

1 **A simple procedure to detect, test for the presence of**
2 **stuttering, and cure stuttered data with spreadsheet programs:**
3 **application to parasites and vectors**

Commenté [FH1]: useful to mention it ? I would delete it

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11 Running title: Stuttering detection and cure

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13 Keywords: Microsatellite markers; Short tandem repeats (STRs); Simple sequence repeats
14 (SSRs); Polymerase chain reaction (PCR); Genotyping errors.

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16

17 **Abstract**

18 Microsatellite are **powerful** markers for empirical population genetics, but may be
19 affected by amplification problems like stuttering that produces heterozygote deficits
20 between alleles with one repeat difference. In this paper, we present a simple procedure
21 that aims at detecting stuttering for each locus overall subsamples and only requires the
22 use of a spreadsheet interactive application on any operating system. We compare the
23 performances of this procedure with the one of MicroChecker on simulations of dioecious
24 pangamic populations, monoecious selfing populations and clonal populations with or
25 without stuttering, and on real data of vectors and parasites. We also propose a cure for
26 loci affected and compare the results with those expected without stuttering. In sexual
27 populations (dioecious or selfers), the new procedure appeared more than three times
28 more efficient than MicroChecker. Cure was able to restore Wright's F_{IS} of stuttered data to
29 the expected value, and particularly so in selfing simulations. In clones, lack of segregation
30 artificially increased false stuttering detection, and only highly significant stuttering tests
31 and loci strongly deviating from others, could be usefully cured, in which case F_{IS} estimate
32 could be much improved. In doubt, and whenever possible, removal of affected and not
33 curable loci may help to shift population genetics parameter estimates towards more
34 reliable values.

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Supprimé: still useful

Supprimé: This is particularly true for non-model and small organisms as parasites and vectors.

Supprimé: dioecious

Supprimé: detection works well and

Supprimé: cure

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Supprimé: parameter estimates but not perfectly so. In selfers, detection and cure work well, providing other confounding factors as null alleles do not interfere.

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Mis en forme : Police :Italique

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Supprimé: Cures are kept only if parameter estimates are improved as well as the behavior of their variation in relation to other factors as number of missing data (null alleles) or specific criteria for clonal populations.

54 Introduction

55 Despite the recent democratization of NGS based techniques, microsatellite loci are
56 still very useful markers, in particular for empirical population genetics of non-model and
57 small organisms, as many parasites and/or their vectors, which are difficult (or impossible)
58 to study with direct methods as direct observation (as for birds) or as mark-release-
59 recapture approaches. Sequencing and single nucleotide polymorphism markers (SNPs)
60 still represent expensive alternatives in time, money and expertise, which lies beyond the
61 reach of many laboratories and most of the time at the expense of sample sizes. Three
62 decades ago, microsatellite markers were presented as the most powerful genetic markers
63 (Jarne & Lagoda, 1996). However, researchers began to detect the different problems that
64 can arise and developed different kinds of cures. The last kind of detection tools and cures
65 only arose very recently (Wang et al., 2012; De Meeûs, 2018; Manangwa et al., 2019; De
66 Meeûs et al., 2021).

67 Stuttering is the result of the Taq polymerase slippage during the PCR amplification
68 of the targeted DNA strand. This generates several PCR products that differ from each
69 other by one repeat and can cause difficulties when discriminating between fake and true
70 homozygotes, such as heterozygous individuals for dinucleotide microsatellite allele
71 sequences with a single repeat difference (De Meeûs et al., 2021). Stuttering produces
72 heterozygote deficits as compared to Castle-Weinberg (CW) expected genotypic
73 proportions (Castle, 1903; Weinberg, 1908), also known as Hardy-Weinberg (HW)
74 expectations (please have a glance at (De Meeûs et al., 2021) for an explanation why we
75 prefer using CW instead of HW). This phenomenon is locus specific and the deviation
76 produced, as measured by wright's F_s (Wright, 1965), proportional to the intensity with
77 which each locus is affected.

78 Today, and to our knowledge, the only procedure to detect stuttering is the one
79 used in MicroChecker (Van Oosterhout et al., 2004). Though it works well enough, it only
80 studies each locus one by one, which is fine because stuttering presence and intensity are
81 expected to be locus specific. Nonetheless, MicroChecker tests for stuttering in each
82 subsample separately, though a global test might be more powerful. Furthermore,
83 MicroChecker was developed under Microsoft® Windows in 2003 (Windows XP), and it
84 begins to display incompatibility issues with most current systems. A simple alternative,
85 which can detect and test for the presence of stuttering at each locus overall subsamples
86 on any platform kind would thus be welcome and timely.

87 In this paper, we present a very simple procedure that only requires the use of a
88 spreadsheet interactive computer application such as Apache® OpenOffice Calc or

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Supprimé: , especially so for non-model organisms

Commenté [FH2]: reference to Guichoux et al. 2011 Balloux, F., Lehmann, L., & Meeûs, T. de. (2003). The Population Genetics of Clonal and Partially Clonal Diploids. *Genetics*, 164(4), 1635–1644.

Supprimé: It is probable that SNPs will experience the same fate. Null alleles are known to exist in SNPs (Vignal et al., 2002) and these markers may display frequent allelic dropouts (Bayerl et al., 2018). Ascertainment bias represent a very serious issue for non-model organisms (Garvin et al., 2010). The number of SNPs needed is at least 200 to compete with microsatellite information (Séré et al., 2017), which raises new problems regarding some testing procedures as neutrality tests or linkage disequilibrium (LD) tests. LD tests are classically undertaken between pairs of loci. Given the very low power of individual LD tests, which is aggravated by false discovery rate (FDR) procedures, such as Benjamini and Yekutieli's (Benjamini & Yekutieli, 2001), required on non-independent tests series, the number of genotyped individuals required to reach a reasonable power might often look prohibitive for most laboratories. This is particularly true for parasitic organisms and their vectors as *Ixodes scapularis* the vector of Lyme disease in North America (De Meeûs et al., 2021), and even more critical regarding the actors of neglected tropical diseases, among which fasciolosis and African trypanosomiasis cause much economic and health issues and represent significant constraint on development (Solano et al., 2010; Hunter, 2014; Correa et al., 2017).

Commenté [FH3]: It could have been interesting to review the different pitfalls, and highlight that stuttering is still an issue. Other biais can be corrected, e.g. null alleles (Chapuis et al. 2007) Chapuis, M.-P., & Estoup, A. (2007). Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution*, 24(3), 621–631. doi: [10.1093/molbev/msl191](https://doi.org/10.1093/molbev/msl191)

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Supprimé: In the most recent version (Windows 10) running the setup program can be tricky. On some computers, the setup program stops at some percent of the process and the user must end it and run again the setup program as many times as necessary (e.g. six times). We may fear that next versions of Windows will be completely incompatible with this program. For other operating systems, MicroChecker requires some specific emulators to work

130 Microsoft® Excel. We compare the performances of this new procedure with the one
131 implemented in MicroCheker on simulated data without (null hypothesis) or with
132 (alternative hypothesis) stuttering, in dioecious populations of various sizes with random
133 mating, hermaphrodites with selfing or clonal populations. We also checked how the cure
134 proposed in De Meeûs et al. (2021) of loci with stuttering signature restore the values
135 expected for some parameters. We finally reanalyzed four real data sets on vectors and/or
136 their parasite: the tick *Ixodes scapularis* in North America (De Meeûs et al., 2021);
137 *Glossina palpalis palpalis*, vector of African trypanosomiasis in Côte d'Ivoire (Berté et al.,
138 2019); the snail *Galba truncatula* and the fluke it transmits, *Fasciola hepatica* in France
139 (Correa et al., 2017); and *Trypanosoma brucei gambiense*, the agent of sleeping sickness
140 in Guinea and Côte d'Ivoire (Koffi et al., 2009). On these datasets, we checked if more loci
141 with stuttering could be diagnosed, cured the loci with suspicion of stuttering, following the
142 technique proposed recently (De Meeûs et al., 2021) to verify if some conclusions could be
143 changed.

144

145 **Material and Methods**

146 **Simulations**

147 Simulations were undertaken with EASYPOP (v. 2.0.1) (Balloux, 2001). We
148 simulated random mating dioecious populations (pangamy), like what probably occurs in
149 the wild for ticks (De Meeûs et al., 2021), Nematocera flies (Prudhomme et al., 2020),
150 Hemipteran bugs (Gomez-Palacio et al., 2013), or tsetse flies (Berté et al., 2019). We also
151 simulated selfing monoecious populations, as flukes and water snails (Correa et al., 2017).
152 The total size of populations was $N_T=10,000$ individuals subdivided into either $n=100$
153 subpopulations of $N=100$ individuals, or $n=500$ and $N=20$, with an even sex ratio (dioecy)
154 or with selfing rate $s=0.3$ (monoecy). The model of migration followed an Island model with
155 migration rate $m=0.01$. We simulated 20 independent loci with a mutation rate of $\mu=0.0001$
156 that followed a mixed model with 70% of mutations following a stepwise mutation model
157 (SMM) and 30% following a KAM model. The maximum possible number of alleles was
158 $K=20$. Each simulation started with maximum variability and was run for 10,000
159 generations. At the end of each simulation, 20 individuals (10 males and 10 females in
160 dioecious populations), when $N \geq 20$, were randomly sampled in 10 subpopulations. [As can](#)

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164 [be seen with the real datasets reanalyzed in the present work, such a sampling design](#)
165 [approximately represents what is classically obtained for most parasites or vectors studies.](#)

166 Simulations of monoecious populations with 30% of selfing allowed checking the
167 interaction of stuttering detection in inbred populations with a high expected heterozygote
168 deficit (here 18%).

169 A subset of simulations with $n=100$, $N=100$ (same values for other parameters as
170 above) but with 100% clonal propagation, was finally undertaken, to fit with diploid clonal
171 pathogens as trypanosomes (Koffi et al., 2009) or yeasts as *Candida albicans* (Nébavi et
172 al., 2006).

173 Each parameter set was replicated 10 times.

174 [In the supplementary material, we provided an example with the results files of the](#)
175 [first simulation, with the root name "TestStutterDioeciousNoStutter-n100N100-1" and](#)
176 [extensions "txt", "equ", "dat", and "gen", for the parameters used, the statistics along the](#)
177 [simulation \(all generations\) and the resulting data files in Fstat and genepop formats,](#)
178 [respectively.](#)

180 Generating stuttering

181 Data were analyzed with Fstat 2.9.4 (Goudet, 2003) updated from (Goudet, 1995) to
182 get information on the [identity](#) of alleles kept at the end of simulations. For each
183 simulation, [input files](#) were imported into a spreadsheet keeping each allele of each locus
184 separated in a single column. We [arbitrarily](#) considered that 10% of possible alleles
185 affected by stuttering was enough. [This](#) means that two alleles (out of 20 [possible ones](#))
186 needed to be recoded for stuttering. Because of genetic drift, not all the 20 possible alleles
187 were present at the end of each [simulation](#). For each locus, [among the allele still present](#),
188 only the first two alleles separated by a single repeat were concerned. For each individual
189 carrying one of these alleles as the second allele, if different by a single repeat from the
190 first allele, the second allele was recoded as identical to the first one. Let us assume that
191 the first two alleles separated by a single repeat were, for instance, allele 5 and 6 for the
192 first locus (Locus1). If allele 5 was in cell B2 and allele 6 in cell C2 in the spreadsheet, the
193 command for generating stuttering in a cell from a free zone (e.g. the first free column after
194 the last column of the data) would be:

195 =B2, for the first allele (no change), and

196 =IF(ABS(B2-C2)=1,IF(OR(B2=5,B2=6),B2,C2),C2), for the second allele of that locus. This
197 way, individuals 4/5, 5/6, or 6/7 are recoded as homozygotes for the first allele.

198 This command was then copied and pasted to transform the whole locus.

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Commenté [FH5]: add a sentence here : For mimicking stuttering we proceeded as follow :

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=SI(ABS(B2-C2)=1;SI(OU(B2=5;B2=6);B2;C2);C2) in French

204 [A template, using the first simulation, can be found in the supplementary material](#)
205 [files as the spreadsheet file "TestStutterDioecious-n1000N100-1-10%Stuttering.xlsx".](#)

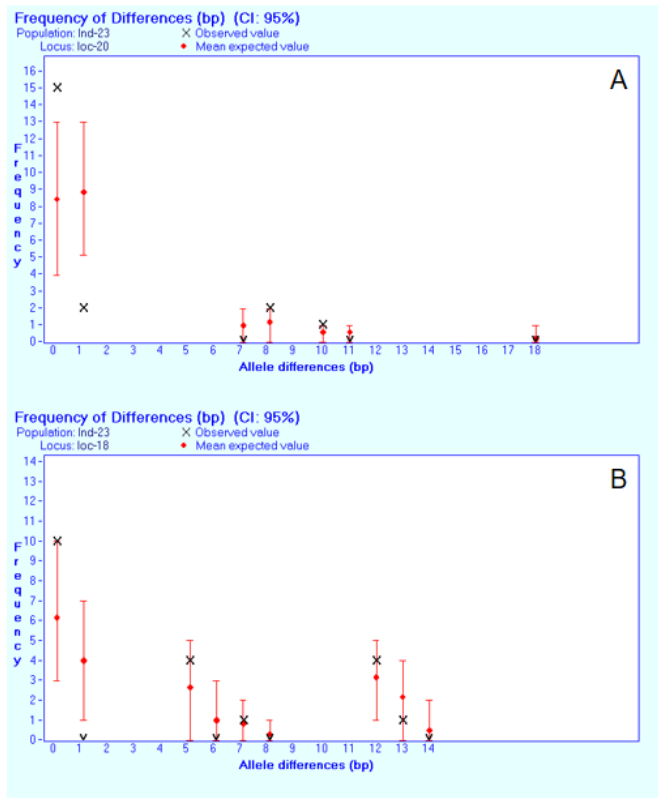
206 Because of drift, some loci in some subsamples did not display allele with one
207 repeat difference. Such manipulation thus generated data with 0% to 100% of alleles
208 displaying stuttering for all of the 20 loci, but with various intensity from one locus to the
209 other, and from one subsample to the other, as expected in real situations. This also
210 allowed checking the kind of variance stuttering can generate on parameter estimates (see
211 below).

213 **Detection of stuttering and testing with MicroChecker**

214 All datasets (raw and with stuttering) were analyzed with MicroChecker with 10,000
215 randomizations. All loci were considered as mononucleotidic, as simulated by Easypop.
216 Stuttering was detected when the observed heterozygosity for alleles with one repeat
217 difference was below the 95% confidence interval (95%CI) for random mating expectation.
218 This was observed from the graphic outputs of MicroChecker (Figure 1).

219

220 Figure 1: Examples of significant stuttering tests using MicroChecker graphic outputs.
 221 Black crosses represent the number of observed genotype of a given class and red
 222 diamonds stand for the corresponding values expected under the null hypothesis
 223 (random mating). The abscissa are the different genotypic classes in terms of size
 224 differences between the two alleles within an individual (e.g. 0 stands for
 225 homozygous genotypes). 1-A: an example with a significant homozygous excess
 226 and a significant deficit of heterozygotes with one repeat size difference between
 227 the two alleles. 1-B: an example where only the deficit of heterozygotes with one
 228 repeat size difference between the two alleles was significant. In the present paper,
 229 both situations are considered significant while only the first one is for
 230 MicroChecker,



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 232
 233

234 For each locus, we summed the number of times MicroChecker found a significant
235 heterozygote deficit probably due to stuttering over the 10 subsamples. We compared this
236 quantity with the expected 5% under the null hypothesis with a one sided exact binomial
237 test with R (R-Core-Team, 2020) (command "binom.test"). The alternative hypothesis was
238 that there are more than 5% significant tests. This test was repeated 20 times across the
239 different loci. To take into account this repetition of independent tests, we used the
240 Benjamini and Hochberg's (BH) false discovery rate (FDR) procedure (Benjamini &
241 Hochberg, 1995) with R (R-Core-Team, 2020) (command "p.adjust") to identify which tests
242 are really significant (see (De Meeûs et al., 2009)).

243 244 **Alternative method to detect and test for stuttering**

245 We needed to compute the expected frequency of individuals heterozygous for two
246 alleles with one repeat difference, for each locus over all subsamples. All allele
247 frequencies outputted and sorted by Fstat were copied in a spreadsheet. Let us assume,
248 for instance, that subsample size was in cell B3, that the size of the smallest allele of the
249 first locus of the first subsample was in cell A4 and its frequency in cell B4, and allele size
250 and allele frequency of the second allele was in cells A5 and B5 respectively. Then the
251 expected frequency of individuals heterozygous for two alleles with one repeat difference
252 was obtained by typing the following command in, for instance, cell C4:

253 `=IF(ABS($A4-$A5)=1,2*B4*B5*B$3,0)`

254 As can be seen, for two successive alleles with more than one repeat difference,
255 this expected frequency was set to 0. Please, note that for a dinucleotidic locus the
256 difference in size must be two (e.g. `ABS($A4-$A5)=2`). For imperfect dinucleotidic loci, the
257 conditional command would be of the form
258 `=IF(OR(ABS($A4-$A5)=1, ABS($A4-$A5)=2),2*B4*B5*B$3,0)` [to include both the cases of](#)
259 [one base difference, which may also generate stuttering, and of two bases \(one repeat\)](#)
260 [difference](#).

261 Now, if the penultimate allele is on line 10 of the spreadsheet, the sum of all
262 expected heterozygotes with one repeat difference for the concerned locus and subsample
263 was obtained by typing the following command in, for instance, cell C12:

264 `=SUM(C4:C10)`

265 Finally, if this sum for the last subsample is in column U, then, the total number of
266 expected heterozygotes with one repeat difference across all subsamples for that locus
267 was obtained with:

268 `=SUM(C12:U12)`

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=SI(ABS(\$A4-\$A5)=1;2*B4*B5*B\$3;0) in French.

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=SI(OU(ABS(\$A4-\$A5)=1; ABS(\$A4-\$A5)=2);2*B4*B5*B\$3;0) in French

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=SOMME(C4:C10) in French

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=SOMME(C12:U12) in French

278 Then, we needed to compute the observed frequency of such heterozygotes. For
 279 this, we copied the raw data (one allele per column) in a spreadsheet. Let us assume that
 280 the first allele of the first locus of the first individual was in cell B2 and the last allele of the
 281 last locus of the first individual was in cell AO2. In cell AQ2 we typed:

282 =IF(ABS(B2-C2)=1,1,0).

283 Please, note again that for dinucleotidic loci, the difference in size would be two,
 284 and it should be one and two for imperfect dinucleotidic loci. We then copied this
 285 command in all remaining cells corresponding to the rest of the dataset. In the cell AQ202
 286 (below the last line of the data), to compute the total of observed heterozygous individuals
 287 for alleles with one repeat difference, we typed:

288 =SUM(AQ2:AQ201).

289 [A template, for the first simulation, is available in the spreadsheet file](#)
 290 ["TestStutterDioeciousNoStutter-n1000N100-1-FstatRes.xlsx"](#).

291 We then compared the observed and expected frequencies with a one sided exact
 292 binomial test with R, the alternative hypothesis being "there are less heterozygote
 293 observed with 1 repeat difference than expected". This provided 20 independent p -values
 294 that we corrected for [False Discovery Rate](#) with the Benjamini and Hochberg's procedure.

295 With selfing, natural homozygosity increase may artificially enhance stuttering
 296 detection. Nevertheless, since there is no easy way to estimate precisely the selfing rate in
 297 case of stuttering, we did not adapt stuttering detection.

298 For clonal propagation, full clonality exhibits specific signature regarding genetic
 299 diversity, F_{IS} and linkage disequilibrium (De Meeûs & Balloux, 2004, 2005; De Meeûs et al.,
 300 2006; Séré et al., 2014). Nevertheless, as shown by the simulations we undertook, the
 301 absence of segregation makes it impossible to predict the expected frequency of specific
 302 heterozygous classes. This led us to entirely modify stuttering detection in that case.
 303 Knowing that the expected proportion of homozygous sites is $Q_i=1/K$ in clonal populations
 304 (De Meeûs, 2015), the expected total heterozygosity should be $H_i=(K-1)/K$ (K is the total
 305 number of possible alleles). The quantity K is never known, so we considered the total
 306 number of alleles observed K_o , as an underestimate, with $H_i'=(K_o-1)/K_o$. Some heterozygote
 307 kinds are expected to be more frequent than others. We thus considered frequencies p_i and
 308 p_j of alleles i and j , to weight expected values by $2p_i p_j$ and built an "expected" heterozygote
 309 frequency between alleles i and j as:

310
$$H_{expij} = \frac{2p_i p_j \left(\frac{K_o - 1}{K_o}\right)}{\left(\sum_{i,j \neq i}^{K_o} 2p_i p_j\right)} = \frac{2p_i p_j \left(\frac{K_o - 1}{K_o}\right)}{\left(1 - \sum_i^{K_o} p_i^2\right)}$$

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 =SI(ABS(B2-C2)=1;1;0) in French

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 =SOMME(AQ2:AQ201) in French

Commenté [FH6]: add reference to Stoeckel & Masson
 2014
 Stoeckel, S., & Masson, J.-P. (2014). The Exact
 Distributions of FIS under Partial Asexuality in Small
 Finite Populations with Mutation. *PLoS ONE*, 9(1),
 e85228. doi: [10.1371/journal.pone.0085228](https://doi.org/10.1371/journal.pone.0085228)

316 We can see that this way the sum of all H_{expij} indeed reaches H' . For each locus,
317 H_{expij} was computed for each heterozygous class with one repeat difference within each
318 subpopulation. We then summed all these expected frequencies over all heterozygote
319 classes with one repeat difference and multiplied it by 20 (subsample size) to obtain the
320 expected number of heterozygotes with one repeat difference for a given subsample. We
321 then summed the results obtained across all the 10 subsamples to obtain the total number
322 of expected heterozygotes with one repeat difference and compared it, for each locus, to
323 the one observed in the simulation. Since $K_0 \leq K$, these expectations may be under-
324 estimates of the real expected frequencies. We might thus expect a deficiency in stuttering
325 detection. Alternatively, since drift should favor particular heterozygous classes by chance,
326 we also expect a total lack of other heterozygous classes, which may lead to **strongly**
327 **significant spurious stuttering signatures**.

Commenté [FH7]: This looks like a stuttering wording pattern (SSSS) :-)

329 **Estimation of Fixation indices and linkage disequilibrium**

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330 Wright's F -statistics (Wright, 1965) estimated with Weir and Cockerham's estimators
331 (Weir & Cockerham, 1984) were computed. F_S measures inbreeding of individuals relative
332 to inbreeding of their subpopulation and F_{ST} measures inbreeding of subpopulations
333 relative to the total inbreeding. We also computed the 95% confidence intervals of
334 bootstrap over loci of these statistics. These were estimated and computed with Fstat
335 2.9.4 (Goudet, 2003) updated from Fstat 1.2 (Goudet, 1995).

336 Amplification problems can increase the variance of F -statistic estimation across
337 loci, and this affects more the F_S than the F_{ST} (De Meeûs, 2018). We used the jackknife
338 over loci estimate of the standard error of F_S and F_{ST} (StdrdErrFIS and StdrdErrFST) of
339 Fstat to measure the effect of stuttering on parameter variation across loci. In particular, in
340 case of null alleles, StdrdErrFIS is at least twice StdrdErrFST (De Meeûs, 2018). We thus
341 measured the ratio $R_{SE} = \text{StdrdErrFIS} / \text{StdrdErrFST}$.

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342 Linkage disequilibrium can be favored by allele miscoring (De Meeûs et al., 2021).
343 We thus tested linkage disequilibrium between all pairs of loci with the G-based
344 randomization test of Fstat over all subsamples because it is the most powerful for
345 combining tests across subsamples (De Meeûs et al., 2009). The **False Discovery Rate** for
346 dependent tests series was computed following Benjamini and Yekutieli procedure
347 (Benjamini & Yekutieli, 2001) with R (command p.adjust).

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352 **Statistical comparisons of method performances**

353 Performance of tests were compared with the Fisher exact test with R-commander
354 package (Fox, 2005; Fox, 2007) for R.

355 We also undertook generalized linear mixed models with the package lme4 (Bates
356 et al., 2015) of R to explain the number of times a test appeared significant. We used a
357 Poisson distribution with a log link. The models were of the form
358 $NSig \sim n + N + Mating + Stuttering + Mating:Stuttering + (1|Rep)$
359 where NSig was the number of loci that outputted a significant stuttering test or the
360 number of locus pairs that appeared in significant LD, n is the total number of
361 subpopulations, N was the size of subpopulations, Mating was the mating system (either
362 pangamic dioecy or Monoecy with 30% selfing), Stuttering was either 0 (no stuttering) or
363 10 (10% stuttering), ":" stood for interaction between two variables, and (1|Rep) was the
364 random effect of replicates.

366 **Cured data sets**

367 Stuttering correction was made for loci that appeared with a significant stuttering at
368 the BH level with the new method described in the present paper. We used the rules
369 described in (De Meeûs et al., 2021): for each incriminated locus, all alleles with one
370 repeat difference were pooled together. Each group of pooled alleles contained an allele
371 the frequency of which was at least 0.05. The main principle behind this rule is that rare
372 alleles should keep small weights in the data. Pooling rare alleles together may artificially
373 create a fairly frequent artificial allele, with a strong though artificial weight. Pooling rare
374 alleles with a reasonably frequent one is supposed to attenuate this problem. If no frequent
375 allele was available, then two solutions were chosen. If the sum of the frequencies of these
376 alleles remained below 0.05, these were not pooled. Otherwise, to minimize the impact
377 that these successive alleles may jointly have on the heterozygote deficit, and to avoid
378 pooling rare alleles together, they were pooled with the closest allele with frequency above
379 or equal to 0.05, even if more than one repeats distant from the closest one.

380 In rare cases, all alleles were one repeat different. To prevent obtaining a
381 monomorphic locus in that case, we pooled alleles two by two, taking care of one of the
382 two alleles pooled displayed a frequency of at least 0.05. In case of uneven number of
383 alleles, the last allele was not left alone and pooled with the previous pair in allele size.

384 Cured data were reanalyzed and statistics compared with the results expected
385 under the null hypothesis (without stuttering). The efficiency of the correction was checked
386 for each locus in each replicate of each simulation. We retained only the corrected loci for

Commenté [FH8]: awkward sentence. Change to :
Each group of pooled alleles contained one allele with a
frequency of at least 0.05%.

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391 which the correction produced a lower F_{IS} as compared to the value obtained with the
392 uncured locus. The average F_{IS} and 95% bootstraps confidence intervals were thus
393 computed on data sets with efficiently cured loci and uncured remaining loci. These values
394 were compared to those obtained under the null hypothesis (no stuttering), and to
395 expected value for Wright's F_{IS} , F_{IS_exp} . For pangamic dioecious populations we used
396 equation 8 from Balloux (2004) (Balloux, 2004): $F_{IS_exp} = -1/(2N+1)$. For selfing populations,
397 we used the classic $F_{IS_exp} = s/(2-s)$ (e.g. (De Meeûs et al., 2007) page 213).

398

399 Real data sets

400 Five data sets were reanalyzed: two regarding dioecious species, two regarding
401 monoecious species and the last regarding a clonal species.

402 The first real data set reanalyzed was on the tick *Ixodes scapularis* the vector of
403 Lyme disease in western USA (De Meeûs et al., 2021). We used the data cured for [short](#)
404 [allele dominance \(SAD\)](#) as explained in the originator paper (De Meeûs et al., 2021) but
405 uncured for stuttering.

406 The second data set concerned the tsetse fly *Glossina palpalis palpalis*, an
407 important vector of sleeping sickness in Côte d'Ivoire (Berté et al., 2019), for which loci
408 X55-3 and pGp23 displaying uncured SAD and locus GPCAG, obviously under selection,
409 were removed. Because some loci were X-linked, only females were kept. Three
410 dinucleotidic loci (pGp20, pGp24, B3) displayed some discrepancies of allele sizes and
411 were marked as mononucleotidic for MicroChecker. For these loci, heterozygotes with
412 single and double nucleotide differences in size were checked for heterozygote deficit due
413 to stuttering.

414 The third and the fourth data sets concerned the highly selfing snail *Galba*
415 *truncatula* and its parasite *Fasciola hepatica*, also monoecious but almost panmictic, in
416 France (Correa et al., 2017). For both species, microsatellite profiles did not fit with the
417 expected pure dinucleotidic motives and, again, stuttering was considered between alleles
418 of 1 base and two bases differences in size.

419 Finally, the clonal species studied was *Trypanosoma brucei gambiense* 1 in
420 Western African foci of sleeping sickness (Koffi et al., 2009), for which one locus
421 (Trbpa1/2), suspected of being under selection, was removed. For this data, we used a
422 derived version of Séré et al superimposition criterion (Séré et al., 2014). In pure clonal
423 populations, the expected value for F_{IS} is $F_{IS_exp} = -(1-H_s)/H_s$, where H_s is Nei's estimator of
424 local genetic diversity (measured within subsamples) (Nei & Chesser, 1983). This criterion
425 can only be used for sufficiently polymorphic loci with $H_s \geq 0.5$. To express the goodness of

426 fit of observed F_{IS} towards this value, we designed a superimposition index $S_c = |F_{IS} -$
427 $F_{IS_exp}| / \max(|F_{IS}|, |F_{IS_exp}|)$, where "max" means the maximum value of the two F_{IS} 's
428 absolute values.

429

430 **Results [and discussion](#)**

431 A synthetic view of simulation results, averages detail computations and test tables
432 are available in the supplementary file S1 for sexual simulations and in the supplementary
433 file S2 for clonal simulations.

434

435 **Detection of stuttering in sexual populations**

436 [The results of these analyses are summarized in Table 1.](#) Stuttering detection per
437 locus was weak in general, with 0‰ and 5‰ significant tests for MicroChecker and the
438 alternative methods, respectively, under the null hypothesis (H0: [there is no stuttering](#)) in
439 monoecious populations. For Microchecker, the total proportion of significant tests over all
440 loci and subsamples was 2‰ in that case. No test stayed significant after [Benjamini and](#)
441 [Hochber](#) correction. In populations with 30% selfing, but still under H0 (i.e. no stuttering),
442 these proportions increased to 10‰ for the alternative method only. It dropped to 2.5‰
443 after [Benjamini and Hochberg](#) correction. For MicroChecker over all loci and subsamples,
444 2‰ only appeared significant under H0 with selfing. With stuttering (H1), the number of
445 significant tests reached 7% and 24% in dioecious populations for MicroChecker and
446 alternative methods respectively (5% and 17% respectively with [Benjamini and Hochberg](#)).
447 This reached 14% and 47% respectively with 30% selfing (10% and 38% respectively with
448 [Benjamini and Hochberg](#)).

449

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Supprimé: In Table 1, we can see that, except under H0 in pangamic dioecious populations, the alternative method appeared much more performant than MicroChecker for the detection of stuttering.¶

459 **Table 1:** Statistical comparisons between MicroChecker and the alternative method to
 460 detect stuttering under the null hypothesis with no stuttering or with 10% of alleles
 461 affected, for different system of mating (dioecious pangamy or 30% of selfing). The
 462 *p*-values obtained correspond to a Fisher exact test comparing number of significant
 463 (S) and not significant (NS) tests (before Benjamini and Hochberg correction)
 464 between the two methods.

Stuttering	Mating	Method	S	NS	<i>p</i> -value
0	Pangamy	MicroChecker	0	600	0.2494
		Alternative method	3	597	
	30% Selfing	MicroChecker	0	400	<0.0001
		Alternative method	41	359	
10%	Pangamy	MicroChecker	43	557	<0.0001
		Alternative method	146	454	
	30% Selfing	MicroChecker	56	344	<0.0001
		Alternative method	187	213	

Commenté [FH9]: Coud you find a way to combine the three tables. It is the same test applied to all contingency tables.

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465
 466
 467 As can be seen from Table 2, selfing significantly increased stuttering detection,
 468 even under H0, where it significantly appeared above the 5% threshold (*p*-value<0.0001).

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469
 470 **Table 2:** Statistical comparisons between dioecious pangamic populations with
 471 monoecious populations with 30% selfing to detect stuttering with the alternative
 472 method under the null hypothesis with no stuttering or with 10% of alleles affected.
 473 The *p*-values obtained correspond to a Fisher exact test comparing number of
 474 significant (S) and not significant (NS) tests.

Stuttering	Mating	S	NS	<i>p</i> -value
0	Pangamy	3	597	<0.0001
	30% Selfing	41	359	
10%	Pangamy	146	454	<0.0001
	30% Selfing	187	213	

475
 476
 477 Expectedly, stuttering was much more easily detected in populations with stuttering
 478 than under H0 (Table 3).

479

483 Table 3: Statistical comparisons of stuttering detection, with the alternative method,
 484 between cases with no stuttering or with 10% of alleles affected, in dioecious
 485 pangamic populations or in monoecious populations with 30% selfing. The p -values
 486 obtained correspond to a Fisher exact test comparing number of significant (S) and
 487 not significant (NS) tests.

Mating	Stuttering	S	NS	p -value
Pangamy	0	3	597	<0.0001
	10%	146	454	
30% Selfing	0	41	359	<0.0001
	10%	187	213	

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488
 489 [The stuttering proportion used here \(10%\) was relatively small, since the realized](#)
 490 [actual proportion of alleles affected at the end of simulations was in general much lower on](#)
 491 [average. This also explains why the power of stuttering detection appeared quite small.](#)
 492 [With higher values, we may expect that the method proposed here will be very accurate,](#)
 493 [especially in inbred populations \(selfers\).](#)

495 Fixation indices and linkage disequilibrium

496 The results for F -statistics are presented in Table 4. With 10% stuttering, we
 497 observed a significant heterozygote deficit of 4% in pangamic dioecious populations. With
 498 30% selfing, F_{IS} expectedly grew much more and reached 20%. Here, the difference
 499 between 0 and 10% of allele submitted to stuttering was not significant (95% CI overlap).

Commenté [FH10]: yes but compared to the initial value, the increase is only 4.4 %. Not very different from the 4% in pangamic population.

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501 Table 4: Results obtained for F_{IS} , its 95% confidence interval (95%CI, 5000 bootstraps
 502 over loci) and for the ratio of jackknife over loci standard error between F_{IS} and F_{ST}
 503 (R_{SE}), for 0 or 10% of stuttering and for pangamic dioecious populations or
 504 monoecious populations with 30% selfing.

Stuttering	Mating	F_{IS}	95%CI F_{IS}	R_{SE}
0	Pangamy	0.0002	[-0.0211, 0.0214]	1.0756
	30% selfing	0.1761	[0.1573, 0.1947]	0.9912
10%	Pangamy	0.0408	[0.0086, 0.0768]	1.8172
	30% selfing	0.2202	[0.1892, 0.2542]	1.6735

505
 506

509 The ratio between standard errors of F_{IS} and F_{ST} was 1.04 on average under H_0 in
510 95% CI=[0.95, 1.14] and reached 1.77 in 95%CI=[1.52, 2] with stuttering. It thus increased
511 slightly with stuttering but rarely reached $R_{SE}=2$ on average, [as was observed in case of](#)
512 [null alleles](#) (De Meeûs, 2018).

513 The proportion of locus pairs in significant LD varied between 8% and 53%
514 depending on the population structure and mating system (average 18%). With [the](#)
515 [Benjamini and Yekutieli False Discovery Rate](#) correction, this varied between 0% (majority
516 of cases) and 15% (average 3%). The effect of stuttering on LD was never significant,
517 whatever the mating system (all p -values>0.388). [This could be expected since in our](#)
518 [simulations, stuttering was not correlated between loci. In real datasets, however, it may](#)
519 [occur that stuttering happen in samples with issues \(poor preservation, mutations affecting](#)
520 [the zone of primers' anchorage, low DNA concentration\). In that case, several loci of the](#)
521 [same individuals will be affected together, then producing fake significant LDs, as was](#)
522 [observed for the tick *I. scapularis*](#) (De Meeûs et al., 2021).

523

524 **Generalized linear mixed models**

525 The generalized linear mixed models confirmed the results seen above with more
526 accuracy.

527 For the number of significant tests, the results figure in Table 5. All parameters
528 appeared to display a significant effect that stayed so after BH correction. The most
529 important parameters were stuttering (positive effect), mating system (selfing increases the
530 effect) and their interaction (more effect of stuttering in random mating dioecious
531 populations). Number of subpopulations and subpopulation sizes displayed a rather weak
532 (though significant) negative effect, but this is probably an artefact due to inconsistencies
533 of results as a function of n or N (see supplementary File S1). For instance, $N=50$, under
534 H_0 with random mating, provided the smallest numbers of significant stuttering while
535 $N=100$ provided more significant results than $N=200$. In the same framework, for
536 subpopulation numbers, it was $n=500$ that provided the smallest number of significant
537 tests, followed by 100 and 50. Similar observations can be done for 10% of alleles affected
538 by stuttering and/or in monocious populations with 30% selfing (see supplementary File
539 S1).

540

541 Table 5: Summary of the generalized linear mixed model for the number of loci found with
 542 a significant stuttering (response variable) with the new alternative multi-
 543 subsamples method. Explanatory variables were: n (number of subpopulations), N
 544 (subpopulation size), mating system (dioecious pangamy or monoecy with 30%
 545 selfing) and stuttering intensity (0 or 10%). In case of qualitative variables (mating
 546 system), the modalities with least positive effects are compared to the one with the
 547 most positive effect (not shown in the output of the analysis). "." stands for the
 548 interaction between two variables. Coefficient estimates (Estimate), standard error
 549 (SE), the Z statistic and its p -value are given.

	Estimate	SE	Z	p -value
(Intercept)	1.2942	0.2787	4.644	<0.0001
n	-0.0024	0.0007	-3.541	0.0004
N	-0.0027	0.0013	-2.043	0.0411
Mating[T.Dioecious]	-2.8398	0.5995	-4.737	<0.0001
Stuttering	0.1518	0.0172	8.8	<0.0001
Mating[T.Dioecious]:Stuttering	0.2367	0.0608	3.892	<0.0001

550
 551
 552 For LD, results are presented in Table 6. The main effects were hold by n , with a
 553 positive impact, and mating system with a strong negative impact of random mating as
 554 compared to selfing. Subpopulation sizes seemed to play a weaker though significant role.
 555 Nevertheless, the pattern of simulations explored introduced a strong collinearity between
 556 n and N . If n is removed from the model, then N become highly significant with a much
 557 stronger (as expected) negative impact (Coefficient of estimate=-0.006). Stuttering did not
 558 influence at all the occurrence of significant linkage disequilibrium between pairs of loci.
 559 These conclusions did not change when the [Benjamini and Hochberg](#) procedure was
 560 applied to these series of p -values.
 561

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563 Table 6: Summary of the generalized linear mixed model for the number of locus pairs
 564 found with a significant linkage disequilibrium. Explanatory variables were: n
 565 (number of subpopulations), N (subpopulation size), mating system (dioecious
 566 pangamy or monoecy with 30% selfing) and stuttering intensity (0 or 10%). In case
 567 of qualitative variables (mating system), the modalities with least positive effects are
 568 compared to the one with the most positive effect (not shown in the output of the
 569 analysis). ":" stands for the interaction between two variables. Coefficient estimates
 570 (Estimate), standard error (SE), the Z statistic and its p -value are given.

	Estimate	SE	Z	p -value
(Intercept)	3.1325	0.1754	17.858	<0.0001
n	0.0040	0.0004	11.164	<0.0001
N	-0.0020	0.0009	-2.15	0.0316
Mating[T.Dioecious]	-0.5683	0.1029	-5.522	<0.0001
Stuttering	-0.0056	0.0104	-0.543	0.5872
Mating[T.Dioecious]:Stuttering	0.0067	0.0134	0.501	0.6162

571
 572
 573 **Clonal populations**

574 The proportion of significant stuttering [detection](#) was very [large](#), even under H0:
 575 53% of significant tests, 50% with [Benjamini and Hochberg](#). [Under H1, these](#) proportions
 576 increased slightly (61 and 57.5 % respectively), but not significantly so (for the uncorrected
 577 tests, the Fisher exact test [outputted](#) a p -value=0.157). Wilcoxon rank sum tests found no
 578 significant difference between data without stuttering and data with 10% stuttering for F_{IS}
 579 or R_{SE} (p -value=0.2481 and p -value=0.4698, respectively). The expectation for F_{IS}
 580 following Balloux et al (2003)'s Equation 10 (Balloux et al., 2003), for an infinite allele
 581 model, and set for full clonality, gives:

$$F_{IS} = \frac{\gamma[q_s - \gamma(q_s - q_d)]}{2N(1 - \gamma)[\gamma(q_s - q_d) - 1] - \gamma[q_s - \gamma(q_s - q_d)]}$$

582 with $\gamma=(1-u)^2$; $q_s=(1-m)^2+m^2/(n-1)$, and $q_d=(1-q_d)/(n-1)$.

583 With the actual parameters, this gave $F_{IS_exp}=-0.3284$. The averages of F_{IS} and 95%
 584 bootstrap confidence intervals (95%CI) [were](#), $F_{IS}=-0.3649$ in 95%CI=[-0.409, -0.315]
 585 without stuttering; and $F_{IS}=-0.3443$ in 95%CI=[-0.3962, -0.2845], with 10% stuttering. [Both](#)
 586 [confidence intervals largely overlapped and contained \$F_{IS_exp}\$](#) .

587 [Clonal populations generated very high proportions of false stuttering detections.](#)
 588 [This was because without segregation of alleles, only some classes of heterozygotes](#)

- Supprimé: signatures
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Déplacé (insertion) [2]

propagate by chance. Heterozygote classes with one repeat difference are quite rare. Indeed, if all 20 alleles were present (which never happened), there would be 19 heterozygote classes with one repeat difference amongst $\binom{20}{2}=190$ possible heterozygous states, hence a proportion of 0.1. Nevertheless, not all allele combinations were kept by drift, which means that in many instances, even if some alleles were one repeat different at the end of simulations, no such heterozygotes were kept by drift. This produced many tests with very small p -values < 0.0001 , but also many with very high p -values > 0.5 : 43 % and 53 % respectively. Put it another way, many simulations ended with no individuals heterozygous for two alleles with one repeat difference, and many others with too many of those (more than 20%): 40 % and 20 % respectively. This also explains why stuttering did not have much impact on the global results, such as F_{IS} estimates. It means that much more than 10% stuttering will be needed to significantly affect parameter estimates in clonal organisms. In such situations, stuttering detection will need being considered with the necessary prudence (see the conclusion) in fully clonal populations. 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.

Cured data

The alleles that were indeed pooled are highlighted with the same color and can be found in excel files that are contained in the supplementary file S3 (zipped file) "PoolingProtocolCureSupFileS3.zip".

In dioecious pangamic simulations, cured data did not entirely fix the stuttering problem. Indeed, using the new method for stuttering detection, eight tests (1.3 %) remained significant after BH correction, which is significantly more than the initial absence of significant test (Fisher exact test, p -value=0.0076), before stuttering was introduced (H0). In monoecious populations with 30% selfing, two tests remained significant (0.5 %), which is not significantly more than the initial result (one significant test) under H0 (p -value=1).

Regarding F_{IS} estimates, in pangamic simulations, the fit between the expected values and the one observed in simulations under the null hypothesis, was not very good and better for larger n 's (not shown). We thus preferred the more complex but more accurate equation 11 in (Vitalis, 2002):

$$F_{IS_exp} = - \frac{\gamma[q_s - \gamma(q_s - q_d)]}{2N[1 - \gamma(q_s - q_d)] + \gamma[q_s - \gamma(q_s - q_d)]}$$

With this equation, the fit was very good, as can be seen in Figure 2.

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Commenté [FH11]: I am not convinced, especially because partial but high clonal rate (above 0.9) would lead to a very twisted distribution of FIS values across loci (see for ex fig 3 in Stoeckel & Masson 2014).

Supprimé: The strategy used to pool close in size alleles followed what was written in the Material and Methods section.

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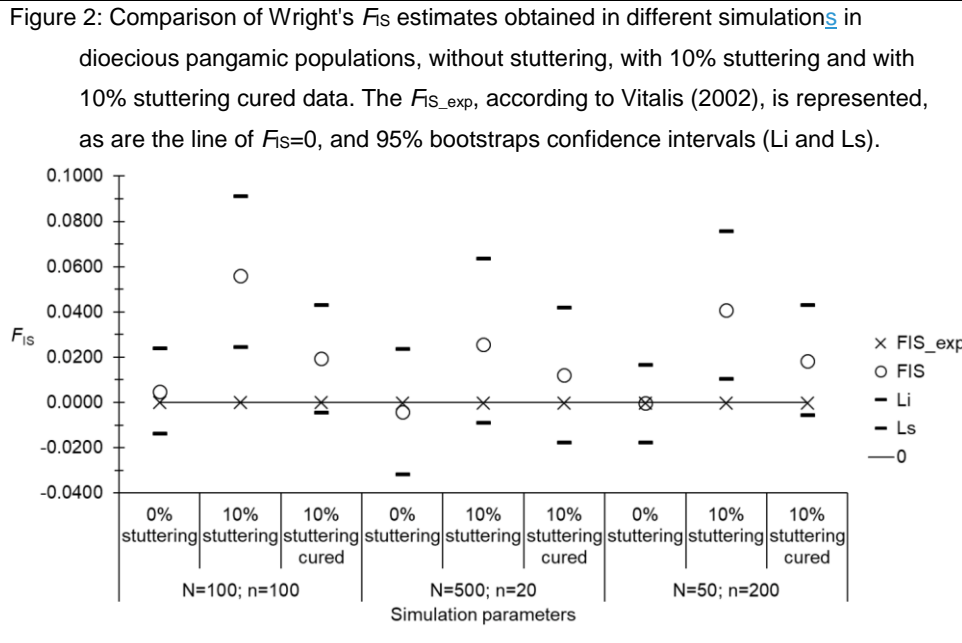
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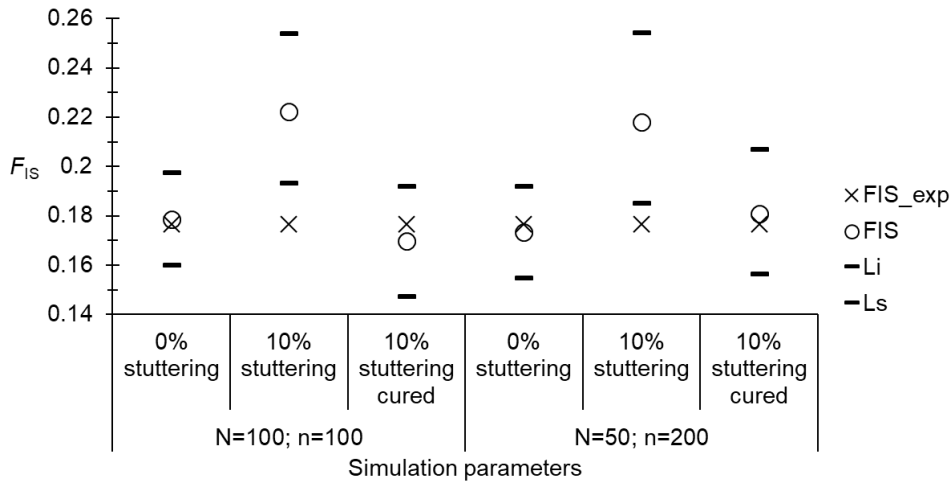
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651

From this figure, we can see that stuttering had a significant impact on F_{IS} estimates, driving it sometimes quite far above the expected value. Cured data, though always providing values higher as compared to H_0 , always included the expected value in their 95% confidence intervals.

As can be seen from Figure 3, in monoecious simulations with 30% of selfing, the fit is almost perfect between F_{IS_exp} and the value obtained under H_0 (no stuttering). Stuttering expectedly significantly increased observed F_{IS} , while cured data presented values that were almost superimposed with expected ones.

652 Figure 3: Comparison of Wright's F_{IS} estimates obtained in different simulations in
 653 monoecious populations with 30% selfing, without stuttering, with 10% stuttering
 654 and with 10% stuttering cured data. The $F_{IS_exp}=s/(2-s)$, is represented, as are the
 655 95% bootstraps confidence intervals (Li and Ls) around observed values.



656

657

658 In conclusion, 10% stuttering significantly increased F_{IS} and the cure used
 659 reasonably restored the F_{IS} expected under the null hypothesis, and particularly so in
 660 inbred populations (with 30% of selfing).

661

662 **Real datasets**

663 For *I. scapularis* from Western USA, MicroChecker test only found one locus with
 664 significant stuttering out of nine (i.e. 11%), after exact binomial tests and Benjamini and
 665 Hochberg correction. Alternatively, the new method developed here found three loci out of
 666 nine loci with a significant stuttering (33%). Cured data provided results in agreement with
 667 a pangamic reproductive strategy, as was already observed by the authors (De Meeûs et
 668 al., 2021).

669 For *Glossina palpalis palpalis* in Côte d'Ivoire, no binomial tests provided a
 670 significant result with MicroChecker statistics. For the new method presented here, two loci
 671 (B3 and XB110) out of seven (22.22%) displayed a significant stuttering. Cured data set
 672 was obtained by pooling alleles following De Meeûs et al (2019)'s rules (De Meeûs et al.,
 673 2021) (see Appendix 2, A2.1). This cure provided a slightly lower $F_{IS}=0.221$ (instead of
 674 0.231) for locus B3, but a higher one for locus XB110 ($F_{IS}=0.277$ instead of 0.252),

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Supprimé : the results were similar between those obtained with our method and the published results with MicroChecker because these were submitted neither to exact binomial tests nor to subsequent BH correction. Nevertheless

Supprimé : 7

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Supprimé : 4.09

Supprimé : After exact binomial tests, only a single locus gave a significant stuttering test with MicroChecker (11%). All these tests stayed significant after BH correction

Supprimé : no stuttering was initially found because author used a different criterion (a significant global heterozygote deficit was necessary to validate a one-repeat heterozygote deficit observed in the graphics of MicroChecker) as compared to what we used here. Nevertheless, only two tests out of 98 (2.04%) gave a significant stuttering with MicroChecker, and

Supprimé : even after BH correction

Déplacé vers le bas [1]: For Locus B3 allele 203 was recoded 201; allele 207 was recoded 205; allele 211 was recoded 209; and alleles 215 was recoded 213. For Locus XB110, alleles 179 was recoded 177; allele 183, was recoded 181; alleles 187 to 199 were recoded 185, allele 203 was recoded 201 and all rare alleles from 205 to 277 were also recoded 203.

703 meaning that the heterozygote deficit at this locus was better explained by another
704 phenomenon (i.e. null alleles).

705 In *G. truncatula*, [after Benjamini and Hochberg correction, 50% of loci displayed a](#)
706 [significant stuttering, while](#) all six loci [were](#) significant [with the new method](#) (all p -
707 $BH < 0.0104$). Allele pooling [for curing the data](#) was as [described in Appendix 2 \(A2.2\)](#). This
708 cure lowered the F_{IS} for three loci, Lt9 (0.778 to 0.776), Lt16 (0.958 to 0.006), and Lt24
709 (0.966 to 0.947) and increased it for the others. However, missing data (assumed null
710 homozygotes) explained almost 50% of F_{IS} variation, with a highly significant Spearman's
711 rank correlation between F_{IS} and number of blank genotypes ($\rho = 0.9411$, p -value = 0.0025),
712 while with the data set cured for loci Lt9, Lt16 and Lt24, the correlation dropped to a non-
713 significant value (p -value = 0.2589). When removing the correction for locus Lt16, which
714 provided an outlier as compared to other loci, the correlation became significant again (p -
715 value = 0.0103), but with a smaller correlation ($\rho = 0.8804$) and only 41% of F_{IS} variation
716 explained by missing genotypes.

717 For *F. hepatica*, [no locus was significant with MicroChecker, while](#) only one locus
718 (Fh28) presented a significant stuttering signature [after Benjamini and Hochberg](#)
719 [correction](#) (p - $BH = 0.0001$). Pooling of alleles at this locus [is described in Appendix 2](#)
720 [\(A2.3\)](#). The F_{IS} of cured data dropped from 0.644 to 0.536. However, we knew that null
721 alleles explained well most of the observed heterozygote deficit (Correa et al., 2017), and
722 the correlation between missing data and F_{IS} , when excluding one locus (Fh25) that
723 displayed too many missing genotypes, was initially significant ($\rho = 1$, p -value = 0.0417), with
724 83 % of the F_{IS} variation explained by blank genotypes. In the cured data, it dropped to a
725 not significant relationship ($\rho = 0.8$, p -value = 0.1667), with 72 % of F_{IS} variation explained by
726 missing genotypes.

727 [These discrepancies strongly suggested that stuttering detection in *G. truncatula*](#)
728 [and *F. hepatica* corresponded to type error I, due to the fact that null alleles better explain](#)
729 [the data and probably interacted with our stuttering detection test. It shows that several](#)
730 [checks need being undertaken before deciding that a locus is significantly affected by](#)
731 [stuttering and requires being cured, especially in selfing species.](#)

732 For the clonal *T. brucei gambiense* 1, [we avoided using MicoChecker \(for obvious](#)
733 [reasons\). With the method expounded in the present paper, three](#) loci displayed a
734 significant stuttering signature: micbg1, misatg4 and misatg9. [These loci were cured as in](#)
735 [Appendix 2 \(A2.4\)](#). These loci presented a lower F_{IS} when cured: from -0.647 to -0.818, from
736 -0.579 to -0.72, and from -0.471 to -0.496 for micbg1, misatg4 and misatg9, respectively.
737 Moreover, several observations suggested an improvement of the quality of the data after

Supprimé: displayed a

Supprimé: stuttering signature after BH correction

Supprimé: followed

Supprimé: : for locus Lt9, 203 with 202, 210 and 212 with 208; for Lt16, 231 to 233 with 230; for Lt21, 107 with 105, 112 with 111, 115 and 116 with 114; for Lt24, 208 and 210 with 207, 215 to 217 with 214, 220 and 221 with 219; for Lt36, 187 to 190 and 192 with 185; and for Lt37, 115 with 113, and 123 with 212

Supprimé: was the following:

Supprimé: Alleles 182 to 183 with 180, allele 188 with 186, and alleles 192 to 194 with 190

Supprimé: indeed

Supprimé: t

Supprimé: , according to the criterion defined in the Material and Methods section

Supprimé: Curing the data implied the

Supprimé: following: for micbg1, allele 164 was pooled with 162, and allele 194 with 192; for misatg4, allele 117 with 115, and 145 with 143; and for misatg9, alleles 130 and 128 with 126, allele 186 with 184, and alleles 194 and 192 with 190

Supprimé: indeed

761 the cure for stuttering. The proportion of significant linkage disequilibrium between locus
 762 pairs increased from 53% (uncured data) to 80% (data cured for the three loci), and the
 763 superimposition S_c increased from 0.9554 to 0.9782, from 0.9800 to 0.9975, and from
 764 0.9539 to 0.9663 for micbg1, misatg4 and lisatg9 respectively. Three loci with missing
 765 genotypes could be suspected to display null alleles, i.e. misatg4, misatg9 and m6c8.
 766 When these loci were removed, [averaged](#) superimposition increased to $S_c=0.9908$. [Here](#)
 767 [again, we can see that some checks allowed deciding that stuttering corrections were valid](#)
 768 [and significantly improved the quality of the data. After stuttering cure, removing other loci](#)
 769 [with suspected null alleles \(i.e. with missing genotypes\) drove the superimposition index](#)
 770 [defined in the Material and Methods section to almost unity, i.e. a perfect fit with the](#)
 771 [expected value under full clonality known to occur in that species](#) (Weir et al., 2016).

773 Conclusions

774 The new method developed here appeared at least three times more efficient (and
 775 often much more) than MicroChecker. [Moreover, the use of spreadsheet programs makes](#)
 776 [its portability universal for any microcomputer.](#)

779 In dioecious pangamic populations, like ticks and tsetse flies, detection works well
 780 and cure improves population genetics parameter estimates but not perfectly so, which
 781 means that, for instance, F_{IS} and F_{ST} will still be slightly overestimated in datasets cured
 782 for stuttering. So, whenever possible, removal of affected loci may help to shift such
 783 estimates towards (slightly) more accurate values.

784 In monoecious selfers, detection works well and cure works very well, providing
 785 other confounding factors as null alleles do not interfere, in which case avoiding stuttering
 786 cure and correct for null alleles appear more appropriate. In doubt, and for subdivision
 787 measures, curing for null alleles may be achieved by the elimination of involved loci for
 788 strong selfers, or applying the INA correction (Chapuis & Estoup, 2007), for reasonably
 789 panmictic populations.

790 In clones, only highly significant stuttering tests with a significant higher F_{IS} as
 791 compared to other loci will need being cured, in which case parameter estimates may be
 792 [much](#) improved.

793 In any way, cure must only be kept if F_{IS} values are improved (lower than it was
 794 initially) and special care must be devoted to the behavior of their variation in relation to

Supprimé: . The average for cured data was then $S_c=0.9718$, which is very important

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Supprimé: Discussion

Supprimé: The power of stuttering detection was weak, except in populations with an increased inbreeding coming from a closed system of mating. In this case, BH correction allowed reducing the false discovery rate to a reasonable level.¶

Supprimé: Selfing significantly increased power detection as discussed above.

Supprimé: The stuttering proportion used here was relatively small, since the realized actual proportion of alleles affected at the end of simulations was in general much lower on average. Indeed, in many subsamples, the two first consecutive allele chosen for stuttering must be present and preceded or followed by an allele with one repeat difference. Otherwise, no stuttering occurs. Nevertheless, this was enough to generate significant heterozygote deficits under the pangamic dioecious mating system. Alternatively, this may also explain why stuttering had no significant impact on the F_{IS} of monoecious selfers. Nevertheless, stuttering detection significantly increased with local inbreeding.¶ Stuttering had a quite small impact on the ratio between the standard error of F_{IS} and F_{ST} , which did not reach the minimum two-fold threshold met with null alleles (De Meeüs, 2018)

Supprimé: Stuttering did not affect linkage disequilibrium. This might have been expected here since how stuttering was generated did not affect more any individual than another. In real life, we may expect amplification problems to affect preferentially some individuals, at several loci, due to several possible and nonexclusive reasons: bad preservation of several DNA extracts, genetically distant individuals (genetically distant populations, subspecies, or cryptic species), and bad amplification conditions. In that case, we may expect some correlation between the affected loci thus generating an increase in linkage disequilibrium between those, as this appeared to be the case for the tick *I. scapularis* in USA (De Meeüs et al., 2021).¶

Subpopulation sizes and number of subpopulations did not affect much stuttering discovery. There is indeed no reason why the effect of stuttering would be increased or decreased by those parameters. The lim(...)

Déplacé vers le haut [2]: . Clonal populations generated very high proportions of false stuttering detections. This was because without segregation of alleles, only some classes of heterozygotes propagate

Supprimé: The strategy to cure the data suffering from stuttering did not allow to entirely restore a completely immune set of population genetics results in pangamic dioecious populations, though the 95% (...)

Déplacé vers le haut [3]: After this cure, removing other loci with suspected null alleles (i.e. with missing genotypes) drove the superimposition index defined in

Supprimé: ¶

¶ Conclusion¶

Supprimé: ;

936 other factors as number of missing data (null alleles) or superimposition index (clonal
937 populations).

938 [The stuttering detection and cure strategies proposed in the present paper](#) will help
939 interpreting [microsatellite data with more accuracy and at the lowest cost. This will be](#)
940 [particularly helpful in non-model organisms, as](#) parasite-vector systems, [for which](#)
941 [microsatellite markers still represent the best cost benefit ratio.](#)

Supprimé: Such measures

Supprimé: in

Supprimé: at the lowest cost, which will be of particular interest in projects involving non-model, neglected, and small organisms that often happened to be economically and medically highly relevant

943 **Author's contributions**

944 All authors read, amended and/or approved the final manuscript.

Supprimé: , except JBR who could not check the last versions

945 Conceptualization: Thierry de Meeûs, Camille Noûs.

946 Data analyses: Thierry de Meeûs.

947 Design of figures: Thierry de Meeûs.

948 Writing of the original draft: Thierry de Meeûs.

949 Supervision: Thierry de Meeûs.

950

951 **Data availability**

952 Scripts used are available in the Appendix 1.

953 [Parameter for the first simulation with Easypop are in the file](#)

954 ["TestStutterDioeciousNoStutter-n1000N100-1.txt"](#); The file

955 ["TestStutterDioeciousNoStutter-n1000N100-1.equ](#) gives population genetics parameters

956 [measured at each generation of the first replicate; "TestStutterDioeciousNoStutter-](#)

957 [n1000N100-1.gen" and "TestStutterDioeciousNoStutter-n1000N100-1.dat" are the](#)

958 [genepop and Fstat files generated by this simulation, respectively. The file](#)

959 ["TestStutterDioecious-n1000N100-1-10%Stuttering.xlsx"](#), is a template for generating 10%

960 [stuttering in the first replicate of the first Easypop simulation; and the file](#)

961 ["TestStutterDioeciousNoStutter-n1000N100-1-FstatRes.xlsx"](#) is a template of stuttering

962 [detection \(together with LD tests and other measured done by Fstat\), for this first replicate](#)

963 [of the fists simulation with 10 % stuttering.](#)

964 Synthetic results for dioecious and monoecious simulations are in Excel format in
965 the supplementary file S1 "SynthesisStutteringTestSexualsSupFileS1.xlsx".

966 Synthetic results for clonal simulations are in Excel format in the supplementary file
967 S2 "StutteringClonesSynthesisSupFileS2.xlsx".

968 Pooling protocols for curing simulated data are in Excel files that were compressed
969 in a zipped file, supplementary file S3 "PoolingProtocolCureSupFileS3.zip".

970

979 **Conflict of interest disclosure**

980 The authors declare that they have no financial conflict of interest with the
981 content of this article. Thierry de Meeûs is one of the PCI Infections administrators.

982

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986

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1111
1112
1113

1114 **Appendices**

1115 **Appendix 1: R-scripts and R-commander menu used to analyse the different**
1116 **datasets**

1117 Regarding R-scripts, most are very small and basic: `binom.test`, for the exact binomial
1118 test; `p.adjust`, for adjusting the p -values in a series of p -values.

1119
1120 For generalized linear mixed models with the package `lme4`, the commands were, for the
1121 number of significant stuttering tests (for instance):

```
1122  
1123 Dataset <-  
1124 read.table("C:/DeMeeus/thierry/StutteringTest/DataMixedModelStutterSimul.txt", header=TRUE)  
1125  
1126 SigStut1<-glmer(StutterSigSperLocus~n+N+Mating+Stuttering+(1|Rep),  
1127 family=poisson(link = "log"), data=Dataset)  
1128 summary(SigStut1)
```

1129
1130
1131 The Fisher exact tests were undertaken with R-commander, in the menu Statistics-
1132 Contingency table-Enter and analyze two-way table

1133
1134
1135 [Appendix 2: Pooling of alleles used to cure real datasets](#)
1136 [A2.1. For *Ixodes scapularis*](#)
1137 [_____ For Locus B3 allele 203 was recoded 201; allele 207 was recoded 205; allele 211](#)
1138 [was recoded 209; and alleles 215 was recoded 213. For Locus XB110, alleles 179 was](#)
1139 [recoded 177; allele 183, was recoded 181; alleles 187 to 199 were recoded 185, allele 203](#)
1140 [was recoded 201 and all rare alleles from 205 to 277 were also recoded 203.](#)

1141
1142 [A2.2. For *Galba truncatula*](#)
1143 [_____ For locus Lt9, 203 with 202, 210 and 212 with 208; for Lt16, 231 to 233 with 230; for](#)
1144 [Lt21, 107 with 105, 112 with 111, 115 and 116 with 114; for Lt24, 208 and 210 with 207,](#)
1145 [215 to 217 with 214, 220 and 221 with 219; for Lt36, 187 to 190 and 192 with 185; and for](#)
1146 [Lt37, 115 with 113, and 123 with 112.](#)

1147
1148 [A2.3. For *Fasciola hepatica*](#)
1149 [_____ Alleles 182 to 183 with 180, allele 188 with 186, and alleles 192 to 194 with 190.](#)

Déplacé (insertion) [1]

1|50
1|51
1|52
1|53
1|54

[A2.4. For *Trypanosoma brucei gambiense* 1](#)

[for micbg1, allele 164 was pooled with 162, and allele 194 with 192; for misatg4, allele 117 with 115, and 145 with 143; and for misatg9, alleles 130 and 128 with 126, allele 186 with 184, and alleles 194 and 192 with 190.](#)