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Phenological and morphological variations of *Oryza rufipogon* and *O. nivara* in Sri Lanka and their evolutionary implications

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Abstract

Phenological and morphological variation are widely viewed as a pivotal driver of ecological adaptation and speciation. *Oryza rufipogon* and *O. nivara*, the wild progenitors of cultivated rice, are most closely related and provide a unique system for investigating ecological adaption and speciation. Here, we investigate variation patterns of flowering phenology and morphological traits within and between *O. rufipogon* and *O. nivara* populations in Sri Lanka by incorporating the *in situ* observation in natural habitats and manipulative experiments in common gardens. First, we observed varying degrees of phenological variation under different temporal and environmental conditions, suggesting that flowering phenology of two *Oryza* species is highly plastic and vary depending on both environment and management practices. Particularly, the Sri Lankan *O. nivara* exhibits high plasticity in flowering phenology with water availability being a primary determinant, implying that *O. nivara* might not be an annual in the strict sense. Second,

we unexpectedly found that flowering time of the two species in Sri Lanka overlapped to a large extent, which suggests that the primary factor to maintain the species divergence in Sri Lanka may not be flowering time but rather environments. Third, we uncovered two distinct morphological groups, corresponding to *O. rufipogon* and *O. nivara* and show that habitat difference rather than spatial isolation is the main explanation for the phenotypic divergence between species. Moreover, our results indicate a substantial link between morphological variation and several major climatic factors including altitude, temperature and rainfall. Finally, our selection analysis suggests that interspecific divergence in the traits related to reproduction and habitat preference is most likely driven by natural selection. Together, our case study on the Sri Lankan *O. rufipogon* and *O. nivara* enhances the understanding of the roles of phenotypic plasticity and environmental factors in the processes of adaptation and speciation.

Keywords: Ecological divergence; flowering phenology; morphological traits; population variation; wild rice

Introduction

Ecological divergence among populations is frequently attributed to local adaptation to contrasting environments and driven mainly by divergent natural selection (Mitchell-Olds et al. 2007; Nosil 2012; Richardson et al. 2014). During the process of ecological adaptation, phenotypic divergence may result in gradual establishment of reproductive isolation as a consequence of divergent natural selection and thus lead to ecological speciation (Erickson et al. 2004; Faria et al. 2014; Nosil 2012; Schluter and Rieseberg 2022). Phenotypic variation influences the survival of plants and a range of interactions between plants and environments on one hand and is the primary source of information for classifying plants and determining their evolutionary relationships on the other (Mitchell-Olds et al. 2007; Nosil 2012; Richardson et al. 2014; Thompson 2005). Therefore, investigations on phenotypic diversity, including phenological and morphological variation, have significant implications for the understanding of evolutionary processes and mechanisms of biological diversity (Franks 2015; Mazer et al. 2022; Mitchell-Olds et al. 2007; Rundle and Nosil 2005; Wadgymar et al. 2022). To date, extensive studies have been conducted on phenotypes and the roles of natural selection on phenological and morphological variation (Erickson et al. 2004; Mitchell-Olds et al. 2007; Peichel and Marques 2017; Richardson et al. 2014; Schluter and Rieseberg 2022). However, the contributions of phenotypic changes to ecological adaptation and speciation in particular environments, the ecological and genetic factors contributing to phenotypic changes as well as the link between the evolution of divergent phenotypes and the build-up of reproductive isolation remain less clear (Franks 2015; Mazer et al. 2022; Schluter and Rieseberg 2022; Seehausen et al. 2014). To address these issues, it is essential to perform studies by integrating multiple approaches, including phenotypic characterization, population genomics and assessment of fitness (Mazer et al. 2022; Richardson et al. 2014; Wadgymar et al. 2022). Of various approaches, *in situ* observation and common garden experiment are classical and irreplaceable methods but have been paid less attention due to the manipulative and observational difficulties, particularly for natural populations of non-model species (Anderson et al. 2012; Mazer et al. 2022; Mitchell-Olds et al. 2007; Park and Post 2022; Sugai et al. 2023).

Oryza rufipogon (Griff.) and *O. nivara* (Sharma and Shastry) are the most closely related species and the direct progenitors of domesticated Asian rice (*O. sativa* L.) (Jing et al. 2023; Khush 1997; Kovach et al. 2007; Oka 1988; Sang and Ge 2007). *Oryza rufipogon* is perennial,

largely cross-fertilized, photoperiod sensitive and inhabits persistently wet areas across southern China, South and Southeast Asia, Papua New Guinea and Northern Australia; while *O. nivara* is annual, predominantly self-fertilized, photoperiod insensitive and occurs in seasonally dry habitats with more restricted distribution in South and Southeast Asia (Kovach et al. 2007; Liu et al. 2015; Sang and Ge 2007; Vaughan et al. 2008; Zheng and Ge 2010). Earlier studies have found significant differentiation between *O. rufipogon* and *O. nivara* for numerous phenotypes at both local and regional scales, involving phenology (flowering time), life history, mating system, and many morphological features (Banaticla-Hilario et al. 2013a; Cai et al. 2004; Cai et al. 2019; Eizenga et al. 2022; Guo et al. 2016; Kuroda et al. 2005; Morishima and Oka 1960; Morishima et al. 1984; Oka 1988; Ren 2019; Samal et al. 2018; Xu et al. 2020). However, several studies revealed a continuum of a few phenotypic traits, particularly for life history, both between and within species (Morishima et al. 1984; Oka 1988; Sano et al. 1980), and geographic isolation has been proposed to be a main factor driving species divergence between the two species (Barbier 1989; Cai et al. 2004; Morishima et al. 1984; Vaughan et al. 2008). These studies raise a question regarding the relative roles of habitats and geographic isolation play driving phenotypic divergence between the two species (Barbier 1989; Cai et al. 2004; Morishima et al. 1984; Vaughan et al. 2008). Moreover, as an accurate indicator of the completion of speciation and a mechanism for maintaining species identity (Abbott 2017; Nosil 2012; Schluter and Rieseberg 2022), flowering time difference was considered to be a major driver of reproductive isolation and thus species divergence between *O. rufipogon* and *O. nivara* (Barbier 1989; Cai et al. 2019; Xu et al. 2020). Although the flowering time of the wild rice species has been amply documented in literatures (e.g., Barbier 1989; Banaticla-Hilario et al. 2013a; Cai et al. 2004; Cai et al. 2019; Eizenga et al. 2022; Grillo et al. 2009; Meng et al. 2024; Oka 1988; Vicentini et al. 2023), it is still less clear about their variation pattern and plasticity across populations because, to our knowledge, no research on flowering phenology of any *Oryza* species has been undertaken based on *in situ* observation for its entire life cycle. Also, few comparative studies of morphological variation have been made on the two wild rice species in terms of ecological adaptation and divergence. Given information available on phenology, morphology and habitat preference of these two species is incomplete, an in-depth study on variation patterns of phenological and morphological traits of *O. rufipogon* and *O. nivara* in different environments/habitats would provide critical insights into the understanding of processes and mechanisms of species

divergence and speciation of these wild species in general.

Sri Lanka exhibited diversified and well-understood ecosystems that consist of three main ecological zones (wet, dry, and intermediate) (Fig. 1) (Liyanage and Senanayake 2010; Sandamal et al. 2018; Seo et al. 2005). Of five *Oryza* species recognized in Sri Lanka, *O. rufipogon* and *O. nivara* are the most common and widely distributed species (Liyanage and Senanayake 2010; Vaughan et al. 2008). Importantly, the two wild rice species are parapatric in distribution, with *O. rufipogon* confined to the wet zone and *O. nivara* to the dry zone of the country (Liyanage and Senanayake 2010; Sandamal et al. 2018; Vaughan et al. 2008). This is in marked contrast to the distribution pattern in many other areas in which the two species are largely sympatric despite different habitats (Cai et al. 2019; Kuroda et al. 2007; Liu et al. 2015; Sano et al. 1980; Vaughan et al. 2008). In addition, based on microsatellite markers, our preliminary study of the *O. rufipogon* and *O. nivara* populations in Sri Lanka found significant genetic differentiation between species and strong genetic structure within species (Sandamal et al. 2018). Therefore, the well-understood climatic zones in Sri Lanka and their close match with the distribution of two wild rice species, along with relatively characterized genetic background (Sandamal et al. 2018, 2021), provide a unique system to investigate adaptive divergence and ecological speciation in plants.

In the present study, we took advantage of the unique system of wild rice in Sri Lanka to investigate the variation patterns of flowering phenology and morphological traits and their implications for ecological adaptation and divergence of *O. rufipogon* and *O. nivara*. By incorporating *in situ* observation and common garden experiment into an integrative assessment, we aimed (1) to comparatively evaluate the life cycle of *O. rufipogon* and *O. nivara*. We were particularly interested in knowing the differentiation and plasticity in flowering phenology of the two species and the factors underlying the variation as well as whether flowering time is an isolation mechanism for species divergence; (2) to address the variation pattern of morphological traits between and within species and the impact of different geographic and environmental factors on the variation of the two species; (3) to determine whether the phenotypic differentiation between the two species is attributed to divergent natural selection, which is usually expected for ecological divergence and speciation (Nosil 2012; Richardson et al. 2014; Rieseberg et al. 2002). These studies not only gain insights into adaptation and speciation process of two wild rice species but also provide an informative case contributing a better understanding of ecological adaptation

and speciation in general. In addition, investigations on wild rice in Sri Lanka would facilitate to develop efficient strategies in rice breeding and conservation of wild rice resources because wild rice species is an important gene pool for rice breeding and the threats to their existence is increasingly serious due to global climate change and urbanization (Eizenga et al. 2022; Jing et al. 2023; Sang and Ge 2007; Vaughan et al. 2008; Warschefsky and Rieseberg 2021).

Materials and Methods

Study populations and experimental design

Fourteen natural populations including six *O. rufipogon* and eight *O. nivara* populations in Sri Lanka were chosen for *in situ* observation and common garden experiment. The *O. nivara* populations were confined to seasonally dry habitats in dry and intermediate zones and the *O. rufipogon* populations inhabited in wide ditches with variable depths of water or in marshy fallows adjacent to deep water in wet and intermediate zones (Fig. 1, Table S1). For each population, a random seed/plant sample of 10 to 16 individuals, spaced at least 5 m apart, was collected to minimize multiple sampling of the same genet.

The *in situ* observation was performed for two *O. rufipogon* populations (SL02-R and SL14-R) in the wet zone and two *O. nivara* populations (SL07-N and SL16-N) in the dry zone (Fig. 1 and 2). The common garden experiment involving five *O. rufipogon* and seven *O. nivara* populations was conducted for two consecutive years (2016-2017) at the Faculty of Agriculture, University of Ruhuna (latitude 06.08 °N and longitude 80.56 °E) in Sri Lanka (Fig. 1, Table S1). Detailed information on all populations and their habitats is provided in Table S1.

For phenological variation, we recorded the date of first flowering and seed setting because flowering time is a crucial adaptive trait that enables plants to coordinate their reproductive processes with favorable environments and seed setting ensures plants to produce fully developed seeds (Mazer et al. 2022; Vicentini et al. 2023). For morphological variation, we measured nine traits (Table S2) that were supposed to be either taxonomically significant or adaptive (Banaticla-Hilario et al. 2013a; Cai et al. 2019; Eizenga et al. 2022; Guo et al. 2016). Of these traits, four including anther length (ANL), panicle neck spikelet length (PNSPL), panicle exsertion (PE) and awn length (AWL) are related to reproduction, three flag leaf traits (FLL, FLW, FLA) are associated with photosynthesis processes, and the other two, culm length (CL) and culm diameter CD), are involved in habitat preference. We assessed ten individuals for *in situ* observation and

fifteen individuals in common garden experiment. Three randomly selected tillers per plant were measured following the descriptors for wild and cultivated rice (Biodiversity International 2007; Cai et al. 2019; Meng et al. 2024).

In situ observations

Preliminary survey showed that, of four populations used for *in situ* observation, two *O. rufipogon* populations (SL02-R, SL14-R) were significantly disturbed by human activities such as canal cleaning, farming, uncontrolled urbanization, and industrialization (Fig. 2a and 2b). The maximum water depth and the length of period of inundation depend on the elevation above the river. Maximum water depth was estimated at the SL02-R site ranged from 1 to 3 m, while at the SL14-R site ranged from 0.5 to 1 m, gradually reducing in August (Table S1). The wet zone habitats of two *O. rufipogon* populations were flooded more deeply and for a longer period due to their deep-water tolerance ability. In contrast, two *O. nivara* populations (SL07-N and SL16-N) grew in lake area together with other grass and weeds and were usually found in the fields that remained dry until the first rain in October or November (Fig. 2c and 2d). For the *O. nivara* populations, seeds germinated at the start of the rainy season, and the plants flowered and produced seeds before the beginning of the dry season. These populations exhibited growth forms typical of the species and occurred in represented habitats of the wet and dry zones.

We established three 1 m^2 quadrats (plots) for all four populations, with quadrats setting up at 3 m intervals around the area of peak plant abundance. Observations were recorded separately in three plots as replicates at two-week intervals for over an entire growth season (March 2016 - June 2017).

Common garden experiment

Seedlings were carefully uprooted from their natural habitats and transplanted in cemented pots (40 cm length × 40 cm width × 45 cm height) containing sandy loam soils free from weeds. Fifteen individuals from each population were planted following a randomized complete block design and kept in open field conditions. Recommended doses of chemical fertilizers were applied at the time of transplanting stage and the water level in the pot was maintained at 15 ± 5 cm during the entire period of plant growth except for transplanting time.

We selected four populations from each species (Fig. 1) for phenological observations. Phenological traits were evaluated in two-week intervals for each plant, involving the

performance at two growth stages (flowering and seed setting) similar to the *in situ* observation. To investigate the morphological variation, we measured nine traits for five *O. rufipogon* and seven *O. nivara* populations (Fig. 1) using three randomly selected tillers per plant following the descriptors for wild and cultivated rice (Biodiversity International 2007; Meng et al. 2024).

Data analysis

All phenotypic analyses were conducted using free software R, version 4.3.1 with supplementary packages and Minitab 17. Phenotypic differentiation between species was also assessed by a standard principal component analysis (PCA) (Banaticla-Hilario et al. 2013a; Uga et al. 2003). Univariate ANOVAs were used to examine the effects of species on the variables using population means and to assess the effects of populations within species using trait values of individuals within populations. To gain a deeper understanding of the impact of habitat and climate on phenotypic variation, we conducted a correlation analysis using altitude, rainfall, and temperature as environmental factors because these factors vary across Sri Lanka and affect the survival and distribution of plant species (Dananjaya 2017). Specially, altitude presents a natural gradient where multiple environmental factors change concurrently, including temperature, oxygen levels, solar radiation, atmospheric pressure, and even soil properties. Different altitudes often harbor distinct ecological niches. Rainfall directly influences water availability in an ecosystem and water availability can influence reproductive period, breeding strategies, and even seed germination. Temperature affects directly the developmental and growth rates and specific life stages and a species has a range of temperatures where they perform optimally. The *t*-test and *F* test were conducted to test for trait divergence between environmental variables and the differences between species, respectively (Banaticla-Hilario et al. 2013a; Nakazato et al. 2008). Morphological similarity and physical distances of pairwise populations were calculated using the dist function and the distGeo functions in R (R Core Team 2020), respectively. The Pearson correlation coefficient between morphological similarity and physical distance was calculated using the corr.test function in R (R Core Team 2020).

We performed the Q_{ST}-F_{ST} analysis, an important and widely used method for testing whether natural selection or genetic drift is the major cause of population divergence for phenotypic traits (Cai et al. 2019; Guo et al. 2016; Merilä and Crnokrak 2001; Nakazato et al. 2008). F_{ST} represents the amount of genetic variance that can be explained by population structure

measured by genetic loci/markers, while Q_{ST} is an analog of F_{ST} but measures genetic differentiation between populations/species for phenotypic trait (Leinonen et al. 2013; McKay and Latta 2002). We used the F_{ST} value published previously for the same set of populations based on SSR markers (Sandamal et al. 2018) and calculated Q_{ST} values for nine quantitative traits following the procedure in Guo et al. (2016) to determine whether the Q_{ST} values were smaller or larger than F_{ST} values. Q_{ST} was calculated using the technique described by Spitze (1993). The variance components between species (V_B) and within species (V_W) for each quantitative trait. Q_{ST} values were obtained by 1,000 bootstrap iterations with each involving resampling across individuals within populations. Hence, Q_{ST} can be computed using the formula $Q_{ST} = V_B / (V_B + 2V_W)$, and the Q_{ST} distribution produced from the 1,000 bootstrap iterations was used to assess the importance of the disparity between Q_{ST} and F_{ST}. If Q_{ST} is significantly larger than F_{ST}, natural selection would be the main factor driving species divergence, whereas a neutral genetic differentiation would be supported if F_{ST} is significantly greater than Q_{ST} (McKay and Latta 2002; Merilä and Crnokrak 2001).

Results

Flowering phenological variation between and within species

We investigated the flowering phenology based on two sets of investigations (*in situ* observation and common garden experiment). First, we performed *in-situ* observation of the life cycle of two *O. rufipogon* and two *O. nivara* populations for an entire year and observed substantial differences in flowering phenology between species, including first heading and flowering duration. It is apparent from Fig. 3 that *O. rufipogon* populations exhibited a much longer life cycle (7 to 10 months) than that of *O. nivara* populations (4 to 5 months). In addition, two peaks of sexual reproductions were found for the *O. rufipogon* populations but only one for the *O. nivara* populations. Unlike two *O. nivara* populations that showed almost the same pattern, a slight difference in life cycle was observed between two *O. rufipogon* populations (SL02-R and SL14-R), likely reflecting the difference of habitats in two sites because SL02-R is situated near the river where water is available year-round, while SL14-R is distributed across marshy land where floods is periodical (Table S1).

Next, based on common garden experiment, we revealed a different pattern of phenological variation to that of the *in situ* observation. As shown in Fig. 4, all *O. nivara* populations exhibited

continuous flowering and seed setting throughout the years with almost all tillers entering the flowering stage, a pattern contrasting to that found in the *in situ* observation in which much shorter life cycle was observed for the *O. nivara* populations. For the *O. rufipogon* populations, a life cycle completed within 7-8 months without two peaks of sexual reproduction found in the *in situ* observation, probably due to different management in the common garden experiment such as frequent watering and weeding. We also found very little variation among populations within species for either *O. rufipogon* or *O. nivara*, probably because of the same management condition. These observations indicated that the flowering phenology of wild rice can vary slightly depending on the growing conditions and that *O. nivara* in Sri Lanka may not be strictly an annual plant and can survive persistently when water is readily available.

An unexpected finding is that flowering time of two species is largely overlapped for either observed populations in the fields or manipulative populations in the common garden despite different variation patterns from two sets of investigations (Fig. 3 and 4), which is incongruent with previous reports that significant difference in flowering time was observed at local scale (Barbier 1989; Cai et al. 2004; Cai et al. 2019; Kuroda et al. 2007; Xu et al. 2020). The overlapped flowering phenology between species and high level of plasticity of flowering time have important implications for divergence and origin of *O. nivara* (see Discussion).

Morphological variation between and within species

We examined variation patterns of morphological traits between and within species based on *in situ* observation and common garden experiment. First, we performed the analyses of nine morphological traits recorded in *in situ* observation. The ANOVA indicated significant differences between species for all traits except for flag leaf angle (FLA) and awn length (AWL) (Table 1). Notably, *O. rufipogon* exhibits significantly larger values than *O. nivara* for seven traits that diverge between species. Significant differentiations were also observed between populations within species for *O. rufipogon* (six traits) and *O. nivara* (four traits), with three traits (FLL, FLW, and AWL) that diverged significantly in both species (Table 1). The PCA analysis further supported the ANOVA results that two distinct groups corresponding to two species are apparent, with intraspecific variation larger in *O. rufipogon* than in *O. nivara* (Fig. 5a). All traits except for two (AL and FLA) exhibited high loadings in PC1 that explains majority of the total variance

310 (52%) (Table S3), suggestive of substantial differentiation between the two species despite high
311 level of variation within species.

312 Next, we analyzed the variation pattern of morphological traits between and within species
313 based on common garden experiment. We found significant differentiation between *O. rufipogon*
314 and *O. nivara* for all nine traits except for two flag leaf traits (FLL and FLW) and significant
315 variation among populations within either *O. rufipogon* or *O. nivara* (Table 2), largely consistent
316 with the results from *in situ* observation on natural populations. PCA also shows clear separation
317 of two species, with PC1 and PC2 accounting for 38.17% and 20.01% of the variance, respectively
318 (Fig. 5b). However, in contrast to the *in situ* observation on natural populations, larger variation
319 within species occurred in *O. nivara* than in *O. rufipogon* (Fig. 5b), with the factor loadings of
320 many traits (Table S4) different from those from *in situ* observation. These results might reflect
321 potential impact of growing/manipulative conditions on the variation patterns of morphological
322 traits and suggest high plasticity for most morphological traits of the wild rice species.

323 **Correlation between morphological traits and environmental variables**

324 To determine the factors that might be associated with morphological differentiation between the
325 two species, we conducted analysis for correlation between each of nine traits and three
326 environmental variables (altitude, rainfall, and temperature) that were potentially variable across
327 Sri Lanka (Dananjaya 2017). We found that all traits except for FLL significantly correlated with
328 at least one of three environmental factors for *O. rufipogon*, with two traits (CD and ANL) affected
329 by all three factors; whereas all traits of *O. nivara* were significantly correlated with at least one
330 environmental factor and four traits of them (CL, CD, AWL, and PNSPL) correlated with all three
331 factors (Table 3), implying that almost all trait were sensitive to the environments or habitats.
332 Specifically, variation of seven, five, and three traits correlated with altitude, rainfall, and
333 temperature, respectively, in *O. rufipogon*; while that of six, seven, and seven traits correlated
334 with altitude, rainfall and temperature, respectively, in *O. nivara* (Table 3). These results suggest
335 that environmental factors play important roles in shaping the variation patterns of morphological
336 traits and contribute to the differentiation between *O. rufipogon* and *O. nivara*.

337 We further tested whether positive correlation exists between morphological similarity and
338 geographic distance for pairwise comparisons within and between species to assess the relative
339 roles of habitat and geography. It is shown that no significant correlations were found between

morphological similarity and geographic distance for population pairs within *O. nivara* (R^2 = 0.0746, P = 0.9553) and between *O. rufipogon* and *O. nivara* (R^2 = 0.0004, P = 0.9125), contrast to the pairs within *O. rufipogon* (R^2 = 0.1987, P = 0.0429) (Fig. S1). These results indicate that the environment/habitat plays more important roles than geographic distance in the morphological divergence between species.

Tests for the role of natural selection in morphological divergence between species

To determine whether natural selection or genetic drift is the primary driver of phenotypic differentiation between the two species, we performed the Q_{ST}-F_{ST} comparison based on measures of nine traits obtained from common garden experiment. By calculating Q_{ST} values for each trait and the F_{ST} value of 0.129 estimated using SSR markers (Sandamal et al. 2018), we detected significantly larger Q_{ST} values than the F_{ST} value for seven out of nine morphological traits (Table 4), suggesting that natural selection plays a role in morphological divergence between *O. rufipogon* and *O. nivara*. Consistently, the observation that habitat instead of geographic distance correlated with the divergence for population pairs of two species in most morphological traits (Fig. S1) suggest that morphological differentiation between *O. rufipogon* and *O. nivara* is adaptive or under natural selection.

Discussion

Ecological adaptation or speciation is a consequence of phenotypic and genetic differentiation, which is driven by divergent selection under different environments (Abbott 2017; Mitchell-Olds et al. 2007; Richardson et al. 2014; Schluter and Rieseberg 2022). Extensive studies have revealed significant differences between *O. rufipogon* and *O. nivara* in flowering time and multiple traits at regional and local scales and indicated that the differentiations were usually associated with habitat differences, as expected in ecological adaptation and speciation (Barbier 1989; Cai et al. 2019; Kuroda et al. 2005; Morishima et al. 1984; Ren 2019; Sharma and Shastry 1965; Xu et al. 2020). However, studies on the relative roles of adaptive plasticity and genetic variation adaptive to the environments as well as the impact of environment factors on phenotypic variation were limited in wild rice because previous studies have predominantly been conducted in artificial settings, such as greenhouses, or in areas that did not accurately reflect the natural habitats of the species (Cai et al. 2004; Cai et al. 2019; Eizenga et al. 2022; Kuroda et al. 2007; Morishima et al. 1961, 1984; Oka 1988). Here, we integrated the *in situ* observation in natural habitats and

manipulative experiment in the common gardens to address the variation and plasticity of phenology and morphological traits under different temporal and environmental conditions, and to examine how seasonal fluctuations and environmental variables impact the phenological and morphological variation of two wild rice species in Sri Lanka.

Flowering phenology is a key factor in the process of ecological adaptation and speciation and has been studied extensively in plant species (Franks 2015; Kay 2006; Mazer et al. 2022; Nakazato et al. 2008; Osborne et al. 2019; Wunder et al. 2023). The adaptation of plants to local environments entails the selection of flowering phenology, which is particularly important for the overall fitness of plants because reproductive success depends largely on the duration of the growing season and the timing of flowering (flowering phenology) (Anderson et al. 2011; Cho et al. 2017; Franks 2015; Wunder et al. 2023). Consequently, flowering phenology has been considered to be a magic trait that facilitates genetic differentiation and triggers the divergence of multiple traits in response to the environments (Lowry et al. 2008; Servedio et al. 2011; Sugai et al. 2023). Although substantial investigations of flowering time have been conducted on rice cultivars (Oka 1988; Vicentini et al. 2023), flowering phenology and its variation within and between wild rice species remain largely elusive despite some efforts (Morishima et al. 1961; Morishima et al. 1984; Oka 1988; Xu et al. 2020; Sandamal et al. 2021). In the present study, we found varying degrees of phenological variation between species, between populations within species, and between *in situ* observation and common garden experiment, which may be caused by both genetic elements and environmental variables such as water availability and temperature (Cai et al. 2019; Kuroda et al. 2005; Morishima et al. 1984; Sang and Ge, 2007; Vaughan et al. 2008). First, we found significant difference between species in flowering phenology, as revealed by either *in situ* observation or common garden experiment. For *in situ* observation, *O. rufipogon* exhibited a prolonged life cycle with two flowering peaks while *O. nivara* displayed a notably shorter life cycle with a single flowering peak, as reported in Sandamal et al. (2021). This reflects the differences in typical environments/habitats where *O. rufipogon* (in wet zone) and *O. nivara* (in dry zone) grow. The bimodal versus unimodal reproductive phases might arise from different adaptive strategies in response to ecological pressures. However, common garden experiment showed a different pattern of flowering phenology, particularly for *O. nivara* that flowers continuously for almost an entire year. This suggests that the flowering phenology or life cycle of *O. nivara* is highly flexible depending on environmental conditions, with water availability being

a primary determinant (Huang et al. 2013; Liu et al. 2015; Sandamal et al. 2021; Sang and Ge 2007; Vaughan et al. 2008). It also implicates that *O. nivara*, at least for the Sri Lankan populations, might not be an annual plant in the strict sense because it can consistently survive and succeed when ample water is available. To some extent, flowering phenology of *O. rufipogon* can also alters depending on environments. Together, these findings suggest that flowering phenology (or life cycle) of two wild rice species is highly plastic and vary depending on environment and management practices, demonstrating previous arguments that plasticity played a crucial role in adaptation of species to varying ecological conditions and could potentially influence their speciation processes (Anderson et al. 2012; Fox et al. 2019; Levin 2009). Second, we unexpectedly found that flowering time of the two species in Sri Lanka overlapped to a large extent based on *in situ* observation and common garden experiment (Fig. 3 and 4). This raises an interesting question about the role flowering phenology plays during the divergence *O. rufipogon* and *O. nivara* because different flowering time is critical to maintaining reproductive isolation between species and has been considered to be a main driver of adaptation and speciation in the two *Oryza* species (e.g., Barbier 1989; Cai et al. 2019; Xu et al. 2020) and many other plant groups (e.g., Briscoe Runquist et al. 2014; Franks 2015; Hall and Willis 2006; Lowry et al. 2008; Nakazato et al. 2008; Sugai et al. 2023). Numerous studies indicated that the divergence or separation between *O. rufipogon* and *O. nivara* is apparent genetically and morphologically at the same locality due to strong barriers to gene flow (e.g., differences in flowering time and mating system) (Banaticla-Hilario 2013b; Barbier 1989; Cai et al. 2019; Kuroda et al. 2007). Our previous study on the Sri Lankan populations of the two species uncovered significant genetic differentiation between species and hypothesized that the Sri Lankan *O. nivara* might be introduced from the Indian subcontinent to the island (Sandamal et al. 2018), implying well-established reproductive isolation (i.e., weak if any gene flow) between the two species in Sri Lanka. Consequently, the primary factor to maintain the divergence of the two species in Sri Lanka may not be flowering time but rather environments because habitat selection is able to restrain gene flow mediated by seed migration and the recruitment of seedlings (Barbier 1989; Cai et al. 2019; Morishima et al. 1984; Richardson et al. 2014). Finally, the observed difference of the Sri Lankan wild rice in flowering phenology and particularly the high levels of plasticity, reflect the strategy how the two species have adapted to their respective environments. Take *O. nivara* as an example, a significantly shorter life span was observed in natural habitats (roughly

six months) (Fig. 3) than in the common garden (the entire year) (Fig. 4). It is understandable that, as a derivative species, *O. nivara* populations evolved a shorter life cycle to cope with the habitat shifts, which took place in both Sri Lanka and many other areas (e.g., Barbier et al. 1989; Cai et al. 2019; Huang et al. 2013; Kuroda et al. 2005). Such a strategy to adapt to dry and stressful habitats through phenological plasticity enables these species to coordinate their reproductive cycles with favorable environmental conditions and has been reported in many other plant species (e.g., Briscoe Runquist et al. 2014; Hall and Willis 2006; Lowry et al. 2008; Nakazato et al. 2008; Sugai et al. 2023).

Phenotypic divergence is the first step towards local adaptation and speciation and has thus been extensively explored across plant species (Mazer et al. 2022; Mitchell-Olds et al. 2007; Richardson et al. 2014; Rieseberg et al. 2002; Wadgymar et al. 2022). In the present study, based on both *in situ* observation and common garden experiment, we demonstrated two distinct morphological groups, corresponding to *O. rufipogon* and *O. nivara*, and substantial phenotypic variation within species in many traits, consistent with previous studies (Banaticla-Hilario et al. 2013a; Cai et al. 2019; Eizenga et al. 2022; Guo et al. 2016; Oka 1988; Sandamal et al. 2021). Specifically, *O. nivara* has a shorter culm, early flowering, shorter and less exserted panicles with more compactness and has more spikelets per panicle with shorter anthers. In addition, we examined the role of spatial isolation in the population/species divergence in terms of isolation by distance (Richardson et al. 2014; Sexton et al. 2014) because the association of morphological divergence with both habitats and geography was reported previously (Banaticla-Hilario et al. 2013a; Cai et al. 2019; Eizenga et al. 2022; Kuroda et al. 2007; Pusadee et al. 2013; Sandamal et al. 2021; Zhou et al. 2008). Analysis of correlation between morphological divergence and habitat and geographic distance suggests that habitat difference rather than spatial isolation is the main explanation for the phenotypic divergence between *O. rufipogon* and *O. nivara* in Sri Lanka. Particularly, our results indicate a substantial link between the variation of morphological traits and several major climatic/environmental factors including altitude, temperature and rainfall, consistent with previous studies (Banaticla-Hilario et al. 2013a; Huang et al. 2013; Liu et al. 2015). In response to these factors, both species have evolved distinct phenotypes to adapt to their environments and expanded in range, which was supported by previous argument that significant range expansions took place for the two species in Sri Lanka since the last interglacial around 120-140 thousand years ago (Sandamal et al. 2018). For instance, predominantly vegetative

propagation, longer and more spreading culms make *O. rufipogon* better adapt to permanently inundated habitats; whereas being annual, shorter and less decumbent culms of *O. nivara* are more suitable for seasonally dry habitats (Banaticla-Hilario et al. 2013a; Cai et al. 2019; Sandamal et al. 2021). Congruent with ecological speciation (Cai et al. 2019; Meng et al. 2024; Sandamal et al. 2018; Zheng and Ge 2010), the differences in morphological traits between *O. rufipogon* and *O. nivara* are mainly explained by distinct habitats they grow and have important implications for their ecological adaptation and evolutionary dynamics.

Natural selection is a fundamental mechanism of evolution that shapes the genetic makeup of populations over time, allowing for adaptations to specific environmental conditions (Mitchell-Olds et al. 2007; Rieseberg et al. 2002; Wadgymar et al. 2022). Although studies of phenotypic divergence between *O. rufipogon* and *O. nivara* have been conducted both on the global and at the local scales (Banaticla-Hilario et al. 2013a; Cai et al. 2019; Eizenga et al. 2022; Kuroda et al. 2007; Pusadee et al. 2013; Sandamal et al. 2021), most of them were unable to examine if the divergence was adaptive or under natural selection. Based on 60 samples that presented two species morphology, Guo et al. (2016) detected significant differentiation between species for 10 morphological traits and suggested that they were adaptive. By further studying six sympatric pairs of *O. rufipogon* and *O. nivara* populations using variance analyses and Q_{ST}-F_{ST} comparison, Cai et al. (2019) revealed significant differentiation between species for 14 of 18 quantitative traits and demonstrated the role of natural selection during phenotypic divergence between species. Nevertheless, it is unknown if similar divergence patterns for morphological traits occur and whether natural selection has played a role in morphological divergence between the two species in Sri Lanka in which parapatric rather than sympatric distribution was found for the *O. rufipogon* and *O. nivara* populations (Fig. 1). Here we identified seven phenotypic traits that exhibited significant differences between the two species and demonstrated that their divergences between species were most likely to be driven by natural selection, as reported previously in studies of sympatric populations of *O. rufipogon* and *O. nivara* (Cai et al. 2019). These traits are related to reproduction and habitat preference and might have evolved in response to varying biotic and abiotic factors, such as reproductive success, water availability, resource competition, or climatic factors. Collectively, the survival and persistence of *O. rufipogon* and *O. nivara* in Sri Lanka are affected by both genetics and environments and natural selection plays an important role in local adaptation and divergence. Further research should focus on elucidation of the genetic

494 basis for the observed phenotypic variation and exploration of the effects of environmental
495 conditions on variation of these traits.

496

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504

505 **Competing interests**

506 No competing interests exist.

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508 **References**

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Figure legends

Fig. 1. Geographic locations of six *O. rufipogon* (blue and dot) and eight *O. nivara* (red and triangle) populations sampled in this study. Population codes with superscripts a and b represent the populations used for the *in situ* observation and common garden experiment, respectively. Detailed information on all populations is provided in Table S1.

Fig. 2. The growth form (upper panels) and habitat (lower panels) of four *O. rufipogon* and *O. nivara* populations used for *in situ* observations. (a, b) Two *O. rufipogon* populations, with SL02-R growing around a river of 3~5 m wide and 1~3 m deep (a) and SL14-R in an abundant marshy land, along with other hydrophytic weeds and fairly dense (b). (c, d) Two *O. nivara* populations, with SL07-N growing nearby a road and separated by paddy fields (c) and SL16-N distributes near to lake area and mix grows with other shrubs and weeds (d).

Fig. 3. Life cycle of *O. rufipogon* and *O. nivara* populations based on *in situ* observation for the natural populations.

Fig. 4. Flowering phenological variation of *O. rufipogon* and *O. nivara* populations based on common garden experiment.

Fig. 5. Principal component analysis (PCA) of *O. rufipogon* (blue) and *O. nivara* (red) populations based on *in situ* observation (A) and common garden experiment (B).

Tables

Table 1. Analysis of variation pattern of nine morphological traits between and within species based on *in situ* observation.

Trait (abbreviation)	*O. rufipogon*		*t*	*O. nivara*		*t*	*F*
	SL02-R mean (SD)	SL14-R mean (SD)		SL07-N mean (SD)	SL16-N mean (SD)		
Anther length (ANL)	3.80 (0.13)	4.20 (0.13)	-11.1***	2.98 (0.43)	2.98 (0.10)	0.02	393.47***
Awn length (AWL)	4.23 (1.33)	6.08 (1.26)	-5.51***	6.27 (0.56)	4.25 (0.83)	10.96***	0.17
Culm diameter (CD)	1.70 (0.60)	1.73 (0.12)	-0.29	1.13 (0.11)	0.89 (0.14)	6.7***	136.14***
Culm length (CL)	150.93 (17.21)	124.20 (11.75)	7.03***	92.13 (11.66)	92.50 (7.32)	-0.2	251.54***
Flag leaf attitude (FLA)	76.80 (25.57)	79.77 (16.33)	-0.54	79.40 (7.77)	75.43 (27.20)	0.77	0.05*
Flag leaf length (FLL)	19.07 (5.75)	32.83 (6.62)	-8.6***	20.42 (2.79)	15.95 (3.84)	5.15***	35.34***
Flag leaf width (FLW)	1.02 (0.26)	1.20 (0.11)	-3.41**	0.90 (0.09)	0.81 (0.14)	2.91**	59.24***
Panicle exsertion (PE)	23.57 (7.51)	24.14 (4.89)	-0.35	8.59 (1.48)	8.25 (3.62)	0.47	302.71***
Panicle neck spikelet length (PNSPL)	2.28 (0.47)	4.31 (1.02)	-9.82***	0.99 (0.19)	0.97 (0.62)	0.2	169.74***

The *t*-test and *F*-test were used for comparing the variation within and between species, respectively. Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 2. Analysis of variation pattern of nine morphological traits between and within species based on common garden experiment.

Population	ANL	AWL	CD	CL	FLA	FLL	FLW	PE	PNSPL
O. rufipogon	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD	mean ± SD
SL01-R	3.45 ± 0.05^{c}	3.58 ± 0.79^{b}	1.45 ± 0.13^{b}	121.9 ± 5.36^{b}	58.87 ± 11.42^{b}	19.27 ± 2.43^{bc}	0.87 ± 0.07^{ab}	10.33 ± 2.25^{b}	1.91 ± 0.40^{c}
SL02-R	3.56 ± 0.05^{b}	4.27 ± 0.28^{b}	1.82 ± 0.28^{a}	131.60 ± 8.80^{ab}	69.97 ± 13.53^{ab}	15.65 ± 1.66^{d}	0.83 ± 0.07^{b}	17.70 ± 5.32^{a}	2.35 ± 0.37^{bc}
SL03-R	3.71 ± 0.06^{a}	6.23 ± 0.46^{a}	1.66 ± 0.11^{ab}	126.50 ± 8.20^{b}	73.13 ± 24.11^{ab}	21.49 ± 2.12^{ab}	0.71 ± 0.05^{c}	10.34 ± 1.52^{b}	4.32 ± 1.72^{a}
SL06-R	3.60 ± 0.06^{b}	5.85 ± 0.65^{a}	1.61 ± 0.14^{ab}	140.10 ± 10.38^{a}	80.70 ± 10.84^{a}	17.58 ± 2.01^{cd}	0.96 ± 0.05^{a}	9.31 ± 1.54^{b}	4.67 ± 0.82^{a}
SL13-R	3.73 ± 0.06^{a}	5.54 ± 0.90^{a}	1.63 ± 0.10^{ab}	141.33 ± 15.43^{a}	74.20 ± 18.69^{ab}	22.78 ± 4.27^{a}	0.86 ± 0.10^{b}	11.45 ±3.02^{b}	3.34 ± 1.27^{ab}
O. nivara									
SL04-N	3.07 ± 0.08^{ab}	8.60 ± 0.28^{a}	1.86 ± 0.03^{a}	114.78 ± 0.48^{a}	41.73 ± 2.39^{c}	20.65 ± 0.97^{ab}	0.85 ± 0.03^{b}	2.32 ± 0.46^{c}	1.76 ± 0.04^{d}
SL07-N	3.04 ± 0.06^{ab}	6.07 ± 0.09^{c}	1.73 ± 0.01^{b}	96.77 ± 1.47^{b}	74.39 ± 1.98^{b}	20.01 ± 1.57^{bc}	0.94 ± 0.04^{a}	7.77 ± 0.16^{a}	1.16 ± 0.04^{e}
SL08-N	2.97 ±0.09^{b}	8.01 ± 0.47^{b}	1.64 ± 0.01^{c}	97.80 ± 1.91^{b}	30.64 ± 3.13^{d}	20.07 ± 0.77^{b}	0.73 ± 0.02^{c}	1.26 ± 0.22^{de}	0.95 ± 0.09^{f}
SL09-N	3.05 ± 0.10^{ab}	6.47 ± 0.26^{c}	1.35 ± 0.04^{d}	83.16 ± 3.77^{cd}	40.24 ± 1.92^{c}	20.09 ± 1.40^{b}	0.87 ± 0.03^{b}	1.00 ± 0.19^{e}	1.89 ± 0.10^{c}
SL10-N	3.11 ± 0.06^{a}	6.32 ± 0.12^{c}	1.63 ± 0.05^{c}	81.05 ± 2.80^{d}	40.30 ± 4.74^{c}	18.31 ± 0.59^{cd}	0.71 ± 0.03^{c}	1.52 ± 0.05^{d}	1.98 ± 0.02^{b}
SL11-N	3.06 ± 0.09^{ab}	3.31 ± 0.62^{e}	1.37 ± 0.01^{d}	79.83 ± 2.72^{d}	34.13 ± 3.59^{cd}	22.18 ± 2.10^{a}	0.66 ± 0.03^{d}	5.36 ± 0.68^{b}	2.30 ± 0.05^{a}
SL12-N	3.04 ± 0.07^{ab}	5.46 ± 0.28^{d}	1.22 ± 0.05^{e}	86.12 ± 4.03^{c}	100.47 ± 13.35^{a}	17.61 ± 0.85^{d}	0.96 ± 0.02^{a}	1.58 ± 0.23^{d}	2.24 ± 0.06^{a}
***F*-Value**	958.61 ± 0.1***	20.96 ± 1.48***	9.64 ± 0.21**	398.48 ± 11.73***	19.28 ± 23.67***	0.79 ± 2.65	1.34 ± 0.11	264.43 ± 3.14***	80.01 ± 0.97***

Within a column, means followed by the same letters of the respective species are not significantly different by Duncan's Multiple Range Test (DMRT) at $P > 0.05$. *F* value with pooled standard deviation. Significance: $P < 0.05$*; $P < 0.01$**; $P < 0.001$***.

Table 3. **Correlation (*r*) between morphological traits and environmental variables based on common garden experiment.**

Trait (abbreviation)	***O. rufipogon***			***O. nivara***		
	Altitude	Rainfall	Temperature	Altitude	Rainfall	Temperature
Anther length (ANL)	-0.454***	0.776***	-0.388**	-0.134	0.232*	-0.196
Awn length (AWL)	-0.412**	0.636***	-0.169	0.642***	-0.332**	0.298**
Culm diameter (CD)	-0.451***	0.422**	-0.393**	0.671***	-0.252*	0.360***
Culm length (CL)	-0.512***	0.235	0.175	0.934***	-0.309**	0.353***
Flag leaf attitude (FLA)	-0.341*	0.264	0.010	0.017	0.448***	-0.700***
Flag leaf length (FLL)	0.173	0.209	-0.077	0.119	-0.453***	0.439***
Flag leaf width (FLW)	-0.158	-0.430**	0.687***	0.259*	0.013	-0.696***
Panicle exsertion (PE)	-0.291*	0.165	-0.381	0.273*	-0.126	-0.176
Panicle neck spikelet length (PNSPL)	-0.324*	0.402**	0.016	-0.612***	0.601***	-0.352***

Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 4. The Q_{ST} values for individual traits and *Z* test for Q_{ST}-F_{ST} comparison.

Trait (abbreviation)	Q_{ST} value	*Z* test
Anther length (ANL)	0.9898	-28.711***
Awn length (AWL)	0.9807	3.794***
Culm diameter (CD)	0.9504	-4.544***
Culm length (CL)	0.9879	-22.798***
Flag leaf attitude (FLA)	0.9659	-4.223***
Flag leaf length (FLL)	0.8848	0.546
Flag leaf width (FLW)	0.9666	-1.369
Panicle exsertion (PE)	0.9808	-13.667***
Panicle neck spikelet length (PNSPL)	0.9534	-7.120***

Z tests for Q_{ST}–F_{ST} comparison for both *O. rufipogon* and *O. nivara* samples. Significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

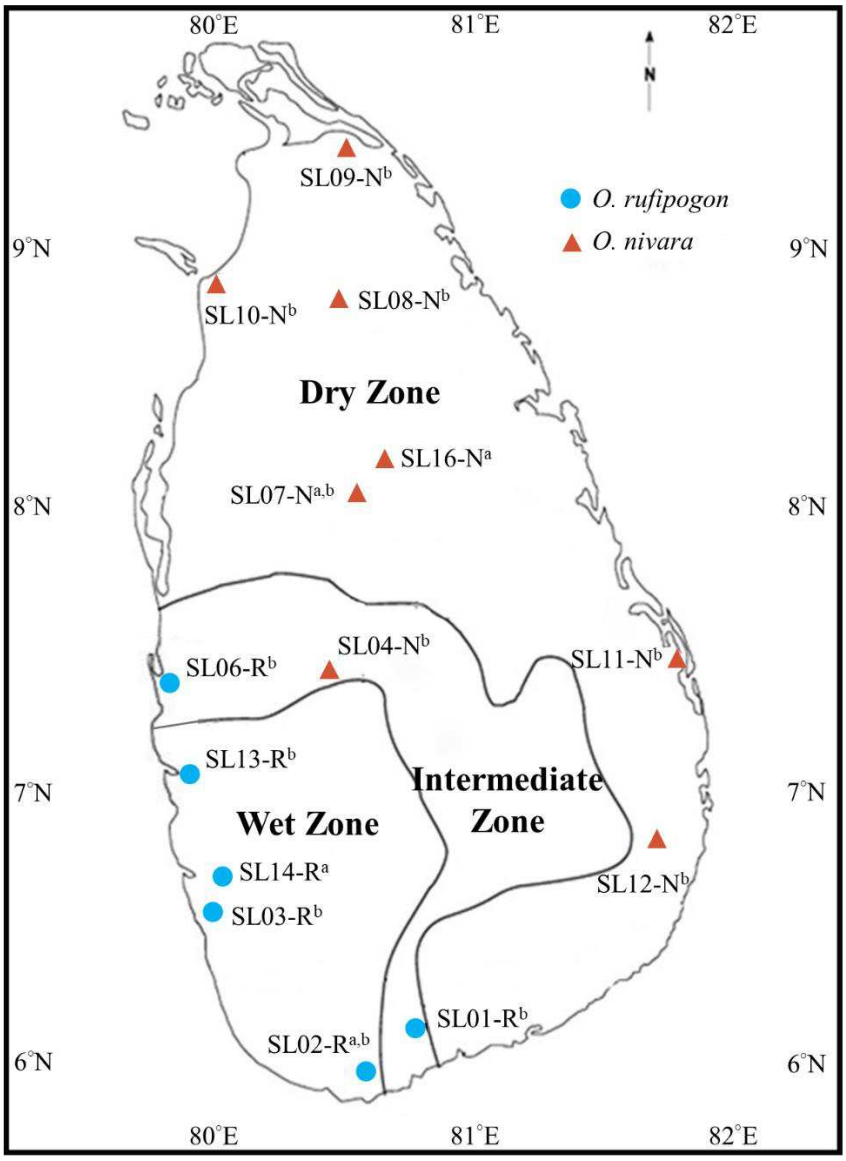
80°E
81°E
82°E
N
9°N
8°N
7°N
6°N
O. rufipogon
O. nivara
SL09-N^b
SL10-N^b
SL08-N^b
Dry Zone
SL16-N^a
SL07-N^a,b
SL04-N^b
SL06-R^b
SL11-N^b
SL13-R^b
Intermediate Zone
Wet Zone
SL14-R^a
SL03-R^b
SL12-N^b
SL01-R^b
SL02-R^a,b

Fig. 1

Fig. 2

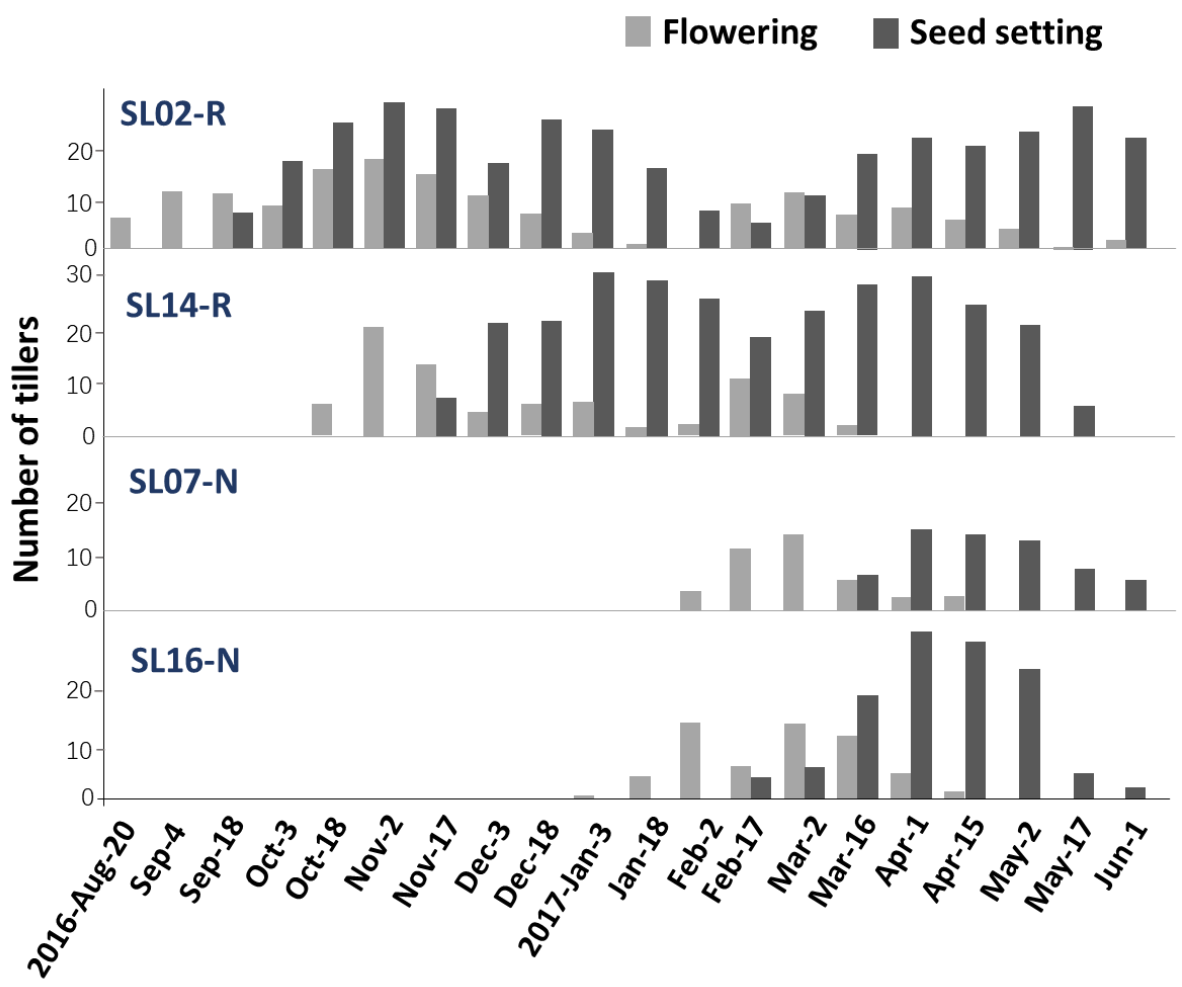
Flowering
Seed setting
SL02-R
SL14-R
SL07-N
SL16-N
Number of tillers
0
10
20
30
2016-Aug-20
Sep-4
Sep-18
Oct-3
Oct-18
Nov-2
Nov-17
Dec-3
Dec-18
2017-Jan-3
Jan-18
Feb-2
Feb-17
Mar-2
Mar-16
Apr-1
Apr-15
May-2
May-17
Jun-1

Fig. 3

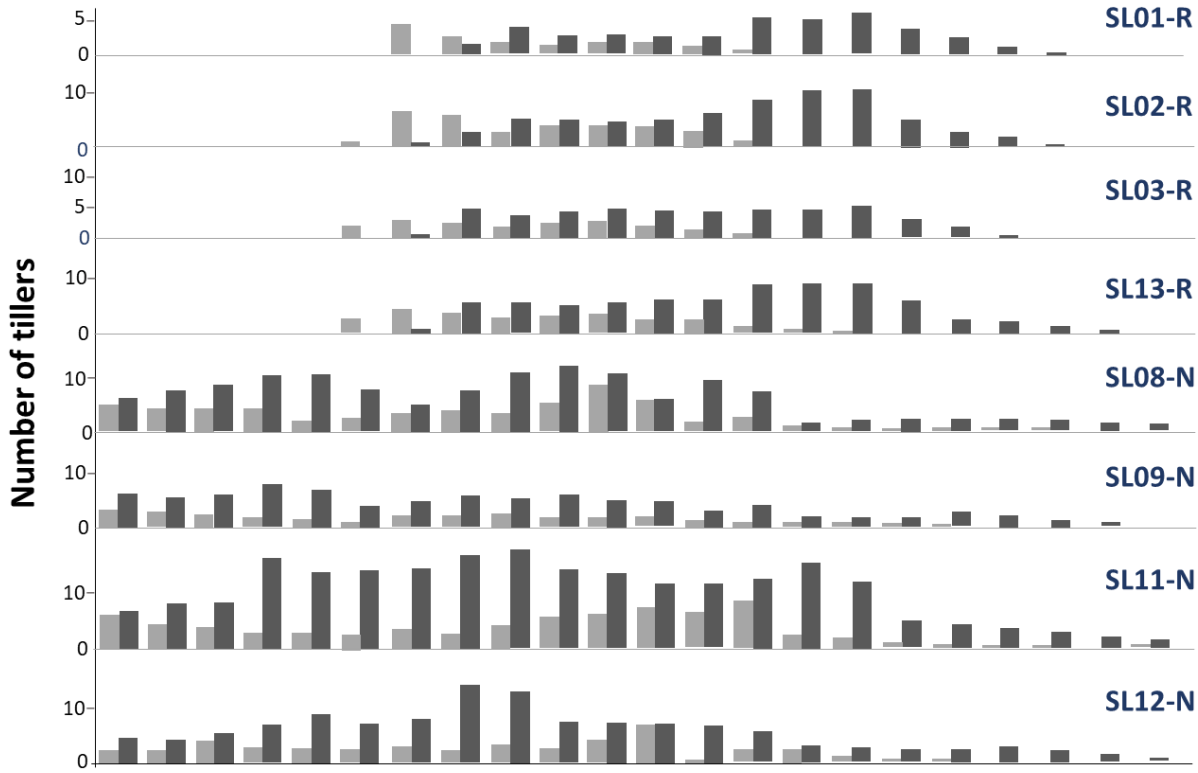
Number of tillers
SL01-R
SL02-R
SL03-R
SL13-R
SL08-N
SL09-N
SL11-N
SL12-N

Fig. 4

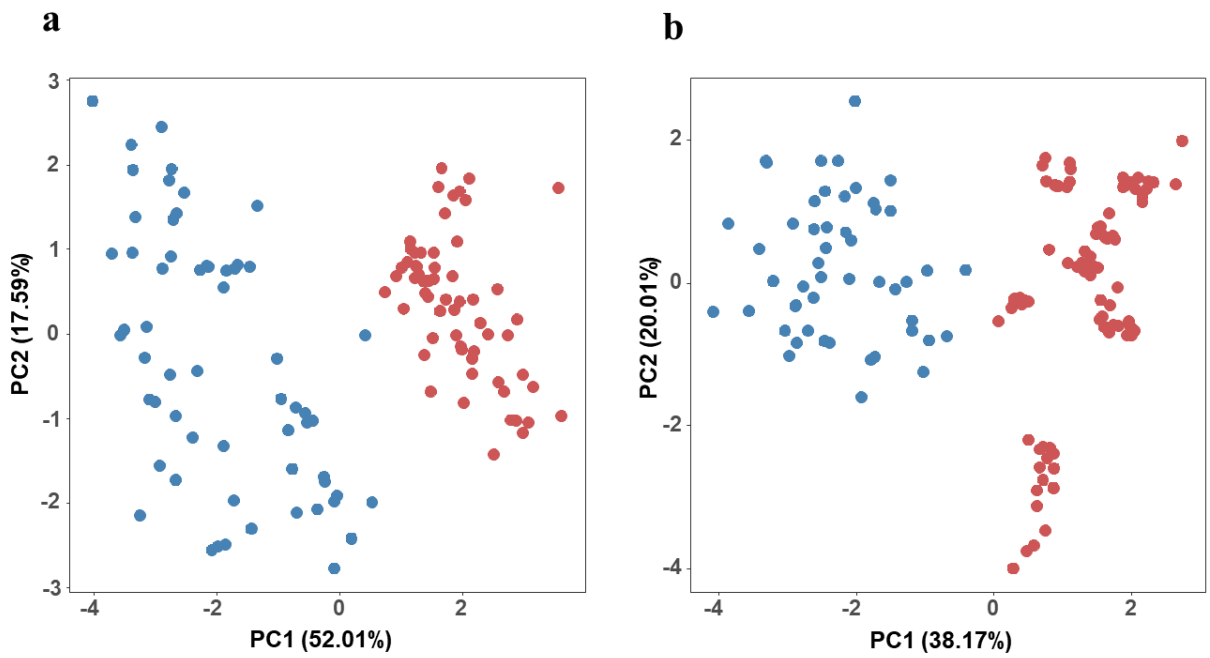

Fig. 5