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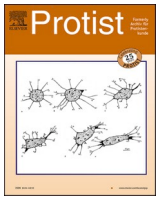

On the host specificity and genetic diversity of *Iodamoeba bütschlii*: Observations from short amplicon-based next-generation sequencing

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ABSTRACT

Iodamoeba is a single-celled intestinal parasite, which is common in humans in certain parts of the world, and also in pigs. For the first time, we provide DNA-based evidence of goat, dromedary, fallow deer, and donkey as hosts of *Iodamoeba* and show that *Iodamoeba*-specific nucleotide sequences from these four hosts do not appear to overlap with those of humans, unlike those from pigs. We moreover show that similar strains of *Iodamoeba* can be found in Madagascar, Western Sahara, and Ecuador and that intra-sample diversity is typically extensive across even small fragments of DNA in both human and non-human hosts.

1. Introduction

Iodamoeba is a genus of intestinal parasitic protists found in humans, non-human primates, and other larger animals. Dobell described the genus in 1919 (Dobell 1919) and gave the name *'Iodamoeba bütschlii'* to the human parasite; since then, *Iodamoeba* found in humans has been assigned to this species. Other species names introduced over the years include *Iodamoeba kueneni*, *Iodamoeba suis*, and *Iodamoeba williamsi* (Noble and Noble 1976); it remains unknown, however, to what extent one or more of these are in fact *Iodamoeba bütschlii*.

Iodamoeba positivity rates reported in surveys of intestinal parasites of humans typically range between 0.1 % and 2.0 %. Of note, however, positivity rates between 7.5 % and 16 % were reported among 381 apparently healthy subjects in Bolivia (Cancrini et al. 1988) and in surveys from Colombia (Pena-Quistial et al. 2020), Venezuela (Incani et al. 2017), and Peru (Maco Flores et al. 2002, Cabrera et al. 2005), suggesting a relatively high transmission rate in the *peri*-equatorial part of South America. It should be noted that these survey data were based on microscopy of faecal concentrates, and if *Iodamoeba*-targeted DNA-based methods had been used, positivity rates might have been even higher due to possibly increased sensitivity.

Apart from humans, *I. bütschlii* has been reported in non-human primates (Stensvold et al. 2012, Pafco et al. 2018, Ray and Banik 1965, Hamad et al. 2014) and pigs/wild boars (Stensvold et al. 2021, Stensvold et al. 2012, Wylezich et al. 2019, Solaymani-Mohammadi et al. 2004, Pakandl 1994, Ray and Banik 1965), in which hosts cysts specific to *Iodamoeba* have been observed (Ray and Banik 1965). Moreover, there are small subunit ribosomal RNA gene (*SSU*) sequences in the NCBI database that have been obtained from samples from sheep and cattle, but these data warrant confirmation. There is limited evidence that other natural hosts could be rodents, camels, and birds (Stensvold et al. 2012), but to our knowledge there are no DNA sequences available for *Iodamoeba* from these hosts yet to confirm the findings.

Together with its sister taxon, *Endolimax nana*, *I. bütschlii* forms part of the Mastigamoebidae B group (Ptackova et al. 2013). The existence of two *SSU* ribosomal lineages (RL) within *Iodamoeba* was demonstrated in 2012 (Stensvold et al. 2012) and has since then been corroborated by independent studies (Chihi et al. 2022, Stensvold et al. 2020, Wylezich et al. 2019), some of which involved metagenomics or metabarcoding.

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While both RL1 and RL2 have been found in humans, to date only RL2 has been found in suid hosts. RL1 has been found in a macaque (Stensvold et al. 2012), whereas the many *Iodamoeba* sequences produced from samples from gorillas by Hamad and colleagues (Hamad et al. 2014) may represent a new ribosomal lineage. Remarkably, when the RL1 and the RL2 lineages were initially described, the genetic distance between them was reported to no less than 31% (Stensvold et al., 2012).

Direct Sanger sequencing of *Iodamoeba*-specific *SSU* PCR products; *i. e.* with no post-PCR steps such as cloning, is of limited use due to intra-genome sequence variation between gene copies – a situation similar to that found in *Endolimax* (Stensvold et al. 2012, Hocke et al. 2022). Meanwhile, amplicon-based next generation sequencing (NGS) using broad-specificity eukaryotic *SSU* primers could be a short-cut to detecting and differentiating *Iodamoeba*-specific nucleotide sequences in faecal DNAs, as was recently shown for *Entamoeba coli* and *Entamoeba hartmanni* (Stensvold et al. 2022).

The aim of the present study was to expand on the genetic diversity and host specificity of *Iodamoeba bütschlii* by analysing data obtained by NGS.

2. Materials and methods

Data from amplicon-based NGS of faecal DNA samples in which *Iodamoeba*-specific nucleotide sequences had been observed were included in the study along with reference sequence extracted from the NCBI GenBank Database (Table 1, Fig. 1). Animal samples were collected from various sites including zoos and farms in northern Algeria, between September and December 2021. To prevent contamination, fresh faeces was carefully gathered from the top layer of the ground, labelled, and then transported to the laboratory for preservation. In April 2023, DNA was extracted using the IndiMag™ Pathogen Kit (INDICAL BIOSCIENCE) according to the manufacturer's protocol.

Moreover, human faecal DNA samples from Ecuador (collected 2019–2022), Western Sahara (collected in 2016), and Madagascar (collected 2016–2017) were available for study. The samples from Ecuador were from schoolchildren (3–11 years) in the Chimborazo and Guayas provinces. The samples from Madagascar come from the rural village of Soavinarivo, schoolchildren (3–11 years), in the central highlands of the island. Soavinarivo is a locality that has an altitude of 1,406 m. The samples from Western Sahara were from individuals residing in refugee camps in Smara, Auserd, and Laayoune. One fresh stool sample was collected per participant. Approval to collect and use the samples was obtained from Human Research Ethics Committee of the University of Valencia (Ecuador procedure number: H1518738039128; Sahara: H1487409978679; and Madagascar: H1506340075678) guaranteeing voluntary participation and respect for the fundamental principles established by the Declaration of Helsinki and Spanish legislation on biomedical research, data protection and bioethics. The samples were provided completely anonymized.

Samples were transported in 70% ethanol to the laboratory and within 1–2 months after collection, the DNA was obtained, which was kept at −20 °C. Starting with 3 g per sample, they were filtered and concentrated via centrifugation (2,500 rpm, 5 min) in Midi Parasep tubes® (Apacor Ltd., Wokingham, UK). The sediment obtained was used for the extraction of DNA with the QIAmp DNA Stool Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's instructions.

Amplicon-based NGS and the generation of consensus sequences were performed as previously described (Stensvold et al. 2022, Chihi et al. 2022, Stensvold et al. 2021). Consensus sequences were submitted to GenBank under the accession numbers PP073378–PP073429.

Study DNA sequences were subject to multiple sequence alignment along with relevant reference sequences from GenBank (Fig. 1), and distance-based phylogenetic analysis was carried out in MEGA7 (Kumar et al. 2016) using the Neighbor-Joining method.

3. Results and discussion

For most of the study samples, more than one *Iodamoeba*-specific consensus nucleotide sequence was produced per sample, indicating either mixed *Iodamoeba* colonisation or intra-strain variation. Hence, a total of 52 consensus sequences were generated from the 14 samples included in the study. The study sequences were typically between 170 and 231 bp long; only the sequence from a donkey was somewhat shorter, only 115 bp.

The phylogenetic analysis revealed a number of interesting findings. Firstly, two major clusters could be identified, corresponding to what are already known as RL1 and RL2. Due to the clear presence of subclusters within each ribosomal lineage, we subdivided RL1 into RL1a and RL1b,

Table 1
Nuclear ribosomal DNA sequences* used in the study according to sample ID, host, country, number of sequences, ribosomal lineage, and reference.

Sample ID/GenBank accession number	Host	Country	Number of sequences	Ribosomal lineage	Reference
B84-BPS046	Donkey	Algeria	1	RL2 (unspecified)	Present study
B26-BPS045	Dromedary	Algeria	1	RL2a	Present study
B33-BPS045	Dromedary	Algeria	5 (a–e)	RL2b and RL2c	Present study
B34-BPS045	Dromedary	Algeria	2 (a, b)	RL2b	Present study
40-BPS007	Human	Western Sahara	4 (a–d)	RL1a	Present study
66-BPS007	Human	Ecuador	3 (a–c)	RL1a	Present study
79-BPS011	Human	Ecuador	3 (a–c)	RL1a	Present study
I17-BPS048	Fallow deer	Algeria	8 (a–h)	RL2b and RL2c	Present study
I18-BPS048	Fallow deer	Algeria	10 (a–j)	RL2b and RL2c	Present study
I5-BPS048	Goat	Algeria	2 (a, b)	RL2b and RL2c	Present study
I6-BPS048	Goat	Algeria	8 (a–h)	RL2b and RL2c	Present study
B14-BPS044	Goat	Algeria	1	RL2a	Present study
1-BPS005	Human	Madagascar	2	RL1a	Present study
32-BPS007	Human	Madagascar	2	RL1a	Present study
JN635744	Pig	United Kingdom	1	RL2a	(Stensvold et al., 2012)
MK801432	Pig	Germany	1	RL2a	(Wylezich et al., 2019)
MK801438	Pig	Germany	1	RL2a	(Wylezich et al., 2019)
MK801454	Pig	Germany	1	RL2a	(Wylezich et al., 2019)
MK801463	Pig	Germany	1	RL2a	(Wylezich et al., 2019)
MK801464	Pig	Germany	1	RL2a	(Wylezich et al., 2019)
JN635740	Human	Cuba	1	RL2a	(Stensvold et al., 2012)
JN635742	Human	Unknown origin	1	RL1b	(Wylezich et al., 2019)
JN635746	Human	Thailand	1	RL1b	(Wylezich et al., 2019)
JN635745	Human	Thailand	1	RL1a	(Wylezich et al., 2019)
JN635741	Human	Unknown origin	1	RL1a	(Wylezich et al., 2019)
AF149916	Human	USA	1	*Endolimax nana*	(Silberman et al., 1999)

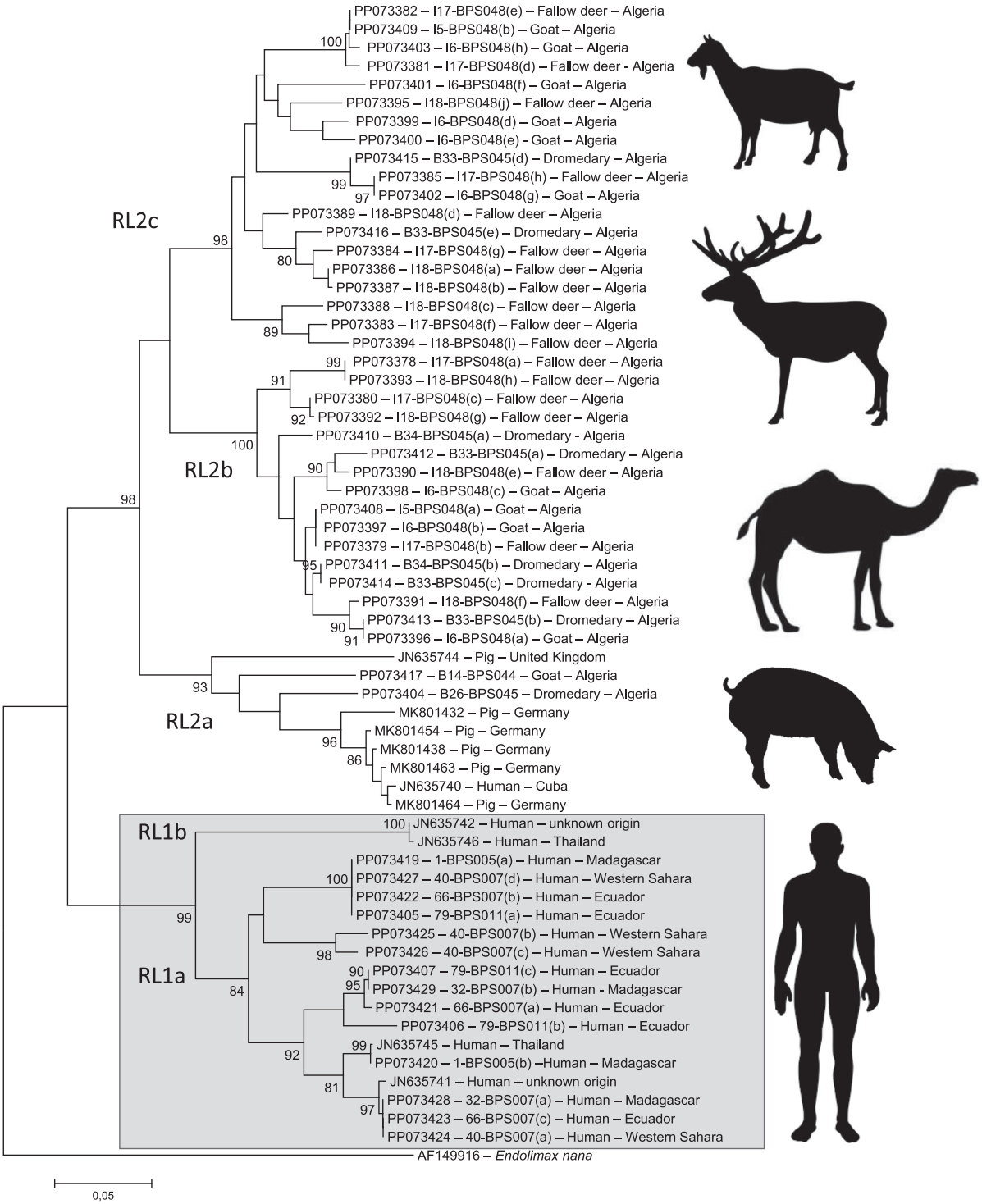

Fig. 1. Evolutionary relationships of study and reference nucleotide sequences. The grey box highlights ribosomal lineage 1 (RL1) sequences, which, to date, have only been found in humans. Three sublineages of RL2 were identified, all of which exhibited moderate host specificity. The evolutionary history was inferred using the Neighbor-Joining method (p-distance model), and Endolimax nana was used as outlier. The analysis involved 63 nucleotide sequences. All ambiguous positions were removed for each sequence pair. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Only bootstrap values ≥ 80 are shown. There were a total of 187 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

and RL2 into RL2a, RL2b, and RL2c (Fig. 1). As seen previously, RL1 comprises *Iodamoeba* nucleotide sequences from humans only, while RL2 holds sequences from non-human hosts, with the exception of JN635740, which was identified in a faecal sample from a person who had travelled to Cuba (Stensvold et al. 2012).

With regard to RL1, all the new sequences fell into RL1a. This lineage appears to be quite diverse, with clear subclusters that include sequences shared between samples. Hence, not only were identical or nearly identical sequences observed in samples from different geographic regions, there were also examples of shared patterns of within-sample diversity, in samples '32' (sequence 32-BPS007(b)) from Madagascar and '79' (sequence 79-BPS011(c)) from Ecuador and in

samples '66' (sequence 66-BPS007(c)) from Ecuador and '40′ (sequence 40-BPS007(a)) from Western Sahara (Fig. 1). For all five human samples analysed in this study, between 2 and 4 sequence variants were observed.

In RL2, subcluster RL2a was observed to hold mainly nucleotide sequences from pigs (from Germany and the UK), but also from a goat and a dromedary (Algeria); only one sequence was available for each of these two latter animals (B14-BPS044 and B26-BPS045). The two remaining subclusters, RL2b and RL2c, were made up of multiple sequences exclusively from goats, fallow deer, and dromedaries. Sample I18-BPS048 produced no less than 10 consensus sequences that were distributed between RL2b and RL2c. We believe these are the first molecular data indicating goat, fallow deer, and dromedary as host of *Iodamoeba*.

Sequences from dromedary, goat, and fallow deer were observed in both of the subclades RL2b and RL2c. The sequence from a donkey (B84-BPS046) was not included in the phylogenetic analysis, since the sequence was short. However, a 73-bp stretch of the sequence had 100 % identity to JN635746 (*Iodamoeba* RL1). In our recent *Endolimax* study (Hocke et al. 2022), we obtained a sequence, 'SW04' (OK483224) from a Swedish wastewater sample, that clustered with *Iodamoeba* RL1 and RL2 with relatively high bootstrap support but that sat on a long branch, which indicates a high degree of genetic divergence. Whether the donkey sequence also represents a novel *Iodamoeba* lineage is uncertain as yet; no morphology data were available for this sample, which could have informed our conclusions.

The life cycle of *Iodamoeba* involves a cyst stage and a trophozoite stage. The name '*Iodamoeba*' reflects the characteristic iodophilic glycogen mass present in *Iodamoeba* cysts often erroneously referred to as a vacuole. Cysts are irregularly shaped, vary in diameter with a mean of approximately 10 μm (Dobell 1919), and usually have a single vesicular nucleus with a large spherical karyosome and very little peripheral chromatin. Unfortunately, no morphological data were available for the study samples, and the identity of the sequence data generated in the present study are based on sequence similarity alone; their origin should be interpreted with this in mind, as it is not known whether cysts of *Iodamoeba* were present in the faeces of the animals sampled. However, since *Iodamoeba* cysts prior to this study had been demonstrated in faecal samples producing nucleotide sequences belonging to RL1 (a and b) and RL2a, the phylogenetic analysis would support the hypothesis that what are here assigned to RL2b and RL2c should also be considered *Iodamoeba*-specific sequences.

Due to lack of overlap, we were not able to compare these sequences with those annotated in GenBank as '*Iodamoeba*' from sheep and cattle (KC922216, KC922238, KC9222276, KC922234, KC922246, KC922268, and KC922300). For the same reason we could not include the sequences from gorillas sampled in Cameroon and published by Hamad et al. (Hamad et al. 2014), due to an overlap of only 31 bp. The sequences from gorillas are all identical apart from unique bp substitutions in two of the sequences. They appear to be more related to RL1 (94.5 % similarity) than to RL2 (90.0 % similarity) and may represent a new ribosomal lineage; however, these sequences do not align well with other *Iodamoeba* sequences, and no data on morphology were provided, so it is not known whether *Iodamoeba* cysts or trophozoites were in fact present in these samples.

The genetic diversity within *I. bütschlii* appears to be remarkably extensive, and it could be argued that the ribosomal lineages—maybe even the sublineages (RL1a, RL1b, etc.)—are distinct species. To help resolve this conundrum, future studies should aim at combining microscopy data with molecular characterisation that involves *SSU* rDNA sequencing, but preferably also sequencing of other marker genes of Iodamoeba in samples from various host species. This would shed light on host specificity and detail our knowledge regarding the genetic diversity with the genus of *Iodamoeba*.

We are aware that the data were generated from samples that had been processed in different ways prior to molecular analysis. However, since the study focused on host specificity and genetic analysis of DNA data rather than methodological aspects, we believe that any differential influence of the different pre-PCR methodologies used might have had minor impact, if any, on the results.

As stated previously (Stensvold et al. 2012), we cannot prove conclusively whether the sequence diversity within samples is because each *Iodamoeba* cell encodes several distinct *SSU*s or because most *Iodamoeba* infections are mixtures of multiple strains, each with a single *SSU* variant. However, in this study we observed that samples from several hosts in geographically distant locations generate multiple consensus sequences and that among these are identical sequences shared between samples. We believe that this supports the first hypothesis, namely that the *Iodamoeba* genome encodes multiple *SSU* genes with distinct sequences.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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