We thank the reviewers for their critical reading and assessment of our manuscript. Below please find our response to individual comments.

***Reviewer: 1***  
  
*Reviewer Report for the Authors  
The manuscript used the high-resolution chromosome conformation capture technique, Micro-C, to investigate 3D genome conformation, particularly topologically associating domains (TADs), in five rice subspecies/ species: Oryza sativa IR64, Oryza sativa Nipponbare, Oryza sativa Azucena, Oryza rufipogon, and Oryza meridionalis. This work advances our understanding of the nature and evolution of TADs in plants. Additionally, the manuscript is well-written, with a logical flow that is clear and easy to follow*.

Thank you for the supportive comments.

*Major Concerns:  
1. Only about one-third of TADs were retained after filtering by overlapping the results of the two methods. How do the authors explain the discrepancy in the remaining TADs? Are these discrepancies considered false positives, or are there alternative explanations? Clarifying the authors' perspective on this would strengthen the manuscript.*

The discrepancies in the number and locations of TADs identified by different callers in the same sample and same experiment have been reported in the literature on 3D genomics and has been a cornerstone of discussion in several reviews, including Liu et al., 2022; Zueffrey et al., 2018; Sefer, 2022. Indeed, some of these TADs may indeed be false positives. There are three major potential explanations of these discrepancies pointed out in the literature: (1) Algorithmic approaches: TAD callers employ diverse methodologies, such as statistical models, clustering techniques, and graph-partitioning methods. (2) Parameter settings: The choice of parameters, including window size and thresholds, can significantly influence TAD detection. (3) Hierarchical TAD structures: Some TAD callers are designed to detect hierarchical structures, identifying both primary TADs and sub-TADs, while others may focus solely on primary TADs. This difference in focus can result in varying sets of detected TADs. In our experience, these are all true, as we observed differing results from Tad calling with different callers and parameter settings. This is why to identify a robust set of TADs, we decided to devise an approach based on using TAD callers that employ different algorithms and hierarchical/ non- hierarchical approaches. We have added a discussion of this issue in the manuscript on lines 192 – 199.

*2. While confident TADs were identified at both 2kb and 5kb resolutions using HiCExplorer and HiTAD, the primary focus is on results from the 5kb resolution. What is the specific rationale for this choice? This point is not clearly explained in the manuscript.*

At 2kb resolution, we obtained 2474 TADs with median size of 44kb, median number of genes 5 per TAD. At 5kb resolution, we identified 1207 TADs with median size of 65kb, median number of genes 7 per TAD. The TADs called at 5 kb resolution had higher median number of genes per TAD, and since we wanted to analyze gene expression within TADs, we chose the 5kb resolution dataset for further analysis to increase the power of the analysis. We agree that this point must be made clear in the paper and added an explanation on lines 209 – 211.

*3. Are there any genes specifically located in TADs with high or low boundary strength, given their distinct genetic and epigenetic features (Figure 5)? Can the authors perform a GO analysis for these genes? The same question applies to genes in conserved TADs (Figure 8A).*

This is an interesting question. We conducted a GO term analysis for genes within strong and weak boundaries but did not observe any statistically significant enrichment.

As for the second question, we were wondering this as well during the manuscript preparation stage. We performed the GO terms analysis on the genes from conserved TADs (which we defined as TADs from groups 4 and 5). We found the following biological processes to be significantly enriched in those groups: negative regulation of phosphorous metabolic processes, negative regulation of phosphate metabolic processes, flavonoid biosynthetic process, flavonoid metabolic process, lipid catabolic process. We thought that these terms were not matching well, and apart from being all related to so called ‘housekeeping’ genes, were of little interest, thus we decided to not mention them in the results section.

*4. The low conservation rate of TADs across genomes is noteworthy (Lines 516-518). However, the manuscript does not clearly explain the process used to identify these high-confidence TADs. It appears the authors used the same method as for Azucena. If so, could the stringent criteria for identifying these TADs contribute to the observed low conservation rate, given that approximately two-thirds of TADs were discarded? Including additional discussion on this point would provide valuable context.*

Thank you for pointing this out: indeed, the same process for identification of high-confidence set of TADs was used for all five genomes. We included this clarification in the Methods section, lines 758 – 760. We recognize that our stringent criteria resulted in retaining only TADs with the highest confidence. This rigorous filtering likely contributes to the observed low conservation rate. Therefore, less pronounced TADs, which may still hold biological significance, were not included in our analysis. However, we decided to ‘sacrifice’ these less pronounced TADs to reduce the likelihood of including false positives or inconsistently defined TADs, leading to more reliable assessments of conservation. In addition, this approach minimizes background noise. In conclusion, we believe that stringent filtering ensures that the evolutionary comparisons are based on the most reliable data. We have included additional discussion on this on lines 674 – 678.

*Minor Concerns:  
1. Figure 2A: The panel showing consensus TADs is unclear. Is the consensus represented by the black regions or the overlap between the blue and green parts? Consider highlighting the black regions in the other two situations and labeling the percentage of overlap. This will make the figure more informative for readers.*

We agree that the labeling is somewhat confusing. The process of TAD annotation was as follows: we identified uniform set of TADs called by both HiCExplorer and HiTAD tools, with the criteria that the TAD body must reciprocally overlap by at least 80% between both tools, and the TAD call made by HiCExplorer tool was retained. Consequently, the consensus TAD would have TAD boundaries identified by HiCExplorer. We chose to do that because the HiCExplorer hicFindTADs tool returns insulation score values for TAD boundaries, while HiTAD does not. To make the figure clearer, we added the following to the figure legend (lines 228 – 229) and to the Methods section (lines 757 – 758): “The TAD body and boundaries identified by HiCExplorer were retained as the consensus.”

*2. Missing Data in Figure 5: There appears to be missing data for H3K27me3 in Figure 5, despite the claim that H3K4me3 is a positive predictor and H3K27me3 a negative predictor of boundary strength (line 384). Can the authors clarify this inconsistency?*

In our analysis, the differences in H3K27me3 enrichment between strong and weak boundaries were not statistically significant and were therefore not reported. However, weak boundaries exhibited lower levels of another repressive epigenetic mark, DNA methylation. This led us to discuss the Arabidopsis H3K27me3 mark, which was identified as a negative predictor of boundary strength. While these are distinct epigenetic marks, both are signatures of repressive chromatin. Thus, we chose to generalize the pattern underlying boundary strength. We added a clarification to make our point clearer to the reader (lines 428 – 430): “In summary, the strength of rice TAD boundaries was found to be associated with active epigenetic marks and negatively correlated with repressive marks.”

*3. Figure 6A X-Axis Scaling: For clearer comparisons, ensure that the x-axis is scaled consistently across all panels in Figure 6A, ideally from -0.75 to 1.25.*

We changed the scaling of the x-axis to *-0.75 to 1.25* where possible. The new version of Fig. 6 has been included with our response.

*4. Group Naming: The labels for Groups 1–5 are not intuitive. Providing descriptive names or additional context would help readers immediately identify which group (e.g., Group 1 or Group 5) is the most conserved.*

We have re-labeled the groups as “Unique” – representing Nipponbare-specific TADs, “Rare” - TADs found in two genomes, "Moderately Conserved” – in three genomes, “Highly Conserved” – in four genomes, and “Core” TADs – in all five genomes. We have re-labeled the pie chart on Fig. 8A and updated group labels in Table S6.

*Reviewer: 2  
Major Concerns: This manuscript provides a rich dataset on the 3D genome organization of cultivated rice and its closely related wild relatives, focusing on topologically associating domains (TADs). The high-resolution chromatin conformation data obtained through Micro-C represents a valuable resource for the research community. While many findings align with those reported in prior studies, the systematic analysis of TAD conservation and evolution addresses a less-explored area and offers intriguing insights. Below are my comments:*

Thank you for reviewing our manuscript. We are grateful for your recognition of our efforts to explore the evolution and conservation of the 3D genome.

*I suggest mapping all samples to the same reference genome to improve the accuracy of conserved TAD identification. Since the goal is to identify conserved regions, lower alignment rates in highly variable regions would have minimal impact on the analysis.*

Thank you for your suggestion. While mapping all samples to the same reference genome could be beneficial in some contexts, we chose to map each sample to its respective reference genome to account for the natural genomic variation between the species. We are using high-quality (“platinum”) genome references for alignments. We believe that this strategy is the most appropriate for the goals of our analysis, as we did not want to introduce biases into our analysis of 3D genome conservation. For instance, if a TAD has a structural variation in one of the genomes, but is conserved, we may lose this TAD if we map all reads to the same genome.

*The use of salinity stress treatment to investigate the consistency of gene expression within TADs may not be representative of broader biological contexts. To strengthen the conclusions, I suggest including additional datasets spanning different tissues and developmental stages. Numerous published datasets could be integrated into the analysis.*

This is great suggestion. Since our gene expression in TADs analysis was performed on Azucena, we decided to search for additional RNA-seq datasets for this variety. The only other available dataset for Azucena gene expression on GEO was for roots under normal and aluminum stress conditions (Arbelaez et al., 2017), with accession number GSE89494. We used the raw read counts and compared the coefficient of variation of gene expression for genes within TAD and non-TAD domains. We found that our previous results were recapitulated for these datasets, namely the CV was lower for TADs than non-TADs under both conditions (Control: meanCV nonTADs = 1.628839; meanCV TADs = 1.309908 (t-test p-value < 2.2e-16); Aluminum Stress: meanCV nonTADs = 1.625955; meanCV TADs = 1.309908 (t-test p-value < 2.2e-16)). We included this finding in the Results section (line 368 – 370) and added a new supplemental figure (Fig. S6) to the manuscript.

*The analyses presented in Figure 5 would benefit from the inclusion of whole-genome controls. Specifically, comparisons with random genomic intervals or broader gene regions would provide a more robust baseline for assessing TAD boundary characteristics.*

We have chosen random (excluding TAD boundaries) 5kb genomic intervals (n=405) to provide controls for boundaries with high insulation scores (n=419) and low insulation scores (n=409), and calculated p-values for pairwise comparisons. What we observed was that gene density at random genomic regions was lower than at strong boundaries, but comparable to that of weak boundaries. TE and gypsy density at random genomic intervals was similar to that of weak boundaries. Both strong and weak boundaries had higher levels of active epigenetic marks H3K27ac and H3K4me3, but lower levels of DNA methylation, than random regions. Gene expression levels, SNP density and sequence conservation scores at random regions were like those at weak boundaries. Overall, these results show that weak boundaries often have characteristics similar to random regions and are in agreement with the observation that the strength of boundaries positively correlates with active and negatively correlates with repressive marks. We have added this information and the corresponding graph as Figure S7.

*The manuscript discusses TAD conservation but does not address the extent to which TADs vary across different tissues or treatments. Including comparative analyses of TAD datasets from diverse tissues would provide a more comprehensive evaluation of TAD conservation and its functional implications. Otherwise, the authors may need to further discuss this.*

We agree that TAD variation across tissues and treatments is an intriguing topic; however, we believe it falls outside the scope of the current project. Our primary focus in this study was the evolutionary conservation of TADs, which necessitated analyzing a single tissue/treatment for meaningful comparisons. Expanding the analysis to include TADs from different tissues would require additional steps, such as tissue isolation, preparation, and sequencing of new Micro-C libraries. We consider this to be a separate project that we could explore in the future.

*The content of Figure 3g appears after the discussion of Figure 4. The text structure needs to be adjusted accordingly.*

Thank you for pointing this out. We have moved the “TAD boundaries are enriched for specific DNA motifs” paragraph to account for this. *The y-axis is not labeled in Figures 3B, 5, 7BDF, 8B, and 9.*

In Figure 3B, the x-axis represents the position relative to TAD. TAD boundaries are marked as ‘0’, and the plots span 20 kb proximal and distal to the boundaries.

In Figures 5, 7BDF, 8B, and 9 the x-axes are hidden during the plotting, but are labeled with categorical values.

*In Figure 8C, the image appears distorted.*

This was a stylistic choice we made during TAD plotting with Coolbox tool. We expanded the y-axis to ensure the image panel integrated more seamlessly into the overall layout.

**References**

Liu K, Li H, Li Y, Wang J, Wang J. A comparison of topologically associating domain callers based on Hi-C data. IEEE/ACM Trans Comput Biol Bioinform. 2022;

Zufferey M, Tavernari D, Oricchio E, Ciriello G. Comparison of computational methods for the identification of topologically associating domains. Genome Biol. 2018 Dec 10;19(1):1–18.

Sefer E. A comparison of topologically associating domain callers over mammals at high resolution. BMC Bioinformatics. 2022 Apr 12;23(1):127.

Arbelaez JD, Maron LG, Jobe TO, Piñeros MA, Famoso AN, Rebelo AR, et al. ALUMINUM RESISTANCE TRANSCRIPTION FACTOR 1 (ART1) contributes to natural variation in aluminum resistance in diverse genetic backgrounds of rice (O. sativa). Plant Direct. 2017 Oct;1(4):e00014.