Rebuttal letter regarding the preprint entitled " Development of nine microsatellite loci for *Trypanosoma lewisi*, a potential human pathogen in Western Africa and South-East Asia, and preliminary population genetics analyses" after the first round of evaluation by PCI Infections

Dear Dr MacLeod,

Please find below the remarks and suggestions, together with our answers and how we dealt with referees' remarks and suggestions. We hope this will meet your and the two referee 's satisfaction.

Thank you for this evaluation. We hope that the new version of our preprint will be suitable for recommendation.

We remain at your disposal for any other suggestion or question you might find necessary to ask.

Best regards

The authors.

Round #1

by Annette MacLeod, 13 Jun 2022 17:15

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accept with minor revision

I think this manuscript will be much improved by adopting the revisions suggested by the reviewers.

Reviews

Reviewed by Gabriele Schönian, 11 May 2022 16:37

This is an interesting study investigating for the first time, at least to my knowledge, the population structure of T. lewisi from West Africa and Southeast Asia using microsatellite markers. The manuscript is well written and all experimental procedures are clearly described.

This study has some limitations, which have been discussed in-depth by the authors. The number of microsatellite markers is not very high and, moreover, there was a problem of amplification of these markers especially in the southeast Asian samples. The sampling procedure also needs to be improved in future studies. Nevertheless, the approach proposed here for population genetic studies in this parasite seems to be promising for future research.

I have only one remark. Is it possible to give more information on the location of the 9 microsatellite markers in the parasite's genome, such as on which chromosome and where exactly on a given chromosome? This could perhaps be added to Table 1.

Answer

We thank Dr Schönian for her interesting remark. Unfortunately, the genome of *Trypanosoma lewisi* has not been sequenced yet. Therefore, we cannot locate the markers we have used for this study.

Reviewed by anonymous reviewer, 02 Jun 2022 07:06

Development of nine microsatellite loci for Trypanosoma lewisi, a potential human pathogen in Western Africa and South-East Asia, and preliminary population genetics analyses

Overall Decision: revise

In this paper the authors set out to develop a system of microsatellite genetic (MS) markers for the understudied parasite Trypanosoma lewisi to provide a tool for investigating population genetic parameters. Overall, the paper describes some ambitious work which

successfully establishes a key set of MS markers. These markers were successfully used on a wide range of samples although amplification problems were found with a subset of samples. The paper reports the outcomes of population genetic analyses on samples that were successfully amplified.

Overall the paper is well constructed, the study conducted in a robust manner and the conclusions are justified by the results. The authors should be congratulated on the detail and robustness with which they describe and conduct the bioinformatic analyses. Due to the constrictions in the number and range of samples used, the authors describe their studies as a preliminary attempt to determine some of the population genetic parameters – this is indeed true but does not deflect from the value of this paper.

I have some comments which the authors need to address.

1. There are errors in syntax and grammar throughout the manuscript that should be addressed. I appreciate the challenges of scientific writing in a non-native language but I would recommend a thorough editing to bring the language in the manuscript up to the professional standard it deserves.

Answer

We have carefully proofread our manuscript and amended all syntaxis and grammatical inaccuracies that we found. We hope these modifications made the style of our amended manuscript more acceptable.

2. In the materials and methods can you please Indicate whether the study has had ethical approval and if so provide an ethical statement, and reference number.

Answer

We concede that ethical considerations had not been detailed in the first version of our manuscript. A dedicated section was added in order to address this issue, and it now reads: "In Benin, researches were conducted within the framework of the research agreement between the Republic of Benin and the French National Institute for Sustainable Development (IRD) that was reapproved on the 6th April 2017, as well as the partnership agreement between IRD and the University of Abomey-Calavi (signed on the 30th September 2010 and renewed on the 3rd July 2019). In Senegal, they were carried out under the framework agreement established between IRD, the Republic of Senegal and the Senegalese Head Office of Waters and Forests (available upon request). At the time of sampling, no ethic agreement was required to investigate pest rodents in these two countries. In Lao Republic and Thailand, ethic agreements were obtained from the National Ethics Committee of Health Research (Ministry of Health Council of Medical Sciences, 51/NECHR) and the Ethical Committee of Mahidol University, Bangkok (0517.1116/661), respectively. Samples from Cambodia were used under the courtesy of the Pasteur Institute of Cambodia (CeroPath project, coord. P. Buchy).

In all countries, explicit oral agreements were systematically obtained from local traditional (e.g. family and household heads, shop, firm and garden owners) as well as administrative (e.g., City Hall services, urban district chiefs) authorities before rodent trapping.

None of the rodent species captured for this study has protected status according to IUCN/CITES. Rodents were captured and brought alive to the lab where they were treated in a respectful manner in accordance with the guidelines of the American Society of Mammalogists (Sikes & Gannon, 2011), sedated and then sacrificed by cervical dislocation as recommended by Mills *et al*. (1995). Handling procedures of African samples were performed under our lab agreement for experiments on wild animals (no. 34-169-1).

Access to and benefit-sharing of genetic resources in Benin produced during the course of the present study was authorized by the Benin national authorities following the Nagoya international protocol (permit 608/DGEFC/DCPRN/PF-APA/SA). The other samples were collected before Nagoya protocol-associated procedures were implemented. Moreover, there is no possibility of commercial

use of any of the genetic diversity evidenced during this work, and the co-authorship with our partners from the countries involved testifies of the access and benefit sharing on the utilization of the genetic diversity studied in this paper.

Biological material transfers to France have been systematically approved by the Regional Head of Veterinary Service Hérault, France).

Samples and associated data were deposited in the Small Mammal Collection at the IRD/CBGP (https://doi.org/10.15454/WWNUPO) as well as at URIB/LARBA/EPAC (Benin) and Kasetsart University (Thailand). They are available upon request."

3. Experimental animals were used, so could you please provide details of how they were maintaned and under what ethical/licensing regulations they were used.

Answer

No animal was kept in experiments. All samples were obtained from direct dissection of wild rats captured in the field.

4. Lines 108 – 112. These are rather old references to infection parameters. It would be preferable to also reference something more recent. For example, a more recent study – see ref later - has determined detailed parameters for the cell cycle and includes data on the timing of infection in mammals (rats) in vivo (Figure S1). (Zhang X, Li SJ, Li Z, He CY, Hide G, Lai DH, Lun ZR. Cell cycle and cleavage events during in vitro cultivation of bloodstream forms of Trypanosoma lewisi, a zoonotic pathogen. Cell Cycle. 2019 Mar;18(5):552-567. doi: 10.1080/15384101.2019.1577651. Epub 2019 Feb 20. PMID: 30712435; PMCID: PMC6464594.)

Answer

We have rewritten this sentence and added this reference: “Incubation in the mammal host lasts five to six days, followed by a multiplication phase of 7 to 10 days (Hoare, 1972; Zhang et al., 2019), , after which non-multiplying adults appear and stay in the blood for weeks if not months ((Hoare, 1972), page 221).”

5. Line 130 - 136. In addition to the references provided. There should be a brief description of the methods used (e.g. primers used, cycling conditions etc.) to avoid readers having to trawl back through other papers.

Answer

Following Referee 1's comment, we have added some clarifications concerning the protocol: “Screening for the presence of *Trypanosoma* in rodent samples were carried out with a 131 bp-long fragment of the 18S rRNA gene qPCR-based assay with two primers (TRYPA1: AGGAATGAAGGAGGGTAGTTCG, TRYPA2: CACACTTTGGTTCTTGATTGAGG) and a pair of hybridization probes (TRYPA3: LCRed640 – AGAATTTCACCTCTGACGCCCAGT – Ph, TRYPA4: GCTGTAGTTCGTCTTGGTGCGGTCT – FITC), using a LightCycler® 480 (Roche Diagnostics). Each reaction was duplicated and set in 10 µL final volume using the LC480 Probes Master Kit (Roche Diagnostics, Meylan, France) with 0.5 µM of each primer, 0.25 µM of each probe and 0.25 µL of uracil-DNA-glycosylase (UDG) (Bilabs, Courtaboeuf, France). After an initial incubation step at 50 °C for 1 min and a denaturation step at 95 °C for 10 min, cycling conditions were performed for 50 cycles with a denaturation step at 95 °C for 10 s, annealing step at 56 °C for 10 s and extension step at 72 °C for 15 s. All qPCR-positive samples were sequenced for 400 bp-long fragments of the SSU rDNA gene to determine

Trypanosoma species, using the primer pair TRYPASEQ1 (ACACTGCAAACGATGACACC) and TRYPASEQ2 (TCAACCAAACAAATCACTCCA). Reaction was carried out in a 50 µL final volume containing 2.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM of each dNTP, 0.2 µM of each primer and 1.25 U of Fast Start Taq DNA polymerase (Roch Diagnostics, Meylan, France). An initial denaturation step was performed for 10 min at 95 °C, than the amplification goes on for 45 cycles with a denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min (Dobigny et al., 2011; Pumhom et al., 2015; Tatard et al., 2017; Cassan et al., 2018; Dobigny et al., 2019)."

6. Line 139. Which strain(s) was/were used in the sequencing project to detect the microsatellites? You state that some of the amplification problems might be due to primer mismatches - did you sequence any of the South East Asian strains to determine whether the Primer sequences were correctly represented in those genomes?

Answer

It is true that we did not specify which strain we used for the sequencing. This information has been added in the amended manuscript: "The DNA library was prepared from the strain Wery L307 24/9/68 68 (isolated in Kinshasa in 1968 by Pr Wéry of Institute of Tropical Medicine in Antwerp Belgium, kindly provided by Etienne Pays, Université Libre de Bruxelles Belgium) and using the Nextera DNA sample kit (Illumina, San Diego, CA, USA)." As regard to Asian isolates, we did not sequence Asian strains to check for the sequences flanking our microsatellite markers. As suggested by our results, South East Asian populations of *T. lewisi* are very distant from African ones, and mismatches with primers

designed from African strains were expected and confirmed. This is why we suggest, in the discussion section, to design specific microstatellite loci for Asian populations.

7. Line 139. Generally, in the interests of repeatability, more detail is required to describe the sequencing methods. Are the data from the sequencing project deposited on public databases? Provision of accession numbers etc. is required.

Answer

We understand the request of Referee 2. However, it is no longer possible to deposit primers on database such as GenBank for example. Instead, we have added the primers sequences obtained after our screening in a specific sheet in the supplementary file S1.

8. The bioinformatic analyses are well described.