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Note

Cryptosporidium occultus in disguise

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ABSTRACT

As data accumulate in GenBank, the difficulties of delineating species of *Cryptosporidium* based on nuclear small subunit ribosomal RNA (*ssu* rRNA) gene information alone becomes increasingly evident. Here, we summarize currently available evidence suggesting that several *ssu* rDNA sequences primarily referred to as *Cryptosporidium suis* (some of them from non-suid hosts) should be considered *Cryptosporidium occultus*.

1. Introduction

Differentiating between species of *Cryptosporidium* based on molecular characterization is not always straightforward, since the genes used to differentiate between the species may not always be or seem sufficiently discriminative. This is particularly the case of the 18S small subunit ribosomal RNA (*ssu* rRNA) gene, a multi-copy gene widely used for the detection of *Cryptosporidium* species but hampered by limited nucleotide diversity. To this end, intra-species variation can be challenging to delimit, which means that in some situations, it may be difficult to decide whether a sequence reflects variation within a species or a different known (or unknown) species. Sometimes, sequencing of additional genes helps facilitate the differentiation.

This situation can be exemplified by the *ssu* rDNA, the 70 kilodalton heat shock protein (*hsp70*), and actin sequences for sample W20486 from a human sampled in the United Kingdom (UK) with the accession numbers HQ822146, HQ822147, and HQ822148, respectively, in GenBank. These sequences were deposited in 2011; i.e., before the description of *Cryptosporidium occultus* (see below) and included in the publication by Robinson and colleagues (Robinson et al., 2011). The team added a comment for W20486 in GenBank saying "that this species should not be referred to as *Cryptosporidium suis*-like, as although similar to *C. suis* at the *ssu* rRNA gene it is distinctly different at the actin and *hsp70* genes".

While *Cryptosporidium parvum* has been known for >110 years (Tyzzer, 1912), *C. occultus* was described only in 2018 by Kváč and colleagues (Kváč et al., 2018). When describing the then new species, the team provided a list of sequences that had already been deposited in GenBank very likely representing *C. occultus* but annotated as '*suis*-like' (typically written 'cf. *suis*') or '*parvum*' or just 'sp.'. None of these were from suid hosts, but there were some from cattle and two from humans (AY030084 and HQ822146; the latter being the *ssu* rDNA sequence mentioned above). Since 2018, sequences have been reported that likely reflect *C. occultus* but that were not deposited in GenBank as such. We here provide additional evidence suggesting that more *ssu* rDNA sequences published in GenBank should be considered *C. occultus* instead

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Table 1
Cryptosporidium occultus sequences published in the NCBI Database (GenBank) to date originally described as *C. occultus* or under different names in various hosts worldwide along with names provided in the original publication where applicable and specified. Bolded accession numbers were already identified by Kváč et al. (2018).

Name in GenBank	Name in original publication	Host	Origin of sample	18S	Actin	*hsp70*	28S	Year of GenBank entry	Reference
C. parvum	*C. parvum* novel genotype	Human	Canada	**AY030084**, AY030085	–	–	–	2001	Ong et al. (2002)
Cryptosporidium sp.	*C. suis*-like genotype	Cattle	Denmark	**DQ182599**	–	**DQ182598**	–	2007	Langkjaer et al. (2007)
Cryptosporidium cf. *suis*	*C. suis*-like genotype	Cattle	India	**GQ345008**	–	–	–	2010	Khan et al. (2010)
Cryptosporidium sp.	*C. suis*-like	Cattle	UK	**HQ822134**	–	–	–	2011	Robinson et al. (2011)
Cryptosporidium sp.	*C. suis*-like	Human	UK	**HQ822146**	**HQ822148**	**HQ822147**	–	2011	Robinson et al. (2011)
C. suis	*C. suis*-like genotype	Rodent	Philippines	**JX485388**, **JX485390**	**JX485409**, **JX485412**, **JX485417**, **JX485418**	–	–	2012/13	Ng-Hublin et al. (2013)
Cryptosporidium sp. genotype RTA368	*Cryptosporidium* new genotype	Cattle	Australia	**KC778530**	–	–	–	2013	Abeywardena et al. (2013a)
Cryptosporidium sp. genotype 3	*Cryptosporidium* genotype 3	Buffalo	Australia	KF019204	–	–	–	2013	Abeywardena et al. (2013b)
Cryptosporidium sp. genotype 9	*Cryptosporidium* genotype 9	Buffalo	Sri Lanka	KF891292	–	–	–	2013	Abeywardena et al. (2014)
Cryptosporidium sp.	*C. suis*-like	Buffalo	Brazil	**JX559850**	**JX559851**	–	–	2015	Aquino et al. (2015)
Cryptosporidium cf. *suis*	*C. suis*-like genotype	Cattle	China	**KM110047**	–	–	–	2015	Ma et al. (2015)
Cryptosporidium cf. *suis*	*C. suis*-like	Deer	China	KX259135	–	–	–	2016	Huang et al. (2018)
Cryptosporidium cf. *suis*	*C. suis*-like	Deer	Australia	KU531660	–	–	KY882326	2016	Koehler et al. (2016)
Cryptosporidium cf. *suis*	*C. suis*-like	Yak	China	**KU052809**	–	–	–	2016	Li et al. (2016)
Cryptosporidium sp.	NS	Rodent	USA	KY644577	–	–	–	2017	Stenger et al. (2018)
C. occultus	NA	Rodent	Czech Republic	**MG699176, MG699177, MG699178, MG699179**	**MG699168, MG699169, MG699170, MG699171**	**MG699172, MG699173, MG699174, MG699175**	–	2018	Kváč et al. (2018)
C. occultus	NA	Rodent	China	MK731963	–	–	–	2019	Wei et al. (2019)
C. occultus	NA	Bactrian camel	China	MT703862	–	–	–	2020	Cao et al. (2020)
C. occultus	NA	Rodent	China	MW092532, MK956930	MW117323	–	–	2020	Li et al. (2020)
C. suis	*C. suis*	Wild boar	Spain	MT114479	–	–	–	2020	Rivero-Juárez et al. (2020)
C. suis	NS	Rodent	China	MT561508	–	–	–	2020	Ni et al. (2021)
C. occultus	NA	Human	China	MH807493	MN177696	MN177697	–	2020	Xu et al. (2020)
Cryptosporidium sp.	*C. occultus*	Alpaca	China	MN876848	–	–	–	2020	Zhang et al. (2020)
C. occultus	NA	Rodent	Austria	MZ314975	–	–	–	2021	Cervero-Arago et al. (2021)
C. suis	*C. occultus*	Rodent	Czech Republic	MT504544	MT507491	MT507485	–	2021	Ježková et al. (2021)
C. occultus	NA	Cattle	China	MW829507	–	–	–	2021	Unpublished
C. occultus	NA	Cattle	Thailand	–	–	MW872749	–	2021	Unpublished
C. occultus	NA	Buffalo	China	OL912797	–	–	–	2021	Unpublished
C. occultus	NA	Cattle	China	OL912798	–	–	–	2021	Unpublished
C. occultus	NA	Cattle	China	OP836384	–	–	–	2023	Feng et al. (2023)
C. occultus	NA	Deer	Portugal	OQ818661	–	–	–	2023	Figueiredo et al. (2023)
C. suis	*C. suis*	Wild boar	Spain	OR030357, OR030358, OR030359, OR030360, OR030361, OR030362	PP467560[a], PP467561[a]	–	–	2023	Martí-Marco et al. (2023)
C. suis	NS	Cattle	China	OR485084	–	–	–	2023	Unpublished
C. occultus	NA	Cattle	Bangladesh	MK982467	–	–	–	2024	Karim et al. (2024)

(*continued on next page*)

Table 1 (*continued*)

Name in GenBank	Name in original publication	Host	Origin of sample	18S	Actin	*hsp70*	28S	Year of GenBank entry	Reference
C. occultus	NA	Lynx	Spain	OR916204	–	–	–	2024	Matas-Méndez et al. (2024)

18S: 18S small subunit ribosomal DNA; 28S: 28S small subunit ribosomal DNA; *hsp70*: 70 kilodalton heat shock protein; NA: Not applicable; NS: Not specified.
[a] Sequences generated in the present study.

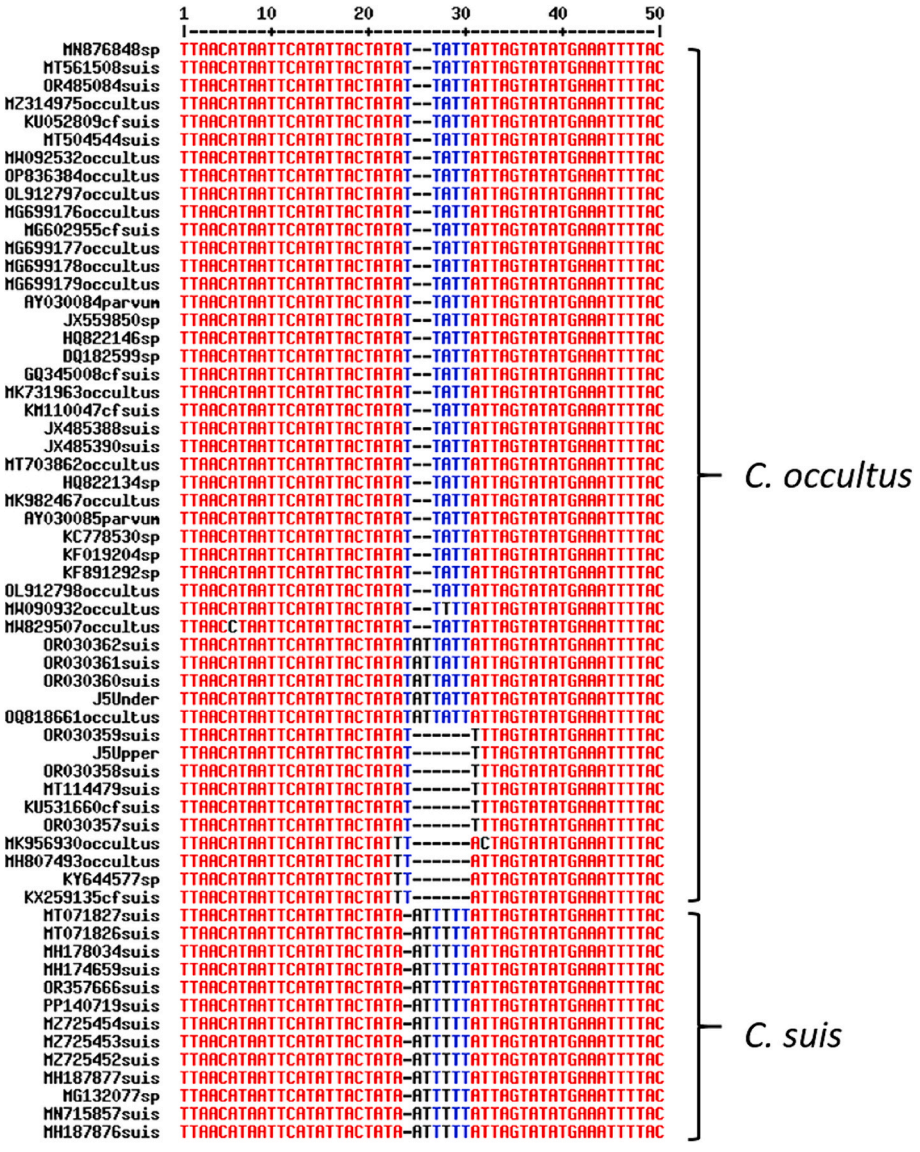

Fig. 1. A 50-bp excerpt of a multiple sequence alignment of *Cryptosporidium ssu* rDNA reference sequences in the NCBI Database (indicated by GenBank accession numbers and the epithet originally provided for each sequence ('cf. *suis*', '*occultus*', '*parvum*', 'sp.', or '*suis*'). The species names to the right of the brackets are those that we would consider applicable to the respective sequences.

of, typically, *C. suis*.

2. Methods

Identification and differentiation of *C. occultus* and *C. suis* were carried out by multiple sequence alignment to identify single nucleotide polymorphisms among the compared sequences, most of which were available in GenBank already. The alignment was produced using MultAlin available online (http://multalin.toulouse.inra.fr/). *Cryptosporidium* actin genes were amplified from faecal DNAs (Table 1) using the protocol described by Sulaiman and colleagues (Sulaiman et al., 2002).

Obtained results were substantiated by phylogenetic analysis and by cross-correlating *ssu* rDNA data with data on other genes, especially actin genes. Phylogenetic analyses of actin and *ssu* rDNA sequences were performed using the Maximum–Likelihood method based on the Kimura 2-parameter model and the Tamura-Nei model respectively (Kimura, 1980). Evolutionary analyses were conducted in MEGA11 (Tamura

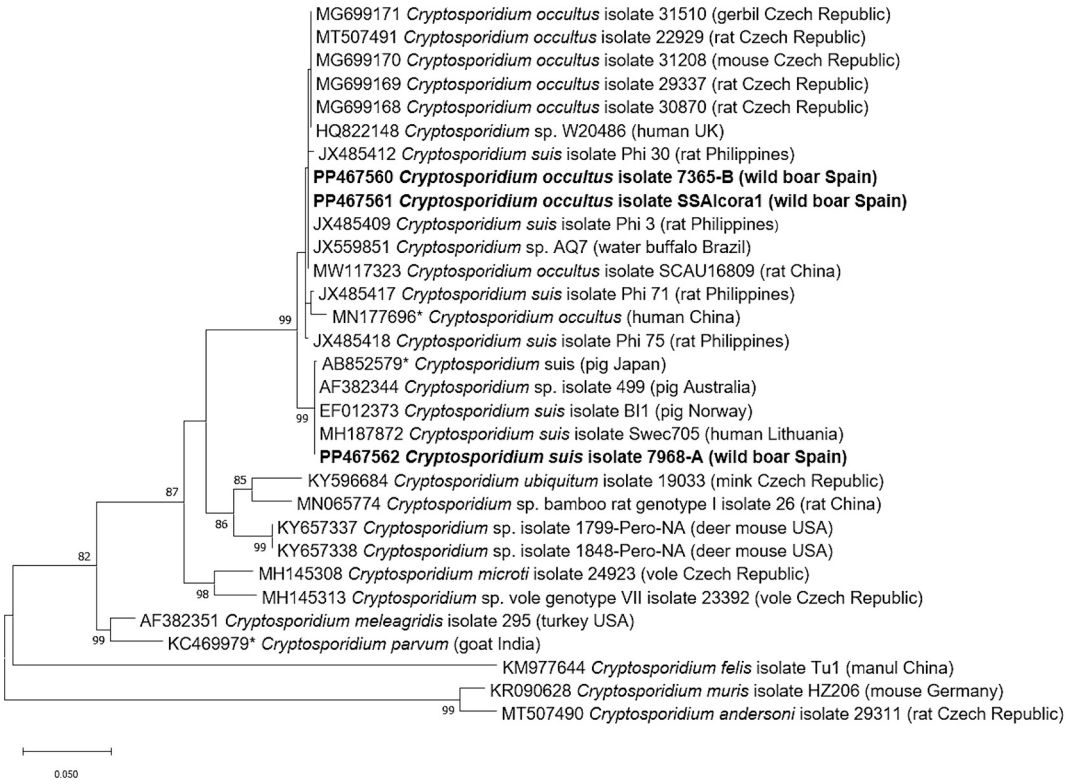

Fig. 2. Phylogenetic analysis of actin sequences of *Cryptosporidium occultus* and *Cryptosporidium suis* along with sequences from genetically related species using the Maximum-Likelihood method. There were a total of 664 positions in the final dataset. Bootstrap values ≥70% from 1000 replicates are shown. *Sequences did not have an isolate (/sample) name. However, in the case of MN177696, it was possible to link the sequence to the sample GX996 in the publication by Xu and colleagues (Xu et al., 2020), which was the origin of MH807493. Actin sequences generated in the present study were highlighted in bold. The two *C. occultus* actin sequences generated in this study were from two different samples that had produced mixed *ssu* rDNA sequences. The 7365-B sample had an 'upper' sequence similar OR030362 and a 'lower' sequence similar to OR030359 (both of which sequences are shown in Fig. 1), and for SSAlcora1, it was the other way around. Moreover, one of the four *C. suis* identical actin sequences produced in the present study was also included (isolate 7968-A).

et al., 2021).

3. Results and discussion

In Table 1, we listed the sequences published in GenBank to date that probably should be considered *C. occultus* based on our analyses. These included those that were already annotated as *C. occultus* and those that were given a different species name or just 'sp.'. Included in the table are those isolates already mentioned by Kváč and colleagues (Kváč et al., 2018) plus 31 additional ones, several of which ($n = 10$) had been referred as *C. suis*. The *ssu* rDNA sequences identified as *C. occultus* by Kváč et al. (Kváč et al., 2018) all have a hallmark sequence motif that reads 'TTACTATATTATTATTAGTATATG' (Fig. 1). However, distinct from this group of sequences are three minor groups of sequences that have group-specific variation in this sequence area, and where the different species names provided could appear to indicate taxonomic uncertainty (Fig. 1). For only two of these sequences, sequences from additional genes were available in GenBank to allow for cross correlation: 1) KU531660 ('*Cryptosporidium* cf. *suis*') could be linked to KY882326 ('*Cryptosporidium* cf. *suis*'), which is a sequence representing the 28S locus, for which the NCBI Database holds very few reference sequences; KY882326 shares up to 98.44% similarity with *Cryptosporidium ubiquitum* and only 97.67% similarity with *C. suis* (data not shown); 2) MH807493 ('*C. occultus*') could be linked to MN177696 (actin) and MN177696 (*hsp70*). MN177697 clusters with *C. occultus*, both in the study by Xu and colleagues (Xu et al., 2020) and in Fig. 2.

Phylogenetic analysis of *ssu* rDNA sequences provided some information (Fig. 3), but due to the relatively short length of some of the sequences, the inferences that could be made were not as robust as those that could be made for the actin sequences. For example, MW090932 that was named '*Cryptosporidium occultus*' in GenBank, clusters with *C. occultus*, although it has the DNA motif of *C. suis* (apart from an 'adenine' that is missing).

To further inform our analyses, we managed to amplify and sequence actin genes from wild boar faecal samples that shared similar *ssu* rDNA sequences with OR030357 and OR030059 (sample 'SSAlcora-1', GenBank acc. no.: PP467561) and OR030360 and OR030362 (sample '7365-B', GenBank acc. no.: PP467560) from the study by Martí-Marco and colleagues (Martí-Marco et al., 2023). Both actin gene sequences were identified as '*C. occultus*' by phylogenetic analysis (Fig. 2). Sequences were 'clean' with no underlying sequence traces that could indicate mixed species infection. Similarly, we amplified *C. suis* actin genes from four wild boar samples also from the study by Martí-Marco and colleagues (Martí-Marco et al., 2023), which were also included in the phylogenetic analysis (GenBank acc. Nos.: PP467562–PP467565); these sequences were from samples that had *ssu* rDNA sequences identical to MT071826 and MT071827. Conspicuously, the amino acid sequences of *C. suis* and *C. occultus* actin genes were identical.

Finally, revisiting the *ssu* rDNA sequence data for sample 'J5' (GenBank acc. no.: MT114479) from a wild boar included in the study (Rivero-Juárez et al., 2020), we noticed a sequence stretch characterized by the presence of double peaks. These could be teased apart into an 'upper' and an 'under' sequence. The double peaks observed turned out to be caused by a 6-bp insertion/deletion. The 'upper' sequence was identical to MT114479, while the 'under' was identical to OQ818661 ('*Cryptosporidium occultus*' from a red deer) (Fig. 1). These observations open up for several possibilities: The two sequences identified in the sequence traces of J5 could reflect 1) a mixed infection of two *C. occultus*

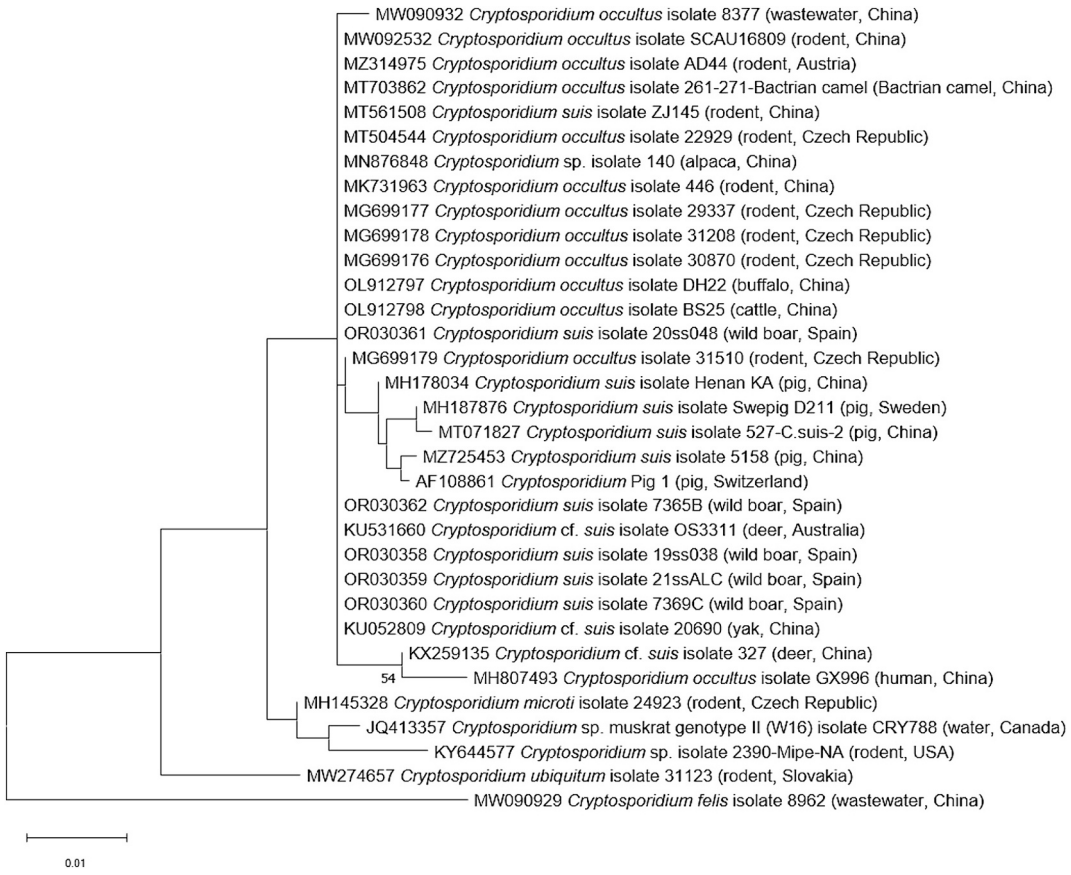

Fig. 3. Phylogenetic analysis of *ssu* rDNA sequences of *Cryptosporidium occultus* and *Cryptosporidium suis* along with sequences from genetically related species using the Maximum-Likelihood method. There were a total of 417 positions in the final dataset. Bootstrap values ≥50% from 1000 replicates are shown. Note that MW090932 was named '*Cryptosporidium occultus*' in GenBank, but, based on its sequence motif ('TTCATATTACTATATTTTTATTAGTATATG'), is only one bp different to *C. suis*.

variants; 2) A and B copies of *C. occultus* – and/or *C. suis* – the way we know them from *C. parvum* and a couple of other species of *Cryptosporidium* (Le Blancq et al., 1997; Xiao et al., 1999); 3) a mixed infection of *C. occultus* and *C. suis*, in case MT114479 is indeed *C. suis*. It would be more likely that the two sequences of J5 reflect A and B copies of *C. occultus* (option 2) than a mixed infection of *C. occultus* variants and *C. suis*, although the latter possibility cannot be completely ruled out at the current stage. Similar observations were made for chromatograms from sample 7365-B and SSAlcora1 (Fig. 2).

The DNA sequence 'TTACTATATTATTATTAGTATATG' appears to be characteristic of *C. occultus*, but it probably should not be considered specific to this species, as this motif also appears in a couple of other sequences that have been termed '*Cryptosporidium* muskrat genotype II' (e.g., JQ413362). And from Fig. 1 it appears that not all *C. occultus* share this sequence signature. Meanwhile, the sequence 'TTCATATTACTATAATTTTTATTAGTATATG' may be specific to *C. suis*.

There is at least one example of a situation where a sequence has been annotated as *C. occultus* but is not. MW090932 (Fan et al., 2021) is one such example, and the sequence should be referred to as *C. suis*.

Conclusively, a number of sequences in GenBank should be referred to as *C. occultus* according to the terminology proposed by Kváč and colleagues (Kváč et al., 2018) and the analyses done by e.g., Xu and colleagues (Xu et al., 2020). It remains a possibility that A and B copies of the *ssu* rRNA gene exists for *C. occultus* and/or for *C. suis*. Experimental infection with *C. occultus* in a suid host was unsuccessful in the study by Kváč and colleagues (Kváč et al., 2018), and whether suids are natural or incidental/transport hosts of *C. occultus* remains to be identified. Wild boars, along with foxes, are the most significant scavengers in Mediterranean ecosystems. Therefore, transmission could potentially occur through scavenging on either rodents or other potential hosts. Transmission could also occur through exposure to *C. occultus* oocysts while rooting. The fact that amino acid sequences of actin genes of *C. suis* and *C. occultus* are identical could indicate that the two species form a species complex. Further sampling will provide more data on any (cryptic) host specificity of the variants, and analysis of *gp60* genes will also inform taxonomy. Long-read next-generation sequencing methods (including nanopore-based DNA sequencing technology or even whole-genome sequencing) are also expected to contribute to the correct identification and assignment of conflictive sequences.

Ethics approval and consent to participate

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CRediT authorship contribution statement

Christen Rune Stensvold: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alba Martí-Marco:** Writing – review &

editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Samantha Moratal:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Marianne Lebbad:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **David Carmena:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Partial actin gene sequences generated in the present study have been deposited in GenBank under accession numbers PP467560–PP467565.

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