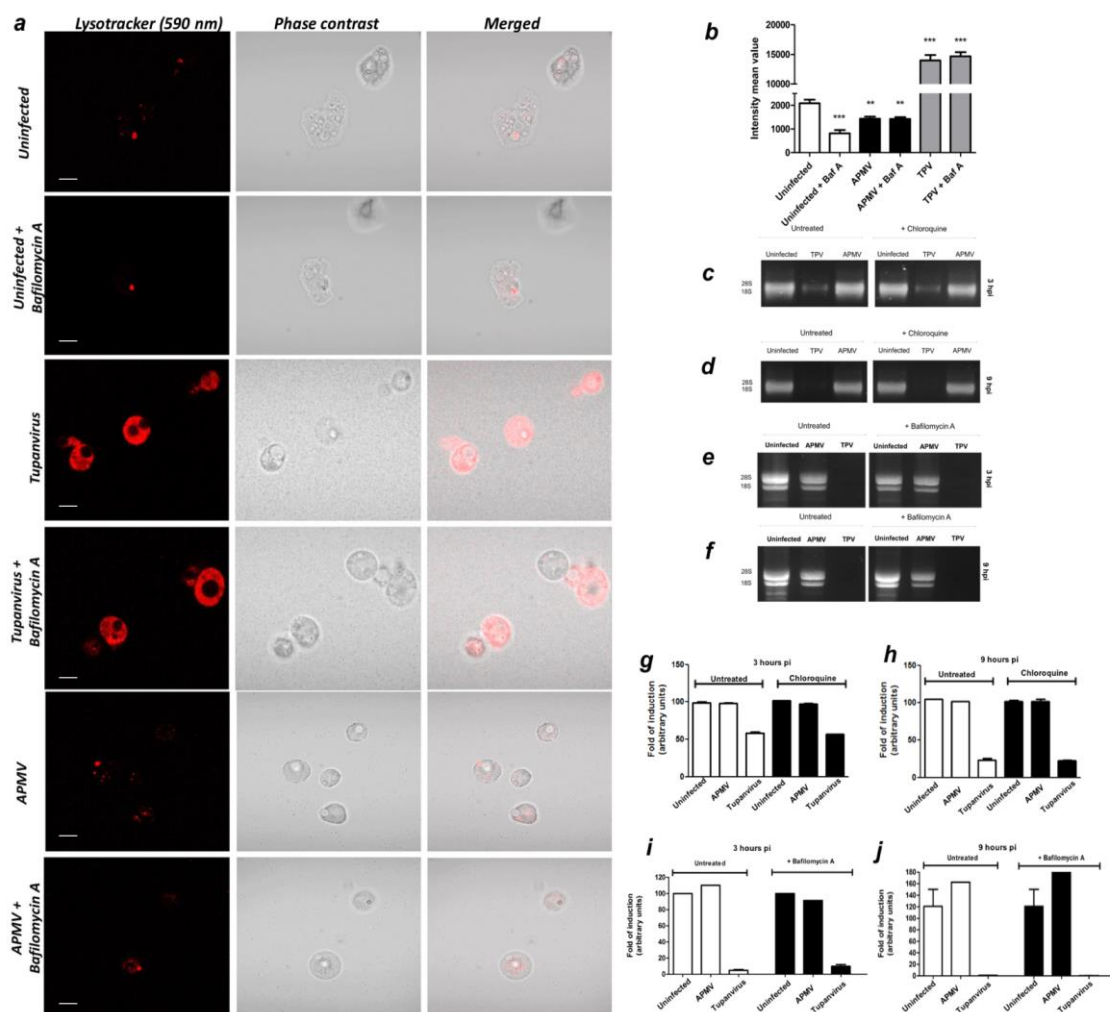
prevented (3 and 9 h.p.i.). (g-j) qPCR results related to the cells infected and treated as

presented in (c-f). Scale bar, 5 μ m. Error bars (b, g-j), standard deviation. Statistical

analyses were performed on the data shown in (b) using a t-test based on control groups.

****: $p < 0.01$; ***: $p < 0.001$. The experiments were performed 3 times independently, with**

two replicates each.



SUPPLEMENTARY TABLE

Supplementary Table 1: Permissiveness profile of Tupanvirus in different cells.

Cell	CPE	Viral titer increasing	Viral genome replication	Infection profile
<i>Acanthamoeba castellanii</i>	+	3log ₁₀ , after 24 hours	5 x 3log ₁₀ , after 24 hours	productive
<i>Acanthamoeba sp E4</i>	+	1log ₁₀ , after 24 hours	6 x 3log ₁₀ , after 24 hours	productive
<i>Acanthamoeba sp. micheline</i>	+	No	2 x 1log ₁₀ , after 24 hours	abortive
<i>Acanthamoeba polyphaga</i>	+	1log ₁₀ , after 24 hours	4 x 1log ₁₀ , after 24 hours	productive
<i>Acanthamoeba royreba</i>	+	No	2 x 1log ₁₀ , after 24 hours	abortive
<i>Acanthamoeba griffini</i>	+	1,5log ₁₀ , after 24 hours	7 x 1,5log ₁₀ , after 24 hours	productive
<i>Vermamoeba vermiformis</i>	+	1log ₁₀ , after 24 hours	6 x 1log ₁₀ , after 24 hours	productive
<i>Dyctiostelium discoideum</i>	+	1log ₁₀ after 48 hours	2 x 1log ₁₀ after 48 hours	productive
<i>Willartia magna</i>	+	0,5log ₁₀ after 24 hours	0,8log ₁₀ after 24 hours	productive
<i>Tetrahymena hyperangularis</i>	+	No	No	cytotoxic
<i>THP-1</i>	+	No	No	cytotoxic
<i>RAW264.7</i>	+	No	No	cytotoxic
<i>Trichomonas tenax</i>	-	No	No	resistant

CPE: cytopathic effect

□