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Divergence in flowering time is a major component contributing to reproductive isolation between two wild rice species (*Oryza rufipogon* and *O. nivara*)

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It is of critical importance for our understanding of speciation process to determine the forms of reproductive isolation and their relative importance in species divergence. *Oryza nivara* and *O. rufipogon* are direct ancestors of Asian cultivated rice and a progenitor-daughter species pair. Investigating the reproductive isolation between them provides insights into plant speciation and helps understanding of the rice domestication. Here, we quantitatively measured the major components of reproductive isolation between the two species based on common garden and crossing experiments for three pairs of sympatric populations in Nepal, Cambodia and Laos.We revealed significant differences in the flowering times between species pairs, with *O. nivara* flowering much earlier than *O. rufipogon*. A very weak reduction in seed set but no reduction in F1 viability and fertility were detected for the crosses between species relative to those within species. Moreover, we detected asymmetrical compatibility between species and found that emasculation significantly decreased pollination success in *O. nivara* but not in *O. rufipogon*. Our study demonstrates that the divergence between *O. nivara* and *O. rufipogon* is maintained almost entirely by the difference in flowering times and suggests that differential flowering times contribute to both habitat preferences and reproductive isolation between species.

reproductive isolation, flowering time, speciation, adaptation, *Oryza* species

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INTRODUCTION

Reproductive isolation, which is the basis for the biological species concept (Mayr, 1942; Coyne and Orr, 2004), is considered the essential reason for biodiversity; thus, the evolution of reproductive isolation is a fundamental question in evolutionary biology given its importance in determining gene flow and understanding the speciation continuum (Widmer et al., 2009; Nosil, 2012; Seehausen et al., 2014; Baack et al., 2015; Abbott, 2017). Reproductive isolation is also referred to as a barrier to gene flow and might be caused by multiple drivers and occur at different stages of the life

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cycle (Ramsey et al., 2003; Coyne and Orr, 2004; Baack et al., 2015; Ostevik et al., 2016; Pereira et al., 2019; Xie et al., 2019). Reproductive barriers are usually divided into those that prevent the formation of hybrids (prezygotic isolation) and those that reduce the fitness of hybrids (postzygotic isolation) (Rieseberg and Carney, 1998; Lowry et al., 2008; Widmer et al., 2009; Richards et al., 2016). Because reproductive isolation is affected by both internal (e.g., crossability, hybrid viability and fertility) and external (e.g., ecological and temporal isolation) barriers, determining the forms of reproductive isolation and the stages in which reproductive isolation occurs as well as the relative importance of the different isolation barriers is of critical importance to our understanding of the process and mechanism of speciation (Coyne and Orr, 2004; Lowry et al., 2008; Via, 2009; Sobel et al., 2010; Nosil, 2012; Baack et al., 2015; Xie et al., 2019).

Oryza rufipogon and *O. nivara* are collectively regarded as the wild ancestors of cultivated rice (*Oryza sativa* L.) and are two incipient species at the early stages of speciation with significant differences in morphology, life history and habitat preferences (Morishima et al., 1961; Sang and Ge, 2007a; Zheng and Ge, 2010; Liu et al., 2015). The perennial *O. rufipogon* is photoperiod sensitive (late flowering), predominantly outcrossing with high vegetative allocation and widely distributed in South China, South and Southeast Asia, Papua New Guinea, and northern Australia. In contrast, the annual *O. nivara* evolved from *O. rufipogon* and is less photoperiod sensitive (early flowering), self-fertilized with high reproductive allocation and co-occurs with *O. rufipogon* throughout South and Southeast Asia (Morishima et al., 1961; Sharma and Shastry, 1965; Morishima, 1985; Sang and Ge, 2007a; Vaughan et al., 2008; Zhou, 2019), *O. rufipogon* grows in areas with year-round water while *O. nivara* is usually found in seasonally dry habitats (Morishima, 1985; Sang and Ge, 2007b; Vaughan et al., 2008). Thus, these two species have sometimes been treated as two ecotypes, which reflects their different life history strategies (i.e., r- and k-selection) (Pianka, 1970; Morishima, 1985).

To date, many studies have been undertaken on *O. nivara* and *O. rufipogon* and have involved morphological variation and taxonomy, crossability and gene flow, and population genetic structure as well as population adaptation and speciation (Morishima et al., 1961; Chu et al., 1969; Morishima et al., 1984; Morishima, 1985; Barbier, 1989; Vaughan, 1989; Naredo et al., 1997; Lu et al., 1998; Cai et al., 2004; Kuroda et al., 2007; Zhou et al., 2008; Zheng and Ge, 2010; Huang and Schaal, 2012; Banaticla-Hilario et al., 2013; Liu et al., 2015; Samal et al., 2018; Cai et al., 2019). These studies clearly indicated that *O. nivara* was recently derived from *O. rufipogon* (about 0.16 million years ago) without apparent genetic differentiation between species, and supported the hypothesis that *O. nivara* originated from the adaptation of *O. rufipogon* to dry habitats during the last glaciations (Barbier, 1989; Zheng and Ge, 2010; Liu et al., 2015). Our recent study, which incorporated population genomics, common garden and crossing experiments further demonstrated that *O. nivara* originated multiple times and was associated with habitat shifts in different areas in South and Southeast Asia, providing a convincing case for parallel speciation in plants (Cai et al., 2019).

Despite these efforts, the forms and extent of reproductive isolation between *O. nivara* and *O. rufipogon* remain largely unclear. Based on population genetic studies, Zheng and Ge (2010) found bidirectional and recurrent gene flow between species, suggesting that these species were still under the process of divergence. Potential interspecific gene flow/introgression was also detected by studies of local populations (Kuroda et al., 2007; Zhou et al., 2008). These estimates of gene flow, however, were based on indirect inference from molecular markers, which were confronted with ancient polymorphism and population dynamics of the species as well as the sampling strategy (Hey, 2006; Zheng and Ge, 2010; Liu et al., 2015). A better understanding of species divergence and speciation requires accurate assessment of the current gene flow or reproductive barrier between species, including prezygotic and postzygotic isolation barriers in terms of the patterns and mechanisms. Although there have been some reports on flowering time variations in wild rice species (e.g., Sharma and Shastry, 1965; Barbier, 1989; Lu, 1996; Cai et al., 2004), no study of prezygotic reproductive isolation has been conducted based on well-designed experiments combined with standard statistical analysis. On the other hand, efforts to quantitatively assess postzygotic reproductive isolation have never been attempted despite numerous crossing experiments involving both cultivated and wild rice (Banaticla-Hilario et al., 2013; Xie et al., 2019). Of the numerous crossing experiments of the *Oryza* species with closely related cultivated rice, Banaticla-Hilario et al. (2013)'s study was thus far the most comprehensive investigation on the crossability patterns between and within *O. nivara* and *O. rufipogon* and provided valuable information on postzygotic barriers to gene flow within and between these species. They found that *O. rufipogon* exhibited high intraspecific crossability and was symmetrically compatible with *O. nivara* with regard to seed set. Nevertheless, this study used a limited number of samples (3 and 10 *O. nivara* and *O. rufipogon* accessions, respectively) and did not take into account the impacts of interplant differences within populations or the potential impacts of manipulation of emasculation during hand pollinations. All these factors might lead to biased evaluation of pre- and postzygotic isolations. Furthermore, in a recent study on parallel speciation of *O. nivara* (Cai et al., 2019), we reported that no significant differences were found for the crosses both between and within species in the overall

crossability, viability and fertility of F1 hybrids. However, a few important questions regarding the forms and relative contributions of individual components of reproductive isolations were not addressed based on in-depth analyses.

In the present study, we analyzed and quantitatively measured major components of reproductive isolation based on common garden and crossing experiments for three pairs of sympatric populations of *O. rufipogon* and *O. nivara* that represented the major areas in which the two species occur. Our objective was to determine the form and strength of reproductive isolation both between and within species by investigating prezygotic and postzygotic isolations for various comparisons. Specifically, we ask: (i) What is the exact strength of the prezygotic reproductive isolation between *O. rufipogon* and *O. nivara* given that strong divergence in flowering times have been reported previously between these two species? (ii) If present, how strong is the postzygotic reproductive isolation between these two species? (iii) What are the relative contributions of the different components of reproductive isolation to the total isolation barriers to gene flow? Resolving these questions not only provides additional insights into the process of population divergence and adaptation in wild rice species, but also facilitates the exploitation of wild rice resources and manipulation in rice improvement and breeding.

RESULTS

Variation in flowering time

We have previously conducted a common garden experiment on 14 *O. rufipogon* and 7 *O. nivara* populations in Nanning City, Guangxi Province, China (Cai et al., 2019; Ren, 2019). In this study, we analyzed the flowering times for three pairs of sympatric populations that were sampled from Nepal (NEP1), Laos (LAO2), and Cambodia (KHM) (Table S1 in Supporting Information). We found marked differences in the first heading and flowering peaks for each of the three population pairs (Figure 1). On average, the *O. nivara* populations flowered much earlier than the *O. rufipogon* populations, with differences of 81 (NEP1; NEP: Nepal), 79 (LAO2; LAO: Laos) and 88 (KHM; KHM: Cambodia) days in 2012 (Table S2 in Supporting Information). This difference in heading dates between pairs of populations was consistent in three consecutive years under the common garden conditions (Figure S1 in Supporting Information), suggestive of almost complete prezygotic reproductive isolation between the population pairs of the two species.

Self-pollinations with and without emasculation

We performed a total of 238 crosses, including 44 intra-population, 58 inter-population within species, 136 between-species crosses and 48 selfing (28 and 20 self-pollinations with and without emasculation, respectively) (Table 1; Table S3 in Supporting Information). First, we found that *O. nivara* generated significantly higher seed set than *O. rufipogon* under self-pollination (Figure 2; Table S4 in Supporting Information). The offspring of the self-pollination also had higher germination and seedling survival rates in *O. nivara* than in *O. rufipogon*, but the difference was not significant (Figure 2). We then compared the seed set of self-pollinations and the germination rate of the offspring of self-pollination with and without emasculation to evaluate the impact of emasculation on pollination success. As shown in Figure 2, the mean seed set of *O. nivara* significantly differed between the self-pollinations with emasculation (0.22±0.16) and those without emasculation (0.57±0.28) (p-value<0.001), while no significant difference in the mean seed set was found between the two types of pollinations (0.07 ±0.12 vs. 0.09±0.10) for *O. rufipogon*. Additionally, we did not find a significant difference in the germination rate of the offspring between the two types of self-pollinations for either species (Figure 2). These observations indicated that emasculation might affect the pollination success for the selfing *O. nivara* to some extent but not for the outcrossing *O. rufipogon*.

Seed set of the crosses within and between populations

Our crossing experiment included 238 cross pollinations (panicles), which were classified into 6 types of combinations (Table 1; Table S3 in Supporting Information). Despite large variations in the seed set for all types of crosses, we did not find a significant difference in seed set between the intrapopulation crosses and the interpopulation crosses for

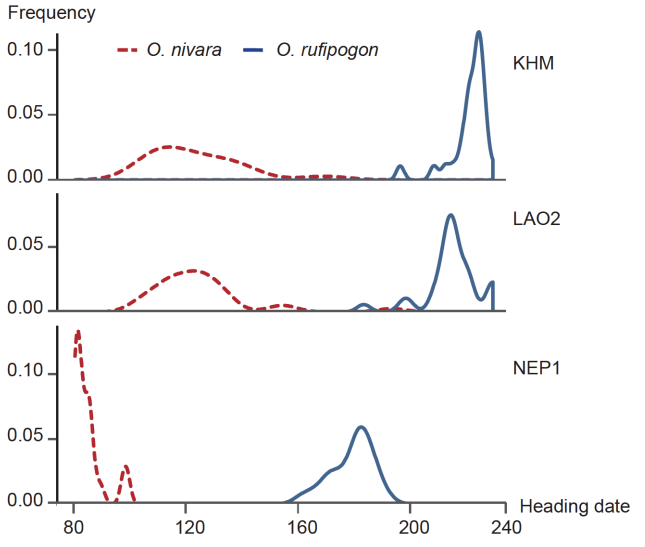

Figure 1 (color online) Frequency distribution of the first heading of three pairs of *O. rufipogon* (solid line) and *O. nivara* (broken line) populations in the common garden experiment in 2012.

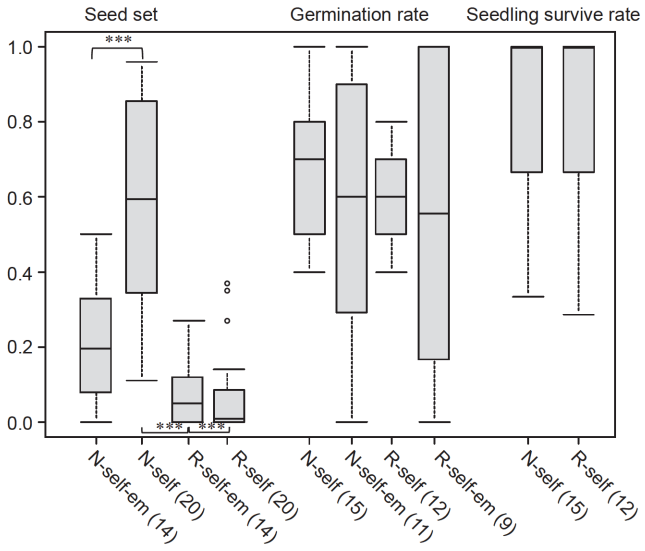

Figure 2 Seed sets and germination rates of the self-pollinations with and without emasculation.

either *O. rufipogon* (R) (i.e., R-R vs. R×R) or *O. nivara* (N) (i.e., N-N vs. N×N) (hereafter, R and N represent *O. rufipogon* and *O. nivara*, respectively) (Figure 3 and 4). In addition, no significant difference in seed set was found between the intraspecies crosses (N-N, N×N, R-R or R×R) and the interspecies crosses (N×R or R×N) (Table S5 in Supporting Information). These results indicated a weak level of the postzygotic barrier between these two species in terms of seed set.

It is noted, however, that the seed set of *O. nivara*×*O. rufipogon* (N×R) was significantly higher than that of R×N (Figure 3; Table S5 in Supporting Information), which implies unequal levels of gene flow between species. Such asymmetrical crossability patterns were reported in previous findings for wild rice (Naredo et al., 1997; Banaticla-Hilario et al., 2013) and in many other studies in which reciprocal crosses were conducted (Tiffin et al., 2001; Lowry et al., 2008; Martin et al., 2017).

Viability and fertility of F1 hybrids

We found large variation in all three components of F1 viability (i.e., germination rate, seedling survival rate and total survival rate) and fertility (i.e., F1 seed set) within each type of cross (Figure 3). The statistical analyses indicated that of the various comparisons, only those between N×N and N×R were significant in seedling survival and total survival rates, while all other comparisons did not have significant differences in any of the three components either within species (intra- and inter-populations) or between species (Table S6 in Supporting Information). Interestingly, we found that on average, the viability of F1 hybrids between species pairs, as measured by the germination and seedling survival rate, were slightly higher than those of their parents (Table S7 in Supporting Information), although the differences were not significant mainly due to the large variation

Table 1 Crossability of different types of artificial pollinations for six *O. nivara* and *O. rufipogon* populations[a)]

Type of crosses	Seed set		Germination rate		Seedling survival rate		Total survival rate		F1 seed set	
	N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE	N	Mean±SE
Self-pollination										
N-self	20	0.57±0.28	15	0.7±0.21	15	0.78±0.3	NA		14	0.33±0.1
R-self	20	0.07±0.12	12	0.53±0.27	12	0.47±0.46	NA		7	0.25±0.16
Total	40	0.32±0.33	27	0.62±0.25	27	0.64±0.4	NA		21	0.3±0.13
Self-pollination with emasculation										
N-self-em	14	0.22±0.16	11	0.56±0.38	NA		NA		NA	
R-self-em	14	0.09±0.10	9	0.54±0.41	NA		NA		NA	
Total	28	0.15±0.15	20	0.55±0.39	NA		NA		NA	
Cross pollination										
N-N	24	0.13±0.09	8	0.71±0.33	8	0.54±0.34	8	0.41±0.25	7	0.32±0.07
R-R	20	0.17±0.21	8	0.69±0.27	8	0.88±0.24	8	0.56±0.32	8	0.27±0.15
N×N	21	0.17±0.15	9	0.68±0.31	9	0.47±0.47	9	0.29±0.33	5	0.53±0.15
R×R	37	0.09±0.12	12	0.63±0.33	12	0.67±0.37	12	0.44±0.27	11	0.38±0.16
N×R	88	0.22±0.19	21	0.77±0.22	21	0.9±0.14	21	0.68±0.21	21	0.39±0.16
R×N	48	0.05±0.09	14	0.65±0.38	14	0.79±0.36	14	0.6±0.35	12	0.36±0.24
Total	238	0.15±0.17	73	0.7±0.3	73	0.74±0.34	73	0.53±0.31	64	0.37±0.18

a) "N-N" and "R-R" represent the crosses between individuals from the same population within *O. nivara* (N) and *O. rufipogon* (R), respectively. "N × N" and "R × R" represent the crosses between individuals from different populations within the same species. "R × N" and "N × R" represent the crosses between individuals from different species (with *O. rufipogon* as the maternal and paternal parents, respectively).

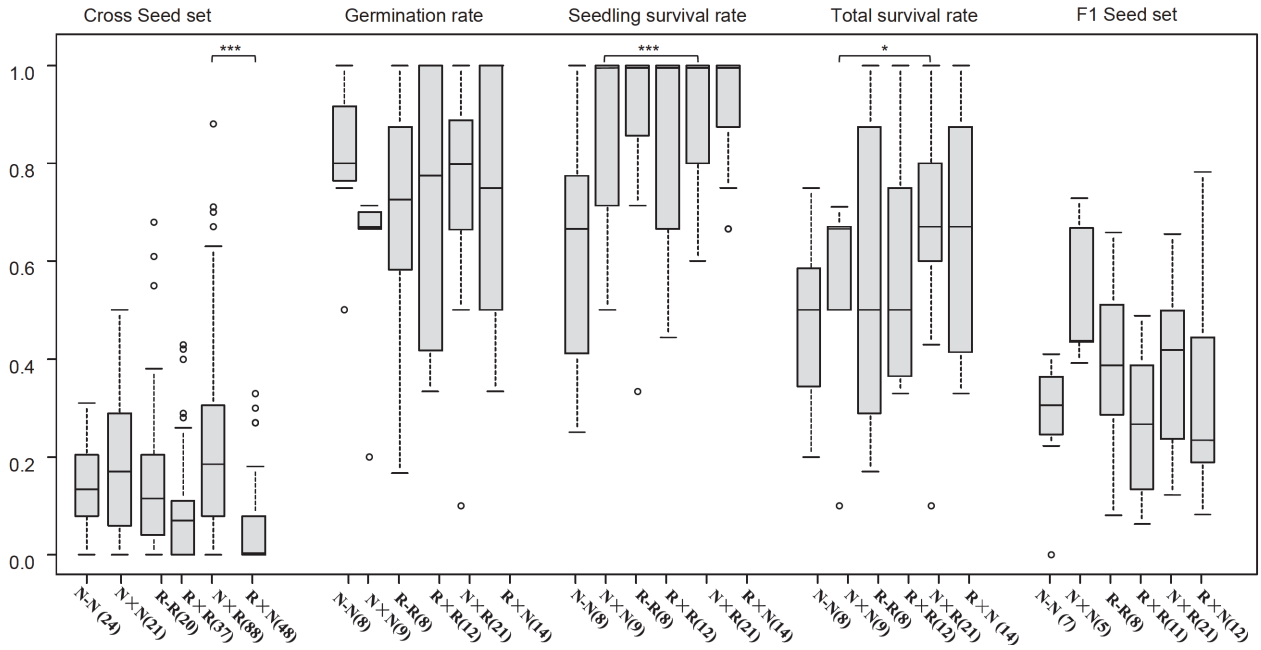

Figure 3 Variations in crossability (seed set), F1 viability (germination rate, seedling survival rate and total survival rate) and F1 fertility (F1 seed set) of different types of the crosses for six *O. rufipogon* and *O. nivara* populations. "N-N" and "R-R" represent crosses between individuals from the same population within *O. 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" represent crosses between individuals from different species (with *O. rufipogon* as the maternal and paternal parents, respectively). Number of the crosses for a specific combination is in parentheses.

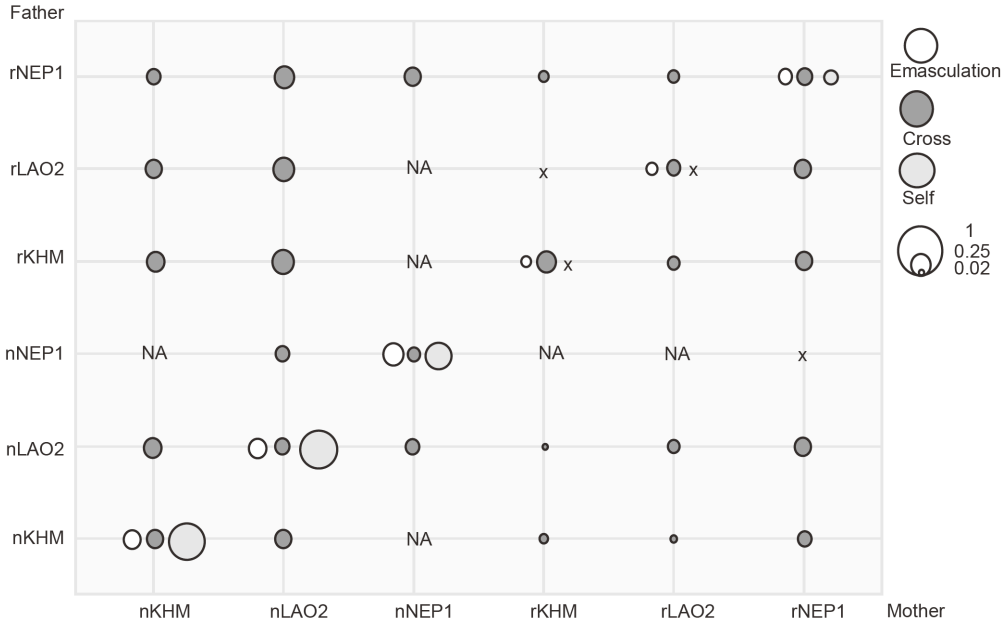

Figure 4 Mean seed sets of the self-pollinations with and without emasculation and of the crosses between and within *O. nivara* and*O. rufipogon* populations.

within combinations.

We further compared the seed sets of the F1 hybrids and their parents to evaluate F1 fertility. We grew the seeds from 14 and 7 self-pollinations for *O. nivara* and *O. rufipogon*, respectively, and calculated the seed set of the self-pollinating plants (Table 1; Table S3 in Supporting Information). We found that the seed sets of the F1 hybrids were higher than those of their parents in two of the three species pairs (i.e., 27% vs. 23% in Nepal and 54% vs. 38% in Cambodia) (Table S7 in Supporting Information). On average, the mean seed sets of the F1 hybrids (33%) were significantly higher than those of their parents (23%) (Table S7 in Supporting

Information) (P=0.02), suggesting higher fertility in hybrids than in their parents. Unlike crossability, we did not find asymmetry in the F1 viability and fertility.

Evaluation of prezygotic and postzygotic reproductive isolation

We first qualified the prezygotic isolation RI_1 (RI, reproductive isolation) using the data collected from the common garden experiment. We observed that the overlap of flowering time between species varied from 0 days (NEP1, RI=1) to 22 days (LAO2, RI=0.9) to 24 days (KHM, RI=0.9), with an average strength of RI_1=0.93 (Table 2), indicating that across all three species pairs, almost complete isolation (only 7% overlap of flowering time) was present between the two species. We further calculated the postzygotic isolation indexes (RI_2 to RI_4) and found that the average strength of isolation varied from weak for crossability (RI_2=0.21) to none for F1 viability (RI_3=0) and F1 fertility (RI_4=0.14) (Table 2; Table S7 in Supporting Information). Particularly, we found negative RI values for some species pairs at the later stage of the life cycle, suggestive of a transgressive advantage (or heterosis) in the F1 hybrids (Table S7 in Supporting Information). Together, our results indicated that very weak and asymmetric postzygotic reproductive barriers existed between *O. nivara* and *O. rufipogon* in seed development at the early stage of the life cycle but not in the production of hybrid progenies at the later stage of the life cycle.

DISCUSSION

Reproductive isolation is well recognized as an indicator of completion of speciation and a mechanism for maintaining species identity (Coyne and Orr, 2004; Widmer et al., 2009; Baack et al., 2015; Abbott, 2017). As wild progenitors of cultivated rice, the Asian wild rice complex, including *O. rufipogon* and *O. nivara*, has been the focus of numerous investigations in terms of interspecific crossability (Nezu et al., 1960; Chu et al., 1969; Naredo et al., 1997; Lu, 1998; Naredo et al., 1998; Banaticla-Hilario et al., 2013). Nevertheless, the exact strengths and individual contributions of different isolation components to the overall reproductive isolation between these two species remain unclear, and particularly, the specific roles of the prezygotic and postzygotic barriers to gene flow between species have not been investigated in depth. Here, we chose to investigate the prezygotic and postzygotic reproductive isolations of the three sympatric pairs of *O. rufipogon* and *O. nivara* populations based on common garden observations and artificial crossing. The resulting findings provided additional insights into the adaptation and divergence of wild rice species and facilitated the management of wild rice resources and rice breeding.

Prezygotic isolation is a major component contributing to reproductive isolation between *O. nivara* and *O. rufipogon*

Of the previous studies on the morphology and reproductive biology of *O. rufipogon* and *O. nivara*, many have investigated their mating systems and flowering phenologies and the reproductive isolation between species, with major goals of revealing the species relationships and taxonomic identity (Oka and Morishima, 1967; Chu et al., 1969; Morishima, 1985; Barbier, 1989; Naredo et al., 1997; Lu et al., 1998; Naredo et al., 1998; Zheng and Ge, 2010; Banaticla-Hilario et al., 2013; Samal et al., 2018). Although it has been widely acknowledged that these two species show contrasting mating systems, different flowering times and weak postzygotic isolation, none of the previous studies have provided quantitative assessment of the strength of both the prezygotic and postzygotic reproductive isolations. Recently, Banaticla-Hilario et al. (2013) conducted a crossing experiment to assess the extent of post-pollination reproductive isolation within and among three *Oryza* species. They observed that two selfing species (*O. meridionalis* and *O. nivara*) had very low seed sets and produced inviable F1 seeds, while the outcrossing *O. rufipogon* exhibited high intraspecific crossability. Although they did not find significant postzygotic reproductive isolation between *O. nivara* and *O. rufipogon*, their results were preliminary and inconclusive because of the small sample size and limited number of crosses undertaken in their crossing experiment. In the present study, we assessed the strength of reproductive isolation at different stages based on three independent species pairs. First, following previous common garden and greenhouse studies (Morishima et al., 1984; Barbier, 1989; Vaughan et al., 2008; Grillo et al., 2009), we found marked between-species differences in the first heading and flowering peaks and a reproductive isolation index RI_1 close to 1, which suggested that the prezygotic reproductive isolation is almost complete (Figure 1 and Table 2). Second, for the first time, we quantitatively evaluated the strength of both the overall and individual components of postzygotic isolation barriers between the two species. The fact that the isolation barriers at various postzygotic stages were either very weak (crossability) or not present and even negative (F1 viability and fertility) indicated that postzygotic reproductive barriers contributed to very little (if at all) to species divergence (Table 2).

It should be noted, however, that there were several caveats in our studies. First, the phenological data we analyzed were exclusively based on common garden experiments, which does not necessarily reflect the performance of these

Table 2 Strength of four reproductive components measured between pairs of species populations[a)]

Population pair	Locality	RI_1	RI_2	RI_3	RI_4
rNEP1/nNEP1	Nepal	1	0.59	0	0.17
rLAO2/nLAO2	Laos	0.9	0.12	0	0.16
rKHM/nKHM	Cambodia	0.9	0.15	0	0.42
Average		0.93	0.21	0	0.14

a) Reproductive isolation indexes of prezygotic isolation (flowering time, RI_1), the crossability (RI_2), F1 viability (RI_3) and F1 fertility (RI_4).

populations in their native environments or habitats. In this context, we further surveyed the literature on the phenology of the wild populations of the two species and found a significant difference in flowering time between species across their entire distribution regions, with *O. rufipogon* flowering significantly later than *O. nivara* (Sharma and Shastry, 1965; Morishima, 1985; Barbier, 1989; Lu, 1996; Kuroda et al., 2007; Zhou, 2019). Specifically, observations on the phenology of wild populations in Nepal, Laos and Cambodia in which this study was involved, indicated that the first heading of *O. rufipogon* was roughly two months later than that of *O. nivara* (Lu, 1996; Lu, 1998; Kuroda et al., 2007), further supporting the conclusion arising from the common garden experiment.

Another problem was that we did not measure several prezygotic barriers, such as habitat and temporal barriers as well as gametic competition (Sobel and Streisfeld, 2015; Ostevik et al., 2016; Delph, 2019). Nevertheless, our conclusion would not be different even if these factors were taken into consideration because the interspecific divergence in flowering time is currently strong enough to maintain strong prezygotic isolation between the species. A final caveat that might impact assessment of the postzygotic barriers was the sample size used in the crossing experiment. Specifically, the representative individuals per population chosen for crossing were still limited, although three species pairs representing different areas were included. This might explain the large variation in the estimates of several postzygotic components. It should be noted, however, despite the limited overall contribution of postzygotic barriers to reproductive isolation (i.e., seed production for interspecific crosses), asymmetrical crossability, as discussed below, might be one of the factors that contributes to interspecific gene flow in the long run and thus had affected the divergence between *O. rufipogon* and *O. nivara*. On the other hand, as a prezygotic isolation barrier, divergence in flowering times between the species is likely to reflect their differences in photoperiodic response and thus ecological requirements (Morishima et al., 1961; Zhou, 2019). Therefore, uncovering the mechanisms underlying photoperiodic sensitivity appears to be critical for understanding the formation of *O. nivara* and represents an interesting but challenging avenue for future research.

Asymmetrical gene flow barrier between speciesand its implications for speciation

Asymmetry in reproductive isolation is widespread in plants (Rieseberg and Carney, 1998; Tiffin et al., 2001; Martin et al., 2017) and may result from various reasons such as parental differences in style/stigma length, breeding system, fruit abortion, segregation distortion and nuclear-cytoplasmic incompatibilities (Tiffin et al., 2001; Lowry et al., 2008; Burton et al., 2013; Li et al., 2019). In the crossing experiment on three *Oryza* species, Banaticla-Hilario et al. (2013) found that the artificial hybridization between *O. rufipogon* and *O. meridionalis* exhibited asymmetrical compatibility, i.e., the crosses with maternal *O. meridionalis* had more reproductive success than those with maternal *O. rufipogon*, while the reciprocal crosses of *O. rufipogon* and *O. nivara* showed comparable crossability success. Unlike Banaticla-Hilario et al. (2013), we observed significantly reduced seed set in the crosses with *O. rufipogon* as the maternal parent relative to those with *O. nivara* as the maternal parent based on multiple crossing combinations (Figure 3 and 4). This incongruence may reflect the limited number of accessions used in the study of Banaticla-Hilario et al. (2013). Notably, that the asymmetry was not detected in the viability or fertility of F1 hybrids between the species. In a study on two subspecies of *Clarkia xantiana*, Briscoe Runquist et al. (2014) also detected an isolation barrier at the stage of hybrid seed development but not in subsequent life stages, with crosses from the selfer to outcrosser failing most frequently. Such asymmetric crossability cannot be explained by cytonuclear interactions as reported in many other studies (Tiffin et al., 2001; Lowry et al., 2008; Widmer et al., 2009; Martin et al., 2017) because the reduction in viability and fertility would be common in the F1 and following generations if cytonuclear interactions were the main reason (Burton et al., 2013; Briscoe Runquist et al., 2014). Differences in style/stigma length, fertility of the female gamete and breeding system might be potential explanations. It would be premature, however, to make any conclusion before in-depth investigations are performed with large samples from different populations.

Despite the strong prezygotic reproductive isolation arising from the differences in flowering time, large variations in the heading dates for the natural populations of the two

species (particularly *O. rufipogon*) provided the possibility of interspecific gene flow to some extent due to overlapping flowering times. Specifically, in some areas where cultivated and wild rice coexist, gene flow is common from cultivated rice to wild rice (Kuroda et al., 2007; Vaughan et al., 2008), which might facilitate introgression between the two wild species. The presence of gene flow between *O. nivara* and *O. rufipogon* has been confirmed in previous studies based on molecular markers (Kuroda et al., 2007; Zhou et al., 2008; Zheng and Ge, 2010). Asymmetrical gene flow between these two species was reported in a previous molecular study that detected a much higher migration rate from *O. rufipogon* to *O. nivara* than in the opposite direction (Zheng and Ge, 2010). To what extent this asymmetric postzygotic barrier of gene flow has contributed to the speciation process of wild rice remains to be uncovered. Furthermore, as indicated previously, selection can favor increased reproductive isolation through the process of reinforcement (Widmer et al., 2009; Baack et al., 2015). The two wild rice species with multiple pairs of sympatric populations provide a unique case to address whether any reinforcement occurs considering that hybridization costs would impose selection for increased prezygotic isolation (Suni and Hopkins, 2018). In summary, understanding the evolution of asymmetries can provide additional insights into the forces and mechanisms that drive reproductive isolation and thus speciation (Tiffin et al., 2001; Lowry et al., 2008; Li et al., 2019).

Fertility of self-pollinations and impact of emasculation on pollination success

Consistent with their mating systems, the selfing *O. nivara* generated higher seed set than the outcrossing *O. rufipogon* under self-pollination. However, no significant difference in the viability (germination and survival rate) of self-pollinated plants was found between *O. nivara* and *O. rufipogon* (Figure 2). It is noted, however, that the artificial emasculation prior to hand pollination significantly decreased the pollination success in *O. nivara*but not in *O. rufipogon*. In a crossing experiment involving three Asia Pacific species of *Oryza*, Banaticla-Hilario et al. (2013) also found that the self-pollinated panicles after emasculation generated much lower seed sets in the two selfing species (i.e., *O. meridionalis* and *O. nivara*) than in the outcrossing *O. rufipogon*. This may explain the large variation in the crossability estimates for the crosses both between and within *Oryza* species in previous studies (Naredo et al., 1997; Naredo et al., 1998; Banaticla-Hilario et al., 2013). Despite these findings, we observed that the viability of the offspring by self-pollination with emasculation was as strong as that by self-pollination without emasculation (Figure 2), suggesting that emasculation did not impact the later stages of plant development. The reasons for the negative impacts of emasculation on pollination success, particularly the different effects on *O. nivara* and *O. rufipogon*, required further investigation. Many factors such as flowering structure due to the different mating systems and ovary damage during emasculation (Martin et al., 2017), might be potential causes.

Emasculation is a routine practice in crossing experiments in many species with self-fertilization systems, but the potential impacts of emasculation on the success of crossing pollinations have received less attention (Brys et al., 2014; Martin et al., 2017). Our results on the negative impacts of emasculation on pollination success remind that caution should be taken in evaluating the strength of reproductive isolation based on crossing experiments with emasculation. This result, together with the findings of asymmetric crossability between two wild rice species, provides valuable information on an avenue for improvement in rice breeding practice.

MATERIALS AND METHODS

Study populations, flowering phenology and design of crossing experiments

In a previous study on phenotypic variation of *O. rufipogon* and *O. nivara*, we conducted a common garden experiment for three consecutive years (2011–2013) in Nanning City, Guangxi Province, China, involving 14 *O. rufipogon* and 7 *O. nivara* populations (Cai et al., 2019; Ren, 2019). In the common garden experiment, we measured 19 phenotypic traits, including the flowering time (heading date), for each individual of all 24 populations. The heading date (on which the first flush of flowers was observed) was censused every day throughout the entire growth season.

In the present study, we chose three pairs of sympatric populations of *O. rufipogon* and *O. nivara* (i.e., NEP1, LAO2, and KHM) (Table S1 in Supporting Information) for the assessment of variations in flowering time within and between populations and for crossing experiments. These populations, with sampling sizes over 30, were collected by the authors during field collections from 2008 to 2011,with the distance between two populations of each pair ranging from 5 km to 18.6 km. Detailed information on these populations was provided in references (Cai et al., 2019; Ren, 2019). The crossing experiments including hand pollinations and subsequent evaluations of F1 plants were conducted in 2016 and 2017 at Lingshui Station (N18°30.6', E110°2.4') in Hainan Province, China. We chose five plants from each population for the hand pollinations, which included crosses of individuals within populations, between populations within species, and between species as well as self-pollinations with and without emasculation (Table 1; Table S8 in Supporting Information). All 30 individuals were identified based on four diagnostic traits (culm height, panicle shape,

anther length, peduncle length) collected from the common garden experiment (Ren, 2019) to ensure that these individuals were typical *O. rufipogon* and *O. nivara*.

Seed germination and artificial pollination

Our field observations showed that *O. nivara* usually headed 30 days earlier than *O. rufipogon* (Zhou et al., 2008). To ensure the concurrence of the flowering time of the two species, we germinated the seeds of *O. nivara* in three batches at an interval of 15 days, with the first batch germinated on June 22, 2016. All *O. nivara* seeds were processed at 50° C for 120 h to break dormancy and dipped in clean water in 6 mm culture dishes; they were moved into paper cups filled with soil from the fields when the leaves emerged. Seedlings at the third-leaf stage were planted in buckets (32 cm diameter and 30 cm height) with field soil in greenhouse. For the perennial *O. rufipogon*, we used the rhizomes maintained in the Lingshui Station for the experiment. Young tillers of the *O. rufipogon* individuals were transplanted into buckets with field soil on June 15, 2016 and planted together with the *O. nivara* individuals in the greenhouse.

To perform the artificial pollinations, we chose at least three healthy tillers of each plant by covering the panicles with sulfate paper bags before anthesis. To avoid self-pollination, we emasculated the panicles prior to hand pollination. We first emasculated the panicles before blossoming of the spikelets, then removed the immature anthers by hand, and finally sprayed water on the emasculated panicles in case of residual pollen.

Qualification of the strength of reproductive isolation

We evaluated the strength of reproductive isolation by calculating an index (*RI*) for both prezygotic and postzygotic isolations. To qualify the prezygotic isolation, we estimated RI_1 following Sobel and Chen (2014): $RI_1=1–H/(H+C)$, where H and C represent the overlapped and non-overlapped proportions, respectively, of the flowering periods between species. RI_1 ranges from zero (complete overlap) to 1 (complete isolation) (Briscoe Runquist et al., 2014; Ostevik et al., 2016). According to our observations in the field and garden, we found that the flowering time of a single plant might last 60 days for *O. rufipogon* individuals and 50 days for *O. nivara* individuals due to many tillers of a single individual. Therefore, the flowering period was estimated to be the duration from the date when the first plant headed to that when the last plant headed plus 60 (for *O. rufipogon*) and 50 (for *O. nivara*) days. It should be noted that we only considered flowering time as the prezygotic barrier because multiple studies have shown that flowering time-based isolation causes almost complete reproductive isolation between these two species (Cai et al., 2019; Zhou, 2019). Such a practice, however, does not imply that other factors such as ecogeography, habitat, dichogamy and pollen competition do not cause a prezygotic barrier to gene flow, as reported in studies on other plant species (e.g., Martin and Willis, 2007; Briscoe Runquist et al., 2014; Sobel and Streisfeld, 2015; Hipperson et al., 2016; Delph, 2019).

We used the crossability (seed set), F1 viability and F1 fertility to measure postzygotic isolation. Crossability was measured by the number of seeds to the number of spikelets emasculated per panicle. F1 viability consists of three components (germination rate, seedling survival rate and total survival rate) (Sobel and Streisfeld, 2015). Germination rate was defined as the number of seedlings at the second-leaf stage to the total number of seeds. Seedling survival rate was defined as the number of plants at the flowering stage to the number of seedlings at the second-leaf stage. Total survival rate was defined as the number of plants in the flowering stage to the total number of seeds. F1 fertility was measured by the mean seed set of the F1 plants (Banaticla-Hilario et al., 2013).

Following Ramsey et al. (2003) and Sobel and Chen (2014), we used three indexes to measure three components of postzygotic isolation, i.e., the crossability (RI_2), F1 viability (RI_3) and F1 fertility (RI_4), based on the formula: $RI=1–(H/P)$, where H and P represent the fitness of F1 hybrids and fitness of their parents, respectively. *RI* ranges from 0 to 1, with the exception that hybrids are fitter than their parents and thus a negative value is obtained. We calculated RI_2 and RI_4 based on mean seed sets for the crosses of two parents and for F1 plants, respectively. To calculate RI_3, we considered two components (germination rate and seedling survival rate). To obtain the effect of previous stages on accumulating isolation, we calculated the absolute contribution (AC) of each component to the total isolation (RI_3) as $AC_n = RI_n\left(1-\sum_{i=1}^{n-1} AC_i\right)$ (Ramsey et al., 2003), where AC_i is the absolute contribution of each component to the total isolation. Pairwise *t*-test was performed by R 3.4.4 (https://www.r-project.org). Box and whisker plots were constructed using the build-in function in R 3.4.4 and bubble plot were constructed with ggplot2 (https://cran. r-project. org/web/packages/ggplot2/index. html).

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

Figure S1 Variation of first heading of three pairs of *O. rufipogon* (blue) and *O. nivara* (red) populations from 2011 to 2013 in the common garden experiment. Boxes and horizontal bars represent the central 50% and median of heading dates, respectively. Dots represent the outliers beyond 1.5 times the interquartile range. Numbers of individuals (*O. rufipogon/O. nivara)* that were observed are in parentheses.

Table S1 Information on the *O. rufipogon* and *O. nivara* populations used in this study. *N*, number of individuals sampled

Table S2 Flowering phenology of three pairs of *O. rufipogon* and *O. nivara* populations in the common garden expriment in three consecutive years in Guanxi, China

Table S3 Summary of different types of crosses for six *O. rufipogon* and *O. nivara* populations

Table S4 Seed set of the self-pollinations with and without emasculation. em, emasculation; ns, no significance; **, $P< 0.01$; ***, $P< 0.001$

Table S5 Significant tests for the seed sets between different types of crosses. ns, no significance; ***, $P<0.001$

Table S6 Significant tests for the viability of F1 hybrids between different types of crosses. Level of significances is listed in the following order: germination rate, seedling survival rate and total survival rate. ns, no significance; *, $P<0.05$; ***, $P<0.001$

Table S7 Viability and fertility of the F1 hybrids between pairs of species populations. *, this index is the absolute contribution (AC) of germination rate (AC1) and seedling survival (AC2) to the total isolation, and zero represents a higher fitness of F1 hybrids relative to their parents

Table S8 Design of crossing experiments. Population codes are the same to those in Table S1 in Supporting Information. Numbers of crosses are in the parentheses

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