Supplementary File S1. Technical notes for manuscript:

Sensitive universal detection of blood parasites by selective pathogen-DNA enrichment and deep amplicon sequencing

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This document provides additional detail on the experiments performed to optimize our universal parasite diagnostic (UPDx) assay.

Additional notes on UPDx optimization

Digestion of neat DNA versus normalized DNA and exclusion of DNA cleanup following digestion

To reduce time and labor requirements, the present UPDx method was optimized for minimal host-derived read recovery and to maximize parasite read recovery. The method described by Flaherty and colleagues involved subjecting a normalized mass of DNA to enzyme digestion (150 ng), followed by a DNA cleanup step that removed restriction enzymes and buffers prior to PCR (Flaherty et al. 2018). While the 150 ng digest was performed for the sake of consistency across samples, it added an additional laborious step. Here, we subjected neat DNA extracts to restriction digestion and did not perform a DNA cleanup step after D1 and/or D2, removing multiple extraneous steps. Exclusion of these steps sometimes enhanced parasite DNA detection, as a loss of DNA during the additional cleanup was avoided; we tested the impact of these alterations on blood containing *P. knowlesi* and *L. infantum*, confirming that these extraneous cleanup procedures resulted in a loss of performance. The number of *P. knowlesi* reads recovered increased when neat DNA vs quantified and diluted DNA (to 150 ng) was tested, though results obtained for *L. infantum* showed little difference. Similarly, cleanup of DNA following restriction digestion and before PCR1 greatly reduced the subsequent number of *P. knowlesi* reads recovered following NGS, but this made little difference for *L. infantum* (Figure S1 - below).

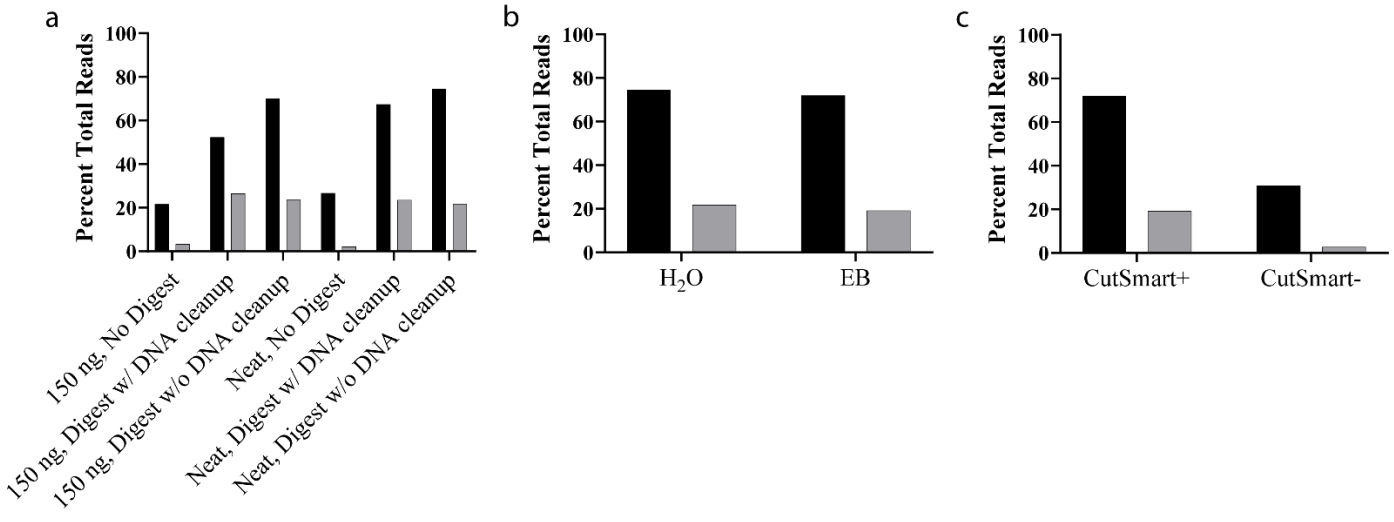
Digestion of DNA extracts in CutSmart Buffer versus water

As part of our assay optimization procedures, the impact of digesting DNA in commercially available CutSmart Buffer (NEB) versus water was also assessed using blood specimens containing *P. knowlesi* and *L. infantum* extracted into Buffer EB. The addition of CutSmart buffer at 1x concentration to the DNA eluate approximately doubled the resultant

number of *P. knowlesi* reads and led to an almost 20 times increase in total *L. infantum* reads, confirming that utilization of CutSmart Buffer, or an equivalent buffer, during restriction enzyme digestion is essential (Figure S1 - below).

The impact of cycle number for PCR1 and length of restriction digest times (D1 and D2)

We observed the recovery of substantially more parasite-specific reads for samples that underwent nested PCR amplification with 15 cycles compared to 10 cycles for PCR1, and when both DNA Digest 1 and DNA Digest 2 (double digestions) were performed, compared to only one of these digestion steps (Figure S1 - below). Restriction digestions were optimized by comparing different digestion times (15 minutes, 1 hour, 2 hours, overnight, or not at all) for both digests (D1 and D2). Restriction digest duration generally had a minor impact beyond 15 minutes. Consequently, the optimal protocol requires Digest 1 for 15 minutes, running PCR1 for 15 cycles, and performing Digest 2 for two hours; though this digest time may be shortened slightly without a major loss of performance.



d

		P. falciparum						*L. infantum*					
Digest 1 (minutes)	**Digest 2 (minutes)**	**No PCR1 (original method)**		**10-cycle PCR1**		**15-cycle PCR1**		**No PCR1 (original method)**		**10-cycle PCR1**		**15-cycle PCR1**	
	120	89.6%	2.6%					41.3%	44.6%				
ND	ND			57.4%	32.4%	41.0%	47.5%			1.8%	89.8%	2.2%	91.8%
ND	15			85.1%	6.1%	83.7%	6.6%			10.3%	74.3%	32.7%	55.9%
ND	60			79.8%	10.8%	85.7%	5.3%			11.7%	71.2%	33.3%	55.6%
15	ND			78.6%	11.2%	83.0%	8.4%			5.3%	84.5%	37.9%	51.2%
15	15			88.1%	3.7%	92.3%	0.4%			14.8%	73.4%	67.3%	22.5%
15	60			87.4%	3.2%	92.3%	0.5%			16.0%	72.3%	65.8%	24.4%
60	ND			77.7%	12.9%	84.3%	7.4%			7.9%	82.0%	40.4%	49.3%
60	15			89.9%	3.2%	92.3%	0.6%			17.2%	70.5%	72.6%	19.2%
60	60			85.1%	3.8%	91.8%	0.4%			19.5%	67.8%	69.6%	21.1%
120	ND			79.2%	12.0%	85.3%	6.3%			7.7%	82.0%	40.2%	48.2%
120	15			90.9%	2.4%	92.9%	0.4%			18.3%	70.8%	78.6%	14.4%
120	60			87.0%	3.6%	92.1%	0.4%			17.7%	67.7%	75.0%	17.8%
ON	ND			76.6%	12.1%	87.2%	4.1%			6.9%	82.3%	45.5%	44.9%
ON	15			83.4%	8.1%	93.1%	0.3%			17.4%	71.0%	79.4%	14.2%
ON	60			88.4%	3.1%	92.3%	0.2%			18.3%	68.8%	73.5%	18.4%

Figure S1. Overview of optimization experiments performed for our UPDx assay

(**a**) 150 ng or 25 µL of neat *P. knowlesi* (black bars) and *L. infantum* (grey bars) DNA extracts in molecular grade water were digested with 10 units of BamHI-HF and BsoBI in 1X CutSmart Buffer for 2 hours. Samples were transferred directly to PCR2 or cleaned before PCR2 amplification and DNA sequencing. No notable difference between 150 ng and neat digestion was observed; DNA cleanup had negative or minimal effects on the final proportion of parasite-derived reads recovered in the sample. (**b**) 25 µL of neat *P. knowlesi* (black bars) and *L. infatum* (grey bars) DNA extracts eluted into molecular grade water (H_2O) or elution buffer (EB) were digested with 10 units of BamHI-HF and BsoBI in 1X CutSmart Buffer for 2 hours and transferred directly to PCR2 for amplification and sequencing. No notable difference was observed between samples eluted into water versus elution buffer. (**c**) 25 µL of neat *P. knowlesi* (black bars) or *L. infantum* (grey bars) DNA extracts in elution buffer were digested with 10 units of BamHI-HF and BsoBI in the presence or absence of 1X CutSmart Buffer for 2 hours and transferred to PCR2 for amplification and sequencing. Substantially higher parasite-specific reads, indicating more efficient digestion of host DNA, were observed for sample digested in the presence of CutSmart buffer. (**d**) DNA extracts of neat *P. falciparum* or *L. infantum* in elution buffer were processed by the original method or one of a variety of conditions including (a) digestion with 10 units PstI-HF for 0, 15, 60, 120 minutes, or overnight (b) PCR1 for 10 or 15 cycles, and (c) digestion with 10 units BamHI-HF and BsoBI for 0, 15, or 60 minutes. Samples were then amplified by PCR2 and sequenced. Optimal results were achieved with 15-cycle PCR1 and any combination of pre-PCR1 (Digest 1) and pre-PCR2 (Digest 2) digestion (n = 1, ND = no digest).

Table S1. Preparation of simulated mixed blood parasite infections

Sample Name	Spiked Analytes	DNA extract of specimen from Table 1*	Volumes of DNA extract mixed	Total volume of final simulated mixture
Mix 1	***P. falciparum***	Specimen 1	4 µL	8 µL
	P. vivax	Specimen 2	4 µL	
Mix 2	***P. ovale***	Specimen 3	4 µL	8 µL
	P. falciparum	Specimen 1	4 µL	
Mix 3	***P. falciparum***	Specimen 1	4 µL	8 µL
	P. malariae	Specimen 4	4 µL	
Mix 4	***P. falciparum***	Specimen 1	4 µL	8 µL
	P. knowlesi	Specimen 5	4 µL	
Mix 5	***P. falciparum***	Specimen 1	2.2 µL	8 µL
	P. vivax	Specimen 2	2.2 µL	
	P. ovale	Specimen 3	2.2 µL	
	P. malariae	Specimen 4	2.2 µL	
	P. knowlesi	Specimen 5	2.2 µL	
Mix 6	***P. falciparum***	Specimen 1	4 µL	8 µL
	T. cruzi	Specimen 10	4 µL	
Mix 7	***P. vivax***	Specimen 2	4 µL	8 µL
	T. cruzi	Specimen 10	4 µL	
Mix 8	***P. falciparum***	Specimen 1	4 µL	8 µL
	T. brucei	Specimen 13	4 µL	
Mix 9	***P. falciparum***	Specimen 1	4 µL	8 µL
	L. loa	Specimen 17	4 µL	
Mix 10	***P. falciparum***	Specimen 1	4 µL	8 µL
	B. malayi	Specimen 16	4 µL	

To produce these simulated mixed infections, DNA extracts from various specimens listed in Table 1 from the main manuscript text* were mixed as described in this table. Prior to testing with UPDx, 3 µL of Qiagen elution buffer was added to all 8 µL of these mixtures (mixes 1 to 10) to bring the final volume to 11 µL. This addition of elution buffer was performed to add extra volume to these mixes (while conserving the original extracts), allowing for sample loss due to evaporation during storage, and to bring the volume of the mix above 8.5 µL. When required for UPDx testing, 8.5 µL of these DNA solutions was processed according to the methods described in the main manuscript text.

Appendix A. Fasta sequences that were aligned to facilitate the design of our 'outer' nested primers

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>HUMRGE - Human 18S rRNA gene, complete
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>AJ439713 - Babesia divergens 18S rRNA gene
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>HQ289870 - Babesia duncani isolate BAB1615 18S ribosomal RNA gene, complete sequence
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>CXZRR18S - Cytauxzoon felis 18S ribosomal RNA
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>GQ332359 - Leishmania infantum 18S ribosomal RNA gene, complete sequence; and internal transcribed
spacer 1, partial sequence
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>AB369994 - Cryptosporidium hominis gene for 18S ribosomal RNA, isolate: Ar108
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>X07773 - Leishmania donovani gene for ribosomal RNA small subunit
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>PFARGEA - P.falciparum 18S ribosomal RNA in asexual parasites
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>PFARRSSU - Plasmodium knowlesi small subunit ribosomal RNA sequence
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>KF696369 - Plasmodium ovale curtisi clone DC-1 18S ribosomal RNA gene, complete sequence
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>AF239980 - Trypanosoma cruzi strain Colombiana 18S ribosomal RNA gene, complete sequence
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>AF227234 - Wuchereria bancrofti 18S small subunit ribosomal RNA gene, partial sequence
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>SMU65657 - Schistosoma mansoni 18S ribosomal RNA gene, complete sequence
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>AY851265 - Trichuris suis 18S ribosomal RNA gene, complete sequence
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>KF250434 - Entamoeba gingivalis strain E 18S ribosomal RNA gene, partial sequence
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>AB426549 - Entamoeba histolytica gene for 18S ribosomal RNA, complete sequence
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>KP722600 - Entamoeba dispar isolate ED_IQ5 18S ribosomal RNA gene, partial sequence
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>Z11976 - S.haematobium gene encoding 18S ribosomal RNA
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>AY618266 - Blastocystis hominis isolate 989 18S ribosomal RNA gene, partial sequence
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>STDRG18S - Strongyloides stercoralis 18S ribosomal RNA gene, complete cds
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>AF222998 - Cryptosporidium parvum 18S ribosomal RNA gene and internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence
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>DQ286403 - Cryptosporidium hominis 18S ribosomal RNA gene, partial sequence
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>TOXRRE - Toxoplasma gondii 18S ribosomal RNA gene, complete sequence
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>FJ236336 - Eimeria maxima clone 158-43 18S ribosomal RNA gene, partial sequence
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>GQ260091 - Taenia solium 18S ribosomal RNA gene, complete sequence
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>JQ609338 - Taenia saginata isolate Dali 18S ribosomal RNA gene, complete sequence
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>GQ260088 - Taenia asiatica 18S ribosomal RNA gene, complete sequence
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>EU344798 - Ancylostoma duodenale 18S ribosomal RNA gene, partial sequence
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>DQ094173 - Loa loa small subunit ribosomal RNA gene, partial sequence
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>AF473852 - Giardia intestinalis small subunit ribosomal RNA gene, complete sequence
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>AY193874 - Hymenolepis nana 18S ribosomal RNA gene, complete sequence
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>KR063346 - Angiostrongylus cantonensis 18S ribosomal RNA gene, partial sequence
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>DQ464371 - Ancylostoma ceylanicum 18S ribosomal RNA gene, partial sequence
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>DQ503458 - Thelazia lacrymalis 18S ribosomal RNA gene, partial sequence
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>JN256973 - Toxocara cati isolate Pb1 18S ribosomal RNA gene, partial sequence
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>JN256977 - Toxocara canis isolate Al2 18S ribosomal RNA gene, partial sequence
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>KM079654 - Ascaris suum isolate PUG-8.2 18S ribosomal RNA gene, partial sequence
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>KM079641 - Ascaris lumbricoides isolate HUG-3.1 18S ribosomal RNA gene, partial sequence
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>Z11590 - S.japonicum small subunit ribosomal RNA gene (partial)
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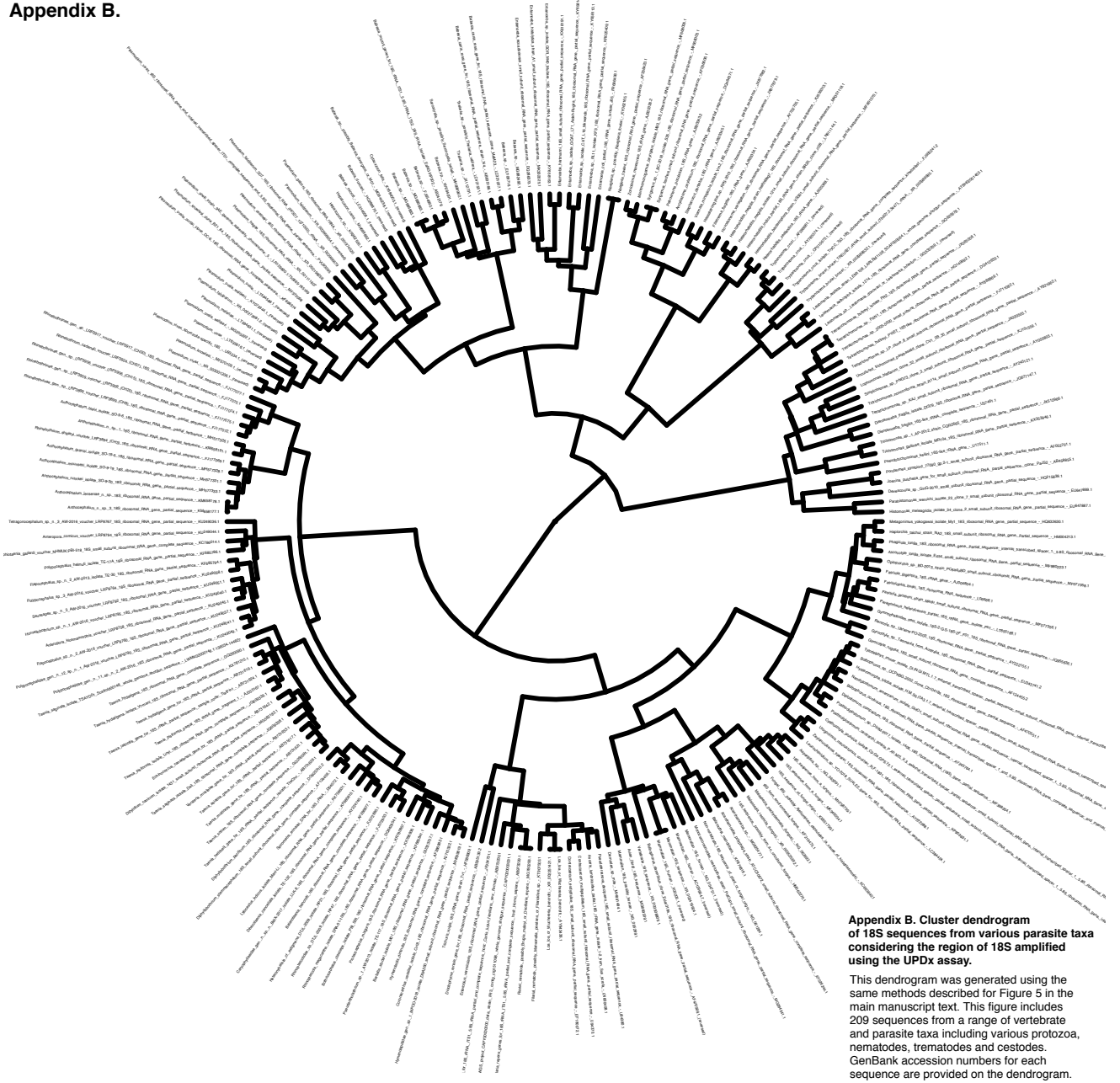
AAGGTATCTGTAGGTGAACCTGCAGATGGATCATCG

Appendix B.



Appendix B. Cluster dendrogram of 18S sequences from various parasite taxa considering the region of 18S amplified using the UPDx assay.

This dendrogram was generated using the same methods described for Figure 5 in the main manuscript text. This figure includes 209 sequences from a range of vertebrate and parasite taxa including various protozoa, nematodes, trematodes and cestodes. GenBank accession numbers for each sequence are provided on the dendrogram.