Expanded host and geographic range of tadpole associations with the Severe Perkinsea Infection group

Vanessa Smilansky[1], Miloslav Jirků[2], David S. Milner[3], Roberto Ibáñez[4,5], Brian Gratwicke[6], Andrew Nicholls[3], Julius Lukeš[2,7], Aurélie Chambouvet[8], Thomas A. Richards[3]

[1]Living Systems Institute and Biosciences, University of Exeter, Exeter, Devon EX4 4QD, UK

[2]Institute of Parasitology, Biology Centre, Czech Academy of Sciences 370 05 České Budějovice, Czech Republic

[3]Department of Zoology, University of Oxford, Oxford OX1 3SZ, UK

[4]Smithsonian Tropical Research Institute, Apartado 0843-03092, Panamá, Republic of Panama

[5]Sistema Nacional de Investigación, SENACYT, Apartado 0816-02852, Panamá, Republic of Panama

[6]Smithsonian National Zoo and Conservation Biology Institute, United States of America

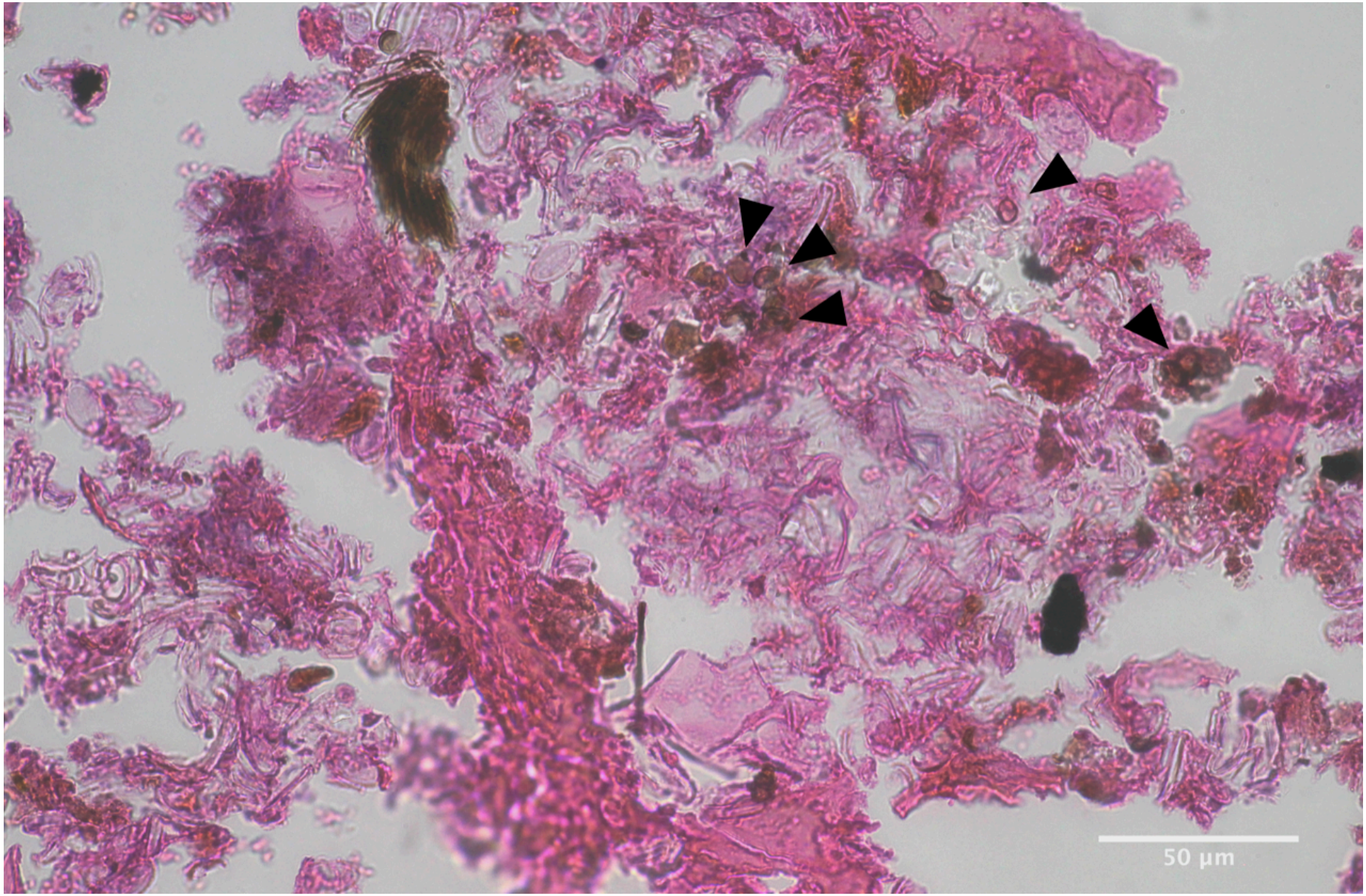
[7]Faculty of Sciences, University of South Bohemia, 370 05 České Budějovice, Czech Republic

[8]CNRS, Univ Brest, IRD, Ifremer, LEMAR, Plouzané, France

Supplementary Materials

Figure S1. Histopathological examination of sectioned tadpole liver tissue taken from a subsample of the UK *Hyla arborea* tadpoles, showing signs of SPI disease, in which we also detected PPC SSU rDNA from parallel liver tissue samples. These samples were preserved in LifeGuard™ preservation solution (Qiagen), kept refrigerated for several days, frozen at -80°C, and then thawed at 4°C before sectioning. As such, these are non-optimal conditions for histopathological examinations. Nonetheless, these micrographs demonstrate several candidate Perkinsea cells (marked with arrow heads) consistent with PPC infections.

Histopathological methodology. Samples were fixed in Davidson's solution for 24h at 4°C (1) and then dehydrated in 70% ethanol for 48h at 4°C. Tissues were dehydrated in ascending ethanol solutions (70%, 80%, 90%, 98% and 100%), cleared with Histoclear (Fisher Scientific), and embedded in paraffin wax. After 4 days of drying, paraffin embedded tissue samples were cut into serial sections of 7 µm (extra thick because of the low tissue quality). Finally, sections were collected onto glass and stained with Harris' hematoxylin and eosin (2). Stained sections were mounted, examined and imaged using an inverted light microscope (VivaTome Zeiss).


50 µm

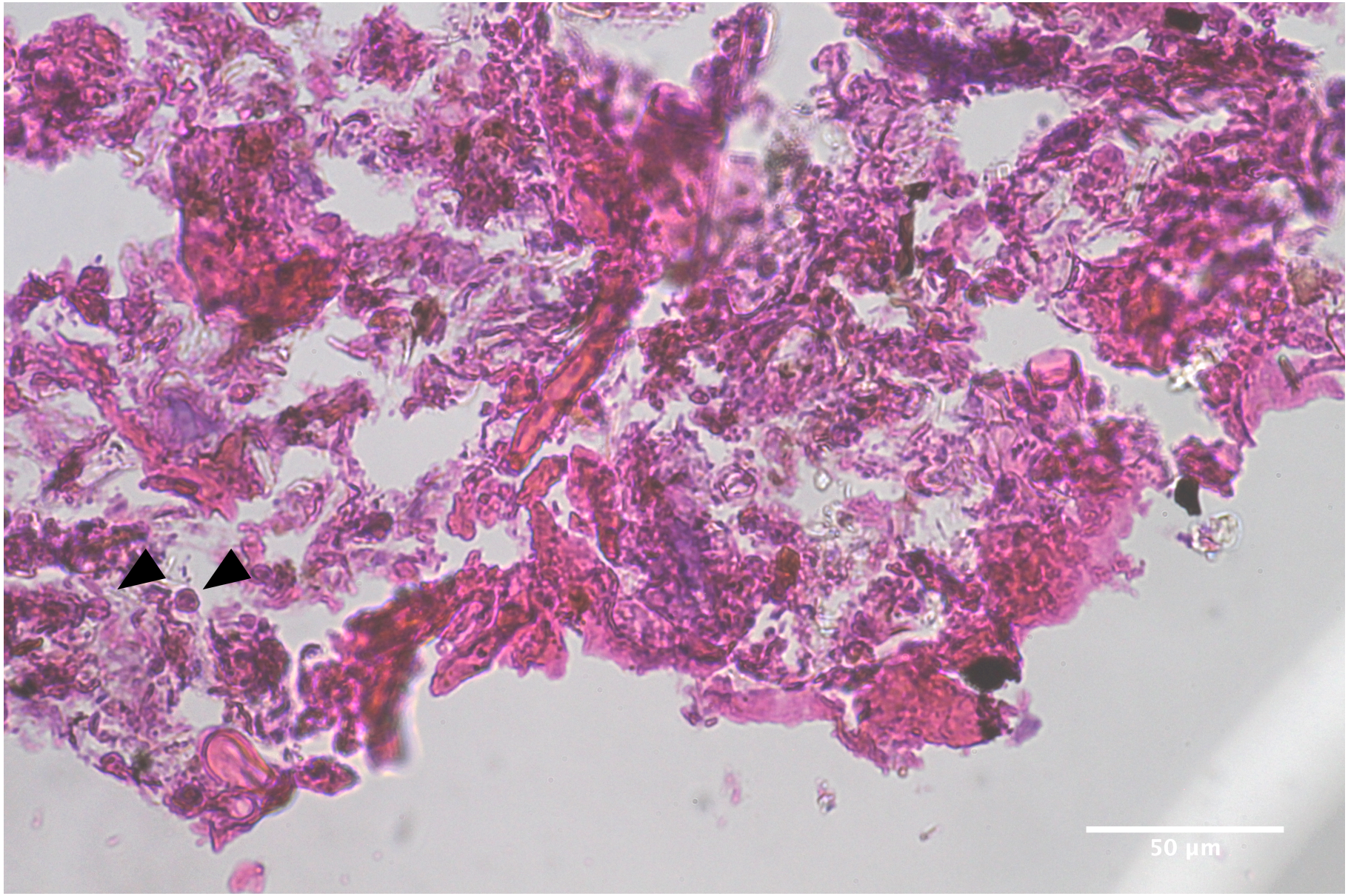

50 µm

Table S1 and Nanopore host taxon sequencing methodology.

We adapted and tested our host sequencing protocol for parallel-MinION™ amplicon sequencing in order to identify tadpole taxonomy for the Panama samples (note that this was not conducted for the UK tadpoles as the taxonomy of these samples was known). We PCR amplified a ~600 bp fragment of the mitochondrial-rDNA using 16Sar-L- and 16Sbr-H-primers (Table S3, Palumbi et al. (1991)). The primers were designed to incorporate 'ID-tags' comprising 12-nts added to the 5' end with a "GC" in between, generating twenty unique identifiers. Each 25 µl reaction mixture included 1x PCR Master Mix (Promega), 500 nM of each primer and 1 µl of template DNA. Each PCR included negative/positive controls and identical cycling conditions to those for the NAG01 screening. PCR products were checked on a 1.5% agarose gel; those that failed to amplify were rerun with 5 µl template DNA.

We then prepared amplicon libraries for MinION™ (Oxford Nanopore) sequencing following the 1D Amplicon by ligation protocol (SQK-LSK108). A total of 69 uniquely-tagged amplicons (~100 ng each) were combined into five pools. These pools were processed for end-repair, then purified with Agencourt AMPure XP beads (Beckman-Coulter) with a ratio of beads to PCR product volume of 0.8. Adapters were ligated to the end-prepped amplicons and re-purified with AMPure XP beads. The libraries were loaded into FLO-MIN106 flow-cells (R9.4.1) and sequenced on a MinION™ Nanopore sequencer using MinKNOW-software.

Reads were demultiplexed based on their ID-tags using MiniBar (version-0.21), allowing for two mismatches between the tags. Low quality reads were filtered via NanoFilt (version-2.5) (3) using the script filter.sh. Reads for the remaining samples were subsampled to 150x (where applicable) and then assembled into contigs (consensus sequences) using Canu (version 1.8) (4) using the script canu.sh. "MC" indicates that the assembly yielded multiple contigs from multiple species, indicating possible contamination, and these samples were excluded from phylogenetic analysis. For samples that failed to assemble into contigs, the demultiplexed, filtered reads were examined. "SE" indicates that these reads contained high rates of sequencing errors in conserved regions. A search of these reads against the NCBI 'nr' database, as well as

their partial alignments (not including the error regions), indicated they were redundant sampling covered by other tadpoles, and they were excluded from phylogenetic analysis.

Only one of the six samples excluded at this stage (marked "SC") was PCR-positive for Perkinsea DNA (i.e. PA_T135). For this sample, the PCR was repeated and the product sequenced using Sanger sequencing methods following the same methods outlined for the NAG01 sequences described in the Materials and Methods section. This sequence was added to the sequence alignment for phylogenetic analysis.

To ensure that an adequate amount of starting material was used for the extractions, the 10 UK liver-tissue-samples were pooled into two groups of five, and 15 of the 81 Panama samples were pooled into three groups of five (the remaining 66 Panama samples were processed as single livers). Panama samples were pooled according to the external morphology of the tadpoles and their collection site (i.e. they shared the same morphology and came from the same site); note that none of the pooled liver-samples contained contigs from multiple species, indicating that the pooling conducted based on morphology did not result in mixed-species pools. Pooling is detailed in the table below.

Table Information. Specimen data for all tadpoles sampled in this study and the corresponding NCBI accession number of the tadpole mitochondrial 16S rDNA sequence, when recovered. Samples highlighted in grey are positive for PCR detection of NAG01 Perkinsea. "Species affiliation" was identified by the BLASTn (Genbank 'nr' database) hit with the highest percentage identity. For some samples, a BLASTn search could either not be completed or could not be relied upon for positive identification (i.e. "NR", "MC", and "SE" in the table). "NR" indicates that no reads were generated.

Sample ID	Single or pooled	Country	Region	Site	GPS	Collection date	Species affiliation (updated taxonomies)	Family	Accession Number
PA_T091	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Leptodactylus savagei*	Leptodactylidae	MW136603
PA_T092	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Leptodactylus savagei*	Leptodactylidae	MW136604

PA_T093	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136605
PA_T094	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136606
PA_T095	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136607
PA_T096	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136608
PA_T097	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136609
PA_T098	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136610
PA_T099	Pooled (x5)	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Boana rosenbergi*	Hylidae	MW136611
PA_T105	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Engystomops pustulosus*	Leptodactylidae	MW136612
PA_T106	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Engystomops pustulosus*	Leptodactylidae	MW136613
PA_T107	Single	PA	Soberania	SNP6	9.071012, -79.656412	17/11/2018	*Engystomops pustulosus*	Leptodactylidae	MW136614
PA_T109	Single	PA	Altos de Campana	AC1	8.681981, -79.928944	20/11/2018	*Silverstoneia flotator*	Leptodactylidae	MW136615
PA_T115	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Dendropsophus ebraccatus*	Hylidae	MW136616
PA_T116	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Dendropsophus ebraccatus*	Hylidae	MW136617
PA_T117	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Dendropsophus ebraccatus*	Hylidae	MW136618
PA_T118	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	NR	NA	NA
PA_T119	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Dendropsophus ebraccatus*	Hylidae	MW136619
PA_T120	Pooled (x5)	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Dendropsophus ebraccatus*	Hylidae	MW136620
PA_T121	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Agalychnis callidryas*	Phyllomedusidae	MW136621
PA_T122	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Allobates talamancae*	Aromobatidae	MW136622
PA_T123	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Rhinella horribilis*	Bufonidae	MW136623
PA_T124	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	SE	NA	NA
PA_T125	Single	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	MC	NA	NA
PA_T126	Pooled (x5)	PA	Altos de Campana	AC2	8.677743, -79.928435	20/11/2018	*Agalychnis callidryas*	Phyllomedusidae	MW136624
PA_T128	Single	PA	El Valle de Anton	EVA3	8.59757, -80.11545	22/11/2018	*Rhinella horribilis*	Bufonidae	MW136625
PA_T129	Single	PA	El Valle de Anton	EVA3	8.59757, -80.11545	22/11/2018	*Rhinella horribilis*	Bufonidae	MW136626
PA_T130	Single	PA	El Valle de Anton	EVA3	8.59757, -80.11545	22/11/2018	NR	NA	NA
PA_T131	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136627
PA_T132	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136628
PA_T133	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136629
PA_T134	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136630

PA_T135	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	SC	NA	MW136631
PA_T136	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136632
PA_T137	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	NR	NA	NA
PA_T138	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136633
PA_T139	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136634
PA_T140	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Allobates talamancae*	Aromobatidae	MW136635
PA_T141	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Rhinella horribilis*	Bufonidae	MW136636
PA_T142	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Smilsca sila*	Hylidae	MW136637
PA_T143	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Boana rosenbergi*	Hylidae	MW136638
PA_T144	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	SE	NA	NA
PA_T145	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	NR	NA	NA
PA_T146	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	MC	NA	NA
PA_T147	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Rhinella horribilis*	Bufonidae	MW136639
PA_T148	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Rhinella horribilis*	Bufonidae	MW136640
PA_T149	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	SE	NA	NA
PA_T150	Single	PA	El Valle de Anton	EVA4	8.601778, -80.115663	22/11/2018	*Rhinella horribilis*	Bufonidae	MW136641
PA_T151	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Smilisca sila*	Hylidae	MW136642
PA_T152	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	NR	NA	NA
PA_T153	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Smilisca sila*	Hylidae	MW136643
PA_T154	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	NR	NA	NA
PA_T155	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Smilisca sila*	Hylidae	MW136644
PA_T156	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	SE	NA	NA
PA_T157	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	SE	NA	NA
PA_T158	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Smilisca sila*	Hylidae	MW136645
PA_T159	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Smilisca sila*	Hylidae	MW136646
PA_T160	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Smilisca sila*	Hylidae	MW136647
PA_T161	Single	PA	Soberania	SNP7	9.07812, -79.65118	24/11/2018	*Boana rosenbergi*	Hylidae	MW136648
PA_T162	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136649
PA_T163	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136650
PA_T164	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136651

PA_T165	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136652
PA_T166	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	NR	NA	NA
PA_T167	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136653
PA_T168	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136654
PA_T169	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136655
PA_T170	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136656
PA_T171	Single	PA	Soberania	SNP8	9.09055, -79.61837	24/11/2018	*Rhinella alata*	Bufonidae	MW136657
UK_HA01-5	Pooled (x5)	UK	Surrey	AME1		July 2019	*Hyla arborea*	Hylidae	NA
UK_HA06-10	Pooled (x5)	UK	Surrey	AME1		July 2019	*Hyla arborea*	Hylidae	NA

Table S2. Description of the regions/sites in Panama sampled in this study, including region-based reports of *Batrachochytrium dendrobatidis* (Bd), the infectious agent of amphibian chytridiomycosis.

Region	**Site**	**GPS**	**Water body**	**Human disturbance**	**Regional Bd detections**
Altos de Campana	AC1	8.681981, -79.928944	Stream	No	2006 (2), 2012 (3, 4)
Altos de Campana	AC2	8.677743, -79.928435	Pond	No	2006 (2), 2012 (3, 4)
El Valle de Anton	EVA3	8.59757, -80.11545	Roadside puddle	Yes	2006 (5), 2012 (4), 2014 (6)
El Valle de Anton	EVA4	8.601778, -80.115663	Pond	No	2006 (5), 2012 (4), 2014 (6)
Soberania	SNP6	9.071012, -79.656412	Roadside puddle	Yes	2007 (2), 2012 (3)
Soberania	SNP7	9.07812, -79.65118	Stream	No	2007 (2), 2012 (3)
Soberania	SNP8	9.09055, -79.61837	Stream	No	2007, 2012 (3)

Table S3. Primers used in this study. Full references provided in the main manuscript.

Primer	Sequence (5' -3')	Specificity	Reference
300F-B	GGG CTT CAY AGT CTT GCA AT	NAG01 Perkinsea	Chambouvet et al. (2015)
NAG01R_1	GCC TGC TTG AAA CRC TCT AA	NAG01 Perkinsea	This study
16Sar-L	CGC CTG TTT ATC AAA ACA T	vertebrates	Palumbi et al. (1991)
16Sbr-H	CCG GTC TGA ACT CAG ATC ACG T	vertebrates	Palumbi et al. (1991)

Table S4. List of Perkinsea SSU-rDNA sequences generated in this study from PPC-positive tadpoles, and their corresponding NCBI accession number.

Sample ID	Sequence/clone	PCR 1	PCR 2	PCR 3	Accession Number
PA_T093	PA_T093_1.1	X			MW136531
	PA_T093_1.2	X			MW136532
	PA_T093_1.3	X			MW136533
PA_T096	PA_T096_1.1	X			MW136534
	PA_T096_1.2	X			MW136535
PA_T097	PA_T097_1.1	X			MW136536
	PA_T097_1.2	X			MW136537
	PA_T097_1.3	X			MW136538
PA_T119	PA_T119_1.1	X			MW136539
	PA_T119_1.2	X			MW136540
PA_T128	PA_T128_1.1	X			MW136541
PA_T135	PA_T135_1.1_T3	X			MW136542
	PA_T135_1.2	X			MW136543
	PA_T135_1.3	X			MW136544
	PA_T135_2.1		X		MW136545
PA_T136	PA_T136_1.1	X			MW136546
	PA_T136_1.2	X			MW136547
	PA_T136_1.3	X			MW136548
PA_T139	PA_T139_1.1	X			MW136549
	PA_T139_1.2_T3	X			MW136550
	PA_T139_1.3	X			MW136551
	PA_T139_2.1		X		MW136552
	PA_T139_2.2		X		MW136553
	PA_T139_2.3		X		MW136554
	PA_T139_2.4		X		MW136555
	PA_T139_2.5_T3		X		MW136556
	PA_T139_2.6		X		MW136557
	PA_T139_2.7		X		MW136558
	PA_T139_2.8		X		MW136559
	PA_T139_2.9		X		MW136560
	PA_T139_3.1			X	MW136561
	PA_T139_3.2			X	MW136562
	PA_T139_3.3			X	MW136563
	PA_T139_3.4			X	MW136564
	PA_T139_3.5			X	MW136565
	PA_T139_3.6			X	MW136566
	PA_T139_3.7			X	MW136567
PA_T140	PA_T140_1.1	X			MW136568
	PA_T140_1.2	X			MW136569
	PA_T140_1.3	X			MW136570
PA_T169	PA_T169_1.1	X			MW136571

UK_HA01-5	UK_HA01-5_54_1.1	X			MW136572
	UK_HA01-5_54_1.2	X			MW136573
	UK_HA01-5_54_1.3	X			MW136574
	UK_HA01-5_54_1.4	X			MW136575
	UK_HA01-5_54_1.5	X			MW136576
	UK_HA01-5_54_1.6	X			MW136577
	UK_HA01-5_54_1.7	X			MW136578
	UK_HA01-5_54_1.8	X			MW136579
	UK_HA01-5_54_1.9	X			MW136580
	UK_HA01-5_54_1.10	X			MW136581
	UK_HA01-5_54_2.1		X		MW136582
	UK_HA01-5_54_2.2		X		MW136583
	UK_HA01-5_54_2.3		X		MW136584
	UK_HA01-5_54_2.4		X		MW136585
	UK_HA01-5_54_2.5		X		MW136586
	UK_HA01-5_54_2.6		X		MW136587
	UK_HA01-5_54_2.7		X		MW136588
	UK_HA01-5_54_2.8		X		MW136589
	UK_HA01-5_54_2.9		X		MW136590
	UK_HA01-5_54_2.10		X		MW136591
	UK_HA01-5_54_2.11		X		MW136592
	UK_HA01-5_54_3.1			X	MW136593
	UK_HA01-5_54_3.2			X	MW136594
	UK_HA01-5_54_3.3			X	MW136595
	UK_HA01-5_54_3.4			X	MW136596
	UK_HA01-5_54_3.5			X	MW136597
	UK_HA01-5_56_1.1	X			MW136598
	UK_HA01-5_56_1.2	X			MW136599
	UK_HA01-5_56_2.1		X		MW136600
	UK_HA01-5_56_3.1			X	MW136601
	UK_HA01-5_56_3.2			X	MW136602

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