Reviewer Report

Title: Development and validation of a multi-locus DNA metabarcoding method to identify endangered species in complex samples

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Reviewer name: Megan Coghlan

Reviewer Comments to Author:

The manuscript entitled 'Development and validation of a multi-locus DNA metabarcoding method to identify endangered species in complex samples' provides a detailed analysis of 12 barcode markers applied to the identification of species within medicinal samples. This study also tests the validity and reproducibility of the developed metabarcoding method across 16 international laboratories. The authors have researched the topic area well, and have taken into account the most pertinent issues involved with attempting to identify species within degraded samples, and with making identifications using incomplete reference DNA databases. This is an invaluable study for the field of wildlife forensics, in particular with regard to endangered species identification in herbal medicines, and will hopefully help enforcement agencies towards prosecuting those involved in the illegal wildlife trade in the near future.

I have just a few questions for the authors in regards to some points in the methods.
1) How were the pooled libraries quantified (lines 642-643) prior to sequencing on the MiSeq? Could this information be added to this section?
2) Were all of the barcoding PCRs carried out using qPCR, and if so, were any of the DNA extracts deemed to be low copy number as evidenced by high CT values?
3) Were extraction controls PCR amplified, and did any contain DNA? If so, were they sequenced as well? Could this be clarified in the methods please?
4) How did the authors choose the 46 reference samples included in the study, and why those species in particular?

It is noted that the authors pooled 8uL of each PCR product to combine into a sample library. I just have a suggestion that in future metabarcoding library set-up, perhaps a method to quantify the concentration of the products could be carried out (e.g., fragment analyser if possible), and then library blending could be adjusted so that each amplicon is pooled in equimolar amounts. This could assist in gaining a more equal number of reads across each sample particularly where there are low read numbers of a genuine taxa that could otherwise be screened out in the bioinformatic filtering stages.

Methods

Are the methods appropriate to the aims of the study, are they well described, and are necessary controls included? Yes

Conclusions

Are the conclusions adequately supported by the data shown? Yes

Reporting Standards

Does the manuscript adhere to the journal's guidelines on [minimum standards of reporting?](#) YesChoose an item.

Statistics

Are you able to assess all statistics in the manuscript, including the appropriateness of statistical tests used? There are no statistics in the manuscript.

Quality of Written English

Please indicate the quality of language in the manuscript: Acceptable

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