Supplemental Figures

A novel fragmented mitochondrial genome in the protist pathogen *Toxoplasma gondii* and related tissue coccidia

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Fig. S1 – Annotated *Toxoplasma gondii* RH sequenced mtDNA PCR products. Fasta format sequences for these products can be found in Dataset_S1.

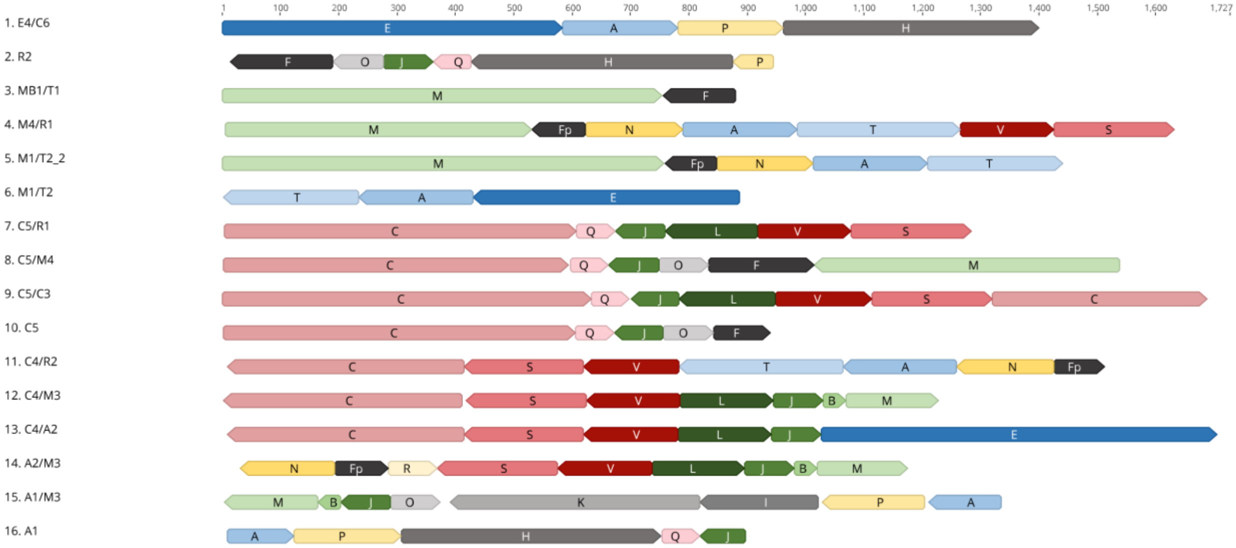
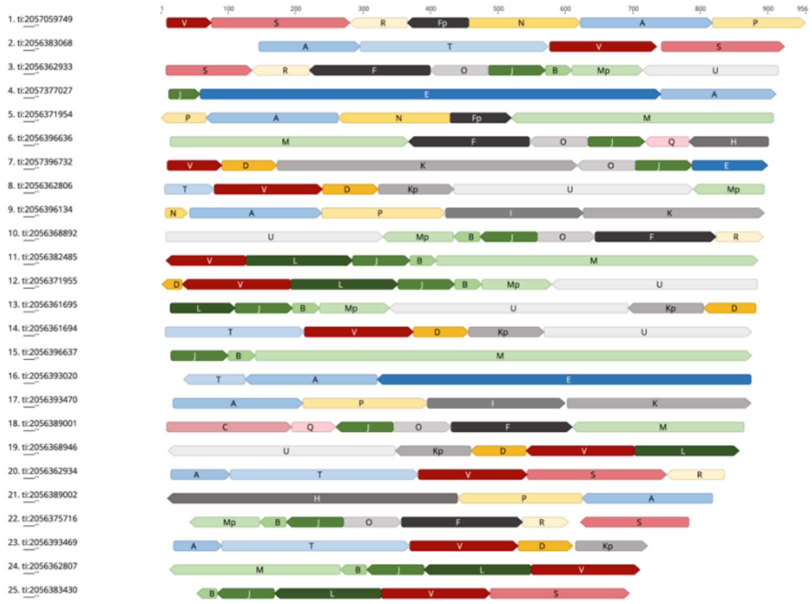
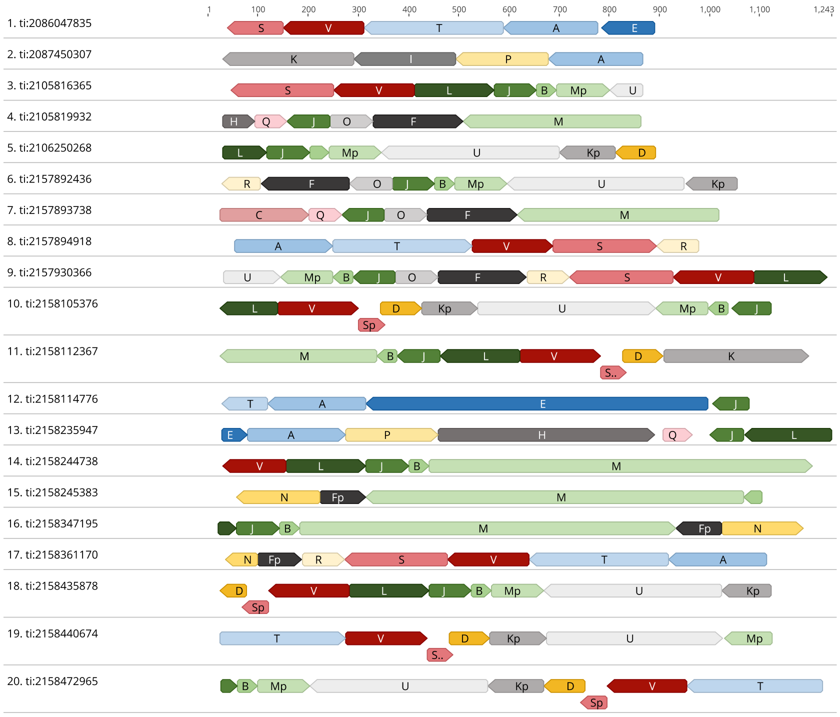


Fig. S2 - Annotation of select A) *T. gondii* and B) *N. caninum* genomic Sanger reads from NCBI SRA database using the 21 sequence blocks from the respective organism

A) *Toxoplasma gondii*



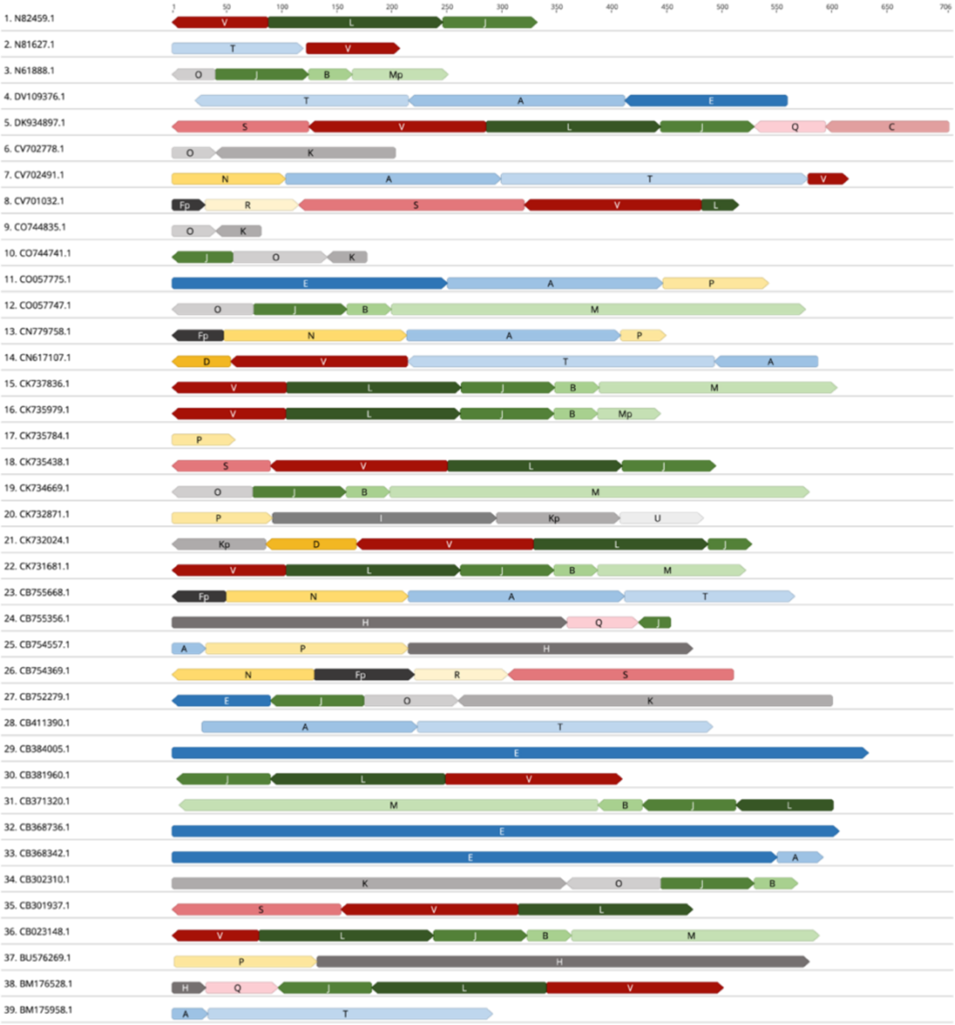
B) *Neospora caninum*



*When block R and F/Fp occur next to each other in *Neospora canin*um, the last 4 bp of F/Fp are missing and instead this junction contains the 2 nt AC/GT depending on orientation

Fig. S3 - Annotation of select A) *T. gondii* and B) *N. caninum* Sanger EST reads from the NCBI EST database using the 21 sequence blocks from the respective organism.

A) Annotated *Toxoplasma gondii* EST reads



B) Annotated *Neospora caninum* Sanger EST reads

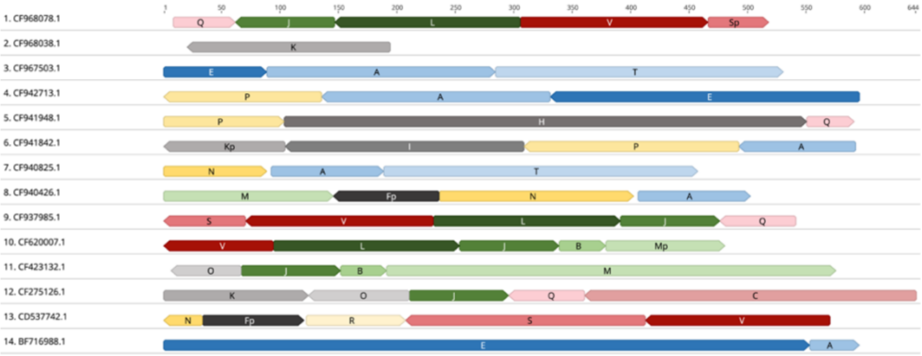
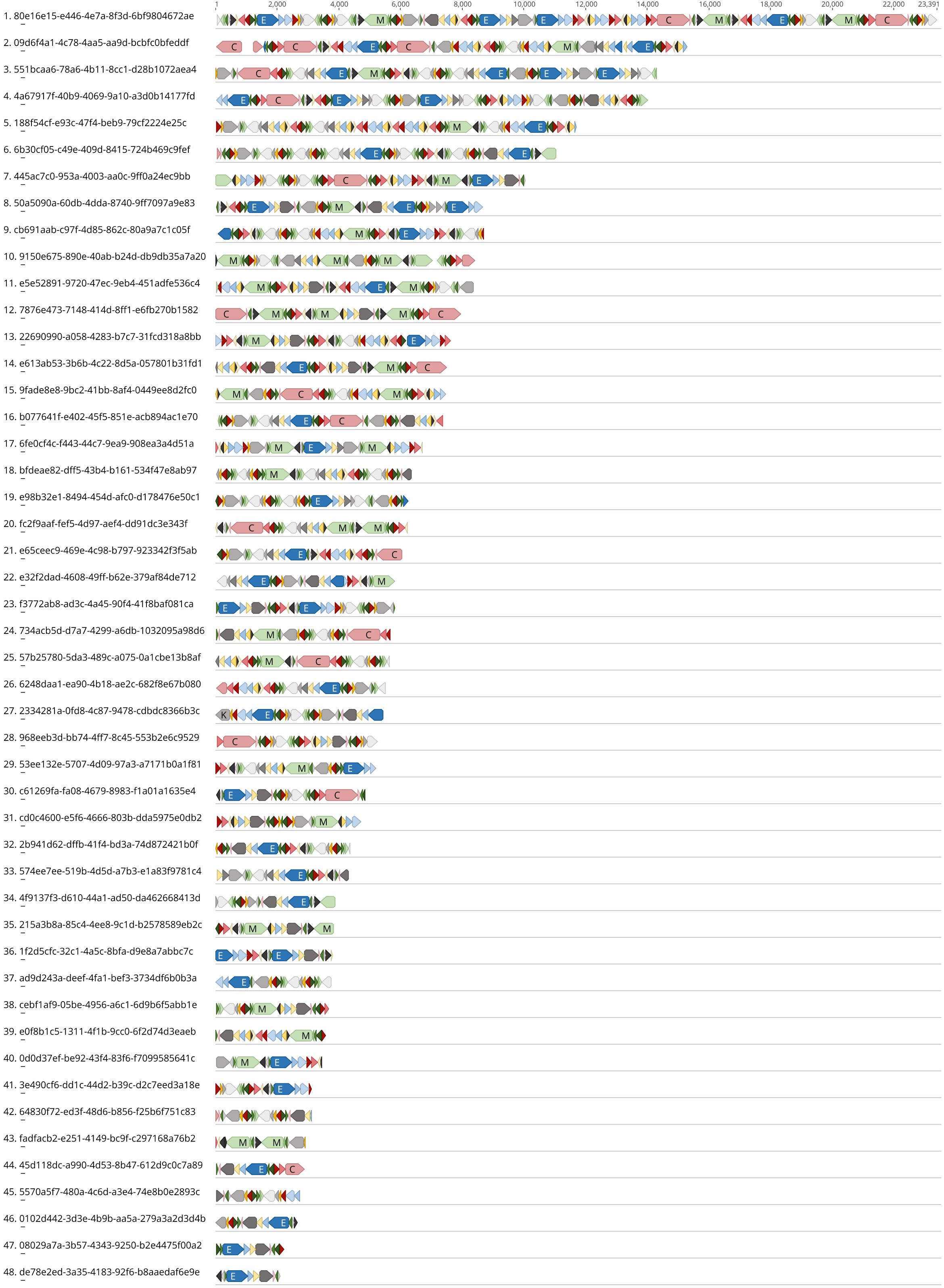
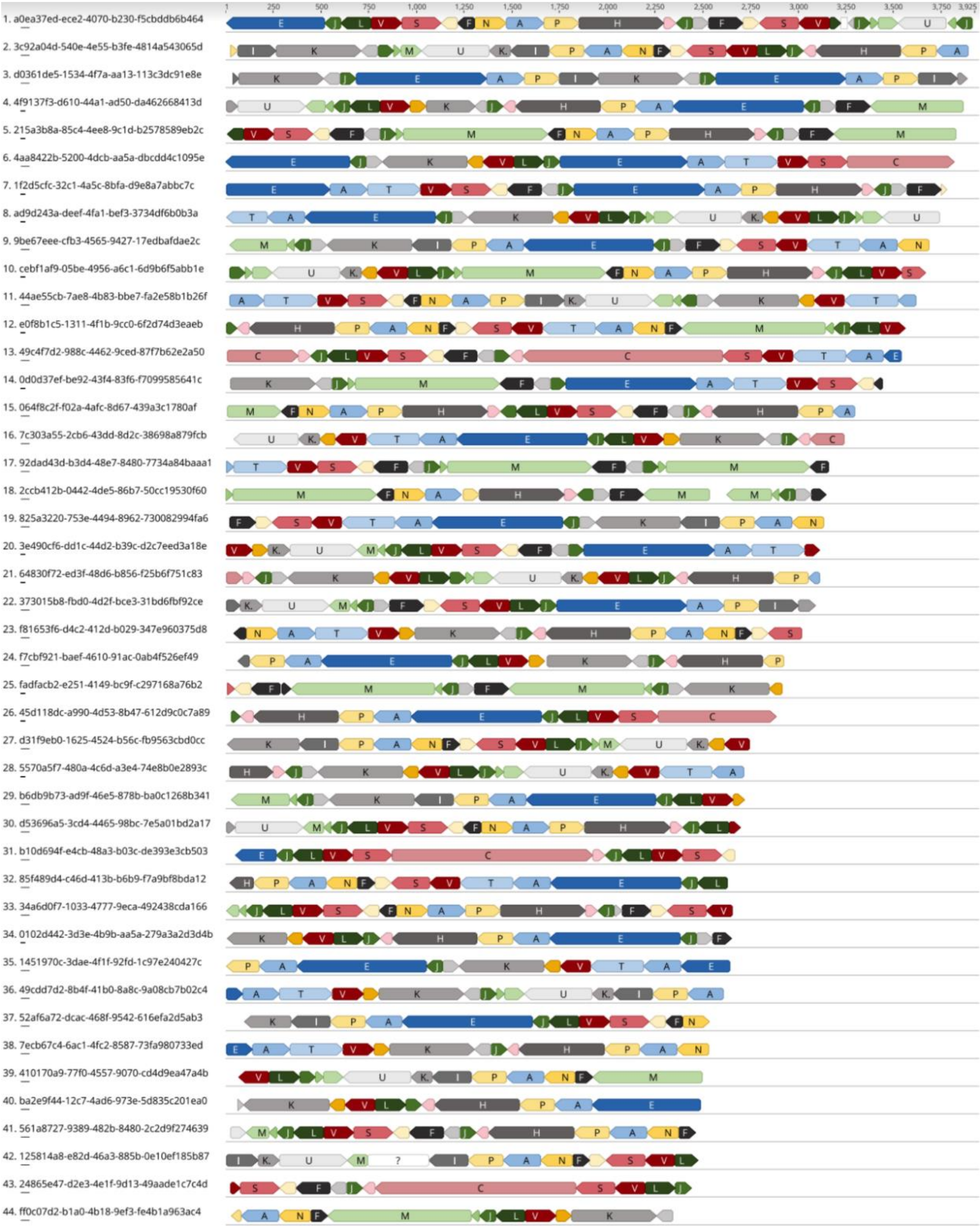


Fig. S4 – Annotated *Toxoplasma gondii* ME49 mtDNA Nanopore reads arranged by length.

a) *Toxoplasma gondii* ME49 Nanopore reads 4-23 kb

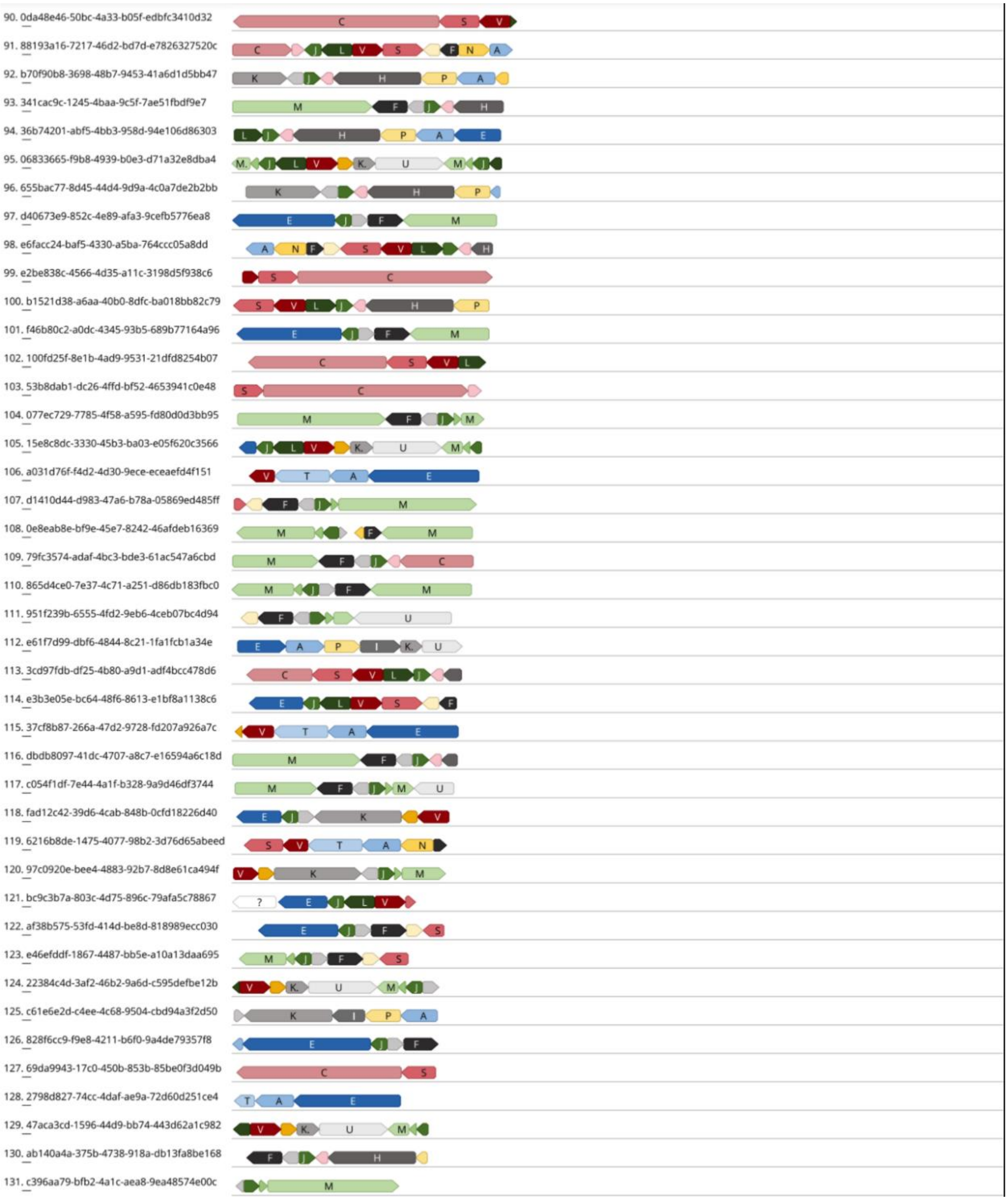


b) *Toxoplasma gondii* ME49 Nanopore reads 1-4 kb



Toxoplasma gondii ME49 Nanopore reads 1-4 kb (continued)

Toxoplasma gondii ME49 Nanopore reads 1-4 kb (continued)



T. gondii	*Th. parva*	*P. falciparum*	mtrRNA segment	*T. gondii* SB	*Th. parva*# Z23263	*P. falciparum*# M76611.1
🟦	🟦	🟦	SSUA	U-204-314	3666-3561	2023-1916
🟦	🟦	🟦	SSUB	K-268-150	4182-4074	505-390
🟦	🟦	🟦	SSUD	M-635-675	3800-3733	5446-5379
🟦	🟦	🟦	SSUE	M-727-754	2095-2057	1638-1680*
🟦	🟦	🟦	SSUF	I-98-156	1840-1900	5507-5447
🟩	🟩	🟩	LSUA	U-37-202	4073-3906	5201-5026
🟩	🟩	🟩	LSUB	W-13-37	3667-3691	4618*-4586
☐	🟩	🟩	LSUC		1824-1839	225*-204
🟩	🟩	🟩	LSUD	H-331-247	3114-3196	5854-5772
🟩	🟩	🟩	LSUE	H-54-244	3199-3377	5771-5577
🟩	🟩	🟩	LSUF	M-366-459	3507-3395	1516-1630
🟩	🟩	🟩	LSUG	M-485-591	5667-5775	389-283
☐	🟧	🟧	RNA1		3827-3905	593-506
☐	🟧	🟧	RNA2		3098-3032	1698-1763
☐	🟧	🟧	RNA3		5658-5584	1830-1910
☐	☐	🟧	RNA4			4625-4696
☐	☐	🟧	RNA5			4716-4802
☐	🟧	🟧	RNA6			4808-4865
☐	🟧	🟧	RNA7		1643-1584	5283-5202
🟧	🟧	🟧	RNA8	F-104-11	1678-1758	5954-5855
☐	🟧	🟧	RNA9		3031-2940	72-125
🟧	🟧	🟧	RNA10	K-445-405;O-17-86	4211-4183	724-625
☐	🟧	🟧	RNA11		4359-4301	5340-5284
☐	🟧	🟧	RNA12		2300-2240	4887-4945
☐	🟧	🟧	RNA13		mtR_52b/35	5025-4996
☐	🟧	🟧	RNA14		4401-4373	5546-5508
☐	🟧	🟧	RNA15		2039-1997	624-594
☐	☐	🟧	RNA16		1759-1790	Mar-33
☐	🟧	🟧	RNA17			126-165
☐	🟧	🟧	RNA18		2192-2154	4964-4988
☐	☐	🟧	RNA19		1518-1494	5576-5547
☐	☐	🟧	RNA20			34-71
☐	☐	🟧	RNA21			1807*-1829
☐	☐	🟧	RNA22			5378-5341

Fig. S5 – Comparative representation of identified mtrRNA segments in the Apicomplexa.

- data from (Feagin et al., 2012). *-inferred coordinates as in Feagin et al., 2012. The *T. gondii* boundaries are inferred. The lack of computationally identified mtrRNAs in *T. gondii* does not mean they are absent. GenBank accession numbers for the mtDNA genome coordinates are as indicated for *Theileria parva* and *Plasmodium falciparum*. SB = sequence block. Blue squares mtSSU segment identified; Green mtLSU segment identified; Orange rRNA segment identified; Blank square, segment not identified (but may be present).

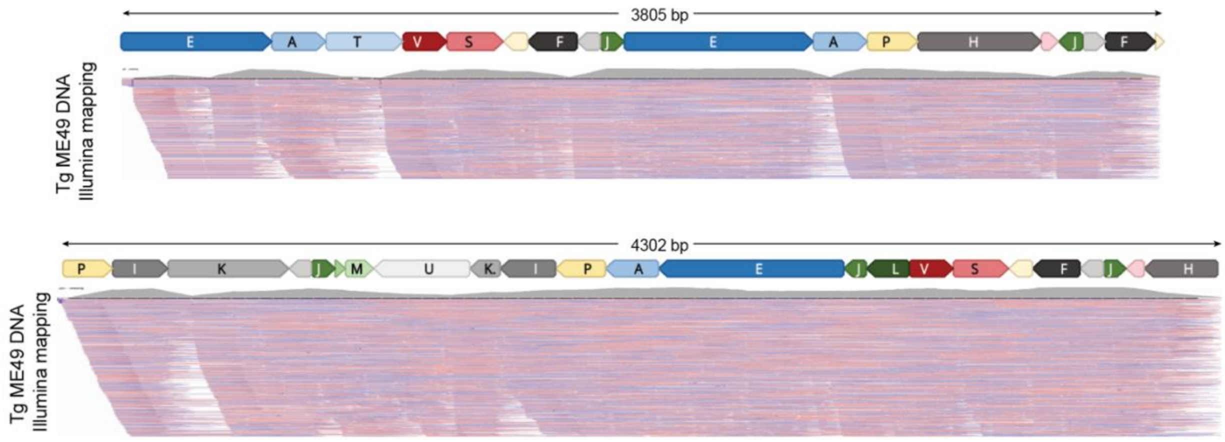
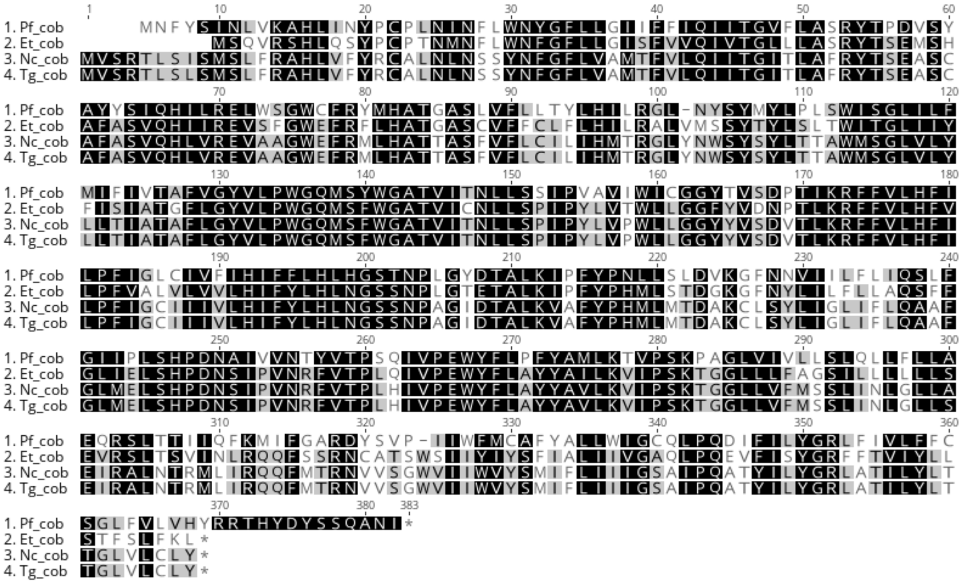


Fig. S6 - Annotated *Toxoplasma gondii* ME49 ONT mtDNA reads with Illumina mapping. Each panel represents the annotation of a single Nanopore read with SBs. There are no intervening nucleotides between SBs. The length of the read is indicated above the annotation. MtDNA-specific paired-end Illumina reads generated from *T. gondii* ME49 DNA (SRR9200762) were mapped to the Nanopore sequences and the alignment was visualized using the Integrated Genomics Viewer (IGV). Both Illumina read ends were required to map at 100% identity. A histogram of read abundance is shown in grey just below each annotated Nanopore read. Red and blue lines below each histogram indicate the mapped paired-end reads. Not all mapped reads are shown.

Fig. S7 - Multiple sequence alignments of mitochondrial cytochrome proteins. Multiple sequence alignment of COXI, COXIII and COB proteins (A-C) and genes (D-F) from the apicomplexan parasites *P. falciparum*, *E. tenella*, *T. gondii* and *N. caninum*. *T. gondii* and *N. caninum* cytochrome sequences were identified as described in the methods and results. Codon 'ATA' was annotated as the start Methionine for the COXI protein in *T. gondii* and *N. caninum*. Amino acid and corresponding CDS nucleotide sequences were downloaded from NCBI for *P. falciparum* (AAC63390.2, AAC63389.2, AAC63391.1) and *E. tenella* (BAJ25753.1, BAJ25754.1, BAJ25752.1).

A) Cytochrome B



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3. Nc_cob    MVSRTLSISMSLFRAHLVFYRCALNLNSSYNFGFLVAMTFVLQIITGITLAFRYTSEASC
4. Tg_cob    MVSRTLSLSMSLFRAHLVFYRCALNLNSSYNFGFLVAMTFVLQIITGITLAFRYTSEASC

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2. Et_cob    AFASVQHIIREVSFGWEFRFLHATGASCVFFCLFLHILRALVMSSYTYLSLTWITGLIIY
3. Nc_cob    AFASVQHLVREVAAGWEFRMLHATTASFVFLCILIHMTRGLYNWSYSYLTTAWMSGLVLY
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3. Nc_cob    EIRALNTRMLIRQQFMTRNVVSGWVIIWVYSMIFLIIIGSAIPQATYILYGRLATILYLT
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1. Pf_cob    SGLFVLVHYRRTHYDYSSQANI*
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4. Tg_cob    TGLVLCLY*
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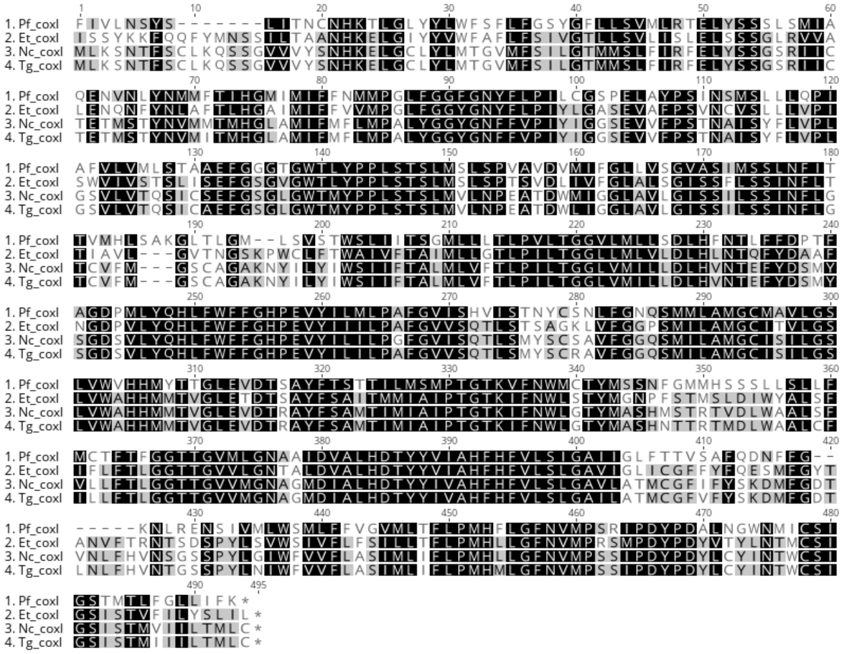
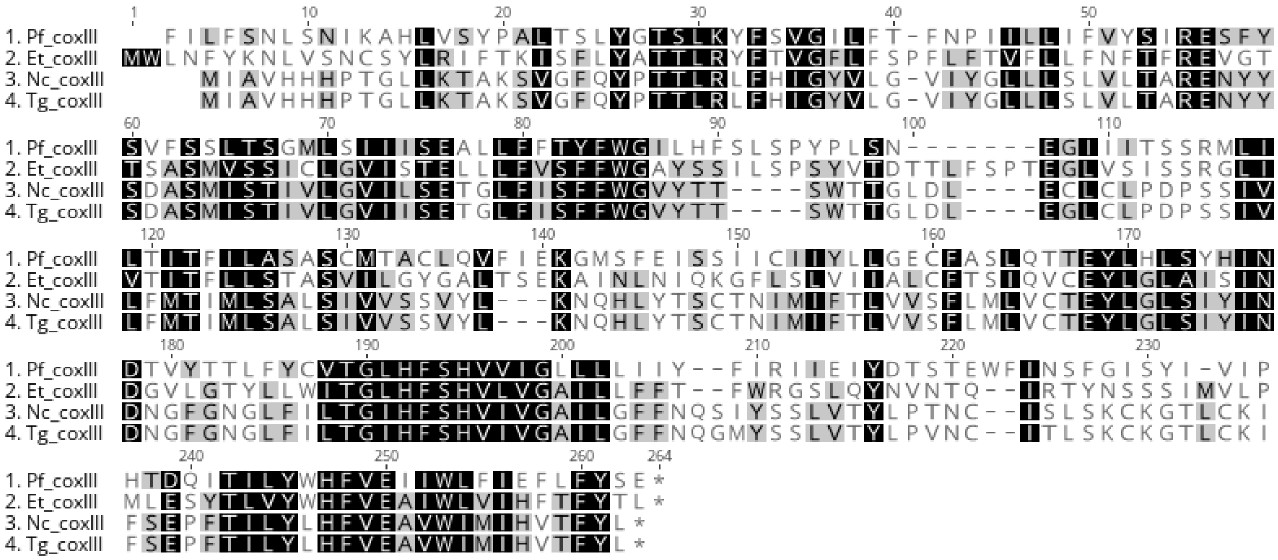
B) Cytochrome oxidase I



C) Cytochrome oxidase III



D) Cytochrome B

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4. Tg_cob ATGGTTTCGAGAACACTCAGTCTATCTATGAGTCTATTCCGGGCACACCTTGTCTTTTATCGGTGTGCTCTAAATCTAAATTCATCTTATAATTTTGGTTTCTTAGTTGCAATGA
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4. Tg_cob CCTTTGTACTCCAAATAATTACAGGTATCACTCTAGCGTTTAGATATACTTCTGAAGCATCTTGTGCATTTGCTAGCGTTCAACATCTAGTTAGAGAAGTAGCAGCAGGATGGGA
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4. Tg_cob GGTTTAGTTTTATATTTACTTACTATAGCCACTGCCTTCCTCGGATATGTACTACCATGGGGACAAATGAGTTTCTGGGGTGCTACAGTCATTACAAACCTCCTTTCTCCAATAC
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4. Tg_cob CATATTTGGTACCTTGGCTACTTGGAGGTTACTACGTATCTGATGTAACATTAAAACGATTCTTTGTATTACATTTTATCTTGCCTTTTATTGGTTGTATTATAATAGTTTTACA
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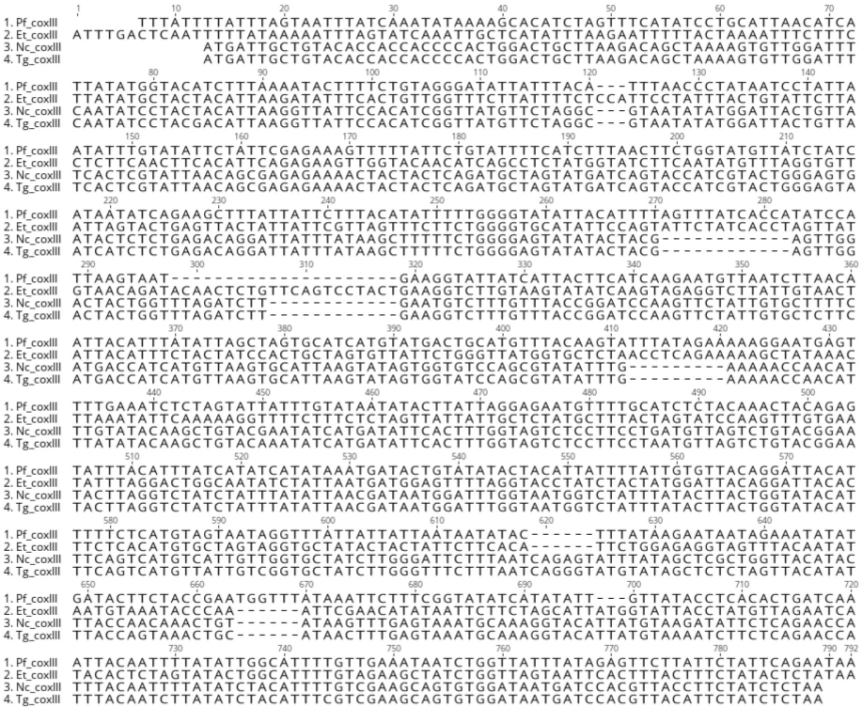
E) Cytochrome oxidase I

F) Cytochrome oxidase III



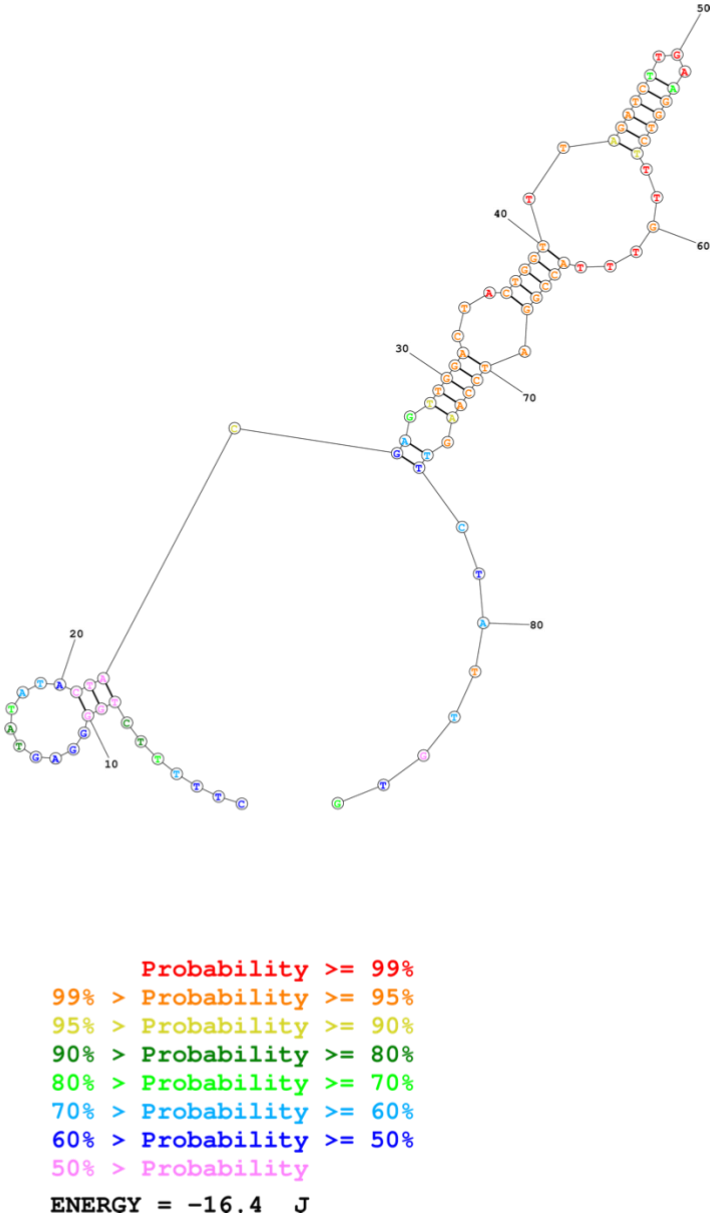


Fig. S8 - Secondary structure of *T. gondii* sequence block J. Structure analysis was performed with RNA structure Web Servers for RNA Secondary Structure Prediction. The Mathews Group. https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html
Settings: MaxExpect partition.pfs MaxExpect.ct --gamma 1 --percent 10 --structures 20 --window 3

Fig. S9 - Multiple sequence alignment of a subset of uncorrected Nanopore reads from the ENU mutant strain ELQ-316. The wt *T. gondii* RH sequence is on the first row. The mutant sequence is on the second row. The mutation, which is an A -> C, is located at position 23.

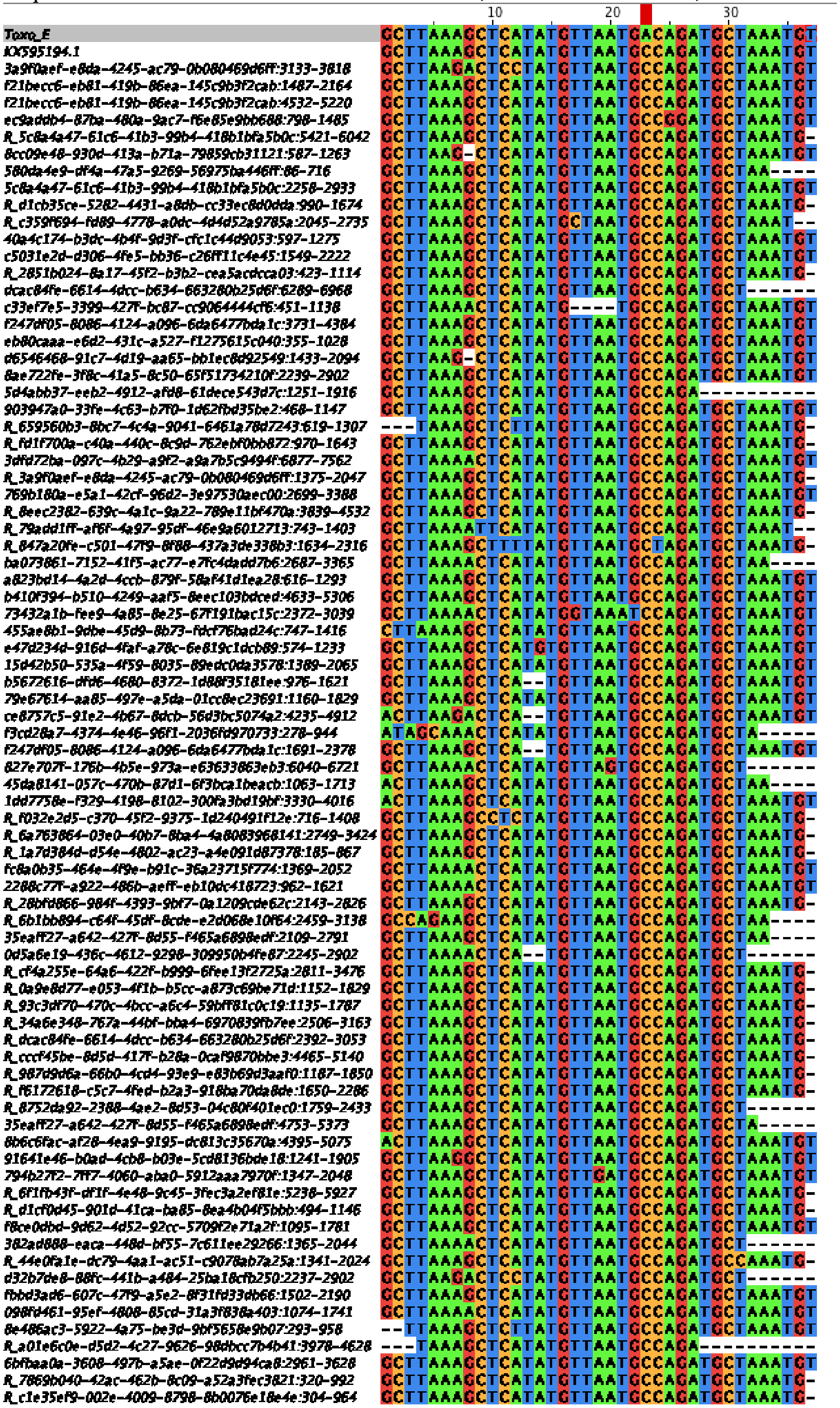


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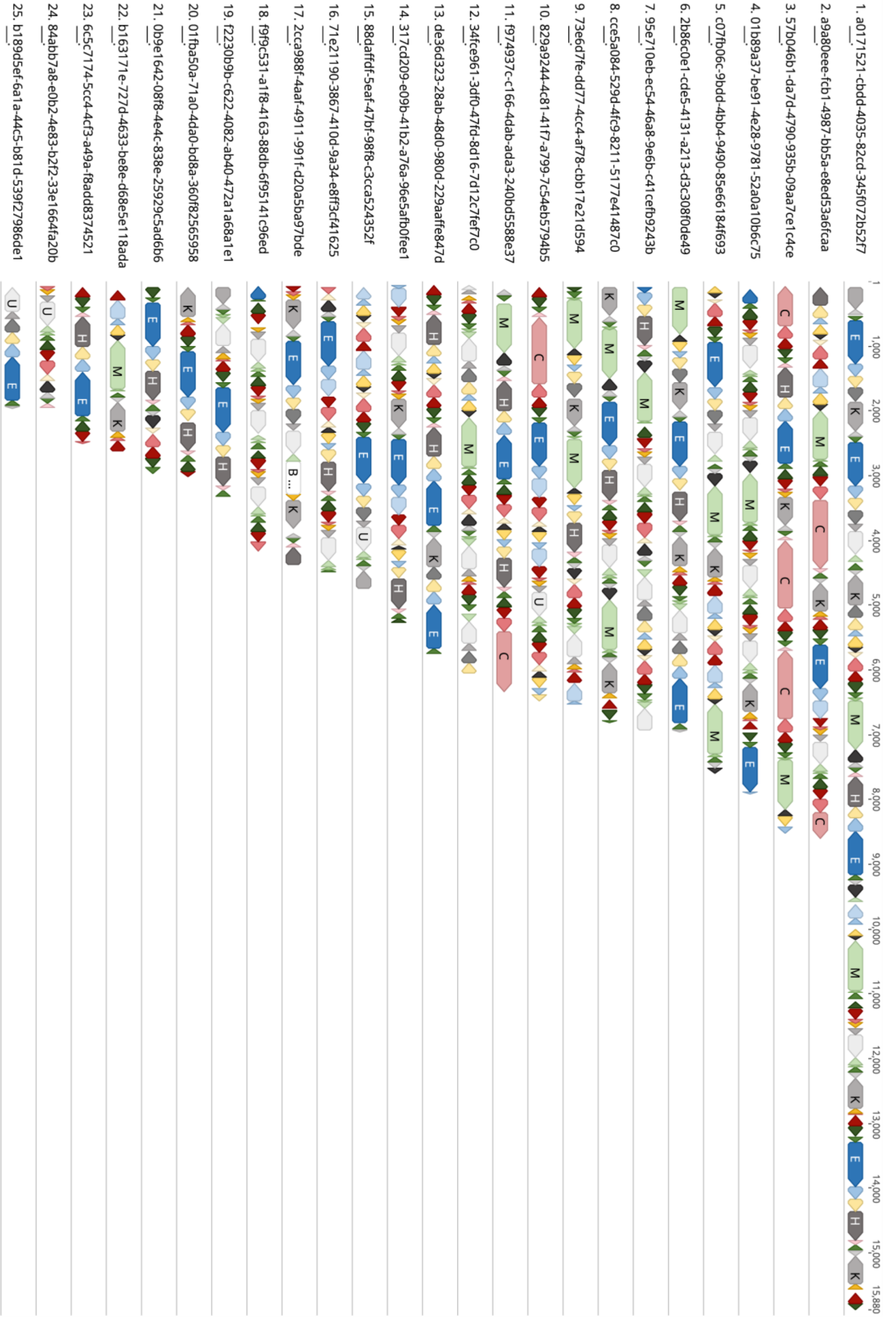
Fig. S10 – Annotated *Neospora caninum* mtDNA Nanopore reads (2-15 kb) arranged by length



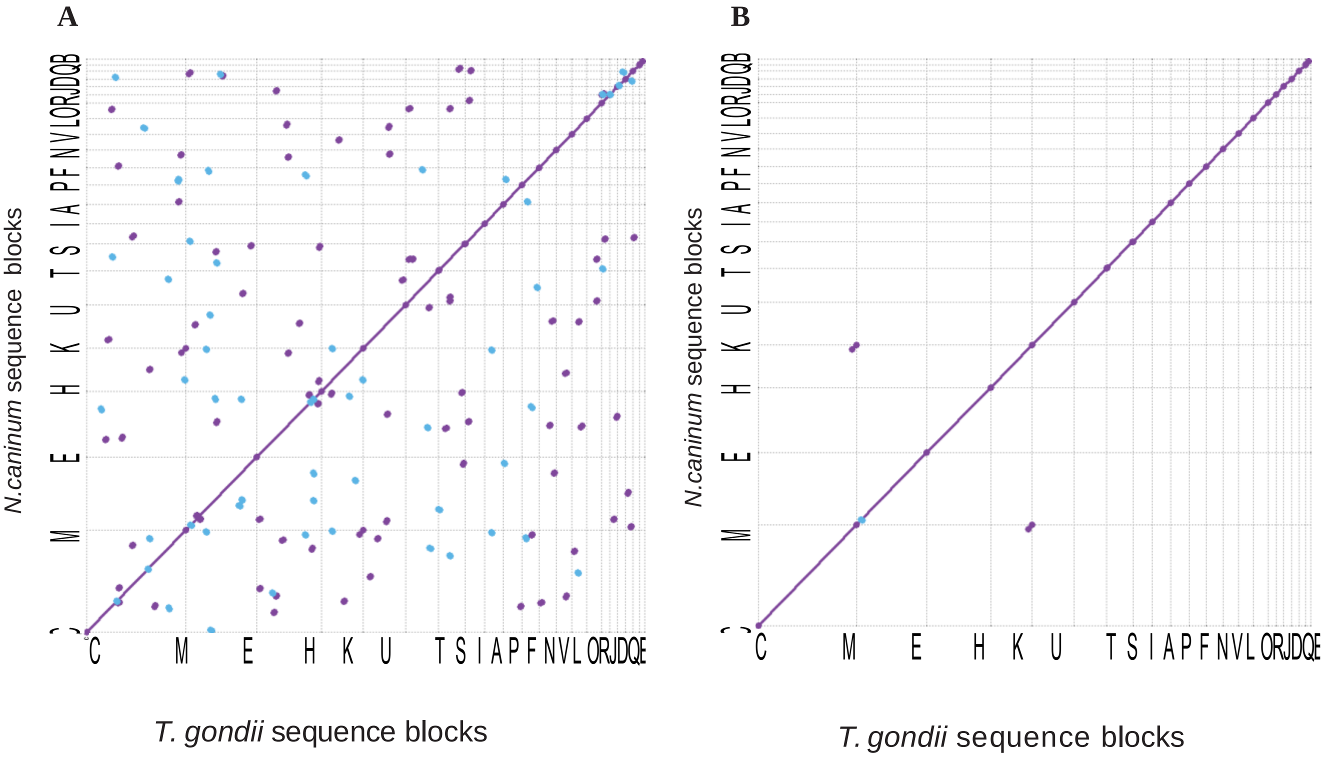
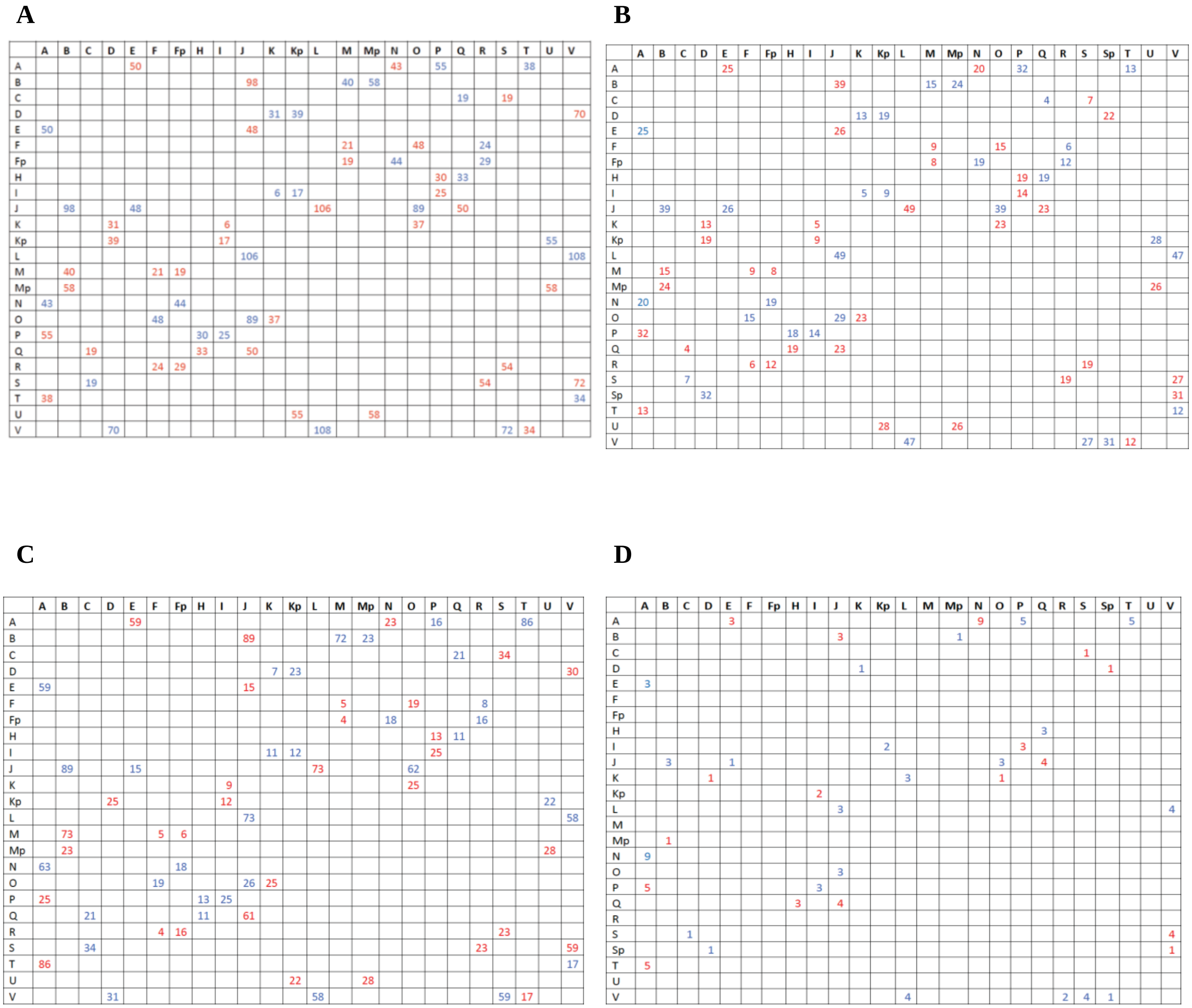


Fig. S11 - Dot plot comparisons of *T. gondii* ME49 and *N. caninum* Nc-1 sequence blocks to detect microhomology. (A-B) The 21 *T. gondii* mtDNA SBs were aligned to the 21 mtDNA blocks from *N. caninum*. Matches detected with window sizes of 10 bp in (A,) and 15 bp in (B) are displayed in blue or purple for forward and reverse matches respectively.



A

	A	B	C	D	E	F	Fp	H	I	J	K	Kp	L	M	Mp	N	O	P	Q	R	S	T	U	V
A					50											43		55				38		
B										98				40	58									
C																			19		19			
D											31	39												70
E	50									48														
F														21			48			24				
Fp														19		44				29				
H																		30	33					
I											6	17						25						
J		98			48								106				89		50					
K				31					6								37							
Kp				39					17														55	
L										106														108
M		40				21	19																	
Mp		58																					58	
N	43						44																	
O						48				89	37													
P	55							30	25															
Q			19					33		50														
R						24	29														54			
S			19																	54				72
T	38																							34
U												55			58									
V				70									108								72	34		

B

	A	B	C	D	E	F	Fp	H	I	J	K	Kp	L	M	Mp	N	O	P	Q	R	S	Sp	T	U	V
A					25											20		32					13		
B										39				15	24										
C																			4		7				
D											13	19										22			
E	25									26															
F														9			15			6					
Fp														8		19				12					
H																		19	19						
I											5	9						14							
J		39			26								49				39		23						
K				13					5								23								
Kp				19					9															28	
L										49															47
M		15				9	8																		
Mp		24																						26	
N	20						19																		
O						15				29	23														
P	32							18	14																
Q			4					19		23															
R						6	12														19				
S			7																	19					27
Sp				32																					31
T	13																								12
U												28			26										
V													47								27	31	12		

C

	A	B	C	D	E	F	Fp	H	I	J	K	Kp	L	M	Mp	N	O	P	Q	R	S	T	U	V
A					59											23		16				86		
B										89				72	23									
C																			21		34			
D											7	23												30
E	59									15														
F														5			19			8				
Fp														4		18				16				
H																		13	11					
I											11	12						25						
J		89			15								73				62							
K									9								25							
Kp				25					12														22	
L										73														58
M		73				5	6																	
Mp		23																					28	
N	63						18																	
O						19				26	25													
P	25							13	25															
Q			21					11		61														
R						4	16														23			
S			34																	23				59
T	86																							17
U												22			28									
V				31									58								59	17		

D

	A	B	C	D	E	F	Fp	H	I	J	K	Kp	L	M	Mp	N	O	P	Q	R	S	Sp	T	U	V
A					3											9		5					5		
B										3					1										
C																					1				
D											1											1			
E	3																								
F																									
Fp																									
H																			3						
I												2						3							
J		3			1												3		4						
K				1									3				1								
Kp									2																
L										3															4
M																									
Mp		1																							
N	9																								
O										3															
P	5								3																
Q								3		4															
R																									
S			1																						4
Sp				1																					1
T	5																								
U																									
V													4								2	4	1		

Fig. S12 - Upstream and downstream sequence blocks in *T. gondii* ME49 and *N. caninum* Nanopore reads. The identity of blocks located immediately at the 5-prime and 3-prime end of each of the 21 sequences blocks and 3 partials were counted, and the counts are shown as a matrix. The number of times a block on the y-axis is present at the 5-prime or 3-prime end in reference to the block on the x-axis is indicated in blue or red respectively. If two blocks were not found next to each other that corresponding square is blank. Only upstream or downstream block occurrences appearing >3 times were counted to avoid anomalies. (A) Data derived from *T. gondii* ME49 Nanopore DNA reads Supplemental Fig. S4; Dataset S3. (B) *N. caninum* Nanopore DNA reads, Supplemental Fig. S10; Dataset S8 (C) *T. gondii* Nanopore RNA reads, Dataset S6 and (D) *N. caninum* Sanger EST reads. Please note the absence of a block relationships in (D) is likely due to missing data since the data set was sparse.

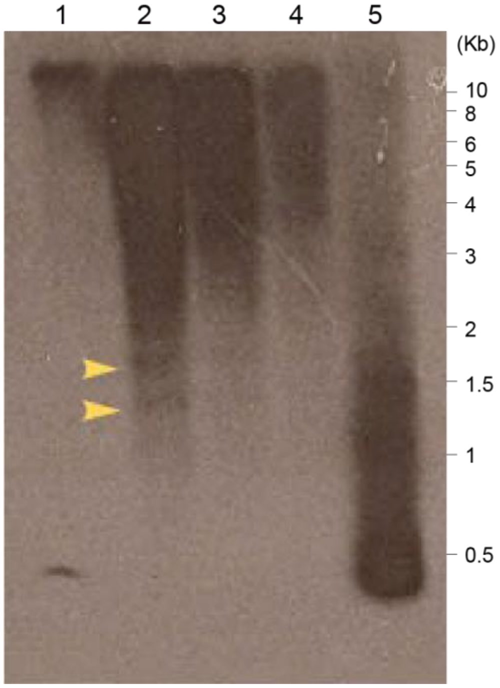


Fig. S13 - *T. gondii* mtDNA is variable in size and does not exist as a tandem repeat unit. Southern blot analysis. Total DNA from the *T. gondii* RH strain was hybridized to a 350 bp *coxI* probe under very stringent conditions. Lane 1: Undigested 2 µg genomic DNA; 2: *XhoI* Digested 15 µg genomic DNA; 3: *XhoI* Digested 5 µg genomic DNA; 4: *XhoI* Digested 2 µg genomic DNA; 5: positive control 350 bp probe fragment. *XhoI* cuts only once in the mitochondrial SBs, it cuts inside sequence block E of the *cob* gene. The yellow arrows point to distinct bands (~1.65 and 1.35 kb) on the blot. Size markers are indicated.

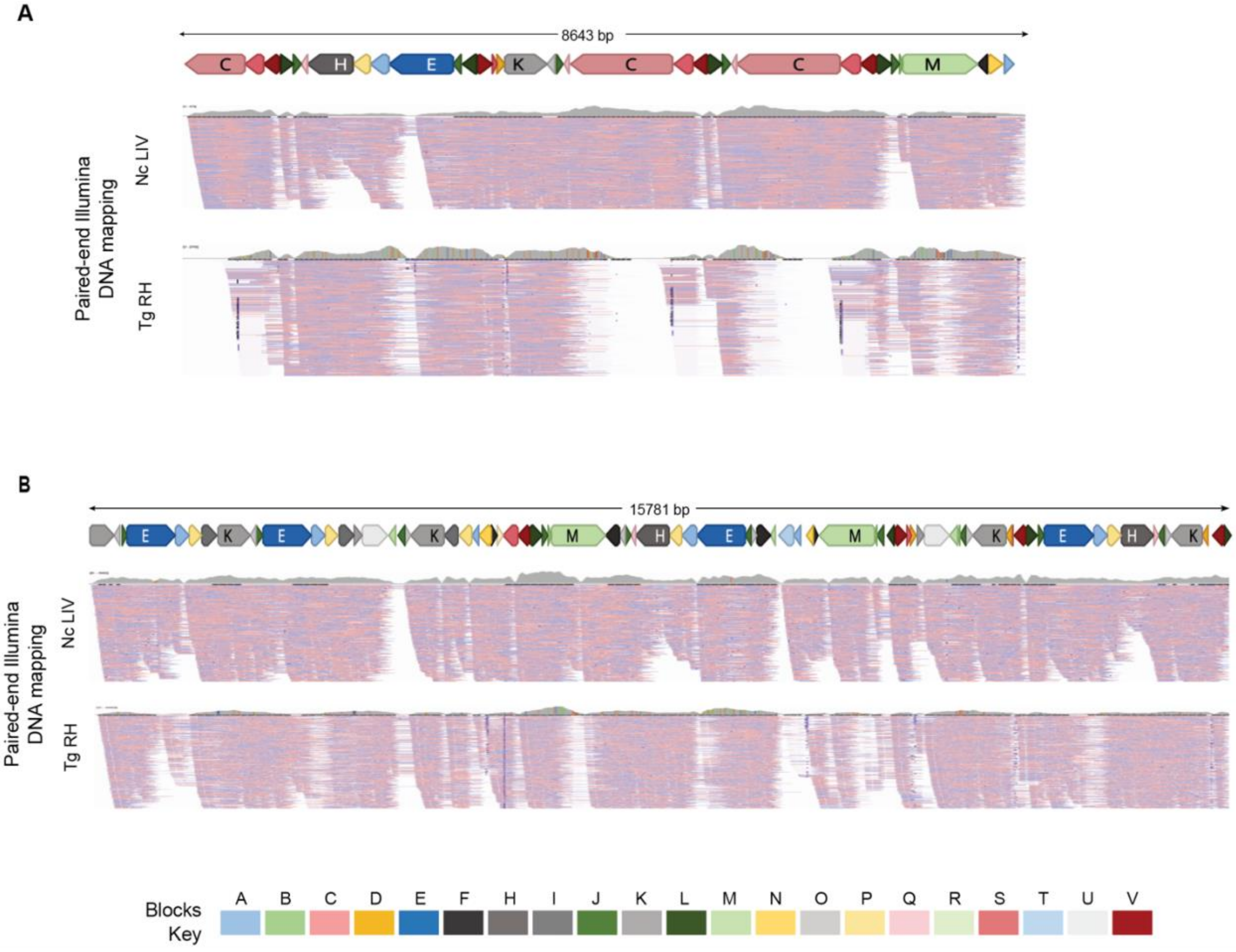


Fig. S14 - *N. caninum* nanopore sequence comparison and degeneration. (A-B) SBs were annotated on two *N. caninum* Nc-1 nanopore sequences reads at scale. The blocks are colored as shown in the key at the bottom of the figure. The two 'Paired-end Illumina DNA mapping' tracks show paired-end read mapping of *N. caninum* (Nc) LIV (ERR012900) and *T. gondii* (Tg) RH (SRR521957) mtDNA-specific reads. Mapping of Nc LIV reads required 100% nucleotide identity whereas 1% mismatch was allowed for mapping *T. gondii* RH reads. Reads were independently mapped to each of the Nanopore mtDNA reads and visualized using IGV. Red and blue lines below each read indicate the mapped Illumina paired-ends.

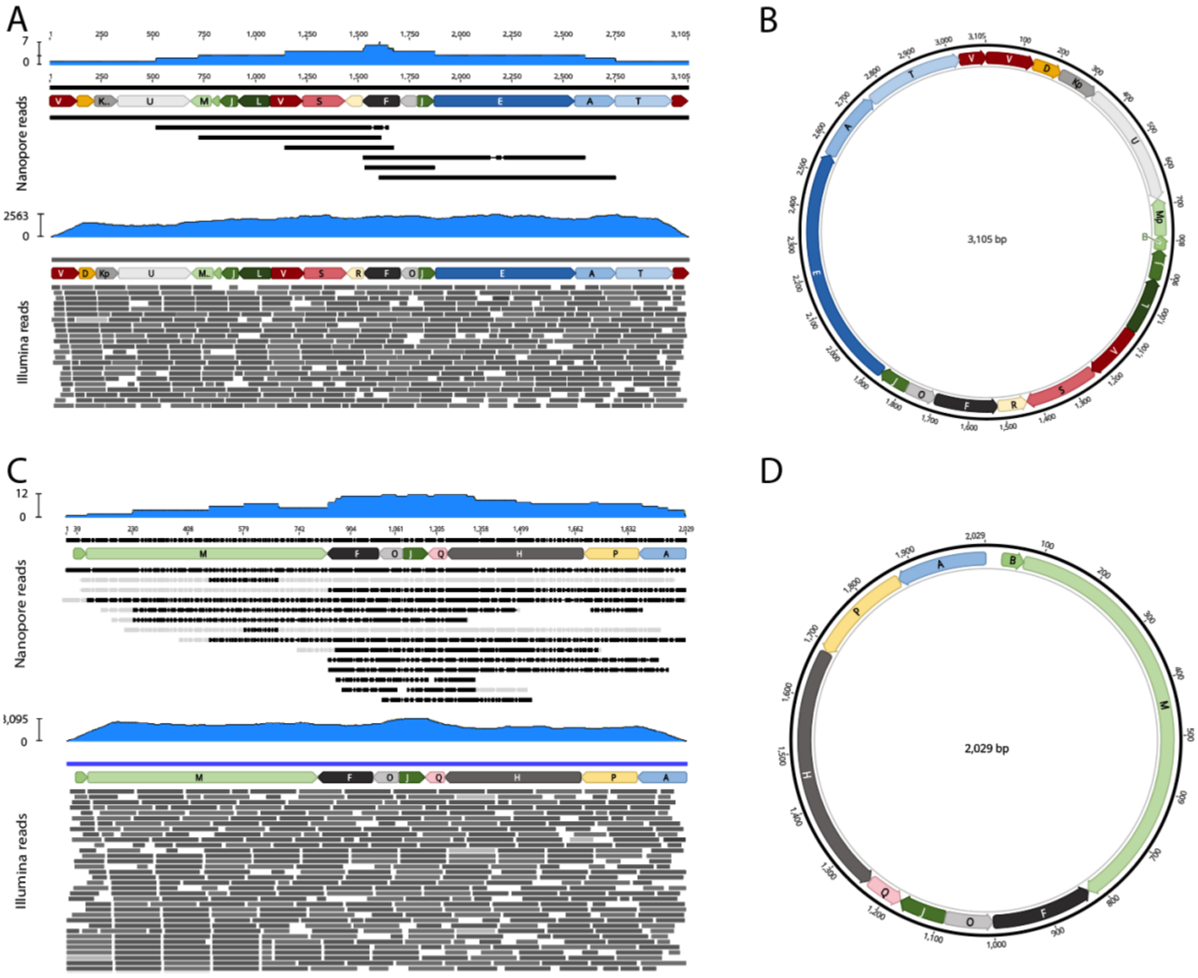


Fig. S15 - Nanopore and Illumina support for possible circular structures. (A, C) Mapped tracks of TgME49 Nanopore (top panels) and TgME49 Illumina reads (bottom panels) to single Nanopore reads identified as possibly existing as circular molecules according to Circlator predictions (B, D). Note the presence of the *cob* gene blocks EAT in panel B. Mapping of single and paired-end Illumina reads was permitted. Not all mapped reads are shown. The depth of read mapping is indicated above each panel in blue. The portions of Nanopore reads that are greyed out in panel C do not map to the annotated Nanopore read above. Additional predictions some of which encode the remaining cytochrome genes are provided in Supplemental Fig. S16.

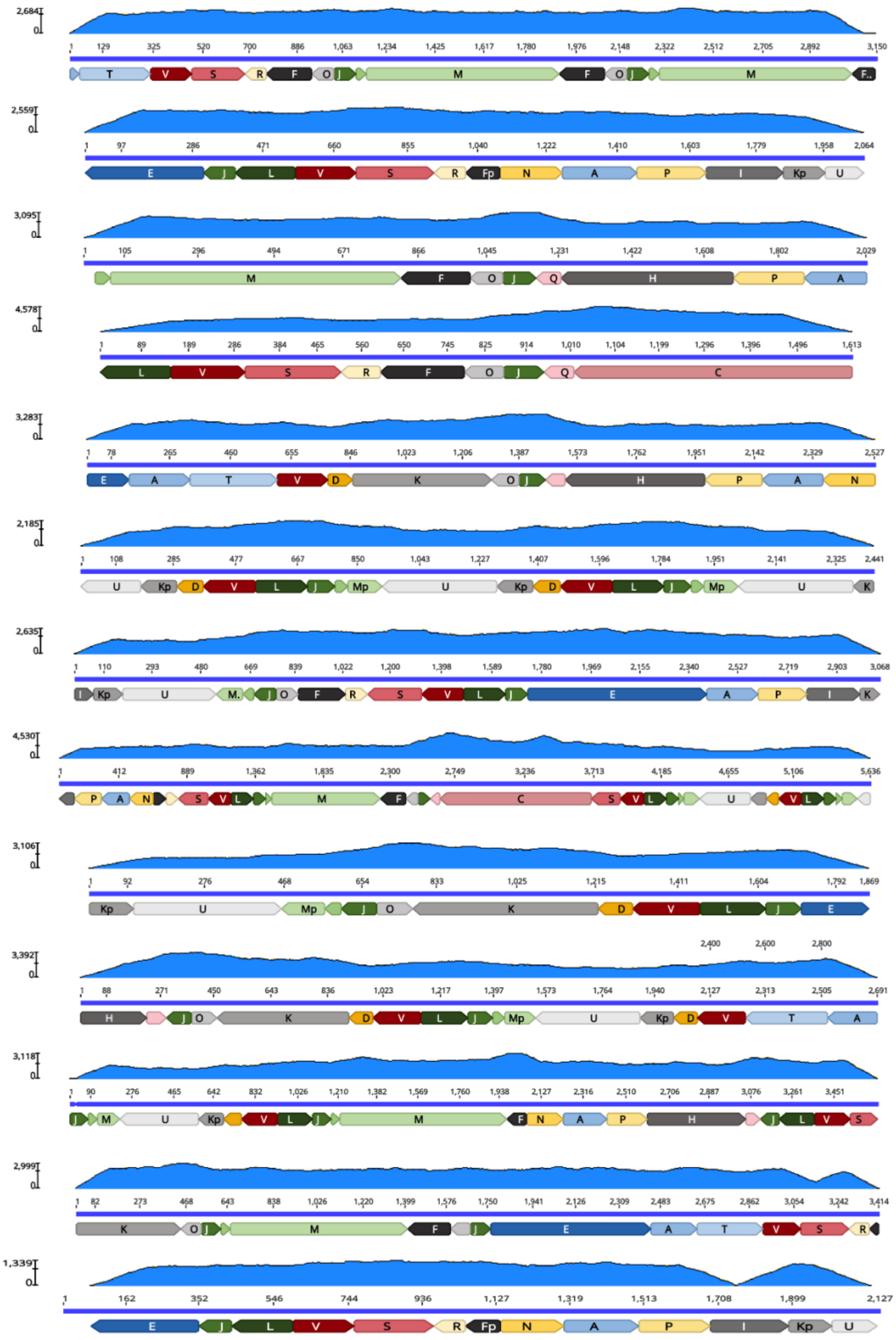


Fig. S16 - Nanopore reads predicted to circularize. *T. gondii* ME49 Illumina mapping frequency support for predicted circular sequences. Mapped read counts are displayed above each Nanopore read in blue. Some reads show fairly uniform coverage and others do not. Scale is as indicated for each read. Full-length cytochrome genes are observed.