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# Comment on Three Papers about Hardy-Weinberg Equilibrium Tests in Autopolyploids

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## Abstract

Three similar manuscripts, by many of the same authors, were recently published describing methods for Hardy-Weinberg equilibrium and random mating testing in autotetraploids [Sun, et al. *Trends in Genetics*, 37(6), 2021. doi:10.1016/j.tig.2020.11.006], autohexaploids [Wang, et al. *Horticulture Research*, 9, 2022. doi:10.1093/hr/uhac104], and autooctoploids [Wang, et al. *Frontiers in Genetics*, 12, 2021. doi:10.3389/fgene.2021.794907]. We found issues with these manuscripts, which we detail here. The main problems that we see are (i) extensive mistakes and implementation errors, (ii) confusion between random mating and equilibrium, (iii) confusion between allo- and autopolyploid inheritance, and (iv) poor hypothesis testing approaches. We provide examples and simulations when appropriate. All of our results are open and reproducible.

## 1 Introduction

This comment pertains to the genotype frequencies of autopolyploids, organisms with more than two sets of homologous chromosomes. We will need a little notation before we discuss the issues here. Let  $\mathbf{q} = (q_0, q_1, \dots, q_K)$  be the genotype frequencies at a single biallelic locus for an autopolyploid population with ploidy  $K$ . That is,  $q_k$  is the proportion of individuals in the population with  $k$  copies of the minor allele. Let  $\mathbf{x} = (x_0, x_1, \dots, x_K)$  be the genotype counts in a random sample of  $n = \sum_{k=0}^K x_k$  individuals. Then  $\mathbf{x}$  is multinomially distributed with size  $n$  and probability vector  $\mathbf{q}$ . One goal in the population genetics of autopolyploids is to model the genotype frequencies. Under random mating, we have [Gerard, 2022b]

$$q_k = \sum_{i=\max(0, k-K/2)}^{\min(k, K/2)} p_i p_{k-i}, \quad (1)$$

where  $\mathbf{p} = (p_0, p_1, \dots, p_{K/2})$  are the *gamete* frequencies of the population. That is,  $p_k$  is the proportion of gametes in the population that have  $k$  copies of the minor allele. Suppose that a population is randomly mating, then there exists a function  $f(\mathbf{q}, \alpha) = (f_0(\mathbf{q}, \alpha), \dots, f_K(\mathbf{q}, \alpha))$  that updates the genotype frequencies from the current generation  $\mathbf{q}$ , to the next  $f(\mathbf{q}, \alpha)$ . Here,  $\alpha$  is called the double reduction rate, a property of meiosis in autopolyploids [Stift et al., 2010]. If the population is at equilibrium, then we have

$$\mathbf{q} = f(\mathbf{q}, \alpha). \quad (2)$$

For each ploidy, there is a  $\mathbf{q}$  that satisfies (2), called the “equilibrium genotype frequencies” [Huang et al., 2019]. These frequencies are a function of the double reduction rate  $\alpha$  and the allele frequency  $r = \frac{1}{K} \sum_{k=0}^K kq_k$ , and have been calculated for ploidies less than or equal to ten [Huang et al., 2019]. If  $\alpha = 0$ , then these equilibrium genotype frequencies reduce to binomial proportions [Haldane, 1930],

$$q_k = \binom{K}{k} r^k (1-r)^{K-k}. \quad (3)$$

Three similar papers were recently released which attempt to distinguish between hypotheses (1)–(3): one for tetraploids [Sun et al., 2021], one for hexaploids [Wang et al., 2022], and one for octoploids [Wang et al., 2021]. These three papers have issues that we have found. We are well situated to provide this comment because we recently published a manuscript distinguishing between (1)–(3) [Gerard, 2022b]. We also recently released a preprint on Bayesian tests for (1) [Gerard, 2022a]. Here, we will list each issue of Sun et al. [2021], Wang et al. [2022], and Wang et al. [2021] in turn, providing examples as needed. If we made any mistakes in the following, we would appreciate any corrections the authors could provide.

## 2 Mistakes and implementation issues

There are many logical and coding issues in Sun et al. [2021], Wang et al. [2021], and Wang et al. [2022]. We list here the ones we found. But the code Sun et al. [2021] is not available, and the code from Wang et al. [2021] and Wang et al. [2022] is verbose and sparsely documented, so there might be more implementation errors that we missed.

The model for meiosis in Wang et al. [2021] is incorrect. This leads to incorrect equilibrium genotype frequencies in their “recursive” test for equilibrium, and thus an incorrect test for equilibrium. We can determine that their model is incorrect by looking at what it implies when  $\alpha = 0$ . In this case, the distribution of gamete dosages is known to follow a hypergeometric distribution [Table 1 from Haldane, 1930, Bever and Felber, 1992, Huang et al., 2019]. If  $X$  is the parental genotype and  $Y$  is the gamete genotype, the reader can see this result by thinking of the probability of obtaining  $Y$  minor alleles out of  $K/2$  chosen alleles from an individual with  $K$  total alleles and  $X$  total minor alleles. Therefore, the correct segregation frequencies are obtained via

$$Pr(Y = y|X = x) = \frac{\binom{x}{y} \binom{K-x}{K/2-y}}{\binom{K}{K/2}}. \quad (4)$$

Table 1 shows that the model for meiosis from Table 1 of Wang et al. [2021] does not equal the probabilities from (4) when  $\alpha = 0$ , indicating that their model for meiosis is incorrect. We can empirically show that their equilibrium frequencies also do not equal binomial proportions when  $\alpha = 0$  (Appendix S2), which they should [Haldane, 1930].

The  $\chi^2$  statistics testing hypotheses (1) and (2) are implemented incorrectly in Wang et al. [2022]. The  $\chi^2$  statistic in equation (1) of Wang et al. [2022] is correct in the manuscript, but in their code they left out the  $N$  term. This affects both their equilibrium testing results and their random mating results. We know this because we reproduced their the 6.602 and 6.649 values on page 5 of their manuscript (Appendix S3). Thus, their tests are improperly implemented.

The random mating test is implemented incorrectly in Wang et al. [2022], even after we include

Parent Genotype	Method	Gamete Genotype				
		4	3	2	1	0
8	Wang et al. [2021]	1	0	0	0	0
8	Correct	1	0	0	0	0
7	Wang et al. [2021]	9/16	3/8	1/16	0	0
7	Correct	1/2	1/2	0	0	0
6	Wang et al. [2021]	225/784	45/98	87/392	3/98	1/784
6	Correct	3/14	8/14	3/14	0	0
5	Wang et al. [2021]	25/196	75/196	285/784	45/392	9/784
5	Correct	1/14	6/14	6/14	1/14	0
4	Wang et al. [2021]	9/196	12/49	41/98	12/49	9/196
4	Correct	1/70	16/70	36/70	16/70	1/70
3	Wang et al. [2021]	9/784	45/392	285/784	75/196	25/196
3	Correct	0	1/14	6/14	6/14	1/14
2	Wang et al. [2021]	1/784	3/98	87/392	45/98	225/784
2	Correct	0	0	3/14	8/14	3/14
1	Wang et al. [2021]	0	0	1/16	3/8	9/16
1	Correct	0	0	0	1/2	1/2
0	Wang et al. [2021]	0	0	0	0	1
0	Correct	0	0	0	0	1

Table 1: Segregation frequencies for an autooctoploid when there is no double reduction, either according to Table 1 from Wang et al. [2021] or according to the correct calculation using the hypergeometric distribution (4). The two approaches are different, so the general model for meiosis in Wang et al. [2021] is incorrect.

the  $N$  term. That is, the authors calculate it differently than what they state in their manuscript. Specifically, in their code, they first estimate the gamete frequencies via maximum likelihood, then put the resulting genotype frequencies through the recursive formula to come up with equilibrium values. However, using this recursive formula just results in the same genotype frequencies as the equilibrium “recursive” test. So, the 6.602 value and the 6.649 value above are different merely because the authors ran the recursive relationship for a different number of iterations.

The degrees of freedom for the both the equilibrium and random mating tests are incorrect in Sun et al. [2021]. They list the degrees of freedom to be four in both tests. But there are already four free parameters under the alternative (since  $q_0 + q_1 + q_2 + q_3 + q_4 = 1$ ). Since Sun et al. [2021] assume the double reduction rate is known, under the null of equilibrium there is one free parameter (the allele frequency), and so the degrees of freedom for the test for equilibrium is  $4 - 1 = 3$ , not 4. Under the null of random mating, there are 2 free parameters (since  $p_0 + p_1 + p_2 = 1$ ), and so the degrees of freedom for the test of random mating is  $4 - 2 = 2$ , not 4.

The degrees of freedom for the random mating test is incorrect in Wang et al. [2022]. On page 4 of Wang et al. [2022], the authors say about their test for random mating that “this test statistic follows the chi-square distribution with an unknown degree of freedom. However, we can empirically determine it as a value between  $7 - 1 - 1 = 5$  to  $7 - 1 = 6$ .” We can theoretically determine the degrees of freedom here. There are 6 free parameters under the alternative (since  $q_0 + q_1 + q_2 + q_3 + q_4 + q_5 + q_6 = 1$ ), and there are 3 free parameters under the null (since  $p_0 + p_1 + p_2 + p_3 = 1$ ), and so the degrees of freedom is  $6 - 3 = 3$ , which is neither 5 nor 6.

The degrees of freedom for the recursive test is incorrect in Wang et al. [2022]. They say, right after their equation (1) that the degrees of freedom is 6. But there are already 6 free parameters under the alternative. Because Wang et al. [2022] assume the double reduction rate is known, there is only 1 free parameter under the null, the allele frequency. Thus, the true degrees of freedom is  $6 - 1 = 5$ , not 6. See Appendix S4 for an empirical demonstration.

The degrees of freedom for the recursive test is incorrect in Wang et al. [2021]. Right after their equation (3), they state that their  $\chi^2$  statistic “is thought to follow a chi-square distribution with eight degrees of freedom.” But there are already 8 parameters under the alternative (since  $\sum_{k=0}^8 q_k = 1$ ). The number of parameters under the null is unclear since they are using a different (incorrect) model for meiosis than we have studied for octoploids, but it likely at least 1 (for the allele frequency). Empirically, it seems the degrees of freedom is 7, not 8 (Appendix S4).

The estimates of  $\alpha$  are implemented incorrectly in Wang et al. [2022]. The authors do not modularize their code into functions, and this led to some logical errors. They have a variable in their simulations called `alpha` that is the true double reduction rate. Their code returns `alpha1`, the estimated double reduction rate. However, their EM algorithm uses `alpha`, *not* the current version of `alpha1`, to update the parental gamete frequencies. Thus, they use the true value of  $\alpha$  in their code that estimates  $\alpha$ . This clearly results in unwarranted advantages. We fixed this bug and reran their simulations, obtaining very biased estimates of  $\alpha$  (Appendix S5). This indicates that either their EM algorithm is wrong, or their code is incorrect. It is hard for us to judge if their EM algorithm is wrong since the EM algorithm used to estimate  $\alpha$  is neither in the manuscript nor in the Supplementary Materials. We also note that when we ran the authors’ “estimate\_alpha.R”, which should produce the simulation results in their Table 3, we were not able to actually reproduce their Table 3.

From what we can understand through their code (in files “table2\_power.R” and “LR.R”), Wang et al. [2021] implement their tests by using the *true* genotype frequencies when constructing their test statistics. Needless to say, researchers would not have access to the true genotype frequencies

in reality. In their “table2\_power.R”, they set some genotype frequencies  $\mathbf{q}_1$  and then obtain the underlying true genotype frequencies via a perturbation of  $\mathbf{q} = f(\mathbf{q}_1, \alpha)$ , where  $\alpha = 0$ . They obtain two equivalently valued variables called `prob` and `prob1`. They use a perturbation of `prob` to generate the data, and `prob1` to construct the test statistic, but both `prob` and `prob1` are equal to  $\mathbf{q}$ . We provide an annotated version of “table2\_power.R” in the Supplementary Material so that it is easier for the reader to see the issue here. Though, the reader is warned, their code is rather verbose and spans 49 8.5×11” pages. Because their test statistic is impossible to calculate in real analyses (because it uses the true genotype frequencies), the simulation results of Wang et al. [2021] are invalid. We also note that when we ran “table2\_power.R” using the authors’ original code, we were not able to actually reproduce their power results in their Table 2.

### 3 Distinguishing between random mating and equilibrium

Sun et al. [2021], Wang et al. [2022], and Wang et al. [2021] suggest that (1) and (2) are the same hypothesis, or at least approximately so. In their manuscripts, they have a “recursive” test and a “gamete-based” test that they claim both test for “asymptotic Hardy-Weinberg equilibrium”. Their “recursive” test does indeed evaluate (2) (assuming  $\alpha$  is known). However, the “gamete-based” test actually evaluates (1).

Since the authors say that (1) is about the same as (2) for any choice of  $\alpha$ , this is worth some exploration. As an extreme counterexample (Appendix S1), let  $\mathbf{p} = (0, 0, 1, 0)$ , then hypothesis (1) states that

$$\mathbf{q}_1 = (0, 0, 0, 0, 1, 0, 0). \quad (5)$$

But  $\mathbf{q}_1$  is not at equilibrium, and we can use  $\mathbf{q}_1$  as the starting point for many rounds of random mating to reach equilibrium (2). When we do, we obtain

$$\mathbf{q}_2 = (0.001, 0.016, 0.082, 0.219, 0.329, 0.263, 0.088), \quad (6)$$

when  $\alpha = 0$ , the lower bound of the double reduction rate. We also obtain

$$\mathbf{q}_3 = (0.005, 0.032, 0.098, 0.204, 0.277, 0.251, 0.133), \quad (7)$$

when  $\alpha = 0.3$ , the upper bound of the double reduction rate [Huang et al., 2019]. Clearly,  $\mathbf{q}_1$ ,  $\mathbf{q}_2$ , and  $\mathbf{q}_3$  are very different. But Sun et al. [2021], Wang et al. [2022], and Wang et al. [2021] suggest that they should be about the same.

As a less contrived example, S1 populations (a single generation of selfing) are technically random mating populations, but hardly any researcher would claim that an S1 population is at equilibrium. See Gerard [2022b] for details.

The only real data example used in Wang et al. [2022] consists of four markers from an F1 population. This is insufficient to explore their methods, as F1 populations exhibit neither random mating (1) nor equilibrium (2). Furthermore, they did not apply their test for random mating on these data, but rather a test for binomial frequencies (3), which is a standard approach, though an incorrect one for F1 populations.

## 4 Confusion between allo- and autopolyploids

Wang et al. [2021] state on page 4 that “The case of no double reduction in the autopolyploid model reduces to allopolyploids if no preferential pairing is assumed.” Sun et al. [2021] states on page 3 that “When  $\alpha = 0$ , the pattern of allelic inheritance reduces from autotetraploids to allotetraploids.” Since allopolyploids exhibit disomic inheritance within each subgenome [Stift et al., 2010], this is true *only* if all subgenomes of an allopolyploid have the exact same allele frequency. This is likely not the case in true allopolyploids. In an extreme example, suppose we have an allooctoploid population with an allele frequency of 0 in two of its subgenomes, and an allele frequency of 1 in the other two subgenomes. Then the overall allele frequency is 0.5, and the allooctoploid equilibrium genotype frequencies are

$$\mathbf{q}_{allo} = (0, 0, 0, 0, 1, 0, 0, 0, 0), \quad (8)$$

because every individual will have two minor alleles each from two subgenomes, and two major alleles each from two subgenomes, and therefore all individuals will have genotype 4. Compare this to the genotype frequencies of an autooctoploid with allele frequency 0.5, at equilibrium when there is no double reduction

$$\mathbf{q}_{auto} = (0.004, 0.031, 0.109, 0.219, 0.273, 0.219, 0.109, 0.031, 0.004). \quad (9)$$

Clearly,  $\mathbf{q}_{allo}$  and  $\mathbf{q}_{auto}$  are very different. This is not a contrived example, as it might be the case that some subgenomes have fixed an allele before the polyploidization event [“fixed heterozygosity”, Cornille et al., 2016].

The tests created in Sun et al. [2021], Wang et al. [2022], and Wang et al. [2021] are only applicable to autopolyploids, but the only real-data example in Sun et al. [2021] and Wang et al. [2021] are allopolyploids. So the authors did not adequately evaluate their method on a reasonable dataset.

## 5 Poor hypothesis testing strategies

The test for equilibrium (2) in Sun et al. [2021], Wang et al. [2021], and Wang et al. [2022] assumes the double reduction rate is known. But it would not be clear to the reader that this is the case from a reading of the manuscripts. The double reduction rate is never known in practice.

The “recursive” approach in Sun et al. [2021], Wang et al. [2021], and Wang et al. [2022] for equilibrium testing is unnecessary. The equilibrium frequencies of tetraploids, hexaploids, and octoploids in the presence of double reduction are well documented in the excellent paper of Huang et al. [2019]. For example, for hexaploids, we have that the equilibrium gamete frequencies are

$$p_0 = \left( 1 - \frac{9(3-\alpha)(6-\alpha)}{(9+\alpha)(9+2\alpha)}r + \frac{27(1-\alpha)(3-\alpha)}{(9+\alpha)(9+2\alpha)}r^2 \right) (1-r), \quad (10)$$

$$p_1 = \left( \frac{9(3-\alpha)(9-4\alpha)}{(9+\alpha)(9+2\alpha)} - \frac{81(1-\alpha)(3-\alpha)}{(9+\alpha)(9+2\alpha)}r \right) r(1-r), \quad (11)$$

$$p_2 = \left( \frac{45\alpha(3-\alpha)}{(9+\alpha)(9+2\alpha)} + \frac{81(1-\alpha)(3-\alpha)}{(9+\alpha)(9+2\alpha)}r \right) r(1-r), \text{ and} \quad (12)$$

$$p_3 = \left( \frac{20\alpha^2}{(9+\alpha)(9+2\alpha)} + \frac{45\alpha(3-\alpha)}{(9+\alpha)(9+2\alpha)}r + \frac{27(1-\alpha)(3-\alpha)}{(9+\alpha)(9+2\alpha)}r^2 \right) r. \quad (13)$$

The equilibrium genotype frequencies are discrete linear convolutions of these proportions. Equations (10)–(13) look complicated, but they are not complicated for a computer. It is easy to implement a likelihood approach to test for equilibrium using these gamete frequencies, and such an approach, advantageously, does not depend on knowing the double reduction rate, which is a huge benefit over the iterative approach of Sun et al. [2021], Wang et al. [2021], and Wang et al. [2022]. Indeed, this likelihood approach is what we did in Gerard [2022b].

Genotype uncertainty is a major issue in polyploids [Gerard et al., 2018, Gerard and Ferrão, 2019, Gerard, 2021a,b], and so methods should be adjusted to account for this uncertainty. The standard approach to do so is using genotype likelihoods [Li et al., 2011], and this is what we did in Gerard [2022b]. However, Sun et al. [2021], Wang et al. [2021], and Wang et al. [2022] approach this by aggregating heterozygous genotypes into a single count, which leaves them with only enough degrees of freedom to test for binomial frequencies (3). They thus provide no way to evaluate hypotheses (1) and (2) in the presence of genotype uncertainty.

## 6 Discussion

We thank the authors of Wang et al. [2022] and Wang et al. [2021] for posting their code to GitHub. Without doing so, our review here would have been much less comprehensive. E.g., our review of Sun et al. [2021] is more limited because their code was not available. Open research is a vital tool for scientific advancement. In the future, however, we would encourage the authors to take greater steps for defensive programming, such as unit testing [Wickham, 2011], continuous integration [Hilton et al., 2016], and code review [Vable et al., 2021]. Other steps the authors could take to improve their computational pipeline include (i) modularizing their code into functions, ideally in a package [Wickham, 2015], (ii) using a workflow management software to aid in reproducibility and decrease the chance for coding errors [Blischak et al., 2019], (iii) providing instructions (ideally automation) on specifically how to reproduce their methods [Heil et al., 2021], and (iv) posting their code on a repository that is committed to permanency and produces DOI’s, such as Zenodo or Figshare, as this extends the lifetime of a work’s reproducibility. We would also encourage greater sanity checks, such as demonstrating that the authors’ test-statistics produce  $p$ -values that are uniform under the null. This alone could have detected the test-statistic and degrees of freedom issues we discuss in Section 2 (Appendix S4).

If the authors would have submitted their manuscript as a preprint, this would have given us time to provide them feedback so that they could correct their work before publication. This is one of the many benefits of preprint culture. However, because this work is now published and bears the mark of “peer reviewed”, we felt the need to prepare a more formal discussion of their work. We exhort all authors to consider preprinting before publication.

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Most analyses were performed using the R statistical language [R Core Team, 2022].



## Supplementary material

All supplementary material is available on GitHub: <https://github.com/dcgerard/hwesupp>.

- The file “hwesupp.Rmd” is an R Markdown file that contains Appendices S1–S5, and is sufficient to reproduce all of the results of this manuscript. It has been knitted into “hwesupp.pdf”.
- The file “sims.csv” contains the simulation output from Appendix S5 of “hwesupp.Rmd”.
- The file “table2\_power.Rmd” contains one iteration of “table2\_power.R” from Wang et al. [2021], annotated to demonstrate the mistakes here. It has been knitted into “table2\_power.pdf”.

Much of the code from Wang et al. [2021] and Wang et al. [2022] was packaged by us in the hexocto package on GitHub <https://github.com/dcgerard/hexocto>.

A fork of the original code from Wang et al. [2021] and Wang et al. [2022] may be found at <https://github.com/dcgerard/hexaploid> and <https://github.com/dcgerard/OctoploidDeer>.

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