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Population genomic analysis unravels the evolutionary roadmap of pericarp color in rice

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ABSTRACT

Pigmented rice stands out for its nutritional value and is gaining more and more attention. Wild rice, domesticated red rice, and weedy rice all have a red pericarp and a comprehensive genetic background in terms of the red-pericarp phenotype. We performed population genetic analyses using 5104 worldwide rice accessions, including 2794 accessions with red or black pericarps, 85 of which were newly sequenced in this study. The results suggested an evolutionary trajectory of red landraces originating from wild rice, and the split times of cultivated red and white rice populations were estimated to be within the past 3500 years. Cultivated red rice was found to feralize to weedy rice, and weedy rice could be further re-domesticated to cultivated red rice. A genome-wide association study based on the 2794 accessions with pigmented pericarps revealed several new candidate genes associated with the red-pericarp trait for further functional characterization. Our results provide genomic evidence for the origin of pigmented rice and a valuable genomic resource for genetic investigation and breeding of pigmented rice.

Key words: red rice, population genomics, pericarp color, re-domestication, divergence time

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INTRODUCTION

Rice has been domesticated for thousands of years and is now grown worldwide as a staple food. Rice produces grains with red, brown, purple, or black pericarps. Pigmented rice, which is rich in phenolic compounds like anthocyanins and proanthocyanidins, plays a role in biological functions such as inhibition of oxidative stress, alleviation of hyperlipidemia, and prevention of obesity (Chu et al., 2019; Yu et al., 2021). With the improvement in societal living standards, growing attention has gradually been focused on red rice because of its benefits to human health.

Red pigmentation of rice grains is attributed to two domestication-related genes, *Rc* and *Rd* (Sweeney et al., 2006; Sweeney et al., 2007). Multiple alleles of the *Rc* gene, which encodes a bHLH protein, have been reported. The recessive *rc* allele contains a 14-bp deletion in exon 6 of *Rc* and is a domesticated loss-of-function allele of *Rc* (Sweeney et al., 2006). *Rc*^{r} and *Rc-g* contain single G-base deletions in distinct locations, both occurring upstream of the 14-bp deletion in the *rc* allele, which restore the reading frame of the gene and thus contribute to the red-pericarp phenotype (Brooks et al., 2008; Lee et al., 2009). The *Rc-s* allele is characterized by a C-to-A transversion within the *Rc* gene, which results in premature termination of the Rc protein and a white pericarp (Sweeney et al., 2007; Furukawa et al., 2007). *Rd* encodes dihydroflavonol-4-reductase, which catalyzes the conversion of dihydroflavonols into leucoanthocyanidins. *Rd* by itself does not generate any pigment, but it regulates accumulation of proanthocyanidins in the pericarp (Sweeney et al., 2006; Furukawa et al., 2007). The *Rc-rd* genotype results in brown rice grains, whereas both *rc-rd* and *rc-Rd* genotypes produce white rice grains (Furukawa et al., 2007).

The evolutionary trajectory of rice has attracted significant attention in the last 10 years (e.g., Huang et al., 2012b; Civáň et al., 2015; Choi et al., 2017; Jing et al., 2023; Wu et al., 2023). Specific efforts have been undertaken to unravel the

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domestication trajectory of red rice (Sweeney et al., 2007; Gross and Olsen, 2010). It has been demonstrated that the 14-bp deletion in *Rc* arose from *Geng-japonica* (GJ) rice and was later introgressed into *Xian-indica* (XI) and *aus* subpopulations. On the other hand, the C-A mutation (*Rc-s*) originated in the *aus* group and did not propagate widely during the domestication process (Sweeney et al., 2007). In the USA, the term ''red rice'' is also used to describe weedy rice, which is found in rice fields worldwide (Gross et al., 2010; Thurber et al., 2010; Wu et al., 2022). The major-effect loci (*qSD7-1* and *qPC7*) that overlap with the *Rc* locus in weedy rice have been studied in several population genomic studies (Li et al., 2017; Qiu et al., 2017). Reversion of *Rc* to a functional state in weedy rice has been attributed to spontaneous back mutations (Sweeney et al., 2007; Cui et al., 2016; Sun et al., 2018), gene flow from wild rice (Song et al., 2014; Wedger et al., 2019; Wu et al., 2023), or standing variation from older landraces that lack the domestication allele (Qiu et al., 2020; Wu et al., 2022).

It is noteworthy that black grain color is not observed in wild rice (*O. rufipogon*), and nearly all wild rice exhibits a red pericarp (Sweeney et al., 2007). Wild plants underwent a pre-domestication cultivation phase, for which certain domestication traits are documented in archaeobotanical records, reflecting varying levels of domestication (Gross and Olsen, 2010). During the initial stage of rice domestication, farmers paid more attention to reducing seed shattering and increasing seed size (Purugganan and Fuller, 2009). Attention shifted toward enhancement and diversification of other traits, including selection for grain color (Gross and Olsen, 2010). Notably, functional changes in genes like *rc* (loss of function) and *Kala4* (gain of function) occurred within a relatively short time frame (Oikawa et al., 2015). The specifics of how, when, and where red and white rice were established during the long domestication process remain elusive.

Although red rice has received less attention owing to shifts in dietary preferences (Ahuja et al., 2007), its significance as a crucial link between the pre- and post-domestication periods is indisputable. Wild rice, domesticated rice, and de-domesticated rice can all exhibit red pericarps. Consequently, red rice encompasses a broad spectrum of rice genetic backgrounds and provides a comprehensive resource for studying the history of rice domestication and genetic improvement. In this study, we sequenced dozens of cultivated red rice accessions from China and compiled additional available genomic data from red and black rice worldwide to form a genomic dataset for 5104 accessions (termed the 5k genomes). The dataset consists of 4315 cultivated, 230 wild, and 559 weedy rice accessions. The genomic data were used in population genomic analyses to investigate the evolutionary history of red rice. To the best of our knowledge, this is the largest collection used to date for studying the population genetics of red rice. Our results provide genomic evidence for the evolutionary trajectory of red rice and a valuable genomic resource for future research and breeding of pigmented rice.

RESULTS

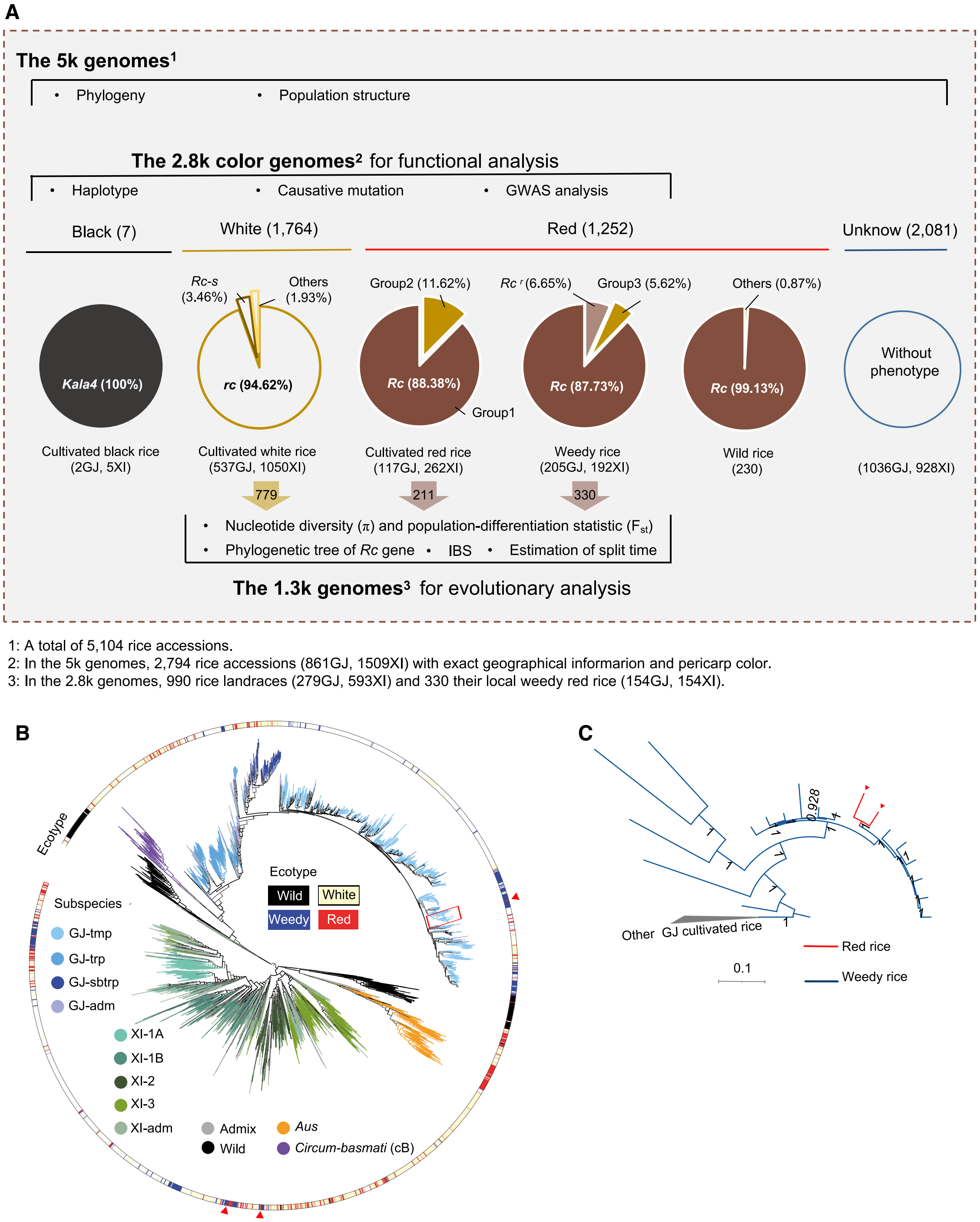
The 5k genomes used in this study

We collected 85 red rice accessions from various regions across China and sequenced them at an average depth of ~15-fold. We also obtained high-depth whole-genome sequencing data for 5019 rice accessions from previous studies (Wang et al., 2018; Xia et al., 2019; Li et al., 2020; Qiu et al., 2020; Zheng et al., 2021). Together, the genomes of 5104 worldwide rice accessions (hereafter referred to as ''the 5k genomes'') were integrated and analyzed (Figure 1A; Supplemental Figure 1; Supplementary Table 1). The 5k genomes include 2437 XI, 1897 GJ, 229 *aus*, 90 *circum*-basmati (cB), 221 admixed, and 230 wild (*O. rufipogon*) accessions (Figure 1A; Supplemental Figure 1). Among them, 2794 (53.30%) accessions (hereafter referred to as ''the 2.8k color genomes'') were classified into black (purple), red (brown), and white groups on the basis of pericarp color (Figure 1A). Alternatively, the 2.8k color genomes could be separated into 542 cultivated red rice, seven cultivated black rice, 481 weedy rice (red pericarp), and 1764 cultivated white rice collected from 117 different countries. In terms of subspecies, the 2.8k color genomes contain 1509 XI, 861 GJ, 202 *aus*, 72 cB, and 150 admixed accessions. From the 2.8k color genomes, 990 landraces and 330 local weedy rice were selected as ''the 1.3k genomes'' dataset for evolutionary analysis (Figure 1A). The 1.3k genomes consist of 747 XI, 433 GJ, 80 *aus*, 26 cB, and 34 admixed accessions.

Phylogeny, population structure, and diversification of the rice populations

We first individually mapped the clean paired-end reads (average 10-fold coverage) of the 5k genomes to the Nipponbare reference genome (IRGSP-1.0). A total of ~46 million single nucleotide polymorphisms (SNPs) and ~5.6 million small insertions/deletions (InDels) were identified. A maximum-likelihood phylogenetic tree based on 4 251 459 high-quality synonymous SNPs clearly showed divergence of subspecies from wild groups (Figure 1B). The phylogenetic tree revealed a mosaic structure of red, black, white, and weedy rice, although specific branches were dominated by single rice types. For example, the *aus* group was clustered into a single clade and closely nested with wild rice (Figure 1B), with 65.6% of the accessions being cultivated red rice collected from South Asia (Supplementary Table 2). Cultivated red and white rice were scattered across the whole tree, and this population structure in the phylogenetic tree was consistent with the results of principal component analysis and ADMIXTURE analysis (Supplemental Figure 2). As expected, weedy rice clustered with both GJ and XI rice in the branches of the phylogenetic tree (Figure 1B). Some cultivated red rice accessions were located within a clade of weedy rice with high bootstrap support (Figure 1C and Supplemental Figure 3), implying that weedy rice may have been re-domesticated into cultivated red rice. On the other hand, within the XI subspecies, several clusters exhibited a mix of weedy and cultivated red rice. Weedy rice was embedded within the cultivated red rice clusters (Figure 1B). Further investigation of derived allele frequency in each range of minor allele frequency in wild rice revealed similar distribution patterns of derived allele frequencies between cultivated red rice (C) and weedy rice (W) but significantly different patterns in wild rice (x axis) (Supplemental Figure 4). These results suggested that weedy rice was likely to have been de-domesticated from cultivated red rice.

Given that landrace genomes contain limited introgression from cultivated white rice, we used the 1.3k genomes, which consist



1: A total of 5,104 rice accessions.
2: In the 5k genomes, 2,794 rice accessions (861GJ, 1509XI) with exact geographical informarion and pericarp color.
3: In the 2.8k genomes, 990 rice landraces (279GJ, 593XI) and 330 their local weedy red rice (154GJ, 154XI).

Figure 1. Overview of the genomic data and workflow used in this study.
(A) Workflow used in this study. Among the 5k genomes, 2794 accessions with colored pericarps and geographical information (the 2.8k color genomes) were used for functional analysis, and 1320 rice accessions (the 1.3k genomes), including landraces and local weedy rice, selected from the 2.8k color genomes were used for evolutionary analysis. The accessions were classified into red, white, and black groups according to their available pericarp color. On the basis of genotypes of two functional genes (*Rc* and *Kala4*), the pigmented rice accessions (*O. sativa*) were further defined as black rice with the *Kala4* genotype or red rice with the *Rc/kala4* genotype. Most wild rice accessions (*O. rufipogon*) have red pericarps and the *Rc/kala4* genotype.
(B) Phylogenetic tree of the 5k genomes inferred from whole-genome SNPs. Different subspecies are indicated by colored lines, and the outermost circle represents the ecotypes of the accessions.
(C) Detailed phylogenetic tree of the rice accessions in the red rectangle in **(B),** showing the relationship between a cultivated red rice (represented by the red lines) and weedy red rice. The cultivated red rice is surrounded by weedy red rice (blue lines). Other cultivated rice is represented by a gray cluster.

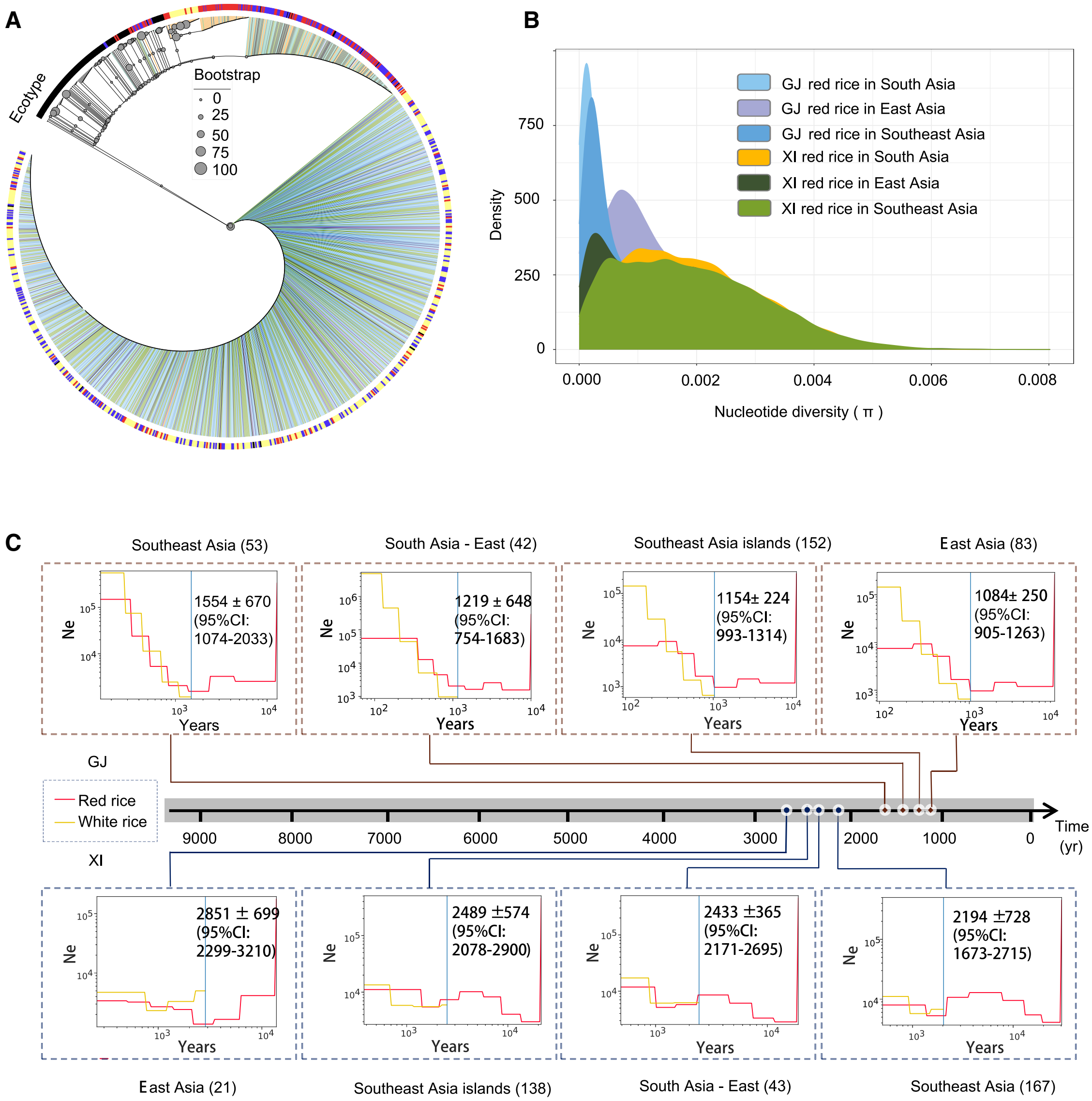


Figure 2. Genetic diversity and differentiation of red rice.

(A) Phylogenetic tree of the 1.3k genomes based on SNPs within the *Rc* gene. The colored lines indicate different subspecies, and the outermost circle represents the ecotypes of the accessions; the color representation is the same as that in Figure 1B.

(B) Distribution of nucleotide diversity density of GJ and XI red rice in different regions. Subspecies and regions are indicated by different colors.

(C) The estimated split time of red rice and white rice. Top: differentiation time of GJ red and white landraces in different regions inferred by SMC++. Bottom: differentiation time of XI red and white landraces in different regions inferred by SMC++.

of landraces and local weedy rice, to investigate the domestication history of red rice. We used variants within the causative gene responsible for the red pericarp, i.e., *Rc*, to construct a phylogenetic tree. The 990 landraces, 330 weedy rice, and 180 wild rice were clearly separated according to different alleles of *Rc* (Figure 2A). Apparently, some red rice were at the positions closest to wild rice. Nearly all *aus* accessions clustered together and nested within wild ancestors, indicating that wild rice was the direct donor of the *Rc* gene in the *aus* rice subgroup (Figure 2A). Several weedy rice accessions were embedded within a group of cultivated red rice but far from wild rice (Figure 2A), suggesting that weedy rice might have been de-domesticated from red rice, retaining the *Rc* gene.

Nucleotide diversity (π) was estimated for different rice groups to compare their genetic diversity (Figure 2B). A density plot showed that GJ red landraces in East Asia (average π = 1.22 × 10^{-3}) and XI red landraces in South Asia (average π = 1.92 × 10^{-3}) were the most diverse (Figure 2B). Red rice had the highest polymorphism in areas of rice origin (i.e., GJ in East Asia and XI in South Asia; Huang et al., 2012b; Civáň and Brown, 2018), indicating a high likelihood of its domestication from wild rice. We also calculated identity by state (IBS) between the red/white landraces and wild rice derived from the same region (Supplemental Figure 5). In all regions, cultivated red landraces exhibited closer genetic proximity to wild rice than to cultivated white landraces (Supplemental Figure 5A), implying that red landraces have greater genetic introgression

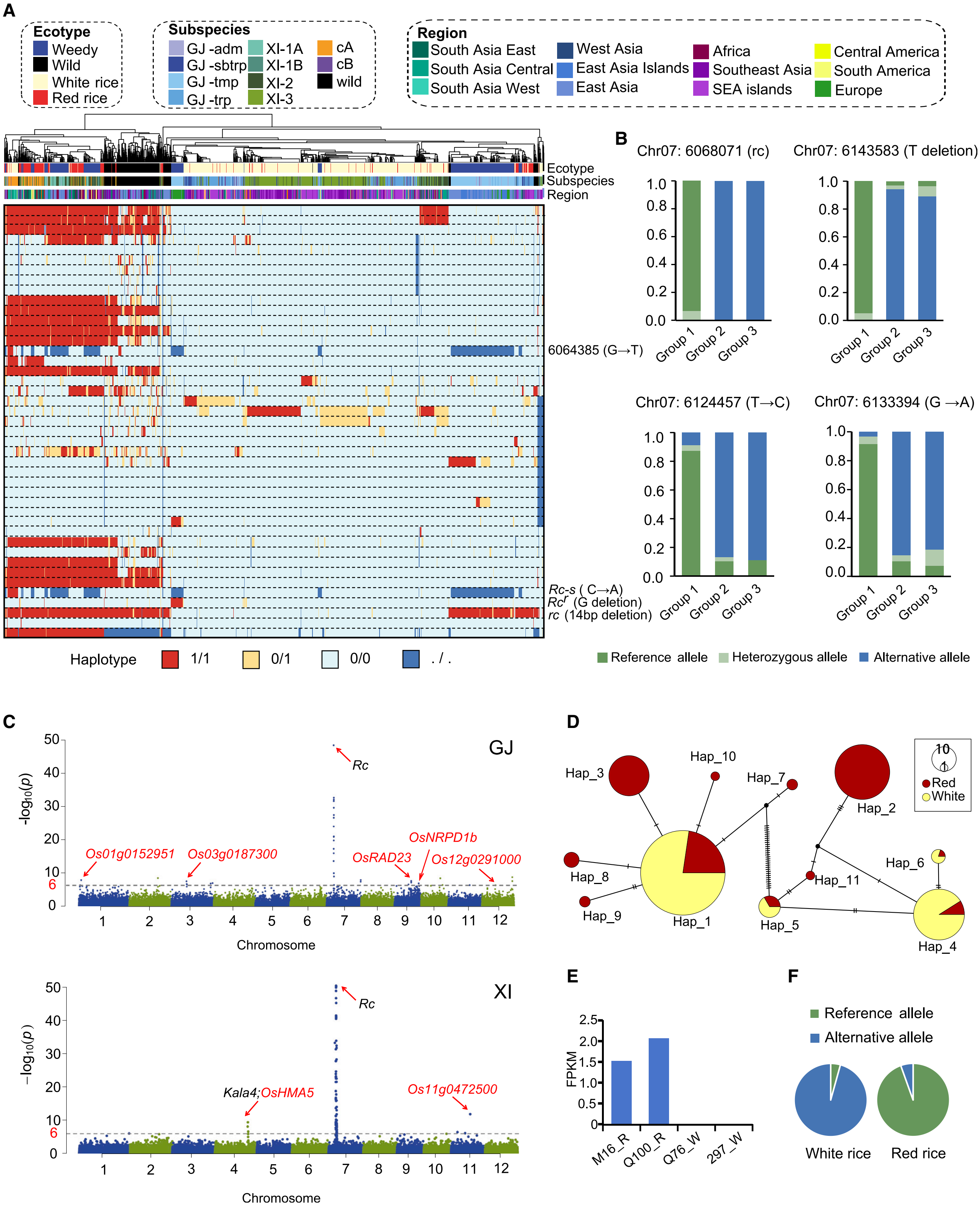


Figure 3. Identification of new candidate genes/alleles for red pericarp in rice.

(A) SNP- and indel-based haplotype map of the *Rc* gene. The ecotype, subspecies, and source region of each accession are indicated by colored lines under the clustered tree, and important mutations, including *rc*, *Rc-s*, and *Rc*^{r}, are marked at their positions. The genotypes (ref, alt, het, and missing) of each variant are color-coded, using the IRGSP-1.0 genome as the reference.

(legend continued on next page)

from wild rice and red landraces are more likely to originate from wild rice.

The split time of red and white rice landraces

To provide a temporal context of differentiation time between red rice and white rice, we used SMC++ (Terhorst et al., 2017) to estimate divergence time between the two groups based on their landraces. SMC++ combines the simplicity of sequentially Markovian coalescent methods and the scalability of site frequency spectrum methods, and its robustness has been demonstrated previously (Zhao et al., 2023). Our results showed that GJ red rice and white landraces from different regions (including East Asia, South Asia, and Southeast Asia) differentiated into different groups within the recent ~2200 yr BP (years before present). The differentiation time of XI red landraces and white landraces in different regions was estimated to be within ~3500 yr BP (Figure 2C and Supplemental Figure 6). These time estimates are consistent with descriptions in ancient Chinese books such as "*Xin Tang Shu*" and "*Song Shi*," which relate that south China had white rice production and entered the era of intensive white-rice farming in the Tang dynasty (about 1500 yr BP), especially in the Taihu lake basin (Li et al., 2021). Our results indicate that the *rc* mutation (e.g., white rice) has been present in the rice population for a long time but was ultimately fixed at a much later stage.

Identification of candidate alleles/genes for rice red pericarp

On the basis of their *Rc* and *Kala4* (contributing to black pericarp) genotypes, pigmented rice accessions (*O. sativa*) were further categorized as either black rice with *Kala4* or red rice with *Rc*/*kala4*. All wild rice accessions showed a red pericarp with the *Rc*/*kala4* genotype. However, a small subset of rice accessions (84/5104, 1.7%) displayed a red pericarp that could not be explained by *Rc* or *Kala4* (Figure 1A). As expected, the majority of cultivated white rice accessions (94.6%) contained the *rc* gene. In addition, 62 (3.5%) accessions harbored the *Rc-s* allele, which contributes to a white pericarp owing to premature termination of the Rc protein caused by a C-A mutation. Intriguingly, 34 (1.9%) accessions with white pericarps harbored the *Rc* gene, and the underlying genetic mechanism for this phenomenon remains to be discovered. We did not find the *Rc-g* allele in any of the accessions, presumably as a result of filtering by a relatively high missing rate of 0.2. To gain insight into the mutation distribution within the *Rc* gene, we constructed a haplotype plot of the *Rc* gene for the 2.8k color genomes and categorized them based on ecotypes, regions, and subspecies (Figure 3A). The haplotype plot indicated that all wild accessions harbored the *Rc* allele, confirming its status as the ancestral allele contributing to the wild-type red-pericarp phenotype. Furthermore, absence of proanthocyanin accumulation in some white rice from the *aus* group was due to the presence of the loss-of-function *Rc-s* allele (with a C-A transversion). In these *aus* white rice, a G-T transition ($r^2 = 0.51$) was identified 3633-bp upstream of the C-A mutation in the *Rc-s* allele. The simultaneous deletion of these two mutations was observed in red rice across all regions except Africa (Figure 3A), perhaps reflecting human selection during the domestication process.

The majority of the 542 cultivated red rice accessions (88.4%, termed "Group1") harbored the *Rc* gene, whereas 63 accessions (11.6%) harbored the *rc* allele but still exhibited a red pericarp (termed "Group2"). Among the 481 weedy rice accessions, the red-pericarp phenotype in most (87.7%) could be attributed to the presence of the *Rc* gene. Thirty-two accessions (6.7%) harbored the *rc* and Rc^r allele, in which a G-base deletion in *rc* restores *Rc* functionality, resulting in a red pericarp. In addition, 27 accessions (5.6%, termed "Group3") contained the *rc* gene but maintained a red pericarp, with the causative mutation(s) for their red-pericarp phenotype remaining unknown (Figure 1A). To further explore the genomic pattern behind the phenotypes and look for variants that contribute to a red pericarp, we focused on rice accessions that exhibited a contradiction between phenotype and genotype at the *Rc* locus (i.e., Group1, Group2, Group3) (Figure 1A). By comparing allele frequencies of SNPs and indels within the 100-kb flanking regions of the *Rc* gene among the three groups, we identified three variations as candidates that could potentially contribute to explaining the contradiction. These included a T deletion 74 266 bp downstream of the *Rc* gene, a G-A mutation 64 077 bp downstream, and a T-C mutation 55 140 bp downstream. The T deletion (chr07:6143583) may affect the function of a transposase-encoding gene (Os07t0212400-01). More than 95% of accessions in Group1 harbored the reference allele (C), whereas over 94% and 89% of accessions in Group2 and Group3, respectively, harbored the alternative allele (T) (Figure 3B). The G-A mutation (chr07:6133394) is an upstream variation in a gene encoding an mRNA-binding protein. More than 91% of accessions in Group1 harbored the reference allele (G), whereas more than 85% and 81% of accessions in Group2 and Group3 harbored the alternative allele (A) (Figure 3B). For the T-C mutation (chr07:6124457), over 87% of accessions in Group1 harbored the reference allele (T), and more than 86% and 88% of accessions in Group2 and Group3 harbored the alternative allele (C) (Figure 3B).

To explore new genes underlying the red-pericarp phenotype, we performed a genome-wide association study (GWAS) for the GJ

(B) Three possible sequence variants related to the contradiction between phenotype and genotype, including a deletion (T) and two single-base mutations (T to C and GnullA**)**. All three variants had a higher rate of the reference allele in Group1 and a higher rate of the alternative allele in Group2 and Group3. Three groups were defined as Group#1 and Group#2, cultivated red rice with the *Rc* or *rc* allele, respectively, and Group#3, weedy red rice with the *rc* allele and without the Rc^r gene.

(C) Manhattan plots for red pericarp color in the GJ (top) and XI (bottom) rice groups. The $-\log_{10}(P)$ values from a genome-wide scan are plotted against position on each of 12 chromosomes. The horizontal dashed lines indicate the genome-wide significance thresholds ($P = 2.9 \times 10^{-7}$ for GJ and $P = 3.0 \times 10^{-7}$ for XI, equivalent to about 6.5 after the logarithm).

(D) Haplotype network of *Os01g00152951* in the GJ group.

(E) Transcriptomic patterns of *Os01g0152951* in the two red rice accessions presented as number of fragments per kilobase of exon model per million mapped reads (FPFM).

(F) *Os04g0556000* in the XI group has a significantly different allele frequency between the red rice and white rice groups.

and XI populations (Figure 3C). Seventeen associated loci ($P = 2.9 \times 10^{-7}$) were identified in the GJ population, and four associated loci were identified in the XI population ($P = 3.0 \times 10^{-7}$) (Supplementary Table 3). Among these loci, one in the GJ group and two in the XI group were associated with known genes involved in proanthocyanin synthesis and other pathways, such as *Rc* (Sweeney et al., 2006) and *Kala4* (Oikawa et al., 2015) (Figure 3C), as supported by previous studies on mutants or recombinant populations. Several new loci were found to be associated with red pericarps in both GJ and XI subspecies (Figure 3C). For example, an association signal ($P = 2.18 \times 10^{-8}$) was found upstream of *Os01g0152951* (annotated as a hypothetical conserved gene) in the GJ population at chr01:2781894. Notably, this locus harbored different dominant haplotypes in the red rice and white rice groups. Haplotype network analysis revealed that 75.7% of the GJ red rice predominantly contained haplotypes hap#2 and hap#3, whereas hap#1 and hap#4 accounted for 79.0% and 90.2% of GJ white rice, respectively (Figure 3D). To better verify the candidate genes, we downloaded RNA-seq data from a previous study of two red rice and two white rice accessions (Zainal-Abidin et al., 2020). We found a significant difference in the expression level of *Os01g0152951* between the two phenotypes (Figure 3E). Further sequence analysis indicated that *Os01g0152951* has been lost in the two white rice (Q76_W and 297_W) (Supplemental Figure 7). Another association signal ($P = 1.49 \times 10^{-9}$) was found upstream of *OsHMA5* (*Os04g0556000*) in the XI population. This gene neighbors *Kala4* and has previously been implicated in xylem loading of copper in rice (Deng et al., 2013). By comparing the allele frequency of each SNP in *OsHMA5* between the red and white rice groups, we found significant difference in allele frequency at chr04:27834266 between red rice (T: 0.95; A: 0.05) and white rice (T: 0.04; A: 0.96) (Figure 3F). The linkage disequilibrium of every single SNP in *Kala4* was calculated, and very low linkage (average $r^2 = 0.019$) was found, suggesting that *OsHMA5* is another promising candidate gene for the red-pericarp phenotype.

DISCUSSION

A red pericarp is an inherent trait of wild rice (Roy and Shil, 2020), and loss of proanthocyanins in the rice pericarp is a domestication-related trait (Xia et al., 2021). Red rice, found across wild, domesticated, and de-domesticated rice, represents the ancient form of rice and preserves rich genomic imprints inherited from its ancestors. Its existence is associated with geographic and cultural contexts, particularly for the red rice landraces. Therefore, red rice maintains an intact genetic background of rice history. The broad spectrum of accessions collected in this study was useful for revealing the domestication history of red rice. Moreover, the population studies carried out here used a balanced number of pigmented and white rice accessions, enhancing the power for illuminating the evolutionary history of rice.

We proposed multiple origins of red rice on the basis of the genome-wide phylogeny results, the phylogenetic tree of the *Rc* gene, and other genetic evidence. We found that red landraces in the *aus* group originated from wild rice, whereas some red rice accessions were re-domesticated from weedy red rice. We found that both cultivated white and red rice could be de-domesticated into weedy rice. Although the utilization of cultivated black rice in this study was limited, the phylogenetic tree seems to support previous findings (Supplemental Figure 8) (Oikawa et al., 2015); i.e., the sequence rearrangement in the promoter of *Kala4* that contributes to the black pericarp phenotype first occurred in tropical GJ and then spread to XI and subsequently to temperate GJ through natural crossing and artificial selection for the black-pericarp trait (Oikawa et al., 2015).

A considerable amount of archaeological and genomic evidence has been proposed to infer the origin of rice (Molina et al., 2011; Huang et al., 2012b; Gross and Zhao, 2014; Civáň et al., 2015; Choi et al., 2017; Choi and Purugganan, 2018; Wang et al., 2018; Zheng et al., 2021; Wu et al., 2023). The evolutionary trajectory of single domestication with multiple origins (i.e., GJ was first domesticated from wild rice, and XI originated from proto-GJ by introgression to local wild rice) has been well documented on the basis of genomic data (Huang et al., 2012b; Choi et al., 2017; Choi and Purugganan, 2018; Wu et al., 2023). The history of rice dispersal has also been reconstructed using whole-genome sequences (Gutaker et al., 2020). In brief, originating around 9000 years ago in the Yangtze Valley, rice diversified into temperate and tropical GJ types after the global cooling event about 4200 yr BP (Figure 4). It was since ∼2500 yr BP that tropical GJ rice reached Southeast Asia and diversified rapidly. GJ rice spread to South Asia by ∼4000 yr BP and led to introgression of domestication alleles into proto-XI or local *O. nivara* populations, leading to the emergence of XI rice (Gutaker et al., 2020). Crop plants acquire characteristics distinct from those of their wild progenitors through a process of stepwise selection, involving collective and progressive changes in several important traits. It has been reported that during the initial period of domestication, humans continued to gather wild rice, leading to slow emergence of the non-shattering trait. Because of the ongoing gene flow into proto-domestications, fixation of the trait took about 2000 years (Purugganan and Fuller, 2009). It is conceivable that rice tends to retain the characteristic red pericarp of wild rice for a long time, until completion of the initial domestication process. Meanwhile, red rice exhibits greater adaptability to local environments (Qi et al., 2022; Gu et al., 2005), which may have encouraged ancient farmers to plant red rice in order to gather more grains.

In addition to human innovation and crop adaptation under the climatic and political conditions of the time (Zhang et al., 2015; Pei et al., 2019), people's preference for certain crops changed agricultural systems (Xhauflair et al., 2017; Overton and Taylor, 2018), as did the red–white shift in rice. At present, white rice varieties dominate in rice production. Our estimates suggest that the red–white shift in rice production occurred about 3500 years ago (Figures 2C and 4 and Supplemental Figure 6). The widespread cultivation of white rice might be attributed to the fact that it is easier to detect insects and eliminate pathogens on a light background. In addition, less energy and time required for cooking also contributed to its popularity (Sweeney et al., 2006). The rapid and extensive adoption of white rice in cultivation may also be attributed, in part, to the recessive nature of the *rc* mutation. Seeds with a white pericarp will faithfully produce offspring with white pericarps (Sweeney et al., 2006). It is conceivable that the favored *rc* allele would not have traveled far beyond its origin if not for selection by humans who picked out the white grain as a preferred food and traded it as a

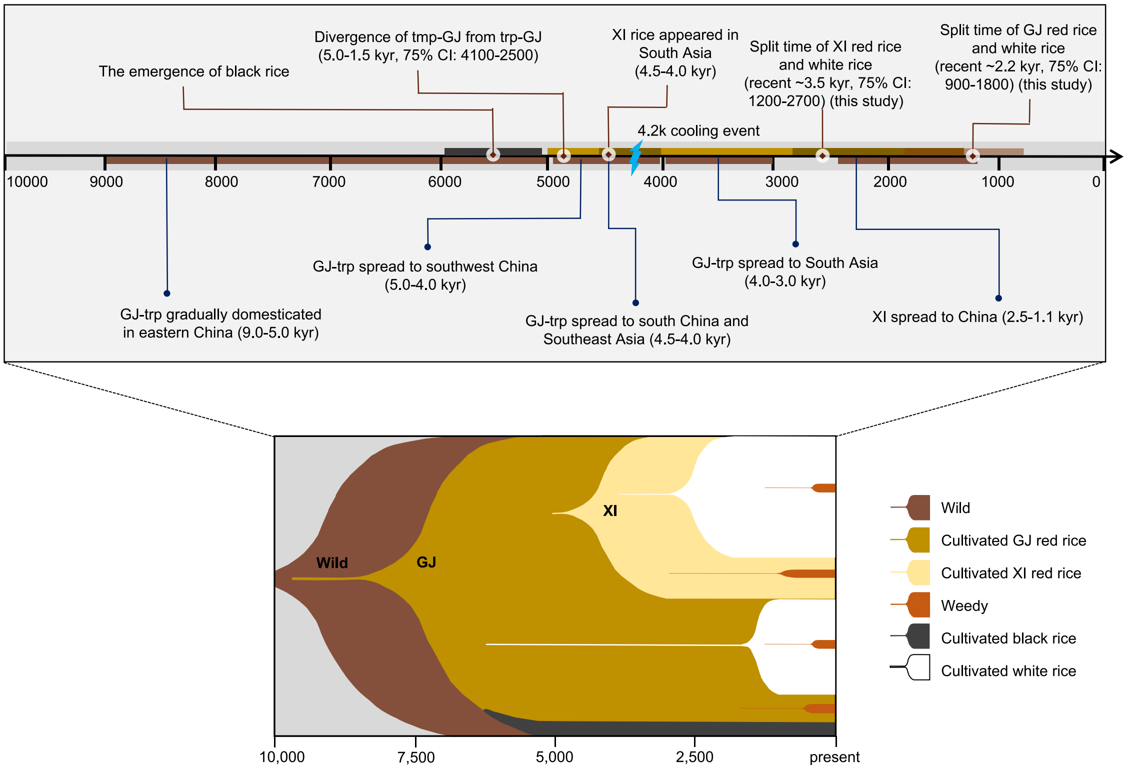

The emergence of black rice
Divergence of tmp-GJ from trp-GJ (5.0-1.5 kyr, 75% CI: 4100-2500)
XI rice appeared in South Asia (4.5-4.0 kyr)
Split time of XI red rice and white rice (recent ~3.5 kyr, 75% CI: 1200-2700) (this study)
Split time of GJ red rice and white rice (recent ~2.2 kyr, 75% CI: 900-1800) (this study)
4.2k cooling event
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
GJ-trp spread to southwest China (5.0-4.0 kyr)
GJ-trp spread to South Asia (4.0-3.0 kyr)
GJ-trp gradually domesticated in eastern China (9.0-5.0 kyr)
GJ-trp spread to south China and Southeast Asia (4.5-4.0 kyr)
XI spread to China (2.5-1.1 kyr)
Wild
GJ
XI
10,000
7,500
5,000
2,500
present
Wild
Cultivated GJ red rice
Cultivated XI red rice
Weedy
Cultivated black rice
Cultivated white rice

Figure 4. A schematic evolutionary trajectory of pigmented rice.

The top panel shows the timeline of rice domestication, including evidence from previous archaeological and genomic studies (Huang et al., 2012b; Gross and Zhao, 2014; Oikawa et al., 2015; Gutaker et al., 2020; this study). The bottom panel shows a wild–red–white model of rice evolution based on the pericarp colors. Ancient wild rice experienced slow domestication and gradually produced GJ rice, most of which had a red pericarp. Over time, the proportion of white-pericarp rice increased, and this was accompanied by the emergence of weedy rice de-domesticated from both red rice and white rice. XI rice was domesticated later and experienced an evolutionary process similar to that of GJ rice.

commodity. Some red landraces seem likely to have served as the intermediate ancestral progenitor of cultivated white rice, having undergone extensive artificial selection and improvement over time. For example, some cultivated white rice accessions were surrounded by red rice accessions in the phylogenetic tree, suggesting their potential origin from red rice (Figure 1B).

Taken together, our results led us to propose a wild–red–white model of rice evolution based on pericarp colors (the lower panel of Figure 4). We believe that rice with a red pericarp existed during the initial domestication period of rice and gradually turned to rice with a white pericarp through a process of diversification. Red rice production predominated for most of the approximately 10 000-year evolutionary history of rice until roughly three thousand years ago, when white rice began gaining in popularity and subsequently predominated in both XI and GJ rice. As suggested by previous work and this study, cultivated white and red rice may feralize to weedy red rice (Qiu et al., 2020; Wu et al., 2022). The red–white shift happened in diverse regions and different historical periods. Therefore, since its domestication from wild rice, rice has entered into a red–white evolutionary cycle and keeps transitioning from one state (e.g., red) to another (e.g., white).

GWAS has been used to study pericarp color for a long time. Larger sample size can greatly enhance the power of GWAS (Huang et al., 2010). The substantial increase in sample size of this study compared with previous studies (Huang et al., 2010, 2012b; Wang et al., 2016; Rana et al., 2022; Yang et al., 2022) enables more comprehensive detection of associated loci. In general, the genetic architecture of the coloration traits exhibited remarkable similarity between the two subspecies, suggesting that visible traits are more susceptible to the influence of introgression between subspecies (Huang et al., 2012a). In summary, our results suggest that in addition to the significant effect of the *Rc* gene, effects of multiple other loci may also contribute to variation in pericarp pigmentation. These newly identified loci are attractive candidates for follow-up investigations that could expand our understanding of the genetic basis of proanthocyanidin synthesis and accumulation. More functional genomics studies are needed to further validate the effects of these genes and identify their functional variants.

METHODS

Sampling of rice accessions

To capture the comprehensive genetic diversity of all rice ecotypes, 5104 rice accessions were used in this study (Supplementary Table 2), including 5019 accessions from published work (Zheng et al., 2021; Wang et al., 2018; Xia et al., 2019; Li et al., 2020; Qiu et al., 2020) and 85 newly sequenced red rice accessions. The newly sequenced accessions were collected from major rice production regions of China.

DNA isolation and genome sequencing

Genomic DNA was extracted from young leaves of each of the 85 red rice accessions using the standard cetyltrimethylammonium bromide–based protocol (Edwards et al., 1991), and 1.5 µg of DNA per sample was used as input for sequencing library construction using the TruSeq Nano DNA HT sample preparation kit (Illumina USA). The libraries were sequenced on the Illumina HiSeq 4000 platform (150-bp, paired-end reads) to generate about 1.21 Tb of raw sequence.

Sequence quality checking and filtering

To eliminate reads with artificial bias (i.e., low-quality paired reads, which primarily resulted from base-calling duplicates and adaptor contamination), raw paired reads from the 85 red rice accessions were first cleaned using NGSQC-toolkit (Patel and Jain, 2012) with default parameters. About 1.21 Tb of high-quality genomic data were retained.

Sequence alignment, variant calling, and annotation

We downloaded raw sequencing data for 5019 rice accessions from each of the cited publications and performed SNP calling from the raw short reads using the same pipeline. First, high-quality reads were mapped to the *O. sativa* reference genome (IRGSP-1.0) by Bowtie 2 (Langmead and Salzberg, 2012) with the default command. Variants were detected (based on the default parameters) and filtered (those with QUAL $\geq$ 30, DP $\geq$ 10, QD $\geq$ 2, and MQ $\geq$ 20 were retained) using GATK software (v3.7) (Mckenna et al., 2010). Next, all variants from different projects, including 3K-RG (Wang et al., 2018), one thousand accessions from China (Li et al., 2020), 185 accessions of wild rice (Zheng et al., 2021), a weedy rice gene pool (Qiu et al., 2020), and the 85 newly sequenced red rice accessions, were integrated into one file in VCF format. The following potential low-quality variants were removed: (1) missing rate >0.2 and (2) minor allele frequency $\leq$0.05. After filtering, ~4 million high-quality SNPs and ~0.5 million indels were retained and annotated with SnpEff (v3.6) (Cingolani et al., 2014) to profile their potential effects on predicted amino acid sequences and gene functions.

Population phylogenetic analysis

To reduce marker redundancy, we randomly picked one SNP from every 10 consecutive SNPs and constructed a SNP dataset consisting of 425 145 sites evenly distributed across the rice genome. Phylogenetic trees were constructed using FastTreeMP (Price et al., 2009) with 1000 bootstrap replicates. iTOL (http://itol.embl.de/) was used to visualize the trees.

Population structure analysis

Structure analysis was performed using ADMIXTURE v1.3.0 software (Alexander et al., 2009) with K values from 5 to 15 to estimate the standard error of parameters. Principal component analysis was performed using PLINK v1.9 (Chang et al., 2015) with the command "–pca 20." Genetic distances were estimated by IBS using PLINK v1.9 both among and within rice subgroups and ecotypes. The distance matrix was imported into R v3.4.1 to plot a boxplot using R scripts.

Fluctuation of effective population size over time

To better understand the historical demographics of red rice compared with white rice, we tried to reconstruct past effective population size using the SMC++ method (Terhorst et al., 2017). Because SMC++ has sufficient power to deal with situations with different coverages and sample sizes, the reconstruction was carried out independently for varying numbers of subgroups of red rice and white rice from different regions around the world. Because sequences of functional genes and regulatory elements do not satisfy neutral selection assumptions and will strongly effect estimation of split time, we masked protein-coding sequences and their 3-kb flanking sequences in the follow-up analyses. Fifteen samples from different regions were randomly selected at a time, and partitioned VCF files were transferred into smc haploblock files, then further divided into 12 chromosomes. The files were used in the ESTIMATE function of SMC++ with a mutation rate of 6.5×10^{-9} to estimate past effective population sizes. Results were scaled in time using an estimated generation time of 1 year and plotted on a smooth linear timescale in R. These demographics were used to calculate split times between red rice and white rice in different regions with the SPLIT function.

Population genetic analysis

The genome of every subpopulation and subgroup was scanned with a 100-kb window size and a 10-kb step size. Population parameters were estimated for each window with VCFtools (Danecek et al., 2011). Nucleotide diversity (π) was calculated with the parameters "–window-pi 100000 –window-pi-step 10000." The average π value in a 100-kb window was taken as the genetic diversity. The population differentiation index (F_{ST}) was measured with the settings "–fst-window-size 100000 –fst-window-step 10000." The minor allele frequency was calculated using PLINK v1.9 with parameters "–noweb –freq" for XI red rice, XI weedy rice, and wild rice, which are all neighboring in the phylogeny. The distribution of the derived allele frequency was calculated in weedy rice (W) and cultivated red rice (C) in each range of minor allele frequency (x axis) in wild rice.

Genome-wide association study

GWAS was performed using a compressed MLM model that could effectively reduce false positives. The equation of the compressed MLM model is $y = X\alpha + P\beta + K\mu + e$, in which y is the phenotype, X is the genotype, P is the population structure matrix (Q matrix), and K is the kinship matrix. The P matrix was built from the top five principal components for population structure correction. The K matrix was built from the matrix of simple matching coefficients. The filtered VCF files were converted to tped format using PLINK v1.9 and entered into the software Efficient Mixed Model Association eXpedited (EMMAX; Kang et al., 2010) for GWAS. The significant *P*-value threshold was determined using the Bonferroni correction method. The Manhattan and QQ plots for GWAS were generated using the R package qqman. To verify the candidate genes, RNA sequences generated by Zainal-Abidin et al. (2020) (accession number PRJEB34340) from two red and two white rice accessions were downloaded and used to compare expression differences of the candidate genes. The two white accessions used in the RNA-seq experiment have the same mutation or allele as that in the candidate genes identified in this study, whereas the two red rice accessions have the wild-type genotype.

DATA AND CODE AVAILABILITY

The raw sequencing data are available at the China National Genomics Data Center (https://ngdc.cncb.ac.cn) under BioProject accession number PRJCA017658.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

L.F. conceptualized and designed the experiments. L.X. performed most of the experiments under the supervision of L.F. and the guidance of D.W. Y.F., C.Y., and X.W. helped to collect the samples for sequencing. L.F., D.W., and Q.-H.Z. revised the manuscript.

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Supplemental information

Population genomic analysis unravels the evolutionary roadmap of pericarp color in rice

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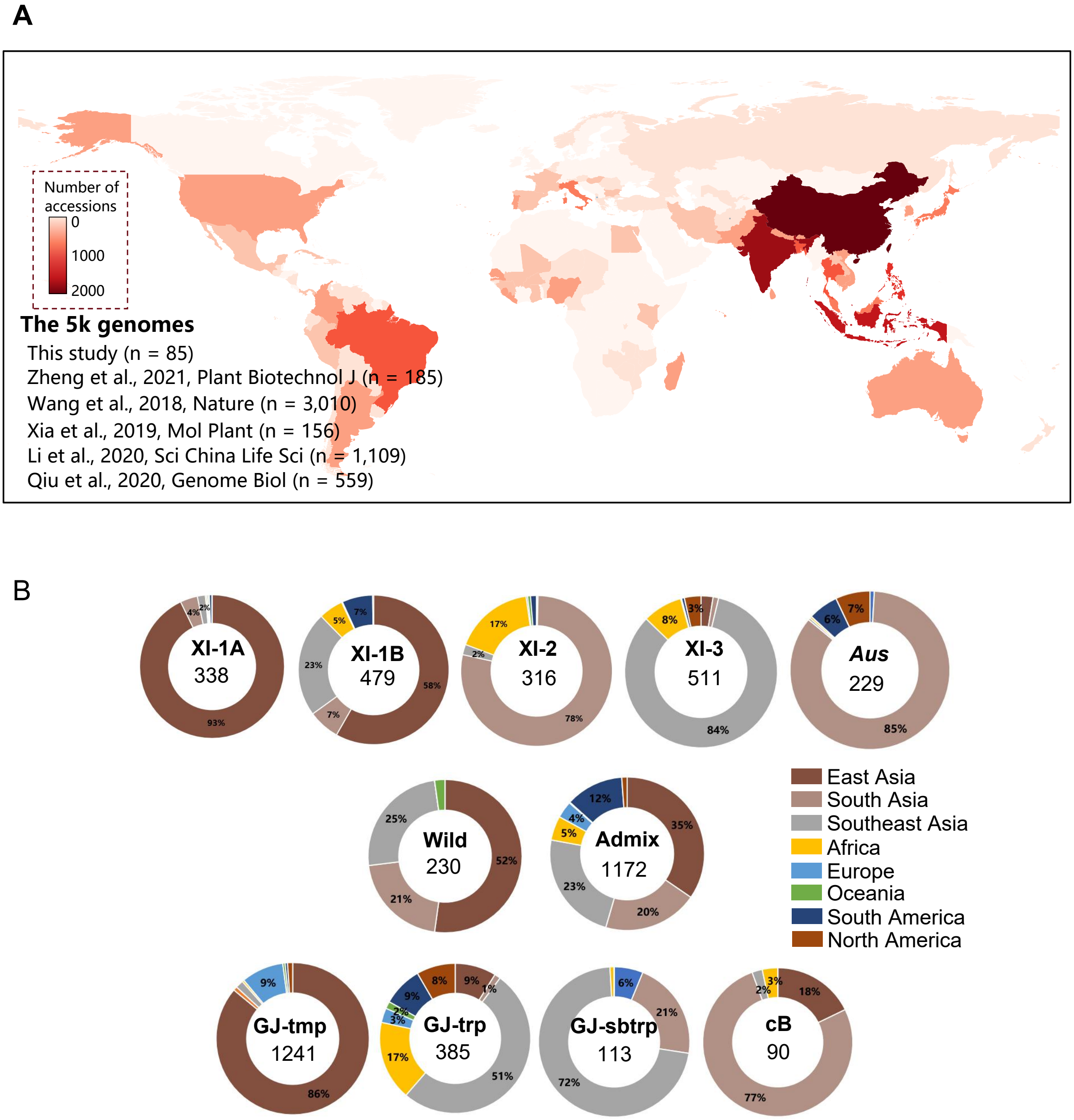
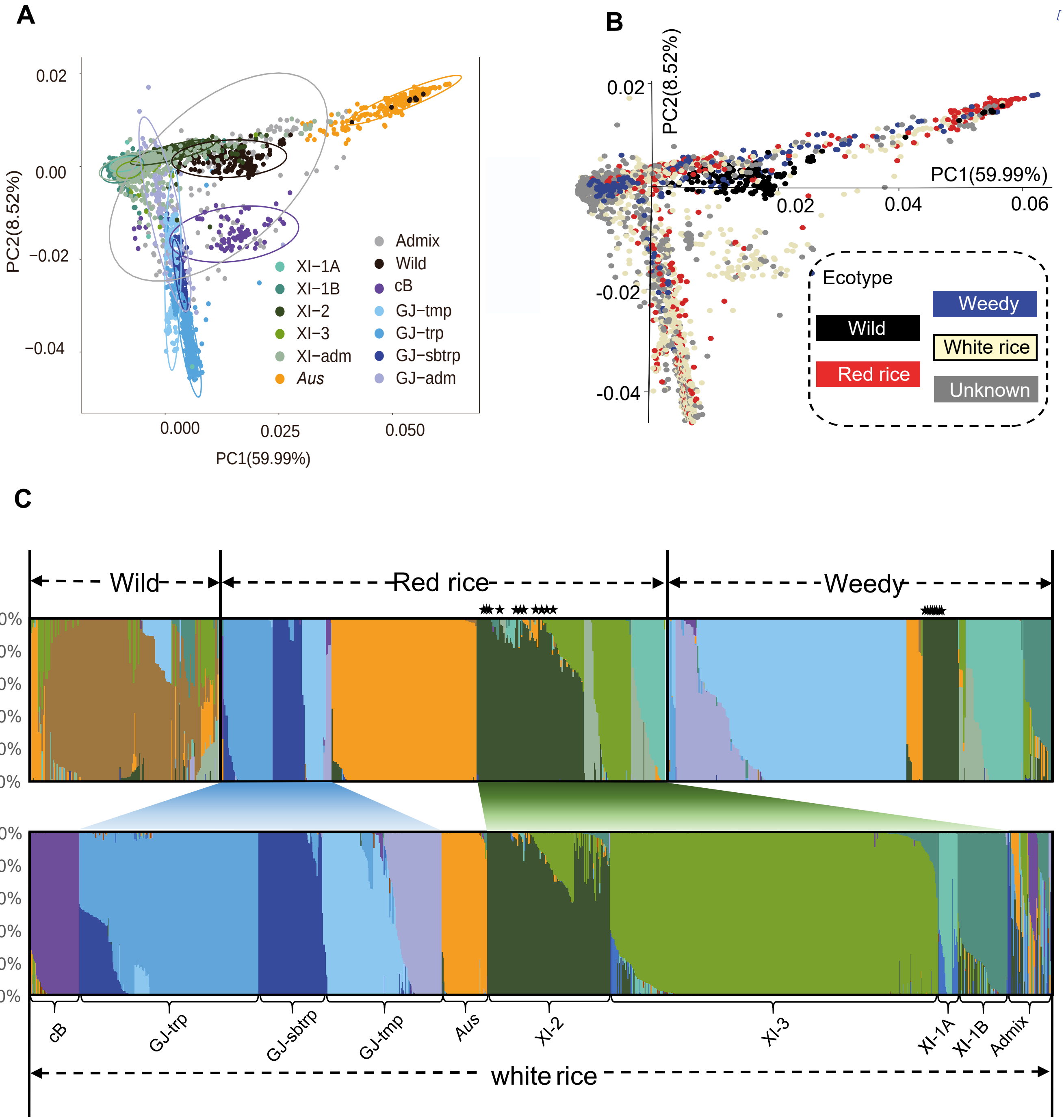
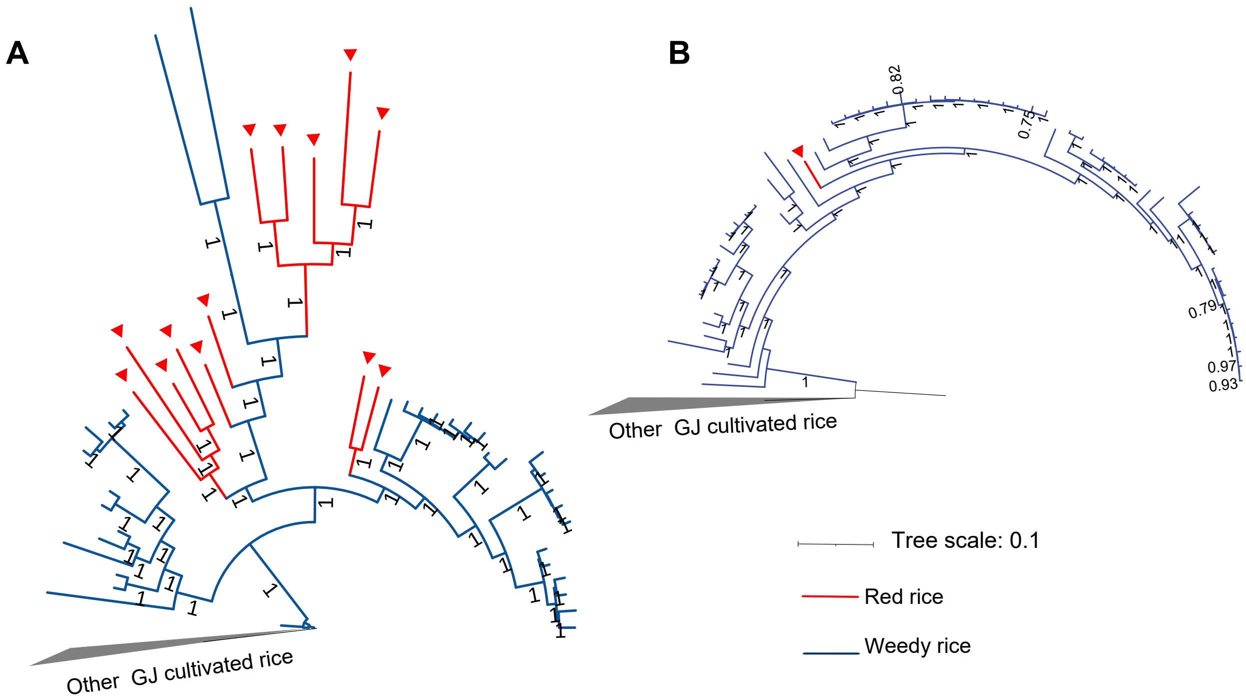


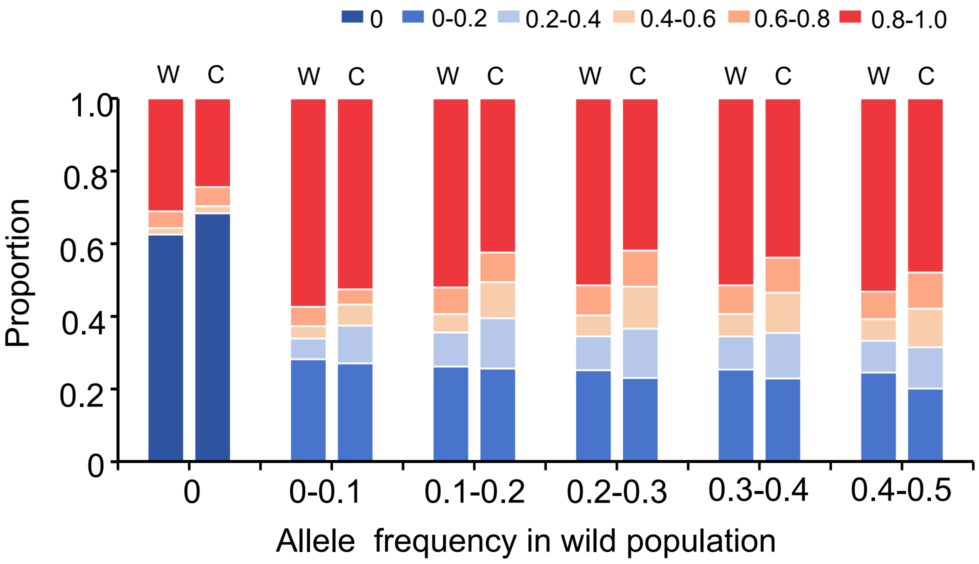
Supplementary Fig.1 Overview of sampling. (A) Geographical distribution and origin of 5,104 rice accessions (termed as 5k genomes) used for this study. (B) Proportion of geographic composition in each subspecies.



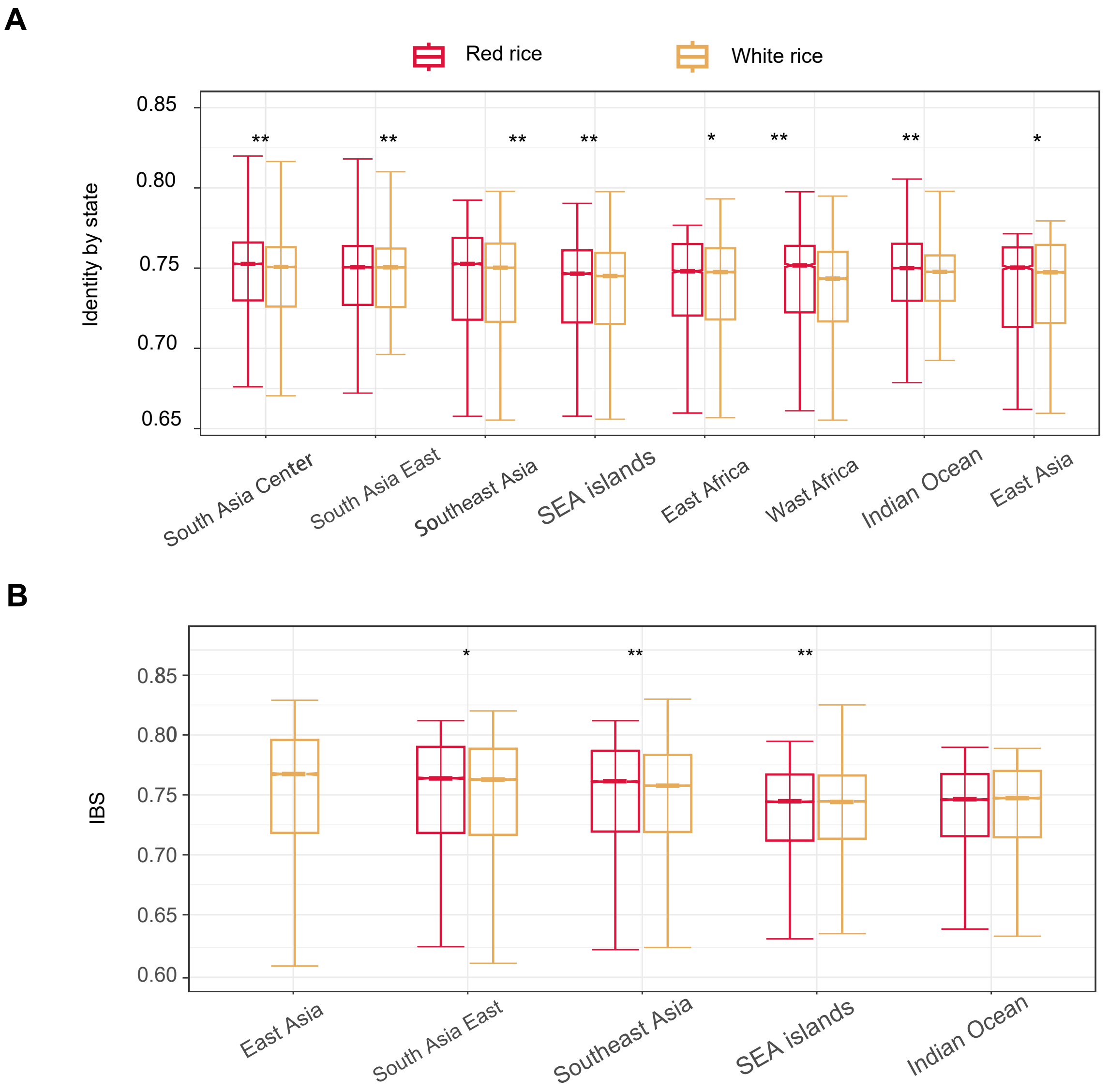
Supplementary Fig.2 Principal-component analyses and population structure of 5,104 accessions. (A) Rice accessions distinguished by subpopulations, including XI rice, GJ rice, aus rice, aromatic rice and wild rice. (B) Rice accessions distinguished by ecotypes, including red rice, white rice, weedy rice, wild rice and unknown type. (C) Individual ancestry coefficients of K = 12 of 154 wild rice, 294 weedy rice, 361 red rice, and 1,238 white rice.



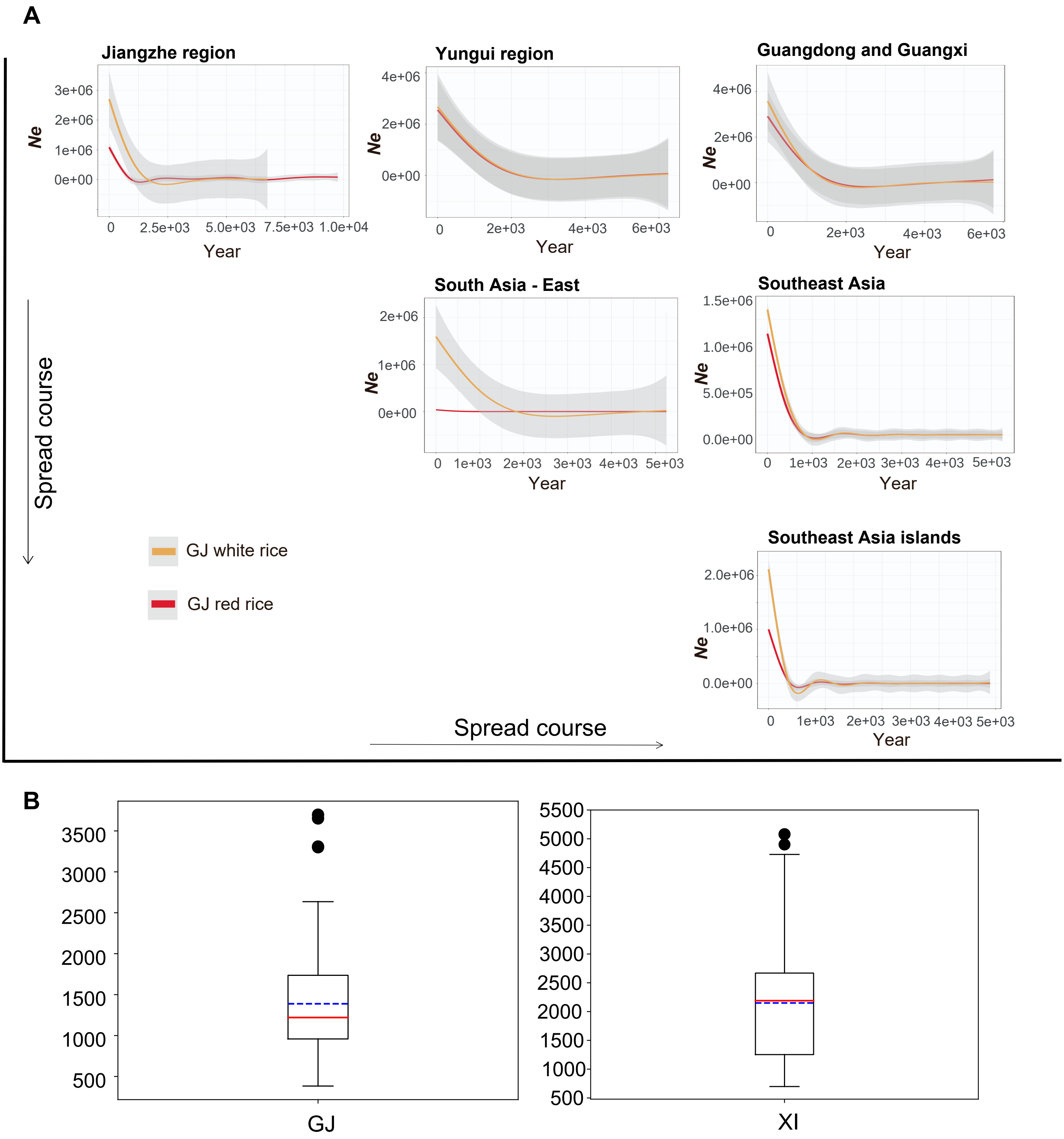
Supplementary Fig.3 Detailed phylogenetic tree of the rice accessions which were marked with red triangle in Figure1B to show the relationship between a cultivated red rice (represented by the red line) with weedy red rice. The cultivated red rice is surrounded by weedy red rice (blue line). Other cultivated rice was represented by a gray cluster.



Supplementary Fig.4 Allele frequency in cultivated red rice and weedy rice, with the frequency range in wild rice shown on the x axis. "C" and "W" refer to cultivated red rice and weedy rice, respectively.



Supplementary Fig.5 (A) Identity-by-state (IBS) distance between wild rice and XI red rice or XI white rice in different regions. (B) Identity-by-state (IBS) distance between wild rice and GJ red rice or GJ white rice in different regions. * and ** indicate significant difference at P < 0.05 and P < 0.01, respectively, determined by the Wilcoxon test.



Supplementary Fig.6 (A) Effective population size history of red rice and white rice subgroups assessed by the coalescent approach SMC++. The thick line represents the mean and the grey-shaded area represents the quartiles of random individuals (n = 15 random pairs). (B) Boxplots of estimated split times of red rice and white rice in GJ and XI population. Center black line: median; Center blue line: mean; box limits: upper and lower quartiles; dots: outliers.

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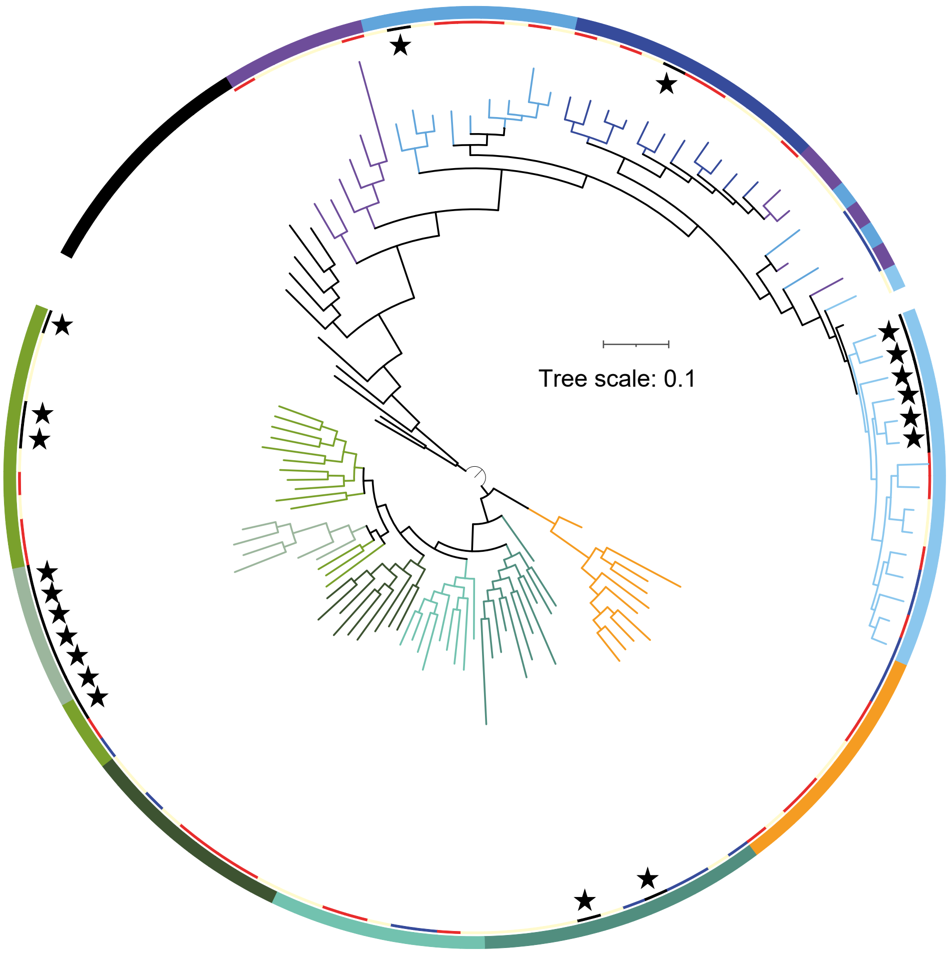
● M16_R  G G A A G G C C G G - - G G G G T T C C C C T T T T G G G G T T A G T T
● Q100_R G G A A G G C C G G T T G G G G T T C C C C T T T T G G G G T T A G T C
● Q76_W  - - - - - -   - - - - - - - - - - - - - - - - - - - - - - - - -   - - -
● 297_W  - - - - - - C C - - - - G G G G T T C C - - - - - - - - - - - - A A T T
```

Supplementary Fig.7 The transcripts of *Os01g0152951* in the two red rice (M16_R, Q100_R) and two white rice (Q76_W, 297_W).



Supplementary Fig.8 Phylogenetic tree of the 120 genomes inferred from whole-genome SNPs, including 10 wild rice, 32 red rice, 20 black rice, 16 weedy rice, and 42 white rice. Different subspecies are indicated by color lines and the intermost circle represents ecotypes of accessions. Red, blue, black, and light yellow represent red rice, weedy rice, black rice, and white rice, respectively.