

Dear Recommender,

You will find below all comments made about the 2<sup>nd</sup> round of our preprint and all answers we made to those.

We hope you will find the new version of our preprint suitable for recommendations for PCI Zoology and remain at your disposal for any question you may find necessary to ask.

Sincerely,

Thierry de Meeûs

### **Minor revision needed**

Dear Authors, the two reviewers of the manuscript during the first round of review are quite convinced about your revision and have just minor issues to be corrected. However, a third reviewer who was not available during the first round of review is also quite convinced but has two issues that could be addressed or at least commented on, 1. the integration of analysis into R incl. an example for a binomial test on two alleles, and 2. a concern related to your simulations and conclusions related to strictly clonal populations. I would be grateful if you could address these smaller issues.

Kind regards

Michael Lattorff

#### **Reviews**

*Reviewed by Thibaut Malausa, 05 Aug 2022 09:00*

The authors have substantially modified the manuscript. I found the updated version, which is significantly shorter, much easier to read. A few adjustments in the abstract, introduction and conclusion also better highlight the strengths of the proposed tool.

The authors have also very carefully taken into account the comments from the two reviewers and I found their responses to the several questions or criticisms convincing.

I just recommend the authors to check the new text parts for minor issues (e.g. line 672 of the version with track change: "occut" should be replaced by "occur";

*Answer*

Done

"need being" is also written several times and I suppose it could be replaced by "need to be" or "will have to be", etc.).

*Answer*

Done

*Reviewed by Thierry Rigaud, 13 Jul 2022 13:09*

I have no further comment. The paper has been improved and made more compact. It has therefore gained in efficiency.

*Reviewed by Fabien Halkett, 26 Jul 2022 12:13*

This manuscript by Dr. T. De Meeûs presents and details a new implementation to test for stuttering pattern among microsatellite locus.

This manuscript is a revision. I did not take part in the first round of review. I have read the revised file and found it to be worthy of recommendation as it addresses an important topic for empirical population genetic studies and is a valuable contribution with the proposed methodology. I have also read the author's response letter and found that the author has convincingly addressed all the concerns raised by the previous reviewers (Dr Thibaut

Malausa and Dr. Thierry Rigaud). I do, however, have a few additional comments and suggestions that might help to further improve the article.

Both reviewers and the authors agree to state that microsatellites are (still) powerful markers to conduct sound population genetic study. One of the strength of microsatellite lies especially in the amount of information we have on the putative bias linked to empirical data acquisition and how to correct for these biases in appropriately curating the data. This curating step (according to relevant procedures) it is at utmost importance in order to perform accurate inference in population genetics. The opinions of the two reviewers diverge on how the method proposed can be applied to genomic data. I agree that it is important that the proposed method be versatile enough to be applied to a handful of microsat markers analyzed on an Excel sheet but also to a bio-informatic pipeline of NGS derived microsat typing. I was curious to test this procedure on my own data set, and even if I handled a reduced dataset I found more convenient to translate the procedure into the R language. I am not a skillfull R programmer, but I manage to write down a small script that do the job quite well, I think. It is convenient to use, as a starting point of this analyses, the output file generated by Genepop that summarize basic information, including the table of genotype frequencies, in the form:

Genotypes	Obs.	Expected
148 , 148	3	2.1801
151 , 148	1	2.1801
151 , 151	2	0.4845

Then in few lines on R it is possible to conduct the binomial test proposed by Dr. T. De Meeûs on the class of heterozygote genotypes that possess two alleles differing by only one repeat.

```
geno_table <- read.table("stutter_test.txt", header = T)
geno_table$diff = geno_table$geno1 - geno_table$geno2
range_repeats <- unique(geno_table$diff)
```

```
Obs <- vector(mode = "numeric", length = length(range_repeats))
Exp <- vector(mode = "numeric", length = length(range_repeats))
```

```
for (i in range_repeats) {
  Obs[i+1] <- sum(geno_table[which(geno_table$diff==i),]$obs)
  Exp[i+1] <- sum(geno_table[which(geno_table$diff==i),]$exp)
}
```

```
binom.test(c(round(Exp[2],0), Obs[2]), n=2, p=0.5)
```

May be it is possible to expand on it (it misses the loop on all locus across populations), but it could worth at least including these few lines in an appendix to the article. Hopefully, someone in the PCI forum will propose a R package but I am not sure it is mandatory for the use of this method in R.

### *Answer*

We thank Dr Halkett for this proposal. Nevertheless, I (TdM) am a much less skilled R programmer than he is, and I must admit that reading this script was rather painful as I understood almost nothing of it. In case of recommendation, all the correspondence between authors, recommender and referees will necessarily be published online and every interested reader will have access to Dr Halkett's script. Instead of including it in the manuscript and take credit for this script, we propose to leave it as such, so that in case of recommendation, it will appear as Dr Halkett's contribution to the topic, and will be citable under his name. We feel this would be more equitable.

*Fabien Halkett*

My second main comment is more scientific. The main strength of the study by Dr. T. De Meeûs is to test the application of his method on simulated data generated after different reproductive modes. The method works very well for selfing, and it is clearly stated. The case of clonality is more puzzling, and I agree that even in the revised version it is still quite hard to follow. It could be worth stating in the introduction that this reproductive mode magnifies the effect of genetic drift due to the lack of segregation (Stoeckel & Masson, 2014; Reichel et al., 2016). This generate a large variability across loci that is indeed a pitfall for this stuttering test.

*Answer*

According to my (TdM) experience, this is really a matter of what we are looking at, and also depends on the parameters set of the population under study. If one looks at heterozygosity (i.e. in terms of eigenvalue effective population size), clonality moderates the effect of genetic drift, so that more alleles can be maintained. In terms of genotypic diversity, this is the reverse as the number of different genotypes tends to decrease in clonal organisms.

As regard to the variance of  $F_{IS}$  across loci, small clonal ( $c=1$ ) populations will tend to display smaller variances as compared to small panmictic populations, while big populations will display higher variances if clonal. Nevertheless, the difference between purely clonal and panmictic populations will generally tend to be small as compared to variances of  $F_{IS}$  observed in weakly sexual populations (i.e.  $c$  in  $[0.9-0.999]$ )

*Fabien Halkett*

With this respect, it is important to note that the author test the case of full clonal populations. The variability across loci is even magnified when considering mixture of clonal and sexual reproduction (clonal rate of 0.9 and above, Stoeckel & Masson 2014; Balloux et al., 2003). I am thus not convinced with the statement lines 632-634 (document with revision marks): "In partial clones, and given the lack of accuracy of the expected number of heterozygotes with one repeat difference, using the panmictic expectations will probably display better performances." I think it is important for practical considerations that the author comment on that point or provide other simulation results.

*Answer*

We understand Dr Halkett's concern. Nevertheless, the main problem here does not come from the variance of  $F_{IS}$  across loci but to the fact that the frequency of a given class of heterozygote is impossible to predict, as long as there is no segregation. It means that the equation we proposed hardly bring any accurate expected frequency of heterozygotes with one repeat difference and other strategies may be suggested. We thus have proposed another way to explain this in the "Result and discussion" and "Conclusions" sections. Detection of stuttering in pure clones appeared uneasy. It will probably be even more difficult in partial clones, at least with  $c>0.5$ .

*Fabien Halkett*

For minor points see the annotated pdf that I upload with the review.

*Answer*

We have followed most Dr Halkett's suggestions, except for the points we discuss below.

*Fabien Halkett*

Introduction, line 64: reference to Guichoux et al. 2011; Balloux, F., Lehmann, L., & Meeûs, T. de. (2003).

*Answer*

We do not understand why it was needed to cite Balloux et al 2003 here. This paper specifically refers to clonal organisms and not to amplification problems. Guichoux et al's paper does not mention all amplification problems, so we have added a few more references.

*Fabien Halkett*

It could have been interesting to review the different pitfalls, and highlight that stuttering is still an issue. Other biases can be corrected, e.g. null alleles

*Answer*

We do not believe that our paper is the right forum to review all amplification issues. Our paper focuses on stuttering only. We have nevertheless listed the different amplification problems in the introduction section of the amended manuscript. We moreover disagree with the fact that all other biases can be corrected as null alleles can. For instance, short allele dominance can only be corrected by reinterpreting the genotypes (peaks), but not from the raw data.

*Fabien Halkett*

Tables 1-3: Could you find a way to combine the three tables? It is the same test applied to all contingency tables.

*Answer*

We could not find a way to produce a single table combining the three tables. We designed a bar chart instead (Figure 2 in the amended manuscript).