

Two divergent haploid nuclei shaped the landscape of population diversity in wheat stripe rust *Puccinia striiformis* f. sp. *tritici*

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This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Wang et al. presented an interesting study on divergence of two nuclei and heterozygosity of Pst. They developed a new pipeline to call genetics variants in dikaryotic fungi. Based on whole-genome-wide genotype, they characterized Pst genome regions as nc-, mc- and hc-hpd regions, and further investigated the formation of hc-hpd. By re-analyzing an inbred Pst population, they found transition of different level of heterozygosity is associated with virulence changes.

I am particularly interested in the proposed method to detect strain mixing. While I totally agree the necessity to detect potential mixtures/contaminations, I have a big concern the methodology the authors used that only rely on distribution of allele balance. The authors stated that the bimodal distribution of AD values is the indicator of mix. I assume some 'pure' isolate will also have this patten: a sample with half of the genome are ref homozygous and the other half are alt homozygous.

Similarly, this method can't distinguish purely high-heterozygous isolates from true mixing strains when Ref Homozygous (AA) mixed with Alt Homozygous (aa).

I kindly request authors to plot AB distributions over several simulations: 1) 'pure' isolate with high heterozygosity; 2) 'true mixing strains', one isolate is pure reference homozygous and the other isolate is pure alternate homozygous; 3) 'pure' isolate with half genome is reference homozygous and the other half genome is alternate allele homozygous. Please note SNPs should be called as cohort, not as individually (as you will loss ref homozygous sites)

I found the authors brought up several new concepts/hypothesis, I recommend authors to better define or formulate them (ie in the context of population genetics), for example 'hpd', otherwise it's hard to follow.

The 'haplotype' is mis-used in most cases in this study. For assembled genomes, the primary and alternate genomes are haplotypes (presumably they are completely phased). But for WGS isolates, they are not phased at all, and any allele switch in any single sites will generate a new haplotype. For my understanding, in most cases, the authors meant to express that the genotype of the isolate is more 'similar' to AA or AB patterns (nuclear genotype?). The authors may consider 'nuclear haplotype' (as used in ref3) or something similar.

Once again, without phasing, no haplotypes.

-related to previous point: the breakpoints in mc-hpd region (Fig4a middle panel) need to be re-interpreted. The authors states these are recombination fingerprints. Again, without phased haplotypes it would be difficult to detect crossover sites, i.e. allele switches in long heterozygosity regions.

Since recombination could be underestimated since no phasing information in long high-heterozygosity regions (i.e. hc-hpd), any conclusion on comparison of recombination between mc-hpd and hc-hpd regions need to be re-evaluated (Lines295-298).

Two serious biology mistakes:

-Line395: aeciospores are dikaryotic (just like urediniospores), not haploid.

-Line246: *Magnaporthe oryzae* is an air-borne pathogen, not soil-borne.

statistics testing of significance is needed in several comparison cases:

-Line296

-Line215

The authors should be cautious to make conclusions. For example Lines 189-94. Before this study, there are sufficient evidence to support that sexual recombination, somatic hybridization, and nucleus exchange contribute genomic diversity in rust fungi (as Ref3 cited in this manuscript).

So the conclusion of different haplotype combinations in causing hpd is not novel but one of the direct genetic consequence of these events. At least authors should discuss this.

Another issue here is that the statement 'high-heterozygosity strains in Pst (AB-type) result from Meselson effect is not entirely correct' is not valid. Firstly, while the authors have evidence to support 'haplotype' combination, authors don't have clear evidence to disprove Meselson effect. It is a basic understanding from population genetics that mutations create new alleles, then sexual recombination, somatic hybridization, and nucleus exchange could shuffle alleles (haplotype blocks).

-Minor

1) Line105: Pr, pheromone receptor gene?

2)Line274, citation Chen et al. (2020) should be Xia et al. (2020)

3)line28, Line393, higher expression in what stage(s)??

4) Any explanation on extremely low mapping rate (7.52% in Supplementary Table 2)?

Reviewer #2

(Remarks to the Author)

In this study, Wang et al., analyse a collection of publicly available Pst datasets to infer the causes of heterozygosity in stripe rust populations. They identify two distinct haplotypes (A and B) and isolates with different combinations of these haplotypes (A/B, A/A, B/B). The main cause of heterozygosity is the combination of A/B haplotypes, whereas A/A and B/B are more homozygous. They identify regions within the genome that contribute more to this diversity and infer recombination based on reference alignments. High divergence genomic regions are enriched for recombination breakpoints and genes potentially associated with host invasion; and shows higher expression. Overall, the manuscript shows the contribution of sexual recombination and somatic hybridisation to driving extensive global population diversity of this pathogen.

While I think that this is an interesting study, which gives more details on the haplotype diversity in stripe rust, I think relying on a not completely phased reference genome and comparing to one haplotype (134pri) is the major limitation of this paper. Making their data and analyses available in a genome browser is very nice!

General comments:

The authors correctly state that there are limitations using the 134pri as reference, as the genome is not completely phased and there will be a reference bias based on which sequences are present/missing in the primary haplotype of this isolate. There are more recent, haplotype phased genomes available now (for example Tam et al., *Genome Research*, 2025). While re-doing the analysis with a properly phased genome may be out of scope for the current study, it would be good to emphasize the limitations a bit more, as there will be a strong bias using one (not completely phased) haplotype as reference. Also note that the naming of haplotypes (A and B) is the opposite in the recently published paper by Tam et al. It is unclear why the B haplotype was used as a reference and not the A haplotype.

"Heterozygosity population divergence" is a signal that is mentioned constantly throughout the manuscript, yet the term was not properly introduced and defined to readers. It is unclear what the initial hypothesis is and what "hpd" signals are to be expected. This makes the manuscript's structure awkward, as some findings of hpd were described to be atypical or "difficult to explain", only later to be inferred as somatic hybridisation. There are many recent genomics work in other wheat rust fungi that have already established this hypothesis. To improve the manuscript, I suggest clustering your samples to propose the AA-AB-BB model first. This would allow the authors to have a better framework and much more specific questions to ask and test. Also please include at least one phylogenetic tree with all isolate names clearly labelled for the best interests of the community.

Many of the population genetic parameters and SNP calling outputs should be described in more detail, so that non-experts in population genetics know what each analysis measures. Some abbreviations are just mentioned without any explanation (F_MISS for example).

The manuscript should be carefully checked for spelling/grammar mistakes and make sure proper citations are added.

Why was 134pri chosen as reference and not 134alt or one of the AZ2 haplotypes?

Are the hc-hpd regions linked to specific TEs or are there any characteristic features?

Specific comments:

Line 37: Which other cereal hosts does Pst infect? Change to wheat?

Line 58-62: L58-62: This whole paragraph feels a bit out of place and can be deleted, as it is not related to this work. Instead, please expand on somatic hybridisation and describe some previous work done on SH (e.g. Sperschneider et al. 2023 on Pt; Henningsen et al. 2024 on Pca).

Line 65: Define and describe hdp in more detail as this is the main term you are using throughout the paper

L69: Describe what "Dfseq" stands for.

L77: Please use correct isolate name "Pst134Epri". Also applies to the rest of the manuscript.

Line 79: What is linkage lossing?

Line 85: Is AD really the abbreviation for allele balance? Brief description what allele balance is and why you are looking at this here.

L103-104: The low SNP density region on chr17 is likely due to rDNA repeats which would be worth mentioning. The authors did not make it clear whether the low SNP density was due to conserved sequences or missingness.

L109-110: Lacks citations, thus I can't see what was "re-discovered".

L115: less fewer

L129-L130: Could you explain how having multiple ancestral components make this region an "origin" of diversity. It indicates there's directionality.

L133-135: Lacks citations

L136: "likely to be products of Meselson effect" How likely? Did you test this? Meselson effect is not the only alternative explanation

L137: "may have" undergone?

L139-142: The missingness could be due to reference bias and a shared haplotype within the AB population. I see this mentioned much later in the manuscript but I don't find this way of structuring arguments helpful. This paragraph is confusing and it's still unclear what are the hypotheses made.

Line 152: A little bit more background on the chosen isolates, are they field isolates, lab strains, crosses?

L152-154: Can you show AZ2 and Pst134E's placements on the tree and their Fis values to further support this?

L166: add an unrooted binary tree or split tree to support this model

Line 181: Explain what IBS measures and why it's important.

L189-194: I understand the conclusion, but the writing is hard to follow

Line 205: What does complete divergence mean? How is that defined?

L211-213, L481-483: Error. Supplementary Table 5 shows non-overlapping windows, which contradicts the stated use of sliding window with 1kbp steps. Please correct or clarify.

L220: This is too vague of a description for a massive transcriptomic dataset and analysis you have. How many Pst genotypes and what are the sporulation stages/conditions included in this RNA-seq dataset? Does this signal remain when you apply different conditions?

Line 220 onwards: Many fungal genes can not be assigned a functional annotation. Could you include how many genes in these regions are not functionally characterized? Fig 4h only mentions the ones with predicted functions, but it would be essential to know what the percentage of functionally characterized vs unknown function in these regions are.

L223: Unsure the point of performing GO term analysis if authors only want to draw conclusions about host invasion. The argument of hc-hpd being crucial for virulence is weak. Need to at least show if these genes are highly expressed only during infections.

L228-240: This paragraph is hard to read. I understand the authors have pointed out that some BB isolates have less shared k-mer content with Pst134pri, indicating there are more divergent haplotypes. Is that why we are seeing more missing sites in BB despite Pst134pri reference being a B? Please make it clear to the readers. Also please consider adding Figure S4 as a main figure.

Line 242 and onwards: Is the comparison between a haploid and dikaryon valid for this analysis?

How can recombination rates be inferred unphased short read datasets, especially in homozygous strains without making too many reference-based assumptions?

L252: "lower baseline in LD decay" is not a proper description for higher recombination, you're looking at r^2 , LD decay is an inference. Please rewrite.

L264-266: Does not make sense

Line 272: How does this show evidence for alternative life cycles?

Line 279: Why were the high heterozygosity isolates removed?

L290: Please show how this was done. The only results/data shown was a haplotype block on chr7 and a virulence table, so it sounds like authors are generalising from a single observation. There is too little analysis done to show evidence for "transition of virulence loci from het to hom could help facilitate virulence".

Line 295 onwards: How are recombination breakpoints identified? How can they be differentiated from transposon insertions, singleton regions that are not in the reference?

L297: Not sure what you mean by hc-hpd "serves as" A/B haploid-inherited region.

Figures:

Fig 2b: It's really difficult to see the leaf node colors.

Figure 2c: Please mark the best-fitting k.

Fig 3d: Adjust so that top is not cut off.

Fig 4: nc-hpd, mc-hpd, hc-hpd should be explained in the figure legend.

Figure 4b: Please label each individual chromosome clearly.

Fig 4e: Change to effector candidates or similar, as there is no validation they are effectors.

Figure 4c,d,g legend: Please describe statistical tests used.

Figure S4e: In Figure 2d, the samples with negative Fis clustered in the middle are already described to be high-

heterozygosity in L133. However in S4e those samples were referred to as AA but I expect it to be AB? Could you clarify?
Figure 5c: Not sure what this is trying to say. Does the right panel show something that doesn't exist? I am a bit confused.
Figure 6b: No description for the p-values.
Table S3: Would be great to add a column for citations.
Figure S1: Please add genotype legend (like the one from Fig S4).
Figure S1c: Could you rotate the clades around for the tree on the right for easier comparison?

Reviewer #3

(Remarks to the Author)

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

Reviewer #4

(Remarks to the Author)

The manuscript by Wang et al. presents the development of a tool to determine divergent nuclei in dikaryotic organism using resequencing approaches and then delves into the characterization of existing data for the stripe rust to determine the complexity and divergence of those individuals and their haplotypes. The paper presents the data on refined pipeline, Dfseq, that allows for dissecting heterozygosity and haplotypes from nuclei. This approach allows for the detection of mixed strains and SNP artifacts, improving comparative population genomics and avoiding misinterpretations.

The paper presents similar data to previous papers, indicating a high heterozygosity, but challenges previous findings. This is based on a large dataset available of over 200 isolates. This paper continues that theme of the high heterozygosity but reevaluates the mechanism and focuses on the evolutionary implications of the dikaryotic nature of rust fungi.

The general gaps are the lack of evidence of the "C haplotype". While the hybrid origin of the B haplotype is a compelling hypothesis, the putative donor lineage (C) remains unidentified, and additional sampling is needed. Also this is only based on resequencing so it lacks fully phased genomes. I wonder how this compare to other methods used by rust research community and how it is similar or different from research using long-read and Hi-C findings?

The paper rightly challenges the Meselson effect explanation for high heterozygosity but downplays how both mechanisms (asexuality and hybridization) might contribute. Present a more nuanced comparison, acknowledging that different mechanisms may dominate in different populations or genomic regions. This would make the argument more balanced.

The hc-hpd regions are described as recombination-suppressed, but the biological basis (structural variation? epistasis? chromatin context?) is unclear.

In the line 39, could you explaining somatic hybridization for non-mycology readers?

Line 54 - 57 Please consider the citation of other studies proposing similar mechanisms:

- <https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1011493>
- <https://10.1094/MPMI-10-24-0122-FI>
- <https://doi.org/10.1101/2025.05.22.655633>

Line 86-87 Do you have any statistics or table that show the proportion of isolates deviating from AD 0.5 among the 266 isolates?

Line 129 Spell out hpd: heterozygosity population divergence. Also, The term "hpd" is introduced as central to the study, but its biological meaning and distinction from general population structure or heterozygosity patterns is vague early on. Define hpd more clearly at first mention (e.g., "hpd refers to divergence between populations that arises due to combinations of divergent haploid genomes within dikaryotic individuals") and reinforce how it differs from prior interpretations.

Line 221 - 223 How was this determined? it is significant based on what test?

Line 249 - 250 are there other systems different from Magnaporthe that could help to make a case for what is observed on rusts? Actually, I wonder if Dfseq is applicable to other systems?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors performed the simulation analysis to test effectiveness of using AlleleBalance in detecting strain mixing, the results are satisfied. All of my previous concerns have been addressed except one very minor issue: all phenotypes (Yr**) in the figures (ie Figure 7, Fig S6) should be italic.

Reviewer #2

(Remarks to the Author)

The revised version of the manuscript addressed most of the concerns raised but there's still a few issues that should be revised or clarified.

Even though the naming of A and B nuclei in this manuscript was somewhat arbitrary, it would be beneficial for further studies to be consistent with the naming. The now published Pst104E genome named A and B haplotypes the opposite of the naming in this manuscript. Classifying isolates by their haplotype combinations and having different naming for the same haplotypes groups in different publications is not going to help.

Fig 5f: The European isolates cluster with BB isolates but are labelled AB? Is that correct?

Fig 6c: Changing the text to "observed" vs "not observed" would be better than "exists" and "no exists", as this is based on available datasets, which may not capture all possible haplotype configurations and possible recombination events. It should also be "linked" and not "linkaged".

The description of RNA-seq dataset analysis is still very vague. Looking at Table S8, mapping stats very greatly between samples. How did multiple mapping affect assigning reads to genes? Could the observed higher expression in hc regions be an artefact of higher mapping efficiency in these regions? What is the difference between reads mapped to "multiple loci" and "too many loci". How does this affect counting?

In response to comment 2.32 the authors state: "We have revised this section to provide the number and proportion of functionally annotated vs. unannotated genes in the regions."

While this information is found in TableS10 now, it is not mentioned. Based on the numbers in TableS10, ~80% of genes do not have an annotated function, which makes the results of the GO analysis questionable in terms of biological meaning as the statistics are done on a very small subset of genes.

Fig 3c, S2, S3: In my understanding, Fis is an index that strictly ranges from -1 to 1. However, those figures display Fis values lower than -1.0. Is this an error in calculation?

L118-119: Could you do a BLAST search to confirm the presence of rDNA?

L124-126: Still unclear what patterns are being referenced here, and not sure whether citations (8) and (45) are appropriate. I suggest deleting this sentence. Authors may consider simply highlighting that e.g. the strict SNP calling strategy would allow for more robust population genetic inference downstream.

L185: If there's a recombinant haplotype of A/B this analysis might not pick this up. Please discuss this limitation.

L255-256: This statement about introgression does not sound convincing without proper tests and warrants an elaboration.

L892: Please provide citations for both genome assemblies.

Reviewer #5

(Remarks to the Author)

Reviewer #4 concerns were mainly about properly defining the scope, terminology, literature and robust interpretations of the authors' findings. I looked at the responses and revisions to the manuscript and these are satisfactory in my view.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Thank you for revising the manuscript again, I know this takes a lot of effort. Especially renaming the haplotypes and revising the figures. This naming is now consistent with another recent preprint, so worth the effort. All of my issues have been addressed.

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Point-by-point Response

Wang et al., Two divergent haploid nuclei shaped the landscape of population diversity in wheat stripe rust.

We would like to express our sincere gratitude to the four reviewers for their careful evaluation of our manuscript. The comments and suggestions were rigorous, constructive, and highly valuable. Guided by these comments and suggestions, we have made extensive revisions to the manuscript.

The major revisions include:

- (1) Validating the reliability of our analytical framework, including confirming the effectiveness of AlleleBalance for assessing strain-mixing and evaluating whether reference genome bias affects the main conclusions of our study. These results have been included in the newly added Supplementary Note.
- (2) Clarifying and refining previously ambiguous concepts, such as the term “hpd”.
- (3) Providing additional original data (Supplementary Data 1-4), including newly added tables (Tables. S4, S10, S16).
- (4) Preparing multiple new figures, with figures (Figs. S3, S10-S13) added in this revision.
- (5) Supplementing previously missing statistical significance (*P*-value) information.
- (6) Adding relevant literature citations.
- (7) Improving wording and clarity throughout the manuscript.

All revised content has been marked in red in the manuscript. After these revisions, we believe the manuscript is now substantially improved, and we hope it is now suitable for reconsideration.

Reviewer #1:

Wang et al. presented an interesting study on divergence of two nuclei and heterozygosity of *Pst*. They developed a new pipeline to call genetics variants in dikaryotic fungi. Based on whole-genome-wide genotype, they characterized *Pst* genome regions as nc-, mc- and hc-hpd regions, and further investigated the formation of hc-hpd. By re-analyzing an inbred *Pst* population, they found transition of different level of heterozygosity is associated with virulence changes.

Review Comments 1.1 I am particularly interested in the proposed method to detect strain mixing. While I totally agree the necessity to detect potential mixtures/contaminations, I have a big concern the methodology the authors used that only rely on distribution of allele balance. The authors stated that the bimodal distribution of AD values is the indicator of mix. I assume some 'pure' isolate will also have this patten: a sample with half of the genome are ref homozygous and the other half are alt homozygous. Similarly, this method can't distinguish purely high-heterozygous isolates from true mixing strains when Ref Homozygous (AA) mixed with Alt Homozygous (aa). I kindly request authors to plot AB distributions over several simulations: 1) 'pure' isolate with high heterozygosity; 2) 'true mixing strains', one isolate is pure reference homozygous and the other isolate is pure alternate homozygous; 3) 'pure' isolate with half genome is reference homozygous and the other half genome is alternate allele homozygous. Please note SNPs should be called as cohort, not as individually (as you will loss ref homozygous sites).

Author response: This is a very good suggestion. A simulation study was conducted in the revised version. We would like to provide a more detailed explanation of the validity of this parameter. First, we sincerely apologize for the misuse of the abbreviation. The parameter we actually used is 'allele balance', abbreviated as AlleleBalance (AB value in VCF file). The AlleleBalance value is widely used in various software tools to evaluate SNP quality.

$$\text{AlleleBalance} = \text{altDepth} / (\text{refDepth} + \text{altDepth})$$

where:

- altDepth = sequencing depth supporting the alternate allele
- refDepth = sequencing depth supporting the reference allele

Interpretation:

- AlleleBalance $\approx$ 0.5 $\rightarrow$ the heterozygous genotype (0/1) is more reliable, as the ratio of reads supporting the reference and alternate alleles is close to 1:1.
- AlleleBalance $\approx$ 0 or AlleleBalance $\approx$ 1 $\rightarrow$ more likely to represent homozygous genotypes (0/0 or 1/1).
- Significant deviation from these expectations may indicate sequencing bias, copy number variation (CNV), or sequencing errors.

In a single pure *Pst* strain, most heterozygous sites should have AlleleBalance values distributed around 0.5. However, we found that some strains show AlleleBalance value distributions deviating from a normal distribution centered at 0.5 (i.e., most heterozygous SNP sites have AlleleBalance values far from 0.5), suggesting possible strain mixtures.

To validate the effectiveness of determining mixture using AlleleBalance distribution, we followed your suggestion and plotted the AlleleBalance value distributions under four scenarios:

- (1) mixed strains as defined in this study,
- (2) highly heterozygous but pure strains as defined in this study,
- (3) NGS reads simulated from a haploid genome, representing an absolutely pure strain,
- (4) artificially mixed NGS reads randomly combined from multiple pure strains, representing an absolutely mixed sample.

The results, shown in Fig. S11, support the reliability of our method, effectively distinguishing between mixed and pure strains (even highly heterozygous ones). Further details are provided in the Supplementary Note.

Review Comments 1.2 *I found the authors brought up several new concepts/hypothesis, I recommend authors to better define or formulate them (ie in the context of population genetics), for example 'hpd', otherwise it's hard to follow.*

Author response: We fully understand and agree with the concerns. Upon reviewing our original manuscript, we also realized that we had indeed introduced too many new concepts and abbreviations, some of which were not sufficiently explained, such as ‘hpd’ and ‘AD’. To address this issue, we have made the following revisions and improvements in the revised manuscript:

Regarding hpd:

The concept of “Heterozygosity population divergence (hpd)” was originally intended to briefly describe the phenomenon of significant differences in heterozygosity levels among subpopulations within *Pst*. However, in the original version (Introduction, line 65), we did not provide a detailed explanation, which made it difficult for readers to understand. In the revised version (lines 72–76), we have provided a clearer definition of this concept and added relevant citations. For example, similar phenomena have been reported in other species by Li et al. and Scally et al. (ref23-24). In addition, we have reduced the frequency of hpd throughout the manuscript. In instances where the abbreviation was unnecessary, we replaced hpd with more intuitive terms (e.g., at line 79, hpd was replaced with heterozygosity, and hc-hpd region was revised to hc region). These changes improve clarity and readability of the manuscript. To assist readers easily get the idea of heterozygosity difference between subpopulations, we also added a schematic figure to illustrate this phenomenon (Fig. S10) and provided detailed explanation in the Supplementary Note.

Regarding AD:

AD is a standard field in VCF files representing allelic depth at each site. In our previous version, we mistakenly abbreviated “Allele Balance” as AD, which is inaccurate. In fact, Allele Balance (AlleleBalance) is derived from AD, and is calculated as follows: $\text{AlleleBalance} = \text{altDepth} / (\text{refDepth} + \text{altDepth})$. We have added the corresponding explanation and references in the revised manuscript (lines 97–99).

Review Comments 1.3 *The 'haplotype' is mis-used in most cases in this study. For assembled genomes, the primary and alternate genomes are haplotypes (presumably they are completely phased). But for WGS isolates, they are not phased at all, and any allele switch in any single sites will generate a new haplotype. For my understanding, in most cases, the authors meant to express*

that the genotype of the isolate is more 'similar' to AA or AB patterns (nuclear genotype?). The authors may consider 'nuclear haplotype' (as used in ref3) or something similar. Once again, without phasing, no haplotypes.

Author response: We thank you for pointing out this mistake. We agree with your comment that in most cases, what we intended to express was the “nuclear haplotype” rather than the phased haplotype. Following your suggestion, we have replaced multiple instances of “haplotype” in the manuscript with “nuclear haplotype.”

Review Comments 1.4 -related to previous point: the breakpoints in mc-hpd region (Fig4a middle panel) need to be re-interpreted. The authors states these are recombination fingerprints. Again, without phased haplotypes it would be difficult to detect crossover sites, i.e. allel switches in long heterozygosity regions. Since recombination could be underestimated since no phasing information in long high-heterozygosity regions (i.e. hc-hpd), any conclusion on comparison of recombination between mc-hpd and hc-hpd regions need to be re-evaluated (lines295-298).

Author response: We understand your concern that the lack of phased data may lead to an underestimation of recombination events. However, since the recombination analysis is based on a selfing line where the parental origin of each individual is fixed and unique, we believe that most recombination sites can still be identified without phasing. Previous studies have also used NGS data from selfed lines to identify recombination breakpoints without phasing, and this approach has been considered feasible (ref35). We have indicated this limitation in the current manuscript (lines 325–327). The content concerning the comparison of recombination between mc-hpd and hc-hpd has been labeled as an ‘inferred’. The Fig. 6c has been revised correspondingly.

Review Comments 1.5 Two serious biology mistakes:

-line395: aeciospores are dikaryotic (just like urediniospores), not haploid.

-line246: Magnaporthe oryzae is an air-borne pathogen, not soil-borne.

Author response: Corrected. We have changed it to pycniospore, another haploid spore stage of *Pst* (line 430 in the current manuscript). Corrected. line 273 revised.

Review Comments 1.6 statistics testing of significance is needed in several comparison cases:

-line296

-line215

Author response: The significance test and its interpretation have been added. Lines 239–241 and 323–324 in manuscript have been revised accordingly.

Review Comments 1.7 The authors should be cautious to make conclusions. For example lines 189-94. Before this study, there are sufficient evidence to support that sexual recombination, somatic hybridization, and nucleus exchange contribute genomic diversity in rust fungi (as Ref3 cited in this manuscript). So the conclusion of different haplotype combinations in causing hpd is not novel but one of the direct genetic consequence of these events. At least authors should discuss this.

Author response: Thanks for your valuable suggestions. We made changes in the revised version as

suggested. Numerous previous studies have supported the role of somatic hybridization and nuclear exchange in contributing to the genetic diversity of rust fungi. We acknowledge that this study has extensively referred to and drawn upon the research methods of ref3 (now ref4). We have revised the manuscript to acknowledge the similar conclusion between our study and ref3 (now ref4) in the current version (line 212-213), noting that the phenomenon of limited nuclear haplotypes indeed occurs in both *Pst* and *Pt* species. Our findings are therefore not entirely novel. However, this does not mean that our study lacks meaning or innovation. As two distinct species, *Pst* also poses a serious threat to wheat production, and understanding the relationship between its nuclear haplotype and genetic diversity is equally important. Our study provides valuable insights into this relationship.

Review Comments 1.8 *Another issue here is that the statement 'high-heterozygosity strains in Pst (AB-type) result from Meselson effect is not entirely correct' is not valid. Firstly, while the authors have evidence to support 'haplotype' combination, authors don't have clear evidence to disprove Meselson effect. It is a basic understanding from population genetics that mutations create new alleles, then sexual recombination, somatic hybridization, and nucleus exchange could shuffle alleles (haplotype blocks).*

Author response: Thanks for your insightful comments. We removed the discussion of the Meselson effect in here. We re-examined and rewrote the subsequent discussion about the Meselson effect's possible contribution to the high heterozygosity observed in the *Pst* population (lines 370–376).

Review Comments 1.9 *line105: Pr, pheromone receptor gene?*

Author response: Yes, we neglected to introduce this gene and also used an incorrect abbreviation (it should be *Pra*). Corrected in the current manuscript (line 117). We added the full name of the gene.

Review Comments 1.10 *line274, citation Chen et al. (2020) should be Xia et al. (2020)*

Author response: Corrected in line 299.

Review Comments 1.11 *line28, line393, higher expression in what stage(s)?*

Author response: Sorry, but we cannot provide an exact answer to this question. The 'higher expression' of genes from certain regions of *Pst* genome was based on the analysis of an integrated set of 250 previously published transcriptome samples (Adams et al., 2021 BMC Genomics). The publicly available transcriptome data we used were obtained from infected leaf samples collected directly from the field across multiple countries and plant developmental time points. Therefore, it is difficult to determine the precise infection or developmental stage of the samples. We have made appropriate revisions to clarify this point. In the current version of the manuscript, the phrases "exhibit higher expression level" at lines 25 and 428 have been revised to "exhibit higher expression level in publicly available transcriptome samples"

Review Comments 1.12 *Any explanation on extremely low mapping rate (7.52% in Supplementary Table 2)?*

Author response: These data were downloaded from public WGS short-read datasets of *Pst* available in the NCBI and BGI databases. Since they are public datasets, we cannot guarantee the quality of every sample, and some may have contamination. The total number of short reads generated for this sample was large enough to cover 75.79% of the reference genome despite only 7.52% of the total amount of short reads being mapped (Table S2). Therefore, we consider the data informative and it will not affect our analyses.

Reviewer #2:

In this study, Wang et al., analyse a collection of publicly available Pst datasets to infer the causes of heterozygosity in stripe rust populations. They identify two distinct haplotypes (A and B) and isolates with different combinations of these haplotypes (A/B, A/A, B/B). The main cause of heterozygosity is the combination of A/B haplotypes, whereas A/A and B/B are more homozygous. They identify regions within the genome that contribute more to this diversity and infer recombination based on reference alignments. High divergence genomic regions are enriched for recombination breakpoints and genes potentially associated with host invasion; and shows higher expression. Overall, the manuscript shows the contribution of sexual recombination and somatic hybridisation to driving extensive global population diversity of this pathogen.

While I think that this is an interesting study, which gives more details on the haplotype diversity in stripe rust, I think relying on a not completely phased reference genome and comparing to one haplotype (134pri) is the major limitation of this paper. Making their data and analyses available in a genome browser is very nice!

Review Comments 2.1 *The authors correctly state that there are limitations using the 134pri as reference, as the genome is not completely phased and there will be a reference bias based on which sequences are present/missing in the primary haplotype of this isolate. There are more recent, haplotype phased genomes available now (for example Tam et al., Genome Research, 2025). While re-doing the analysis with a properly phased genome may be out of scope for the current study, it would be good to emphasize the limitations a bit more, as there will be a strong bias using one (not completely phased) haplotype as reference. Also note that the naming of haplotypes (A and B) is the opposite in the recently published paper by Tam et al. It is unclear why the B haplotype was used as a reference and not the A haplotype.*

Author response: We fully understand your concerns regarding the choice of reference genome, especially when reference genome had not been fully phased. However, at the start of this study, high-quality phased genomes were not available in public databases. Genomes such as AZ2 and the Pst104E (Tam et al., Genome Research, 2025), only became publicly accessible around the time we were close to finish our study. Therefore, we had to use the relatively high-quality Pst134 genome available at that time for our analyses.

Regarding the choice of A versus B as the reference genome, we acknowledge that our decision was somewhat arbitrary. To address your concerns, we have now used more reference genomes, re-aligned the reads for variant detection, and repeated several analyses from this study. These validations confirmed the correctness of our main conclusions. The results are shown in Fig. S12, with further explanations provided in the Supplementary Note.

Review Comments 2.2 *“Heterozygosity population divergence” is a signal that is mentioned constantly throughout the manuscript, yet the term was not properly introduced and defined to readers. It is unclear what the initial hypothesis is and what “hpd” signals are to be expected. This makes the manuscript’s structure awkward, as some findings of hpd were described to be atypical or “difficult to explain”, only later to be inferred as somatic hybridisation. There are many recent*

genomics work in other wheat rust fungi that have already established this hypothesis. To improve the manuscript, I suggest clustering your samples to propose the AA-AB-BB model first. This would allow the authors to have a better framework and much more specific questions to ask and test.

Author response: The concept of “Heterozygosity population divergence (hpd)” was originally intended to briefly describe the phenomenon of significant differences in heterozygosity levels between subpopulations of *Pst*. However, in the original manuscript (Introduction, line 65), we did not provide a detailed explanation, which made it difficult for readers to understand. In the revised manuscript (lines 72–76), we introduced this concept by simply describing it and added references to relevant studies. For example, Li et al. and Scally et al. reported similar phenomena in other species. In addition, we have reduced the use of the abbreviation hpd throughout the manuscript. In instances where the abbreviation was unnecessary, we replaced hpd with more intuitive terms (e.g., at line 79, hpd was replaced with heterozygosity, and hc-hpd region was revised to hc region). These changes improve clarity and readability of the manuscript. To help readers to better understand this concept, we created a schematic diagram illustrating this phenomenon (Fig. S10) and provided further explanation in the Supplementary Note.

As you suggested that we should cluster samples to propose the AA–AB–BB model. We did not emphasize the presentation of this model in the Results section of the manuscript. The remaining ambiguity or atypical impression for readers may relate to how the results were presented. We performed extensive analyses of this type. Fig. S4e and S4f show sample clustering using different methods, which we believe sufficiently support our conclusions. As you suggested in a later comment, we have now added Fig. S4 as the main figure (Fig. 5) to provide readers with a clear view of the AA–AB–BB model.

Review Comments 2.3 *Also please include at least one phylogenetic tree with all isolate names clearly labelled for the best interests of the community.*

Author response: Added, with the new Fig. S3 showing a phylogenetic tree labelled with isolate accession number.

Review Comments 2.4 *Many of the population genetic parameters and SNP calling outputs should be described in more detail, so that non-experts in population genetics know what each analysis measures. Some abbreviations are just mentioned without any explanation (F_MISS for example).*

Author response: We have provided explanations for F_MISS, VCF, TPM, and FPKM in the revised manuscript, including lines 121–122, 468, and 523–524.

Review Comments 2.5 *The manuscript should be carefully checked for spelling/grammar mistakes and make sure proper citations are added.*

Author response: We have corrected multiple errors and added the missing references with reviewers’ valuable assistance.

Review Comments 2.6 *Why was 134pri chosen as reference and not 134alt or one of the AZ2 haplotypes?*

Author response: This issue has been addressed above. We acknowledge that our choice of reference genome was arbitrary. As mentioned earlier, we have reanalyzed the data using multiple reference genomes and confirmed our main conclusions do not depend on a specific reference genome (Supplementary Note).

Review Comments 2.7 *Are the hc-hpd regions linked to specific TEs or are there any characteristic features?*

Author response: This is an interesting question that deserves further analysis. However, we believe it is quite distant from the focus of this study. Our focus is on difference in heterozygosity between subpopulation and A/B nuclear haplotype. Nonetheless, we have provided the physical positions of the hc-hpd regions in Table S6 to support future study of relationship between TE and hc-hpd by other researchers.

Review Comments 2.8 *line 37: Which other cereal hosts does Pst infect? Change to wheat?*

Author response: We changed the text as suggested, since *Pst* infects only one cereal crop.

Review Comments 2.9 *line 58-62: L58-62: This whole paragraph feels a bit out of place and can be deleted, as it is not related to this work. Instead, please expand on somatic hybridisation and describe some previous work done on SH (e.g. Sperschneider et al. 2023 on Pt; Henningsen et al. 2024 on Pca).*

Author response: Thanks for this insightful advice! We revised the text as suggested. Lines 58–62 in the original manuscript (now line 65–69) provide an example that helps readers understand how organisms can acquire heterozygosity through other mechanisms rather than Meselson effect. We agree with your suggestion to include additional discussion of studies related to somatic hybridization. We added a reference to Henningsen et al. (2024) on *Pca* in now lines 45–49 to help readers understand somatic hybridization and the progress of research in the community.

Review Comments 2.10 *line 65: Define and describe hdp in more detail as this is the main term you are using throughout the paper*

Author response: We have defined the ‘hpd’ and added a clearer description here (now line 72–76).

Review Comments 2.11 *L69: Describe what “Dfseq” stands for.*

Author response: “Dfseq” has been removed. Its introduction in the main text has been deleted. It is now described only as an SNP-calling pipeline in the Methods section.

Review Comments 2.12 *L77: Please use correct isolate name “Pst134Epri”. Also applies to the rest of the manuscript.*

Author response: Corrected.

Review Comments 2.13 line 79: *What is linkage lossing?*

Author response: The description here was overly abrupt. What we actually intended to describe is the phenomenon of unusually weak linkage among closely adjacent SNPs within a genomic region. We have revised this description (lines 90–91).

Review Comments 2.14 line 85: *Is AD really the abbreviation for allele balance? Brief description what allele balance is and why you are looking at this here.*

Author response: The abbreviation “AD” refers to Allelic Depth in VCF files, but in our manuscript, we incorrectly used it when we actually meant Allele Balance (AB, or AlleleBalance), which is derived from AD. The correct definition is: $\text{AlleleBalance} = \text{altDepth} / (\text{refDepth} + \text{altDepth})$. Typically, $\text{AlleleBalance} \approx 0$ or 1 indicates homozygosity (0/0 or 1/1), while $\text{AlleleBalance} \approx 0.5$ indicates heterozygosity (0/1). This clarification and definition have been added to the revised manuscript (lines 97–99).

Review Comments 2.15 L103-104: *The low SNP density region on chr17 is likely due to rDNA repeats which would be worth mentioning. The authors did not make it clear whether the low SNP density was due to conserved sequences or missingness.*

Author response: We agree with your concern regarding this issue and its importance. Therefore, we have revised this section and added your suggested speculation in lines 118–119.

Review Comments 2.16 L109-110: *Lacks citations, thus I can't see what was “re-discovered”.*

Author response: Added. Please see line 125 in the revised version.

Review Comments 2.17 L115: *less fewer*

Author response: Corrected in line 130. Thank you for pointing out this error.

Review Comments 2.18 L129-L130: *Could you explain how having multiple ancestral components make this region an “origin” of diversity. It indicates there's directionality.*

Author response: We recognized the lack of rigor in the original statement. In general, determining the origin of diversity requires using metrics such as π to assess subpopulation diversity. We have removed this description (line 145).

Review Comments 2.19 L133-135: *Lacks citations*

Author response: Added. Please see line 150 in the revised version.

Review Comments 2.20 L136: *“likely to be products of Meselson effect” How likely? Did you test this? Meselson effect is not the only alternative explanation*

Author response: What we intended to state is a factual observation: we observed the same phenomenon as reported previously. *Pst* strains from non-Asian regions exhibit higher

heterozygosity, which was attributed to the Meselson effect in previous studies. We simply aimed to indicate that our observations of heterozygosity are consistent with prior findings, while noting that they interpreted it as largely driven by the Meselson effect. In the subsequent part of our manuscript, we explain that high heterozygosity is not solely attributed to the Meselson effect—indeed, as you mentioned, the Meselson effect is not the only possible explanation. We have revised the misleading texts accordingly (lines 151–153).

Review Comments 2.21 L137: “may have” undergone?

Author response: Revised as suggested.

Review Comments 2.22 L139-142: *The missingness could be due to reference bias and a shared haplotype within the AB population. I see this mentioned much later in the manuscript but I don’t find this way of structuring arguments helpful. This paragraph is confusing and it’s still unclear what are the hypotheses made.*

Author response: We have revised this paragraph to clarify our logic and explicitly present the core hypothesis. Specifically, we now emphasize that the observed heterozygosity patterns may be influenced by both biological processes (such as sexual vs. asexual reproduction) and technical or population-structural factors (e.g., reference bias and shared haplotypes). Line155-164 in revised version are rewritten. This revision clarifies the logic: we first describe the unexpected observation, then connect it with potential explanations, and finally outline the implication (need for further investigation).

Review Comments 2.23 line 152: *A little bit more background on the chosen isolates, are they field isolates, lab strains, crosses?*

Author response: Added in manuscript (now lines 169–171). AZ2 is a laboratory-generated strain, derived from the sexual progeny of experimentally induced *Pst* isolates A153 and XZ-2. *Pst*134E is a field isolate sampled from Wagga Wagga, New South Wales, Australia.

Review Comments 2.24 L152-154: *Can you show AZ2 and Pst134E’s placements on the tree and their Fis values to further support this?*

Author response: Our WGS data do not include AZ2 and *Pst*134E, so we cannot tell their exact placements on the phylogenetic tree. Their heterozygosity is demonstrated in Fig. 3a, which shows the number of SNP variations between the haploid nuclei of these two strains. The data on Fig. 3a reflects the heterozygosity of the strains—the higher number of SNPs, the higher heterozygosity, therefore, the *Pst*134E isolate exhibits high heterozygosity, while the AZ2 isolate exhibits comparatively low heterozygosity.

Review Comments 2.25 L166: *add an unrooted binary tree or split tree to support this model*

Author response: Thanks for your suggestion. We added Fig. 5f of the revised manuscript to support this model. The phylogenetic tree of global *Pst* isolates viewed from the perspective of haplotype

combination, where AA, AB, and BB grouped distinctly. This figure clearly illustrates the AA – AB – BB model.

Review Comments 2.26 line 181: *Explain what IBS measures and why it's important.*

Author response: The IBS was calculated for the 239 samples included in the genetic variation map of this study, generating IBS values between all pairs of samples. This analysis was used to assess the genetic similarity among strains with different nuclear haplotype combinations. We want to primarily address the question of whether strains with the same nuclear haplotype combination are more similar to each other and whether strains with different nuclear haplotype combinations are less similar. This description has been revised (lines 202–204).

Review Comments 2.27 L189-194: *I understand the conclusion, but the writing is hard to follow*

Author response: Thanks for your point. This paragraph was indeed abrupt and difficult to follow. Previous studies have also suggested that different nuclear haplotype combinations influence heterozygosity. Here, to help reader better understand, we added references to these studies, and explained how different nuclear haplotype combinations contribute to heterozygosity in *Pst* populations. Revised in lines 212-218.

Review Comments 2.28 line 205: *What does complete divergence mean? How is that defined?*

Author response: Our intention is to describe that the hc-hpd regions show no signals of recombination, with the SNPs in these regions remaining homozygous for one allele (alt) in AA isolates, homozygous for the other allele (ref) in BB isolates, and heterozygous in AB isolates. The wording here was inaccurate, as “complete divergence” was not defined. We have removed this statement (line 229).

Review Comments 2.29 L211-213, L481-483: *Error. Supplementary Table 5 shows non-overlapping windows, which contradicts the stated use of sliding window with 1kbp steps. Please correct or clarify.*

Author response: Thank you for pointing out this issue. We acknowledge that our description in this section was incorrect. While we performed sliding window statistics using a 1 kb step size, the windows presented in the results are non-overlapping, which is inappropriate. The estimation of region types was performed using only non-overlapping windows. We clarified in manuscript (line 521).

Review Comments 2.30 L220: *This is too vague of a description for a massive transcriptomic dataset and analysis you have. How many Pst genotypes and what are the sporulation stages/conditions included in this RNA-seq dataset? Does this signal remain when you apply different conditions?*

Author response: Thanks for your point. The description of this analysis is indeed too vague. All of the 250 RNAseq samples comes from a previous study (Adams et al., 2021 BMC Genomics), where infected leaf samples were collected directly from the field across multiple countries and different

plant developmental time points. All samples are *Pst*-infected wheat leaf as stated by Adams *et al.* thus the information of the precise sporulation stages/conditions of each RNAseq sample not available. The purpose of this analysis is to provide a general idea about the genomic activity in chromosome regions of different heterozygosity through an average expression level of a diverse set of transcriptomic samples. Accordingly, we revised the main text in lines 245-246 of the revised version. For readers interested in the expression data, we deposit the gene expression matrix and metadata in Supplementary data 1, Table S8.

Review Comments 2.31 line 220 onwards: *Many fungal genes can not be assigned a functional annotation. Could you include how many genes in these regions are not functionally characterized? Fig 4h only mentions the ones with predicted functions, but it would be essential to know what the percentage of functionally characterized vs unknown function in these regions are.*

Author response: We have revised this section to provide the number and proportion of functionally annotated vs. unannotated genes in the regions. This information is presented in Table S10. At the same time, we released the functional annotation of the Pst134E genome gene generated in this study, which are available in Supplementary data 1.

Review Comments 2.32 L223: *Unsure the point of performing GO term analysis if authors only want to draw conclusions about host invasion. The argument of hc-hpd being crucial for virulence is weak. Need to at least show if these genes are highly expressed only during infections.*

Author response: We presented the GO enrichment of genes in different genomic regions, among which some are related to virulence. Fig. 4g shows the distribution of gene expression levels in the three types of regions across 250 samples. These expression levels were generated from field *Pst*-infected wheat leaf samples and reflect pathogen's growth and developmental activity. However, they can't specifically reflect the expression during the infection period. We have revised this section, removed the argument of hc-hpd being crucial for virulence (line 252).

Review Comments 2.33 L228-240: *This paragraph is hard to read. I understand the authors have pointed out that some BB isolates have less shared k-mer content with Pst134pri, indicating there are more divergent haplotypes. Is that why we are seeing more missing sites in BB despite Pst134pri reference being a B? Please make it clear to the readers. Also please consider adding Figure S4 as a main figure.*

Author response: There was an error in the statement. BB isolates actually share a considerable number of kmers with Pst134pri, at least more than AA strains. When using Pst134pri (B nuclear haplotype) as the reference genome, BB isolates also show fewer missing sites compared to AA strains. We have revised this paragraph (line 261-263). Fig. S4 was now added into a main figure (Fig. 5).

Review Comments 2.34 line 242 and onwards: *Is the comparison between a haploid and dikaryon valid for this analysis? How can recombination rates be inferred unphased short read datasets, especially in homozygous strains without making too many reference-based assumptions?*

Author response: We compared LD decay between *Magnaporthe oryzae* and *Pst* using the same computational pipeline. The calculation assesses the extent of linkage decay caused by recombination based on the genotypes in the population, and is valid.

We identified recombination breakpoints by detecting the boundaries between homozygous and heterozygous genotype regions, and each haplotype block was required to be supported by at least five consecutive SNPs sharing the same genotype. Since the recombination analysis is based on a selfing line where the parental origin of each individual is fixed and unique, we believe that most recombination sites can still be identified without phasing. Previous studies have also used NGS data from selfed lines to identify recombination breakpoints without phasing, and this approach has been considered feasible (ref35). We have indicated this limitation in the current manuscript (lines 325–327). The content concerning the comparison of recombination between mc-hpd and hc-hpd has been labeled as an inference lacking sufficient supporting evidence, and Fig. 6c has been revised. We must acknowledge that this issue objectively exists, but it is difficult to resolve due to the lack of hifi/hic data.

Review Comments 2.35 L252: *“lower baseline in LD decay” is not a proper description for higher recombination, you’re looking at r^2 , LD decay is an inference. Please rewrite.*

Author response:

Thanks for your comment. We rewrote this part in the revised version of the manuscript (lines 279-280).

Review Comments 2.36 L264-266: *Does not make sense*

Author response: Deleted in line 291. This section represents a biological hypothesis regarding A/B haploid-inherited regions and lacks evidence. It should not appear in the Results section and has been removed.

Review Comments 2.37 line 272: *How does this show evidence for alternative life cycles?*

Author response: The alternation of generations in *Pst* has been confirmed by previous studies, and we have added a citation here (now in line 297).

Review Comments 2.38 line 279: *Why were the high heterozygosity isolates removed?*

Author response: Because it contradicts basic biological principle. In a selfing line, after a sexual reproduction event, heterozygous sites undergo recombination and may turn into homozygous while homozygous sites may not turn into heterozygous site. Therefore, the offspring could not have a higher heterozygosity than the parent. Offspring segregants with heterozygosity higher than their parents may be contaminated. We have added this explanation to the manuscript, along with references supporting this point.

Review Comments 2.39 L290: *Please show how this was done. The only results/data shown was a haplotype block on chr7 and a virulence table, so it sounds like authors are generalising from a*

single observation. There is too little analysis done to show evidence for “transition of virulence loci from het to hom could help facilitate virulence”.

Author response: Thanks for your careful reading. We indeed lacked a description of the existing results. Figs. 6 support the idea that “the transition of virulence loci from heterozygous to homozygous contributes to enhanced virulence.” The figures show heatmaps of genotypes in selfed populations for candidate intervals. We can see differences in virulence between heterozygous and homozygous haplotypes. We added citation to the figures and supplementary tables after this sentence (line 318).

Review Comments 2.40 line 295 onwards: *How are recombination breakpoints identified? How can they be differentiated from transposon insertions, singleton regions that are not in the reference?*

Author response: We identified recombination breakpoints by detecting the boundaries between homozygous and heterozygous sites. Each haplotype block was required to be supported by at least five consecutive SNPs sharing the same genotype. We acknowledge that this approach is not precise, as it is based on unphased genomic data and cannot distinguish transposon insertions or haplotype regions absent from the reference genome, as you pointed out. Therefore, we have revised the description of these results to clarify these limitations (line 325-327). The content regarding the comparison of recombination between mc-hpd and hc-hpd has been marked as inference based on insufficient evidence.

Review Comments 2.41 L297: *Not sure what you mean by hc-hpd “serves as” A/B haploid-inherited region.*

Author response: The original phrasing was inaccurate, and the use of “serves as” is ambiguous. Our intention was to describe that hc-hpd regions exhibit nearly complete inter-chromosomal linkage, maintaining one haplotype in the A haploid genome and another haplotype in the B haploid genome. Therefore, we define these regions as A/B haploid-inherited regions. The speculation that such regions are recombination suppressed has no direct evidence, so this sentence has been deleted (line 323).

Review Comments 2.42 Fig 2b: *It’s really difficult to see the leaf node colors.*

Author response: Revised, and black borders have been added on the tree of Fig 2b for easier viewing.

Review Comments 2.43 Figure 2c: *Please mark the best-fitting k.*

Author response: Marked in red color, where $k=9$.

Review Comments 2.44 Fig 3d: *Adjust so that top is not cut off.*

Author response: Revised.

Review Comments 2.45 Fig 4: *nc-hpd, mc-hpd, hc-hpd should be explained in the figure legend.*

Author response: Added. Thank you for pointing out this issue. We also checked the figure legends for other figures to ensure that no important information or *P*-value tests were omitted. The legends of Fig. 2, 4, and 6 have been revised accordingly.

Review Comments 2.46 *Figure 4b: Please label each individual chromosome clearly.*

Author response: Added.

Review Comments 2.47 *Fig 4e: Change to effector candidates or similar, as there is no validation they are effectors.*

Author response: Revised.

Review Comments 2.48 *Figure 4c,d,g legend: Please describe statistical tests used.*

Author response: Added in legend.

Review Comments 2.49 *Figure S4e: In Figure 2d, the samples with negative *F_{is}* clustered in the middle are already described to be high-heterozygosity in L133. However in S4e those samples were referred to as AA but I expect it to be AB? Could you clarify?*

Author response: There was an error in the annotation here. AB strains in Fig. S4d and S4e were incorrectly labeled as AA. Corrected.

Review Comments 2.50 *Figure 5c: Not sure what this is trying to say. Does the right panel show something that doesn't exist? I am a bit confused.*

Author response: This figure serves as a schematic illustration to explain the concept of A/B haploid-inherited regions. The right panel you referred to as “something that doesn’t exist” are actually depicted with high transparency to indicate nuclear haplotypes that do not exist in the population. These represent hypothetical nuclear haplotypes in which recombination has occurred in the *hc* region, but such types were not observed in our dataset. The figure illustrates that certain regions within the A/B nuclear haplotypes have not undergone recombination. They exhibit inter-chromosomal linkage and A/B haploid-inheritance, maintaining A haplotype in the A nuclear haplotype and B haplotype in the B nuclear haplotype. We have added these descriptions to the figure legend to improve readability (lines 802–806).

Review Comments 2.51 *Figure 6b: No description for the *p*-values.*

Author response: Added in legend.

Review Comments 2.52 *Table S3: Would be great to add a column for citations.*

Author response: Added.

Review Comments 2.53 *Figure S1: Please add genotype legend (like the one from Fig S4).*

Author response: Added.

Review Comments 2.54 *Figure S1c: Could you rotate the clades around for the tree on the right for easier comparison?*

Author response: Rotated.

Thank you for your meticulous comments and suggestions. We believe the manuscript greatly improved after this extensive revision.

Reviewer #3:

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

Thank you for your review! With your guidance, we were able to correct several issues in the manuscript and make it more rigorous and complete.

Reviewer #4:

The manuscript by Wang et al. presents the development of a tool to determine divergent nuclei in dikaryotic organism using resequencing approaches and then delves into the characterization of existing data for the stripe rust to determine the complexity and divergence of those individuals and their haplotypes. The paper presents the data on refined pipeline, Dfseq, that allows for dissecting heterozygosity and haplotypes from nuclei. This approach allows for the detection of mixed strains and SNP artifacts, improving comparative population genomics and avoiding misinterpretations.

The paper presents similar data to previous papers, indicating a high heterozygosity, but challenges previous findings. This is based on a large dataset available of over 200 isolates. This paper continues that theme of the high heterozygosity but reevaluates the mechanism and focuses on the evolutionary implications of the dikaryotic nature of rust fungi.

Review Comments 4.1 *The general gaps are the lack of evidence of the "C haplotype". While the hybrid origin of the B haplotype is a compelling hypothesis, the putative donor lineage (C) remains unidentified, and additional sampling is needed.*

Author response: We indeed lack direct evidence for the presence of the C haplotype. This part of the content was primarily mentioned in the Discussion as a hypothesis. However, we realized that similar descriptions also appeared elsewhere in the manuscript, such as in the Abstract. As unsupported hypotheses should not be presented as main results, we have removed and revised these parts of the Abstract. At present, the term "C haplotype" appears only in the Discussion section. In addition, we have emphasized once again that the "C haplotype" is described solely as a hypothesis lacking supporting evidence. lines 21–23 and 417–418 have been revised.

Review Comments 4.2 *Also this is only based on resequencing so it lacks fully phased genomes. I wonder how this compare to other methods used by rust research community and how it is similar or different from research using long-read and Hi-C findings?*

Author response: Yes, this study was conducted based on resequencing and RNA-seq data, without involving any Hi-C or long-read data. No phasing was performed in our study. Nuclear haplotype was inferred only from short-read sequencing data, which has been demonstrated as feasible in ref4. Recently, the rust research community has generated an increasing amount of public long-read and Hi-C sequencing data. These datasets are typically used in genome assembly or pan-genome analyses, and in some cases, for nuclear haplotype analysis. We have prepared a schematic diagram (Fig. S13) to illustrate what these types of data can and cannot do, as well as their respective advantages and limitations, which addresses the question you raised. Further explanations are provided in the supplementary note, which are also provided in the following for convenience:

"Analyses achievable with short-read and long-read sequencing strategies"

In this study, we used haploid genomes and short-read data for our analyses. We present a schematic (Fig. S13) to illustrate what analyses can and cannot be performed when defining *Pst* nuclear haplotype using different sequencing data types.

In recent years, multiple high-quality haplotype-resolved genomes of rust fungi have been released. These studies typically rely on long-read sequencing combined with Hi-C sequencing. Using long-

read data enables de novo genome assembly, and Hi-C data assists in haplotype phasing. By using these data, we can directly reconstruct both nuclear genomes from a single isolate, thus providing an accurate definition of its nuclear haplotype. However, long-read sequencing remains costly.

In contrast, next-generation sequencing (NGS) is more cost-effective. Due to the short read length, NGS data are not suitable for direct genome assembly. A recent study used NGS data to infer nuclear haplotype combinations in *Pt* isolates. By evaluating the shared k-mer counts and identity between the NGS reads and several *Pt* haploid genomes, they confirmed nuclear exchange events in *Pt*.

Our study adopted the same strategy to define the nuclear haplotype combinations of *Pst* isolates. However, while this method allows the inference of nuclear haplotype combinations, it also has limitations. Without access to high-quality haplotype-resolved reference genomes, such analyses are difficult to perform.”

Review Comments 4.3 *The paper rightly challenges the Meselson effect explanation for high heterozygosity but downplays how both mechanisms (asexuality and hybridization) might contribute. Present a more nuanced comparison, acknowledging that different mechanisms may dominate in different populations or genomic regions. This would make the argument more balanced.*

Author response: Thanks for your constructive suggestion. We rewrote the discussion about the Meselson effect’s possible contribution to the high heterozygosity observed in the *Pst* population (now in lines 369–376). We explained the potential origins of different types of heterozygous sites and acknowledged that multiple mechanisms may jointly contribute to heterozygosity.

Review Comments 4.4 *The hc-hpd regions are described as recombination-suppressed, but the biological basis (structural variation? epistasis? chromatin context?) is unclear.*

Author response: The hc-hpd regions have been redefined as hc regions (regions highly contributing to heterozygosity differentiation). The hc regions exhibit linkage across chromosomes, with no obvious recombination fingerprint. Therefore, we speculate that recombination suppression may occur in these regions. However, we acknowledge that this phenomenon is purely speculative, and the biological basis of such genomic features remains unclear in this study. Accordingly, we have removed the discussion of the biological basis of recombination suppression (lines 291) and the description of recombination suppression (lines 323).

Review Comments 4.5 *In the line 39, could you explaining somatic hybridization for non-mycology readers?*

Author response: Explained. A description of somatic hybridization has been added in lines 37–42.

Review Comments 4.6 *line 54 - 57 Please consider the citation of other studies proposing similar mechanisms:*

- <https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1011493>

- <https://10.1094/MPMI-10-24-0122-FI>

- <https://doi.org/10.1101/2025.05.22.655633>

Author response: Added in line 63.

Review Comments 4.7 line 86-87 *Do you have any statistics or table that show the proportion of isolates deviating from AD 0.5 among the 266 isolates?*

Author response: First, we apologize for the misuse of the abbreviation. The parameter we actually used is allele balance, which is abbreviated as AlleleBalance in the revised manuscript. Supplementary Table 1 includes a column indicating whether a strain is mixed. Among the 266 strains, there are 27 mixed strains, accounting for 10.1%. We have added this description in lines 102–103.

Review Comments 4.8 line 129 *Spell out hpd: heterozygosity population divergence. Also, The term "hpd" is introduced as central to the study, but its biological meaning and distinction from general population structure or heterozygosity patterns is vague early on. Define hpd more clearly at first mention (e.g., "hpd refers to divergence between populations that arises due to combinations of divergent haploid genomes within dikaryotic individuals") and reinforce how it differs from prior interpretations.*

Author response: Thank you for your suggestion. This issue was raised by multiple reviewers. The intention of using “Heterozygosity population divergence (hpd)” was to briefly describe the phenomenon of differences in heterozygosity levels between subpopulations within the *Pst* species. However, because we did not provide a detailed explanation when introducing this concept, it resulted in reduced readability. We have now added a detailed explanation as you advised (line 72–76) and included references to studies of other species, such as those by Li *et al.* and Scally *et al.*, which describe similar phenomena. In addition, we have reduced the frequency of the abbreviation hpd throughout the manuscript. In instances where the abbreviation was unnecessary, we replaced hpd with more intuitive terms (e.g., at line 79, hpd was replaced with heterozygosity, and hc-hpd region was revised to hc region). These changes improve clarity and readability of the manuscript. To assist readers without a computational background, we also added a schematic diagram to illustrate this phenomenon (Fig. S10) and provided further explanation in the Supplementary Note.

Review Comments 4.9 line 221 - 223 *How was this determined? it is significant based on what test?*

Author response: We quantified the mean expression levels of genes in the three types of regions across 250 samples, measured in FPKM. Then we used t-test to examine whether the distributions of mean gene expression levels differed significantly among the three regions. We have added this description in lines 245–246 and indicated in the Fig. 4 legend that t-test was used as the significance test.

Review Comments 4.10 line 249 - 250 *are there other systems different from Magnaporthe that could help to make a case for what is observed on rusts? Actually, I wonder if Dfseq is applicable to other systems?*

Author response: Before addressing your question regarding Dfseq, we would like to clarify that we have accepted the editor’s suggestion to revise the description of Dfseq. We presented it only as an SNP-calling pipeline. The main text no longer includes a detailed introduction of this method. The Dfseq pipeline can be used to distinguish true heterozygosity from mixed isolates within a WGS

sample. It is suitable for variant detection in highly heterozygous dikaryotic fungi, effectively reducing the risk of strain mixing. However, due to limitations in computational resources and time, we did not test it in other systems. We did not test other systems different from *Magnaporthe*. However, we conducted simulations and confirmed its validity in the *Pst* system. The results are presented in the Supplementary Note and Fig. S11.

Point-by-point Response

Wang et al., Two divergent haploid nuclei shaped the landscape of population diversity in wheat stripe rust.

We would like to express our sincere gratitude to the reviewers. With their review and suggestions, we have made thorough revisions to the manuscript, making it more rigorous.

The major revisions include:

- (1) Based on the reviewers' suggestion, we revised our A/B nuclear haplotype naming convention to align with the nuclear haplotype nomenclature used in previous studies.
- (2) Validating the accuracy of the RNA-seq analysis used in this study, addressing the reviewers' concerns regarding false positives caused by multiple mapping.
- (3) Improving wording and clarity throughout the manuscript.
- (4) Preparing new figure and table (Fig. S14, Table. S17).

All revised content has been marked in red in the manuscript.

Reviewer #1:

*The authors performed the simulation analysis to test effectiveness of using AlleleBalance in detecting strain mixing, the results are satisfied. All of my previous concerns have been addressed except one very minor issue: all phenotypes (Yr**) in the figures (ie Figure 7, Fig S6) should be italic.*

Thank you for your review. With your help, our manuscript has become more rigorous and complete. We appreciate the minor issues you raised, and we have replaced the 'Yr**' in Fig. 7 and S6 with italics.

Reviewer #2:

The revised version of the manuscript addressed most of the concerns raised but there's still a few issues that should be revised or clarified.

Review Comments 2.1 *Even though the naming of A and B nuclei in this manuscript was somewhat arbitrary, it would be beneficial for further studies to be consistent with the naming. The now published Pst104E genome named A and B haplotypes the opposite of the naming in this manuscript. Classifying isolates by their haplotype combinations and having different naming for the same haplotypes groups in different publications is not going to help.*

Author response: We fully understand your concerns on this issue. Based on your suggestion, we swapped the naming of the A and B nuclear haplotypes in this manuscript to align with the Pst104E genome haplotype nomenclature. The manuscript, figures, and tables have been revised accordingly (lines 177-179, 197-201, 414-417, Fig. 3-6, etc.).

Review Comments 2.2 *Fig 5f: The European isolates cluster with BB isolates but are labelled AB? Is that correct?*

Author response: We apologize for the confusion caused by our unclear figure. The annotation of the nuclear haplotype in this figure was not accurate. We added annotation lines to better indicate the nuclear haplotype information of the isolates. Fig. 5f and its legend (lines 811-812) have been revised.

Review Comments 2.3 *Fig 6c: Changing the text to “observed” vs “not observed” would be better than “exists” and “no exists”, as this is based on available datasets, which may not capture all possible haplotype configurations and possible recombination events. It should also be “linked” and not “linkaged”.*

Author response: Thank you for pointing out the inappropriate wording. We revised the text in Fig. 6c, changing "exists" to "observed," "no exists" to "not observed," and "linkaged" to "linked," to more accurately reflect the observations based on the available dataset and avoid misunderstandings. Fig. 6c has been revised.

Review Comments 2.4 *The description of RNA-seq dataset analysis is still very vague. Looking at Table S8, mapping stats vary greatly between samples. How did multiple mapping affect assigning reads to genes? Could the observed higher expression in hc regions be an artefact of higher mapping efficiency in these regions? What is the difference between reads mapped to “multiple loci” and “too many loci”. How does this affect counting?*

Author response: The issues you raised are indeed thoughtful. First, the description here is not clear. We didn't clearly describe what RNA-seq samples were used here. The RNA-seq sample is quite unique, which causes the differences in alignment statistics. These RNA-seq data are from field genomics samples collected using the methods proposed in Adams et al., based on field-sampled wheat and *Pst* spores (ref36). These samples contain RNA reads from both wheat

and *Pst*, and the proportion of reads from each is not clear, leading to substantial discrepancies in alignment statistics. However, prior study have shown the feasibility of using such samples for gene expression quantification (ref36), so we still consider these data valuable for our study. We have made revisions to lines 247-248 in this manuscript to clearly specify the characteristics of the RNA-seq data.

You later raised questions about how multiple mappings affect gene expression quantification and asked multiple questions. Let us address them one by one.

(1) How does multiple mapping affect the assignment of reads to genes? Could higher expression in high-coverage regions be an artifact of better mapping efficiency in these regions?

Here, we only used uniquely mapped reads for re-quantification, and the analysis remains unchanged. The results were consistent with previous findings, with genes in the hc region showing higher expression than those in other regions (Fig. S14). This addresses your concern, and the conclusion "We found that genes in the hc region generally had higher expression than genes of other regions" is not an artifact caused by multiple mapping. Interestingly, when multiple mapping is removed, the difference of expression levels between nc and mc regions become non-significant, which suggests that multiple mapping does have an effect on gene expression quantification. Your concern is valid, but we have still proven the accuracy of our conclusions.

(2) What is the difference between "mapped to multiple loci" and "too many loci," and how do these affect counts?

These terms are parameters given by the STAR software during the alignment process.

Reads mapped to multiple loci: This means STAR found multiple valid alignment positions for the read in the reference genome, but the number of alignments does not exceed the parameter `--outFilterMultimapNmax` (default is 10). These alignments are retained, and STAR outputs one as the "primary" alignment while marking the others as secondary, or records the multiple mapping information in the SAM format.

Reads mapped to too many loci: This means the number of valid alignment positions for the read exceeds the threshold set by `--outFilterMultimapNmax`, so STAR considers it "too many loci" and discards it (without outputting specific mapping positions). These reads are not counted as valid mappings.

The key difference is that the former is accepted multiple mappings, while the latter exceeds the threshold and is discarded.

Review Comments 2.5 *In response to comment 2.32 the authors state: "We have revised this section to provide the number and proportion of functionally annotated vs. unannotated genes in the regions." While this information is found in TableS10 now, it is not mentioned. Based on the numbers in TableS10, ~80% of genes do not have an annotated function, which makes the results of the GO analysis questionable in terms of biological meaning as the statistics are done on a very small subset of genes.*

Author response: Regarding your concern that the GO analysis may lack biological significance due to the 80% of genes lacking functional annotations, we acknowledge this limitation. Currently, the number of functionally annotated fungal genes is limited, and this issue is common in many

fungal genomics studies. We can only perform this analysis with the limited annotation information available. We have updated the text to refer to the information in Table S10 and acknowledged the potential limitations of this analysis (lines 256-257).

Review Comments 2.6 *Fig 3c, S2, S3: In my understanding, Fis is an index that strictly ranges from -1 to 1. However, those figures display Fis values lower than -1.0. Is this an error in calculation?*

Author response: Thank you for pointing out this issue. The Fis index is indeed strictly between -1 and 1, but we found that in our 239 isolates, four had Fis values < -1: CRR651295, CRR651298, SRR24010205, SRR27320883. This may be due to the lack of outlier filtering during calculation with VCFtools. We have corrected the extreme values for these four isolates to -1. Figures 3c, S2, and S3 have been revised.

Review Comments 2.7 *L118-119: Could you do a BLAST search to confirm the presence of rDNA?*

Author response: Of course, we extracted the 1.4-2.1mb region from chr17 and performed a BLAST search, using rRNA sequences from Bioproject PRJNA39195 (18S), PRJNA51803 (28S) as references. This confirmed the presence and distribution of rDNA in this genomic region (Table S17).

Review Comments 2.8 *L124-126: Still unclear what patterns are being referenced here, and not sure whether citations (8) and (45) are appropriate. I suggest deleting this sentence. Authors may consider simply highlighting that e.g. the strict SNP calling strategy would allow for more robust population genetic inference downstream.*

Author response: This statement was unclear. We deleted the sentence as your suggestion and revised it to: "In conclusion, the genetic map obtained through the strict SNP calling strategy would allow for more robust population genetic inference downstream." (line124-126)

Review Comments 2.9 *L185: If there's a recombinant haplotype of A/B this analysis might not pick this up. Please discuss this limitation.*

Author response: Yes, as you mentioned, when recombinant haplotypes of A and B exist, it is difficult to distinguish which nuclear haplotype the analysis assigns them to, and they cannot be detected. To investigate whether recombinant A/B haplotypes exist, other analytical methods are required, such as genotype heatmaps or genomic collinearity analysis. We added a note about this limitation in lines 190-192: "It is also worth noting that nuclear haplotypes determination based on k-mers cannot clearly distinguish the recombinant haplotype of the A/B."

Review Comments 2.10 *L255-256: This statement about introgression does not sound convincing without proper tests and warrants an elaboration.*

Author response: You are correct that without proper testing (e.g., D-statistics), gene introgression inferences are not conclusive. This issue arose due to our overly simplified description. Our

intention was simply to propose a hypothesis, suggesting that the hc region shares some features of introgressed segments (e.g., higher SNP density) and could be considered a potential introgression region, rather than concluding it is an introgressed segment. We have rewritten this section (line260-262) to provide the reasoning behind the hypothesis and softened the tone of the inference, to avoid misinterpretation.

Review Comments 2.11 L892: Please provide citations for both genome assemblies.

Author response: References been provided, and we added the citation in line 912.

Reviewer #4:

Reviewer #4 concerns were mainly about properly defining the scope, terminology, literature and robust interpretations of the authors' findings. I looked at the responses and revisions to the manuscript and these are satisfactory in my view.

Thank you for your review! Your review has allowed our work to progress further.

Point-by-point Response

Wang et al., Two divergent haploid nuclei shaped the landscape of population diversity in wheat stripe rust *Puccinia striiformis* f. sp. *tritici*.

We would like to express our sincere gratitude to the reviewers. With their review and suggestions, we have made thorough revisions to the manuscript, making it more rigorous.

Reviewer #2:

Thank you for revising the manuscript again, I know this takes a lot of effort. Especially renaming the haplotypes and revising the figures. This naming is now consistent with another recent preprint, so worth the effort. All of my issues have been addressed.

Thank you for your review. With your help, our manuscript has become more rigorous and complete. Our efforts are worthwhile.