nature portfolio

Peer Review File

Interplay between large low-recombining regions and pseudo-overdominance in a plant genome

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
See attached file "Comments on Salson et al"

Reviewer #2

(Remarks to the Author)
The manuscript "Pervasive interplay between large inversions and pseudo-overdominance in a plant genome" presents a case for the persistence of genetic diversity in populations due to pseudo-overdominance. I greatly enjoyed reading this paper, finding the arguments clear and very well supported by the different approaches used, which, I have to say, are impressively varied. I am unaware of any other works that have so rigorously shown that observed heterozygosity within non-recombining regions is most probably due to pseudo-overdominance, and I believe their results leave little doubt as to the conclusions. I have some very minor comments/queries and highly recommend that this paper be accepted for publication.

Minor comments:

There are some typos and a couple of not quite comfortably worded sentences in the manuscript. It's not a huge problem, just pointing it out. I didn't copy all of them but here are some of them:
line 104: The sentence seems incomplete - "As a result, the authors of these studies hence more frequent testing of pseudo-overdominance patterns in genomic analyses"

line 680: The SNPs and genotypes identifications -> The methods of identification of SNPs and genotypes are fully described ..

line 755: DNAs were quantified -> DNA was quantified

Question on methodology: In the last part of the methodology, "Associations between the chromosome 3 haplotypes and phenotypic traits" the authors mention imputing missing genomic data. As I am quite naive when it comes to this methodology, how much would it have affected the results not to do that? Would it be possible to include an analysis that uses "exclusion of incomplete data" instead? So as to attempt to evaluate whether a bias may have been introduced through this process.

Reviewer #3

(Remarks to the Author)

The paper presents evidence that the genomes of 126 accessions of cultivated pearl millet from West Africa share at least 6 large (41-88 Mb) regions of low recombination that contain elevated heterozygosity, strong fitness effects, and high ratios of non-synonymous and often deleterious variants (including at least one synthetic lethal). Exome sequencing reveals that

these regions represent large inversions that largely prevent recombination, ensuring tight linkage. In other contexts, tight linkage can accelerate background selection or selective sweeps that act to reduce genetic variation. In contrast, the high variability being maintained in these regions and the many results presented here strongly support the authors' interpretation that pseudo-overdominance (POD) derived from many deleterious mutations linked in repulsion generating balancing selection.

The authors present additional data and analyses to further explore the causes and consequences of this POD. I was surprised by the size of these inverted regions (though they are in line with the review by Wellenreuther and Bernantchez 2018) and that they cover so much of the genome (17% here). It was also remarkable that the authors were able to identify three (chromosomes 4 and 7) or six (chromosomes 2, 3, and 6) distinct clusters of haplotype variants characterized by large differences in heterozygosity (and later, fitness). These findings support their interpretation that strong POD exists within these regions. These convincing results are well-presented and further dissected within the largest inversion on chromosome 3 (e.g., Figs. 3 and 4). Further tests within this region reveal introgression of wild genetic variation into cultivated populations. The authors also performed self-fertilization experiments to analyze segregation ratios of a key marker within the chromosome 3 inversion. They found that one haplotype homozygote is almost completely missing, confirming the high segregational load due to POD and thus how balancing selection can sustain the inversion polymorphism.

It was also impressive to see the authors reach back in time to find accessions from 1976 to trace how chromosome 3 haplotype frequencies have evolved since then. The stable persistence in haplotype population structure (for all but a rare cluster) further supports the idea that POD and suggests that such polymorphisms may be very long lived (as suggested by results from other studies). In another example of how thorough this work is, the authors tested for associations between the inversion haplotypes and 11 traits. Again, they found clusters that differ in traits related to fitness. They also make a very interesting suggestion in the Discussion that (lines 477-479)

In sum, the paper addresses topics of considerable current interest with comprehensive data and a careful set of original analyses. The paper is also well-written and provides a thoughtful discussion. Their results suggest that POD may often be expected in genomic regions with restricted recombination, setting the stage for additional work on non-model organisms as long-read technology enables more of these kinds of analyses. The authors present a clear, convincing, and complete story with broad implications in both evolutionary biology and plant and animal breeding. I thus have only very minor suggestions to improve the paper a bit more (below).

Minor suggestions

Lines 86 et seq. Casting natural selection and neutral evolution as "either – or" alternatives is simplistic given that most evolutionists accept both as important and seek to integrate them.

L. 104 – suggested rewording: replace "hence . . " with "urge others to test more frequently for pseudo-overdominance patterns in genomic analyses particularly since this phenomenon could be . . "

L 265 & Disc L. 476, etc. – The authors find "nested rearrangements." Do they consider these to occur commonly? Does having a few 'divergent haplotypes' possibly limit the total expression of segregational load by segregating many mutations together into these blocks?

Reviewer #4

(Remarks to the Author)
In the manuscript "Pervasive interplay between large inversions and pseudo-overdominance in a plant genome" the authors search for signatures of pseudo-overdominance in the pearl millet. Pseudo-overdominance occurs when the mutational load of two haplotypes (caused by deleterious recessive mutations) are complemented giving the heterozygote increased fitness (i.e. overdominance). When this process occurs in relation to structural variants, it is generally referred to as associative overdominance (*sensu* Ohta) as the presumptively neutral structural variant is "associated" with the deleterious alleles that cause the variance.

The authors use a combination of previously published exome capture data, whole genome resequencing, optical mapping and laboratory crosses to:

1. Identify seven candidate inversions in the genome, some with more than 2 arrangements. 6/7 include the centromere so were already regions of low recombination.

2. Show negative F_{IS} stats (6/7 regions), high pn/ps ratios (3/7 regions), and a higher ratio of deleterious to tolerated mutations (2/7 regions).

3. Take a closer look at the chromosome 3 region, confirm the presence of an inversion there and show evidence for the introgression of one of the arrangements from divergent wild populations.

4. Using three selfed parents, show unusual segregation patterns pointing toward lethality of one homozygote and overdominance of the heterozygote.

The authors suggest that these results indicate that pseudo-overdominance is prevalent in the pearl millet genome and a major force maintaining variation in this species. While pseudo-overdominance is a fascinating force that is often overlooked, myself and my co-reviewer were not convinced by the evidence shown here. Below we will go through this point by point but this can be summarized by three main issues: 1. Different figures often point towards different conclusions, 2. Large amounts of data are not shown and reasoning is not given as to why, and 3. Neutral expectations are not properly laid out. In our opinion these issues make the paper currently unsuitable for publication.

General/Overall concerns

1. Writing and vocabulary.
1a. The Introduction felt a bit vague and detached from the rest of the manuscript, many terms are not properly explained and some parts are difficult to follow, e.g.,
- Lines 81 and 86 suggest that selection used to be (initially) considered a major driving force but that is no longer the case…
- Line 104: Not sure what is meant by: "As a result, the authors of these studies hence more frequent testing of pseudo-overdominance…"
- Line 122: Or by: "diversity evolution pattern"

1b. Specifically, when talking about putative inversions the vocabulary is very confusing. Please refer to arrangements (e.g. A and B) and then genotypes (AA, AB, and BB) rather than genotypes A, B, and C where B is a heterozygote.
1c. It is hard to keep track of the different data sets, when which is used and what type of SNP subset. Make sure these are always clearly stated.
1d. Please clarify the differences between pseudo-overdominance and associative overdominance. The manuscript talks about both processes.

2. Analyses on the chromosome 3 region do not paint a clear picture.
2a. Figure 3C-D is quite confusing. Based on C one would suggest a large inversion encompassing the entire area (101-189) and then perhaps another inversion from ~120-170. But based on D the major inversion is from 155-175 mb.
2b. The recombination rate (Figure S3) looks strange, one would usually expect varying recombination and then a large section of low recombination in the inversion. In several chromosomes, it seems that the recombination is not necessarily lower than other parts outside the putative inversion/structural variant.
Surprising that several of the p-values are significant.
Would not the centromere to a large extent have the same effect? Is it possible to disentangle the two?
2c. Figures S3-4 are not convincing, none of the synteny patterns shown are characteristic of an inversion, where one would expect a flipped section resulting in an X. In fact it seems that it was impossible to determine synteny for a large portion of the region?
2d. The authors say on lines 264-265 that it appears that haplotype structuring is occurring. However, they do not explain this process and additionally, this does not appear to be haplotype structuring which describes a within arrangement phenomenon, whilst this appears more like multiple structural variants. To determine if haplotype structuring is happening the arrangements need to be clearly defined.
2e. What do the haplotype patterns outside the identified region look like? The authors show 236/1000 SNPs in figure 3. What do all the 1000 SNPs look like?

3. A neutral explanation for F_{IS} in inversions is missing. What would the F_{IS} of an inversion without overdominance look like? Given high heterozygosity in heterokaryotypes (higher than in collinear regions) would you expect a negative F_{IS} for an inversion even without overdominance? It is clear that F_{IS} is lower in these regions compared to the collinear genome but this is not the appropriate comparison.
3a. What would the F_{IS} of this region be if all three haplotypes were segregating under HWE? You can generate a neutral expectation using the defined genotypes (A-E). I.e. genotypes -> haplotype frequencies -> expected genotype frequencies -> expected F_{IS} values.
3b. Line 221: "Negative F_{IS} are very uncommon and notably indicative of a selective heterozygote advantage"
- We would argue that it is not that uncommon and that heterozygote advantage only is one possible explanation.

4. More population level genotype information is missing. Under pseudo-overdominance you would expect more heterokaryotypes than under HWE. The authors don't seem to have tested this. Is it true?

5. The segregation analysis is difficult to follow
5a. The authors start with 96 parents but then narrow them down to three. How come only 3 of the 96 parents were heterozygous when you suspect this region to be pseudo-overdominant? Is there another reason the other parents were excluded?
5b. How did you go from 1,500 SNPs to 10 to 5 to 24 and then 5 SNPs again? Which 5 are you in the end showing?

More minor concerns

6. Significance labels are not clear, especially for 0.05 <p <0.1, consider using something else instead.
7. Please be more specific in your language. For example, say that you used local PCA instead of “We employed a population genomics approach…”
8. The WGS sequencing data is underutilized. Could the population genetic analyses also include these individuals?

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
They have responded conscientiously to my comments on the previous version, although I still have reservations about the evidence for POD.

I am, however, very concerned about one important aspect of the results. In Table S6, they show values of synonymous site and nonsynonymous site diversities (piN and pS). piN is consistently as big or bigger than piS , as is the diversity for all sites. Indeed, the diversities are very small (of the order of 0.001 to 0.0004, despite they’re saying that this is a high diversity species. I’ve never seen datasets with mean piN as big or bigger than piS. This suggests to me that there is something wrong with their procedures for analysing the population genomics data; this needs to be cleared up before the paper can be considered further.

Reviewer #2

(Remarks to the Author)
The manuscript “Interplay between large structural variants and overdominance in a plant genome ?” examines whether overdominance or pseudo-overdominance could explain the excess of heteroyzgosity observed in 7 distinct genomic regions. They find that in most cases, the FIS observed in these regions does correspond to an excess of heterozygosity that is most likely linked to a form of (pseudo-)overdominance. One of these regions on Chromosome 3 harbours two variants that are most probably introgressed from wild populations, with one of the two variants most likely being lethal at the homozygote state (H2).

Upon reading the other reviewers' comments and the corresponding replies, I have learned much, and realised that I missed some points in my previous reading/review. This has left me (unfortunately) less enthusiastic with regards to the results, specifically concerning what is observed on Chr 3. I have added my queries below. In its current form, the manuscript is much clearer, I greatly appreciated the inclusion of the expectations of F_IS values in different selection regimes, and the classifications added. Though I think some small points still need to be addressed (included below), I still do believe this work is an interesting contribution to understanding how structural variants may influence genetic diversity and in general whether pseudo-overdominance can be a plausible mechanism to explain excess heterozygosity.

Comments:

From their (extensive) analyses, the authors come to the conclusion that ``Genotypes visualization suggests that the H1 haplotype resulted from a double crossing-over event between 120 and 175 Mb involving the H2 haplotype and the standard one, indicating haplotype structuring." So, this implies that H2 is the initial introgressed haplotype, and H1 a recombinant that was generated either following an inversion or the inversion appeared after the generation of the recombinant (I personally think the first is more likely). Now, I agree that the absence of H2H2 homozygotes and higher frequencies of heterozygotes, confirmed by the significantly negative F_IS could point to pseudo-overdominance. Under classical overdominant selection, Glémin 2021 (doi:10.1111/evo.14194) showed that in populations where heterozygotes are always favoured over all homozygotes, several haplotypes can indeed segregate in ouctrossing populations. However, in pseudo-overdominance it is not so clear that this would be stable on long evolutionary time scales. Recombinants are expected to have lower fitness when in the heterozygote state, compared to the initial haplotypes because they carry deleterious mutations present on both haplotypes. Seeing that this seems to be a relatively recent event, pseudo-overdominance may nevertheless be plausible. However, in Fig. 3c, we see that there are some SNPs on H2 that are shared with R, more than those shared with H1 (or so it seems). If recombination is that prevalent between R and H2, why is H2 still so lethal? If this heterozygosity is only due to pseudo-overdominance, why was it not broken up? This is really puzzling to me. And either it is explained and I missed it (though I did look for it...), or it is not explained well enough for me to get my head around it. In my opinion, there are possibly several things happening here. H2 homozygotes are not viable in domesticated populations for reasons that do not necessarily imply only fixed deleterious mutations (i.e. there could be genomic incompatibilities elsewhere in the genome). Whereas H1, having a smaller introgressed region, doesn't suffer as much at the homozygous phase, maybe because the loci involved in the H2 lethality are not on H1.

Even though inversions do reduce rates of recombination, I'm not fully convinced that it is only a question of inversions. Specifically in the case of Chr3, I think more generally it is a question of introgression. The inversion probably enhances the effect, but is not the main player. I may be wrong, but this is the impression I am under reading this new version of the manuscript, and works I have come across since last reading the manuscript. These kinds of selection dynamics depend on the frequency of the genomic region and what it actually contains, not the inversion itself per se.

Minor comments :

Table 1: Name of second test missing for pN/ps

Lines 591 - 595: “Our empirical findings present a possible narrative, suggesting an evolutionary trajectory wherein sex chromosomes evolve from pseudo-overdominant inverted regions. This novel model posits a prerequisite for recombination suppression (and pseudo-overdominance) preceding sexual determinism, offering fresh insights into the complex interplay between genetic mechanisms and evolutionary outcomes.”

I had let this slip by during the last round of review, but upon re-reading the manuscript, I don't see why this is here. The inversions studied here have nothing to do with sex chromosomes or even sex determinism. Why is this shout-out necessary? To me, this part is off subject and it should be removed. On a side note, recent works on S-loci have shown that the even though there may be some sheltering of deleterious mutations close to super-loci (like those found in butterflies) linked to the mating type, the sheltered load does not extend very far, there are no inversions, and there is barely a reduction in recombination. This, along with other recent works, seems to therefore disagree with the hypothesis that it is sheltering load that favours the evolution of sexual chromosomes.

Reviewer #3

(Remarks to the Author)
The authors have successfully addressed the technical and methodological points raised in the reviews, making a strong and interesting paper even stronger and more interesting.

L 542 et seq. Do the authors have any suggestions on this chicken vs. egg question – which came first, the load surrounding the centromere or the inversion augmenting the load?

The authors also raise the issue of whether POD represents an “evolutionary trap” (L 582, as a prelude to the evolution of sex chromosomes). They might also consider an alternative idea – that POD could create an “evolutionary escape” from the steady accumulation of load in low recombination regions like around centromeres. That is, Muller’s ratchet might at some point select for ‘fixing’ the load via POD – capping the load at a maximum of 50% per region even when such regions contain multiple lethal equivalents. In the presence of substantial identity disequilibrium, POD regions might also benefit each other by promoting heterozygosity, reducing the segregating load in other regions as well.

Minor point:
L 221 change “significant” to “significantly”

Reviewer #4

(Remarks to the Author)
We (myself and the junior reviewer) previously reviewed this manuscript. We found that the authors have made many positive changes: The introduction reads much better, it is easier to follow the arrangement terminology, and it is easier to keep track of the different data sets. The overall story is much easier to follow now.

This work is truly impressive, extending across multiple datasets and countless analyses. However, despite all of this we remain unconvinced of the authors central point: that pseudo-overdominance (POD) is a primary mechanism maintaining diversity in this system. Due to this we do not think it is suitable for publication in Nature Communications. Below we go through several issues that contributed to this conclusion.

First, the authors rely heavily on heterozygosity at the SNP level. They argue that increased heterozygosity in these regions compared to the collinear genome is a signature of POD. However, increased heterozygosity is a natural consequence of any segregating structural variant. This heterozygosity can be caused by either neutral or deleterious mutation. Only deleterious mutation will lead to POD. The authors examine three measurements for mutation load in Table 1: (A) Pi-N/Pi-S, (B) pN/pS, and (C) Ratio of deleterious to tolerated mutations. However, statistical significance for their seven test regions is rarely reached (1/7 for A, 3/7 for B, and 2/7 for C). Additionally, not a single region was significant for all three measures. Overall, the SNP data points towards a potential role of POD but in our opinion is not strong enough evidence to back the

authors claims.

We also found ourselves wondering at the lack of genotype (i.e. karyotype) level data. If POD is in fact occurring, we would expect HWE at the genotype with more heterokaryotypes than expected. We have performed this test ourselves using the 1976 data from table S13

Genotype | Observed | Expected
RR | 61 | 63
RH1| 69 | 36
H1H1| 23 | 20
RH2 | 26 | 10
H1H2 | 9 | 6
H2H2 | 0 | 2

The chi-sq test on this is highly significant. We suggest the authors add more of these types of analyses utilizing all their datasets. However, we want to note that still don't feel that this is enough to support the strong conclusions of the authors given the mixed results of the other data sets.

In particular, we were not convinced that POD was maintaining the diversity on Chr3. To start, is introgression ongoing? This would explain continued maintenance of the variation and the size of the haplotype that is not contained within an inversion. Overall the patterns on Chr3 confused us. The authors show a full 88 mb haplotype that appears to be in LD but only 20 mb of this is an inversion. It would be very unusual for such a large block to be maintained over the long time period necessary for POD to develop. Also what does the pattern look like outside of these coordinates (see minor point 5)? A discussion of the full haplotype of Chr3 is needed.

We believe that H2 appears to be lethal based on what the authors have shown but that does not mean that it is caused by the accumulation of recessive deleterious alleles. Given that it is a double crossover event that generated it, it is likely that a gene may have been disrupted. Most lethal arrangements in nature are caused by issues such as this rather than mutation accumulation. For examples see the Ruff (e.g., Kupper et al. 2016 or Lamichhaney et al. 2016) and the fire ant (Hallar BL et al. 2007). Also from figure S11C it appears that H2 does not even contain the inversion?

Finally, we very much disagree with the authors that haplotype structuring is occurring here and ask for that text to be removed. First, the authors indicate that H2 evolved through a double crossover event meaning that there was no slow natural split from H1 as in haplotype structuring. Additionally, it seems that H2 may not even contain an inversion? Finally, the pattern predicted by haplotype structuring, SNPs differentially fixed in either sub-arrangement by selection (see figure 4F and 4G from Berdan et al 2021) does not appear in figure 3C, at least not in the inverted region.

Minor comments:

1. 3A use different colors or shapes for different groups of heterokaryotypes

2. S5D – not all windows seem to be outside the normal distribution of Pi- Recombination relationship. And Table S5 does not take recombination rate into account.

3. Were there any corrections for multiple testing for any of the population genetic measures?

4. H2 could easily have interrupted a gene. No reason to suspect this is accumulation of deleterious alleles which is a slow process.

5. What does the region just outside of the identified region on Chr3 look like? Does the pattern continue at all?

6. While some of the other 6 regions do appear to be inversions the authors should be careful as they have not fully been identified as such.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Major comments

I did not conduct a full review of the previous version, due to my concerns about their diversity estimates. The authors appear to have resolved the problems with these, so I have now gone over the paper in detail.

The paper presents an impressive wealth of detail on patterns of variability around centromeric regions in pearl millet populations, and is probably the first detailed analysis of such patterns in a plant species. This is unusually favourable material compared with, say, Drosophila or humans, since the pericentric regions are very large, containing hundreds of genes, and have low levels of genetic recombination.

There is an a priori expectation that strong effects of selection at linked sites should be observed for these regions. However, a major finding is that genetic diversity is not reduced in the low recombination regions around centromeres, in contrast with what is usually observed, starting with 35-year old observations on Drosophila populations (it's a bit surprising that they relegate the relevant figure to the SI). This has, however, been found before in some outcrossing plant systems, often interpreted in terms of lower gene density leading to reduced hitchhiking effects (e.g. Wright et al. 2006 Genetics 174: 1421-1430). While their interpretation in terms of associative/pseudo- overdominance is reasonable in principle,given their evidence for strong haplotype structuring in these regions, there are two concerns about how to interpret this observation.

One is their interpretation of their haplotype structure in terms of inversions. This is based purely on finding that there is strong haplotype structuring in the centromeric regions; they do not describe any direct evidence for inversions in the genome sequences themselves. They are strong advocates of pseudo-overdominance (POD), and this is indeed quite plausible for large non-recombining genomic regions with thousands of sites subject to purifying selection. So why is it necessary to postulate the existence of inversions ? Indeed, if there is little or no recombination, there is no especial reason why inversions should be favoured in pericentric regions- the fact that the putative inversions are all found there is very suspicious.

The main objection to this interpretation is the case when three haplotypes coexist, one of which appears to be homozygous lethal (chr. 3). However, they have evidence that one of these is derived by introgression from wild populations; if small local populations are evolving in isolation, different haplotypes could easily be generated (see ref.7). The lethality is, of course, harder to explain, but this is also true of the inversion interpretation. Here they do seem to have evidence that their haplotype H1 has an inversion relative to the reference sequence (Fig 3d and Fig S10); the evidence for H2 looks weaker to me, but I lack expertise in interpreting such data. If I understand Fig 3d correctly, the inversion is only 25Mb, which is much smaller than the size of the whole region (Table 1). A critical question is whether the haplotype divergence is confined to the region covered by this inversion, or does it extend across the whole pericentric region? Fig S2 suggests that the latter; if so, then the inversion may not mean very much as far as causing the observed pattern. In addition, the frequency of the inversion among the haplotype concerned has not been established.

I thus feel that the authors need to be much more cautious about their interpretation, and consider carefully to what extent they really need to invoke inversions to explain their data. The problem with inferring inversions simply from haplotype data is, of course, not unique to their system.

The other problem is technical. Centromeric regions are often enriched for gene duplications, which are quite abundant in plant genomes. Divergence between duplicates can easily be confounded with diversity for a single copy gene. For their results on this point to be fully convincing, I feel that they need to exclude this possibility, e.g. by looking carefully at coverage for the genes they are using compared with the rest of the genome.

Minor comments

The references are carelessly formatted, with many inappropriate capital letters etc.

Placing the legends to the SI figures in a different file is unhelpful; each figure should have its own legends.

l.36 'insights which helped to' is better.

l.63 'likely to prevail' seems rather strong- there is little evidence for this; 'may occur' is more accurate in the light of current theory and empirical knowledge (e.g. ref 10).

l.66 'may' not 'might'

l.68 'low rates of recombination,'

l.72 add ‘multiple genomes’

l.74 Delete ‘, suggesting ..state’; it’s redundant.

l.75 ‘exhibits’

l.78 ‘potential’ is better than ‘complex’

l.88 Ref. 4 is concerned with inbreeding depression, not diversity; please find another reference.

l.94 Gilbert et al. characterization of POD as a form of balancing selection is rather silly; POD is just a consequence of purifying selection. As a matter of historical accuracy, the 1st model for a random mating population was done by Charlesworth & Charlesworth 1997 Genet. Res. 70: 63-73, followed by S. Palsson (e.g. Palsson, S., and P. Pamilo (1999). Genetics 153: 475-483. Glémin’s article is just a comment on Abu Awad and Waller.

l.99 Delete comma;

l.100 ‘to’ not ‘of’. It also retards the loss of variability at the neutral loci (see also Charlesworth B 2022 Genetics 221: iyac027)

l.103 ‘recessive’ needs qualification; the process requires partial recessivity (dominance coefficients less than one-half).

l.104 The effect of finite population size is not a form of inbreeding; it happens when there is purely random mating.

l.105 ‘recombination rates’.

l.109 ‘contribute to higher than expected’ is more accurate than ‘a major means’. The present statement ignores the fact that background selection effects may also act in low recombination regions, due to the fact that AOD/POD works only for relatively weakly selected variants (see ref. 18).

l.110 ‘genomic regions’.

l.116 ‘variability’ is better than ‘selective process’.

l.121 ‘with the detection of local adaptation’

l.129 ‘characterized’

l.130 comma after ‘patterns’

l.132 delete ‘mechanism’ and put ‘including POD’ in parentheses.

l.136 ‘with respect to the wild type’ is baffling; do they mean ‘gene flow from wild populations’? (wild type in genetics refers to the normal allele at a locus).

l.141 ‘wild population’

l.144 ‘and’ before ‘composed’

l.146 ‘into’ not ‘in’

l.147 ‘a’ not ‘the’

l.152 ‘is’ not ‘could be’

l.155 ‘results’ not ‘might result’

l.166-169 Since recombination rates are estimated from LD, the two patterns are totally correlated. If an inversion is present, it’s not clear that the recombination rates are very meaningful; in any case, the statistic estimated is the recombination rate scaled by the effective population size, so differences in the latter (e.g. caused by background selection) could be responsible for the differences.

Table 1: Why display both N/ S and pN/pS ? The latter is not very informative, since it does not correct for the numbers of sites in the two categories. It would be helpful to include the genome-wide average, and to state whether it’s that goes N up, S that goes down, or both.

l.214 ‘Signatures of overdominance and…’

l.216 ‘or’ not ‘and’

l.221-222. ‘a signature of heterozygote excess’

l.247-248 ‘departure from Hardy-Weinberg’

l.254-255 This statement is confusing, as it suggests that there is something special about the centromeres. There is no need for the multiplicity of recent references to this phenomenon- it’s been known for nearly 35 years. The point is that various hitchhiking processes reduce variability there, due to the low recombination rates around centromeres (see the references on l.108-111). It would have been clearer if they had referred to centromeres at that point in the paper.

l.245 ‘recombination rate’ (recombination is a process, quantified by its rate).

l.256-7 The correlation between diversity and estimates of recombination rate is unfortunately not so easy to interpret, since the latter is presumably estimated by correcting the scaled recombination rate by dividing by an estimate of local Ne, which in turn is estimated from the diversity and a value for the mutation rate (they should include a brief account of the method; readers should not have to hunt down a reference).
This probably leads to an underestimation of the relation, so their test is actually on the conservative side, which might be worth mentioning.
I don’t understand the difference between Figs S5 and 6; it seems like the former is for all SNPs and the latter for synonymous ones. Why do it both ways? This also applies to the remarks in the text; it’s much cleaner to use S than for all sites.

l.260-1 ‘the positive selection on heterozygotes’ is a peculiar term. Why not just say ‘overdominance or associative overdominance’? A paragraph break after ‘heterozygotes’ on l.261 would help the reader.
l.262 ‘homokaryotypes’

l.261-284 The rationale for these analyses seems to me to be somewhat confused. They are in fact tackling two problems: the effects of low recombination around centromeres, and the effects of hypothetical inversions on diversity. As stated in my general comments, the evidence for inversions is weak, except for H1 on chr. 3, and the data could potentiall be explained in terms of of haplotype structuring produced by POD.

They could state more clearly that the homokaryotypes are likely to have lower diversity than when all karyotypes are pooled; this is a necessary consequence of divergence between karyotypes (this is also true if the so-called karotyopes are just haplotypes). If an inversion polymorphism is maintained for a sufficiently long time in a centromeric region, for whatever reason, divergence between the arrangements will cause an increase in overall diversity in the region covered by the inversion (for a recent theoretical analysis, see Charlesworth B 2023 Genetics 224:iyad116). Both S and N could be affected in this way. This effect could counteract the effects of background selection etc in the centromeric regions.
Fig S6b and S7B suggest that this is likely to be going on: except for the right-hand pair, the homokaryotypes seem to have much reduced diversity compared with the rest of the population.
On the inversion hypothesis, there is no solid logical basis for ascribing this effect to overdominance or POD; any mechanism that maintained the inversions for a substantial time (e.g. frequency-dependent selection) would have the same effect. This section needs to be rewritten to make this point clear; of course, the patterns could simply be generated by POD without any inversions.

l.292 ‘Homozyous’ not ‘homozygote’

l.407 It’s not really ‘non-Mendelian’; lack of homozygotes for recessive lethals was discovered early in Mendelian genetics.

l.410 ‘absent’

Discussion

I feel that this needs to be extensively revised, in line with my comments on the interpretation in terms of inversions. I have therefore not reviewed it in detail.

Reviewer #2

(Remarks to the Author)
The new version of the manuscript addresses all of my remaks and I find it is clearer and better balanced. I enjoyed reading it through and believe it would make an important contribution to the study of POD.

Minor remarks:

L273 - The increase was not correlated with low recombination rates (Fig. S7c,d). When only homokaryotes are considered, πN reduced by 45% on average (Fig. S7b, Table S5). This reduction was significant for three regions, even though a small effect of sample size may occur as for πS (Fig. S7e, Table S7).

L563 - Notably, questions persist regarding how much genetic load needs to accumulate to enable the emergence of POD

and how this process interacts with structural variants

L564 - Sentence repeated twice - Most studies have focused on the emergence of variants and genetic load within a single population. - repeated

Reviewer #3

(Remarks to the Author)

The paper presents convincing evidence for anomalous high heterozygosity in 6 genomic regions in pearl millet from 126 Senegal accessions and significantly elevated ratios of non-synonymous to synonymous variation in a 7th (Chr1) region (Table 1). This leads the authors to explore whether selection via pseudo-overdominance (POD) may be acting to maintain the heterozygosity in terms of several hallmarks. Haplotypes from these regions on chromosome 2 and 7 confirm strong heterozygote advantage (Fig. 1) and regions that include centromeres with low recombination do not show the reductions in genetic diversity expected. These results and the higher deleterious load levels within the candidate regions all support the conclusion that selection is acting to maintain variation in these regions. The evidence that these structural variants have remained stable over 40 years further supports the idea that stabilizing selection, probably via POD, sustains this anomalously high variation.

The authors have done a conscientious job of responding to the second round of reviews, re-considering conceptual points, correcting a few errors, conducting further analyses, and qualifying several of their conclusions. Although several evolutionary forces may be at play in these genomes, the authors' original hypothesis that genomic POD could act to sustain variability and mutational load in these regions remains more supported than undermined.

The authors also thoroughly respond to questions of how introgression and structural variants may contribute to sustaining highly heterozygous regions, noting that recent simulation studies suggest introgression could be a key engine driving POD. Whether this requires recurrent introgression (because POD regions are short-lived) or only rare or intermittent introgression (because POD can persist) remains unresolved, but this was never the paper's focus.

The authors have important results to share, having done detailed and thorough analyses of several interesting data sets highly relevant to understanding how genetic diversity is sustained in general and the particular selective forces maintaining anomalously high genetic variation within these 7 genomic regions in cultivated pearl millet. The biological significance of their findings, the use of complementary innovative genomic approaches, the fact that anomalously high heterozygosity occurs across 17% of the genome, and the authors' thorough analyses fully justify publication in Nature Communications (as does the interest these results have stirred up among the reviewers). The existence of alternative hypotheses that might explain some of these results is now fully acknowledged and addressed and should by no means preclude publication of the current paper. This is how science advances. Critics are free to publish counter-arguments or pursue additional work if they feel that is needed.

Minor points

Line 546 -- There is a tandem duplication of a sentence.

Line 566 et seq. – Ref 7 might also be cited alongside Ref 25 as it, too, explored how population hybridization enhances opportunities for generating POD and the importance of balanced load for sustaining it for thousands of generations (and so might also be cited at L 585 and/or lines 589-590).

Reviewer #4

(Remarks to the Author)
A junior researcher and I previously reviewed this manuscript. We find that the authors have addressed our previous comments, but we remain uncertain about the conclusions. There appears to be a strong case for the role of continued gene flow in maintaining the chromosome 3 polymorphism, and we do not see compelling evidence to support POD over ongoing gene flow for the H2 haplotype. Below are a few specific points for further consideration:

1. While the authors have moderated their conclusions in parts of the manuscript, we suggest that they also consider adjusting the conclusions in the abstract to align with this tone.

2. The authors describe H2H2 as strongly lethal despite observing some homozygotes in nature. We recommend rewording this from "strongly lethal" to "strongly deleterious."

3. Figure S12 shows elevated FST across the entire chromosome for H2 heterozygotes. H2 heterozygotes are also separated in the local PCA outside of the H2 region (see figure for reviewers and Figure S1, which should be colored by genotype). This suggests that the pattern is not limited to a specific region but spans much of the chromosome, supporting the idea of ongoing gene flow. We do not believe the region defined by the authors exhibits different population genetic patterns from the rest of the genome.

4. We apologize for our earlier miscalculation of HWE. However, we were surprised to see that all of the karyotype-level data were in HWE, which we feel weakens the POD argument. Additionally, negative Fis values are not uncommon in wild datasets, particularly in the context of introgression. We do not consider Fis alone to be a strong argument for POD, especially in the presence of structural variants (SVs).

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
See attached file.

Version 4:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
See attached file.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Comment. This paper presents some interesting results on newly-discovered polymorphic inversion systems in pearl millet, based on large-scale genomic analyses. It makes a useful contribution to the increasingly long list of cases where such polymorphisms have been found outside the classical examples in Drosophila, which were studied cytologically using polytene chromosomes. The finding that one inversion haplotype appears to be nearly lethal when homozygous is especially interesting (clearly, this one has to be maintained by selection), although there are precedents for this in other systems. I am not an expert in genome analysis, so I cannot critically evaluate this aspect of the paper; as far as I can tell, this work has been done competently and their evidence for the inversions seems solid.

We appreciate the overall positive comment on our study. We really appreciate this lengthy review to help us building a better version of the manuscript In order to address each point thoroughly and constructively, we have divided comments into several paragraphs. This will help us respond more effectively and incorporate your suggestions into an improved version of our manuscript.

Comment 1.1. I believe, however, that there are serious problems with their interpretation in terms of "pseudo-overdominance" (POD) as the mechanism for maintaining their inversions, and with the nature of the evidence for this process in their system. For the reasons stated below, the authors need to be much more cautious in their claims about the reasons for the maintenance of the inversions. First, let's try and clarify what is meant by POD. I think it's most useful to use the term in relation to a situation in which partially recessive or recessive deleterious mutations are present in repulsion with each other, so that an individual heterozygous for a pair of haplotypes carrying different complements of mutations has a higher fitness than the homozygotes for these haplotypes.

Answer1.1. We more clearly defined the term and we agree with this definition. We have now clarified this definition in the introduction part :

L94-101 : POD is defined by Gilbert et al.[8] as a "*form of balancing selection that maintains complementary deleterious haplotypes, effectively masking the effects of recessive deleterious mutations*". Consequently, the complementation of deleterious alleles in repulsion results in a heterozygote advantage[4–6]. There is considerable confusion in the literature regarding the distinction between pseudo-overdominance and associative overdominance[9–11]. Overdominant and deleterious alleles, as well as POD, can lead through hitchhiking to associative overdominance (AOD)[12,13], i.e. an apparent heterozygote advantage at nearby neutral loci[8,10,14].

Comment 1.2. This easily arises in crosses between inbred lines, fixed by chance for deleterious mutations at different loci, as discussed in ref. 4, or within a very small population. The authors don't seem to distinguish between what happens such situation versus large random mating populations (see their citations of ref. 13); only the latter is relevant to their study of millet populations.

Answer 1.2. We partially agree. Yes, our observations come from a large population but this population is structured and subject to introgression from a sympatric wild subspecies. Our model is a partially outcrossing domesticated species and we sampled landraces. Typically, F_{IS} between landraces

are around 0.1-0.2, and F_{ST} between landraces around 0.05 to 0.1 (Diack et al. 2017 doi: 10.3389/fpls.2017.00818, Olodo et al. 2020 https://doi.org/10.1371/journal.pone.0239123). Our model is therefore, as many domesticated species, in between the "inbred lines" (i.e., small population size) and a random mating natural population.

However, an important context to consider, especially regarding chromosome 3, is that the sequence originates from a wild, divergent population. Different deleterious alleles may have accumulated and become fixed in each population prior to hybridization. This scenario creates favorable conditions for the process leading to POD described by Sturtevant & Mather (1938) and theoretically investigated by (Abu-Awad & Waller 2023). We have now elaborated on this situation and its distinctions from theoretical models in greater detail within the discussion section:

L559-580 : Distinguishing true overdominance from POD can be challenging[4]. While GWAS analysis failed to unveil any association between SNPs and fitness-related traits on chromosome 3, the presence of true beneficial alleles cannot be discarded. True overdominance is expected to be rare[4] and does not necessarily require low recombination. In contrast, POD requires regions of strong linkage between deleterious loci, which maintain correlation in homozygosity, a phenomenon known as identity disequilibrium (ID)[60]. Hence, the role of inversions in POD was theoretically proposed earlier[17]. Some theoretical studies suggest that POD alone could not maintain inversions[22] or only under very specific conditions[10]. Most of these studies simulated the emergence of inversions within either a population or a meta-population with gene flow under a symmetric island model. However, a recent study[7] tested a scenario where genetic drift fixed alternative sets of recessive deleterious mutations among isolated populations. Under such a scenario, POD zones were able to persist for hundreds to thousands of generations when strong linkage and mildly deleterious mutations were involved[7]. The latter authors suggested, like others, that centromeres and inversions may be susceptible regions for POD to occur. In our study, we demonstrated, at least for the large candidate region from chromosome 3, that the origin is likely to be due to introgression from a divergent wild relative population and the presence of an inversion. This finding closely matches the scenarios and assumptions proposed by Abu-Awad & Waller[7].

More generally, we would argue that cultivated plant species may be prone to POD because: i) cultivated plants, in particular annual plants, often undergo severe population bottlenecks[61,62] leading to a high cultivated genetic load[63]; and ii) cultivated species show frequent hybridization in secondary contact zones propelled by human migrations[64–66].

Comment 1.3. POD is closely related to the phenomenon of associative overdominance (AOD), in which a neutral locus linked to a locus (or loci) subject to mutation to partially recessive or recessive deleterious alleles, or to heterozygote advantage, itself experiences an apparent heterozygote advantage. Confusingly, Ohta & Kimura (1969 Genetics 63:229) used the term pseudo-overdominance, when they were in fact studying AOD caused by a single locus with heterozygote advantage.

Answer 1.3. We are very grateful and deeply thankful to reviewer #1 for providing references, insights and explanations for better clarification and distinction between the processes of associative and pseudo-overdominance. It's also in line with how Charlesworth still refers in a recent study (Charlesworth 2024). We fully agree that the use of both terms, in the literature, has sometimes been very confusing and we have tried to ensure that the manuscript is clear throughout to avoid confusion as much as possible.

Comment 1.4. Even more confusingly, their conditions for apparent heterozygote advantage in a randomly mating population do not generate selection for maintaining variability at the neutral locus; if you use their equations for the apparent fitnesses at the neutral locus, you get no change in allele frequency at this locus (Zhao & Charlesworth 2016 Genetics 203:1315). However, there are quite stringent conditions on tightness of linkage, dominance and strength of selection that can lead to a reduction in the effectiveness of genetic drift at the neutral locus; Nes values of the order of 1 are needed for this single-locus effect in randomly mating populations (Zhao & Charlesworth 2016); otherwise, background selection (ref. 11) operates, causing an enhanced effect of drift. Multilocus simulations (e.g. Latter 1998 Genetics 148: 1142) show that there can be significant retarding effects of partially recessive deleterious mutations on the loss of neutral variability in small populations, consistent with observations on lab populations of Drosophila (e.g. Schou et al 2017 Mol Ecol 26:6510). However, it is questionable whether this is likely to happen in large natural populations, except when there is limited gene flow between isolated populations (as modelled in ref. 7; ref. 5 is just a commentary on this paper, by the way), or in genomic regions with very low rates of recombination (as in refs. 8 and 10). Even in the latter cases, the observations and theory suggest that there is just a bit more variability and less distortions of gene geneaologies by the presence of deleterious mutations than would otherwise be the case. The authors need to provide a much more nuanced account, in the light of these comments. The authors need to provide a much more nuanced account, in the light of these comments

Answer 1. 4. We agree with the comments of reviewer #1 in that the emergence of POD requires specific conditions, as noted in the comments and references therein. We have nuanced our comments at several points in the discussion (highlighted text). However, as stated, this is unlikely to happen except when there is limited gene flow between isolated populations. A scenario that was theoretically confirmed by Abu-Awad & Waller (2023) and that closely matches the case of chromosome 3, where the sequence of the haplotype originates from the wild subspecies and has been introgressed into cultivated pearl millet.

Comment 1.5: the original proposal for this process (POD) is, however, not cited (Sturtevant & Mather 1938 Am Nat 72:747), and neither is Ohta'sproposal re inversions based on her AOD work (1971 Genet Res 18:227).

Answer 1.5. Thank you for pointing out the omission of this early work. We now cite the foundational literature by Sturtevant & Mather (1938).

Comment 1.6. The question of whether this type of effect of deleterious mutations can maintain inversion polymorphisms has, as the authors say, been raised several times in the literature, mainly as purely verbal speculation. However, recent theoretical investigations do not support to this idea. First, studies of the introduction of inversions into populations subject to mutation and selection at multiple loci suggest that autosomal inversions are unlikely to experience a long term selective advantage as a result of the presence of such mutations, in the absence of any other effects of inversions on fitness (Connallon & Olio 2021 Mol Ecol 31:3267; refs 56; 57 and 58- the last two refs are incomplete in the bibliography). Empirical studies of the fitness effects of Drosophila inversions are hard to reconcile with the POD idea (Charlesworth 2024), and imply that other factors are involved in their maintenance by selection, including cases of strong heterozygote advantage, although POD may contribute to the later. Overall, therefore, it seems unlikely on theoretical grounds that the inversions described here could be maintained by POD, alone, although POD may contribute a small effect for the inversions that are at approximately equal frequencies. Second, it has recently been shown that autosomal inversions in freely recombining genomic regions subject to mutation, selection and drift only acquire a long-term

net heterozygote advantage if they are present at nearly equal frequencies, and the size of this advantage is likely to be small (Charlesworth 2024 Genetics 226:iyad218). Otherwise, the rarer arrangement accumulates a higher mutational load than the alternative, and homozygotes for it are less fit than the heterozygotes and homozygotes for the alternative.

Answer 1.6. Recent literature (Berdan et al., 2021; Connallon & Olito, 2021; Charlesworth, 2024) has developed theoretical frameworks to assess the impact of deleterious mutations on the evolution of inversions and the conditions under which polymorphism could be maintained by these effects. For instance, Connallon & Olito (2021) stated that: "*When deleterious mutations are partially recessive (h < ½), an established inversion may either proceed to fixation under positive selection or remain polymorphic under balancing selection due to associative overdominance (see Appendix B). The likelihood of each outcome depends on the fitness benefit of the inversion (s b) relative to the initial number of mutations that it carries »*

However, most of these models have considered an inversion arising from the population itself, gene flow through an island model which is a symmetric population structure, or non-fluctuating selection across time and space. Nonetheless, they have also begun identifying factors that could delay the fate of inversions, such as gene conversion, which is expected to increase in low recombination regions and inversions (Korunes & Noor, DOI: 10.1111/mec.14921), or haplotype structuring (Berdan et al. 2021). For haplotype structuring, many studies, including ours, have shown a significant number of 6-clusters patterns rather than 3-clusters patterns (see section L508-516). This suggests that haplotype structuring is an important factor for the evolutionary outcomes of inversions, or at least allows them to be maintained longer in the population. It is noteworthy that the chromosome 3 region showed a pattern suggesting a double crossing-over event that has led to two divergent haplotypes segregating in the populations, and thus it might be subjected to haplotype structuring effects. Overall, our study is experimental, and our conditions might actually differ from those explored in most of simulation studies. This is particularly true for chromosome 3, where the genetic sequence within the inversion likely arises through introgression from a divergent population. While the importance of introgression in species evolution is increasingly recognized, to our knowledge, there are no simulation studies that have investigated the extent to which AOD (Associative Overdominance) or POD (Pseudo-Overdominance) could help maintain introgression, especially in low recombination regions or those captured by inversions.

Based on simulation, even though it has not always been thoroughly studied and certainly needs more work, if POD is created by introgression, it could be observed for hundreds to many thousands of generations (Abu-Awad and Waller 2023). Using populations sampled 40 generations apart, we have only been able to show a likely heterozygote advantage over this time period. We cannot determine if it confers a long-term net heterozygous advantage, as we could not estimate the age of the phenomenon on chromosome 3 due to part of the diversity originating from the wild population. However, we tend to agree with the hypothesis suggested by reviewer #1: it may have reached its final stage where one of the rarer arrangements appears to have accumulated a higher mutational load and is approaching lethality. We believe that POD, based on the different evidence we have, is playing a role in the maintenance of chromosome 3, but we acknowledge that POD might not be the only force at work. We previously discussed this in the discussion, but we certainly did not emphasize it enough. We now include greater detail in the discussion section and more strongly state the possibility that other forces might also be contributing (see Answer 1.2.). The question remains as to the relative strength of POD compared to other factors. We hope our study will encourage more simulation and experimental work to better assess the role POD might play, or not, in low recombination regions and the outcomes of introgression.

Comment 1.7. In addition, the authors' evidence suggests that most of their inversions are in pericentric regions of the genome. They provide little detail about what these regions contain in the way of genes versus repetitive sequences; it is well known plant genomes often contain large non-recombining pericentric regions with low gene densities (Haenel et al. 2018 Mol Ecol 27:2477; Brazier & Glémin 2022 PLoS Genet 18: e1010141).

Answer 1.7. We now have added this information in Table 1 and a figure (Fig S3) showing the number of genes present along the candidate regions. As indicated, this number is indeed lower compared to other regions of the genome. However, thousands of genes are still present across the candidate regions, with over 650 genes in the chromosome 3 region alone.

Comment 1.8. It is possible that the effects of low recombination in generating patterns suggesting associative overdominance, reported in refs 8 and 10, could be operating in these regions. However, the lack of recombination would mean that an inversion with no other fitness effects would behave just like a random haplotype, since it has no recombination-suppression effect. It thus unclear whether any long term maintenance of an inversion by selection could be produced by deleterious mutation**s** in this region.

Answer 1.8. Yes, it is unclear, and to our knowledge, there is no theoretical model that has investigated this question. However, it is striking that we found nearly all centromeres of pearl millet involved with structural variants. The role of inversions in centromere evolution has been extensively demonstrated across various clades. This topic was already discussed in the first version of the manuscript (L528-536).

In the case of chromosome 3, we believe that divergence from the haplotype introgressed have significantly alleviated the genetic load of this region. It is important to note that domesticated species are hypothesized to have a higher genetic load (demonstrated in some cases but counterexamples also exist) attributed to the domestication bottleneck which increases drift, compared to their wild relatives (a phenomenon referred to as the cost of domestication; see Liu et al., 2017, https://doi.org/10.1093/molbev/msw296).

Comment 1.9. Furthermore, the evidence provided by the authors for POD is not conclusive. The main evidence comes from the analyses presented in Fig. 2 and Table 1, where they show that SNPs associated with the inversions have a deficiency of homozygotes relative to Hardy- Weinberg expectations (i.e. negative FIS values), whereas there is little departure from random mating expectation for SNPs in the genome as a whole. This does suggest an overall heterozygote advantage for at least some of their inversions (provided that the population can be assumed to be in equilibrium. However, no conclusions can be drawn concerning the causes of this heterozygote advantage from this observation alone (see ref. 18 for a discussion of possible selective advantages to inversions). It is, however, curious that Fig. 2 shows only a small effect for chromosome 3, which has the haplotype that is apparently recessive lethal.

Answer 1.9: We agree that F_{IS} supports the hypothesis of an overall heterozygote advantage. The question of whether this advantage stems from direct heterozygote advantage or pseudo-overdominance (POD) remains open, and we have extensively discussed and enhanced this discussion in our manuscript. Additional evidence, such as temporal analysis, further supports a heterozygote advantage for chromosome 3. Identification of a haplotype with a strong deleterious signature (segregation) suggests counter-selection against at least one haplotype. This implies that this haplotype has accumulated or harbors deleterious alleles that are not evident in the heterozygous state. Therefore, considering all

these indicators (F_{IS}, diversity, presence of structural variants), particularly for chromosome 3, we propose that our hypothesis regarding the significant impact of POD warrants discussion. Nevertheless, we acknowledge that other forces, such as direct overdominance, may also contribute.

Regarding the F_{IS} value on chromosome 3, as queried by reviewer 4, we have elaborated a statistical framework to derive the F_{IS} for a SNP found inside an advantageous heterokaryotype (see comment **QR4.10** and Supplementary text 1). Depending on the segregation frequency of the SNP in the haplotype, the frequency of the karyotype but also on the relative heterokaryotype advantage, F_{IS} will vary. A recessive lethal allele might lead to a more negative F_{IS} (estimated on SNP) compared to others, but it might depend on a large number of variables. For example, F_{IS} is calculated based on all SNPs, including those with low allele frequencies. Given the extensive size of the chromosome 3 region (and thus the number of low-frequency SNPs), this might result in an underestimated average value of F_{IS}.

Comment 1.10. Table 1 does seem to show evidence for relaxed purifying selection on nonsynonymous sites in the regions covered by the inversions. However, it is not clear how to interpret this, given that the inversions are mostly contained in pericentric regions, where (at least in Drosophila) there is abundant evidence for relaxed purifying selection against deleterious mutations (e.g. ref. 10 and citations therein). There is thus a good deal of confounding of the effects of location in pericentric regions with the presence of inversions. In addition, such relaxation of selection is expected purely due to the separation of the population into smaller subpopulations by the inversions, without any significant heterozygote advantage necessarily accruing (Charlesworth 2024), so finding evidence for relaxed selection does not entail the operation of POD.

Answer 1.10:

We agree that the fact that the candidate regions are mainly pericentromeric and are localized in low-recombining regions is a confounding factor. We acknowledged this aspect more clearly in the paper, and proposed a strategy to deal with it for the study of diversity, which could indicate POD if diversity is maintained across the candidate regions. We however agree that this confounding factor is difficult to deal with.

We added new results showing that we observe a clear relationship between recombination and diversity: Figs. S5c, S6c, S7c. To disentangle the effects of POD and low recombination, we computed the diversity π, π_S at synonymous sites, and π_N at non-synonymous sites for the most frequent homokaryotype as the expected centromeric diversity without the influence of the structural variants. For each candidate region, we compared the diversity of the most frequent homokaryotypes with the diversity observed considering the whole population, i.e. considering all the haplotypes segregating in the population: Figs. S5b, S6b, S7b, and Table S6. We showed that the diversity tends to be higher for most of the candidate regions when all the haplotypes are considered, with significance for four of the regions tested. We showed that the subsampling of the population did not affect the diversity of the genome outside of the candidate regions: Figs. S5e, S6e, S7e.

These different approaches are imperfect to deal witht the confounding effect of lower recombination of the centromeric region, but we showed this new results now in the revised version of the manuscript.

Sentence added in the manuscript: L255-276 (see below in **QR1.4)**

- QR1.1 : There are some more minor general points. First, some more information should be given about the study system. We are not told its scientific name its genome size, numbers of chromosomes, whether they are metacentric or telocentric, and what the gene density in the pericentric regions is (several relevant tables seem to be missing from the Supplementary Information). Second, the authors are not native English speakers, and there are many errors that need to be corrected. I give a few examples below.

The last version of the manuscript has been proof read by a professional English editor.

Specific comments

- QR1.2 :

l.59 ‘anecdotal’ is not appropriate here.

Corrected L59-60

l.65 ‘outcrossing’ would be more familiar to most people.

Corrected L65

l.66 I presume they mean ‘studied’ here, as the whole genome was ‘identified’.

Corrected L65

l.83 ‘selective’ not ‘adaptive’- selection and adaptation are not the same thing.

Corrected L85

- QR1.3 :

l.70-75 They have no solid evidence for POD, as discussed above.

We have taken the reviewer's comments into account and have moderated our language accordingly.

l.90-125 See the general comments on POD above.

We have taken the reviewer's comments into account and have moderated our language accordingly.

- QR1.4 : l.131 How high is high diversity? It would be useful to have a number for silent nucleotide site diversity, and a comparison between the pericentric regions and the genome as a whole.

We performed analyses of the diversity π using all the sites, and at synonymous sites (π_S) or non-synonymous sites (π_N): see Tables S5-S7, and Figs. S5-S9. The neutral diversity is expected to be lower in low recombining regions, notably due to background selection. The expectation was thus to have a lower diversity around the centromere, around which we identified the candidate regions. We thus performed the analysis with all the population first, and then with a subset of the most frequent homokaryotypes for each candidate region, to assess the effect of the presence of the different

haplotypes in the diversity of the population. We also estimated the correlation between the recombination rate and the diversity. This allowed to assess if the candidate regions followed the same pattern and present a diversity similar as the rest of the genome for a given recombination rate. We notably showed that the diversity at non-synonymous sites is significantly higher on the candidate region of chromosome 3 when including all the haplotypes in comparison with the diversity of the most frequent haplotype only. To ensure robustness, we confirmed that these results were not influenced by reduced sample size by conducting significance testing with a matched sample size from the rest of the genome. These findings suggest that pseudo-overdominance may be occurring in the candidate region of chromosome 3, potentially contributing to the maintenance of neutral diversity (Gilbert et al., 2020).

Sentence added in the manuscript:

L255-276: A second hallmark of POD is an increase in diversity. However, five of the six candidate regions contain the centromere, which is expected to exhibit low diversity. Indeed, as observed in other species[36–40], a positive correlation between diversity (π) and recombination is observed (Fig. S5c). Consequently, low recombination regions confound the analysis of the impact of POD on diversity. Based on the diversity-recombination relationship, one would expect a significant decrease in diversity in these regions. Contrary to this expectation, six of the seven regions showed no significant reduction in diversity (Table S5, Fig. S5a; red dots in Fig. S5d). To disentangle the effects of POD and low recombination, we also calculated the diversity π of the most frequent homokaryotype as the expected centromeric diversity without the influence of structural variants. These values tended to be lower than those observed when all karyotypes are considered (Fig. S5b; Table S6), and were significant for four of the six regions we could test. We verified that this lower diversity π is not simply a consequence of subsampling (Fig. S5e). These results suggested that diversity π was maintained; however, the confounding factor of being in a low recombination region is not fully accounted for. The results for π_S and π_N tended to be lower than those observed when all karyotypes are considered (Figs. S6b and S7b, Table S6), and were significant for some comparisons on chromosomes 3 and 6. The results for π_S and π_N and their ratio might suffer from loss of power because of the ten-fold decrease in the number of SNPs annotated as synonymous and non-synonymous (Figs. S6-S9 and Tables S5 and S6). For the large chromosome 3 candidate region, we observed a significant reduction in π_S for one comparison with the rest of the genome (Table S5 and Fig. S7a), and a tendency to an increase in π_N (Table S5 and Fig. S6a), resulting in a significantly higher π_N/π_S ratio (Figs. S8 and S9, Tables 1 and S7). We also used the *pN/pS* metrics. A

And L739-777 in Materials and Methods.

- QR1.5 : Table 1. Presenting Pn/Ps values without standardizing the values per nucleotide sites is not very helpful, as it does not give the reader a sense of whether nonsynonymous nucleotide site diversity relative to synonymous site diversity is unusually large in the pericentric regions. Also, we ought to be told whether synonymous site diversity is low in these regions compared with the genome as a whole, as one might suspect from other studies (e.g. ref 10). It's also not clear to me how you can do G-tests on ratios, as they appear to have done; I would have thought a resampling procedure would be a safer type of test for differences. In addition, given the high LD in these regions, there is a issue with lack of independence among the different loci involved that needs to be addressed; it's not clear that each locus can be treated in isolation. The same applies to the Wilcoxon tests.

We have added the analysis of the πN/πS ratio and values are reported in Table 1. We conducted both the G-test and the resampling procedure for all comparison (Tables S3 and S4). Both tests yielded

similar results. Indeed, as the reviewer noted, high LD is a recurring problem in these types of regions, but to our knowledge, there is no test that can address perfectly this issue.

Sentence added in the manuscript:

L273-276 : For the large chromosome 3 candidate region, we observed a significant reduction in π_S for one comparison with the rest of the genome (Table S5 and Fig. S7a), and a tendency to an increase in π_N (Table S5 and Fig. S6a), resulting in a significantly higher π_N/π_S ratio (Figs. S8 and S9, Tables 1 and S7).

- **QR1.6 :** l.155-6 It's not clear that their claim for reduced recombination rates in the pericentric regions are meaningful (Fig S3) are meaningful. Their method is not explained in detail, but I believe it is based on population genomics methods that give estimates of the product of effective population size (Ne) and recombination rate. Since Ne is likely to be reduced in the pericentric regions, the effect of Ne is confounded with recombination rate. In addition, these methods assume random mating, but this assumption is violated if the genomic region is segregating for inversions. The higher LD in these regions (Fig S3) is probably a compound of a real reduction in recombination rate and a reduction in Ne (see ref. 10 for a discussion of this type of situation in Drosophila), but they haven't distinguished these effects as far as I can tell.

Yes, as noted by reviewer 1, since our structural variants are in pericentric regions, it is highly likely that the estimated recombination rate is a combination of a real reduction in recombination rate and a reduction in Ne. However, our aim was simply to confirm that the variants we detected were in low recombination regions prone to AOD or POD processes, not to disentangle the contributions of each process to the reduction in recombination rate.

- **QR1.7 :** l.215-228 See the general comments for a critique of the interpretation in terms of POD.

We have taken the reviewer's comments into account and have moderated our language accordingly.

- **QR1.8 :** l.329 It would be good to have quantitative estimates of the differentiation between haplotypes in terms of statistics such as dxy and FST, as has been done for studies of inversions in other systems (e.g. Kapun et al. 2023 MBE 40: msad118).

We added supplementary figures (Fig. S13 and S14 and Tables S7 and S8) displaying F_{ST} and D_{xy} statistics estimated between different pairs of sample clusters. We tested the significance of these statistics using a resampling procedure.

Sentences added in the manuscript:

L310-316 : The low sequence identity observed in the central region of the chromosome, as indicated by both synteny plots and optical mapping, suggested substantial sequence divergence between H_1 and H_2 and the reference genome. This genetic divergence was also supported by F_{ST} estimates. All pairwise F_{ST} between clusters were significantly higher in the candidate region than in the rest of the genome (Fig. S13 and Table S9). Although less pronounced, the same trend was observed when using D_{XY} (Fig. S14 and Table S10).

L499-501 : The second 88 Mb alternate haplotype seems not to carry a major inversion, but to show a

strong sequence divergence revealed by sequence alignments and genetic distances.

- QR1.9 l.430-431 This statement should be much stronger; there is essentially no other way for the polymorphism for the lethal haplotype to be maintained, other than by negative assortative mating, which is implausible for a plant.

We corrected the sentence.

Discussion

I have not reviewed the Discussion in detail, given my general criticisms of their interpretations in terms of pseudo-overdominance and some important aspects of their analyses

Reviewer #2 (Remarks to the Author):

The manuscript "Pervasive interplay between large inversions and pseudo-overdominance in a plant genome" presents a case for the persistence of genetic diversity in populations due to pseudo-overdominance. I greatly enjoyed reading this paper, finding the arguments clear and very well supported by the different approaches used, which, I have to say, are impressively varied. I am unaware of any other works that have so rigorously shown that observed heterozygosity within non-recombining regions is most probably due to pseudo-overdominance, and I believe their results leave little doubt as to the conclusions. I have some very minor comments/queries and highly recommend that this paper be accepted for publication.

We would like to thank reviewer 2 for taking the time to revise our study and for his enthusiasm. We have included his comments in the new version of the manuscript.

Minor comments:

There are some typos and a couple of not quite comfortably worded sentences in the manuscript. It's not a huge problem, just pointing it out. I didn't copy all of them but here are some of them:

- **QR2.1 :**line 104: The sentence seems incomplete - "As a result, the authors of these studies hence more frequent testing of pseudo-overdominance patterns in genomic analyses"

Corrected L119-122

- **QR2.2 :** line 680: The SNPs and genotypes identifications -> The methods of identification of SNPs and genotypes are fully described ..

Corrected L906

- **QR2.3 :** line 755: DNAs were quantified -> DNA was quantified

Corrected L812

- **QR2.4 :** Question on methodology: In the last part of the methodology, "Associations between the chromosome 3 haplotypes and phenotypic traits" the authors mention imputing missing genomic data. As I am quite naive when it comes to this methodology, how much would it have affected the results not to do that? Would it be possible to include an analysis that uses "exclusion of incomplete data" instead? So as to attempt to evaluate whether a bias may have been introduced through this process.

Part of the data has already been used in a previous publication: Faye et al. 2022. In the latter, we used three different models for the GWAS analysis. We obtained similar results with all three methods. Our findings in this study are consistent with those obtained by Faye et al. 2022. Thus, we do not believe that a significant bias could have been introduced by imputing missing genomic data.

Reviewer #3 (Remarks to the Author):

The paper presents evidence that the genomes of 126 accessions of cultivated pearl millet from West Africa share at least 6 large (41-88 Mb) regions of low recombination that contain elevated heterozygosity, strong fitness effects, and high ratios of non-synonymous and often deleterious variants (including at least one synthetic lethal). Exome sequencing reveals that these regions represent large inversions that largely prevent recombination, ensuring tight linkage. In other contexts, tight linkage can accelerate background selection or selective sweeps that act to reduce genetic variation. In contrast, the high variability being maintained in these regions and the many results presented here strongly support the authors' interpretation that pseudo-overdominance (POD) derived from many deleterious mutations linked in repulsion generating balancing selection.

The authors present additional data and analyses to further explore the causes and consequences of this POD. I was surprised by the size of these inverted regions (though they are in line with the review by Wellenreuther and Bernantchez 2018) and that they cover so much of the genome (17% here). It was also remarkable that the authors were able to identify three (chromosomes 4 and 7) or six (chromosomes 2, 3, and 6) distinct clusters of haplotype variants characterized by large differences in heterozygosity (and later, fitness). These findings support their interpretation that strong POD exists within these regions. These convincing results are well-presented and further dissected within the largest inversion on chromosome 3 (e.g., Figs. 3 and 4). Further tests within this region reveal introgression of wild genetic variation into cultivated populations. The authors also performed self-fertilization experiments to analyze segregation ratios of a key marker within the chromosome 3 inversion. They found that one haplotype homozygote is almost completely missing, confirming the high segregational load due to POD and thus how balancing selection can sustain the inversion polymorphism.
It was also impressive to see the authors reach back in time to find accessions from 1976 to trace how chromosome 3 haplotype frequencies have evolved since then. The stable persistence in haplotype population structure (for all but a rare cluster) further supports the idea that POD and suggests that such polymorphisms may be very long lived (as suggested by results from other studies). In another example of how thorough this work is, the authors tested for associations between the inversion haplotypes and 11 traits. Again, they found clusters that differ in traits related to fitness. They also make a very interesting suggestion in the Discussion that (lines 477-479)

In sum, the paper addresses topics of considerable current interest with comprehensive data and a careful set of original analyses. The paper is also well-written and provides a thoughtful discussion. Their results suggest that POD may often be expected in genomic regions with restricted recombination, setting the stage for additional work on non-model organisms as long-read technology enables more of these kinds of analyses. The authors present a clear, convincing, and complete story with broad implications in both evolutionary biology and plant and animal breeding. I thus have only very minor suggestions to improve the paper a bit more (below).

We sincerely appreciate the reviewer for dedicating time to review our study. We are delighted that they agree that our research can offer new insights in evolutionary biology and the fields of plant and animal breeding. We have carefully incorporated their comments into the revised manuscript.

Minor suggestions

- **QR3.1 :** Lines 86 et seq. Casting natural selection and neutral evolution as “either – or” alternatives is simplistic given that most evolutionists accept both as important and seek to integrate them.

Yes, we agreed that is too simplistic. We have modified the sentence accordingly:

L88-91 : Although natural selection was initially considered to be a major driver in shaping diversity, theory and evidence suggest neutral processes have a considerable impact on the genetic diversity that prevails in any population[2].

- **QR3.2 :** L. 104 – suggested rewording: replace “hence . . ” with “urge others to test more frequently for pseudo-overdominance patterns in genomic analyses particularly since this phenomenon could be . . “

Corrected L119-122

- **QR3.3 :** L 265 & Disc L. 476, etc. – The authors find “nested rearrangements.” Do they consider these to occur commonly? Does having a few ‘divergent haplotypes’ possibly limit the total expression of segregational load by segregating many mutations together into these blocks?

The complexity of arrangements, or haplotype structuring as observed in our study, can lead to a 6-cluster pattern instead of the classical 3-cluster pattern. It is conceivable that greater complexity could result in even more clusters. In the literature, the 6-cluster pattern, similar to our findings, appears to be quite common. One of the pioneering studies in this regard was conducted by Faria et al. (2018), who identified two overlapping inversions. However, most studies do not delve into the level of detail we explored for chromosome 3, making it challenging to assess the diversity of structures and their impacts. Indeed, the implications for segregational load are a pertinent question, but to our knowledge, few studies have investigated this aspect. Nevertheless, simulations conducted by Berdan et al. (2021) demonstrated that haplotype structuring, where divergent haplotypes segregate, reduces the detrimental impact of deleterious load on fitness and delays the evolutionary decay of inversions.

Reviewer #4 (Remarks to the Author):

In the manuscript “Pervasive interplay between large inversions and pseudo-overdominance in a plant genome” the authors search for signatures of pseudo-overdominance in the pearl millet. Pseudo-overdominance occurs when the mutational load of two haplotypes (caused by deleterious recessive mutations) are complemented giving the heterozygote increased fitness (i.e. overdominance). When this process occurs in relation to structural variants, it is generally referred to as associative overdominance (*sensu* Ohta) as the presumptively neutral structural variant is “associated” with the deleterious alleles that cause the variance.

The authors use a combination of previously published exome capture data, whole genome resequencing, optical mapping and laboratory crosses to:

1. Identify seven candidate inversions in the genome, some with more than 2 arrangements. 6/7 include the centromere so were already regions of low recombination.

2. Show negative F_{IS} stats (6/7 regions), high pn/ps ratios (3/7 regions), and a higher ratio of deleterious to tolerated mutations (2/7 regions).

3. Take a closer look at the chromosome 3 region, confirm the presence of an inversion there and show evidence for the introgression of one of the arrangements from divergent wild populations.

4. Using three selfed parents, show unusual segregation patterns pointing toward lethality of one homozygote and overdominance of the heterozygote.

The authors suggest that these results indicate that pseudo-overdominance is prevalent in the pearl millet genome and a major force maintaining variation in this species. While pseudo-overdominance is a fascinating force that is often overlooked, myself and my co-reviewer were not convinced by the evidence shown here. Below we will go through this point by point but this can be summarized by three main issues: 1. Different figures often point towards different conclusions, 2. Large amounts of data are not shown and reasoning is not given as to why, and 3. Neutral expectations are not properly laid out. In our opinion these issues make the paper currently unsuitable for publication.

We would like to thank both reviewers 4 and 5 for taking the time to revise our study and for helping us to clarify several points of the manuscript.

General/Overall concerns1. Writing and vocabulary.

- **QR4.1:** 1a. The Introduction felt a bit vague and detached from the rest of the manuscript, many terms are not properly explained and some parts are difficult to follow, e.g.,
■ Lines 81 and 86 suggest that selection used to be (initially) considered a major driving force but that is no longer the case…

Yes, we agreed that is too simplistic. We have modified the sentence accordingly:

L88-91 : Although natural selection was initially considered to be a major driver in shaping diversity, theory and evidence suggest neutral processes have a considerable impact on the genetic diversity that prevails in any population[2].

■ Line 104: Not sure what is meant by: "As a result, the authors of these studies hence more frequent testing of pseudo-overdominance…"

Corrected L119-122

■ Line 122: Or by: "diversity evolution pattern"

Corrected

- **QR4.2 :** 1b. Specifically, when talking about putative inversions the vocabulary is very confusing. Please refer to arrangements (e.g. A and B) and then genotypes (AA, AB, and BB) rather than genotypes A, B, and C where B is a heterozygote.

We have modified this in figures and throughout the manuscript.

- **QR4.3 :** 1c. It is hard to keep track of the different data sets, when which is used and what type of SNP subset. Make sure these are always clearly stated.

We have added this information throughout the manuscript.

- **QR4.4 :** 1d. Please clarify the differences between pseudo-overdominance and associative overdominance. The manuscript talks about both processes.

Because a related issue was mentioned by reviewer #1, we have clarified the difference between the two terms and added some literature in section:

L94-101 : POD is defined by Gilbert et al.[8] as a "*form of balancing selection that maintains complementary deleterious haplotypes, effectively masking the effects of recessive deleterious mutations*". Consequently, the complementation of deleterious alleles in repulsion results in a heterozygote advantage[4–6]. There is considerable confusion in the literature regarding the distinction between pseudo-overdominance and associative overdominance[9–11]. Overdominant and deleterious alleles, as well as POD, can lead through hitchhiking to associative overdominance (AOD)[12,13], i.e. an apparent heterozygote advantage at nearby neutral loci[8,10,14].

2. Analyses on the chromosome 3 region do not paint a clear picture.

- **QR4.5 :** 2a. Figure 3C-D is quite confusing. Based on C one would suggest a large inversion encompassing the entire area (101-189) and then perhaps another inversion from ~120-170. But based on D the major inversion is from 155-175 mb.

We acknowledge that our observations and interpretations regarding chromosome 3 may not have been sufficiently clarified. We have provided additional explanations in our response to QR4.7 to address this issue.

- **QR4.6 :**2b. The recombination rate (Figure S3) looks strange, one would usually expect varying recombination and then a large section of low recombination in the inversion. In several chromosomes, it seems that the recombination is not necessarily lower than other parts outside the putative inversion/structural variant. Surprising that several of the p-values are significant. Would not the centromere to a large extent have the same effect? Is it possible to disentangle the two?

Indeed, other regions of the genome could also have low recombination rates. Possible explanations include strong selection (hard sweep), the presence of structural variants that were not detected because they were fixed in our samples, or small structural variants that did not pass our filter size. In this species, many of the large structural variants we detected were found in centromeres. Centromeres have been shown to be prone to large structural variants (see discussion L528-537). Centromeres, as well as structural variants such as inversions or highly divergent introgressions, will reduce recombination. To our knowledge, we have not found any methods that would allow us to disentangle the role of each process, centromere vs. structural variants, in reducing recombination.

- **QR4.7 :**2c. Figures S3-4 are not convincing, none of the synteny patterns shown are characteristic of an inversion, where one would expect a flipped section resulting in an X. In fact, it seems that it was impossible to determine synteny for a large portion of the region? **QR.4.6** Figure 3C-D is quite confusing. Based on C one would suggest a large inversion encompassing the entire area (101-189) and then perhaps another inversion from ~120-170. But based on D the major inversion is from 155-175 mb.

We thank the reviewer for this comments that make us realize that our text lacked clarity. We will take the time to clarify it below and the two figures needed for the understanding are Figures 3, and S10 and S11.

Figure 3C is the representation of the genotype for the most structuring SNPs (238) along the chromosome 3 region detected. The whole 1000 SNPs of the region are shown in the new supplementary figure asked in **QR4.9** (Fig. S10e). Both figures are consistent and showed that there is significant diversity between and within haplokaryotypes.

Fig. 3C identified based on genotypes, two haplotypes carrying different structural variants:

- a smaller one from 120-175 Mb (referred to as H_1)
- a larger one from 101-189 Mb (referred to as H_2)

Genotypes visualization suggests that the H_1 haplotype resulted from a double crossing-over event between 120 and 175 Mb involving the H_2 haplotype and the standard one, indicating haplotype structuring.

The optical mapping and long reads were obtained for an individual from group H_1H_2, heterozygote for almost all the region except between 155-175Mb (Fig. 3c). The synteny plot of the long reads scaffolds for the two haplotypes of this individuals are presented in Fig S11c for H_1 (left panel) and H_2 (right panel), and Fig. 3d H_1 only.

The synteny plot for H_1 showed the X pattern between 155 to 175 Mb (and Fig S11c left panel and Fig 3.d highlighted by arrows) indicative of an inversion. However, it is possible to see on the same haplotype, 3 others small inversions (around 130, 140 and 190 Mb), encompassing the 120-170 Mb region seen in the individuals of the group H_1H_1.

The optical mapping synteny plot for the H_2 (Fig. S11c, on the right) showed a large divergent segment as seen by the orange dots that represent alignments with identity between 25 and 50% - This zone goes approximately from 101-190 Mb. Divergence, and particularly in this region can be seen in the optical mapping alignment shown in Fig. S11b. Briefly, blue colors means that sufficient number sites matched. While yellow, means that sequences are too divergent. This is accordance with other statistics estimates (introgression analysis, Fst measures). A highly divergent fragment introgressed into a species is a structural variant which will also reduce recombination as in the case of an inversion.

Therefore, because our discourse was not enough specific, reviewer #4 was expecting that the two structural variants were inversion, while in reality our results suggest that H_1 shows divergence coupled with several inversions including the 155-175 Mb one and H_2 is mainly composed on a large divergent introgression. We have modified the manuscript accordingly :

L497-503: For chromosome 3, we confirmed the presence of a large inversion of approximately 20 Mb in size within one of the alternate haplotypes. The second 88 Mb alternate haplotype seems not to carry a major inversion, but to show a strong sequence divergence revealed by sequence alignments and genetic distances. The method of local PCA[27,29] has been commonly used to detect inversions in previous studies[28,30] but it is also effective to detect large and strongly divergent sequences[30,45.].

L300-316: We sequenced and assembled one heterozygous individual, Autof-Pod103sr8, belonging to cluster H_1H_2, using long-read sequencing and optical mapping (Table S8). This assembled individual confirmed the presence of at least one large inversion on H_1 with putative breakpoints at 155 and 175 Mb (Figs. 3d and S11c). Other smaller inversions were present between 125 and 150 Mb (Figs. 3d and S11c). The second haplotype, H_2, spans the entire candidate region from approximately 101 to 189 Mb and aligns with the reference genome with low sequence identity ranging from 25% to 50% across this region (orange dots in Fig. S11c). The two largest scaffolds obtained from optical mapping showed good alignment to the reference genome at the beginning and end of the chromosome, but showed low congruence in the central region, approximately from 100 to 200 Mb (highlighted in yellow in Figs. S11b and S12a), in contrast to an inbred line used as a control (Fig. S12b). The low sequence identity observed in the central region of the chromosome, as indicated by both synteny plots and optical mapping, suggested substantial sequence divergence between H_1 and H_2 and the reference genome. This genetic divergence was also supported by F_{ST} estimates. All pairwise F_{ST} between clusters were significantly higher in the candidate region than in the rest of the genome (Fig. S13 and Table S9). Although less pronounced, the same trend was observed when using D_{XY} (Fig. S14 and Table S10).

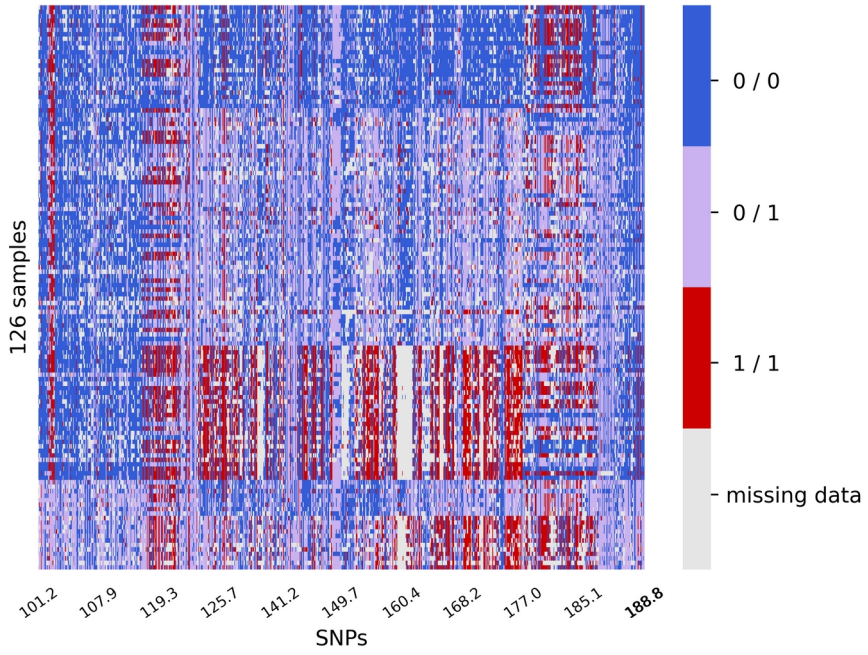
- **QR4.8 :**2d. The authors say on lines 264-265 that it appears that haplotype structuring is occurring. However, they do not explain this process and additionally, this does not appear to be haplotype structuring which describes a within arrangement phenomenon, whilst this appears more like multiple structural variants. To determine if haplotype structuring is happening the arrangements need to be clearly defined.

Berdan et al. (2021) introduced the term 'haplotype structuring' to describe situations where an inverted arrangement '*splits into two or more divergent haplotype clusters that carry partially complementary sets of deleterious recessive alleles*'. Their simulations considered the impact of gene flux through gene conversion, but did not include double crossovers, which are also important (Korunes & Noor, 2018, DOI: 10.1111/mec.14921). In our previous version, we did not clearly explain that genotype visualization suggests the H_1 haplotype resulted from a double crossover event between 120 and 175 Mb involving the H_2 haplotype and the standard one. This phenomenon suggests an intra-arrangement process, supporting the consideration of haplotype structuring.

We added: L516-526: Genotype visualization suggests that the H_1 haplotype might result from a double crossing-over event between 120 and 175 Mb involving the H_2 haplotype and the standard one. Berdan et al.[24] proposed the term of haplotype structuring to refer to cases where the inverted arrangement "*splits into two or more divergent haplotype clusters that carried partially complementary sets of deleterious recessive alleles*". We proposed that double-crossovers could lead to haplotype structuring, a process also recently suggested by other authors[52]. Simulations show that haplotype structuring would mitigate the negative fitness effect of deleterious mutations accumulating within arrangements[24]. The observation of frequent patterns suggestive of haplotype structuring[31,35,47-49] could indicate that the latter phenomenon is an important factor in the long-term maintenance of these structural variants.

- **QR4.9 :**2e. What do the haplotype patterns outside the identified region look like? The authors show 236/1000 SNPs in figure 3. What do all the 1000 SNPs look like?

We provide the figure with 1000 SNPs in Fig. S10e. Results are consistent between the two figures.



- **QR4.10 :**3. A neutral explanation for F_{IS} in inversions is missing. What would the F_{IS} of an inversion without overdominance look like? Given high heterozygosity in heterokaryotypes (higher than in collinear regions) would you expect a negative F_{IS} for an inversion even without overdominance? It is clear that F_{IS} is lower in these regions compared to the collinear genome but this is not the appropriate comparison.

We develop a mathematical theory for the karyotype F_{IS} (Supplementary text 1, see below) and for a SNP inside one of the haplotype. Without overdominance (s=0, below) F_{IS} = 0 both for the karyotype and any SNP segregating inside one of the haplotype. So as we develop below the theory for a SNP segregating inside an haplotype showing a karyotype with (s>0) or without overdominance (s=0), it demonstrate that if overdominance occur a SNP will exhibit a negative FIS, and if not will be equal to zero.

We tested now both for 1) does F_{IS} is lower than the expected value across the genome? And also add 2) does the F_{IS} is negative?

Mathematical formula and modelisation of F_{IS} in the context of relative overdominance for 2 haplotypes

We considered two haplotypes A and B and considered that heterokaryotype gave a relative selective advantage compare to homokaryotypes. This selective advantage is set to 1+s, s>0
We considered p and q being the allele frequency, p+q=1.

Under Hardy-Weinberg equilibrium, the allele frequencies are before selection:

Karyotypes	AA	AB	BB
Frequency before selection	p^2	2pq	q^2
Frequency after selection	$p^2/(1+2pqs)$	$2pq(1+s)/(1+2pqs)$	$q^2/(1+2pqs)$

After selection the frequency of karyotype AB will be

$$H_o = \frac{2pq(1+s)}{2pq(1+s)+p^2+q^2} = \frac{2pq(1+s)}{2pqs+2pq+p^2+q^2} = \frac{2pq(1+s)}{1+2pqs}$$ as $p^2+q^2+2pq=1$

The frequency of both homozygote karyotype is just rescale by 1/(1+2pqs)
Allele frequency p' of haplotype A estimated after selection is

$$p' = \frac{p^2+pq(1+s)}{(1+2pqs)} = \frac{p^2+pq+pqs}{(1+2pqs)} = \frac{p(p+q)+pqs}{(1+2pqs)} = \frac{p+pqs}{(1+2pqs)} = \frac{p(1+qs)}{(1+2pqs)}$$

and for B

$$q' = \frac{q^2 + pq(1+s)}{(1+2pqs)} = \frac{q(1+ps)}{(1+2pqs)}$$

Consequently, the expected heterozygosity for karyotype under hardy-Weinberg will be $H_e = 2p'q' = \frac{2pq(1+ps)(1+qs)}{(1+2pqs)^2}$

The defined the F_{IS} value for karyotype as:

$$F_{IS} = \frac{H_e - H_o}{H_e} = \frac{\frac{2pq(1+ps)(1+qs)}{(1+2pqs)^2} - \frac{2pq(1+s)}{(1+2pqs)}}{\frac{2pq(1+ps)(1+qs)}{(1+2pqs)^2}}$$

$$= \frac{2pq(1+ps)(1+qs) - 2pq(1+s)(1+2pqs)}{2pq(1+ps)(1+qs)}$$

$$= \frac{(1+ps)(1+qs) - (1+s)(1+2pqs)}{(1+ps)(1+qs)}$$

$$= \frac{1+ps+qs+pqs^2 - 1 - 2pqs - s - 2pqs^2}{(1+ps)(1+qs)}$$

$$= \frac{1-1+s(p+q) - s - 2pqs + pqs^2 - 2pqs^2}{(1+ps)(1+qs)}$$

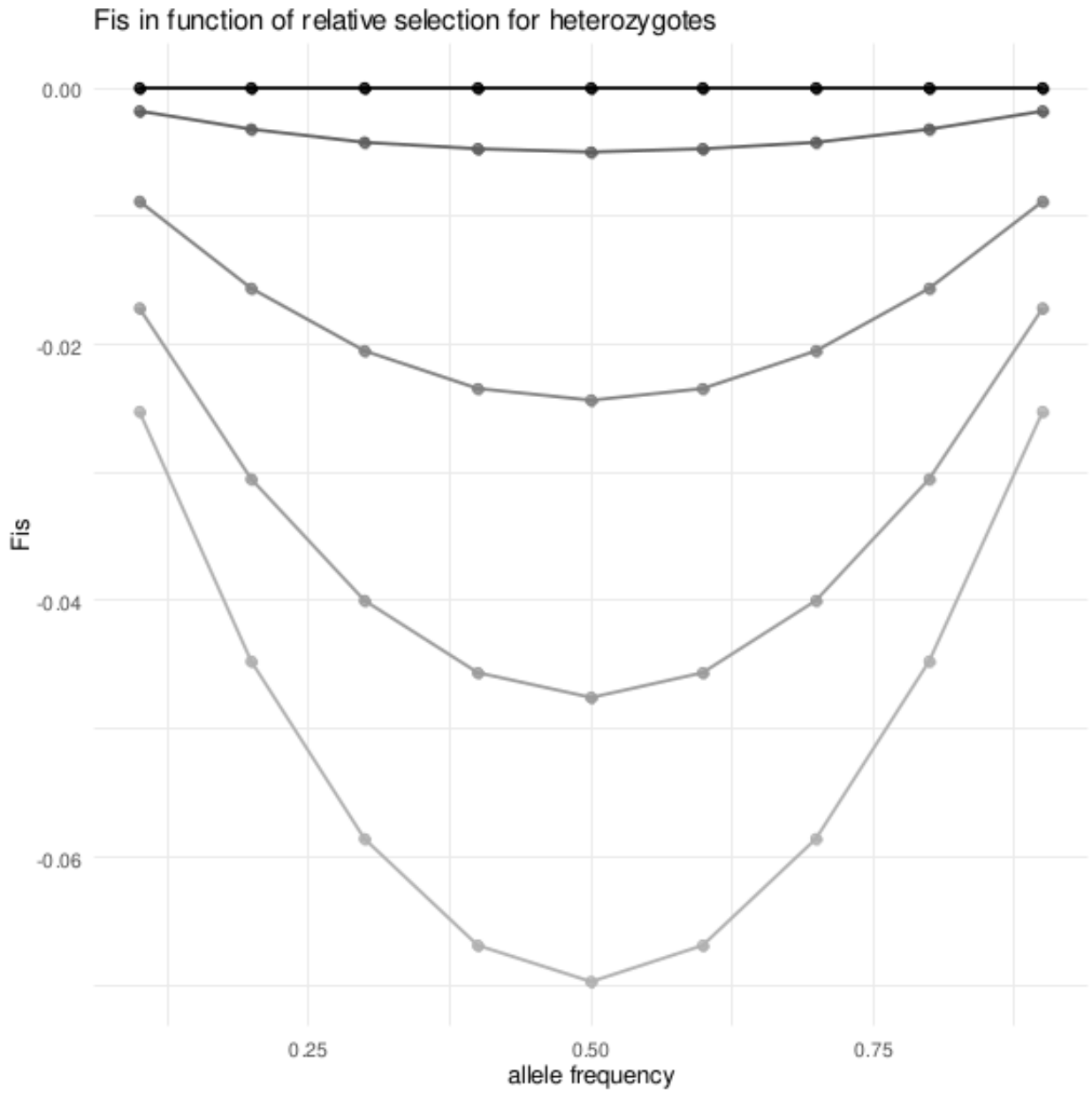
$$= \frac{-2pqs - pqs^2}{(1+ps)(1+qs)}$$

$$= \frac{-pqs(2+s)}{(1+ps)(1+qs)}$$

So $F_{IS} < 0$ if $s > 0$ and $p > 0$.

$F_{IS} = 0$ si $s = 0$ or $p = 0$ (no polymorphism).

Figure 1. F_{IS} in function of haplotype frequency with different value of relative overdominance.



The figure represents the F_{IS} value with selection coefficient s=0 (dark) and s increasing 0.01 (dark grey), 0.05, 0.10, 0.15 (lighter grey).

Now, we consider a SNP segregating only in one of the haplotypes. We only consider a situation where the SNP appear in one haplotype only and its frequency evolve by drift. Recombination or other phenomenon (gene flux, …) do not allow this SNP to be transfer to the other haplotype. We consider the probability the exact same variant appearing in the other haplotype by mutation is very low.

So if consider the variant of the SNP to be named "a" to be present on haplotype A with a proportion of α, αϵ]0, 1]. Its alternate "b" is present in all B haplotype, and in the haplotype A with a proportion of (1- α). If α=0, there is no variation i.e. no SNP, this case is excluded. If α=1 the SNP is perfectly aligned with the two haplotypes, the SNP a is in all haplotype A, the SNP b is in all haplotype B.

The overdominance is create by the heterozygote karyotype (not the SNP), so we now will estimate the F_{IS} for the SNP. To do so, we need to calculated how many karyotype AA, AB et BB led to the SNP genotype aa, ab, and bb.

If the karyotype is BB, all SNP genotype are bb

If the karyotype is AB:

-a proportion α of the haplotype A harbor the allele "a", and consequently their genotype is ab.

-a proportion (1- α) of the haplotype A harbor the allele "b", and consequently their genotype is bb

If the Karyotype is AA

-a proportion will be aa: α^2

-a proportion will be ab : $2\alpha (1- \alpha)$

-a proportion will be bb : $(1- \alpha)^2$

So, now we could derive the aa, ab and bb genotypes based on the probability of the different frequency of the karyotypes AA, AB and BB.

Genotype of SNP	aa	ab	bb
Frequency after selection	$\alpha^2p^2/(1+2pqs)$	$\alpha\ 2pq(1+s)/(1+2pqs)+ 2\alpha (1- \alpha)\ p^2/(1+2pqs)$	$q^2/(1+2pqs)+(1- \alpha)2pq(1+s)/(1+2pqs)+ (1- \alpha)^2p^2/(1+2pqs)$

The observed heterozygosity will be for the SNP:

$$H_o = \frac{2\alpha pq(1+s) + 2\alpha(1-\alpha)p^2}{1+2pqs}$$

The expected heterozygosity necessitates to recalculate the allele frequency of the "a" allele

$$p'_a = \frac{\alpha^2p^2 + \frac{1}{2}(2\alpha pq(1+s) + 2\alpha(1-\alpha)p^2)}{1+2pqs}$$

$$= \frac{\alpha^2p^2 + \alpha pq(1+s) + \alpha(1-\alpha)p^2}{1+2pqs}$$

$$= \frac{\alpha^2 p^2 + \alpha pq(1+s) + \alpha p^2 - \alpha^2 p^2}{1+2pqs}$$

$$= \alpha \frac{pq(1+s) + p^2}{1+2pqs}$$

$$= \alpha \frac{p(1+qs)}{1+2pqs}$$

be $H_e = 2p'_a(1-p'_a) = 2\alpha \frac{p(1+qs)}{1+2pqs}\left(1 - \alpha \frac{p(1+qs)}{1+2pqs}\right) = \frac{2\alpha p(1+qs)}{1+2pqs}\left(\frac{1+2pqs-\alpha p(1+qs)}{1+2pqs}\right)$

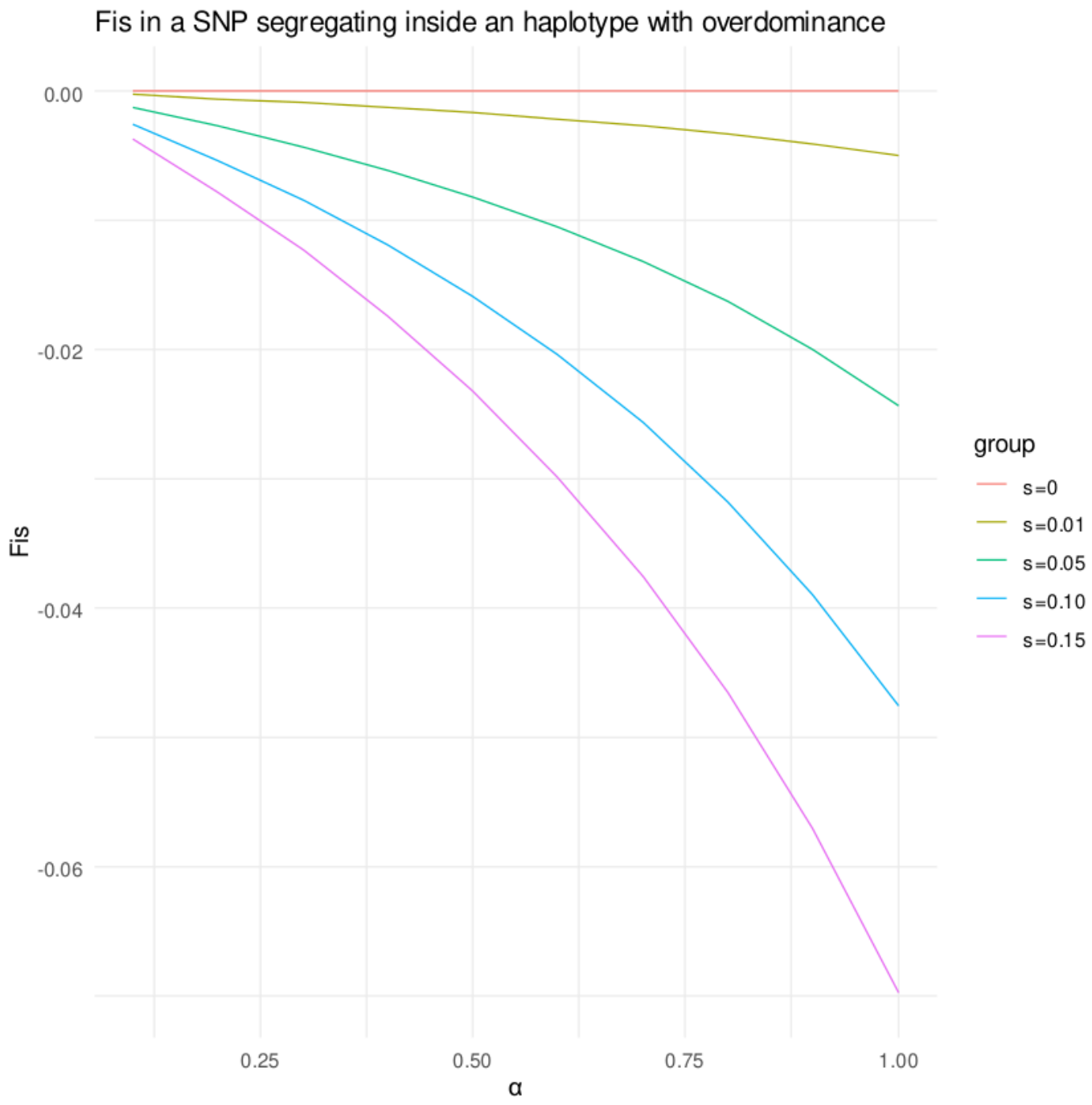
$$= \frac{2\alpha p(1+qs)(1+2pqs-\alpha p(1+qs))}{(1+2pqs)^2}$$

So using this formula we could derived $F_{IS} = \frac{H_e - H_o}{H_e}$

This framework allows us to understand how the F_{IS} value of the SNP related to allele frequency of the haplotypes A and B, and the relative advantage of the heterokaryotype AB.

We modeled in the next graph the F_{IS} (Figure 2) based on these formulae for different values of α, for p=q=0.5 (for simplicity, but we test the range of frequencies p and q ranging from 0.10 to 0.90 with similar results) to illustrate how the F_{IS} in the inversion will led to a F_{IS} in a given SNP. As expected all F_{IS} values are negative or null (null if s=0). A SNP generally exhibits negative but less negative F_{IS} than the karyotype F_{IS}, and both F_{IS} give the exact same values when α=1.

Figure 2. F_{IS} in a SNP segregating inside a haplotype.



The simulations were done for p=q=0.5. We consider two haplotype A and B, with the heterokaryotype having a relative overdominance of intensity 1+s, s>0. We study here the F_{IS} for a SNP segregating in the A haplotype only. The value of α represent the proportion of "A" haplotype carrying the "a" allele. The proportion of α varied in the interval]0, 1]. The alternate allele b is present in the other "A" haplotype and in all "B" haplotype.

- **QR4.11 :**3a. What would the F_{IS} of this region be if all three haplotypes were segregating under HWE? You can generate a neutral expectation using the defined genotypes (A-E). I.e. genotypes -> haplotype frequencies -> expected genotype frequencies -> expected F_{IS} values.

We developped the modelisation for the case of three haplotypes:

Mathematical formula and modelisation of F_{IS} in the context of relative overdominance for 3 haplotypes

We consider three haplotypes A,B,C and considered that heterokaryotes have a relative selective advantages compare to homokaryotes. The selective advantage is 1+s. For simplicity we considered each heterokaryote to have a similar selective advantage but generalization will be easy.

We considered the frequency of the three haplotypes p,q and r respectively for A,B,C with p+q+r=1

Under Hardy-Weinberg equilibrium, before selection, the genotype allele frequencies are :

Karyotype	AA	AB	BB	AC	BC	CC
Frequency before selection	p^2	2pq	q^2	2pr	2qr	r^2
Frequency after selection	p^2/ (1+2pqs+2prs+2qrs)	2pq(1+s)/ (1+2pqs+2prs+2qrs)	q^2/ (1+2pqs+2prs+2qrs)	2pr(1+s)/ (1+2pqs+2prs+2qrs)	2qr(1+s)/ (1+2pqs+2prs+2qrs)	r^2/ (1+2pqs+2prs+2qrs)

After selection, heterogaryote frequencies increase by a factor 1+s.

The different frequencies are rescaled by the total sum D, so theit total sum is still 1.

$D= p^2+q^2 +r^2+2pq(1+s)+ 2pr(1+s)+ 2qr(1+s)= = p^2+q^2 +r^2+2pq+2pr+2qr+2pqs+2prs+2qrs$

as $p^2+q^2 +r^2+2pq+2pr+2qr=1$

$D=(1+2pqs+2prs+2qrs)$

The observed heterokaryotype frequency is

$H_o=(2pq+2pr+2pq)\ (1+s)/(1+2pqs+2prs+2qrs)$

We could infer the frequency p' of A haplotype after selection:

$p'=(p2+pq(1+s)+pr(1+s))/(1+2pqs+2prs+2qs)=(p(p+q+r)+pqs+prs)/(\ 1+2pqs+2prs+2qs)$

$p'=p(1+qs+rs)/\ (1+2pqs+2prs+2qs)$

for B haplotype:

$q'= q(1+ps+rs)/(1+2pqs+2prs+2qs)$

for C haplotype :

$r'=r(1+ps+qs)/(1+2pqs+2prs+2qs)$

Using these expected frequency p', q' and r', we could deduce the expected frequency of heterokaryotypes, assuming HW. The frequency of expected heterokaryotypes is 1 minus the frequency of expected homokaryotypes:

$$\mathrm{H}_e = 1 - \frac{p^2(1+qs+rs)^2}{(1+2pqs+2prs+2qrs)^2} - \frac{q^2(1+rs+ps)^2}{(1+2pqs+2prs+2qrs)^2} - \frac{r^2(1+ps+qs)^2}{(1+2pqs+2prs+2qrs)^2}$$

Consequently, we could deduce heterokaryotype F_{IS}

$$F_{IS} = \frac{H_e - H_o}{H_e}$$

In case s=0, H_e simplify to $H_e=1-p^2-q^2+r^2=2pq+2rp+2rq$ and HO to 2pq+2rp+2rq

Consequently, as expected, $F_{IS}=0$

In case r=0,

$$\mathrm{H}_e = 1 - \frac{p^2(1+qs+rs)^2}{(1+2pqs+2prs+2qrs)^2} - \frac{q^2(1+rs+ps)^2}{(1+2pqs+2prs+2qrs)^2} - \frac{r^2(1+ps+qs)^2}{(1+2pqs+2prs+2qrs)^2}$$

$$H_e = 1 - \frac{p^2(1+qs)^2}{(1+2pqs)^2} - \frac{q^2(1+ps)^2}{(1+2pqs)^2} = \frac{(1+2pqs)^2 - p^2(1+qs)^2 - q^2(1+ps)^2}{(1+2pqs)^2}$$

$$= \frac{1+4pqs+4p^2q^2s^2 - p^2 - 2p^2qs - p^2q^2s^2 - q^2 - 2q^2ps - q^2p^2s^2}{(1+2pqs)^2}$$

$$= \frac{(1-p^2-q^2) + 2p^2q^2s^2 + 2pqs - 2p^2qs + 2pqs - 2q^2ps}{(1+2pqs)^2}$$

$$= \frac{(2pq) + 2p^2q^2s^2 + 2pqs(1-p) + 2pqs(1-q)}{(1+2pqs)^2}$$

$$= \frac{2pq + 2p^2q^2s^2 + 2pqsq + 2pqsp}{(1+2pqs)^2}$$

$$= 2pq\frac{1+2pqs^2+sq+sp}{(1+2pqs)^2}$$

$$= 2pq\frac{(1+sp)(1+sq)}{(1+2pqs)^2}$$

As expected, we founded the expected heterozygosity for two haplotypes (r=0).

We could simulate for different values of s, the F_{IS} in case of three haplotypes. Here we reported an estimation of F_{IS} for r=p=q=1/3 but it is easy with the given formulae to derive F_{IS} for any particular haplotype frequencies:

For s=0.01, F_{IS} =-0.00331

For s=0.05, F_{IS}=-0.01613

For s=0.10, F_{IS}=-0.03125

For s=0.15, F_{IS}=-0.04545

Based on the initial parameters, it is easy if necessary to extend to heterokaryote having different selective advantage s_{AB}, s_{AC}, s_{BC}.

- **QR4.12 :**3b. Line 221: "Negative F_{IS} are very uncommon and notably indicative of a selective heterozygote advantage"
■ We would argue that it is not that uncommon and that heterozygote advantage only is one possible explanation.

Indeed, heterozygote excess, indicated by negative F_{IS}, can have several potential causes. One cause might be a small reproductive population size. When only a few breeders contribute to the next generation, allelic frequencies can differ between male and female parents, as seen in crosses between highly inbred varieties. Negative F_{IS} can also result from negative assortative mating, also called disassortative mating, where individuals mate with others who are more dissimilar than by chance. This can occur in species with kin recognition systems, such as the major histocompatibility complex or self-incompatibility systems, which avoid consanguineous mating.

Another explanation for negative F_{IS} is asexual reproduction, which can maintain or even increase heterozygosity through mutations over generations. In many species, functional genes exist on both sex chromosomes in what are known as pseudo-autosomal regions. These genes often behave like autosomes unless they are tightly linked to the sex-determining gene. With tight sex linkage, pseudo-autosomal genes can have different allele frequencies between males and females, leading to an excess of heterozygotes in the heterogametic sex and the population as a whole. The last explanation for heterozygote excess involves positive selection for heterozygotes, due to true overdominance or processes such as AOD (Associative Overdominance) or POD (Pseudo-Overdominance).

Our sentence may be somewhat awkward, as we intended to note that positive F_{IS} values are generally observed more frequently than negative F_{IS} values. Given the biology of pearl millet, explanations involving asexuality and small populations can be excluded. Although there is no information about self-incompatibility or sex chromosomes in this species, we cannot entirely rule out these explanations. However, combined with other evidence, the most probable explanation seems to be some form of positive selection for heterozygotes. We have included this section in the discussion:

L552-557 : Here, we observed significantly negative F_{IS} values in five candidate regions, suggestive of an advantage for heterozygotes. In empirical studies, positive F_{IS} values are observed more frequently than negative F_{IS} values[35]. Negative F_{IS} values can be observed in cases of asexuality, disassortative mating, small populations and overdominance. Given the biology of pearl millet, negative F_{IS} values are more likely a signature of overdominance.

- **QR4.13 :**4. More population level genotype information is missing. Under pseudo-overdominance you would expect more heterokaryotypes than under HWE. The authors don't seem to have tested this. Is it true?

We added a test to see if F_{IS} values were significantly negative compare to HWE expectations (Table 1):

L221-223 : Five of them were significantly negative, i.e. a heterozygote excess signature compared to HWE expectations (Fig. 2, Table 1).

5. The segregation analysis is difficult to follow

- **QR4.14 :** 5a. The authors start with 95 parents but then narrow them down to three. How come only 3 of the 96 parents were heterozygous when you suspect this region to be pseudo-overdominant? Is there another reason the other parents were excluded?

We did genotyped 95 parents, among them we obtained 27 % that carried the whole structural variant which is roughly of the same frequency of the one estimated in the population (Tab S8), 8 % that showed recombination and 65 % that were homozygotes reference. We just selected 3 parents within the 27 % that carried the inversion because of budget limitation.

We have added the sentence:

L856-857 : Subsequently, we selected three parents among those being heterozygous for the five diagnostic SNPs.

- **QR4.15 :** 5b. How did you go from 1,500 SNPs to 10 to 5 to 24 and then 5 SNPs again? Which 5 are you in the end showing?

Given the cost of Kaspar sequencing and the fact that we only wanted to estimated recombination and segregation, we estimated that only few SNPS would be enough.

1) First set "diagnostic set" was to characterize the structural variant in Chr3:

We just randomly selected SNPs among the 1500 SNPs that contributed the most to the identification of the structural variants (i.e. the PC2 axis). Due to cost limitation, we decided to select randomly only 10 SNPs that were distributed along the H_2 haplotype. Among those 10, some did not feet these two criteria: i) had fewer than 20% missing data for genotyping, and ii) were polymorphic within at least one of the progenies. We ended up with 5 SNPs

2) Second set "control set" was to characterize the reference Chr3 as a control

Unlike the first set, where we were fairly certain of having heterozygotes, a significant number of the 2nd set of SNPs could be homozygotes, so we decided to randomly select 24 SNPs and not 10 as in the first set. We then get back to all SNPs, but not in the 1500 ones, and selected the 24 SNPs that were located in the same regions that the one from the first set. As expected, many of them were homozygotes in our parent lines, and other did not meet the criteria established previously. We ended up with 5 SNPs.

So at the end, we have two different sets of SNPs that were constitute independently from each other, each one is composed of five SNPs (see Table S11). Therefore, we went 1) from 10 to 5, and 2) from 24 to 5.

We have added the sentence:

L859-862 : For these segregations, we designed a new set selected from among all SNPs excluding the on identified by Faye et al.[33] (see above). Given the expectation of greater homozygosity than observed in the previous set, the decision was taken to select 24 SNPs.

More minor concerns

- **QR4.16 :**6. Significance labels are not clear, especially for 0.05 <p <0.1, consider using something else instead.

We changed to:

The statistical significance levels are indicated as follows: *** for p-values < 0.001, ** for p-values < 0.01, * for p-values < 0.05, ns for non-significant p-values.

- **QR4.17 :** 7. Please be more specific in your language. For example, say that you used local PCA instead of “We employed a population genomics approach…”

We have modified the sentence accordingly.

L128 and L142-14

- **QR4.18 :** 8. The WGS sequencing data is underutilized. Could the population genetic analyses also include these individuals?

The WGS sequencing data only include 27 Senegalese cultivated samples with few samples within each cluster, in comparison with 126 samples for the exome sequencing dataset. For this reason, we mainly used the exome sequencing dataset for this study.

Reviewer #5 (Remarks to the Author):I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Responses to reviewers' comments

Reviewer #1 (Remarks to the Author):

They have responded conscientiously to my comments on the previous version, although I still have reservations about the evidence for POD.

Rev1-1:I am, however, very concerned about one important aspect of the results. In Table S6, they show values of synonymous sites and non-synonymous sites diversities (piN and pS). piN is consistently as big or bigger than piS , as is the diversity of all sites. Indeed, the diversities are very small (of the order of 0.001 to 0.0004, despite the fact that they're saying that this is a high diversity species. I've never seen datasets with mean piN as big or bigger than piS. This suggests to me that there is something wrong with their procedures for analysing the population genomics data; this needs to be cleared up before the paper can be considered further.

Ans1-1: We are grateful to the reviewer for pointing out this inconsistency. Upon re-evaluating our calculations, we identified a bug in our pipeline on the rescaling of the diversity statistics per site: πN, πS, pN and pS. We recalculated the statistics with a publish tool (and added details in the material and methods section L727-763). The corrected genomic averages are now higher, with genomic πS: 0.0119 and πN: 0.0037. The overall mean πN/πS ratio of 0.31. In Chen et al. (2017, https://doi.org/10.1093/molbev/msx088), π4 across the tree of life ranged from 0.00064 to 0.0082, with values of 0.0177 for drosophila and 0.0097 for maize. The πN/π4 ratio ranged from 0.08 to 0.4, with lower range values for insects like a value of 0.09 for drosophila, and higher range values for plants like 0.2 for maize. Similarly, in Burgarella et al. (2024, https://doi.org/10.1093/evlett/qrae039), estimates in wheat species ranged from 0.001 to 0.02 for πS and from 0.1 to 0.25 for πN/πS. Therefore, our revised estimates are consistent with previously published values in plants.

We adjusted the results section from L257-277 and corrected Table 1, Figs. S6-S8 and Tables S4-S7.

Reviewer #2 (Remarks to the Author):

The manuscript “Interplay between large structural variants and overdominance in a plant genome ?” examines whether overdominance or pseudo-overdominance could explain the excess of heterozygosity observed in 7 distinct genomic regions. They find that in most cases, the FIS observed in these regions does correspond to an excess of heterozygosity that is most likely linked to a form of (pseudo-)overdominance. One of these regions on Chromosome 3 harbors two variants that are most probably introgressed from wild populations, with one of the two variants most likely being lethal at the homozygote state (H2). Upon reading the other reviewers' comments and the corresponding replies, I have learned much, and realized that I missed some points in my previous reading/review. This has left me (unfortunately) less enthusiastic with regards to the results, specifically concerning what is observed on Chr 3. I have added to my queries below. In its current form, the manuscript is much clearer, I greatly appreciated the inclusion of the expectations of F_IS values in different selection regimes, and the classifications added. Though I think some small points still need to be addressed (included below), I still do believe this work is an interesting contribution to understanding how structural variants may influence genetic diversity and in general whether pseudo-overdominance can be a plausible mechanism to explain excess heterozygosity.

We are delighted that our writing efforts have helped to clarify our findings. As discussed in the paper, in most cases, POD or overdominance alone cannot be ruled out. However, like you, we believe these results may open new perspectives for understanding these processes and for developing methodologies that will better address these questions.

Comments:

Rev2-1: From their (extensive) analyses, the authors come to the conclusion that ``Genotypes visualization suggests that the H1 haplotype resulted from a double crossing-over event between 120 and 175 Mb involving the H2 haplotype and the standard one, indicating haplotype structuring." So, this implies that H2 is the initial introgressed haplotype, and H1 a recombinant that was generated either following an inversion or the inversion appeared after the generation of the recombinant (I personally think the first is more likely). Now, I agree that the absence of H2H2 homozygotes and higher frequencies of heterozygotes, confirmed by the significantly negative F_IS could point to pseudo-overdominance. Under classical overdominant selection, Glémin 2021 (doi:10.1111/evo.14194) showed that in populations where heterozygotes are always favored over all homozygotes, several haplotypes can indeed segregate in ouctrossing populations. However, in pseudo-overdominance it is not so clear that this would be stable on long evolutionary time scales. Recombinants are expected to have lower fitness when in the heterozygote state, compared to the initial haplotypes because they carry deleterious mutations present on both haplotypes. Seeing that this seems to be a relatively recent event, pseudo-overdominance may nevertheless be plausible. However, in Fig. 3c, we see that there are some SNPs on H2 that are shared with R, more than those shared with H1 (or so it seems). If recombination is that prevalent between R and H2, why is H2 still so lethal? If this heterozygosity is only due to pseudo-overdominance, why was it not broken up? This is really puzzling to me. And either it is explained and I missed it (though I did look for it...), or it is not explained well enough for me to get my head around it. In my opinion, there are possibly several things happening here.

Ans2-1: Thank you for the relevance of the comments. The way we previously wrote this section was not sufficiently clear and may have suggested that H_1 resulted from a recombination event between H_2 and R. While this remains plausible, the overall findings tend to suggest that H_1 and H_2 originated from two distinct introgression events, as evidenced by the F_{ST} values between the two haplotypes. We have clarified this point in the paragraph below added to the manuscript. This scenario of two introgression events would explain the pertinent and valid concerns exposed by the reviewer regarding the absence of reduced fitness in recombinants and the persistence of H_2 lethality.

Section corrected from L470-481:

Considering the efficiency of genomics-based approaches and the growing availability of genomic data, we anticipate that an increasing number of studies will reveal the prevalence and diversity of large structural variations in genomes. We conducted a detailed analysis of the candidate region on chromosome 3. Our findings suggest that this region likely originated through introgression from a divergent wild relative population. The two haplotypes, H_1 and H_2, exhibit significant divergence with the reference genome, and some evidence of lower recombination rates. The H_1 haplotype carries an inversion and appears to have resulted from a double crossover event between 120 and 175 Mb, involving the reference haplotype and a divergent one. However, it remains unclear whether this latter haplotype corresponds to the haplotype H_2 currently segregating in the population. F_{ST} values between H_1 and H_2 are high within the candidate region, suggesting substantial genetic differentiation. Therefore, H_1 and H_2 might both originate from different introgression events.

Rev2-2: H2 homozygotes are not viable in domesticated populations for reasons that do not necessarily imply only fixed deleterious mutations (i.e. there could be genomic incompatibilities elsewhere in the genome). Whereas H1, having a smaller introgressed region, doesn't suffer as much at the homozygous phase, maybe because the loci involved in the H2 lethality are not on H1.

Ans2-2: We fully agree with these observations and apologize if our writing was misleading. It is indeed highly plausible that the deleterious load does not solely account for the observed lethality. To clarify this point, we have added the following sentences L540-544.

Notably, one of the two haplotypes H_2 appears to be strongly lethal in homozygous state. While the accumulation of recessive deleterious mutations may partially account for this lethality, the observed deleterious load might not be sufficient to fully explain the phenomenon. Other phenomena leading to half-lethal systems have been observed like gene disruption or genomic incompatibilities[60], and could not be ruled out.

Rev2-3: Even though inversions do reduce rates of recombination, I'm not fully convinced that it is only a question of inversions. Specifically in the case of Chr3, I think more generally it is a question of introgression. The inversion probably enhances the effect, but is not the main player. I may be wrong, but this is the impression I am under reading this new version of the manuscript, and works I have come across since last reading the manuscript. These kinds of selection dynamics depend on the frequency of the genomic region and what it actually contains, not the inversion itself per se.

Ans2-3: We agree with this observation. In the revised version of the manuscript, we place less emphasis on inversions and highlight the importance of introgression, particularly the divergence of this introgression, in maintaining the polymorphism of structural variants and establishing POD (L552-560, L575-577, L597-599).

L552-560: Recent simulation studies[25] explored scenarios in which POD emerge through introgression. The simulation results demonstrated that POD can readily emerge following introgression from divergent populations[25]. Interestingly, at the onset of introgression, fitness for all karyotypes will increase due to the reduction of the load outside the region[25]. The heterozygote advantage was found to be positively associated with the divergence between populations and the deleterious load of the recipient population, provided that the deleterious loads of the two haplotypes are not too dissimilar. If the two haplotypes are maintained over an extended period, one of the likely fates is the evolution towards a half-lethal system[25]. These favorable conditions align with the scenario observed for the region on chromosome 3, where haplotypes likely originated in a divergent wild relative population.

L575-577: However, many parameters remain unexplored, such as the number of migrants or introgression events whether involving the same rearrangement or a novel one.

L597-599: More generally, we believe that future theoretical studies should delve deeper into the role of gene flow and divergence in the emergence of POD.

Minor comments :

Table 1: Name of second test missing for pN/ps

Corrected

Lines 591 - 595: “Our empirical findings present a possible narrative, suggesting an evolutionary trajectory wherein sex chromosomes evolve from pseudo-overdominant inverted regions. This novel model posits a prerequisite for recombination suppression (and pseudo-overdominance) preceding sexual determinism, offering fresh insights into the complex interplay between genetic mechanisms and evolutionary outcomes.” I had let this slip by during the last round of review, but upon re-reading the manuscript, I don't see why this is here. The inversions studied here have nothing to do with sex chromosomes or even sex determinism. Why is this shout-out necessary? To me, this part is off subject and it should be removed. On a side note, recent works on S-loci have shown that the even though there may be some sheltering of deleterious mutations close to super-loci (like those found in butterflies) linked to the mating type, the sheltered load does not extend very far, there are no inversions, and there is barely a reduction in recombination. This, along with other recent works, seems to therefore disagree with the hypothesis that it is sheltering load that favours the evolution of sexual chromosomes.

Corrected: We agreed and have remove this section from the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have successfully addressed the technical and methodological points raised in the reviews, making a strong and interesting paper even stronger and more interesting.

We thank the reviewer for its positive feedbacks and enthusiasm.

Rev3-1: L 542 et seq. Do the authors have any suggestions on this chicken vs. egg question – which came first, the load surrounding the centromere or the inversion augmenting the load?

Ans3-1: It is difficult to answer the question of anteriority of one phenomenon versus the other.

Rev3-2: The authors also raise the issue of whether POD represents an "evolutionary trap" (L 582, as a prelude to the evolution of sex chromosomes). They might also consider an alternative idea – that POD could create an "evolutionary escape" from the steady accumulation of load in low recombination regions like around centromeres. That is, Muller's ratchet might at some point select for 'fixing' the load via POD – capping the load at a maximum of 50% per region even when such regions contain multiple lethal equivalents. In the presence of substantial identity disequilibrium, POD regions might also benefit each other by promoting heterozygosity, reducing the segregating load in other regions as well.

Ans3-2: We thank you for this highly relevant comment. We have incorporated this perspective into the discussion in the following new paragraph (L563-564).

What are the evolutionary consequences of maintaining polymorphism of non-recombining structural variants when POD is involved? POD could be viewed as an 'evolutionary trap', where the relative advantage of heterozygotes perpetuates haplotypes within populations despite their deleterious effects in homozygous state. Conversely, POD might act as an 'evolutionary escape' from the steady accumulation of genetic load in regions of low recombination, such as those near centromeres, by partitioning the load between haplotypes ultimately fixing half of the deleterious mutations in each haplotype. Simulations suggest that states where both homokaryotypes are maintained are typically transient but can persist for thousands of generations[25]. Evolutionary outcomes such as half-lethal systems (where one homokaryotype is lost) or balanced-lethal systems (where arrangements persist only in heterozygous state) may persist longer but occur only under specific conditions for the latter one. In this latter case, population fitness decreases. Overall, simulation studies indicate that POD alone is unlikely to maintain polymorphism over long evolutionary timescales[22,25]. However, many parameters remain unexplored, such as the number of migrants or introgression events whether involving the same rearrangement or a novel one.

Minor point:
L 221 change "significant" to "significantly"

Corrected

Reviewer #4 (Remarks to the Author):

We (myself and the junior reviewer) previously reviewed this manuscript. We found that the authors have made many positive changes: The introduction reads much better, it is easier to follow the arrangement terminology, and it is easier to keep track of the different data sets. The overall story is much easier to follow now.This work is truly impressive, extending across multiple datasets and countless analyses.

We thank the reviewer for their numerous insightful comments.

Rev4-1: However, despite all of this we remain unconvinced of the authors central point: that pseudo-overdominance (POD) is a primary mechanism maintaining diversity in this system. Due to this we do not think it is suitable for publication in Nature Communications. Below we go through several issues that contributed to this conclusion.

Ans4-1: Throughout the manuscript, we have tempered our assertions by emphasizing that the regions exhibit all the necessary conditions for POD to emerge, but neither overdominance nor POD can be conclusively ruled out. As highlighted by the reviewer's remarks, the complexity of these processes makes them difficult to distinguish. However, we believe that our study is one of the few shedding lights on this complexity, which warrants further exploration both empirically and in terms of simulations/theories. Notably, our results provide evidence that both overdominance and POD might represent more common forms of selection than previously thought, involving large genomic regions and a significant fraction of the genome.

Rev4-2: First, the authors rely heavily on heterozygosity at the SNP level. They argue that increased heterozygosity in these regions compared to the collinear genome is a signature of POD. However, increased heterozygosity is a natural consequence of any segregating structural variant. This heterozygosity can be caused by either neutral or deleterious mutation. Only deleterious mutation will lead to POD. The authors examine three measurements for mutation load in Table 1: (A) Pi-N/Pi-S, (B) pN/pS, and (C) Ratio of deleterious to tolerated mutations. However, statistical significance for their seven test regions is rarely reached (1/7 for A, 3/7 for B, and 2/7 for C). Additionally, not a single region was significant for all three measures. Overall, the SNP data points towards a potential role of POD but in our opinion is not strong enough evidence to back the authors claims.

Ans4-2: We agree with the reviewer's observation that increased heterozygosity is a natural consequence of any segregating structural variant, as well as the associated deleterious load. Furthermore, there are confounding effects on diversity, such as the increase in PiN/pN as well as in PiS/pS, which can render the ratios insignificant compared to other regions. What is unexpected in structural variants, however, is the significant negative F_{IS} values, although overdominance could also account for these. On the other side, simulation studies showed that POD emerges very easily when introgression is involved which has been confirmed for chromosome 3. Therefore, as the reviewer aptly stated, our results suggest a potential role for POD. This is why we have tempered our assertions from the first version of the manuscript regarding POD being the sole mechanism at play.

Rev4-3:We also found ourselves wondering at the lack of genotype (i.e. karyotype) level data. If POD is in fact occurring, we would expect HWE at the genotype with more heterokaryotypes than expected. We have performed this test ourselves using the 1976 data from table S13

Genotype | Observed | Expected
RR | 61 | 63
RH1| 69 | 36
H1H1| 23 | 20
RH2 | 26 | 10
H1H2 | 9 | 6
H2H2 | 0 | 2

The chi-sq test on this is highly significant. We suggest the authors add more of these types of analyses utilizing all their datasets. However, we want to note that still don't feel that this is enough to support the strong conclusions of the authors given the mixed results of the other data sets.

Ans4-3: We thank the reviewer for their suggestion and have conducted the test for each of the regions. None of the regions were found to be higher than the significance level, including the one tested by the reviewer. It appears there may have been an error in the estimation of the expected number of heterozygotes in the reviewer's example (homozygotes should be counted twice for karyotype frequency estimation, and once for heterokaryotes). The lack of significancy is likely due to the fact that the cluster inference is based on a few highly discriminating SNPs, causing the variance within the group itself to be minimal during the PCAadapt analysis. Therefore, using a large number of SNPs to estimate the deviation from HWE seems to be more powerful than using karyotypes.

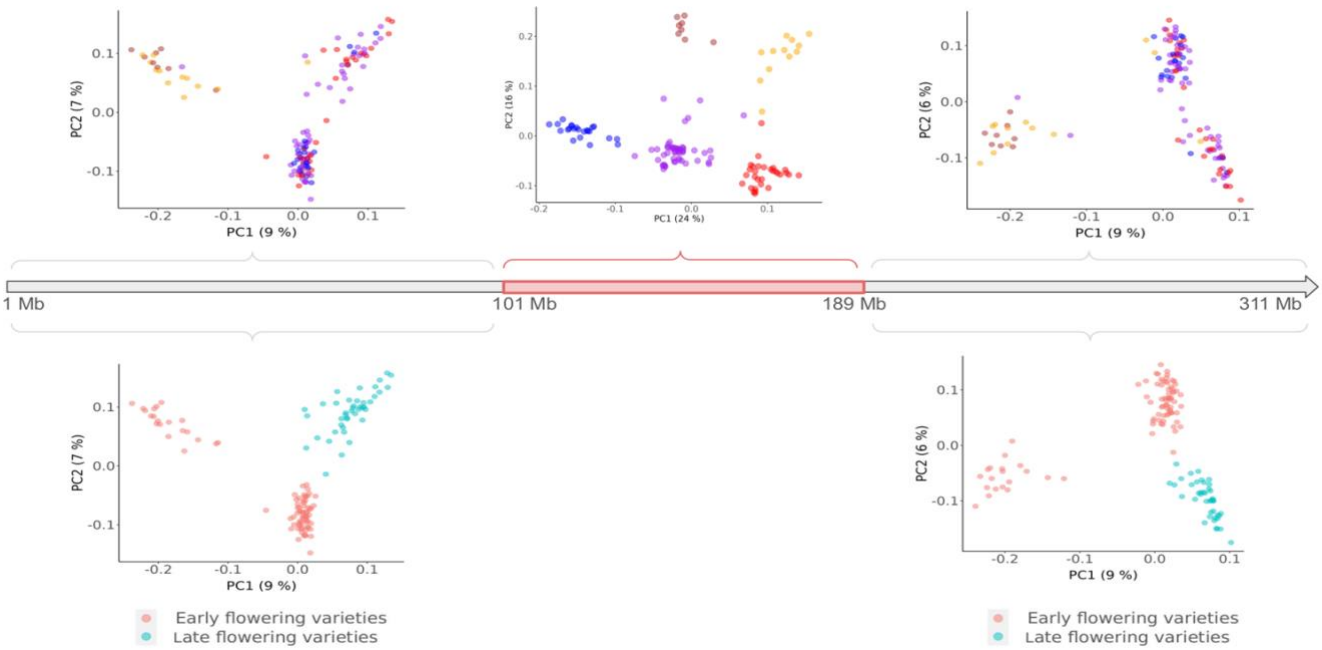
Rev4-4: In particular, we were not convinced that POD was maintaining the diversity on Chr3. To start, is introgression ongoing? This would explain continued maintenance of the variation and the size of the haplotype that is not contained within an inversion. Overall the patterns of Chr3 confused us. The authors show a full 88 mb haplotype that appears to be in LD but only 20 mb of this is an inversion.

Ans4-4: We have added a section on the Chr3 region in the discussion (L473-L481, see Ans2-1) and more details in our response to comment on haplotype structuring (Ans4-7). The population structure pattern outside this region is the same as the genome-wide pattern (see figure below). Indeed, the local PCA approach identifies regions that deviate from the global structure.

Defining "ongoing" introgression is challenging. Wild and cultivated pearl millet are subspecies with limited reproductive isolation, meaning that several introgression events could have occurred. This is indeed one of the hypotheses for the Chr3 region (L479-480).

L473-481: Our findings suggest that this region likely originated through introgression from a divergent wild relative population. The two haplotypes, H_1 and H_2, exhibit significant divergence with the reference genome, and some evidence of lower recombination rates. The H_1 haplotype carries an inversion and appears to have resulted from a double crossover event between 120 and 175 Mb, involving the reference haplotype and a divergent one. However, it remains unclear whether this latter haplotype corresponds to the haplotype H_2 currently segregating in the population. F_{ST} values

between H_1 and H_2 are high within the candidate region, suggesting substantial genetic differentiation. Therefore, H_1 and H_2 might both originate from different introgression events.



Population structure pattern for regions outside the candidate region on chromosome 3

Rev4-5: It would be very unusual for such a large block to be maintained over the long period necessary for POD to develop. Also what does the pattern look like outside of these coordinates (see minor point 5)? A discussion of the full haplotype of Chr3 is needed.

Ans4-5: Yes, it would be much more difficult for such a large block to be maintained over the long period necessary for POD to develop if it was within the population. This is a key aspect. In our case, the load had time to emerge within each population separately, both the cultivated and the wild one. In addition, this would favor the "complementarity" of loads. Thus, when introgression occurred, all the conditions were in place for POD to emerge. Simulations of such a scenario by Berdan et al. (2022) showed that POD emerges very easily. In this study, they simulated only a single migrant. This led to many simulations where the introgressed fragment was lost due to drift. In our manuscript, we advocate for future theoretical studies that investigate more deeply the impact of gene flow on the emergence of POD.

Rev4-6: We believe that H2 appears to be lethal based on what the authors have shown but that does not mean that it is caused by the accumulation of recessive deleterious alleles. Given that it is a double crossover event that generated it, it is likely that a gene may have been disrupted. Most lethal arrangements in nature are caused by issues such as this rather than mutation accumulation. For examples see the Ruff (e.g., Kupper et al. 2016 or Lamichhaney et al. 2016) and the fire ant (Hallar BL et al. 2007).

Ans4-6: We fully agree with these observations and apologize if our writing was misleading. It is indeed highly

plausible that the deleterious load does not solely account for the observed lethality. To clarify this point, we have added the following sentences (L540-544).

Notably, one of the two haplotypes H_2 appears to be strongly lethal in homozygous state. While the accumulation of recessive deleterious mutations may partially account for this lethality, the observed deleterious load might not be sufficient to fully explain the phenomenon. Other phenomena leading to half-lethal systems have been observed like gene disruption or genomic incompatibilities[60], and could not be ruled out.

Rev4-7: Also from figure S11C it appears that H2 does not even contain the inversion? First, the authors indicate that H2 evolved through a double crossover event meaning that there was no slow natural split from H1 as in haplotype structuring. Additionally, it seems that H2 may not even contain an inversion?

Ans4-7: Indeed, both haplotypes are identified using an approach that seeks for non-recombining variants, but we were able to prove through assembly that only the H1 haplotype contains an inversion. In line with genetic distance estimates, figures S11b (yellow section) and S11c (orange dots) show divergence from 120–180 Mb on Chr3. Regardless of the alignment, both haplotypes show rather strong divergence, enough to make alignment with the Reference haplotype somehow more difficult. Divergence from a sequence introgressed from wild relatives certainly limits recombination.

Rev4-8: Finally, we very much disagree with the authors that haplotype structuring is occurring here and ask for that text to be removed. Finally, the pattern predicted by haplotype structuring, SNPs differentially fixed in either sub-arrangement by selection (see figure 4F and 4G from Berdan et al 2021) does not appear in figure 3C, at least not in the inverted region.

Ans4-8: We have removed this section.

Minor comments:

1. 3A use different colors or shapes for different groups of heterokaryotypes

Corrected

2. S5D – not all windows seem to be outside the normal distribution of Pi- Recombination relationship. And Table S5 does not take recombination rate into account.

Diversity (π) estimates were performed on 10 Mb windows, not based on SNP numbers. As a result, some heterogeneity in data power can be expected, along with heterogeneity in recombination rates across the candidate regions, which are quite large. Accounting for the effect of recombination is challenging. We try to address part of this by estimating diversity values using only individuals from the most frequent homokaryotype. This approach reflects the “expected” diversity while taking the recombination rate into account.

3. Were there any corrections for multiple testing for any of the population genetic measures?

As we were not implementing a very large genome scale number of tests, we have only few regions, we did not use a FDR.

4. H2 could easily have interrupted a gene. No reason to suspect this is accumulation of deleterious alleles which is a slow process.

Corrected. This has been added in L540-544.

5. What does the region just outside of the identified region on Chr3 look like? Does the pattern continue at all?

See previous answer.

6. While some of the other 6 regions do appear to be inversions the authors should be careful as they have not fully been identified as such.

Corrected. We had put less emphasis into inversion in the new version and we used the term “non-recombining”.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Major comments

I did not conduct a full review of the previous version, due to my concerns about their diversity estimates. The authors appear to have resolved the problems with these, so I have now gone over the paper in detail.

The paper presents an impressive wealth of detail on patterns of variability around centromeric regions in pearl millet populations, and is probably the first detailed analysis of such patterns in a plant species. This is unusually favorable material compared with, say, Drosophila or humans, since the pericentric regions are very large, containing hundreds of genes, and have low levels of genetic recombination.

There is an a priori expectation that strong effects of selection at linked sites should be observed for these regions. However, a major finding is that genetic diversity is not reduced in the low recombination regions around centromeres, in contrast with what is usually observed, starting with 35-year old observations on Drosophila populations (it's a bit surprising that they relegate the relevant figure to the SI). This has, however, been found before in some outcrossing plant systems, often interpreted in terms of lower gene density leading to reduced hitchhiking effects (e.g. Wright et al. 2006 Genetics 174: 1421-1430). While their interpretation in terms of associative/pseudo- overdominance is reasonable in principle, given their evidence for strong haplotype structuring in these regions, there are two concerns about how to interpret this observation.

One is their interpretation of their haplotype structure in terms of inversions. This is based purely on finding that there is strong haplotype structuring in the centromeric regions; they do not describe any direct evidence for inversions in the genome sequences themselves. They are strong advocates of pseudo-overdominance (POD), and this is indeed quite plausible for large non-recombining genomic regions with thousands of sites subject to purifying selection. So why is it necessary to postulate the existence of inversions ? Indeed, if there is little or no recombination, there is no especial reason why inversions should be favoured in pericentric regions- the fact that the putative inversions are all found there is very suspicious.

The main objection to this interpretation is the case when three haplotypes coexist, one of which appears to be homozygous lethal (chr. 3). However, they have evidence that one of these is derived by introgression from wild populations; if small local populations are evolving in isolation, different haplotypes could easily be generated (see ref.7). The lethality is, of course, harder to explain, but this is also true of the inversion interpretation. Here they do seem to have evidence that their haplotype H1 has an inversion relative to the reference sequence (Fig 3d and Fig S10); the evidence for H2 looks weaker to me, but I lack expertise in interpreting such data. If I understand Fig 3d correctly, the inversion is only 25Mb, which is much smaller than the size of the whole region (Table 1). A critical question is whether the haplotype divergence is confined to the region covered by this inversion, or does it extend across the whole pericentric region? Fig S2 suggests that the latter; if so, then the inversion may not mean very much as far as causing the observed pattern. In addition, the frequency of the inversion among the haplotype concerned has not been established.

I thus feel that the authors need to be much more cautious about their interpretation, and consider carefully to what extent they really need to invoke inversions to explain their data. The problem with

inferring inversions simply from haplotype data is, of course, not unique to their system.

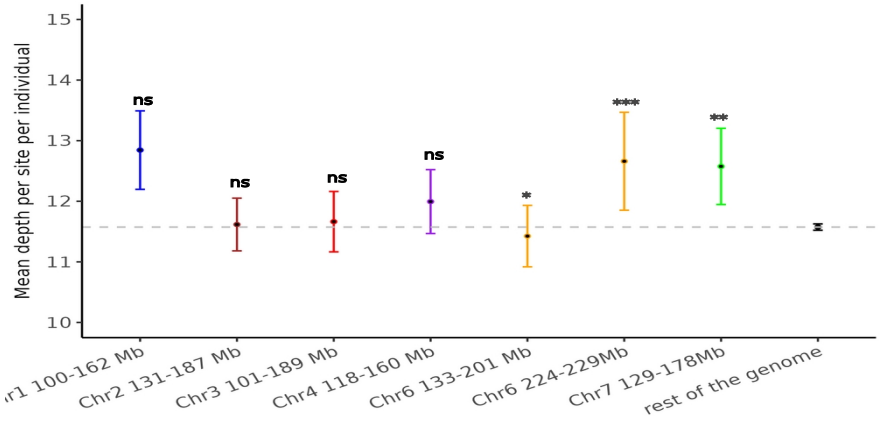
The other problem is technical. Centromeric regions are often enriched for gene duplications, which are quite abundant in plant genomes. Divergence between duplicates can easily be confounded with diversity for a single copy gene. For their results on this point to be fully convincing, I feel that they need to exclude this possibility, e.g. by looking carefully at coverage for the genes they are using compared with the rest of the genome.

Responses to comments

We appreciate the positive comment on the impressive wealth of details in this paper. Indeed, working with models other than Drosophila and human may sometimes challenge expectations, but it also offers the opportunity to shed new light on how diversity is shaped, particularly in less studied contexts.

We have previously softened the initial emphasis on inversions from the first version of the manuscript. We now acknowledge that we are focusing on structural variants in low-recombining regions (non-recombining structural variants). While inversions may be involved, other forms of variants, such as divergent introgression or haplotype structuring, can also play a role.

Regarding the potential bias of gene duplication in the centromeric regions, we conducted the necessary verification. If these regions were strongly enriched for gene duplications, we would expect at least twice the coverage observed in the rest of the genome (two duplicate genes mapping on one gene in the reference). Even if we expected half the genes to exhibit this tendency, it would 1.5 times higher. So, we would expect a very strong higher sequencing depth to have an impact on our F_{IS} values. However, as shown in the figure below, only two out of seven regions exhibit a very slight increase in depth, making an artifactual effect due to gene duplication very unlikely. In fact, one of the regions shows lower depth than the rest of the genome.



The references are carelessly formatted, with many inappropriate capital letters etc.
Corrected

l.36 ‘insights which helped to’ is better.
Corrected
l.63 ‘likely to prevail’ seems rather strong- there is little evidence for this; ‘may occur’ is more accurate in the light of current theory and empirical knowledge (e.g. ref 10).
Corrected
l.66 ‘may’ not ‘might’
Corrected
l.68 ‘low rates of recombination,’
Corrected
l.72 add ‘multiple genomes’
Corrected
l.74 Delete ‘, suggesting ..state’; it’s redundant.
Corrected
l.75 ‘exhibits’
Corrected
l.78 ‘potential’ is better than ‘complex’
Corrected
l88 Ref. 4 is concerned with inbreeding depression, not diversity; please find another reference.
Corrected
l.94 Gilbert et al. characterization of POD as a form of balancing selection is rather silly; POD is just a consequence of purifying selection. As a matter of historical accuracy, the 1st model for a random mating population was done by Charlesworth & Charlesworth 1997 Genet. Res. 70: 63-73, followed by S. Palsson (e.g. Palsson, S., and P. Pamilo (1999). Genetics 153: 475-483. Glémin’s article is just a comment on Abu Awad and Waller.

We are merely citing the sentence from a published study, ensuring proper attribution of the statement to its respective author.

l.99 Delete comma;
Corrected
l.100 ‘to’ not ‘of’. It also retards the loss of variability at the neutral loci (see also Charlesworth B 2022 Genetics 221: iyac027)
Corrected
l.103 ‘recessive’ needs qualification; the process requires partial recessivity (dominance coefficients less than one-half).
Corrected
l.104 The effect of finite population size is not a form of inbreeding; it happens when there is purely random mating.
Corrected
l.105 ‘recombination rates’.
Corrected
l.109 ‘contribute to higher than expected’ is more accurate than ‘a major means’. The present statement ignores the fact that background selection effects may also act in low recombination regions, due to the fact that AOD/POD works only for relatively weakly selected variants (see ref. 18).
Corrected

l.110 ‘genomic regions’.
Corrected
l.116 ‘variability’ is better than ‘selective process’.
Corrected
l.121 ‘with the detection of local adaptation’
Corrected
l.129 ‘characterized’
Corrected
l.130 comma after ‘patterns’
Corrected
l.132 delete ‘mechanism’ and put ‘including POD’ in parentheses.
Corrected
l.136 ‘with respect to the wild type’ is baffling; do they mean ‘gene flow from wild populations’? (wild type in genetics refers to the normal allele at a locus).
Corrected
l.141 ‘wild population’
Corrected
l.144 ‘and’ before ‘composed’
Corrected
l.146 ‘into’ not ‘in’
Corrected
l.147 ‘a’ not ‘the’
Corrected
l.152 ‘is’ not ‘could be’
Corrected
l.155 ‘results’ not ‘might result’
Corrected
l.166-169 Since recombination rates are estimated from LD, the two patterns are totally correlated. If an inversion is present, it’s not clear that the recombination rates are very meaningful; in any case, the statistic estimated is the recombination rate scaled by the effective population size, so differences in the latter (e.g. caused by background selection) could be responsible for the differences.

Yes, we agree. One of the reasons for the comparison was somehow to show the “expected LD,” which includes the expected effect of background selection in the region. As previously discussed, and noted by everyone, it is not possible to differentiate the contribution of each process.

Table 1: Why display both piN/piS and pN/pS ? The latter is not very informative, since it does not correct for the numbers of sites in the two categories. It would be helpful to include the genome-wide average, and to state whether it’s that goes piN up, piS that goes down, or both.

We aimed to be comprehensive by presenting all statistics.

l.214 ‘Signatures of overdominance and…’
Corrected
l.216 ‘or’ not ‘and’
Corrected
l.221-222. ‘a signature of heterozygote excess’
Corrected

l.247-248 ‘departure from Hardy-Weinberg’
Corrected

l.254-255 This statement is confusing, as it suggests that there is something special about the centromeres. There is no need for the multiplicity of recent references to this phenomenon- it’s been known for nearly 35 years. The point is that various hitchhiking processes reduce variability there, due to the low recombination rates around centromeres (see the references on l.108-111). It would have been clearer if they had referred to centromeres at that point in the paper.

We have added these sentences in response to the previous comment. While we do not disagree that this might be considered a very “obvious” phenomenon, the diverse readership of this journal may not always be familiar with such results. Therefore, placing them in the context of existing literature may not be a significant issue.

l.254 ‘recombination rate’ (recombination is a process, quantified by its rate).
Corrected

l.256-7 The correlation between diversity and estimates of recombination rate is unfortunately not so easy to interpret, since the latter is presumably estimated by correcting the scaled recombination rate by dividing by an estimate of local Ne, which in turn is estimated from the diversity and a value for the mutation rate (they should include a brief account of the method; readers should not have to hunt down a reference). This probably leads to an underestimation of the relation, so their test is actually on the conservative side, which might be worth mentioning.

We have described how the ReLERNN estimates recombination rates in the Material and Methods L707-710.

I don’t understand the difference between Figs S5 and 6; it seems like the former is for all SNPs and the latter for synonymous ones. Why do it both ways? This also applies to the remarks in the text; it’s much cleaner to use piS than pi for all sites.

We aimed to be comprehensive by presenting all statistics. Figure S5 illustrates Pi, Figure S6 focuses on piNS, and Figure S7 presents piS.

l.260-1 ‘the positive selection on heterozygotes’ is a peculiar term. Why not just say ‘overdominance or associative overdominance’? A paragraph break after ‘heterozygotes’ on l.261 would help the reader.
Corrected
l.262 ‘homokaryotypes’
Corrected
l.261-284 The rationale for these analyses seems to me to be somewhat confused. They are in fact tackling two problems: the effects of low recombination around centromeres, and the effects of hypothetical inversions on diversity. As stated in my general comments, the evidence for inversions is weak, except for H1 on chr. 3, and the data could potential be explained in terms of of haplotype structuring produced by POD.

Yes, that is exactly the case. As we have already stated, we cannot disentangle the centromere effect from the structural variant effect. Moreover, in this revised version, we have explicitly clarified that our

focus is on the effects of non-recombining structural variants. As mentioned in our manuscript, this could correspond to an inversion, as in H1, or a highly divergent region, potentially introgressed, as in H2. Therefore, while the effect of H2 could indeed be amplified by haplotype structuring induced by POD, providing clear evidence for such structuring remains challenging. This is likely why Reviewers 4 and 5 suggested not addressing this aspect further.

They could state more clearly that the homokaryotypes are likely to have lower diversity than when all karyotypes are pooled; this is a necessary consequence of divergence between karyotypes (this is also true if the so-called karotyopes are just haplotypes). If an inversion polymorphism is maintained for a sufficiently long time in a centromeric region, for whatever reason, divergence between the arrangements will cause an increase in overall diversity in the region covered by the inversion (for a recent theoretical analysis, see Charlesworth B 2023 Genetics 224:iyad116). Both piS and piN could be affected in this way. This effect could counteract the effects of background selection etc in the centromeric regions. Fig S6b and S7B suggest that this is likely to be going on: except for the right-hand pair, the homokaryotypes seem to have much reduced diversity compared with the rest of the population. On the inversion hypothesis, there is no solid logical basis for ascribing this effect to overdominance or POD; any mechanism that maintained the inversions for a substantial time (e.g. frequency-dependent selection) would have the same effect. This section needs to be rewritten to make this point clear; of course, the patterns could simply be generated by POD without any inversions.

The last sentence referred to the whole paragraph, including the FIS estimates. We have clarified this point.

l.292 ‘Homozyous’ not ‘homozygote’
Corrected

l.407 It’s not really ‘non-Mendelian’; lack of homozygotes for recessive lethals was discovered early in Mendelian genetics.
Corrected

l.410 ‘absent’
Corrected

Discussion

I feel that this needs to be extensively revised, in line with my comments on the interpretation in terms of inversions. I have therefore not reviewed it in detail.

In previous rounds of reviews, we addressed and softened the use of the term "inversion," opting instead for the broader term of "non-recombining structural variant." Throughout this review process, we have been grateful to the reviewer for identifying mistakes and potential flaws. As Reviewer 3 pointed out, we have addressed many of these concerns by developing a conceptual framework for FIS of SNPs in inversions, calculating new statistics, and estimating SNP depth, among other improvements. At this stage, however, we find that conducting once again a partial review is somewhat unfair. This approach delays the opportunity for our work to reach a broader scientific community and be discussed publicly.

Reviewer #2 (Remarks to the Author):

The new version of the manuscript addresses all of my remaks and I find it is clearer and better balanced. I enjoyed reading it through and believe it would make an important contribution to the study of POD.

Minor remarks:

L273 - The increase was not correlated with low recombination rates (Fig. S7c,d). When only homokaryotes are considered, πN reduced by 45% on average (Fig. S7b, Table S5). This reduction was significant for three regions, even though a small effect of sample size may occur as for πS (Fig. S7e, Table S7).
Corrected

L563 - Notably, questions persist regarding how much genetic load needs to accumulate to enable the emergence of POD and how this process interacts with structural variants
Corrected

L564 - Sentence repeated twice - Most studies have focused on the emergence of variants and genetic load within a single population. - repeated
Corrected

Reviewer #3 (Remarks to the Author):

The paper presents convincing evidence for anomalous high heterozygosity in 6 genomic regions in pearl millet from 126 Senegal accessions and significantly elevated ratios of non-synonymous to synonymous variation in a 7th (Chr1) region (Table 1). This leads the authors to explore whether selection via pseudo-overdominance (POD) may be acting to maintain the heterozygosity in terms of several hallmarks. Haplotypes from these regions on chromosome 2 and 7 confirm strong heterozygote advantage (Fig. 1) and regions that include centromeres with low recombination do not show the reductions in genetic diversity expected. These results and the higher deleterious load levels within the candidate regions all support the conclusion that selection is acting to maintain variation in these regions. The evidence that these structural variants have remained stable over 40 years further supports the idea that stabilizing selection, probably via POD, sustains this anomalously high variation.

The authors have done a conscientious job of responding to the second round of reviews, re-considering conceptual points, correcting a few errors, conducting further analyses, and qualifying several of their conclusions. Although several evolutionary forces may be at play in these genomes, the authors' original hypothesis that genomic POD could act to sustain variability and mutational load in these regions remains more supported than undermined.

The authors also thoroughly respond to questions of how introgression and structural variants may contribute to sustaining highly heterozygous regions, noting that recent simulation studies suggest introgression could be a key engine driving POD. Whether this requires recurrent introgression (because POD regions are short-lived) or only rare or intermittent introgression (because POD can persist) remains unresolved, but this was never the paper's focus.

The authors have important results to share, having done detailed and thorough analyses of several interesting data sets highly relevant to understanding how genetic diversity is sustained in general and the particular selective forces maintaining anomalously high genetic variation within these 7 genomic regions in cultivated pearl millet. The biological significance of their findings, the use of complementary innovative genomic approaches, the fact that anomalously high heterozygosity occurs across 17% of the genome, and the authors' thorough analyses fully justify publication in Nature Communications (as does the interest these results have stirred up among the reviewers). The existence of alternative hypotheses that might explain some of these results is now fully acknowledged and addressed and should by no means preclude publication of the current paper. This is how science advances. Critics are free to publish counter-arguments or pursue additional work if they feel that is needed.

Minor points

Line 546 -- There is a tandem duplication of a sentence.
Corrected
Line 566 et seq. – Ref 7 might also be cited alongside Ref 25 as it, too, explored how population hybridization enhances opportunities for generating POD and the importance of balanced load for sustaining it for thousands of generations (and so might also be cited at L 585 and/or lines 589-590).
Corrected

Reviewer #4 (Remarks to the Author):

A junior researcher and I previously reviewed this manuscript. We find that the authors have addressed our previous comments, but we remain uncertain about the conclusions. There appears to be a strong case for the role of continued gene flow in maintaining the chromosome 3 polymorphism, and we do not see compelling evidence to support POD over ongoing gene flow for the H2 haplotype. Below are a few specific points for further consideration:

1. While the authors have moderated their conclusions in parts of the manuscript, we suggest that they also consider adjusting the conclusions in the abstract to align with this tone.
Corrected

2. The authors describe H2H2 as strongly lethal despite observing some homozygotes in nature. We recommend rewording this from "strongly lethal" to "strongly deleterious."

Corrected

3. Figure S12 shows elevated FST across the entire chromosome for H2 heterozygotes. H2 heterozygotes are also separated in the local PCA outside of the H2 region (see figure for reviewers and Figure S1, which should be colored by genotype). This suggests that the pattern is not limited to a specific region but spans much of the chromosome, supporting the idea of ongoing gene flow. We do not believe the region defined by the authors exhibits different population genetic patterns from the rest of the genome.

The PCA figure sent in with the previous report clearly showed that there is not the same pattern of structuring in the rest of the chromosome and genome.

4. We apologize for our earlier miscalculation of HWE. However, we were surprised to see that all of the karyotype-level data were in HWE, which we feel weakens the POD argument. Additionally, negative Fis values are not uncommon in wild datasets, particularly in the context of introgression. We do not consider Fis alone to be a strong argument for POD, especially in the presence of structural variants (SVs).

We support POD as a hypothesis for the observed patterns in cultivated landraces - not wild populations - with several lines of evidence, not just FIS. In particular, on chromosome 3, we show the likely deleterious effect of one of the haplotypes in the homozygous state in three different crosses, enrichment of the deleterious allele and maintenance over 40 generations.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Please find an answer to the last set of review: we modified the term “structural variant” to “low recombining region” thorough the text to be more accurate. It is a more neutral description of what the method we used is able to detect. This low recombining region could be linked or associated with structural variant (inversion, chromosome translocation, …), centromere, pericentromeric region.

We answer to the different comments below:

Comment 1.
Major comments
The authors have made quite a few changes in response to my comments and those of the other reviewers, and the MS is substantially improved.

I do not think, however, that the authors have adequately responded to the point that I made in my previous review – that they do not have strong evidence for structural variants. A structural variant implies that there is some form of rearrangement of the DNA, most likely an inversion in the present case (it’s not clear what other kind of structural variant they have in mind; this needs to made explicit). They certainly have strong evidence for differentiation of pericentromeric regions into distinct haplotypes, reflecting strong linkage disequilibrium over a large physical difference. However, these are regions known to have very low recombination rates; it is thus possibly for pseudo-overdominance to produce strongly differentiated haplotypes in the complete absence of any chromosomal rearrangements distinguishing the haplotypes, as has been shown theoretically.

The fact that the method they use was devised in order to detect inversions does not mean that the patterns they detect imply the existence of structural variants.
Despite this being pointed out in my previous review, their discussion and conclusion contains repeated references to structural variants (e.g. l.476, 479, 483-484, 498-500,576,601), although they have toned down the abstract a bit. This is misleading, as they have only one case where there is direct evidence for an inversion. They seem to be confusing patterns that are consistent with inversions with evidence that there actually are inversions.

The paper thus still needs to be revised appropriately, especially the Discussion, to make it clear that it is entirely possible that their data can be explained without appealing to structural variants in the sense that is normally understood in genetics. In fact, the existence of such patterns in the absence of structural variants is a much more interesting discovery than yet another case of inversions.

We would like to thank reviewer 1 for his comment and and for helping us to clarify the discussion.

Answer 1. We now clarify that appealing to structural variant is not necessary. We use the term of low recombining region thorough the paper.

Comment 2.

2. I was asked by the editor to consider their responses to reviewer 4. Here is my evaluation (it would have been helpful if the authors had indicated the relevant line numbers).

1. I can’ t see what change has been made to the abstract; introgression is still mentioned on l.71-72.

2. This has been done.

3 and 4. I think the authors’ replies are reasonable.

Answer 2. Thanks for the check on this answer.

Minor comments
There are still quite a few minor corrections to the writing that are needed. In addition, there are still some problems with inappropriate capitals in the references

Comment 3.
l.74 ‘in’ not ‘at’
Answer: Corrected

Comment 4.
l. 75 ‘or’ not ‘and’
Answer: Corrected

Comment 5.
l.86 I still don’t think ref 4 is appropriate; there are several much later papers that review the genomic evidence for balancing selection.
Answer: We added more appropriate and more recent references.

Comment 6.
l.87 ‘patterns’
Answer: Corrected

Comment 7.
l.102 ‘requires’ not necessitates.
Answer: Corrected

Comment 8.
l.104 Selfing is a form of inbreeding, so this is redundant.
Answer: Corrected

Comment 9.
l.116 Delete ‘the’ before ‘variability’
Answer: Corrected

Comment 10.
l.129 Delete ‘the’
Answer: Corrected

Comment 11.
l.140 ‘gene flow from wild populations’
Answer: Corrected

Comment 12.
l.262 The reference to ‘structural variants’ is confusing, as they don’t have evidence for structural variants at this point.
Answer: Corrected.
We changed the wording “structural variant “ to “low recombining region”.

Comment 13.
l.273 ‘was reduced’
Answer: Corrected

Comment 14.
l.276 ‘the pi-N/pi-S…’
Answer: Corrected

Comment 15.
l.478 ‘the’ not ‘those’
Answer: Corrected

Comment 16.
l.487-488 Lower recombination rates than what? Extensive LD of course implies lack of recombination.
Answer: Corrected

Comment 17.
l.519 ‘apparently’, ‘negative F IS ..’; ‘in the homozygous state’
Answer: Corrected

Comment 18.
l.525 I think they have misunderstood ref 4. In that paper, the discussion of POD was purely about what happens in crosses between inbred lines, not what is going on in a population; the plant breeders showed that in many cases the superiority of the F1 was could be accounted for by lines being fixed for different recessive or partially recessive deleterious mutations. The population phenomenon was first described by Charlesworth & Charlesworth 1997 Genet. Res. 70: 63-73, followed by S. Palsson (e.g. Palsson, S., and P. Pamilo (1999 Genetics 153: 475-483), as I pointed out in my previous review (for some reason, they persist in ignoring these references). There is little connection between the two, apart from the involvement of deleterious mutations and recessivity; you can easily get the former without any involvement of the latter. This needs to be cleared up.
Answer: We clarified.
We also cited the two initial articles.

Comment 19.
l.533-535, 539 This is another misunderstanding. Identity disequilibrium arises from associations in homozygoity generated by non-random mating in a purely neutral context.
Answer: Corrected.
We suppressed reference to identity equilibrium.

Comment 20.
l.537 The role of POD in maintaining inversions in random mating populations is not well supported theoretically (ref.25 and Charlesworth B. 2024 Genetics 226:iyad218).l.538-9 If there is already low recombination, there is unlikely to be any selection pressure for inversions. They could, of course, accumulate by drift and become associated with haplotypes maintained by POD.
Answer: Corrected, we added a sentence specifying it.

Comment 21.
l.538-9 If there is already low recombination, there is unlikely to be any selection pressure for inversions. They could, of course, accumulate by drift and become associated with haplotypes maintained by POD.
Answer: Sentence corrected.

Comment 22.
l.540-42 This is a confusing description; POD is the presence of complementary haplotypes.
Answer: Sentence corrected.

Comment 23.
l.544 ‘variants such as inversions’ (delete the ‘such.. inversions on l.545). What other structural variant could be involved?
Answer: Corrected.

Comment 24.
l.545 They don’t give a reference to back up this assertion; it is, in fact, validated by the study mention on l.537.
Answer: Corrected, reference added.

Comment 25.
l.546 It is unclear whether they mean that it’s the benefical alleles or the deleterious variants that cause the inversions to rise to high frequencies.
Answer: Corrected, clarified.

Comment 26.

l.556 By gene disruption, do they mean that a gene was broken at the site of a rearrangement? ‘Genomic incompatibilities’ is a vague term. It’s simply possible that a rearrangement arose on a lethal-containing haplotype; there would be little selection to oppose it’s initial spread if mating is random.

Answer: Corrected. We agreed.

Comment 27.

l.567-573 I don’t think they accurately represent the conclusions of ref. 25; the abstract says “However, this polymorphism was easily destabilized by further mutation accumulation, which was often asymmetric, disrupting the quasi-equilibrium state.” Given the weakness of the theoretical support for a role of POD in maintaining inversions, they need to tone down their statements about this.

Answer: Corrected, We are now more precise, and reported more accurately that under very restricted conditions they observed half lethal system as an outcome.

ROUND 1 REVIEWER 1 ATTACHMENT:
General Comments on Salson et al.

This paper presents some interesting results on newly-discovered polymorphic inversion systems in pearl millet, based on large-scale genomic analyses. It makes a useful contribution to the increasingly long list of cases where such polymorphisms have been found outside the classical examples in Drosophila, which were studied cytologically using polytene chromosomes. The finding that one inversion haplotype appears to be nearly lethal when homozygous is especially interesting (clearly, this one has to be maintained by selection), although there are precedents for this in other systems. I am not an expert in genome analysis, so I cannot critically evaluate this aspect of the paper; as far as I can tell, this work has been done competently and their evidence for the inversions seems solid.

I believe, however, that there are serious problems with their interpretation in terms of "pseudo-overdominance" (POD) as the mechanism for maintaining their inversions, and with the nature of the evidence for this process in their system. For the reasons stated below, the authors need to be much more cautious in their claims about the reasons for the maintenance of the inversions.

First, let's try and clarify what is meant by POD. I think it's most useful to use the term in relation to a situation in which partially recessive or recessive deleterious mutations are present in repulsion with each other, so that an individual heterozygous for a pair of haplotypes carrying different complements of mutations has a higher fitness than the homozygotes for these haplotypes. This easily arises in crosses between inbred lines, fixed by chance for deleterious mutations at different loci, as discussed in ref. 4, or within a very small population. The authors don't seem to distinguish between what happens such situtation versus large random mating populations (see their citations of ref. 13); only the latter is relevant to their study of millet populations.

POD is closely related to the phenomenon of associative overdominance (AOD), in which a neutral locus linked to a locus (or loci) subject to mutation to partially recessive or recessive deleterious alleles, or to heterozygote advantage, itself experiences an apparent heterozygote advantage. Confusingly, Ohta & Kimura (1969 Genetics 63:229) used the term pseudo-overdominance, when they were in fact studying AOD caused by a single locus with heterozygote advantage. Even more confusingly, their conditions for apparent heterozygote advantage in a randomly mating population do not generate selection for maintaining variability at the neutral locus; if you use their equations for the apparent fitnesses at the neutral locus, you get no change in allele frequency at this locus (Zhao & Charlesworth 2016 Genetics 203:1315). However, there are quite stringent conditions on tightness of linkage, dominance and strength of selection that can lead to a reduction in the effectiveness of genetic drift at the neutral locus; $N_e s$ values of the order of 1 are needed for this single-locus effect in randomly mating populations (Zhao & Charlesworth 2016); otherwise, background selection (ref. 11) operates, causing an enhanced effect of drift. Multilocus simulations (e.g. Latter 1998 Genetics 148: 1142) show that there can be significant retarding effects of partially recessive deleterious mutations on the loss of neutral variability in small populations, consistent with observations on lab populations of Drosophila (e.g. Schou et al 2017

Mol Ecol 26:6510). However, it is questionable whether this is likely to happen in large natural populations, except when there is limited gene flow between isolated populations (as modelled in ref. 7; ref. 5 is just a commentary on this paper, by the way), or in genomic regions with very low rates of recombination (as in refs. 8 and 10). Even in the latter cases, the observations and theory suggest that there is just a bit more variability and less distortions of gene geneaologies by the presence of deleterious mutations than would otherwise be the case. The authors need to provide a much more nuanced account, in the light of these comments.

The question of whether this type of effect of deleterious mutations can maintain inversion polymorphisms has, as the authors say, been raised several times in the literature, mainly as purely verbal speculation; the original proposal for this process is, however, not cited (Sturtevant & Mather 1938 Am Nat 72:747), and neither is Ohta's proposal re inversions based on her AOD work (1971 Genet Res 18:227) . However, recent theoretical investigations do not support to this idea. First, studies of the introduction of inversions into populations subject to mutation and selection at multiple loci suggest that autosomal inversions are unlikely to experience a long term selective advantage as a result of the presence of such mutations, in the absence of any other effects of inversions on fitness (Connallon & Olio 2021 Mol Ecol 31:3267; refs 56; 57 and 58- the last two refs are incomplete in the bibliography). Second, it has recently been shown that autosomal inversions in freely recombining genomic regions subject to mutation, selection and drift only acquire a long-term net heterozygote advantage if they are present at nearly equal frequencies, and the size of this advantage is likely to be small (Charlesworth 2024 Genetics 226:iyad218). Otherwise, the rarer arrangement accumulates a higher mutational load than the alternative, and homozygotes for it are less fit than the heterozygotes and homozygotes for the alternative. Empirical studies of the fitness effects of Drosophila inversions are hard to reconcile with the POD idea (Charlesworth 2024), and mply that other factors are involved in their maintenance by selection, including cases of strong heterozygote advantage, although POD may contribute to the latter.

Overall, therefore, it seems unlikely on theoretical grounds that the inversions described here could be maintained by POD, alone, although POD may contribute a small effect for the inversions that are at approximately equal frequencies. In addition, the authors' evidence suggests that most of their inversions are in pericentric regions of the genome. They provide little detail about what these regions contain in the way of genes versus repetitive sequences; it is well known plant genomes often contain large non-recombining pericentric regions with low gene densities (Haenel et al. 2018 Mol Ecol 27:2477; Brazier & Glémin 2022 PLoS Genet 18**:** e1010141). It is possible that the effects of low recombination in generating patterns suggesting associative overdominance, reported in refs 8 and 10, could be operating in these regions. However, the lack of recombination would mean that an inversion with no other fitness effects would behave just like a random haplotype, since it has no recombination-suppression effect. It thus unclear whether any long term maintenance of an inversion by selection could be produced by deleterious mutations in this region.

Furthermore, the evidence provided by the authors for POD is not conclusive. The main evidence comes from the analyses presented in Fig. 2 and Table 1, where they show that

SNPs associated with the inversions have a deficiency of homozygotes relative to Hardy-Weinberg expectations (i.e. negative F_{IS} values), whereas there is little departure from random mating expectation for SNPs in the genome as a whole. This does suggest an overall heterozygote advantage for at least some of their inversions (provided that the population can be assumed to be in equilibrium. However, no conclusions can be drawn concerning the causes of this heterozygote advantage from this observation alone (see ref. 18 for a discussion of possible selective advantages to inversions). It is, however, curious that Fig. 2 shows only a small effect for chromosome 3, which has the haplotype that is apparently recessive lethal.

Table 1 does seem to show evidence for relaxed purifying selection on nonsynonymous sites in the regions covered by the inversions. However, it is not clear how to interpret this, given that the inversions are mostly contained in pericentric regions, where (at least in Drosophila) there is abundant evidence for relaxed purifying selection against deleterious mutations (e.g. ref. 10 and citations therein). There is thus a good deal of confounding of the effects of location in pericentric regions with the presence of inversions. In addition, such relaxation of selection is expected purely due to the separation of the population into smaller subpopulations by the inversions, without any significant heterozygote advantage necessarily accruing (Charlesworth 2024), so finding evidence for relaxed selection does not entail the operation of POD.

There are some more minor general points. First, some more information should be given about the study system. We are not told its scientific name its genome size, numbers of chromosomes, whether they are metacentric or telocentric, and what the gene density in the pericentric regions is (several relevant tables seem to be missing from the Supplementary Information). Second, the authors are not native English speakers, and there are many errors that need to be corrected. I give a few examples below.

Specific comments

l.59 ‘anecdotal’ is not appropriate here.

l.65 ‘outcrossing’ would be more familiar to most people.

l.66 I presume they mean ‘studied’ here, as the whole genome was ‘identified’.

l.70-75 They have no solid evidence for POD, as discussed above.

l.83 ‘selective’ not ‘adaptive’- selection and adaptation are not the same thing.

l.90-125 See the general comments on POD above.

l.131 How high is high diversity? It would be useful to have a number for silent nucleotide site diversity, and a comparison between the pericentric regions and the genome as a whole.

Table 1. Presenting Pn/Ps values without standardising the values per nucleotide sites is not very helpful, as it does not give the reader a sense of whether nonsynonymous nucleotide site diversity relative to synonymous site diversity is unusually large in the pericentric regions. Also, we ought to be told whether synonymous site diversity is low in these regions compared with the genome as a whole, as one might suspect from other studies (e.g. ref 10). It's also not clear to me how you can do G-tests on ratios, as they appear to have done; I would have thought a resampling procedure would be a safer type of test for differences. In addition, given the high LD in these regions, there is a issue with lack of independence among the different loci involved that needs to be addressed; it's not clear that each locus can be treated in isolation. The same applies to the Wilcoxon tests.

l.155-6 It's not clear that their claim for reduced recombination rates in the pericentric regions are meaningful (Fig S3) are meaningful. Their method is not explained in detail, but I believe it is based on population genomics methods that give estimates of the product of effective population size (N_e) and recombination rate. Since N_e is likely to be reduced in the pericentric regions, the effect of *Ne* is confounded with recombination rate. In addition, these methods assume random mating, but this assumption is violated if the genomic region is segregating for inversions. The higher LD in these regions (Fig S3) is probably a compound of a real reduction in recombination rate and a reduction in *Ne* (see ref. 10 for a discussion of this type of situation in Drosophila), but they haven't distinguished these effects as far as I can tell.

l.215-228 See the general comments for a critique of the interpretation in terms of POD.

l.329 It would be good to have quantitative estimates of the differentiation between haplotypes in terms of statistics such as d_{xy} and F_{ST}, as has been done for studies of inversions in other systems (e.g. Kapun et al. 2023 MBE 40: msad118).

l.430-431 This statement should be much stronger; there is essentially no other way for the polymorphism for the lethal haplotype to be maintained, other than by negative assortative mating, which is implausible for a plant.

Discussion

I have not reviewed the Discussion in detail, given my general criticisms of their interpretations in terms of pseudo-overdominance and some important aspects of their analyses.

ROUND 4 REVIEWER 1 ATTACHMENT:

Major comments

The authors have made quite a few changes in response to my comments and those of the other reviewers, and the MS is substantially improved.

I do not think, however, that the authors have adequately responded to the point that I made in my previous review – that they do not have strong evidence for structural variants. A structural variant implies that there is some form of rearrangement of the DNA, most likely an inversion in the present case (it's not clear what other kind of structural variant they have in mind; this needs to made explicit). They certainly have strong evidence for differentiation of pericentromeric regions into distinct haplotypes, reflecting strong linkage disequilibrium over a large physical difference. However, these are regions known to have very low recombination rates; it is thus possibly for pseudo-overdominance to produce strongly differentiated haplotypes in the complete absence of any chromosomal rearrangements distinguishing the haplotypes, as has been shown theoretically.

The fact that the method they use was devised in order to detect inversions does not mean that the patterns they detect imply the existence of structural variants. Despite this being pointed out in my previous review, their discussion and conclusion contains repeated references to structural variants (e.g. l.476, 479, 483-484, 498-500,576,601), although they have toned down the abstract a bit. This is misleading, as they have only one case where there is direct evidence for an inversion. They seem to be confusing patterns that are *consistent* with inversions with evidence that there actually *are* inversions.

The paper thus still needs to be revised appropriately, especially the Discussion, to make it clear that it is entirely possible that their data can be explained without appealing to structural variants in the sense that is normally understood in genetics. In fact, the existence of such patterns in the absence of structural variants is a much more interesting discovery than yet another case of inversions.

I was asked by the editor to consider their responses to reviewer 4. Here is my evaluation (it would have been helpful if the authors had indicated the relevant line numbers).

1. I can' t see what change has been made to the abstract; introgression is still mentioned on l.71-72.
2. This has been done.

3 and 4. I think the authors' replies are reasonable.

Minor comments

There are still quite a few minor corrections to the writing that are needed. In addition, there are still some problems with inappropriate capitals in the references

l.74 'in' not 'at'

l. 75 'or' not 'and'

l.86 I still don't think ref 4 is appropriate; there are several much later papers that review the genomic evidence for balancing selection.

l.87 ‘patterns’

l.102 ‘requires’ not necessitates.

l.104 Selfing is a form of inbreeding, so this is redundant.

l.116 Delete ‘the’ before ‘variability’

l.129 Delete ‘the’

l.140 ‘gene flow from wild populations’

l.262 The reference to ‘structural variants’ is confusing, as they don’t have evidence for structural variants at this point.

l.273 ‘was reduced’

l.276 ‘the pi-N/pi-S…’

l.478 ‘the’ not ‘those’

l.487-488 Lower recombination rates than what? Extensive LD of course implies lack of recombination.

l.519 ‘apparently’, ‘negative F_{IS}..’; ‘in the homozygous state’

l.525 I think they have misunderstood ref 4. In that paper, the discussion of POD was purely about what happens in crosses between inbred lines, not what is going on in a population; the plant breeders showed that in many cases the superiority of the F1 was could be accounted for by lines being fixed for different recessive or partially recessive deleterious mutations. The population phenomenon was first described by Charlesworth & Charlesworth 1997 Genet. Res. 70: 63-73, followed by S. Palsson (e.g. Palsson, S., and P. Pamilo (1999 Genetics 153: 475-483), as I pointed out in my previous review (for some reason, they persist in ignoring these references). There is little connection between the two, apart from the involvement of deleterious mutations and recessivity; you can easily get the former without any involvement of the latter. This needs to be cleared up.

l.533-535, 539 This is another misunderstanding. Identity disequilibrium arises from associations in homozygoity generated by non-random mating in a purely neutral context.

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l.540-42 This is a confusing description; POD *is* the presence of complementary haplotypes.

l.544 'variants such as inversions' (delete the 'such.. inversions on l.545). What other structural variant could be involved?

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l.556 By gene disruption, do they mean that a gene was broken at the site of a rearrangement? 'Genomic incompatibilities' is a vague term. It's simply possible that a rearrangement arose on a lethal-containing haplotype; there would be little selection to oppose it's initial spread if mating is random.

l.567-573 I don't think they accurately represent the conclusions of ref. 25; the abstract says "However, this polymorphism was easily destabilized by further mutation accumulation, which was often asymmetric, disrupting the quasi-equilibrium state." Given the weakness of the theoretical support for a role of POD in maintaining inversions, they need to tone down their statements about this.

ROUND 5 REVIEWER 1 ATTACHMENT:

The authors have responded well to my comments on the previous version, but I still have s ome suggestions for improvements in their interpretation of the data, as well some minor c hanges to the writing.

Title: I think ‘pseudo-overdominance’ or ‘associative overdominance’ would be more accurate than ‘overdominance’.

I don’ t want to be too pedantic about this point, but my comment is based on the following considerations. The authors state on l.60 that pseudo-overdominance is a special case of overdominance. This probably reflects the perspective of some plant breeders studying the causes of heterosis in crosses between inbred lines, but is not really appropriate for the population genetics of natural populations, where overdominance has long been used as meaning heterozygote advantage at the level of a single allelic difference (e.g. p.56 of the 2010 textbook by the Charlesworths). It is also used in this sense by many leading investigators of heterosis, who use the term ‘dominance hypothesis’ for what would be described here as pseudo-overdominance (e.g. Crow 1988 Genetics 148:923). I think it would be helpful to the reader to keep a clear distinction between true overdominance in this sense and pseudo-overdominance, and thus to delete the phrase ‘a particular case of overdominance’ on l.60-61, and to make appropriate changes elsewhere, avoiding conflating overdominance and pseudo-overdominance.

l.68 add ‘a larger’ before ‘ratio’

l.72 add ‘to be’ before ‘highly’

l.86 ‘diversity’ not ‘diversitys’

l.140 ‘gene flow from wild populations’ not ‘wild population gene flow’

l.142 delete ‘and’

l.219 ‘is’ not ‘are’

l.267 delete ‘diversity’

l.279 comma after ‘Overall’

l.340 add ‘been’ before ‘derived’

l.392 ‘alternative’ not ‘alternate’

l.478 ‘these’ not ‘theses’

l.481 ‘from’ not ‘with’

l.482 ‘a’ before ‘low’

l. 496 This is found in *H. numata*, which is unusual in having a within-population polymorphism.

l.503 ‘on’ not ‘to’

l.509 It would be worth mentioning that the near absence of recombination in these regions means that inversions could accumulate by a purely neutral process. In addition, if gene flow is involved, as the authors postulate later on, it seems possible that more than two haplotypes could be maintained in low recombination regions without any inversions, although this has not been examined in simulations.

l.516 The population size has to be very small to result in significantly negative F_{IS}.

l.519 Change ‘POD….’ to ‘The conditions for the occurrence of POD in natural populations have been studied theoretically.’

l.528 Delete ‘emergence’

l.533 ‘recessive or partially recessive’ not ‘recessive’

l.538 ‘sweeps’ I don’t understand what is meant here; selective sweeps can actually cause deleterious mutations to rise in frequency. This statement seems to be based on loose thinking.

l.539-41 How can hitchhiking cause an increase in diversity? This again seems like loose thinking.
I recommend replacing the two sentences on l.541-542 starting with ‘In our case..’ with ‘In our case, interpreting π_N/π_S ratios is particularly challenging, as a reduction in effective population size due to any cause will lead to an increase in π_N/π_S [12].”

l.547 ‘such as’ not ‘like’

l.561 ‘probably’ not ‘likely’