DNA barcoding of *Cryptosporidium*

JAN ŠLAPETA*

Sydney School of Veterinary Science, Faculty of Science, University of Sydney, Sydney, New South Wales, Australia

(*Received 13 April 2017; revised 25 August 2017; accepted 12 September 2017; first published online 8 November 2017*)

SUMMARY

Cryptosporidium spp. (Apicomplexa) causing cryptosporidiosis are of medical and veterinary significance. The genus *Cryptosporidium* has benefited from the application of what is considered a DNA-barcoding approach, even before the term 'DNA barcoding' was formally coined. Here, the objective to define the DNA barcode diversity of *Cryptosporidium* infecting mammals is reviewed and considered to be accomplished. Within the *Cryptosporidium* literature, the distinction between DNA barcoding and DNA taxonomy is indistinct. DNA barcoding and DNA taxonomy are examined using the latest additions to the growing spectrum of named *Cryptosporidium* species and within-species and between-species identity is revisited. Ease and availability of whole-genome DNA sequencing of the relatively small *Cryptosporidium* genome offer an initial perspective on the intra-host diversity. The opportunity emerges to apply a metagenomic approach to purified field/clinical *Cryptosporidum* isolates. The outstanding question remains a reliable definition of *Cryptosporidium* phenotype. The complementary experimental infections and metagenome approach will need to be applied simultaneously to address *Cryptosporidium* phenotype with carefully chosen clinical evaluations enabling identification of virulence factors.

Key words: DNA barcoding, DNA taxonomy, systematics, *Cryptosporidium*, Apicomplexa, public health.

CRYPTOSPORIDIUM AND CRYPTOSPORIDIOSIS

Cryptosporidium spp. causing cryptosporidiosis are of medical and veterinary significance because the parasite leads to diarrhoeal disease for which no treatments or vaccines exist (Fayer, 2008; Checkley *et al*. 2015). A study involving 22 500 children from Africa and Asia revealed that the protozoan parasite *Cryptosporidium* is one of four major pathogens causing diarrhoea in infants and toddlers (Kotloff *et al*. 2013). Of the four, rotavirus, *Cryptosporidium*, *Shigella* bacteria and enterotoxigenic *Escherichia coli,* only *Cryptosporidium* has no effective drug or vaccine (Checkley *et al*. 2015). In animals, *Cryptosporidium* spp. are highly prevalent, with close to 100% in young calves, for example (Santín *et al*. 2008; Santín, 2013).

Cryptosporidium species infect almost all vertebrate species (Santín, 2013). The genus has over 30 species formally described, with many potentially awaiting formal descriptions (Šlapeta, 2013). The majority of cases of human cryptosporidiosis are caused either by *Cryptosporidium hominis* or *Cryptosporidium parvum* 'bovine genotype' [=*C. pestis*]. Identification can be difficult because (i) species of *Cryptosporidium* vary in their host specificity and (ii) the morphological characteristics of the excreted oocysts are insufficient for species identification. Historically, two morphologically distinct parasites of the gastrointestinal tract were originally described by Ernest E. Tyzzer from mice (*Cryptosporidium muris* and *C. parvum*) (Tyzzer, 1910; Tyzzer, 1912; Šlapeta, 2009). Later, these two parasites were assumed to infect all mammals, including cattle (O'Donoghue, 1985; Upton and Current, 1985). The default species affecting the mammalian intestine became *C. parvum* and for the mammalian stomach, *C. muris* (Upton and Current, 1985). However, such a scheme became untenable and it was suggested that each of the intestinal and gastric groups are assemblages of many different species that are virtually indistinguishable using oocyst characteristics (Morgan *et al*. 1997; Peng *et al*. 1997; Xiao *et al*. 1999*b*).

In this review, I aim to show that the advances in species identification for the genus *Cryptosporidium* have benefited from the application of what is considered a DNA-barcoding approach, even before the term 'DNA barcoding' was formally coined. I will outline how both DNA barcoding and DNA taxonomy for *Cryptosporidum* are currently applied and demonstrate the limitations of using such an approach by concentrating on recent examples from the literature. I will conclude with the prospects of using DNA approaches in species identification for *Cryptosporidium* in formal taxonomical works. I argue that the objective to define the DNA barcode diversity of *Cryptosporidium* has been accomplished.

DNA-BARCODING WORKFLOW FOR *CRYPTOSPORIDIUM*

DNA barcoding is an approach to species identification that uses the statistical probability of nucleotide sequence homology, enabled by the relative ease of

* Corresponding author: Sydney School of Veterinary Science, Faculty of Science, University of Sydney, Sydney, New South Wales, Australia. E-mail: jan.slapeta@sydney.edu.au

Parasitology (2018), **145**, 574–584.
doi:10.1017/S0031182017001809

DNA isolation, PCR amplification and DNA sequencing. The approach starts with the selection of voucher specimens of formal taxa within a particular studied group of organisms, such as the genus *Cryptosporidium*, followed by DNA sequencing of a selected marker gene(s). DNA barcoding is viewed as a supporting tool of formal taxonomy, not a replacement (Hebert *et al*. 2003; Hajibabaei *et al*. 2007; Hansen *et al*. 2007; Ondrejicka *et al*. 2014). The final phase of the approach for established taxa is an evaluation of the 'genetic distance' of the selected marker sequence for the given species and the genetic distance that separates species from each other. These criteria are termed, respectively, the 'within'- and 'between'-species distance(s). Once formal taxa are characterized by DNA sequences or DNA barcode(s) and the within- and between-species distance parameters are known, can a set of unknown specimens be DNA barcoded and identified, if they match the existing DNA barcode criteria associated with these formal species. If their DNA barcode(s) do not match any of the DNA barcodes, but are positioned within the range expected for within-species DNA distance, they can be considered a new genotype or a variant of that existing species. If the DNA barcode does not match the sequences of formal taxa, and falls outside the expected within-species DNA distance of any known species, this may suggest the existence of a novel species (Besansky *et al*. 2003; Siddall *et al*. 2012).

DNA-barcoding projects are built around formally identified specimens, hence Linnaean taxonomy (Fig. 1) (Hebert *et al*. 2003; Hajibabaei *et al*. 2007; Hansen *et al*. 2007; Ondrejicka *et al*. 2014). DNA barcodes or DNA sequences are generated and evaluated on whether they are within and between the congeneric Linnaean species, which fall into a quadrant that delineates well-defined species (Fig. 1, inset). Such an approach tests whether the DNA locus chosen for the barcoding successfully resolves the formal Linnaean taxa. Assuming the marker is suitable, Scenario 1 (Fig. 1) depicts a situation that all formal Linnaean taxa represent well-defined species using the DNA barcode marker. On the other hand, Scenario 2 (Fig. 1) shows a situation where only one taxon is well defined (top left quadrant; Fig. 1, inset); while the other taxa within the remaining three quadrants are considered not well defined. It has been argued that plotting the minimum between congeneric taxon DNA marker distances against maximum within taxon DNA marker distances enables observers to identify composite or cryptic species (top right quadrant) (Hebert *et al*. 2003). In addition, low minimum between taxa distance suggests either recently emerging species (bottom left quadrant) or probable specimen misidentification, particularly if the maximum within taxon distance is exceeded (bottom right quadrant). The fundamental question is: where to draw these thresholds in order to split the graph into the four quadrants? Ultimately, the threshold lies where the expert consensus opinion of those studying the group will consider biologically appropriate. For many parasites, such threshold is highly subjective and is epitomized in the discussion of 'lumpers and splitters' within the formal Linnaean taxonomy.

SUPERIORITY OF USING THE DNA APPROACH IN IDENTIFICATION OF *CRYPTOSPORIDIUM*

For the entire *Cryptosporidium* genus, neither formal Linnaean taxonomy nor expected within- and between-species distances were agreed upon or known. In fact, those studying *Cryptosporidium* have looked to DNA sequencing to resolve suspected controversies (Carraway *et al*. 1996; Peng *et al*. 1997; Xiao *et al*. 1999a, b, 2000).

Early studies into *Cryptosporidium* were loosely applying a DNA-barcoding approach; the taxonomy, however, has always been in a state of flux, because of limited morphological or/and biological distinctness (Fig. 2). In the mid/late-1990s an initial consensus had been reached that *C. muris* and *C. parvum* are collective species of all specimens located in the stomach or intestine of mammals, respectively, and that *C. baileyi* parasitizes birds, and *C. serpentis* parasitizes reptiles. A study that first brought consistent DNA sequences of *C. muris*, *C. parvum*, *C. baileyi* and *C. serpentis* for SSU rDNA presented what could be considered DNA barcodes (Fig. 2) (Xiao *et al*. 1999a, b). Generation of DNA sequences for some *Cryptosporidium* isolates and strains was welcomed, because it provided presumably unambiguous and easy to interpret patterns previously suggested by those that were detecting the parasites in animal feces and for medical or veterinary professionals dealing with the clinical condition (Carraway *et al*. 1996; Peng *et al*. 1997). Once the SSU rDNA barcodes were available, it enabled confirmation of two suspected distinct transmission routes for what was then thought to be a single species called *C. parvum* (Fig. 2).

The early data immediately called into question the formal Linnaean taxonomy (specifically *C. parvum*, see Fig. 2) and within- and between-species DNA distances. Leaving aside the taxonomy, the within- and between-species distances for *Cryptosporidum* presented a challenge, because the two distinct transmission cycles for human cryptosporidiosis were caused by what appeared to be two distinct *Cryptosporidium* DNA barcodes. The presence of two distinct DNA barcodes, for what was then called *C. parvum*, established what could be an acceptable within-species distance. Later, they were re-considered to be sufficient to

https://doi.org/10.1017/S0031182017001809 Published online by Cambridge University Press

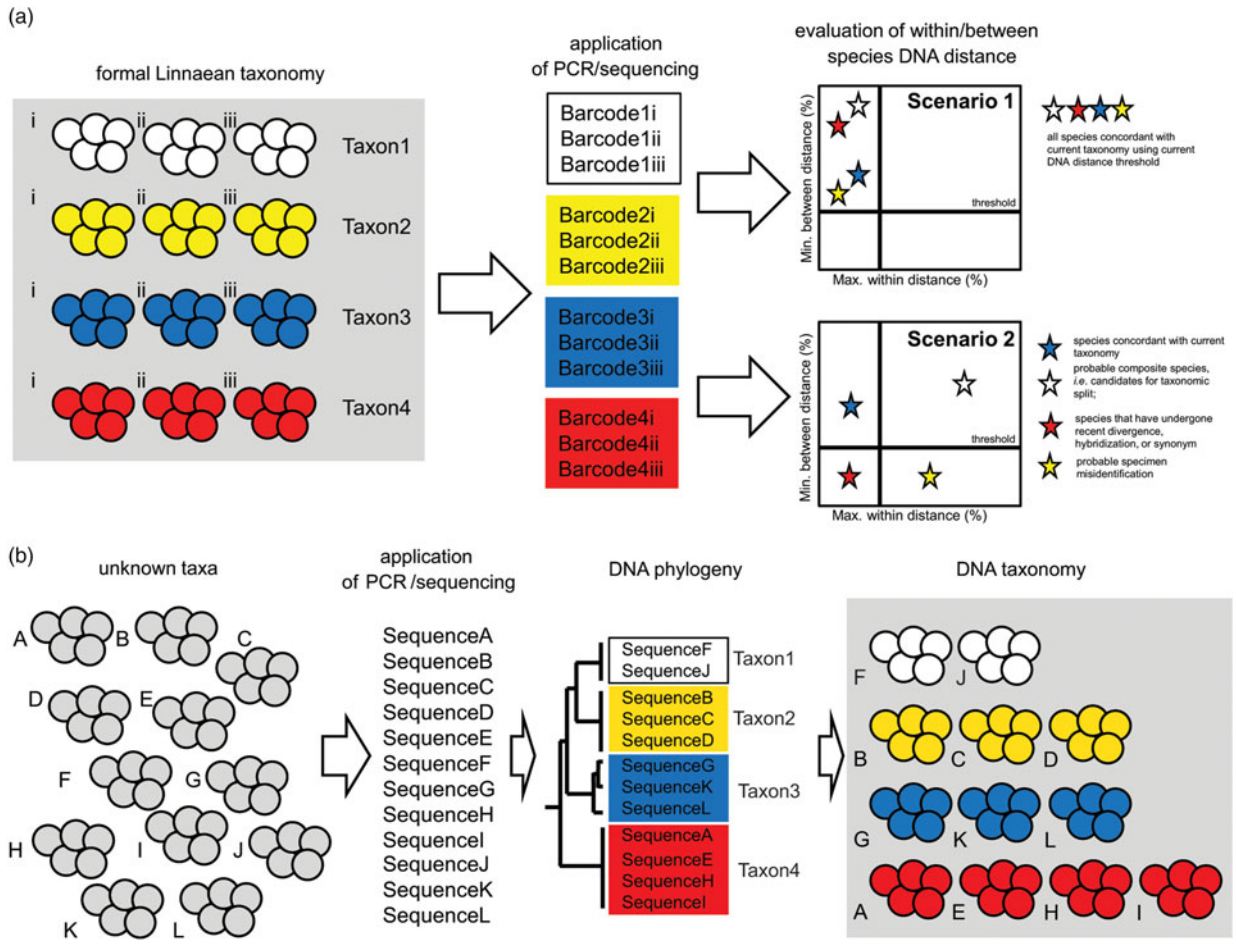

Fig. 1. Differences between the DNA barcoding and DNA taxonomy workflows for *Cryptosporidium*. (a) DNA barcoding starts with taxonomically well-defined specimens/vouchers for which DNA barcodes are generated and within and between distances are evaluated for congruence with formal Linnaean taxonomy. For isolates assigned to a known species/taxon (groups of coloured oocysts), DNA barcodes are generated (i.e. for Taxon 1 there are Barcode1i-iii) followed by plotting maximum within Taxon1 genetic distance against minimum distance from all the other DNA barcodes from either Taxon2 or Taxon3 (between taxa distance). See the text for explanation of Scenario 1 and Scenario 2. Ideally the DNA barcodes are concordant with the existing taxonomy (Scenario 1). DNA barcoding, however, enables discovery of cryptic or misdiagnosed species (Scenario 2). (b) DNA taxonomy starts with unknown *Cryptosporidium* samples of which we generate DNA sequences that are then processed *via* phylogenetic workflow and *Cryptosporidium* species retrospectively assigned. On the left, groups of oocysts (grey circles) represent individual isolates. These isolates are of unknown taxonomical identity (grey). For an unknown species (taxa), DNA sequences are generated and phylogeny inferred and only then species (taxon) assigned.

establish a new formal taxon *C. hominis* (Morgan-Ryan *et al*. 2002).

The most studied host for *Cryptosporidium* species is human and the human *Cryptosporidium* spp. isolates were to define the taxonomy of the genus. The SSU rDNA of *C. parvum* 'bovine' genotype is 99·49% identical with *C. parvum* 'human' genotype across the alignment spanning 1753 nucleotide residues (Figs. 2 and 3) (Morgan-Ryan *et al*. 2002; Xiao *et al*. 2002). Elevating *C. parvum* 'human' genotype to a species status (=*C. hominis*) meant that the between genotype distance, became between-species distance. Moreover, no genetic distance within what was called *C. parvum* 'human' genotype at SSU rDNA meant that the newly established *C. hominis* had no within-species variation at SSU rDNA.

The precedent with *C. hominis* opened a new '*can of worms*'. While epidemiology has prompted the recognition of the species, it was the DNA sequences that provided the heaviest weight in the species description. The *Cryptosporidium* field entered the phase of DNA taxonomy. The availability of molecular methods to sequence *Cryptosporidium* marker genes from animal samples, as well as environmental samples, led to hundreds of publications determining the extent of diversity (Xiao *et al*. 1999a; Sulaiman *et al*. 2000); hence what could informally be called the *Cryptosporidium* diversity initiative. Over the past 15 years, we have seen new *Cryptosporidium* species molecularly characterized and formally described (Xiao *et al*. 2004; Šlapeta, 2009, 2013; Fayer, 2010). The issues that remain to be resolved are, 'are there noticeable within-

https://doi.org/10.1017/S0031182017001809 Published online by Cambridge University Press

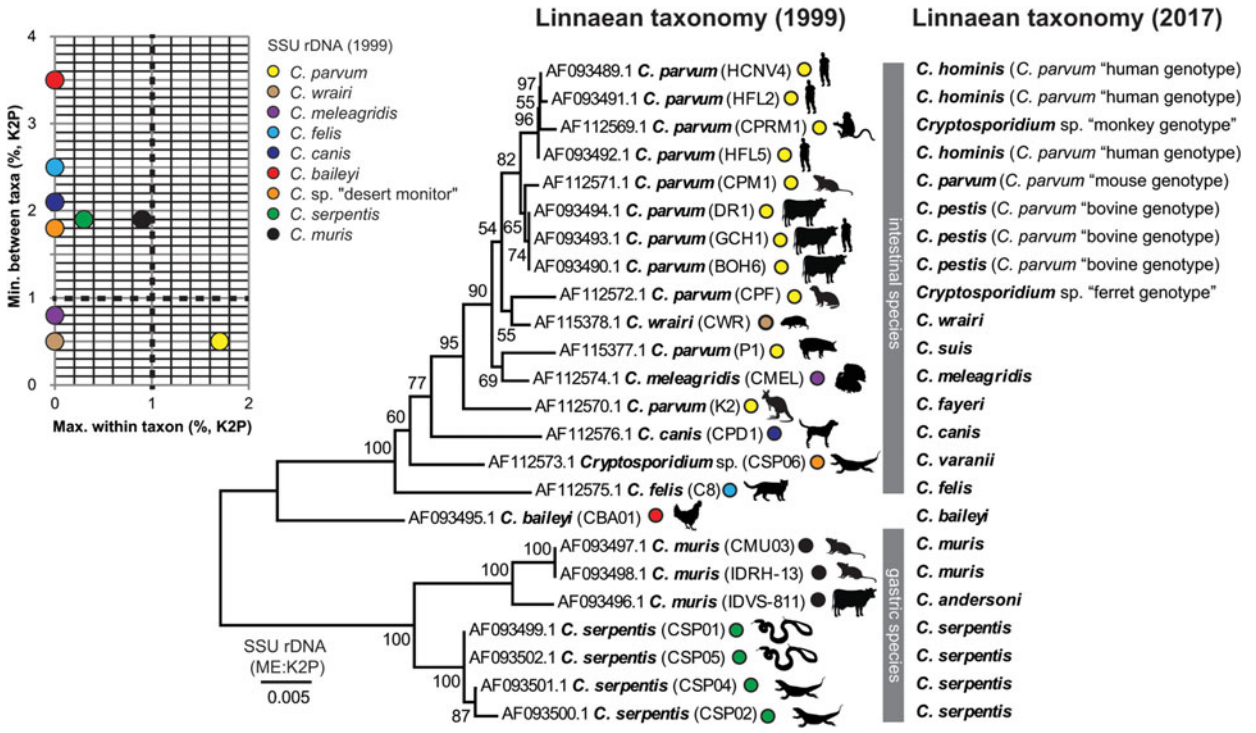

Fig. 2. *Cryptosporidium* DNA barcoding in practice. The first consistent DNA sequences of *C. muris*, *C. parvum*, *C. baileyi* and *C. serpentis* for SSU rDNA presented what could be considered DNA barcodes (Xiao *et al*. 1999a, b). DNA sequences for some *Cryptosporidium* provided presumably unambiguous detection of the parasites in animal and human feces. Between and within taxon (inset) DNA distance demonstrated what was considered *C. parvum* needed a thorough re-evaluation. The principal host is plotted next to the name and both the 1999 taxonomy as well as the current 2017 version is provided. The phylogenetic tree is based on Clustal aligned SSU rDNA sequences. The tree was inferred using the Minimum Evolution method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The pairwise genetic distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

species distances, or do the SSU rDNA barcodes all represent un-described novel species?'

CRYPTOSPORIDIUM SPECIES IN THE AGE OF DNA TAXONOMY

The DNA taxonomy approach argues that the superior data used for taxonomic decisions is DNA sequences and proponents argue that DNA sequencing should be utilized, rather than traditional morphology or biology (Fig. 1). The approach applies a universal system based solely on reference DNA, therefore species are simply defined by sequence identity (Hajibabaei *et al*. 2007; Ondrejicka *et al*. 2014).

There are 35 named species of *Cryptosporidium* that can be considered valid with valid names according to the International Code of Zoological Nomenclature (Table 1, Fig. 3). Only two are lacking a DNA barcode, both representing *Cryptosporidium* species originally described from fish (*C. nasoris*, *C. scophthalmi*). Because of the absence of comparative material, the Roman numeral system was suggested to address the potential unstable taxonomy (Šlapeta, 2013). The Roman numeral system is only introduced for the named species of *Cryptosporidium* and together with the accession numbers in primary DNA databases (GenBank: www.ncbi.nlm.nih.gov/genbank, EMBL: www.ebi.ac.uk/ena and DDBJ: www.ddbj.nig.ac.jp) serve as a straightforward DNA reference system, therefore a reliable barcoding platform for species identification (Table 1) (Šlapeta, 2017). The SSU rDNA reference alignment including all 33 DNA barcodes is publically available at http://dx.doi.org/10.6070/H43T9F9G.

DNA BARCODING *VS* DNA TAXONOMY: EXAMPLES FROM THE LATEST ADDITIONS TO THE SPECTRUM OF *CRYPTOSPORIDIUM* SPECIES

Within the *Cryptosporidium* literature the distinction between DNA barcoding and DNA taxonomy is unclear (Fig. 1). To illustrate the mixing of DNA barcoding and DNA taxonomy concepts I will examine the latest additions to the growing spectrum of *Cryptosporidium* named species. I will revisit the within-species and between species-identity to reflect on the taxonomy issues using the five recently (re-)described *Cryptosporidium* species – *C. proliferans* (Species XXXIV), *C. testudinis* (Species

https://doi.org/10.1017/S0031182017001809 Published online by Cambridge University Press

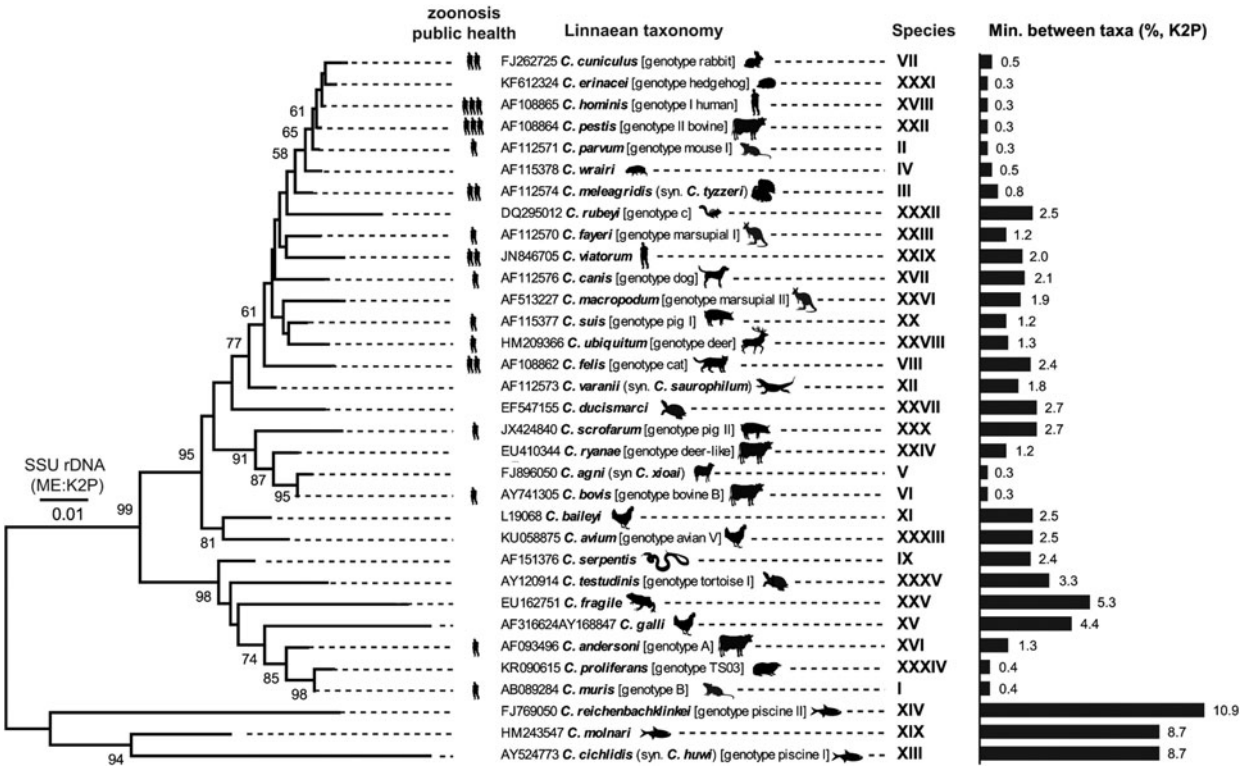

Fig. 3. Phylogeny of 33 named *Cryptosporidium* species using reference dataset of SSU rDNA sequences. The tree was inferred using the Minimum Evolution method (bootstrap test with 1000 replicates). All ambiguous positions were removed for each sequence pair. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter (K2P) method and are in the units of the number of base substitutions per site. A minimum distance (%) to the closest *Cryptosporidium* species is indicated by a bar plot on the right (between taxa SSU rDNA genetic distance). GenBank accession number accompanies all species names, as well as public health significance. There is no SSU rDNA sequence available for *C. nasoris* (Species X) and *C. scophthalmi* (Species XXI). The alignment is publically available at http://dx.doi.org/10.6070/H43T9F9G.

XXXV), *C. avium* (Species XXXIII), *C. rubeyi* (Species XXXII) and *C. huwi* (=*C. cichlidis* Species XIII) (Table 1).

The recently described *C. proliferans* (Species XXXIV, Table 1) belongs to a gastric invading species (Fig. 3) (Kváč *et al*. 2016). The description is based on thorough comparative work with morphological, histopathological and experimental studies. Authors argue for morphological distinctness from its closest sister species *C. muris* (Species I, Table 1) (Kváč *et al*. 2016). The only meaningful comparative morphology applied in the genus *Cryptosporidium* are oocysts' size and shape, therefore, authors argue for difference between *C. proliferans* strain TS03 oocysts (6·8–8·8 × 4·8–6·2 μm) and *C. muris* strain HZ206 oocysts (6·3–8·1 × 5·0–6·6 μm) (Kváč *et al*. 2016). Despite the thorough biological data delivered, authors applied the concept of DNA taxonomy stating that their, and previous, genetic work has demonstrated the presence of a new species. The DNA sequences originally obtained for *C. proliferans* coupled with minute 0·4% genetic distances from *C. muris* at SSU rDNA, initiated their thorough descriptive and taxonomical work (Kváč *et al*. 2016). The biology of *C. proliferans* is defined by a single laboratory maintained isolate; however, it remains to be tested if the claimed biological differences are shared by other isolates of the same species that are now defined by the DNA barcode.

The latest addition to the *Cryptosporidium* taxa is *C. testudinis* (Species XXXV, Table 1) (Ježková *et al*. 2016). Its identity was first revealed in a study from 2002 that demonstrated large numbers of undefined DNA barcodes/sequences (including SSU rDNA genotypes) (Xiao *et al*. 2002). The 2002 study could be considered a DNA-barcoding study, because the authors aimed to match generated DNA barcodes with those of known taxa (Xiao *et al*. 1999*a*; b). Unlike the above *C. proliferans*, *C. testudinis* is clearly delineated by 3·3% DNA distance at SSU rDNA from its closest *Cryptosporidium* species (Fig. 3). In the description, the valuable DNA-barcoding information is that *C. testudinis* from eight different species of tortoises was essentially identical at SSU rDNA (99·8% identity; 0·2% within-species identity) (Ježková *et al*. 2016). As described above for *C. proliferans*, authors reported

https://doi.org/10.1017/S0031182017001809 Published online by Cambridge University Press

Table 1. Current taxonomic summary of *Cryptosporidium* species

Species number	Valid species name	SSU rDNA		Public health significance	Host range	Human	Cattle	Genotype designation
		Accession numbers*	Minimum between taxa distance (%)					
I	*C. muris* Tyzzer, 1907	AB089284	0·4	Minor	MB	Yes		*C. muris* B genotype
II	*C. parvum* Tyzzer, 1912	AF112571	0·3	Minor	M	(Yes)		Mouse I genotype
III	*C. meleagridis* Slavin, 1955 (syn. *C. tyzzeri* Levine, 1961)	AF112574	0·8	Moderate	MB	Yes	(Yes)	
IV	*C. wrairi* Vetterling, Jervis, Merrill, Sprinz, 1971	AF115378	0·5	None	M		(Yes)	
V	*C. agni* Barker, Carbonell, 1974 (syn. *C. xiaoi* Fayer, Santín, 2009)	FJ896050	0·3	None	M			*C. bovis*-like genotype
VI	*C. bovis* Barker, Carbonell, 1974	AY741305	0·3	None	M	(Yes)	Yes	Bovine B genotype
VII	*C. cuniculus* Inman, Takeuchi, 1979	FJ262725	0·5	Moderate	M	Yes		Rabbit genotype
VIII	*C. felis* Iseki, 1979	AF108862	2·4	Moderate	M	Yes	(Yes)	Cat genotype
IX	*C. serpentis* Levine, 1980	AF151376	2·4	None	RM		(Yes)	
X	*C. nasoris* Hoover, Hoerr, Carlton, Hinsman, Ferguson, 1981	n/a	n/a	None	F			n/a
XI	*C. baileyi* Current, Upton & Haynes, 1986	L19068	2·5	None	B			
XII	*C. varanii* Pavlásek, Lávičková, Horák, Král, Král, 1995 (syn. *C. saurophilum* Koudela, Modrý, 1998)	AF112573	1·8	None	R			Desert monitor genotype
XIII	*C. cichlidis* (Paperna, Vilenkin, 1996) (syn. *C. huwi* Ryan, Paparini, Tong, Yang, Gibson-Kueh, O'Hara, Lymbery, Xiao, 2015)	AY524773	8·7	None	F			Piscine genotype 1
XIV	*C. reichenbachklinkei* (Paperna, Vilenkin, 1996)	FJ769050	10·9	None	F			Piscine genotype 2
XV	*C. galli* Pavlásek, 1999	AF316624 AY168847	4·4	None	B			Finch genotype
XVI	*C. andersoni* Lindsay, Upton, Owens, Morgan, Mead, Blagburn, 2000	AF093496	1·3	Minor	M	Yes	Yes	*C. muris* A genotype
XVII	*C. canis* Fayer, Trout, Xiao, Morgan, Lal, Dubey, 2001	AF112576	2·1	Minor	M	Yes	(Yes)	Dog genotype
XVIII	*C. hominis* Morgan-Ryan, Fall, Ward, Hijjawi, Sulai accession numbers man, Fayer, Thompson, Olson, Lal, Xiao, 2002	AF108865	0·3	Major	M	Yes	Yes	Human (I) genotype
XIX	*C. molnari* Alvarez-Pellitero, Sitjà-Bobadilla, 2002	HM243547	8·7	None	F			
XX	*C. suis* Ryan, Monis, Enemark, Sulaiman, Samarasinghe, Read, Buddle, Robertson, Zhou, Thompson, Xiao, 2004	AF115377	1·2	Minor	M	(Yes)	Yes	Pig genotype II
XXI	*C. scophthalmi* Alvarez-Pellitero, Quiroga, Sitjà-Bobadilla, Redondo, Palenzuela, Padrós, Vázquez & Nieto, 2004	n/a	n/a	None	F			n/a
XXII	*C. pestis* Šlapeta, 2006	AF108864	0·3	Major	M	Yes	Yes	Bovine (II) genotype

Table 1. (*Cont.*)

Species number	Valid species name	SSU rDNA		Public health significance	Host range	Human	Cattle	Genotype designation
		Accession numbers*	Minimum between taxa distance (%)					
XXIII	*C. fayeri* Ryan, Power, Xiao, 2008	AF112570	1·2	Minor	M	(Yes)		Marsupial genotype I
XXIV	*C. ryanae* Fayer, Santín,Trout, 2008	EU410344	1·2	None	M		Yes	Deer-like genotype
XXV	*C. fragile* Jirků, Valigurová, Koudela, Křížek, Modrý, Šlapeta, 2008	EU162751	5·3	None	A			
XXVI	*C. macropodum* Power, Ryan, 2008	AF513227	1·9	None	M			Marsupial genotype II
XXVII	*C. ducismarci* Traversa, 2010	EF547155	2·7	None	R			
XXVIII	*C. ubiquitum* Fayer, Santín, Macarisin, 2010	HM209366	1·3	Minor	M	Yes	Yes	Deer genotype
XXIX	*C. viatorum* Elwin, Hadfield, Robinson, Crouch, Chalmers, 2012	JN846705	2·0	Moderate	M	Yes		
XXX	*C. scrofarum* Kváč, Kestřánová, Pinková, Květoňová, Kalinová, Wagnerová, Kotková, Vítovec, Ditrich, McEvoy, Stenger, Sak, 2013	JX424840	2·7	Minor	M	(Yes)	(Yes)	Pig genotype II
XXXI	*C. erinacei* Kváč, Hofmannová, Hlásková, Květoňová, Vítovec, McEvoy, Sak, 2014	KF612324	0·3	None	M			Hedgehog genotype
XXXII	*C. rubeyi* Li, Pereira, Larsen, Xiao, Phillips, Striby, McCowan, Atwill, 2015	DQ295012	2·5	None	M			Genotype-c
XXXIII	*C. avium* Holubová, Sak, Horčičková, Hlásková, Květoňová, Menchaca, McEvoy, Kváč, 2016	KU058875	2·5	None	M			Avian genotype V
XXXIV	*C. proliferans* Kváč, Havrdová, Hlásková, Daňková, Kanděra, Ježková, Vítovec, Sak, Ortega, Xiao, Modrý, Chelladurai, Prantlová, McEvoy, 2016	KR090615	0·4	None	M			TS03 genotype
XXXV	*C. testudinis* Ježková, Horčičková, Hlásková, Sak, Květoňová, Novák, Hofmannová, McEvoy, Kváč, 2016	AY120914	3·3	None	M			Tortoise genotype I

Host range: M – mammal; B – bird; R – reptile; F– fish. n/a – no applicable, because it has not been characterised using any DNA signature. (Yes) – indicates extremely rare or experimental evidence; * accession numbers in primary DNA databases (GenBank: www.ncbi.nlm.nih.gov/genbank, EMBL: www.ebi.ac.uk/ena and DDBJ: www.ddbj.nig.ac.jp).

that oocysts are larger compared to the only other tortoise *Cryptosporidium* species, but ultimately, DNA sequencing must be used to differentiate the taxa, thereby arguing in favour of DNA taxonomy.

The description and naming of *C. avium* (Species XXXIII, Table 1) is the latest case in support of the direct use of DNA taxonomy (Holubová *et al*. 2016). While the experimental and morphological work is valuable, it did not elucidate the problem of cryptosporidiosis and diversity of *Cryptosporidium* species in birds. The study elevated a previously known genotype to a species level from a single host species and locality (Holubová *et al*. 2016). Therefore, the within-species DNA distance remains ambiguous, especially, because a known avian genotype, genotype II, is closely related to both *C. avium* and *C. baileyi* (Species XI, Table 1). Similar to *C. avium*, the newly introduced species *C. rubeyi* (Species XXXII, Table 1) that was described and named from ground squirrels is another case where its distance of 2·5% from the closest species is deceivingly strong (Fig. 3) (Li *et al*. 2015). On the other hand, there are several closely related DNA barcodes or genotypes obtained from closely related squirrels (Pereira *et al*. 2010). Therefore, the within-species *C. rubeyi* DNA distance is ambiguous. The DNA taxonomy approach is even more obvious in the description and naming of *Cryptosporidium* species in fish, *C. huwi* (Species XIII, Table 1; the name is considered to be synonymous with *C. cichlidis*) (Šlapeta, 2013; Ryan *et al*. 2015). The genotype (DNA barcode, known as piscine genotype I) was simply elevated to the species status, using a demonstration of *Cryptosporidium* developmental stages in a histological section from an affected fish (Ryan *et al*. 2015). Similar to bird *Cryptosporidium* species, the *Cryptosporidium* fish species diversity is largely unknown (Yang *et al*. 2015).

Together these five descriptions demonstrate issues surrounding the taxonomy and DNA barcoding of *Cryptosporidium* spp. in animals, and in particular the neglect towards understanding the within-species distance for a defined *Cryptosporidium* species. Currently, there are 35 named species considered valid (Table 1) (Šlapeta, 2009, 2011, 2012, 2013). If we accept that within- and between-species genetic distances are as low as 0·3%, as demonstrated for some of the zoonotic and human-infecting species, all the unique genotypes/DNA barcodes can be considered new candidate species. It appears that the intentions are there to name more species based on DNA sequences, with the only obstacle being a demonstration of oocysts/or tissue development. If such DNA taxonomy is the way forward for *Cryptosporidium*, the focus should be to aim at detecting the parasite in as the large proportion of potential susceptible hosts as has been done for *C. testudinis* (Ježková *et al*. 2016). Using a single strain to justify new description should not be adequate as it does not allow evaluation of the within-species DNA diversity. The within-species DNA diversity is especially valuable for hosts, such as birds and fish, where the diversity is less well known. The past 20–30 years have primarily focused on domestic animals and humans and their *Cryptosporidium* species, and the assessment of diversity preceded the taxonomical work. The DNA-barcoding approach is a favourable approach for non-farm or pet animals as well. The outstanding question is the absence of a universal catalogue that would be a proxy to the detected barcodes, enabling synthesis of past and current efforts to understand the species diversity of the genus *Cryptosporidium*.

All latest additions to the *Cryptosporidium* species list come from non-farm animals, implying that we have largely defined the diversity in human and farm animal cryptosporidia at species level (Li *et al*. 2015; Ryan *et al*. 2015; Holubová *et al*. 2016; Ježková *et al*. 2016; Kváč *et al*. 2016). In human and farm animals, the attention now focuses on the within-host subspecies diversity of *Cryptosporidium* species and their possibility to exchange genetic material.

CRYPTOSPORIDIUM SPP. INFECTING MAMMALS: OBJECTIVE ACCOMPLISHED

Has the *Cryptosporidium* species diversity initiative met its milestone to define such diversity? It can be concluded that, as far as mammalian species, efforts have been exhaustive and the majority of dominant, if not all, *Cryptosporidium* species and genotypes are defined and their DNA sequence (i.e. SSU rDNA) is already in the public domain. The objective has been enabled by the ease of DNA isolation, PCR amplification and DNA sequencing. In fact, for humans as the host, the efforts have been exhaustive and apart from identification of *C. viatorum* in recent years, there has been no major revelation or change in *Cryptosporidium* species involved (Elwin *et al*. 2012; Bouzid *et al*. 2013). DNA barcoding, and to some extent DNA taxonomy, has provided the needed framework to address questions revolving around the host susceptibility to infection as well as retrospectively zoonotic potential of individual *Cryptosporidium* species. Similarly, it can be concluded for major production animals such as cattle and swine (Kváč *et al*. 2013; Santín, 2013).

On the other side are *Cryptosporidium* species in birds and fish, of which there are a limited number of studies, some largely based on anecdotal evidence (Morgan *et al*. 2001; Alvarez-Pellitero and Sitjà-Bobadilla, 2002; Ng *et al*. 2006; Zanguee *et al*. 2010; Barugahare *et al*. 2011). In birds, *C. baileyi* and *C. meleagridis* are well characterized from chicken and turkeys (Current *et al*. 1986; Akiyoshi

https://doi.org/10.1017/S0031182017001809 Published online by Cambridge University Press

et al. 2003), but some studies have demonstrated unprecedented diversity of avian *Cryptosporidium* even in single host species (Jellison *et al*. 2004; Zhou *et al*. 2004). Fish infecting *Cryptosporidium* species are potential of production value in aquaculture (Sitjà-Bobadilla and Alvarez-Pellitero, 2003; Barugahare *et al*. 2011). Experimental infections under controlled conditions will be relevant in establishing the relevance of cryptosporidiosis in fish, as well as resolving the complex diversity of *C. molnari* and related genotypes/species in fish. Laboratory models for fish species would be particularly valuable to elucidate age susceptibility and association of *Cryptosporidium* species involved. Utilizing economically significant farmed fish could be considered positively by the aquaculture industry.

GENOME SEQUENCE AS THE PROGRESSION FROM DNA-BARCODING OBJECTIVE

Although the objective of DNA barcodes for species identification was accomplished in human and farm animals, a new objective has emerged. It is not clear if the DNA-barcoded species/isolates remain stable as far as their genomic and phenotypic characteristics are concerned. In other words, is there the possibility to exchange genetic material between individual species or strains infecting concurrent hosts resulting in genotypic and phenotypic variants? The role of super-infection and the challenge or emergence of more virulent isolates is not understood (Grinberg and Widmer, 2016). Experimental infections are potentially achievable with recently developed gene manipulation technologies, but require biosafety controlled environments (Vinayak *et al*. 2015).

Ease and availability of whole-genome DNA sequencing of the relatively small *Cryptosporidium* genome offers an initial perspective on the intra-host diversity (Hadfield *et al*. 2015; Ifeonu *et al*. 2016; Troell *et al*. 2016; Feng *et al*. 2017). Highly complete genomes of the principally isolated species of *Cryptosporidium* and their isolated variants have recently been made public, thereby enabling determination of the *status quo* for an assumed single strain/isolate. Single cell strains of *Cryptosporidium* are technically unachievable due to the absence of reliable culture techniques, thus field isolates are often assumed as strains if passed in the next host animal, or even directly assumed as strains if other evidence suggests no other strain was involved. The opportunity emerges to apply a metagenomic approach to purified field/clinical *Cryptosporidum* isolates and such metagenomes established to define the degree of heterogeneity or plasticity of the *Cryptosporidium* metagenome. The whole-genome perspective has the opportunity to move from the potentially biased single gene/locus genotyping to the discovery of regions of metagenome synteny that are particularly variable in field isolates or as the infection progresses (Oberstaller *et al*. 2013; Andersson *et al*. 2015; Guo *et al*. 2015; Hadfield *et al*. 2015; Ifeonu *et al*. 2016; Troell *et al*. 2016; Feng *et al*. 2017).

The outstanding problem remains that a reliable definition of a *Cryptosporidium* phenotype – a measurable outcome of the genetically identified strain of the parasite within its host (niche) – is lacking. Unlike a genotype that is defined by the nuclear DNA sequence of *Cryptosporidium* spp. strain, the phenotype is the combination of the genotype and the circumstances of the infection inside the host as well as the circumstances preceding the infection. The complementary experimental infections and metagenome approach will need to be applied simultaneously to address issues surrounding the definition of the *Cryptosporidium* phenotype. Careful attention to chosen clinical evaluations enabling evaluation of oocyst output, morbidity and identification of virulence factors or other characteristics should also be focused on in order to resolve the matter.

Cryptosporidium and cryptosporidiosis research has entered a new phase of opportunities, because of the renewed public health sector interest (Checkley *et al*. 2015). The traditional parasitology experimental procedures that include parasite purification and enumeration, as well as experimental infections are highly relevant to both animal models for cryptosporidiosis as well as culture models (Vinayak *et al*. 2015). The parasitology community has to offer intervention solutions for relevant species of *Cryptosporidium* of public health significance, similarly to the period of intense leadership in defining the routes of transmission during the 1980s and 1990s (Fayer, 1997). The DNA-barcoding objective for *Cryptosporidium* was accomplished; the success should be a motivation for the new objective of associating the phenotype with the genotype of *Cryptosporidium*.

FINANCIAL SUPPORT

This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

REFERENCES

Akiyoshi, D. E., Dilo, J., Pearson, C., Chapman, S., Tumwine, J. and Tzipori, S. (2003). Characterization of *Cryptosporidium meleagridis* of human origin passaged through different host species. *Infection and Immunity* **71**, 1828–1832.

Alvarez-Pellitero, P. and Sitjà-Bobadilla, A. (2002). *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L. and *Dicentrarchus labrax* L. *International Journal for Parasitology* **32**, 1007–1021.

Andersson, S., Sikora, P., Karlberg, M. L., Winiecka-Krusnell, J., Alm, E., Beser, J. and Arrighi, R. B. (2015). It's a dirty job – A robust method for the purification and de novo genome assembly of *Cryptosporidium* from clinical material. *Journal of Microbiological Methods* **113**, 10–12.

https://doi.org/10.1017/S0031182017001809 Published online by Cambridge University Press

Barugahare, R., Dennis, M. M., Becker, J. A. and Šlapeta, J. (2011). Detection of *Cryptosporidium molnari* oocysts from fish by fluorescent-antibody staining assays for *Cryptosporidium* spp. Affecting humans. *Applied and Environmental Microbiology* **77**, 1878–1880.

Besansky, N. J., Severson, D. W. and Ferdig, M. T. (2003). DNA barcoding of parasites and invertebrate disease vectors: what you don't know can hurt you. *Trends in Parasitology* **19**, 545–546.

Bouzid, M., Hunter, P. R., Chalmers, R. M. and Tyler, K. M. (2013). *Cryptosporidium* pathogenicity and virulence. *Clinical Microbiology Reviews* **26**, 115–134.

Carraway, M., Tzipori, S. and Widmer, G. (1996). Identification of genetic heterogeneity in the *Cryptosporidium parvum* ribosomal repeat. *Applied and Environmental Microbiology* **62**, 712–716.

Checkley, W., White, A. C., Jr, Jaganath, D., Arrowood, M. J., Chalmers, R. M., Chen, X. M., Fayer, R., Griffiths, J. K., Guerrant, R. L., Hedstrom, L., Huston, C. D., Kotloff, K. L., Kang, G., Mead, J. R., Miller, M., Petri, W. A., Jr, Priest, J. W., Roos, D. S., Striepen, B., Thompson, R. C., Ward, H. D., Van Voorhis, W. A., Xiao, L., Zhu, G. and Houpt, E. R. (2015). A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for cryptosporidium. *The Lancet Infectious Diseases* **15**, 85–94.

Current, W. L., Upton, S. J. and Haynes, T. B. (1986). The life cycle of *Cryptosporidium baileyi* n. sp. (Apicomplexa, Cryptosporidiidae) infecting chickens. *Journal of Protozoology* **33**, 289–296.

Elwin, K., Hadfield, S. J., Robinson, G., Crouch, N. D. and Chalmers, R. M. (2012). *Cryptosporidium viatorum* n. sp. (Apicomplexa: Cryptosporidiidae) among travellers returning to Great Britain from the Indian subcontinent, 2007–2011. *International Journal for Parasitology* **42**, 675–682.

Fayer, R. (ed.) (1997). *Cryptosporidium and Cryptosporidiosis*. Boca Raton, FL: CRC Press.

Fayer, R. (2008). General biology. In Fayer, R. and Xiao, L. (eds). Cryptosporidium and Cryptosporidiosis. Boca Raton, FL: CRC Press, pp. 1–42.

Fayer, R. (2010). Taxonomy and species delimitation in *Cryptosporidium*. *Experimental Parasitology* **124**, 90–97.

Feng, Y., Li, N., Roellig, D. M., Kelley, A., Liu, G., Amer, S., Tang, K., Zhang, L. and Xiao, L. (2017). Comparative genomic analysis of the IId subtype family of *Cryptosporidium parvum*. *International Journal for Parasitology* **47**, 281–290.

Grinberg, A. and Widmer, G. (2016). *Cryptosporidium* within-host genetic diversity: systematic bibliographical search and narrative overview. *International Journal for Parasitology* **46**, 465–471.

Guo, Y., Li, N., Lysen, C., Frace, M., Tang, K., Sammons, S., Roellig, D. M., Feng, Y. and Xiao, L. (2015). Isolation and enrichment of *Cryptosporidium* DNA and verification of DNA purity for whole-genome sequencing. *Journal of Clinical Microbiology* **53**, 641–647.

Hadfield, S. J., Pachebat, J. A., Swain, M. T., Robinson, G., Cameron, S. J., Alexander, J., Hegarty, M. J., Elwin, K. and Chalmers, R. M. (2015). Generation of whole genome sequences of new *Cryptosporidium hominis* and *cryptosporidium parvum* isolates directly from stool samples. *BMC Genomics* **16**, 650.

Hajibabaei, M., Singer, G. A., Hebert, P. D. and Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics* **23**, 167–172.

Hansen, H., Bakke, T. A. and Bachmann, L. (2007). DNA taxonomy and barcoding of monogenean parasites: lessons from *Gyrodactylus*. *Trends in Parasitology* **23**, 363–367.

Hebert, P. D., Cywinska, A., Ball, S. L. and deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings Biological Sciences/The Royal Society* **270**, 313–321.

Holubová, N., Sak, B., Horčičková, M., Hlásková, L., Květoňová, D., Menchaca, S., McEvoy, J. and Kváč, M. (2016). *Cryptosporidium avium* n. sp. (Apicomplexa: Cryptosporidiidae) in birds. *Parasitology Research* **115**, 2243–2251.

Ifeonu, O. O., Chibucos, M. C., Orvis, J., Su, Q., Elwin, K., Guo, F., Zhang, H., Xiao, L., Sun, M., Chalmers, R. M., Fraser, C. M., Zhu, G., Kissinger, J. C., Widmer, G. and Silva, J. C. (2016). Annotated draft genome sequences of three species of *Cryptosporidium*: *Cryptosporidium meleagridis* isolate UKMEL1, *C. Baileyi* isolate TAMU-09Q1 and *C. hominis* isolates TU502_2012 and UKH1. *Pathogens Disease* **74**, ftw080.

Jellison, K. L., Distel, D. L., Hemond, H. F. and Schauer, D. B. (2004). Phylogenetic analysis of the hypervariable region of the 18S rRNA gene of *Cryptosporidium* oocysts in feces of Canada geese (*Branta canadensis*): evidence for five novel genotypes. *Applied and Environmental Microbiology* **70**, 452–458.

Ježková, J., Horčičková, M., Hlásková, L., Sak, B., Květoňová, D., Novák, J., Hofmannová, L., McEvoy, J. and Kváč, M. (2016). *Cryptosporidium testudinis* sp. n., *Cryptosporidium ducismarci* Traversa, 2010 and *Cryptosporidium* tortoise genotype III (Apicomplexa: Cryptosporidiidae) in tortoises. *Folia Parasitology* (*Praha*) **63**, 035.

Kotloff, K. L., Nataro, J. P., Blackwelder, W. C., Nasrin, D., Farag, T. H., Panchalingam, S., Wu, Y., Sow, S. O., Sur, D., Breiman, R. F., Faruque, A. S., Zaidi, A. K., Saha, D., Alonso, P. L., Tamboura, B., Sanogo, D., Onwuchekwa, U., Manna, B., Ramamurthy, T., Kanungo, S., Ochieng, J. B., Omore, R., Oundo, J. O., Hossain, A., Das, S. K., Ahmed, S., Qureshi, S., Quadri, F., Adegbola, R. A., Antonio, M., Hossain, M. J., Akinsola, A., Mandomando, I., Nhampossa, T., Acacio, S., Biswas, K., O'Reilly, C. E., Mintz, E. D., Berkeley, L. Y., Muhsen, K., Sommerfelt, H., Robins-Browne, R. M. and Levine, M. M. (2013). Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the global enteric multicenter study, GEMS): a prospective, case-control study. *Lancet* **382**, 209–222.

Kváč, M., Kestřánová, M., Pinková, M., Květoňová, D., Kalinová, J., Wagnerová, P., Kotková, M., Vítovec, J., Ditrich, O., McEvoy, J., Stenger, B. and Sak, B. (2013). *Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (*Sus scrofa*). *Veterinary Parasitology* **191**, 218–227.

Kváč, M., Havrdová, N., Hlásková, L., Daňková, T., Kanděra, J., Ježková, J., Vítovec, J., Sak, B., Ortega, Y., Xiao, L., Modrý, D., Chelladurai, J. R., Prantlová, V. and McEvoy, J. (2016). *Cryptosporidium proliferans* n. sp. (Apicomplexa: Cryptosporidiidae): molecular and biological evidence of cryptic species within gastric *Cryptosporidium* of mammals. *PLoS ONE* **11**, e0147090.

Li, X., Pereira, M., Larsen, R., Xiao, C., Phillips, R., Striby, K., McCowan, B. and Atwill, E. R. (2015). *Cryptosporidium rubeyi* n. sp. (Apicomplexa: Cryptosporidiidae) in multiple *Spermophilus* ground squirrel species. *International Journal of Parasitology Parasites Wildlife* **4**, 343–350.

Morgan-Ryan, U. M., Fall, A., Ward, L. A., Hijjawi, N., Sulaiman, I., Fayer, R., Thompson, R. C., Olson, M., Lal, A. and Xiao, L. (2002). *Cryptosporidium hominis* n. sp. (Apicomplexa: Cryptosporidiidae) from *Homo sapiens*. *Journal of Eukaryotic Microbiology* **49**, 433–440.

Morgan, U. M., Constantine, C. C., Forbes, D. A. and Thompson, R. C. (1997). Differentiation between human and animal isolates of *Cryptosporidium parvum* using rDNA sequencing and direct PCR analysis. *Journal of Parasitology* **83**, 825–830.

Morgan, U. M., Monis, P. T., Xiao, L., Limor, J., Sulaiman, I., Raidal, S., O'Donoghue, P., Gasser, R., Murray, A., Fayer, R., Blagburn, B. L., Lal, A. A. and Thompson, R. C. (2001). Molecular and phylogenetic characterisation of *Cryptosporidium* from birds. *International Journal for Parasitology* **31**, 289–296.

Ng, J., Pavlasek, I. and Ryan, U. (2006). Identification of novel *Cryptosporidium* genotypes from avian hosts. *Applied and Environmental Microbiology* **72**, 7548–7553.

O'Donoghue, P. J. (1985). Cryptosporidium infections in man, animals, birds and fish. *Australian Veterinary Journal* **62**, 253–258.

Oberstaller, J., Joseph, S. J. and Kissinger, J. C. (2013). Genome-wide upstream motif analysis of *Cryptosporidium parvum* genes clustered by expression profile. *BMC Genomics* **14**, 516.

Ondrejicka, D. A., Locke, S. A., Morey, K., Borisenko, A. V. and Hanner, R. H. (2014). Status and prospects of DNA barcoding in medically important parasites and vectors. *Trends in Parasitology* **30**, 582–591.

Peng, M. M., Xiao, L., Freeman, A. R., Arrowood, M. J., Escalante, A. A., Weltman, A. C., Ong, C. S., Mac Kenzie, W. R., Lal, A. A. and Beard, C. B. (1997). Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human transmission cycles. *Emerging Infectious Diseases* **3**, 567–573.

Pereira, M., Li, X., McCowan, B., Phillips, R. L. and Atwill, E. R. (2010). Multiple unique *Cryptosporidium* isolates from three species of ground squirrels (*Spermophilus beecheyi*, *S. beldingi*, and *S. lateralis*) in California. *Applied and Environmental Microbiology* **76**, 8269–8276.

Ryan, U., Paparini, A., Tong, K., Yang, R., Gibson-Kueh, S., O'Hara, A., Lymbery, A. and Xiao, L. (2015). *Cryptosporidium huwi* n. sp. (Apicomplexa: Eimeriidae) from the guppy (*Poecilia reticulata*). *Experimental Parasitology* **150**, 31–35.

Santín, M. (2013). Clinical and subclinical infections with *Cryptosporidium* in animals. *New Zealand Veterinary Journal* **61**, 1–10.

Santín, M., Trout, J. M. and Fayer, R. (2008). A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. *Veterinary Parasitology* **155**, 15–23.

Siddall, M. E., Kvist, S., Phillips, A. and Oceguera-Figuero, A. (2012). DNA barcoding of parasitic nematodes: is it kosher? *Journal of Parasitology* **98**, 692–694.

Sitjà-Bobadilla, A. and Alvarez-Pellitero, P. (2003). Experimental transmission of *Cryptosporidium molnari* (Apicomplexa: Coccidia) to gilthead sea bream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.). *Parasitology Research* **91**, 209–214.

Šlapeta, J. (2009). Centenary of the genus *Cryptosporidium*: from morphological to molecular species identification. In Ortega-Pierres, M. G., Cacciò, S., Fayer, R., Mank, T., Smith, H. and Thompson, R. C. A. (eds), Giardia and Cryptosporidium: From Molecules to Disease. Wallingford, Oxfordshire, UK: CAB International, pp. 31–50.

Šlapeta, J. (2011). Naming of *Cryptosporidium pestis* is in accordance with the ICZN code and the name is available for this taxon previously recognized as *C. parvum* 'bovine genotype'. *Veterinary Parasitology* **177**, 1–5.

Šlapeta, J. (2012). The name *Cryptosporidium tyzzeri* Ren, Zhao, Zhang, Ning, Jian, Wang, Lv, Wang, Arrowood and Xiao, 2012 is permanently invalid. *Experimental Parasitology* **130**, 306–307.

Šlapeta, J. (2013). Cryptosporidiosis and *Cryptosporidium* species in animals and humans: a thirty colour rainbow? *International Journal for Parasitology* **43**, 957–970.

Šlapeta, J. (2017). *Cryptosporidium*: identification and genetic typing. *Current Protocols Microbiology* **44**, 20B.1.1–20B.1.17.

Sulaiman, I. M., Morgan, U. M., Thompson, R. C., Lal, A. A. and Xiao, L. (2000). Phylogenetic relationships of *Cryptosporidium* parasites based on the 70-kilodalton heat shock protein (HSP70) gene. *Applied and Environmental Microbiology* **66**, 2385–2391.

Troell, K., Hallstrom, B., Divne, A. M., Alsmark, C., Arrighi, R., Huss, M., Beser, J. and Bertilsson, S. (2016). *Cryptosporidium* as a testbed for single cell genome characterization of unicellular eukaryotes. *BMC Genomics* **17**, 471.

Tyzzer, E. E. (1910). An extracellular coccidium, *Cryptosporidium muris* (gen. et sp. nov.) of the gastric glands of the common mouse. *Journal of Medicine Research* **23**, 487–509.

Tyzzer, E. E. (1912). *Cryptosporidium parvum* (sp. nov.), a coccidium found in the small intestine of the common mouse. *Archives Protistenkunde* **26**, 394–412.

Upton, S. J. and Current, W. L. (1985). The species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) infecting mammals. *Journal of Parasitology* **71**, 625–629.

Vinayak, S., Pawlowic, M. C., Sateriale, A., Brooks, C. F., Studstill, C. J., Bar-Peled, Y., Cipriano, M. J. and Striepen, B. (2015). Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*. *Nature* **523**, 477–480.

Xiao, L., Escalante, L., Yang, C., Sulaiman, I., Escalante, A. A., Montali, R. J., Fayer, R. and Lal, A. A. (1999a). Phylogenetic analysis of *Cryptosporidium* parasites based on the small-subunit rRNA gene locus. *Applied and Environmental Microbiology* **65**, 1578–1583.

Xiao, L., Morgan, U. M., Limor, J., Escalante, A., Arrowood, M., Shulaw, W., Thompson, R. C., Fayer, R. and Lal, A. A. (1999b). Genetic diversity within *Cryptosporidium parvum* and related *Cryptosporidium species*. *Applied and Environmental Microbiology* **65**, 3386–3391.

Xiao, L., Alderisio, K., Limor, J., Royer, M. and Lal, A. A. (2000). Identification of species and sources of *Cryptosporidium* oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool. *Applied and Environmental Microbiology* **66**, 5492–5498.

Xiao, L., Sulaiman, I. M., Ryan, U. M., Zhou, L., Atwill, E. R., Tischler, M. L., Zhang, X., Fayer, R. and Lal, A. A. (2002). Host adaptation and host-parasite co-evolution in *Cryptosporidium*: implications for taxonomy and public health. *International Journal for Parasitology* **32**, 1773–1785.

Xiao, L., Fayer, R., Ryan, U. and Upton, S. J. (2004). *Cryptosporidium* taxonomy: recent advances and implications for public health. *Clinical Microbiology Reviews* **17**, 72–97.

Yang, R., Palermo, C., Chen, L., Edwards, A., Paparini, A., Tong, K., Gibson-Kueh, S., Lymbery, A. and Ryan, U. (2015). Genetic diversity of *Cryptosporidium* in fish at the 18S and actin loci and high levels of mixed infections. *Veterinary Parasitology* **214**, 255–263.

Zanguee, N., Lymbery, J. A., Lau, J., Suzuki, A., Yang, R., Ng, J. and Ryan, U. (2010). Identification of novel *Cryptosporidium* species in aquarium fish. *Veterinary Parasitology* **174**, 43–48.

Zhou, L., Kassa, H., Tischler, M. L. and Xiao, L. (2004). Host-adapted *Cryptosporidium* spp. in Canada geese (*Branta canadensis*). *Applied and Environmental Microbiology* **70**, 4211–4215.