Parallel Speciation of Wild Rice Associated with Habitat Shifts

Zhe Cai,[†,1,2] Lian Zhou,[†,1,2] Ning-Ning Ren,[†,1,2] Xun Xu,[†,1,2] Rong Liu,[1] Lei Huang,[1] Xiao-Ming Zheng,[1] Qing-Lin Meng,[1,2] Yu-Su Du,[1,2] Mei-Xia Wang,[1,2] Mu-Fan Geng,[1,2] Wen-Li Chen,[1] Chun-Yan Jing,[1,2] Xin-Hui Zou,[1,2] Jie Guo,[1] Cheng-Bin Chen,[3] Hua-Zhong Zeng,[3] Yun-Tao Liang,[3] Xing-Hua Wei,[4] Ya-Long Guo,[1,2] Hai-Fei Zhou,[1] Fu-Min Zhang,[1,2] and Song Ge*[,1,2]

[1]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing, China
[2]University of Chinese Academy of Sciences, Beijing, China
[3]Guangxi Academy Agricultural Sciences, Nanning, Guangxi, China
[4]State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou, China
[†]These authors contributed equally to this work.
*Corresponding author: E-mail: gesong@ibcas.ac.cn.
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Abstract

The occurrence of parallel speciation strongly implies the action of natural selection. However, it is unclear how general a phenomena parallel speciation is since it was only shown in a small number of animal species. In particular, the adaptive process and mechanisms underlying the process of parallel speciation remain elusive. Here, we used an integrative approach incorporating population genomics, common garden, and crossing experiments to investigate parallel speciation of the wild rice species *Oryza nivara* from *O. rufipogon*. We demonstrated that *O. nivara* originated multiple times from different *O. rufipogon* populations and revealed that different *O. nivara* populations have evolved similar phenotypes under divergent selection, a reflection of recurrent local adaptation of ancient *O. rufipogon* populations to dry habitats. Almost completed premating isolation was detected between *O. nivara* and *O. rufipogon* in the absence of any postmating barriers between and within these species. These results suggest that flowering time is a "magic" trait that contributes to both local adaptation and reproductive isolation in the origin of wild rice species. Our study thus demonstrates a convincing case of parallel ecological speciation as a consequence of adaptation to new environments.

Key words: parallel speciation, reproductive isolation, adaptation, divergent selection, wild rice.

Introduction

The occurrence of parallel evolution, or the repetition of evolutionary changes in similar environments strongly implicates the action of natural selection in biological diversification because random genetic drift is unlikely to generate such a pattern (Rundle et al. 2000; Schluter 2009; Nosil 2012; Ostevik et al. 2012). As a special form of parallel evolution, parallel speciation involves independent formations of reproductive isolation in separate but closely related lineages/populations as by-products of adaptation to similar divergent environments, providing a convincing case for ecological speciation and for natural selection in generating reproductive isolation (Schluter and Nagel 1995; Johannesson 2001; Nosil 2012).

Nevertheless, demonstrating parallel speciation has been a challenging task because it requires in-depth analyses involving phylogenetics, phenotypes, ecology, and particularly the repeated formation of reproductive isolation between species (Schluter and Nagel 1995; Johannesson 2001; Rieseberg and Willis 2007; Nosil 2012). As noted in previous studies, a convincing example of parallel speciation would meet the following criteria (Schluter and Nagel 1995; Abbott and Comes 2007; Nosil 2012; Ostevik et al. 2012): 1) the populations in similar environments must be phylogenetically distinct and must arise from multiple origins rather than from gene flow caused by secondary contact of allopatric populations; 2) reproductive isolation must have established between descendent and ancestral populations; 3) reproductive isolation must not have been established between descendent populations; and 4) the shared characteristics in descendent populations must be proved to have evolved by natural selection. Despite well-characterized cases of parallel speciation in animals such as sticklebacks (Rundle et al. 2000; Colosimo et al. 2005), stick insects (Nosil et al. 2002; Soria-Carrasco et al. 2014), finches (Ryan et al. 2007), marine snails (Butlin et al. 2014; Ravinet et al. 2016), and cichlid fishes (Elmer et al. 2014; Meier et al. 2017), only a few cases have been documented in plants (Abbott and Comes 2007; Roda et al. 2013; Richards et al. 2016; Comes et al. 2017; Trucchi et al. 2017). Moreover, the adaptive process and mechanisms underlying parallel speciation remain largely unknown (Rundle et al. 2000; Schluter 2009; Nosil 2012).

The wild rice *Oryza rufipogon* and *O. nivara* offer a unique opportunity for studying adaptive divergence and ecological speciation (Sang and Ge 2007; Zheng and Ge 2010). They are the most closely related species in the rice genus *Oryza* and are collectively regarded as the wild progenitors of cultivated rice (*Oryza sativa* L.) (Morishima et al. 1992;

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Sang and Ge 2007; Vaughan et al. 2008). As a perennial, *O. rufipogon* is widely distributed throughout southern China, South and Southeast Asia, Papua New Guinea, and northern Australia, and grows in areas with year-round water, such as swamps and lakes; whereas *O. nivara* is an annual found within a more restricted distribution in South and Southeast Asia and usually inhabits ponds and swamps that dry up completely during the dry season (Sharma and Shastry 1965; Morishima et al. 1992; Vaughan et al. 2008). Within the same geographical area, the two species can be found to occur in close physical proximity, ranging from several kilometers to <100 m (Morishima et al. 1992; Kuroda et al. 2007; Vaughan et al. 2008). Therefore, the geographic distribution of the two species is of typical sympatry or parapatry, where the populations of the species pairs are found in different habitats of the same localities. Biologically, *O. rufipogon* is characterized by its tall stature, a mixed mating system, photoperiod sensitivity (late flowering), and profligate vegetative reproduction. In contrast, *O. nivara* is characterized by its short stature, predominant self-fertilization, and photoperiod insensitivity (early flowering) (Morishima et al. 1992; Sang and Ge 2007; Banaticla-Hilario et al. 2013). Despite marked differences in gross morphology and ecology, the two species are cross-compatible (Lu et al. 2000; Banaticla-Hilario et al. 2013) and exhibit little interspecific genetic differentiation (Barbier 1989; Zhu et al. 2007; Zheng and Ge 2010); thus their speciation process is incomplete.

Many lines of evidence suggest that the annual *O. nivara* originated from the perennial *O. rufipogon* as an adaptation to dry habitats (Barbier 1989; Morishima et al. 1992; Zheng and Ge 2010). In this process, many changes such as flowering time and mating system promoted assortative mating and acted as premating barriers to genetic exchanges between species (Morishima et al. 1992; Zheng and Ge 2010; Liu et al. 2015). A recent population genetic study (Liu et al. 2015) suggested that *O. nivara* might have originated multiple times, implicative of parallel speciation. Here, we studied all four criteria that must be fulfilled for parallel speciation using multiple approaches and provide a convincing case of parallel speciation in wild rice. In addition, our genomic and phenotypic data, in conjunction with demographic modeling demonstrate that multiple origins of *O. nivara* populations are associated with habit shifts during the last glaciation and that a change of flowering time in the descendant species is a key step during this process.

Results

Whole-Genome Resequencing and SNP Validation

We performed whole-genome resequencing of pooled samples (Pool-Seq) for 15 *O. rufipogon* and 9 *O. nivara* populations (fig. 1; supplementary table S1, Supplementary Material online; see Materials and Methods). We chose over 20 individuals per population for DNA pooling, except for seven populations for which 14–18 individuals were available (supplementary table S1, Supplementary Material online). To acquire sufficient high-quality mapping data, we used the latest version IRGSP 1.0 of the *Oryza sativa* genome

(Kawahara et al. 2013) as the reference and retained reads that were uniquely mapped to this reference with a mapping quality score >30. We produced 268-Gb high-quality data after trimming with an average sequencing depth of 30× per pooled sample and finally retained 6,168,151 polymorphic sites for subsequent analyses (supplementary table S2, Supplementary Material online; see Materials and Methods).

To assess the potential mapping bias in our data, we compared the mapping rate, genome coverage (with sites where the mapping depth ≥10), mismatch, and indel rate between mapped reads and the reference (supplementary table S2, Supplementary Material online) and did not find significant differences between species in these features (t-test, $P = 0.075$ for mapping rate, $P = 0.203$ for genome coverage, $P = 0.317$ for mismatch, and $P = 0.172$ for indels). Previous studies also showed that the two species had comparable genetic distances to the reference *O. sativa* without interspecies mapping bias (Huang et al. 2012; Guo et al. 2016). These observations indicated that mapping bias did not influence SNP calling.

To further validate the reliability of our SNP calling and, hence, our allele frequency estimation, we genotyped 271 individuals sampled from four population pairs (i.e., NEP1, NEP2, KHM, and LAO1) and one Chinese *O. rufipogon* population (rGX-bh) using Illumina Infinium BeadChip (Illumina Inc.). After excluding the sites in which calling rate <90% or Hardy–Weinberg equilibrium was rejected (Silva-Junior and Grattapaglia 2015), we successfully genotyped a total of 1,096 sites that were also detected by the Pool-Seq approach. For this subset of SNPs, we compared allele frequency estimates by Pool-Seq with estimates based on genotyping, and found high consistency between the two estimates (Pearson's correlation coefficient $r = 0.90$, ranging from 0.86 to 0.91 for individual populations; determination coefficient in linear regression $R^2 = 0.82$, ranging from 0.74 to 0.98), which validated the accuracy of allele frequencies determined by the Pool-Seq approach (Rellstab et al. 2013). On the other hand, because of the inherent limitation of distinguishing rare alleles from sequencing errors in Pool-Seq (Futschik and Schlötterer 2010; Lynch et al. 2014), we also applied different cut-off levels (i.e., 0.03, 0.05, 0.07) to rare alleles of the Pool-Seq data and obtained the same results in terms of the tree topology.

Phylogenetic and Population Analyses Revealed Multiple Origins of *O. nivara*

We performed phylogenetic analyses to distinguish between single and multiple origin hypotheses of *O. nivara*. Under the single origin (SO) hypothesis, populations of the two species should indicate reciprocal monophyly; in contrast, for the multiple origin (MO) hypothesis, populations from the same region should form a distinct clade (Schluter and Nagel 1995; Quesada et al. 2007). First, we conducted phylogenetic analyses based on Pool-Seq data and revealed that the *O. nivara* populations did not form a monophyletic clade but rather grouped together in accordance to the geographic region where they were collected (fig. 2a). This result did not change when different levels of missing data (supplementary fig. S1, Supplementary Material online) and various distance parameters (supplementary fig. S2, Supplementary
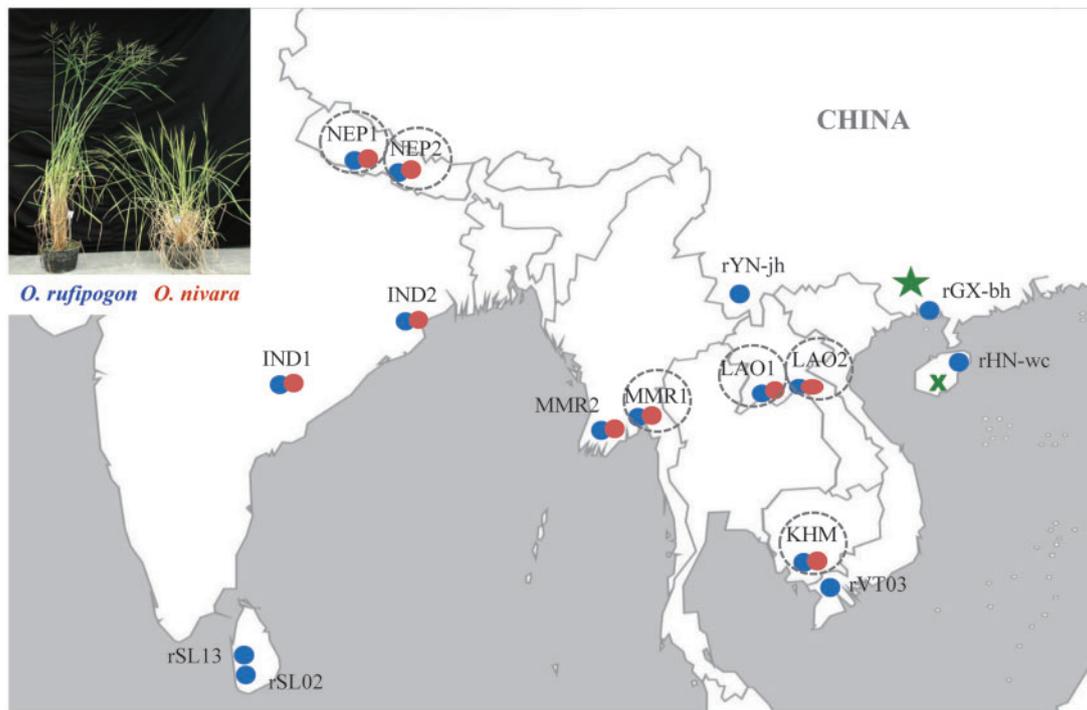
Fig. 1. Sampling locations of *Oryza rufipogon* (blue) and *O. nivara* (red) populations used in this study. Inset photo of perennial *O. rufipogon* (left) and annual *O. nivara* (right) plants originally published in *Journal of Systematics and Evolution* (2013, 51: Cover) and adapted with permission. Circles denote the six population pairs of *O. rufipogon* and *O. nivara* used in the common garden experiment, of which three pairs (i.e., NEP1, LAO2, and KHM) were used for artificial crossing. The green star and cross show the locations where the common garden experiment and the artificial crossing study were conducted, respectively. Detailed information on all the populations is listed in supplementary table S1, Supplementary Material online.

Material online; see Materials and Methods) were used, suggesting that *O. nivara* might have originated multiple times in different regions.

Given the potential limitation of the Pool-Seq approach in detecting low-frequency alleles (Lynch et al. 2014; Fracassetti et al. 2015), we then used the combined sequences of 16 neutral genes to reconstruct the phylogenetic relationship of six population pairs of the two species (see Materials and Methods). The resulting trees showed that the *O. nivara* populations did not form a monophyletic clade; instead, they were more closely related to geographically neighboring *O. rufipogon* populations (fig. 2d and supplementary fig. S3, Supplementary Material online). Separate analyses on individual genes clearly indicated that none of genes supports the reciprocal monophyly of the two species (supplementary fig. 4a, Supplementary Material online) although exact relationships among populations were unclear due to low resolutions of genes, which arose from recent species divergence and ancient polymorphisms (Zheng and Ge 2010). In addition, all *O. nivara* populations clustered with the neighbored *O. rufipogon* populations in the species tree that inferred from gene trees (supplementary fig. 4b and c, Supplementary Material online). STRUCTURE analysis showed that the best supported clustering ($K = 2$) suggested the deepest divergence between two regions (South Asia vs. Southeast Asia) rather than between the two species (fig. 2d and supplementary fig. S5a, Supplementary Material online), again supporting the MO hypothesis of the *O. nivara* origin. Principal

coordinate analysis (PCoA) based on these sequences also indicated that all the samples were organized into two distinct groups that corresponded to their geographic origins rather than their species identity (fig. 2e), suggesting that the *O. nivara* populations have evolved in parallel in different regions. Together, these results indicated that at least two independent origins of *O. nivara* occurred, with one in South Asia and the other in Southeast Asia.

Test for Alternative Hypotheses for Nonmonophyly

Although nonmonophyly of populations, that is, clustering of individuals from different populations by region rather than by species, is generally interpreted as multiple (parallel) evolution, a single origin with subsequent gene flow between species cannot be excluded entirely (Johannesson 2001; Schluter 2009; Nosil 2012). As shown in figure 3a, under the SO model, an ancestral population diverged into different species before they colonized in multiple regions, with or without an initial period of allopatry; in contrast, under the MO model, an ancestral population colonized multiple regions and then diverged into different species within regions.

To distinguish between the alternative models, we followed the methods by Roda et al. (2013) and Ravinet et al. (2016) to investigate the phylogenetic patterns at neutral, outlier, and linked loci which were assumed to reflect different origin models (fig. 3b). The outlier loci (candidates of selection) were defined as the sites where allele frequency was significantly different between species and the linked
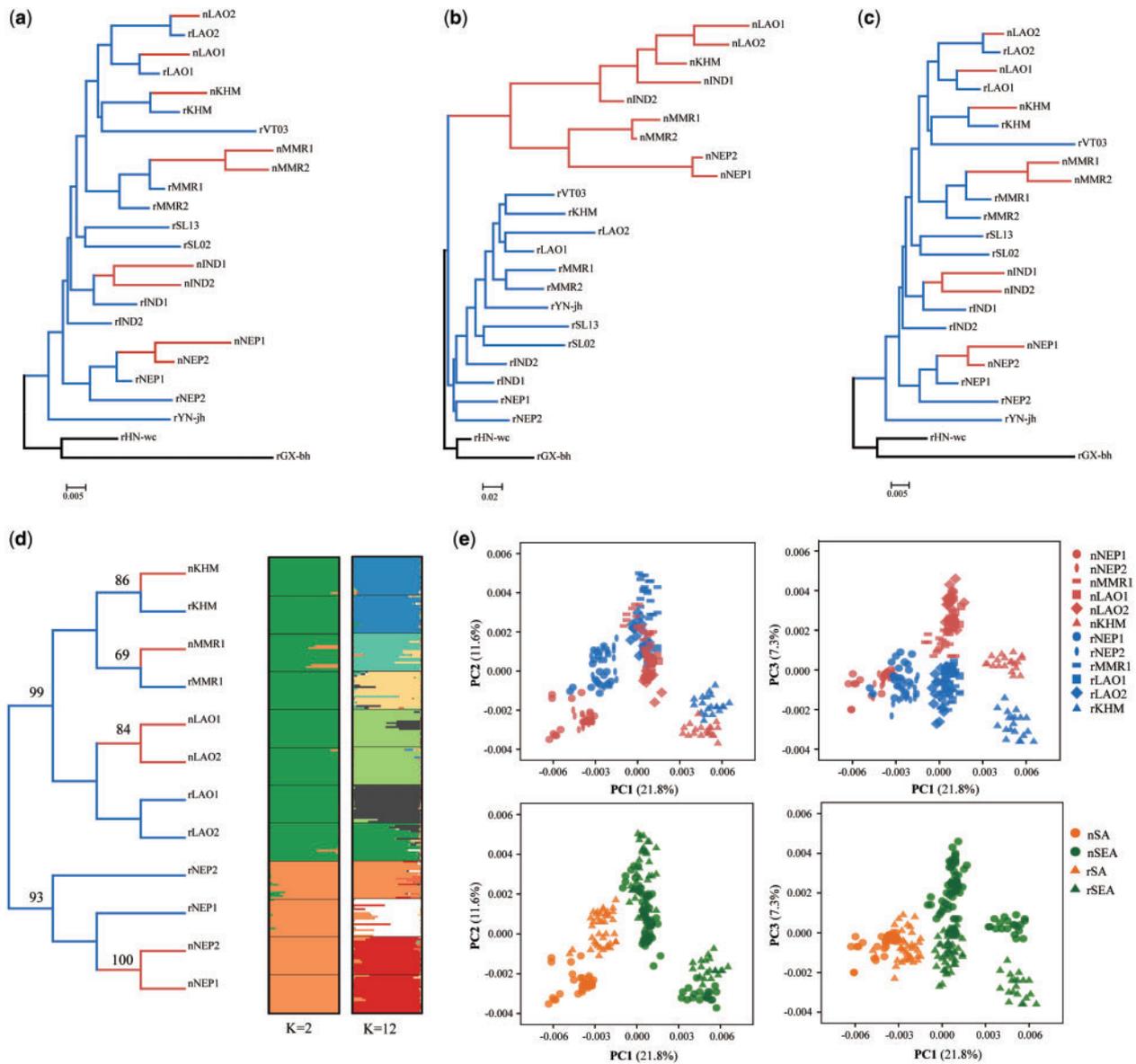
FIG. 2. Phylogeny of 15 *Oryza rufipogon* (blue) and 9 *O. nivara* (red) populations based on whole-genome and population sequencing data. (*a*–*c*) Neighbor-joining trees constructed with Wright's F_{ST} distance based on neutral (*a*), outlier (*b*), and linked (*c*) SNPs of whole-genome resequencing data. (*d*) Neighbor-joining tree inferred based on concatenated sequences of 16 nuclear neutral genes (bootstrap values are shown for nodes with >60% support), and the model-based population assignment at $K = 2$ set to the highest ΔK value and $K = 12$ set to the number of populations, where each horizontal line represents an individual, with its assignment probability to genetic clusters represented by different colors. (*e*) Principal coordinate analysis (PCoA) of all individuals of six population pairs based on sequences of 16 neutral genes indicate that 12 populations form two groups corresponding to geography rather than to species. SA and SEA represent South Asia and Southeast Asia, respectively. Population names correspond to the population IDs in figure 1 and supplementary table S1, Supplementary Material online.

loci were those tightly linked to outlier loci (see Materials and Methods). The results indicate that the two rice species show reciprocal monophyly in the tree constructed from the outlier SNPs (fig. 2*b*); whereas the tree that was constructed based on linked SNPs (fig. 2*c*) generated a similar phylogeny to that based on neutral SNPs (fig. 2*a*), that is, populations from the same region clustered together, which is a pattern consistent with the MO model (fig. 3*b*). These results remained the same when different genetic distances, different criteria of grouping SNPs, and various missing levels were used (supplementary figs. S1, S2, and S6, Supplementary Material online; see Materials and Methods).

To further verify that the nonmonophyly of the *O. nivara* populations represents multiple origins rather than local gene flow due to secondary contact, we used the sequence data of 16 neutral genes to perform approximate Bayesian computation (ABC) for distinguishing the alternative models of origin for two groups of populations: one group consists of two population pairs in Nepal (NEP1 and NEP2) (hereafter, South Asia) and the other group consists of three population pairs in Cambodia and Laos (KHM, LAO1, and LAO2) (hereafter, Southeast Asia). The two groups of populations, geographically separated by >2,000 km (fig. 1), are independent lineages in the trees and thus represent two independent origins
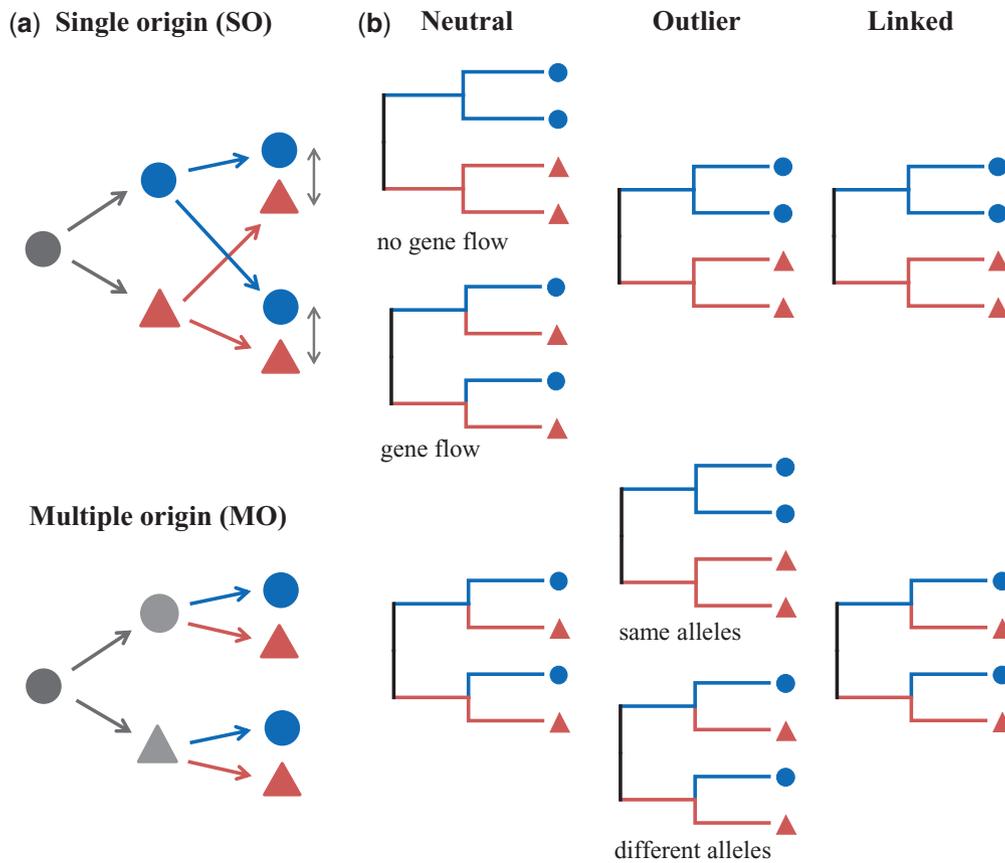
FIG. 3. Schematic diagram and test for single origin (SO) and multiple origin (MO) models. (*a*) Species divergence may arise in face of gene flow after secondary contact between two species that were already divergent in allopatry (the SO model) or may occur multiple times in sympatry (the MO model). (*b*) Test for alternative models of species origin. Both neutral and outlier SNPs generate similar phylogenies because of gene flow and different alleles at a locus; whereas linked SNPs give rise to different phylogenetic trees under different models of origin. Blue dots and red triangles represent *Oryza rufipogon* and *O. nivara* populations, respectively. Single-headed arrows stand for colonization or migration, and double-headed arrows indicate the gene flow between populations.

of *O. nivara* (fig. 2d). For either MO or SO model, we considered different scenarios involving population split events only (Standard scenarios), fluctuating population sizes through time (Demography scenarios), and gene flow between paired populations (Migration scenarios) (supplementary fig. S7, Supplementary Material online; see Materials and Methods). We applied these models to different combinations of population pairs in the two regions (i.e., South Asia and Southeast Asia) from which two independent origins of *O. nivara* were assumed. In all six combinations of population pairs between regions, the posterior probability (PP) was much higher for the MO models than for the SO models (supplementary table S3, Supplementary Material online; see Materials and Methods). To further evaluate which MO model was more likely, we compared the PP values among three MO models and found that the Standard scenario (mean PP of 0.47, ranging from 0.31 to 0.64) and Demography scenario (mean PP of 0.51, ranging from 0.33 to 0.67) were equally supported with much greater possibility than that of the Migration scenario (mean PP was only 0.02), suggesting a negligible level of gene flow between the paired populations.

Adaptive Divergence of Phenotypic Traits and the Role of Natural Selection

To examine which phenotypic traits are significantly differentiated between species and if the divergence is an adaptive response to divergent habitats, we conducted a common garden experiment for six pairs of populations sampled from different localities (fig. 1). Linear mixed-effect models detected that 14 of 19 traits showed significant differences, and only three grain traits (weight, length, and thickness of grains), awn length, and the flag leaf length were not significantly different between species (fig. 4a and supplementary table S4, Supplementary Material online). Similarly, PCA showed that the combined samples are organized into two distinct groups (or species), with PC1 and PC2 accounting for 46.1% and 16.9% of the variance, respectively (fig. 5a). A three-way analysis of variance (ANOVA) (Butlin *et al.* 2014) on first two PCs and all 18 traits further indicate that all traits except for PC2 showed significant differences between species and the differences remain significant after correction for multiple testing, except for three traits (panicle exsertion, number of spikelets per panicle, and attitude of flag leaf). By contrast, no significant differentiation was found between regions within
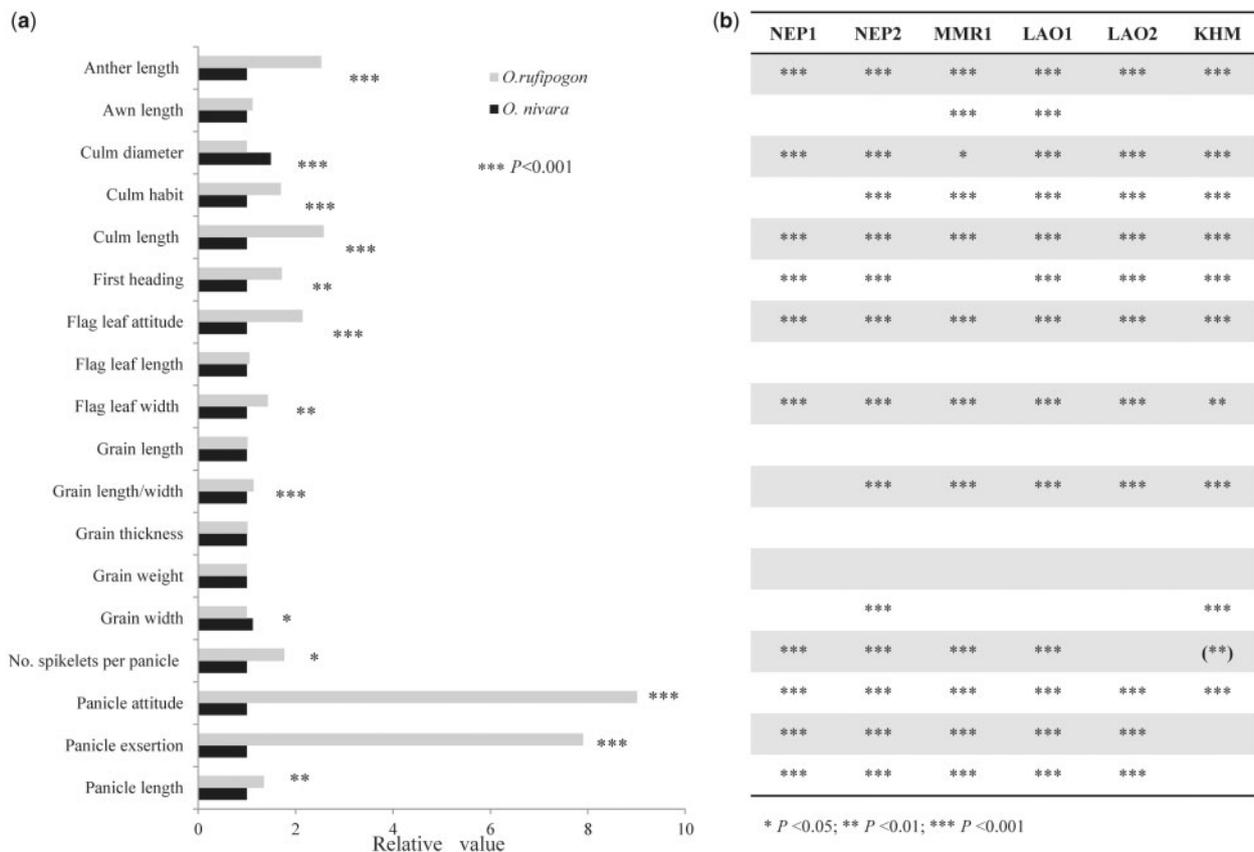
FIG. 4. Phenotypic divergence between *Oryza rufipogon* and *O. nivara* based on 18 quantitative traits recorded in the common garden experiment. (*a*) Divergence in mean phenotypic values of the combined samples. The species differences were standardized to the smaller value of the two species and thus the scale represents the relative difference between species for individual traits. (*b*) Significance (after correction for multiple testing) and direction of phenotypic divergence in six population pairs of *O. rufipogon* and *O. nivara*. Parentheses indicate that the direction of phenotypic divergence was opposite to that found in the combined samples.

species and between localities within regions for all traits (table 1). In general, relative to the perennial *O. rufipogon*, the annual *O. nivara* is shorter and thicker, flowers earlier, has shorter and less exserted panicles with more compactness, and has more spikelets per panicle with shorter anthers and awns (fig. 4a), consistent with previous reports (Grillo et al. 2009; Banaticla-Hilario et al. 2013; Guo et al. 2016).

Separate analyses on individual pairs of populations based on one-way ANOVA and χ^2 test obtained more striking differences between species (fig. 5b and supplementary table S4, Supplementary Material online), suggesting that phenotypic difference between the paired populations is associated with habitat differentiation. Importantly, almost all the traits that diverged between species showed remarkable concordance in the direction of phenotypic differentiation for all six population pairs (fig. 4b). It is noted that the six *O. nivara* populations consist of two groups originating independently from different areas as indicated earlier. These results imply the role of natural selection during phenotypic divergence between *O. rufipogon* and *O. nivara*.

To test the hypothesis that phenotypic differentiation between species is an adaptive response to divergent habitats, we performed Q_{ST}–F_{ST} analysis, a method that has been widely used to examine whether natural selection or genetic drift is the main cause of population differentiation in

phenotypic traits (McKay and Latta 2002; Leinonen et al. 2013). Based on the sequences of 16 neutral genes, we obtained an average F_{ST} value of 0.300 between species across six paired populations, with individual pair values ranging from 0.195 to 0.393 (supplementary fig. S8, Supplementary Material online). For the combined test, all 18 traits showed significantly higher Q_{ST} than F_{ST} (Z-test, $P < 0.0001$; supplementary table S5, Supplementary Material online). Separate analyses on individual population pairs revealed that 11 traits showed significantly higher values for Q_{ST} than for F_{ST} across all six pairs (ranging from 14 to all 18 traits) (supplementary fig. S9 and table S5, Supplementary Material online). The Q_{ST}–F_{ST} comparison therefore indicated that the parallel phenotypic divergence between species was an adaptive response to divergent habitats and driven by natural selection.

Premating Isolation Contributes to the Barrier of Gene Flow between Species

To determine whether reproductive isolation is established between the ancestral *O. rufipogon* and descendent *O. nivara* populations and between populations within species, we investigated both premating and postmating isolations for various comparisons of populations based on common garden and crossing experiments (see Materials and Methods). We found marked between-species differences in first heading
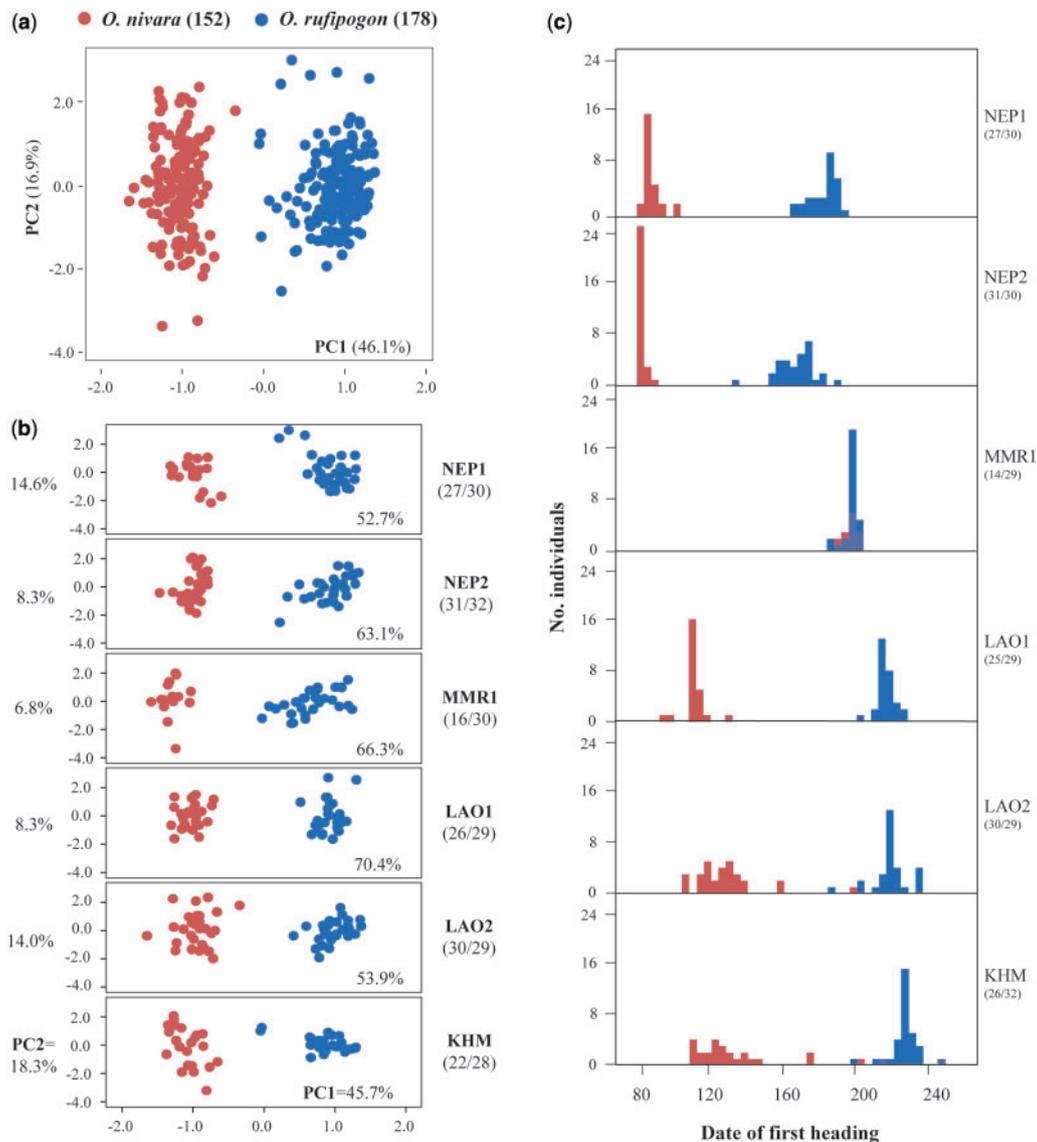
FIG. 5. Phenotypic variation and premating reproductive isolation between species. (*a* and *b*) Principal component analysis (PCA) of six pairs of *Oryza rufipogon* (blue) and *O. nivara* (red) populations based on 19 morphological traits recorded in the common garden experiment. All combined samples (*a*) and separate analyses on each of six paired populations (*b*) show the clear parallel phenotypic differentiation between species. Number of individuals (*O. nivara/O. rufipogon*) observed for the paired populations are in parentheses. (*c*) Frequency distribution of first heading of six population pairs in the common garden experiment. Bars indicate the number of individuals initiating flowering for *O. rufipogon* (blue) and *O. nivara* (red). Number of individuals (*O. nivara/O. rufipogon*) observed for the paired populations are in parentheses.

and flowering peak for five out of the six population pairs (fig. 5c). One exception is the population pair in Myanmar (MMR1), where the flowering time of the two species entirely overlapped (fig. 5c and supplementary fig. S10 and table S4, Supplementary Material online). A plausible reason is that the *O. nivara* populations in Myanmar represent a third origin that is different from the two origins we discussed here (i.e., one in South Asia and the other in Southeast Asia). This explanation is in agreement with the phylogenetic trees based on the Pool-Seq data (fig. 2a), in which two *O. nivara* populations in Myanmar (nMMR1 and nMMR2) formed a distinct lineage with two *O. rufipogon* populations in Myanmar (rMMR1 and rMMR2). An in-depth study of the hypothesis on the origin of the Myanmar *O. nivara* is currently underway and should provide further insights into the origin of this species.

On an average, the *O. nivara* populations flowered much earlier than the *O. rufipogon* populations, with the differences ranging from 84.3 (NEP2) to 105.6 (LAO1) days (supplementary table S4, Supplementary Material online). This difference in flowering time between species was consistent in two consecutive years under standard common garden conditions (supplementary fig. S10, Supplementary Material online). Notably, within each species, flowering time showed a latitudinal cline and overlapped, with first heading date depending on the latitude of the populations (fig. 5c), suggesting potential gene flow between populations/localities within species. Further analysis based on a three-way ANOVA revealed significant differences in flowering time for populations between species but not for populations within species whether they were from same or different regions (table 1). The Overlap

Table 1. Three-Way ANOVA for PC1, PC2, and 18 Phenotypic Traits.

Traits	Mean ± SE		Source of Variation				
	Oryza rufipogon	*O. nivara*	Species/Habitat	Region	Locality	Species × Region	Species × Locality
PC1	0.67±0.34	−1.31±0.36	2.08E-04***(*)	0.7	0.97	0.39	4.76E-11***(***)
PC2	0±0.82	0.01±1.29	0.99	0.56	0.95	0.31	1.38E-16***(***)
First heading	203.69±22.62	118.61±35.51	9.58E-05***(**)	0.25	0.33	0.05	1.68E-03**(-)
Attitude of panicle branches	40.82±12.81	4.53±12.29	3.40E-04***(*)	0.26	0.32	0.2	0.18
Panicle exsertion	10.43±5.58	1.32±3.46	5.81E-03**(-)	0.87	0.67	0.17	8.21E-04***(-)
Anther length	0.48±0.07	0.19±0.04	2.56E-07***(***)	0.55	0.96	0.07	0.21
Panicle length	27.56±4.38	20.41±4.51	3.20E-04***(*)	0.88	0.81	0.38	3.33E-12***(***)
Awn length	5.62±1.4	5.05±1.3	4.90E-05***(**)	0.41	0.95	0.12	9.04E-03**(-)
Culm length	179.78±32.13	69.77±22.09	5.37E-05***(**)	0.72	0.56	0.3	1.44E-12***(***)
No. spikelets per panicle	76.77±34.96	43.44±15.59	2.37E-03**(-)	0.76	0.62	0.48	3.52E-16***(***)
Grain weight	0.13±0.02	0.13±0.04	1.67E-04***(*)	0.4	0.51	0.37	6.82E-04***(-)
Grain length	8.03±0.5	7.94±0.69	7.32E-05***(**)	0.98	0.33	0.18	3.80E-06***(***)
Grain width	2.08±0.17	2.33±0.22	6.61E-06***(***)	0.8	0.95	0.43	1.38E-10***(***)
Grain length/width	3.91±0.37	3.44±0.19	0.0***(***)	0.89	0.39	0.96	1.16E-06***(***)
Grain thickness	1.49±0.1	1.47±0.15	1.30E-06***	0.65	0.52	0.59	1.31E-07***(***)
Culm habit	79.33±13.65	46.67±15.15	1.89E-05***(**)	0.44	0.63	0.44	1.29E-03**(-)
Culm diameter at basal internode	0.51±0.11	0.76±0.17	6.34E-06***(***)	0.72	0.4	0.22	0.03*(-)
Attitude of flag leaf	73.71±22.97	34.44±19.52	8.43E-04***(-)	0.82	0.55	0.76	3.87E-05***(**)
Flag leaf length	25.04±5	23.85±8.79	1.42E-04***(*)	0.7	0.74	0.23	8.44E-07***(***)
Flag leaf width	1.23±0.22	0.86±0.16	1.07E-05***(***)	0.38	0.67	0.07	0.04*(-)

Significance after correction for multiple testing is in the parentheses.
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Index (OI) (Pianka 1973) also shows no significant difference in flowering time between populations within species (supplementary table S6, Supplementary Material online).

We further chose three population pairs to conduct a crossing experiment to detect potential postmating isolation (see Materials and Methods). Based on a total of 238 artificial crosses involving those within populations, between populations within species, and between species, we found that the average seed set and F1 viability of the crosses between species were comparable to those of the crosses within species, although significant differences in seed set were found between the crosses with *O. rufipogon* as the maternal parent and those with *O. nivara* as the maternal parent (fig. 6 and supplementary table S7, Supplementary Material online). In addition, neither premating nor postmating reproductive barriers were observed between populations within both *O. rufipogon* and *O. nivara* (figs. 5c and 6), in agreement with previous reports (Lu et al. 2000; Banaticla-Hilario et al. 2013). Similarly, statistical analyses did not detect significant differences in average seed set and F1 viability between populations within species (supplementary table S7, Supplementary Material online). Together, these results indicate that the difference between species in flowering time caused almost completed premating reproductive isolation between species and contributed to the divergence of two wild species.

Discussion

Parallel speciation has been reported in animals (Johannesson 2001; Nosil 2012) and strongly suggests the action of natural selection in the process of speciation. However, few cases of parallel speciation have been demonstrated in plants, which might arise from the possibility that plants are less prone to parallel speciation than animals or reflect a lack of empirical studies involving rigorous testing (Abbott and Comes 2007; Ostevik et al. 2012). This study demonstrates an unequivocal case of parallel speciation in plants, in which all four criteria for parallel speciation are satisfied. One particular challenge to demonstrate parallel speciation is to distinguish between the multiple origin and the single origin following gene flow between species (Quesada et al. 2007; Nosil 2012; Roda et al. 2013; Butlin et al. 2014; Faria et al. 2014), which was overlooked in many previous studies. Using whole-genome resequencing and Sanger sequencing of population samples, we performed both phylogenetic analyses and ABC modeling to obtain solid evidence for supporting the multiple origin of the derived species (i.e., *O. nivara*). Our study of wild rice, together with an accumulating number of other cases in plants (Roda et al. 2013, 2017; Richards et al. 2016; Ru et al. 2016; Comes et al. 2017; Trucchi et al. 2017) imply that parallel speciation might not be uncommon in plant species.

The evolution of reproductive isolation is the most important step in the formation of new species (Rieseberg and Willis 2007; Nosil 2012). In addition to our common garden study that identified almost complete premating reproductive isolation between species, a literature survey on the phenology of wild populations further indicates marked differences in flowering time between the species across their entire distributional regions, with *O. nivara* flowering significantly earlier than *O. rufipogon* (Sharma and Shastry 1965; Barbier 1989; Lu 1998; Kuroda et al. 2006). Such a premating isolation mechanism in plant speciation has been reported in many previous investigations (Hall and Willis 2006; Lowry et al. 2008; Moyers and Rieseberg 2016; Ferris et al. 2017),
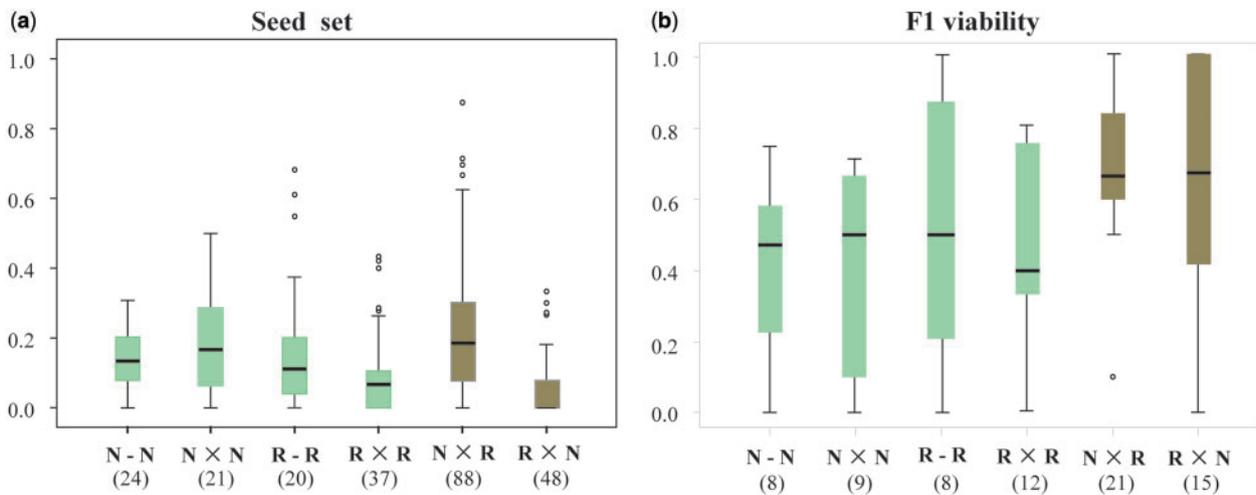
Fig. 6. Postmating reproductive isolation between species. Boxplots showing average seed set (*a*) and F1 viability (*b*) of crosses for various combinations of the three pairs of populations, respectively. Boxes and horizontal bars represent the central 50% and the median of the data, respectively. Dots represent outliers beyond 1.5 times the interquartile range. "N-N" and "R-R" represent crosses between individuals from the same population within *Oryza nivara* (N) and *O. rufipogon* (R), respectively. "N × N" and "R × R" represent crosses between individuals from different populations within the same species. "R × N" and "N × R" (dark columns) represent crosses between individuals from different species (with *O. rufipogon* as the maternal and paternal parents, respectively). Number of crosses for a specific combination is in parentheses.

supporting the notion that prezygotic isolation was either a more important or earlier-evolving barrier to gene flow than was postzygotic isolation in plant speciation (Rieseberg and Willis 2007; Nosil 2012). It is evident that the phenotypic divergence and reproductive isolation between *O. rufipogon* and *O. nivara* are associated with habitat differences, as expected in ecological speciation (Schluter 2009; Nosil 2012; Moyers and Rieseberg 2016). Based on a quantitative trait locus (QTL) analysis of eight traits involving life history, mating system, and flowering time, Grillo et al. (2009) found that a total of 30 QTLs, with effect sizes ranging from 2.9% to 36.5%, contributed to the major phenotypic differentiation between *O. rufipogon* and *O. nivara*, with >80% QTL alleles of *O. nivara* acting in the same direction of phenotypic evolution. Our recent genome-wide expression investigation of *O. rufipogon* and *O. nivara* (Guo et al. 2016) revealed that of the 21,415 expressed genes across three tissues, ~8% (1,717 genes) differed significantly in expression levels between species and that 62% of the differentially expressed genes exhibited a signature of directional selection. These results demonstrate a complex genetic basis of the phenotypic divergence between the two species and suggest that these genetic changes were fixed under directional selection.

The parallel speciation in wild rice raises an interesting question of what drives the formation of new species. Previous studies hypothesized that *O. nivara* originated from *O. rufipogon* in association with an ecological shift from a persistently wet to a seasonally dry habitat during recent glaciations (Barbier 1989; Morishima et al. 1992; Zheng and Ge 2010; Banaticla-Hilario et al. 2013; Huang et al. 2013). This hypothesis is consistent with the estimated time of *O. nivara* origin (Zheng and Ge 2010) and species distribution modeling which suggested that precipitation and temperature were the main climate variables contributing to

the distribution of *O. nivara* (Liu et al. 2015), as well as the fact that many annual grasses evolved in response to the dry climate in monsoonal Asia in recent glaciations (Morishima et al. 1992; Liu et al. 2015). Of the three major mechanisms (escape, avoidance, and tolerance) through which plants adapt to drought, drought escape is to develop rapidly and reproduce before the onset of drought and thus is optimal for annual plants in environments with short growing seasons (Juenger 2013; Kooyers 2015). As such, flowering time is usually investigated as a measure of drought escape because earlier flowering results in greater fitness during the shortened growing season (Kooyers 2015). Flowering time changes can have widespread ecological consequences and adaptive responses to drought through flowering time shifts have been well-characterized in plant species (Hall and Willis 2006; Franks 2015; Kooyers 2015; Moyers and Rieseberg 2016; Ferris et al. 2017). For example, strong selection for earlier flowering was reported in two *Brassica rapa* populations following drought (Kooyers 2015). In a study of two ecotypes of *Mimulus gutttatus*, Hall and Willis (2006) found that early flowering was favored in an area where annual plants grew and was characterized by dry soils, demonstrating that divergent selection on flowering time contributed to local adaptation. Therefore, early flowering is a classic adaptation of plants to dry habitats and allows plants to complete reproduction before seasonal drought (Franks 2015; Kooyers 2015; Ferris et al. 2017). Similarly, flowering time in our case is a typical "magic trait" (Servedio et al. 2011; Nosil 2012; Moyers and Rieseberg 2016) that contributes both to adaption and to reproductive isolation. The nearly complete isolation in flowering time together with a difference in mating system (Sang and Ge 2007; Vaughan et al. 2008) provide strong premating barriers to gene flow between species and play critical roles in the *O. nivara* origin. Previous studies also found that an QTL with relatively large effect contributed to the

loss of photoperiod sensitivity in *O. nivara* (Grillo et al. 2009) and that genetic changes of seed storage protein genes in *O. nivara* provided an advantage for seedling establishment and survival in dry soil conditions (Huang et al. 2013). To summarize, this system provides an outstanding scenario for testing the underlying mechanisms of ecological speciation as well as what genes and gene networks and to what extent selection acts repeatedly during speciation.

Materials and Methods

Study System and Population Sampling

We sampled 15 *O. rufipogon* and 9 *O. nivara* populations that covered the major areas where the two species are sympatric (fig. 1). Of them, 18 populations were sampled by the authors during field collections from 2008 to 2011, in which both the leaf and seed samples were collected individually, with the population sizes ranging from 14 to 32. The remaining six populations were obtained from the International Rice Research Institute (IRRI) in Los Banos, Philippines, with sample sizes >14 individuals per population (supplementary table S1, Supplementary Material online). These IRRI samples were collected from localities with the same latitudes and longitudes and thus were treated as "populations" similar to previous investigations (Zheng and Ge 2010; Banaticla-Hilario et al. 2013; Liu et al. 2015). We conducted whole-genome sequencing of pooled DNA samples (Pool-Seq) for all 24 populations. Of them, six and three pairs of sympatric populations of the two species were used in a common garden experiment in Guangxi Province and an artificial crossing experiment in Hainan Province, respectively (fig. 1 and supplementary table S1, Supplementary Material online).

Common Garden Experiment and Analyses of Morphological Traits

To investigate phenotypic variation in populations within and between species and its correlation with habitats, we conducted a common garden experiment using six sympatric pairs of *O. rufipogon* and *O. nivara* populations at the Guangxi Academy of Agricultural Sciences (GAAS) in Nanning City (N22°50.72′, E108°14.85′), Guangxi Province, China, for three consecutive years (2011–2013) (fig. 1). These natural populations were sampled across four localities (Nepal, Myanmar, Laos, and Cambodia) by the authors, with the distance between two populations of each pair ranging from 1.3 to 44.9 km (supplementary table S1, Supplementary Material online).

Over 30 individuals, each descended from separately collected maternal plants, were used for all populations (supplementary table S1, Supplementary Material online). Seeds were germinated at 30°C in an incubator, and the young seedlings were then transplanted to pots at three-leaf stage and finally planted randomly in the experiment garden of GAAS. We measured 19 morphological traits (supplementary table S8, Supplementary Material online) for each individual based on the methods described in Bioversity International et al. (2007). To obtain detailed information on phenotypic

variation of all populations, we conducted censuses every day throughout the entire growth season during the three years. All measurements were taken on three tillers/culms and averaged for each trait, with exceptions of first heading, seed traits and stolon trait (supplementary table S8, Supplementary Material online).

We used the linear mixed-effects models (*nlme* package in R) to analyze divergence of morphological traits at the species level. The species served as fixed effect and population as random effect in linear mixed model. For each population pair, phenotypic divergence was analyzed using ANOVA and χ^2 test (Guo et al. 2016). Phenotypic differentiation between species was also assessed by a standard principal component analysis (PCA) based on the combined and individual data sets. To examine how the phenotypic variation is partitioned between species/habitat, among localities within species and among individuals within populations, we further performed a three-way analysis of variance (ANOVA) (Butlin *et al.* 2014) on first two PCs and all 18 phenotypic traits, with habitat/species and region (Nepal, Myanmar, Laos, and Cambodia) as fixed factors, and locality (six sites where pairs of species populations were sampled) as a random factor within the interaction between fixed factors. To quantitatively assess the extent of flowering time-based reproductive isolation between populations, we calculated an Overlap Index (OI) (Pianka 1973) as detailed in Lobo et al. (2003).

Whole-Genome Resequencing, Variant Calling and SNP Validation

Genomic DNA was extracted from fresh or silica-gel dried leaves using the standard CTAB method. DNA pools were created for each of the 24 populations by mixing equal amount of individual DNA that was quantified using a Qubit 2.0 fluorometer. For sequencing, we sheared the pooled DNA and constructed ∼500-bp paired-end libraries using a Paired-End Sample Preparation Kit (Illumina, San Diego, CA) following the manufacturer's instructions. After removing the adaptor and primer sequences (Bolger et al. 2014), the reads were trimmed with a minimum base quality score 30 (error rate 0.001) and a minimum length 50 bp (Kofler, Orozco-terWengel, et al. 2011). The reads that lost their mates after trimming were also dropped. As a result, a total of 268-Gb high-quality data with a depth of 1.35× for each individual was generated (supplementary table S2, Supplementary Material online); this amount of data ensures the accurate estimation of allele frequencies according to empirical and theoretical studies (Futschik and Schlötterer 2010; Kofler, Orozco-terWengel, et al. 2011). The summary statistics of the raw data were calculated using *FastQC* v0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

The mapping and variant calling processes were conducted following the protocol proposed by Schlötterer (Schlötterer et al. 2014). The read mapping tool *bwa* v0.6.2-r126 (Li and Durbin 2009) and *samtools* v0.1.19 (Li et al. 2009) were used to map the reads to the reference genome (IRGSP 1.0 of *Oryza sativa* genome; Kawahara et al. 2013), allowing a maximum of three mismatches and one gap for each mapping read. Only the reads that were uniquely mapped to the

reference and had a mapping quality score >30 were retained. Two different pipelines, *PoPoolation2* (Kofler, Pandey, et al. 2011) and *VarScan 2* (Koboldt et al. 2012) were adopted for variant calling. We removed duplicates showing identical 5′ coordinate (Fracassetti et al. 2015) using *Picard* (http://broadinstitute.github.io/picard/) and indels together with ten flanking nucleotides to further reduce false positives in variant calling. To validate the reliability of these SNPs, an Illumina Infinium BeadChip (Illumina Inc.) was customized using the probes designed from rice whole-genome resequencing data (Huang et al. 2012). All the manipulations were carried out following the manual. Genotyping calling was analyzed using software Genome Studio (Illumina Inc.). The markers whose call rate <90% or violating Hardy–Weinberg equilibrium were excluded (Silva-Junior and Grattapaglia 2015). The consistency of allele frequencies between Pool-Seq and genotyping data was evaluated by Pearson's correlation coefficient and linear regression.

Sequencing of 20 Neutral Genes and Population Genetic Analysis

We sequenced fragments of 20 single-copy nuclear genes for 20 individuals from each of the 12 populations that were used for the common garden experiments (supplementary table S1, Supplementary Material online). These nuclear genes are unlinked loci randomly distributed on all 12 chromosomes of rice, and most of them were demonstrated to evolve neutrally in previous studies (Zhu et al. 2007; Zheng and Ge 2010; Liu et al. 2015); thus, they were suitable for a population genetics study. Additional information on the sequenced regions of all loci and the primers used for amplification is provided in supplementary table S9, Supplementary Material online.

Genomic DNA extraction, polymerase chain reaction (PCR) amplification and sequencing of the genes followed our previous studies (Zheng and Ge 2010; Liu et al. 2015). Sequencing was performed on an ABI3730XL automatic sequencer (Applied Biosystems Corp.). Purified PCR products were sequenced either directly or after cloning into pGEM T-easy vectors (Promega Corp.) if dual peaks were found due to some heterozygous individuals (Zhu et al. 2007; Zheng and Ge 2010). Singletons produced by direct sequencing were confirmed through repeated PCR amplification and sequencing (Zheng and Ge 2010; Liu et al. 2015). The sequenced fragments were assembled by ContigExpress (Informax Inc., North Bethesda, MD) and edited manually. All sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/), with the accession numbers KX275412–KX276089.

We tested the neutrality of all 20 loci in our samples using Tajima' D (Tajima 1989) and Fu and Li's D* and F* (Fu and Li 1993). Although all these fragments did not show significant derivation from neutrality at the population level, we found significant values for either Tajima's D or Fu and Li's D* and F* at one or a few of the specific populations for four loci (GELP80, P14, PK, T4) (supplementary table S10, Supplementary Material online). Despite the fact that significant values for the parameters might arise from different factors other than nonneutrality, we conducted our population genetic analyses by excluding these four loci. Based on the sequences of the 16 neutrally evolved loci, we calculated the number of segregating sites (S), number of haplotypes (h), nucleotide diversity π, θ_w, and F_{ST} by DNASP v5.1 (Librado and Rozas 2009).

To examine the genetic subdivision of six pairs of populations, we conducted Bayesian clustering analysis using STRUCTURE 2.23 (Pritchard et al. 2000) and performed principal coordinate analysis (PCoA) implemented in DARWIN (Perrier and Jacquemoud-Collet 2006). For STRUCTURE analysis, we modeled K values from 1 to 15, with 50,000 burn-in iterations followed by 300,000 Markov chain Monte Carlo (MCMC) iterations for accurate parameter estimates. To verify the consistency of the results, we performed 20 independent runs for each K value using an admixture model with correlated allele frequencies. The uppermost hierarchical level of the genetic structure was estimated using the statistic ΔK following the procedure described by Evanno et al. (2005).

Phylogenetic Analysis

We performed phylogenetic analyses based on the Pool-Seq and population sequencing data sets to reveal the genetic affinities among populations and to distinguish between the SO and MO hypotheses of *O. nivara*. Under the SO hypothesis, populations of the two species should indicate reciprocal monophyly; in contrast, for the MO hypothesis, populations from the same region should form a distinct clade.

For the Pool-Seq data, we generated the Neighbor-Joining (NJ) tree, as implemented in *phylip* v3.69 (Felsenstein 1989), based on the neutral SNPs (see below); this method was used because the neutral loci were desirable for inferring the shared ancestry and were free of the distortion role of selection on the affinity among populations (Beaumont and Balding 2004). For generating the population trees, we calculated the widely used genetic distances based on the allele frequency of SNPs, including the classic Wright's F_{ST} (Hudson et al. 1992), Nei's G_{ST} (Nei and Chesser 1983), Hedrick's standardized G_{ST} (generally called G′st) (Hedrick 2005) and Jost's D (Jost 2008). As these relative measures relied on the diversity within population, the absolute measure of divergence d_{XY} was also calculated by dividing the number of pairwise nucleotide differences by the total length of the variable and invariable sites between populations (Nei and Li 1979). We also generated the trees under different missing levels (from 10% to 60%) that were defined as the proportion of missing data in 276 pairwise comparisons of the 24 populations.

For combined sequences of the 16 neutral genes, population trees were constructed using NJ and UPGMA methods by Mega 5 (Tamura et al. 2011). A maximum likelihood (ML) tree of single genes was also constructed using the RAxML program (Stamatakis 2014), which were used to generate estimated species-tree by using ASTRAL (Mirarab et al. 2014) at the individual and population levels (fig. S4).

Test for Alternative Hypotheses for Nonmonophyly

We used two approaches to distinguish between the SO and MO models. First, we test for the alternative models by generating phylogenies using different types of SNPs across the

genome (Roda et al. 2013; Ravinet et al. 2016). Specifically, we divided the genome-wide SNPs into three categories: outlier SNPs (candidates of selection), linked SNPs (tightly linked to outlier SNPs), and neutral SNPs. The outlier SNPs were defined as the sites where allele frequency was significantly different between species for one/three/five pairs of *O. rufipogon/O. nivara* populations (Fisher's exact test, FDR-corrected *P* value ≤0.05). Here, three criteria (one/three/five pairs) were used to define outliers because some SNPs met the criteria by chance. When the criterion was set to one pair, more SNPs would be defined as outlier even they were neutral in most other pairs; however, when five pairs used as the criterion, more real outliers will be omitted. For the linked SNPs, we empirically defined them as the SNPs that were <100/300/500 bp away from the nearest outlier SNPs. The neutral SNPs were then defined as the remaining SNPs. Phylogenetic trees were then constructed using the data sets from different combinations of the SNP grouping criteria. In the MO model, outlier sites would group populations either by species or by geography; whereas the neutral and linked sites would produce a phylogenetic pattern in which populations are clustered by geography rather than by species (Roda et al. 2013) (fig. 3*b*). We found that data sets with different combinations obtained the same conclusion that the *O. nivara* populations originated multiple times (supplementary fig. S6, Supplementary Material online). Thus, we retained in the main text only the result from the combination of the criterion "three pairs" and "300 bp", in which the number of three types of SNPs is 4,292,150 (neutral), 561,218 (outlier), and 1,314,783 (linked), respectively (fig. 2). Furthermore, phylogenetic analyses under different distances and various levels of missing data (from 10% to 60% of 276 pairwise comparisons of 24 populations) did not change our conclusion (supplementary figs. S1 and S2, Supplementary Material online).

In the second method, as described by (Butlin et al. 2014), we performed approximate Bayesian computation (ABC) that is likelihood-free and widely used in the parameter estimation and model selection of complicated evolutionary scenarios (Beaumont 2010; Csilléry et al. 2012) to compare different models for demographic history of the populations of two species based on the sequences of 16 neutral genes. Because two major models of *O. nivara* origin (fig. 3*a*) involve different scenarios of demographic history, the extent and direction of gene flow, and the different split times between paired populations, three kinds of scenarios were considered for each major model with a total of 12 models (supplementary fig. S7, Supplementary Material online). In the first scenario, only the split event between populations was modeled (i.e., the standard scenario) (supplementary fig. S7, top panel, Supplementary Material online) and in two additional more complex scenarios, the history of population size (i.e., the demography scenario) and gene flow (i.e., the migration scenario) were modeled separately (supplementary fig. S7, middle and bottom panels, Supplementary Material online). The prior distribution of the model parameter was defined based on previous studies (Zheng and Ge 2010; Ai et al. 2012; Liu et al. 2015) and is listed in supplementary table S11, Supplementary Material online. Shared parameters between models had the same prior, and all the parameters were log uniform, with the exception of the split time and mutation rate, which were uniform. Generation time was set to one year. For each model, we performed 1.0e7 coalescent simulations using *fastsimcoal2* (Excoffier et al. 2013) by randomly drawing the parameter sets from the parameter space.

The summary statistics, calculated by *arlsumstat* in *Arlequin* v3.11 (Excoffier et al. 2005), were used to compare the simulated and observed data. These statistics included the number of segregating sites (S), nucleotide diversity (π), Tajima's D, and pairwise F_{ST} among populations. As a result, a total of 18 summary statistics were used. To capture the main information in the summary statistics and to avoid the noise introduced by large number of statistics, we tried to transform the summary statistics using partial least squares (PLS) method (Wegmann et al. 2010). However, we found that the information on variation from the summary statistics could not be recovered with subset of the PLS components, probably because of the lack of multicollinearity of our statistics. Thus, the raw summary statistics were used in our ABC analysis.

Selection between the MO and SO models was performed under each scenario by comparing their posterior probabilities (Wegmann et al. 2010; Csilléry et al. 2012; Butlin et al. 2014), which were estimated by the multinomial logistic regression method ("mnlogistic") with a tolerance rate of 0.001. The posterior probabilities among three MO models were further compared with test which MO model was more likely. As the posterior probability was an approximation in ABC method, we performed additional analysis to test the goodness of fit between our models and the data. We first evaluated whether ABC could distinguish between the MO and SO models by performing cross-validation under each scenario. The confusion matrices of cross-validation indicated that most models were assigned to their own categories, except for the migration scenario, in which the three SO models were slightly confused (supplementary fig. S11, Supplementary Material online). These plots demonstrated that the models were distinguishable by the ABC method in our analysis. We then adopted three methods to test the goodness of fit for the model: 1) to compute a *P* value according to the null distribution of the mean distance between observed and accepted samples (supplementary table S12, Supplementary Material online); 2) to make the posterior predictive checks by sampling 1,000 sets of parameters from their posterior distribution and simulating the summary statistics a posteriori using *fastsimcoal2*. The resulting distribution of each summary statistic should reproduce the observed value if the preferred model fits our data well (as judged by the *P* value) (supplementary table S13, Supplementary Material online); and 3) we plotted the first two PCs based on PCA of the accepted summary statistics of the preferred models (supplementary fig. S12, Supplementary Material online), and the result should match the observed summary statistics if the model fits our data. R package *abc* (Csilléry et al. 2012) was used to implement the ABC method.

Test for Natural Selection for Phenotypic Divergence between Species

We performed the Q_{ST}–F_{ST} comparison to test whether natural selection or genetic drift was the main cause of population differentiation in the phenotypic traits (McKay and Latta 2002; Leinonen et al. 2013). We first used the sequences of the neutral genes to measure genetic differentiation between species or between paired populations by F_{ST}, implemented in DNASP v5.1. Then, we estimated Q_{ST}, an analogue of F_{ST}, to measure the amount of variance among populations relative to the total variance in the quantitative traits (Leinonen et al. 2013). Specifically, we followed the method proposed by Spitze (1993) to calculate Q_{ST} according to $Q_{ST} = V_B/(V_B + 2V_W)$ for each trait, where V_B and V_W are the components of variance between and within species, respectively. The Q_{ST} values were obtained by 1,000 bootstrap iterations with each involving resampling across individuals within populations or species using nested ANOVA. The distribution of Q_{ST} was used to evaluate the significance of difference between Q_{ST} and F_{ST}. If the Q_{ST} value significantly exceeded F_{ST}, the hypothesis of natural selection driving species divergence was supported; in contrast, the neutral genetic differentiation between species was assumed if the Q_{ST} values did not differ significantly from F_{ST} (McKay and Latta 2002; Whitlock and Guillaume 2009; Leinonen et al. 2013).

Crossing Experiment and Analysis of Postmating Isolation

To examine the strength of postmating isolation, we conducted artificial crossing, including crosses within populations, between populations within species, and between species in 2016 and 2017 in the Lingshui Station (N18°30.6′, E110°2.4′) in Hainan Province, China (fig. 1). For this purpose, we choose to grow the seeds from the three pairs of *O. rufipogon* and *O. nivara* populations (i.e., NEP1, LAO2, and KHM) that were used in the common garden experiment, with each population consisting of five individuals (for a total of 30 plants). A total of 238 crosses were performed, including 44 intrapopulation, 58 interpopulation within species, and 136 interspecies crosses. Selfing of the panicles with (28 crosses) and without (40) castration were also conducted (supplementary fig. S13, Supplementary Material online).

Seeds were exposed to 50°C for five days to break dormancy and then soaked in culture dishes after the coats were peeled. Seedlings were transplanted into soil cultures until the trefoil stage and then were planted in buckets (32 cm in diameter and 30 cm in height) filled with field soil. Since *O. nivara* started flowering >30 days earlier than *O. rufipogon* in Hainan Province, we grew three lots of *O. nivara* seeds to ensure the flowering times of the two species matched. We emasculated the mother panicles between ∼7:30 and 10:00 AM, enclosed them in paper bags, and then pollinated emasculated panicles when the spikelets of paternal donors were producing pollens.

We examined the extent of postmating isolation by determining the seed set of crosses and the viability of F1 hybrids following the methods of Sobel and Streisfeld (2015). Seed set was calculated by counting the number of seeds per spikelet, and F1 viability was measured by the survival rate of the F1 hybrids of 73 combinations (399 seeds) representing various crossing types. Seeds that germinated were transplanted into soil culture material in the same way mentioned earlier. Seedlings were transplanted into the garden at the tillering stage. We defined the survival rate of F1 hybrids as the percentage of seeds that germinated and grew to the flowering stage with more than half of tillers heading. We calculated average seed set and F1 viability at the population and species levels and performed the *t*-test for significance by R language v3.1.2 (https://www.R-project.org/).

Supplementary Material

Acknowledgments

Author Contributions

S.G. designed the study. Z.C., L.Z., N.-N.R, and X.X. performed the research. Z.C. and S.G. analyzed the Pool-Seq data and conducted various simulations. L.Z., N.-N.R., R.L., Y.-S.D., M.-X.W., C.-B.C., H.-Z.Z., and Y.-T.L. performed the common garden experiments in Guangxi. N.-N.R., L.Z., L.H., C.-Y.J., J.G., and F.-M.Z. conducted the analyses of phenotypic and sequencing data. X.X., Q.-L.M., and M.-F.G. conducted the artificial crossing in Hainan. X.-M.Z., H.-F.Z., W.-L.C., R.L., L.Z., X.-H.Z., Y.-L.G., and X.-H.W. joined the field survey, sample collections, and lab assistances. S.G., and Z.C. wrote the manuscript.

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