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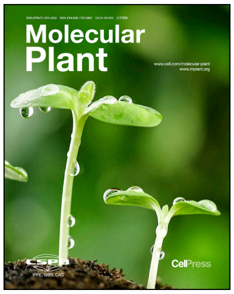

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Origination and establishment of a trigenic reproductive isolation system in rice

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Running title: Origin of a reproductive isolation system in rice

Dear Editor,

Reproductive isolation is both the indicator and a primary force of speciation, and plays a key role in maintaining species identity. Understanding the origin and mechanisms of reproductive isolation is of fundamental importance in evolutionary biology. In recent years, a number of genes that induce reproductive barriers have been identified in several model organisms such as Drosophila, rodents, yeast, Arabidopsis, rice and other species, which have greatly advanced our understanding of the mechanisms regulating reproductive isolation (Chae et al., 2014; Lafon-Placette and Kohler, 2015; Maheshwari and Barbash, 2011; Ouyang and Zhang, 2013 review thereof). However, little is known about how the genomes evolve to form reproductive barriers and how such systems become established in the populations, although such reproductive barriers are widely observed and demand understanding for both biological study of speciation and practical application in crop improvement.

The Asian cultivated rice (*Oryza sativa* L.) comprises two subspecies, *indica* and *japonica*. Hybrid sterility between *indica* and *japonica* represents one of the best-characterized examples of postzygotic reproductive isolation in plants. We previously reported a killer-protector system at the *S5* locus composed of three tightly-linked genes, *ORF3*, *ORF4*, and *ORF5*, which together regulated the female gamete fertility of *indica-japonica* hybrids (Chen et al., 2008; Yang et al., 2012). Typical *indica* and *japonica* varieties contain the haplotypes of *ORF3+ORF4-ORF5+* and *ORF3-ORF4+ORF5-*, respectively. The killer is made of *ORF4+* and *ORF5+*, which work together to kill the gamete, while *ORF3+* confers protection to the gametes, such that female gametes without *ORF3+* are selectively eliminated, causing hybrid sterility and segregation distortion.

Reciprocal BLASTN search using *ORF3*, *ORF4*, and *ORF5* as queries identified a unique set of three tightly-linked genes on chromosome 5 (designated as *Ospara3-5*) showing 73.72%, 67.74%, and 71.37% identities to *ORFs3*, *4*, *5* (chromosome 6), respectively (Supplemental Table S1). TBLASTN search identified another set of three genes locating adjacent to each other on chromosome 12 (designated as *Os345-like*), which showed less similarity to either *ORFs3–5* or *Ospara3–5*.

Sequences with significant similarity to either *ORFs3–5*, *Ospara3–5* or *Os345-like* were identified in other species by reciprocal blast (Supplemental Table S1). These similar sequences also located adjacent to each other, although they have frequently changed their relative orientations. One group of similar sequences showed higher similarity with *ORFs3–5* or *Ospara3–5* (labeled as para), and the other group was more similar to *Os345-like* (labeled as like). Therefore, ORF3–5/Ospara3–5 and Os345-like belonged to two different lineages, which led us to focus our attention to the ORF3–5/Ospara3–5 pair relationship in subsequent pursuit for the origin of S5. Based on phylogenetic trees, the Ospara3–5 were more closely related to most hits in the outgroup species than to ORFs3–5 (Supplemental Figures S1A, B, C). Thus the Ospara3–5 are likely to be more ancestral than the S5 locus, suggesting that ORFs3–5 were derived from Ospara3–5. Interestingly, we found a TBLASTN hit in *Z. latifolia* (ZlORF4), which belonged to the Oryzeae tribe, showed higher similarity to ORF4 than to other sequences (sequences labeled as para). Therefore, the three-gene fragment of the S5 complex might have originated from *Ospara3–5* after the formation of the Oryzeae tribe (Supplemental Figure S2).

A question thus arose: how did the *Ospara3–5* fragment duplicate to form the *ORFs3–5* block? We investigated the sequence similarity between the *ORFs3–5* and *Ospara3–5* fragments, which identified several intergenic regions showing high similarity between the two homologous blocks (Figure 1A, Supplemental Table S2). These regions contained multiple repetitive sequences of the RPO_OS *Helitron* transposon elements (http://www.girinst.org/) (Kapitonov and Jurka, 2007). We detected *Helitron* termini bracketing *Ospara4* and *Ospara5* (Hel_Os05g-A). And similarly, *ORF4* and *ORF5* were also flanked by *Helitron* termini (Hel_Os06g-A). This suggested a possibility that the *ORFs3–5* segment was derived by *Helitron* movement, carrying the internal *Ospara4–5* fragment on chromosome 5 and capturing the 3’-terminal *Ospara3*, to insert them on chromosome 6 thus generating a new fragment containing *ORFs3–5* (Supplemental Figure S3). This inference was partly supported by previous observations that *Helitron* transposition mediated by a rolling circle mechanism may fail to recognize the assumed 3’ palindrome termination signal and to capture the DNA fragment downstream (Kapitonov and Jurka, 2007). After duplication through *Helitron* transposition, a combination of mutational mechanisms acted together to recreate the three genes of the *S5* locus from *Ospara3–5* (Supplemental Figures S4A, B, C), thus conferred functional divergence to the newborns giving rise to reproductive barriers.

To learn how the three genes evolved into a reproductive barrier, we analyzed patterns of variation and diversity of *ORFs3–5* in 635 rice accessions from 62 geographically diverse countries, representing two cultivated and 11 wild species (Supplemental Table S3, S4). The *ORF3+ORF4+ORF5+* was the most frequent type (56.94%) in the wild species (Supplemental Table S5), and *ORF3+*, *ORF4+*, and *ORF5+* were the most frequent alleles in both *O. sativa* (0.6735, 0.4822, and 0.5797) and wild species (0.9306, 0.7222, and 0.7639) (Supplemental Table S6), suggesting that they may be either the ancestral alleles or under selection. We further analyzed the functional Indels/SNPs of *ORFs3–5* in their parent genes. Interestingly, the 11-bp sequence characteristic of *ORF4+* and functional SNP of C819 characteristic of *ORF5+* were identified in *Ospara4* and *Ospara5*, respectively, suggesting that they were the ancestral alleles (Supplemental Figure S4D) (Yang et al., 2012).

The ancestral *ORF3+ORF4+ORF5+* represented a balance between killing and protecting. Therefore, mutation in the protector *ORF3+* alone would be unable to survive because of a functional killer. Thus the mutation of *ORF3+* to *ORF3-* must have occurred after the emergence of non-functional killer, either *ORF4-* or *ORF5-* (including *ORF5*n). Based on our data, a mutation that occurred in *ORF4+* in the ancestral populations resulted in *ORF3+ORF4-ORF5+* giving rise to the typical *indica* rice. Similarly, *ORF5-* emerged in the ancient population with *ORF3+ORF4+ORF5+* to generate the intermediate population carrying *ORF3+ORF4+ORF5-*. An important question was whether *ORF3-* arose in the population carrying *ORF3+ORF4-ORF5+* or the one with *ORF3+ORF4+ORF5-*. The absence of *ORF3-ORF4-ORF5+* ruled out the first possibility. In contrast, *ORF3-ORF4+ORF5-* was observed in high frequency indicating that the *ORF3-* allele arose in the population carrying *ORF3+ORF4+ORF5-*, thus generating the typical *japonica* genotype *ORF3-ORF4+ORF5-* and subsequently spread in the population (Supplemental Figure S5). The inference was well supported by the phylogenetic tree (Supplemental Figure S6) and the co-existence of *ORF3+ORF4+ORF5-* and *ORF3-ORF4+ORF5-* in wild rice accessions from Jiangxi Province of China (Supplemental Table S3). Consequently, reproductive barrier occurred in hybridization between the populations carrying *ORF3+ORF4-ORF5+* and ones with *ORF3-ORF4+ORF5-*.

An interesting question now is: what are the evolutionary forces that drive the mutually incompatible haplotypes, *ORF3+ORF4-ORF5+* and *ORF3-ORF4+ORF5-*, to high frequencies to establish such at the population level? We investigated variation patterns of 4000 SNPs in regions flanking *S5* (~154-kb) in 529 accessions of *O. sativa* (http://ricevarmap.ncpgr.cn/django/home/) (Figure 1B). A dramatic decrease in genetic variation was observed around *S5* region (~44kb) in the *indica* subgroup, which is in contrast to the patterns observed in other rice groups especially compared to the temperate *japonica* subgroup. We further conducted tests for selection in different rice lineages (Supplemental Table S7). Significant negative values were detected for all the three genes at *S5* locus in *indica* rice using Fay and Wu's H, Fu and Li's D* and F* tests, suggesting that the spread of the *indica* haplotype might be driven by natural selection, possibly due to fitness advantage. We also determined whether demography is responsible for the departure from neutrality of *S5* locus in *indica* rice. The coalescent simulation results revealed significant values for Fay and Wu's H, Fu and Li's D* and F* tests in *indica* populations for all the three genes at *S5* locus (Supplemental Table S8). Taken together, the results suggested that the *indica* haplotype of *S5* locus was under strong positive selection during the evolution and/or rice breeding. Such positive selection may have provided a driving force leading to the establishment of *ORF3+ORF4-ORF5+* to dominate the *indica* group.

By contrast, the *japonica* haplotype *ORF3-ORF4+ORF5-* also reached a high frequency after origination within a short period of time. However, using the allele frequency spectrum tests and demographic simulations, not all the tests returned significant values for the three genes, suggesting that selection might not be responsible for the spread of the *japonica* haplotype. Therefore, the preservation of typical *japonica* genotype (*ORF3-ORF4+ORF5-*) might be due to the founder effect resulting from domestication of the *japonica* rice. This inference was highly plausible when considering that *japonica* subspecies had experienced a severe bottleneck during domestication of rice (Huang et al., 2012; Zhu et al., 2007).

In summary (Supplemental Figure S7), the *S5* complex originated by duplication from *Ospara3–5* after the formation of the Oryzeae tribe, most likely through *Helitron* transposition. A combination of mutational steps generated incompatible *indica* and *japonica* alleles in pre-differentiated rice groups, giving rise to the trigenic reproductive isolation system. Natural selection in *indica* rice and founder effect associated with domestication in *japonica* populations increased the frequencies of incompatible alleles to form a functional reproductive barrier between the *indica* and *japonica* subspecies, eventually result in genetic differentiation and restructuring of rice genetic composition. This also suggests the possibility that domestication and artificial breeding in crops can lead to the early stages of speciation.

ACCESSION NUMBERS

XXXX to XXXX

AUTHOR CONTRIBUTIONS

YO and QZ conceived and designed the experiments; YO, GL, JM, CX, and HD performed the experiments, including the PCR amplification, DNA sequencing, and sequence assembling; YO, GL, JM, CX, and CZ analyzed the data; WX, XL, JX, and HS contributed reagents/materials/analysis tools; YO and QZ wrote the paper.

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CONFLICT OF INTEREST

All the authors declare that they have no conflict of interests.

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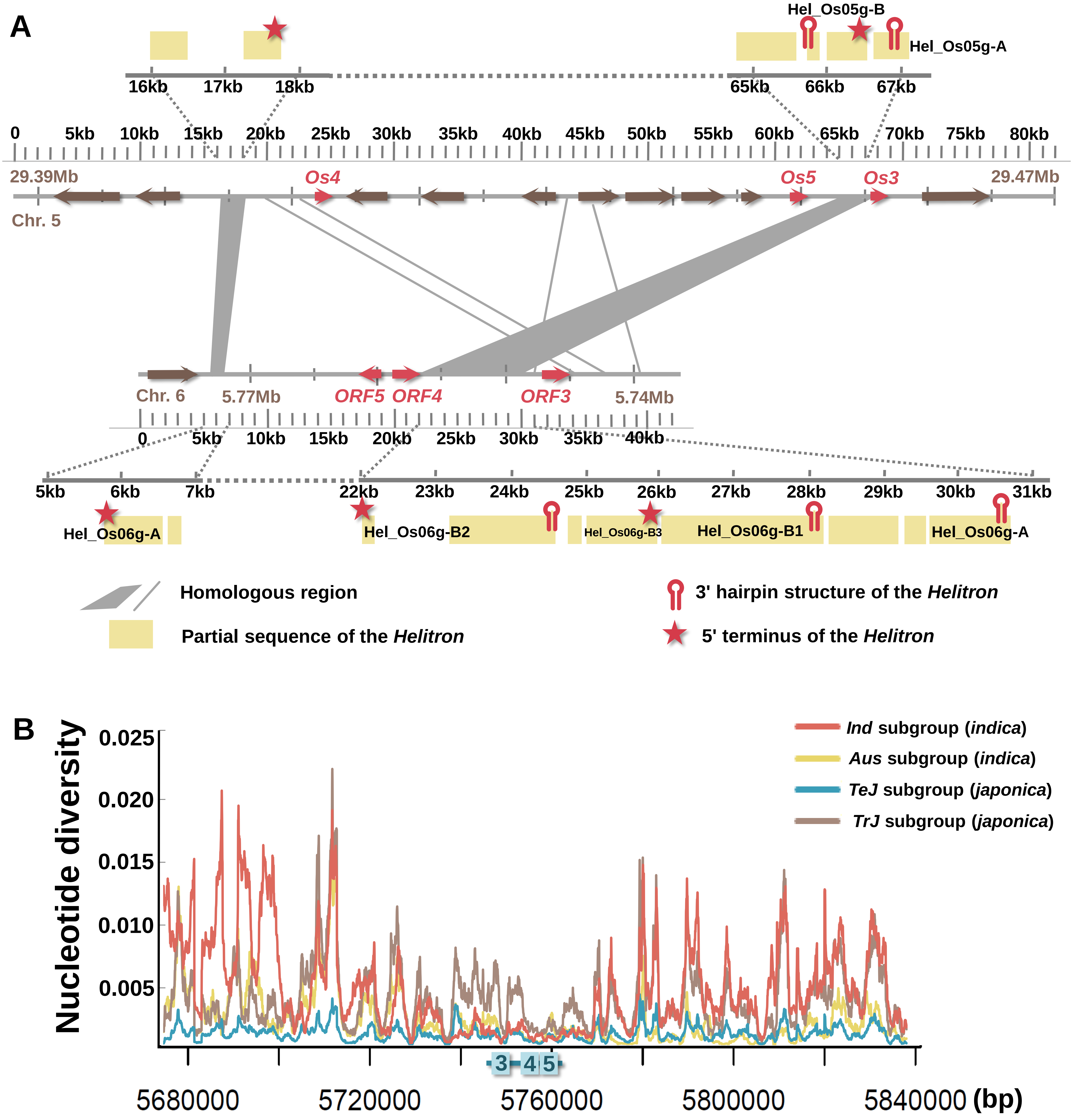
FIGURE LEGENDS

Figure 1. Sequence (A) and genetic variation (B) analysis in S5 region. Sequence analysis is performed between S5 and its paralogous regions in rice. The numbers in brown and black indicate genomic positions on the chromosomes (in Mb) and the relative scales (kb) in the analyzed regions, respectively. Nucleotide diversity is calculated based on polymorphism data flanking *S5* locus (4000 SNPs) in 529 rice accessions of *O. sativa*. The position of the *S5* region is shown at the bottom. See methods for details.



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