**Topologically associating domains and the evolution of 3D genome architecture in rice**

**by**

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**ABSTRACT**

We examined the nature and evolution of three dimensional (3D) genome conformation, including topologically associating domains (TADs), in 5 genomes within the genus Oryza. These included three varieties from subspecies within domesticated Asian rice *O. sativa* as well as their closely related wild relatives *O. rufipogon* and *O. meridionalis.* We used the high-resolution chromosome conformation capture technique Micro-C which we modified for use in rice. Our analysis of rice TADs shows that TAD boundaries have high transcriptional activity, low methylation levels, low TE content and increased gene density. We also find significant correlation of expression levels for genes within TADs, suggesting that they do function as genomic domains with shared regulatory features. Our findings indicate that animal and plant TADs may share more commonalities than was initially thought, as evidenced by similar genetic and epigenetic signatures associated with TADs and boundaries. To examine 3D genome divergence, we employed a computer vision-based algorithm for the comparison of chromatin contact maps and complemented this analysis by assessing the evolutionary conservation of individual TADs and their boundaries. We conclude that overall chromatin organization is conserved in rice, and 3D structural divergence correlates with evolutionary distance between genomes. We also note that individual TADs are not well conserved, even at short evolutionary timescales.

**INTRODUCTION**

In recent years, researchers have begun to explore the nature of three dimensional (3D) genome organization (1–3) , which affects gene regulation (4–7) and may play a central role in the evolution of genomic architecture (8). The study of higher-order structures has been facilitated by advances in chromosome conformation capture (3C) techniques, which have led to the development of methods such as Hi-C (9), Omni-C, and Micro-C (10), allowing the genome-wide mapping of 3D genome organization. These molecular approaches have enabled the discovery of large chromatin structural elements, such as A/B compartments, as well as smaller self-interacting regions of chromosomes referred to as topologically associating domains (TADs), which are kilo- to megabase-sized genomic regions that interact within the nucleus and are believed to represent a fundamental structural/functional unit of the genome. TADs appear to play a prominent role in gene regulation (4) and in genome replication by synchronizing origins of replication (11,12). Enhancers that regulate gene expression in regulatory domains are associated with the presence of TADs (4), and genes located within the same TAD have been found to have correlated expression patterns (5–7).Moreover, TAD boundaries often coincide with breakpoints of chromosomal rearrangements in mammals and fruit flies (13,14), leading to the hypothesis that TADs are maintained during evolution as intact units.

In mammals, TADs are thought to be formed by the cohesion-CTCF mediated loop extrusion mechanism (15), whereas in fruit flies other mechanisms have been proposed, including boundary pairing (16) and chromatin compartmentalization driven by phase separation (17). These TADs are delimited by TAD boundaries, which in animals are chromatin accessible regions enriched for active transcription marks and housekeeping genes (18). In mammals these boundaries bind CCCTC-binding factor (CTCF), and in fruit flies by various insulator proteins including BEAF-32, Chromator, CP190, or M1BP (18).

In plants, there is no consensus on the nature and nomenclature of these interacting chromosomal regions. Certain studies refer to these interacting sequences as chromatin domains (CDs) (19), chromatin folding domains (20), TAD-like domains (2,21,22), or more conventionally TADs (23)(Liu *et al.*, 2017). We will refer to such domains as TADs. Until recently, it was thought that TADs exist only in plants with genomes larger than 400 Mb, such as maize (24,25). However, the application of high-resolution Hi-C and Micro-C technologies for plant genomes has shown that plants with small genomes such as Arabidopsis also possess TAD-like domains, albeit smaller (19,26). Evidence has begun to accumulate that plant TADs might also play a functional role in gene regulation; for example, clusters of co-expressed biosynthetic pathway genes in different plants, including rice, are co-localized within TADs (27). Despite the presence of these genomic features, however, plants do not have the CTCF proteins associated with TAD boundaries in animals, although they do have cohesins (28).

Genome studies have indicated that TADs may be conserved functional building blocks of the genome, since rearrangement breakpoints typically observed between species do not typically disrupt TADs (13,14,20). Evolutionary TAD conservation is important for the preservation of *cis*-regulatory environments necessary for the control of gene expression, which suggests that TAD reorganization may be important for the evolution of novel traits. In the bobtail squid *E. scolopes*, for example, conserved gene neighborhoods involved in the origin cephalopod-specific traits are located within TADs (29). TAD boundaries, however, paradoxically have a dual nature. On the one hand, these boundaries appear to be evolutionarily constrained (21,30) and their deletion rare and under negative selection (31,32). Nevertheless, synteny breakpoints in flies and mammals (13,33) are also enriched at boundaries, Thus, TAD boundaries are conserved elements which preserve gene regulation within specific domains, yet they are prone to be sites of chromosomal breakage, highlighting their role in genome rearrangements during evolution (34).

It is thus unclear the degree to which TADs and their boundaries evolve. For example, a number of direct comparisons of TADs between species have shown that they are strongly conserved in some phylogenies (e.g., mammals) (35). In contrast, very little conservation of mammalian TAD boundaries was found in a recent study, with only 14% of human boundaries conserved between four primate and 4 rodent species (36). More recent studies similarly present contrasting results: for example, only 43% TADs were found to be conserved between human and chimpanzees (37). Dixon *et al*. reported that 76% of mouse TAD boundaries are conserved in humans (38), although a careful re-evaluation of their data concluded that only 31% of boundaries were conserved between the species (39), and other studies indicate that TADs were generally not conserved (37). A possible explanation for these contrasting results was suggested by Torosin *et al*. (40), who proposed that different types of TADs evolve under different evolutionary forces, with highly conserved TADs in vertebrates and flies enriched for developmentally regulated genes, while TADs enriched for broadly expressed genes evolve rapidly (40,41).

Like animals, plants appear to have varying levels of TAD conservation. Only 8.23% of the foxtail millet domains were conserved in sorghum, and similar results were found in a sorghum and maize comparison (42). Between *Brassica rapa* and *Brassica oleracea* 25% of TADs were found to be conserved (43), and 40-48% of TADs were found to be conserved between two poplar species (44). However, these studies assessed TAD conservation at different map resolutions and used different definitions of TAD conservation, which prevents us from generalizing about the overall level of TAD conservation in plants.

To explore the nature and evolution of plant TADs, we investigated the patterns of global 3D genome evolution in domesticated Asian rice (*Oryza sativa*) and two of its closest wild relatives (*O. rufipogon* and *O. meridionalis*). Unlike most other studies, we chose species/subspecies that have evolved over a relatively recent timescale. Rice was initially domesticated in China from *O. rufipogon* around 9,000 years ago as *O. sativa* ssp*. japonica*, which eventually evolved about 4,000 years ago into temperate and tropical *japonica*. A separate domestication of rice occurred in the Indian subcontinent ~4,500 years ago, leading to *O. sativa* ssp*. indica*, which traces its ancestry to *O. nivara* and diverged from the *japonica* lineage ~550,000 years. *O. meridionalis* is the most basal species in the AA group of the genus Oryza and diverged from *O. sativa* and *O. rufipogon* ~2.4 million years ago (45). Applying Micro-C method (10) to these species/subspecies, we show that TADs do appear to be functional units of the rice genome, and use gene expression, genomic and epigenomic data to identify signatures associated with TAD boundaries. Our investigation also examines 3D genome conservation and evolution in closely related *Oryza* genomes and provides insights into chromatin conformation patterns at different scales, showing that global 3D genome evolution closely tracks sequence evolution. Finally, we suggest that while higher-order chromosome organization tracks genome sequence evolution, individual TADs can evolve rapidly.

**RESULTS AND DISCUSSION**

**Generating Micro-C chromatin contact maps of five rice genomes**. We first generated high-resolution chromatin contact maps from five *Oryza* genomes. We selected three varieties of domesticated rice *Oryza sativa* that represent the two main subspecies, *indica* (IR64) and *japonica*; for the latter we used a tropical *japonica* (Azucena) and a temperate *japonica* (Nipponbare). We also worked with two wild species - *O. rufipogon*, the wild ancestor of domesticated *japonica*, and *O. meridionalis* (Fig. 1B). To generate these contact maps, we applied the recently developed Micro-C technology (10), which is a modification of Hi-C that includes a micrococcal nuclease (MNase) digestion step to assess proximity between chromosomal regions. We adapted a Micro-C protocol for use in rice that bypasses nuclei extraction prior to cross-linking and proximity ligation, allowing for faster generation of sequencing data (see Methods).

We assessed the reproducibility of the maps obtained by comparing two biological replicates for each genome (46). The replicates showed good concordance (see Methods), and we merged the replicates to obtain high-resolution genome contact maps. We used the definition of Rao *et al*. (47) to estimate maximum contact map resolution with HiCRes (48), which was close to 1 kb for all genomes except for *O. meridionalis*, for which we obtained a resolution of 4 kb (Table S1).

The merged chromatin contact maps for the five genomes are shown in Fig. 1A, C. Visual inspection allowed us to observe similar chromosome structures between genomes at low resolution, with contact density strongly concentrated along the main diagonals. Distance-dependent interaction frequencies represented as probability versus distance [P(s)] curves (49), reveal that intra-chromosomal contact frequencies decay rapidly as genomic distance increases (Fig. S1). Overall, we conclude that the Micro-C maps we generated closely align with the reported overall chromosome structure of rice, and we are able to distinguish chromosome territories, compartments, TADs and loops (Fig. 1A). In accordance with previous Hi-C maps of the rice genome (23,42,50,51), we also found weak intra- and inter-chromosomal clustering of telomeres, represented as off-diagonal areas of higher contact frequencies (Fig. 1A). However, no clustering of centromeres was observed, and like others we conclude that rice chromosomes do not adopt a Rabl conformation characterized by telomeres and centromeres clustering at opposite poles of the nucleus. This is in line with previous cytological (52,53) and Hi-C studies (23,42,50,51).

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**Figure 1. Micro-C analysis of chromatin interactions in rice.** (A) Micro-C contact map of *O. sativa* Azucena variety at different resolutions allows for the detection of various features of the 3D genome. The arrow in the rightmost map shows features associated with loops. (B) Neighbor-joining tree of the selected Oryza genomes created using synonymous site substitutions (dS). Branch labels represent the synonymous substitution rate; estimated divergence times were obtained from Stein et al., 2018 (45). (C) Genome-wide Micro-C contact maps of rice genomes. Maps are colored according to contact frequencies between bins, with darker red representing more contacts. Lines bounding the 12 chromosomes are shown, with chromosome 1 in the upper left and the numbering proceeding to the right and down, up to chromosome 12.

**Identification of TADs from Azucena Micro-C data**. To gain insight into the nature of TADs in the rice genome, we decided to dissect the genome of the Azucena rice variety and annotate TADs at high resolution. We chose Azucena given that as a tropical *japonica* it may represent the oldest lineage of domesticated rice, and it has been used in multiple functional genomic and systems biology studies (54–56).

We initially employed three widely used tools for TAD annotation [HiCExplorer (6), Arrowhead (47), and HiTAD (57)] to call TADs at three resolutions (1 kb, 2 kb and 5 kb). All tools clearly identified TAD-like structures, although we observed considerable variation in TAD calls between the tools (Table S2). TADs called by HiCExplorer and HiTAD showed better concordance in TAD number and size. We identified 4,650, 6,420, and 3,630 HiCExplorer domains with median TAD size of 52, 48, and 75 kb, while using HiTAD, we found 868, 6,535, and 3,534 domains with median TAD size of 177, 40, and 70 kb at 1 kb, 2 kb and 5 kb resolutions, respectively. We therefore decided to proceed with these two tools. To validate the TAD calls from these tools, we also took advantage of the fact that TAD boundaries are enriched for active chromatin marks and depleted for repressive chromatin marks and DNA methylation in different organisms, including rice (21,23,38,42,58) (see below). Based on these criteria, we concluded that HiCExplorer accurately called TADs at 1, 2, and 5 kb resolution, whereas HiTAD accurately called TADs only at 2 and 5 kb resolution (see Methods).

The discrepancies in the number and locations of TADs identified by different callers in the same sample and same experiment have been extensively reported in the 3D genomics literature ((59,60)). There are three major potential explanations of these discrepancies: different algorithmic approaches, various parameter settings, and the presence of hierarchical TAD structures: 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. HiCExplorer relies on calculating insulation scores for calling TADs, while HiTAD uses a directionality index (DI), and considers hierarchical TADs. The insulation score measures the number of interactions spanning a given genomic region, while DI assesses the shift in the directionality of contacts upstream and downstream of a region. While strong DI values are a signature of a TAD boundary, they can be associated with many regions in the genome (61), and we reasoned that TADs called by both methods would represent the most robust set of domains. We therefore identified 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 (Fig. 2A, B). With these criteria we obtained 2,474 TADs at 2 kb resolution. These TADs had a median size of 44 kb and a median number of 5 genes per TAD, covering 32.3% of the genome (Fig. 2C, E, G). At 5 kb resolution, we identified 1,207 TADs with median size of 65 kb, median number of 7 genes per TAD, covering 23.6% of the genome (Fig. 2D, F, H). 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 (see below), we chose the 5kb resolution dataset for further analysis to increase the power.

In plant and animal genomes, A and B compartments are genomic regions characterized by similar chromosomal features and interaction patterns. A compartments have been shown to contain mainly euchromatin, while B compartments contain mainly heterochromatin (42,62). To characterize the distribution of TADs between euchromatic and heterochromatic regions of the genome, we first identified the corresponding regions by calling compartments. We did that at the 160kb resolution using fanc compartments tool of the FAN-C package (63). Compartment calling was done based on the GC content, where regions with low GC content corresponded to B compartment, and regions with high GC content – to A compartments. We then analyzed the relative distribution of TADs between A and B compartments by using Chi-squared test to compare observed and expected number of TADs in compartments. The results suggested no strong compartmentalization bias for TADs in terms of association with euchromatin and heterochromatin.

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**Figure 2. TAD identification from Azucena Micro-C data.** (A) The approach used to call TADs using the two tools HiCExplorer and HiTAD. A TAD call is arrived at by consensus if the reciprocal overlap between the TADs called by the two methods is > 80%. The TAD body and boundaries identified by HiCExplorer were retained as the consensus. (B) An example of an ~1 Mb region of Azucena chromosome 1 with TADs called by the two tools independently, and with the approach depicted in the previous panel. Position in the region is indicated by the numbers above. The directionality index values, and the insulation scores are indicated. Contact map resolution is 5 kb. (C), (D) Overlap of TADs called with HiCExplorer and HiTAD tools at 2 kb and 5 kb resolutions, respectively. (E), (F) Size distribution of Azucena TADs at 2 kb and 5 kb resolutions, respectively. The median TAD sizes in kb are indicated. (G), (H) Distribution of the number of genes per TAD called at 5 kb and 2 kb resolutions, respectively. The median gene number per TAD is indicated.

**Epigenetic and genetic properties of TADs and TAD boundaries.** We wanted to better characterize genomic features associated with TADs and TAD boundaries in Azucena (Fig. 3A) and created genome-wide profile plots for a set of genetic and epigenetic features (54) for these TAD regions (see Methods) (Fig. 3B). We found that boundaries of TADs called at 5 kb resolution are enriched in the active promoter-associated marks H3K27ac and H3K4me3. These histone marks play similar roles in metazoans and were previously found to be associated with TAD boundaries in different organisms (38,42,64). The repressive epigenetic mark H3K27me3 was found to be neither enriched nor depleted at TAD boundaries, consistent with previous findings in pepper (20) and cotton (64) and in contrast to Drosophila boundaries (14). Despite it being a repressive mark, it is found at the promoters of ‘bivalent’ genes, where both H3K27me3 and H3K4me3 mark the transcription start site and is also observed at the promoter of genes associated with active transcription (65,66). This suggests a complex relationship between boundaries and H3K27me3 enrichment. H3K18ac is a mark associated with enhancers in Drosophila (67). We found H3K18ac to be depleted at boundaries, which seem to be associated with promoters and not enhancers; this is, to our knowledge, the first report of the H3K18ac pattern around plant TAD boundaries. We also find that TAD boundaries are enriched with active transcription signals as measured by precision nuclear run-on and sequencing (PRO-Seq), which maps transcriptionally engaged polymerase activity at base-pair resolution. Finally, we observe a depletion for transposable elements at TAD boundaries (Fig. 3B).

We found that boundaries are associated with higher gene density and lower DNA methylation (Fig. 3B). To quantify the differences in gene coverage, we first classified the genome into three categories – TAD boundary (5 kb segments as above, abbreviated as TADbr), TAD body (TADbody, TAD domains identified above minus the TADbr overlap), and non-TAD body (nonTADbody, genomic regions not identified as a TADbr or a TADbody) (Fig. S2A). We found that TAD boundaries are enriched for protein-coding genes whereas there was no difference between TADbody and nonTADbody (Fig. 3C). This is consistent with previous findings in rice which found TAD boundaries have higher gene density, but contrasts with the claim that TADs are depleted for protein-coding genes (23). This disparity could be due to their measure of gene density, which was defined as the fraction of chromatin annotated as protein-coding genes, which can be confounded by the length of genes or transcript. Indeed, we found a significant number of shorter gene transcripts in TAD boundaries (Fig. 3D).

Gene and genomic GC content was significantly higher within TAD boundaries (Fig. 3E, Fig. S2B); this is not surprising given the high gene density in TAD boundaries and that genes have high GC content compared to intergenic regions (68). We also found that TAD boundaries have lower methylation levels compared to TAD bodies and non-TAD bodies (Fig. 3F). All of this taken together indicates that TAD formation in rice may be linked to high density of short genes with high GC content and low DNA methylation levels.

Lastly, in Drosophila the majority of TAD boundaries identified at high resolution (77%), co-localize with promoters, and those boundaries tend to be flanked by divergently oriented gene promoters (6). We wanted to see if a similar phenomenon exists in the rice genome. We observed that in our set of boundaries identified at 5 kb resolution, 54.5% overlap with promoters, which is more than would be expected by chance (*P* < 2.74 x 10-121, binomial test). However, we did not observe an orientation preference for gene promoters flanking rice boundaries, as observed in Drosophila. We also found through GO term analysis that genes overlapping TAD boundaries are significantly enriched for genes linked to translation, and molecular functions such as RNA binding and ribosome-associated categories (Fig. S3). These are typically highly expressed housekeeping genes, which correlates with the fact that boundaries are enriched with active transcription signatures and have low methylation levels, which is consistent with previous work in rice (23) and other plant species (20,21,44,69–74), as well as fungi (75), insects (6), and vertebrates (76); this suggests common principles governing 3D genome conformation across both plants and animals.

Given the structural and functional role of TADs, it is plausible that their boundaries could be evolutionarily conserved at the sequence level. Indeed, studies show that TAD boundaries are depleted for SNPs in mammals (31) and in plants (20). However, a previous study in rice did not find a decrease in sequence variation at TAD boundaries, but a dip ~5 kb before boundaries was observed (77). We asked whether SNP density is lower at boundaries in our dataset and used publicly available data from the Rice SNP-Seek Database (78) which combines variant data from a large panel of rice varieties. We observe a clear reduction of SNP density at boundaries called at 5 kb resolution (Fig. 3B), suggesting that rice TAD boundaries are depleted for genomic variation. The differences observed between our and previously published data could be due to different TAD annotation methods used [Armatus in previous study (77) and HiTAD/HiCExplorer in our study].

A number of studies provide evidence for selection against structural variations (SVs) at TAD boundaries in different species, including human (31), pepper, tomato (20), soybean (79), and cotton (80) genomes. We used the data from Rice SNP-Seek Database (78) to look at possible SV breakpoint enrichment at TAD boundaries. However, we did not observe differences in the enrichment of structural variant breakpoints around TADs and their boundaries (Fig. 3B).

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**Figure 3. Genetic and epigenetic properties of TADs and TAD boundaries in Azucena.** (A) A representative example of TADs in a 200 kb region on chromosome 1 in Azucena. The panels below show genomic and epigenomic feature distribution. TAD boundaries are highlighted. Genes, promoters and TEs are indicated by thick arrows on top. (B) Distribution of genomic and epigenomic features across TADs called at 5 kb resolution. TADs were linearly transformed to align the panel’s borders. TAD boundaries are marked as ‘0’, and the plots span 20 kb proximal and distal to the boundaries. (C) Gene density, defined as the number of genes within a feature normalized by the length of the feature, (D) transcript length in kilobases (kb), (E) gene GC content, (F) Methylation score normalized by the length of the feature, among three classes of genomic regions: TAD body, 5 kb TAD boundary, and non TAD body (segments of genome not recognized as TAD or boundary). Significance of two-tailed t-test depicted as ns (non-significant), \*(P < 0.05), \*\*(P < 0.01), \*\*\*(P < 0.001), and \*\*\*\*(P < 0.0001). (G) Sequence motifs enriched at rice TAD boundaries, as detected by HOMER and STREME.

(81)

(82)(83)(84)(84)(1,85)

**TADs are gene expression regulatory units.** The idea that TADs represent not only structural, but also functional genomic units, finds support in several observations made in animals. In mammals, histone modifications are often similar within genes of the same TAD (5,47). In addition, genes within the same TADs change their expression during cell differentiation in a similar way (5), and co-regulated genes often are located within a TAD (86). Higher expression correlation between human and mouse orthologs was observed for genes within TADs compared with non-TAD genes (33), and co-expressed gene pairs are significantly enriched within TADs in 12 vertebrate species (76). This co-expression occurs because TADs restrict chromatin interactions between genes and distal regulatory elements. There is conflicting evidence, however, of such gene expression correlation in plants. Nutzmann *et al*. (27) found that genes within four biosynthetic gene clusters in Arabidopsis, maize, tomato and rice were co-expressed, although this study did not include a genome-wide analysis. Indirect evidence for possible plant gene co-expression also comes from a poplar study, where pairs of paralogs located in conserved TADs showed more similar expression levels (44). Only one genome-wide study, however - in maize - has investigated the relationship between gene expression and co-localization within TADs and concluded that genes within TADs were not co-expressed (87).

We tested whether TADs within rice are significantly co-expressed, using expression data from plants grown in greenhouse under control and salinity stress conditions. We focused on identified TAD and non-TAD domains (remaining segments of the genome not identified as a TAD) (Fig. 4A, Table S3). We found that genes within TAD domains show significantly lower levels of variation in expression compared to non-TAD regions (Fig. 4B). In normal greenhouse conditions, the mean coefficient of variation (CV) of gene expression in TADs is 1.69, while in non-TAD regions it is 2.49; in saline conditions the mean CV for TADs = 1.68 and non-TAD regions = 2.46 (two-tailed t-test *P* < 0.0001). This result was also replicated under stress conditions in the greenhouse and field across multiple timepoints, as well as for TAD and non-TAD regions located within euchromatin and heterochromatin (Fig. S4, Fig. S5). To gain further support for our finding, we leveraged the publicly available Azucena root gene expression data measured under normal and aluminum stress conditions (88), and were able to replicate our result (Fig. S6). The lower variance of gene expression within TADs is consistent with the role of TADs in restricting the activity of regulatory elements (41,89), indicating that these inferred rice TADs may indeed represent functional units of gene co-expression.

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**Figure 4. Gene co-expression in TAD domains.** The coefficient of variation (CV) of gene expression in TAD and non-TAD regions are measured using plants in normal and saline conditions in the greenhouse. Three biological replicates were used for each condition. Significance of two-tailed t-test depicted by ns (non-significant), \*(*P* < 0.05), \*\*(*P* < 0.01), \*\*\*(*P* < 0.001), and \*\*\*\*(*P* < 0.0001).

**TAD boundary strength is associated with distinct genetic and epigenetic features**. TAD boundaries may exhibit varying levels of insulation as reflected in their insulation scores. Lower insulation scores indicate reduced contact frequencies between upstream and downstream loci and reflect a strong TAD boundary; these tend to be more conserved during evolution and harbor highly expressed genes, as shown in the recent comparative analysis of TADs in 4 different primates and 4 rodents which indicated that ultraconserved TADs have higher insulation strength (36). Similarly, stronger TAD boundaries have genes with higher expression than weak boundaries in the fungal plant pathogen *Verticillum dahlia* (75).

We classified boundaries based on their insulation scores and examined the relative enrichment of genetic and epigenetic marks at boundaries with low (“strong” boundaries) and high insulation scores (“weak” boundaries). We defined low and high insulation scores as ones falling into the lower and upper quartiles of the score distribution, respectively. We found that stronger boundaries have significantly higher levels of transcription (measured by PRO-seq signals), although the difference is modest. They are also strongly associated with lower DNA methylation and active promoter-associated chromatin marks H3K27ac and H3K4me3. Interestingly, they are also depleted for H3K18ac, a histone mark associated with enhancers in Drosophila, that we showed is overall depleted at rice TAD boundaries. In summary, the strength of rice TAD boundaries was found to be associated with active epigenetic marks and negatively correlated with repressive chromatin. Our observations are corroborated by a recent analysis of Arabidopsis Micro-C data, which showed that the active histone mark H3K4me3 is a positive while repressive mark H3K27me3 is a negative predictor of boundary strength (19), suggesting these features may be generalizable across flowering plants.

In addition, we obtained PhastCons and fitCons (r) scores for the rice genome (54), which are evolution-based measures of potential genomic function. PhastCons scores represent tribe-level interspecies sequence conservation estimates, and fitCons scores provide probabilities that mutations at individual nucleotide sites have fitness consequences (54). We find that stronger boundaries also have higher fitness consequence and PhastCons scores, suggesting purifying selection on these genomic regions. They also had higher gene density, lower SNP density, and lower TE content (Fig. 5). Comparisons of boundaries with random genomic controls showed that weak boundaries share some characteristics with random regions (Fig. S7). Interestingly, we found that stronger boundaries are specifically depleted for *gypsy* retrotransposons (Fig. 5).

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**Figure 5. TAD boundary strength is associated with distinct genetic and epigenetic features.** Box plots show comparison of genetic and epigenetic features for strong (low insulation scores) and weak (high insulation scores) boundaries. Nipponbare TAD boundaries identified at 5 kb resolution. Significance of Wilcoxon rank-sum test depicted by ns (non-significant), \*(P < 0.05), \*\*(P < 0.01), \*\*\*(P < 0.001), and \*\*\*\*(P < 0.0001).

**Global chromatin structure conservation correlates with evolutionary divergence time between genomes**. In order to quantitatively compare the global 3D structure of different rice genomes, we utilized Comparison of Hi-C Experiments using Structural Similarity (CHESS), a computer vision-based algorithm for the comparison of chromatin contact maps (90). CHESS provides a quantitative measure for similarity between a pair of normalized chromatin contact matrices, SSIM, with a value of 1 indicating identity between matrices, while 0 indicates no similarity. CHESS has previously been used to compare global chromatin conformation and extract differential features from *Drosophila melanogaster* wild type and mutant lines (91) or *Fusarium graminearum* genomes under different conditions (92), and determine levels of chromatin conformation conservation between human and mouse (90), different species of fungal pathogens (93), and homologous blocks between X and Y chromosomes in primates (94).

We started by ensuring that the various *Oryza* genomes were sufficiently colinear by aligning individual chromosomes (95) and visualizing alignments as dotplots (Fig. S7). We observed good colinearity between all genome pairs and detected a small number of large SVs more than 500 kb in size (Table S4). Of note, we detected a prominent large inversion on chromosome 6 that has previously been reported in *indica* genotypes (96). We masked large SVs and did a 500 kb sliding window analysis comparing Micro-C submatrices binned at 25 kb resolution across the genome between pairs of genomes; we generated similarity score (SSIM) and signal-to-noise (SN) ratios using CHESS for each pair of colinear genomic windows (Fig. 6A, S7).

We found that colinear genome regions share overall structural similarity between species/subspecies, but the degree of similarity differed between comparisons. To quantify overall similarity between genomes, we calculated the mode of the kernel density estimate (KDE) plot of similarity scores for colinear regions and corrected for random similarity by subtracting the modal value of SSIM for random regions, obtaining genome-wide normalized SSIM values (GN-SSIM). GN-SSIM values were highest for comparisons between biological replicates, and lowest for the interspecies comparisons (Table S5).

Whether 3D genome conservation levels correlate with evolutionary distances between species remains largely unknown. Two Drosophila species separated by ~15 million years of divergence were found to share only 25% of TADs (40), while in another study two Drosophila species separated by ~49 million years were found to share 30-40% of TADs (14). In our study, we examine chromosome conformation divergence between more closely related species/subspecies; the genotypes analyzed have diverged between ~4,000-5,000 years ago (temperate vs. tropical *japonica*) (97) to ~2.4 million years (between *O. sativa* and *O. meridionalis*) (45). To compare structural vs. nucleotide sequence similarity between our Oryza genomes, we calculated pairwise synonymous substitution levels (dS) between coding sequences and found a significantly negative correlation between SSIM and dS (*P* < 0.0371) [Fig. 6B, C]. A neighbor-joining tree using the GN-SSIM values recapitulates the topology of the evolutionary tree of the Oryza species generated using sequence data (Fig. S9), (45). Overall, the degree of global structural similarity was correlated with the degree of sequence similarity between genomes, and this result is in contrast with previous studies. For example, 3D genome conservation was assessed in distantly related *Anopheles* species, and the level of conservation was found to be similar for all pairwise species comparisons and did not correlate with evolutionary distance (92). In another study, chromatin conformation conservation was found to be independent of sequence conservation in fungi (93). Our results may indicate distinct principles of chromatin conformation evolution in plant genomes.

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**Figure 6. Global chromatin structure conservation correlates with evolutionary diversification time between genomes.** (A) Distributions of empirically determined CHESS scores for pairs of colinear (magenta) regions and 100 random permutations of region pairs (grey) for inter-species/subspecies comparisons. (B) 3D sequence similarity correlates with 1D sequence divergence estimates. dS values were calculated from whole genome coding sequences. 95% regression confidence intervals are indicated. (C) Same as (B), but with *O. meridionalis* excluded from pairwise comparisons.

**Genomic blocks with different structural similarity levels have distinct genetic and epigenetic properties**. Our CHESS comparisons show that different genomic windows have different degrees of structural similarity (Fig. 6A), indicating that they may be subject to different evolutionary constraints. This is especially evident in the *O. sativa* ssp*. japonica* (Nipponbare)/*O.meridionalis* comparison results, where we observed a bimodal distribution of SSIM scores (Fig. 7A). To examine the nature of the genomic regions in these two modes of 3D chromosome similarity, we focused on 500 kb windows corresponding to each mode and analyzed the distribution of genetic and epigenetic features between these genomic regions.

We found that structurally-similar genomic regions have higher sequence similarity, lower DNA methylation, higher gene density, higher H3K27ac and H3K4me3 content and lower H3K27me3 content (Fig. 7B). We also observe that these high-similarity genomic regions have higher FitCons and PhastCons scores, lower SNP density and lower TE content (specifically *gypsy*, *copia* and SINE elements) (Fig. S10). We then performed similar analysis on genomic windows from the lower and upper tails of the SSIM distribution (Fig. 7C) and found that 3D-similar regions have higher sequence similarity and lower DNA methylation levels (Fig. 7D). We found a similar pattern when we analyzed the lower and upper tails of the *O. sativa* ssp*. japonica* (Nipponbare)/*O. rufipogon* SSIM distributions (Fig. 7E, F). Taken together, our results show that genomic regions with conserved 3D structure across species are characterized by higher DNA sequence similarity, enriched in active gene-rich chromatin and depleted for repressive marks.

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**Figure 7. Genomic blocks with different structural similarity levels have distinct genetic and epigenetic properties**. (A) Distribution of empirically determined CHESS scores for the Nipponbare – *O. meridionalis* comparison with the regions around each mode are highlighted. (C) Distribution of empirically determined CHESS scores for the Nipponbare – *O. meridionalis* comparison with the upper and lower tails of the distribution highlighted. The first and third quartile values are indicated. (E) Distribution of empirically determined CHESS scores for the Nipponbare – *O. rufipogon* comparison with the tails of the distribution highlighted. The first and third quartile values are indicated. (B), (D), (F) Comparison of genetic and epigenetic features for genomic windows corresponding to the highlighted areas on the distribution plots. Significance of Wilcoxon rank-sum test depicted by ns (non-significant), \*(P < 0.05), \*\*(P < 0.01), \*\*\*(P < 0.001), and \*\*\*\*(P < 0.0001).

**Conserved TADs are gene-rich, actively transcribed regions**. Our evolutionary analysis thus far focused on global 3D genome comparisons. We decided to look specifically at individual TADs and investigate their evolution within the five genomes. We employed two approaches: a widely used liftover-based (38,40,76,98), and a BLAST-based approach (see Methods) [Fig. S11A, S12]. In the first approach, we identified sets of high-confidence TADs, which resulted in the identification of TADs for Azucena (1,187 TADs), Nipponbare (1,425 TADs), IR64 (1,352 TADs), *O. rufipogon* (1,112 TADs)and *O. meridionalis* (1,072 TADs) at 5 kb resolution. We considered TADs to be syntenic if the coordinates of the TADs in the other genomes reciprocally overlapped at least 50% with the Nipponbare TAD; this part of the analysis used Nipponbare as the anchor reference genome given its high level of annotation. After performing pairwise comparisons, we assigned each TAD to five conservation group based on how many genomes the TAD was found in. “Unique” represents Nipponbare-specific TADs, “Rare” encompasses TADs found in two genomes, "Moderately Conserved” – in three genomes, “Highly Conserved” – in four genomes, and “Core”– TADs found in all five genomes. (Table S6). We analyzed TAD size, number of genes per TAD, mean gene expression, GC content, SNP/SV density, but found no significant differences between conservation groups. However, conserved TADs have significantly higher gene density and lower TE content (Fig. S11B).

We complemented this analysis by implementing a BLAST-based approach (Fig. S12). Briefly, we aligned Nipponbare TAD sequences to a database of Azucena TAD sequences, recording all hits per TAD and the total coverage per Nipponbare TAD. If the total coverage of the orthologous TAD was ≥50% of the reference TAD, it was recorded as conserved. We performed this for 4 pairs of genomes, using Nipponbare as the reference, and assigned Nipponbare TADs to 5 conservation groups following the logic previously described (Fig. 8A, Table S6).

Using this approach, we found that more conserved TADs have higher gene content, lower TE content, and are enriched for the active chromatin mark H3K4me3 (Fig. 8B). In agreement with the elevated gene content, conserved TADs also exhibit higher sequence conservation scores (Fig. 8B, S12). Conserved TADs have been found to be enriched for genes and active chromatin marks in Drosophila (99) and legumes (21). These and our findings highlight the general nature of 3D genome organization and TAD functionality across taxa.

Of note, in *O. sativa* inter-subspecies comparisons, only 38.9% of TADs were found to be conserved between Nipponbare and Azucena genomes, and 38.1% of TADs were conserved between Nipponbare and IR64. Thus, rice 3D genome organization shows low levels of intraspecific TAD conservation, similar to the 36% reported for two maize inbred lines (73); this suggests that plant TADs may be dynamic fast evolving structures. We note that some conserved TADs that have contracted/expanded in size have their boundaries coincident with structural variants (Fig. 8C), implicating the role of SVs in rice TAD reorganization and possibly generating new gene expression patterns.

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**Figure 8. Rice TAD conservation**. (A) Nipponbare TADs assignment to five conservation groups using BLAST-based approach. (B) Comparison of gene and transposable element (TE) density, H3K4me3 mark and fitcons scores for TAD conservation groups. The Wilcoxon rank-sum tests were performed for all pairwise comparisons between groups. Compact letters represent groups whose distributions of features were not significantly different from each other. (C) Structural variants affect boundary rearrangements between *O. sativa* and *O. rufipogon*. Contact matrices with TADs (triangles) were plotted over a 400 kb section of chromosome 1 of *O. sativa* Nipponbare variety (top) and *O. rufipogon* (bottom). Conserved TADs are connected. Orthologous genes are in green, non-orthologous genes are in yellow. Matrix resolution is 5 kb.

**Boundaries of conserved TADs are actively transcribed sequences enriched for sequence conservation signatures**. Previous studies in vertebrates revealed that genetic and epigenetic properties of TAD boundaries vary depending on their level of sequence conservation. Conserved boundaries were found to have stronger insulation strength, enrichment of older TEs and higher gene densities (36,76). There is some indication of similar behavior of plant TAD boundaries; for example, cultivar-specific TAD boundaries in cotton harbor more TEs than conserved boundaries (71,80). To investigate whether similar trends can be observed for rice, we grouped TAD boundaries identified in Nipponbare based on whether they are conserved in other species/subspecies - a ‘conserved’ group where boundaries of TADs shared between at least 4 of our study genomes, and a ‘non-conserved’ group that are Nipponbare-specific.

We found that conserved boundaries had lower DNA methylation levels and H3K27me3 levels, but higher levels of active histone marks H3K27ac and H3K4me3.They also had higher gene content, lower TE content (specifically, DNA transposons and *gypsy* elements), higher GC content, and higher gene expression levels as measured by RNA-seq (55), indicating that conserved TAD boundaries are transcriptionally active gene-dense regions (Fig. 9, Fig. S14). We also found that conserved TAD boundaries have higher FitCons and PhastCons values and are characterized by low SNP and SV breakpoint density (Fig. 9), suggesting the action of purifying selection. Overall, boundaries conserved within rice genomes are gene-rich, active transcription regions with low DNA sequence variation, similar to what has been observed in cotton (70), human and mouse boundaries (36).

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**Figure 9. Boundaries of conserved TADs are actively transcribed sequences enriched for sequence conservation signatures**. Box plots show comparison of genetic and epigenetic features for conserved (yellow) and non-conserved (blue) Nipponbare TAD boundaries identified at 5 kb resolution. Significance of Wilcoxon rank-sum test depicted by ns (non-significant), \*(P < 0.05), \*\*(P < 0.01), \*\*\*(P < 0.001), and \*\*\*\* (P < 0.0001).

**The nature of rice TADs and their evolution**. The nature of three-dimensional genome structure has been of increasing interest in recent years, as higher-order structures of the genome associated with functional features have been defined (4,38,86). A key 3D feature of genomes are TADs, which have been shown to represent discrete genomic units that appear to serve as regulatory domains that allow for some level of co-regulated gene expression, at least in mammals (4,5,7) and Drosophila (6).

The nature of plant TADs, however, is less well understood, and indeed their functional significance in plants have been disputed (27,44,87). Our analysis of TADs and their boundaries in rice provide evidence that these genomic elements share features with functional TADs in metazoan systems, including high transcriptional activity, enrichment for active histone marks, low methylation levels, low TE content and increased gene densities at TAD boundaries. Moreover, we find significant correlation of . levels for genes within these rice TADs, suggesting that they indeed function as genomic domains with shared regulatory features. Our findings emphasize that animal and plant TADs may share more commonalities than initially thought, as evidenced by similar genetic and epigenetic signatures associated with TADs and their boundaries.

Conservation of 3D genome topology is a highly contested topic in the field of comparative genomics (39), and different computational approaches for TAD calling and arbitrary definitions of conservation status has resulted in a lack of consensus of whether 3D genome is conserved between species. Nevertheless, a prevailingview withincomparative 3D genomics is that TADs are highly conserved across species, although this has been challenged and has led to skepticism about the functional and evolutionary importance of TADs (39). To address this problem, we quantitatively assessed global 3D genome topology conservation and diversity, as well as specific dissection of TADs and TAD boundaries. We showed that, on a global scale, chromosome topology between Oryza genomes that have diverged between ~4,000 to 2.4 million years ago is largely conserved, and that 3D structural divergence is observed that correlates with evolutionary distance between genomes. However, we also show that individual TADs display low levels of conservation, even between subspecies, supporting previous findings made in other plant species such as foxtail millet, sorghum, legumes and Arabidopsis (21,42,71). We also demonstrate that different TAD groups have different levels of conservation, with conserved TADs being gene-dense, enriched for active chromatin marks, and depleted for transposable elements. We should note that we restricted evolutionary analysis to high-confidence TADs, identified with two callers independently, to ensure that the comparisons are based on the most reliable data. This approach helps ensure that observed differences reflect true evolutionary changes rather than artifacts of data noise or algorithmic variability.

How to explain this apparent discrepancy between TADs being functional units and yet generally not conserved between closely related species? One explanation may stem from the phenomenon of conserved functional neighborhoods with changing genes (100). It was shown that the chromosomes of higher eukaryotes, including plants, contain genes arranged in functional neighborhoods with gene co-expression, similar to genes arranged into clusters within a TAD (100). The function of a cluster is constrained within a neighborhood, and if a chromosomal rearrangement breaks a neighborhood, selective pressure will lead to the formation of another neighborhood with similar function through additional chromosomal rearrangements (100). Similar mechanisms could explain the seeming instability of TADs in evolution. In addition, high levels of presence/absence variation known for plant genomes, including rice, could also contribute to the highly dynamic nature of the TAD genomic landscape. Future studies can indeed examine the evolutionary mechanisms underlying the divergence of TADs in eukaryotic genomes and their functional consequences.

**METHODS**

**Plant materials and growth conditions for Micro-C.** Seeds of *O. sativa* landraces Nipponbare (IRGC 12731, temperate japonica), Azucena (IRGC 328, tropical japonica), and IR64 (IRGC 66970, indica) were provided by the International Rice Research Institute (Los Baños, Philippines). Seeds of *O. rufipogon* (W1943) and *O. meridionalis* (W2112) were provided by the National Institute of Genetics (Mishima, Japan). Seeds were incubated for 5 d at 50° C and germinated in water in the dark for 48 h at 30° C. These were subsequently sown on hydroponic pots suspended in 1× Peters solution and 1.8 mM FeSO4 (pH = 5.1–5.8) (JR Peters). Plants were grown in growth chambers (12-h days; 30°C/20°C day/night; 300–500 μmol quanta m−2 s−1; relative humidity: 50–70%). Leaf tissue was collected from 14-day-old plants.

**Micro-C library preparation and sequencing.** For each species/variety, we generated two replicate Micro-C datasets (Table S1). The Micro-C libraries were prepared using the Dovetail Micro-C Kit for animal tissue, which we adapted for plant tissues. In brief, 50 mg of frozen leaf tissue were ground to a fine powder in liquid nitrogen. Ground tissue was fixed with disuccinimidyl glutarate and formaldehyde. The cross-linked chromatin was then digested using micrococcal nuclease (MNase) until an optimal digestion profile of 40-70% mononucleosomes was achieved. The sample was then lysed with SDS, and the chromatin ends were repaired and ligated to a biotinylated bridge adapter followed by proximity ligation. The cross-links were then reversed, the proteins were degraded, and the DNA was purified and ligated with Illumina-compatible adaptors. Biotinylated DNA was pulled down on streptavidin beads and then PCR amplified. Two biological replicates were used. The libraries were sequenced on the Illumina NextSeq 500 platform with 150-bp paired-end reads at the NYU CGSB Genomics Core facility.

**Micro-C data analysis.** Chromatin contact maps were generated using the Dovetail Genomics pipeline (<https://micro-c.readthedocs.io/en/latest/index.html>). Briefly, sequenced read pairs were mapped to the reference genomes (see below) using BWA-MEM v.0.7.17 (101), then low quality reads (MAPQ<40) and PCR duplicates were removed by pairtools v.1.0.2 (102). Hic and mcool files (that included multiple bin sizes) were generated by juicer v.1.6 (103) and cooler v.0.9.0 (104), respectively. Hic files were normalized by KR method in Juicebox v.2.20.00 (105), and cooler balance was applied to normalize mcool files. CoolBox v.0.3.8 (49) was used for plotting the contact maps at 5kb resolution together with genetic/epigenetic tracks and for plotting the distance-dependent decay of chromatin contacts. We used HiCRep (46) to calculate the stratum-adjusted correlation coefficient (SCC) at 5 kb resolution with the following parameters: h=30, lbr=0, ubr=1000000. We calculated SCC values between biological replicates for each chromosome of each species, then averaged the values for all chromosomes. The average SCC values ranged from 0.76 to 0.88. HiCRes (48) was used to estimate the maximum contact map resolution.

**Compartment calling.** We called A/B compartments by implementing fanc compartments tool from the FAN-C package (63). The fanc compartments command produces a correlation matrix from a contact matrix file binned at 160kb resolution. Compartment matrices are calculated on a per-chromosome basis. Then, the eigenvector of the correlation matrix was used to make compartment calls. The average GC content of regions with positive and those with negative eigenvector entries was used to determine the compartment type of genomic regions. As GC content has previously been shown to correlate well with compartmentalisation, the eigenvector was oriented such that negative entries correspond to B (low GC content) and positive entries to A (high GC content) compartments. TAD and non-TAD regions were assigned to A/B compartments if more than 50% of the region fell within the respective compartment.

**TAD annotation.** We compared performance of three tools for TAD identification: hicFindTADs from HiCExplorer v.3.7.2 package (6), HiTAD from TADLib (57), and Arrowhead from the juicer package (103). We called TADs in the Azucena genome at three resolutions: 1 kb, 2 kb, and 5 kb. HiTAD detects hierarchical TADs, including TADs, sub-TADs and smaller domains. For our analysis, we used only TADs level 0. The number of TADs called by each tool can be found in Table S2, and we noted that they were similar for HiCExplorer and HiTAD. We then generated metagene plots for the repressive (DNA methylation, H3K27me3) and active (H3K4me3, H3K27ac) marks using deepTools v.3.5.2 (106). We expected TAD boundaries to be enriched for active chromatin marks and depleted for repressive marks. Based on the distribution of signal (Fig. S15), we concluded that TADs were most accurately called by HiCExplorer at 1, 2, and 5 kb resolutions, and by HiTAD at 2 and 5 kb resolutions. We then identified uniform set of TADs called by both HiCExplorer and HiTAD tools at 2 kb and 5 kb resolutions, with the criteria that the TAD body must reciprocally overlap by at least 80% between both tools. The TAD body and boundaries identified by HiCExplorer were retained as the consensus. TAD boundaries were defined as 2 kb and 5kb genome fragments identified by hicFindTADs from HiCExplorer. The same approach for calling TADs and TAD boundaries was applied to the remaining Oryza genomes, except that only the 5kb resolution was considered.

**Genetic and epigenetic features of TADs and boundaries.** We used *pybedtools* v.0.9.1, Python wrapper for BEDTools (107) to analyze enrichment of genetic and epigenetic features between TADs/nonTADs/TAD boundaries and TAD conservation groups. We performed Wilcoxon rank sum tests for all pairwise comparisons between the groups and corrected the resulting *p* values using the Benjamini–Hochberg procedure.Metagene plots of the distribution of features across TADs were generated with deepTools (106).

**Repeat masking of genomes.** We performed two rounds of repeat masking of the five genomes used with RepeatMasker v.4.1.2 ((<https://www.repeatmasker.org/>), using default repeat libraries and the Oryza Repeat Database from Rice Genome Annotation Project ([http://rice.uga.edu](http://rice.uga.edu/)), which resulted in about 43% of genomes masked.

**Identification of conserved TADs.** We used two approaches to identify conserved TADs. For liftOver-based approach, we first generated chain files for pairs of repeat-masked genomes using custom scripts based on UCSC pipeline (<http://genomewiki.ucsc.edu/index.php/LiftOver_Howto>). We then lifted over the genomic coordinates of TADs from the query to the target (Nipponbare) genome with UCSC liftOver tool (108). To be successfully lifted over, TADs in one genome require a 20% minimum ratio of bases (−minMatch= 0.20) to be remapped in the other genome. Lifted-over TADs from the query genome that reciprocally overlapped a TAD in the target genome by at least 50% were identified with BEDTools (109) *intersect* (-r 0.5) and recorded as conserved. We performed this for 4 pairs of genomes and assigned Nipponbare TADs to 5 conservation groups.

For BLAST-based approach, we first generated a database of TAD sequences with BEDTools *getfasta*. Then, the database of the query species was aligned to the target species database using BLASTn (blast+ v.2.13.0) (110) , and the results were filtered with custom Python code so that only TADs on the same chromosome in query and target remained, and all hits per TAD and the total coverage was recorded. If the total coverage of the target species’ TAD was ≥50% of the query species’ TAD, it was recorded as conserved. We performed this for 4 pairs of genomes and assigned Nipponbare TADs to 5 conservation groups.

**Boundary motif enrichment.** TAD boundaries identified at resolutions 2 kb and 5 kb were used to identify motifs enriched at boundaries.We used HOMER v.4.11 (82) with two sets of parameters (*-len 10 -size given* and *-len 8,10,12 -size 200*). We also used STREME from the MEME Suite v.5.3.0 (83) with default parameters. For background, randomly chosen non-boundary sequences were used. The motifs found to be significantly enriched by both tools in both sets of boundaries were reported.

**GO enrichment.** We analyzed whether genes at TAD boundaries are enriched for specific functional categories using the enrichGO function in clusterProfiler v.4.0 (111). We ran clusterProfiler for all three ontologies: biological process, molecular function, and cellular component.

**TE annotation.** The IRGSP rice6.9.5.liban TE library (112) was complemented with additional, high-confidence TE consensuses from wild rice genome (*Oryza rufipogon*). In order to do this, EDTA was run on *O. rufipogon* GCA\_000817225.1 assembly, retaining only TE consensuses without homology to rice6.9.5.liban library. These novel sequences were further filtered to avoid false positives, by retaining only those longer than 200 bp and carrying a conserved TE domain (as identified by TEsorter (113)) or containing more than three full-length copies in the genome (more than 90% of consensus length). The combined library was used to annotate IRGSP and Azucena genome assemblies using RepeatMasker v.4.1.2 (<https://www.repeatmasker.org/>).

**RNA-seq analysis.** We grew three replicates of Azucena in Yoshida culture solution (based on (114)) in the Lloyd T. Evans Plant Growth Facility (PGF) of the International Rice Research Institute, Los Baños, Philippines greenhouse under normal (0 dS/m-2 equivalent to 0mM NaCl) and saline (10 dS/m-2 equivalent to 100mM NaCl solution) conditions. Tissue samples were collected from the third leaf at specific time points and immediately soaked in RNAlater™ Stabilization Solution (Invitrogen). Total RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Cat No. 74904). Contaminating DNA was removed from the total RNA samples by treatment with RNase-free DNase (Qiagen, Cat No. 79254). RNA quality was determined by gel electrophoresis. Strand-specific RNA-seq libraries were synthesized using the NEBNext® Ultra™ II Directional RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA). The libraries were sequenced at nine libraries per lane using standard methods for paired-end 51 base pair reads on an Illumina HiSeq 2500 at the NYU CGSB Genomics Core facility. Demultiplexed reads were aligned to transcripts from the *Oryza sativa* Japonica assembly IRGSP-1.0 (GCA\_001433935.1). and counted with kallisto (v0.46.0) and then processed into a gene count matrix. The read counts obtained were normalized using the TMM (trimmed mean of M values) method with edgeR v4.2.1 (115), and then averaged over the replicates for each timepoint per environment. Transcripts that were expressed (read count > 0) in at least eight of the ten samples were chosen for downstream analyses, leading to a total of 34,716 expressed transcripts.

**Co-expression analysis.** We estimated the coefficient of variation, defined as the standard deviation (sd) by mean (CV = sd/mean), as a measure of gene co-expression. For this we chose TAD and nonTAD domains with at least five genes expressed, giving us a total of 1,247 domains (678 TAD domains and 569 nonTAD domains). Here, we focus on the results from normal conditions timepoint 1 (0 mins) in the main manuscript and attach the results from all other timepoints and conditions in the supplementary. Additionally, we estimated the CV for TAD and nonTAD domains using previously published leaf tissue drought and salinity stress data from the field [(55) and Gupta et al., under review)], and aluminum stress data for roots (88).

**Genome alignments.** The following reference genomes were used: *O.sativa* Nipponbare (IRGSP-1.0), *O. sativa* Azucena (PRJNA424001), *O. sativa* IR64 (PRJNA509165), *O. rufipogon* (PRJEB4137), *O. meridioinalis* (PRJNA48433). To make sure the genomes are colinear, we aligned the individual chromosomes pairwise using the nucmer utility (with parameters --mum -l 100 -c 1000 -d 50) from the MUMmer4 software package v.4.0.0 (95) and visualized the alignments as dotplots using mummerplot -postscript command (Fig. S7). We observed good collinearity between all pairs of comparisons and detected a small number of large SVs (defined as more than 500 kb in size). To extract the coordinates of large SVs (Table S4), we first filtered the alignments with delta-filter utility of MUMmer (-m -i 90 -l 100), then applied show-coords utility and custom scripts.

**Detection of structural variants.** We used MUM&Co v3.8 (116) to detect insertions, deletions and duplications in fragments of chromosomes between genomes. The three classes of SVs from output tsv files were converted into bed files using custom Python code and visualized in CoolBox (49).

**Identification of colinear genomic blocks.** To generate pairs of genomic windows for CHESS analysis, we first masked the identified large SVs from the genomes. We then split one of the genomes in the pair into 500 kb fragments with a step size of 250 kb. Next, we lifted over the coordinates of the start and end of the blocks using UCSC *liftOVer* utility, generating bedpe files with pairs of colinear genomic blocks.

**Comparison of colinear genomic blocks.** We used CHESS v.0.3.8 (90) to compare global genome conformation between species/varieties. We compared Micro-C submatrices binned at 25 kb resolution across the genome between pairs of genomes to generate a similarity score (SSIM) and signal-to-noise (SN) ratio for each pair of colinear genomic windows. We then filtered out the genomic windows with SN < 0.5 and plotted the distribution of SSIM for resulting windows together with the distributions of SSIM for 100 random permutations of region pairs. Using this approach, we compared all combinations of five genomes (10 comparisons). For control, we compared the Micro-C submatrices of biological replicates for each accession (Fig. S8). To quantify the overall similarity between genomes, we calculated the mode of the KDE plot of the similarity scores for colinear regions and subtracted from that the mode of the KDE plot of SSIM for random regions, obtaining a genome-wide normalized SSIM value (GN-SSIM). All GN-SSIM values for the interspecies/varieties comparisons were significantly different from the biological replicates’ GN-SSIM values (t-test).

We compared the enrichment for genetic and epigenetic features between groups of genomic windows with *pybedtools* (107). To calculate sequence similarity between pairs of windows, we first extracted the sequences of 500 kb windows in FASTA format using Biopython (<https://github.com/biopython/biopython>), aligned them pairwise with EMBOSS Stretcher (117) and recorded the sequence identities using custom code. To control for possible biases introduced by the different mappability of the genomic windows, we calculated mappability scores using GenMap (with parameters -K 30 -E 2) (118), which computes the uniqueness of k-mers for each position in the genome. We compared the mappability scores for the groups of genomic windows analyzed and found that they were not significantly different. To compare the level of structural similarity with the level of sequence similarity between genomes, we calculated synonymous substitution levels (dS) between coding sequences using orthologr v.0.4.2 package (119).

**DATA ANALYSIS TOOLS**

HOMER v.4.11

STREME v.5.3.0

BWA-MEM v.0.7.17

pairtools v.1.0.2

GenMap

HicRes

HicRep

Orthologr v.0.4.2

DeepTools v.3.5.2

MUMmer4

MUM&Co v.3.8

CoolBox v.0.3.8

HiCExplorer v.3.7.2

HiTAD

Arrowhead

juicer v.1.6

Juicebox v.2.20.00

cooler v.0.9.0

Pybedtools v.0.9.1

BEDTools v.2.30.00

EMBOSS Stretcher

Biopython

blast+ v.2.13.0

UCSC liftOver

RepeatMasker v4.1.2

edgeR v.4.2.1

FAN-C v.0.9.1

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**SUPPORTING INFORMATION**

**Additional file 1: Figure S1. Chromatin contact probabilities as a function of genomic distance.**

**Additional file 2: Figure S2. Genome GC content by TAD features.** (A) Partition of the genome into three classes of genomic regions. Triangles on top depict the extent of the TAD, while bidirectional arrows at the bottom show the different classes of TAD features. (B) TAD boundaries (TADbr) have the highest GC content followed by nonTAD bodies (nonTADbody) and then TAD bodies (TADbody). Significance of two-tailed t-test depicted by ns (non-significant), \* (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), and \*\*\*\* (P < 0.0001).

**Additional file 3: Figure S3. Gene ontology enrichment analysis for genes at Azucena TAD boundaries identified at 5 kb resolution**. CC – Cellular component, MF – molecular function, BP – biological process.

**Additional file 4: Figure S4. Gene co-expression in TAD domains**. Shown here is the coefficient of variation (CV) measured using plants in the normal (A) and saline (B) in the greenhouse at multiple timepoints (60 mins, 180 mins, 240 mins, and 5 days), and in the field exposed to drought stress (C) and salinity stress (D). Significance of two-tailed t-test depicted by ns (non-significant), \* (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), and \*\*\*\* (P < 0.0001).

**Additional file 5: Figure S5. Gene co-expression in TAD domains within A and B compartments**. Shown here is the coefficient of variation (CV) measured using plants in the normal (A, C) and saline (B, D) in the greenhouse at multiple timepoints (60 mins, 180 mins, 240 mins, and 5 days). TAD and non-TAD regions were assigned to A/B compartments if more than 50% of the region falls within a respective compartment. Significance of two-tailed t-test depicted by ns (non-significant), \* (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), and \*\*\*\* (P < 0.0001).

**Additional file 6: Figure S6. Gene co-expression in TAD domains in root tissue**. Shown here is the coefficient of variation (CV) measured using plants in the normal and aluminum stress conditions. Significance of two-tailed t-test depicted by ns (non-significant), \* (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), and \*\*\*\* (P < 0.0001).

**Additional file7: Figure S7. Comparison of strong and weak TAD boundaries with random genomic regions.** Box plots show comparison of genetic and epigenetic features for strong (low insulation scores) and weak (high insulation scores) boundaries, as well as random 5kb genomic regions. Significance of Wilcoxon rank-sum test depicted by ns (non-significant), \*(P < 0.05), \*\*(P < 0.01), \*\*\*(P < 0.001), and \*\*\*\*(P < 0.0001).

**Additional file 8: Figure S8. Genome collinearity within Oryza visualized using MUMmer.** The nucmer utility with parameters --mum -l 100 -c 1000 -d 50 was used to align individual chromosomes pairwise.

**Additional file 9: Figure S9. Distributions of empirically determined CHESS scores.** Plots are for indicated pairs of colinear (magenta) regions and 100 random permutations of region pairs (grey) for comparisons of biological replicates.

**Additional file 10: Figure S10. Neighbor-joining tree constructed using the GN-SSIM values recapitulates the topology of the evolutionary tree of the Oryza species**. (A) Calculation of GN-SSIM value from the distribution of empirically determined CHESS scores for pairs of colinear (magenta) regions and 100 random permutations of region pairs (grey). (B) Rate of global chromatin structure evolution. GN-SSIM values were used to create a distance matrix to construct the neighbor-joining tree. (C) Evolutionary tree of the Oryza species constructed using genome-wide median dS values. dS values were computed for all coding sequences with orthologr. Median dS values were calculated for all genomes pairwise and then used to create a distance matrix.

**Additional file 11: Figure S11. Comparison of genetic and epigenetic features for genomic windows corresponding to the two modes of the KDE plot of SSIM values for the Nipponbare-*O. meridionalis* comparison**. Structurally-similar genomic regions have higher fitcons and PhastCons scores, lower SNP density and lower TE content (specifically *gypsy, copia* and SINE elements). Significance of Wilcoxon rank-sum test depicted by ns (non-significant), \* (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), and \*\*\*\* (P < 0.0001).

**Additional file 12: Figure S12. Detecting conserved TADs using liftover-based approach.** (A) Schematic representation of the liftover-based conserved TADs identification method. (B) Conserved TADs have higher gene coverage and lower TE content.

**Additional file 13: Figure S13. TAD conservation analysis with BLAST: detecting conserved TADs with SVs.** (A) Schematic representation of the BLAST-based approach to identify conserved TADs. (B) Distribution of conserved TAD coverages per genome pair analyzed. (C) Distribution of conserved TAD coverages per genome pair analyzed, but only the coverages above 50% plotted.

**Additional file 14: Figure S14. Comparison of PhastCons scores for TAD conservation groups.** Group 5 represents TADs with orthologs in all 5 genomes, group 1 represents Nipponbare-specific TADs. The Wilcoxon rank-sum tests were performed for all pairwise comparisons between groups. Compact letters represent groups whose distributions of features were not significantly different from each other.

**Additional file 15: Figure S15. Conserved TAD boundaries have lower density of *gypsy* elements and DNA transposons.** Shown here are comparisons of TE density for strong (low insulation scores, yellow) and weak (high insulation scores, blue) Nipponbare TAD boundaries identified at 5kb resolution. Significance of Wilcoxon rank-sum test depicted by ns (non-significant), \* (P < 0.05), \*\* (P < 0.01), \*\*\* (P < 0.001), and \*\*\*\* (P < 0.0001).

**Additional file 16: Figure S16. The distribution of epigenomic features across TADs called with different tools at different resolutions.** TADs were linearly transformed to align the panel’s borders. Boundaries were marked as ‘0’, and the plots span 20 kb proximal and distal to the boundaries.

**Additional file 17: Table S1.** Micro-C libraries and contact map statistics. **Table S2.** Number of TADs identified with different tools in Azucena. **Table S3.** Properties of spatial chromatin features. **Table S4.** Large (> 500 kb) structural variants detected with mummer4. **Table S5.** GN-SSIM scores. **Table S6.** Number of TADs in conservation groups.

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**DATA AVAILABILITY**

The Micro-C and RNA-seq data have been deposited to NCBI SRA under the accession numbers PRJNA1121389 and PRJNA1198503, respectively.

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