

Phenotypic Differentiation between Weedy Rice Populations Associated with their Local Adaptation in Early-and Late-Season Rice Fields

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Abstract

Background: Temperatures and photoperiods can profoundly affect plant growth and development and play vital roles in the local adaptation of plant species. Weedy rice (*Oryza sativa* f. *spontanea*) is a conspecific weed of cultivated rice, and it was found in the same rice fields (sympatry) of early and late rice-cultivation seasons in Leizhou, Guangdong Province of China. Generally, the phenological conditions, such as temperature and photoperiod, are different in the two seasons. Therefore, the early- and late-season weedy rice populations in the same rice fields provide a perfect system for estimating sympatric divergence in plant species. The previous study had demonstrated considerable genetic divergence between the early- and late-season weedy rice populations. Here, we designed *in situ* common garden experiments to estimate the phenotypical differences between the two-season weedy rice populations and disclose the local adaptation in weedy rice populations associated with their ambient temperature and photoperiod.

Results: Distinct air temperature and day length variation patterns were recognized between the early and late rice-cultivation seasons, based on the 10-year historical climate data. More stressful conditions, indicated by low air temperature and long day length, were found for weedy rice growth in the early seasons. Noticeably, significant differences in plant heights, the number of tillers, flowering time, and reproductive traits were detected between the two-season weedy rice populations according to the early-season common garden experiment. The early-season populations showed evident higher plant heights, more tillers, and earlier flowering time than the late-season populations. However, such differences were not detected in the late-season common garden experiment. In addition, evident local adaptation represented by the traits such as plant heights, flowering time, and reproductive traits was only detected in the early-season weedy rice populations. The principal component analysis also showed clear population clusters between the two-season populations using the phenotypical data.

Conclusions: This study provided clear evidence of phenotypic differentiation between the sympatric early- and late-season weedy rice populations, probably associated with the local adaptation to their ambient temperature and photoperiod. Our findings also have potential roles in facilitating the design of strategies for effective weedy rice control practices.

Introduction

Temperatures and photoperiods can profoundly affect plant growth and development [1, 2] and contribute to seed germination, flowering time, and reproductive traits of plants [3, 4]. Generally, each species has a specific temperature range represented by minimum, maximum and optimum [5]. For example, the optimal temperature for rice cultivation is between 25 and 35 °C [6], and temperature beyond optimum may have adverse effects on rice growth and development. However, responses to temperatures differ among plant species throughout their life cycle and are primarily the phenological responses [5]. For instance, a prolonged period of cold, called the vernalization response, can promote plants to flower in *Arabidopsis thaliana* [7], and a high temperature can shorten the grain filling period in rice [8]. In

addition, the defined range of maximum and minimum temperatures form the boundaries of observable growth, vegetative development increases as temperatures rise to the species optimum level [5].

However, the temperature is often an unreliable marker of seasonality. Therefore, most plant species native to areas outside the tropics have evolved a second line of safeguarding them against misleading temperature conditions—photoperiod, which is defined as the developmental responses of plants to the change of day length over the years [9]. In other words, the response to photoperiod has evolved in plants because day length is a reliable indicator of the time of year [10, 11], enabling developmental events to be scheduled to coincide with particular environmental conditions [12]. Photoperiod plays a significant role in synchronizing flowering in plant populations, thus effectively ensuring reproductive success and preventing emergence in a risky environment due to the misleading signal from the following temperature [9]. Noticeably, the interaction between temperature and photoperiod also plays an essential role during the life of plant species [1, 13]. For example, the floral transition of plants always depends on the accurate measurement of changes in photoperiod and temperature. Thus, photoperiod and temperature are two pivotal regulatory factors of plant flowering [13].

Individual plants are sessile, and therefore have to develop the means to detect and respond to environmental changes as they occur. Consequently, plants continuously monitor their surroundings and adjust their growth to daily and seasonal cues [14]. Weed species, such as agricultural weeds, have been rapidly evolved to adapt to changes during farming practices [15, 16]. In the light of the intrinsic capacity of rapid adaptation, weedy species that occur over a relatively short period become an appealing system to study evolutionary processes. Generally, the agricultural weed syndrome includes rapid growth, high nutrient-use efficiency, seed dormancy, efficient seed dispersal, crop mimicry, and herbicide resistance [15]. Therefore, agricultural weeds must possess traits that permit them to survive and thrive in the recently created environment. The evolution of herbicide resistance is probably the most emblematic and well-documented case of rapid evolution in weeds [17]. In addition, the climate change, such as temperature and moisture fluctuations, directly effects on the survival, distribution, and competition of weedy species in cropping systems [18]. For example, Xia et al. [19] found that weedy rice seeds can germinate at a lower temperature than its co-occurred cultivated rice, and the germination ratio showed a latitudinal gradient pattern between weedy rice populations from north China down to Jiangsu Province.

Weedy rice (*Oryza sativa* f. *spontanea*, WR, Additional file 2: Figure S1a) is a noxious agricultural weed infesting worldwide rice fields [20]. It is a conspecific weed that belongs to the same biological species of cultivated rice (*O. sativa*) but with strong seed shattering and prolonged seed dormancy. In typical tropic rice cultivation regions, such as Guangdong, Guangxi, and Hainan Provinces, farmers cultivate rice for two seasons: the early and late rice-cultivation seasons. In both the two seasons, weedy rice was found in the same rice fields (sympatry). Generally, phenological conditions, such as temperature and photoperiod, between the two seasons are considerably different. Noticeably, differential genetic diversity and considerable genetic differentiation between the early- and late-season WR populations had been reported by Kong et al. [21]. Therefore, we believe that such considerable genetic differentiation is

accompanied by an inevitable phenotypic divergence between the two-season WR populations in the same rice fields, probably caused by the adaptive evolution in the weedy rice populations.

The common garden experiment is an efficient tool to study adaptation and the genetic bases of the adaptive traits by growing individuals from different populations in a shared environment [22, 23], and it has been used extensively with plant species [24, 25]. In this study, we conducted in situ common garden experiments to estimate the phenotypic divergence between the early- and late-season WR populations in early and late rice-cultivation seasons, respectively. The central questions addressed are as follows: (1) What are the temperature and photoperiod variation patterns in different rice-cultivation seasons in Leizhou? (2) Do the vegetative and reproductive growth traits diverge between the sympatric two-season weedy rice populations? (3) Has the local adaptation developed in weedy rice populations? Answers to the above questions can support the viewpoint of genetic divergence between the sympatric two-season WR populations from another perspective and provide solid evidence of rapid adaptive evolution associated with ambient surroundings in plant species.

Results

Differences in air temperature and day length between the two rice-cultivation seasons

Different air temperature variation patterns were found between the early (ES) and late (LS) rice-cultivation seasons (Figure 1a, grey lines), based on the analysis of daily air temperature data (from 2011 to 2020) of Leizhou in Guangdong Province, China. The average air temperature raised gradually in ES and decreased in LS. In addition, the average air temperature was significant 1.7 °C lower in ES (25.9 vs. 27.6 °C, $p < 0.001$) than in LS (Figure 1b). Noticeably, a much lower air temperature was detected in the ES during the initial growth stages (Figure 1b). For example, during the seedling stage (S), the average air temperature of ES was significant 8.2 °C lower (20.9 vs. 29.1 °C, $p < 0.001$) than that of LS, and it was supported by the measured air and soil temperature in the common garden experiments (Additional file 2: Figure S2). Similarly, the average air temperature was 4.6 °C lower (24.2 vs. 28.8 °C, $p < 0.001$) in ES during the tillering stage (T). These results indicated a significantly lower air temperature in ES than in LS, particularly during the initial growth stages.

Results also showed different patterns of day length variation between ES and LS (Figure 1a, black lines). The average day length increased gradually in ES and decreased in LS. The average day length was significant 0.45 h longer (12.76 vs. 12.31 h, $p < 0.001$) in ES than that in LS (Figure 1c). In addition, significant differences in day length were detected at different rice growth stages, particularly during the flowering (F) and ripening stage (R) (Figure 1c). For example, the average day length was 0.98 h longer (13.11 vs. 12.13 h, $p < 0.001$) in ES during the flowering stage, and was 1.81 h longer (13.37 vs. 11.56 h, $p < 0.001$) in ES during the ripening stage. These results suggested significantly longer day lengths in ES than that in LS, particularly during the reproductive growth stages.

Effects of populations and population pairs on early-season WR growth and development

Analytical results based on two-way ANOVA showed that population (WRE or WRL) had significant ($p < 0.05$) effects on vegetative growth traits, including plant height, number of tillers per plant and leaf length/width, and reproductive growth traits, including flowering time and reproductive traits in the early rice-cultivation season (Table 1). However, no significant effects of the population were detected in the late rice-cultivation season (Additional file 1: Table S4). These results indicated significant differences in these vegetative and reproductive growth traits between the early- and late-season WR populations in the early rice-cultivation season. In addition, population pairs (CDE/L, HJE/L, and DCE/L) also showed significant effects on some of the traits at different stages in the early and late rice-cultivation season (Table 1, Additional file 1: Table S4). Noticeably, a pronounced effect of population pair in the early season was detected, for example, the highly significant effects ($p < 0.001$) on flowering time and reproductive traits (Table 1). These results showed variations of the phenotypic differences estimated between the early- and late-season WR populations among different population pairs, and suggested that the micro-environments in different rice fields also played some roles in weedy rice growth and development.

In addition, transplant season (ES and LS) also had profound effects on almost all of growth and development traits in both the early- and late-season weedy rice populations (Additional file 1: Table S5 and S6). This result suggested that differences in ecological elements, such as temperature and day length, between ES and LS may directly contribute to the further growth and development status of weedy rice, although in the same rice field.

Growth advantages in vegetative and reproductive traits of early-season WR

Significant differences ($p < 0.001$) in plant height were detected between the early- and late-season WR populations according to the early-season common garden experiment (Figure 2). The early-season WR populations showed a clear higher height than the late-season populations at different growth stages in all population pairs. In addition, significant differences were also detected in the number of tillers per plant between the two-season WR populations at the early growth stages in the early season (Figure 3, $p < 0.001$). However, no significant differences in plant height and the number of tillers per plant were detected in the late-season common garden experiment (Figure 2 and 3). Furthermore, the leaf length and width differences between the two-season WR populations were also detected (Additional file 2: Figure S4 and S5). These results suggested significant differences in vegetative growth between the two-season WR populations in the early rice-cultivation environment and evident growth advantages in the early-season WR populations.

Noticeably, apparent differences in flowering time were observed in the WR population pairs (CDE/L and DCE/L) in the early-season common garden experiment (Figure 4). The early-season populations exhibited early flowering time compared to their corresponding late-season populations. In addition, results of flowering time at the stages of different proportions of flowering plants also supported the significant early flowering of the early-season populations in CDE/L and DCE/L population pairs (Figure

5). However, only minor differences in flowering time in CDE/L population pair were detected in the late-season common garden experiment (Additional file 2: Figure S3), although no such differences in flowering were found in the early-season populations at different stages (Figure 5). These results indicated considerable divergences in flowering time between the two-season WR populations, and the early-season population was significantly early flowering in the native environment.

Also, results showed a relatively greater grains per plant, higher seed setting rate, and 100-seeds weight in the early-season WR populations, in the early-season common garden experiment (Figure 6). For example, the early-season WR populations had significantly more grains per plant (Figure 6a) and a significantly higher rate of seed sets than the corresponding late-season populations in CDE/L and DCE/L population pairs (Figure 6b). However, no such significant differences in reproductive traits were detected in the late-season common garden experiment (Figure 6a and b). These results suggested significant differences in reproductive traits between the two-season WR populations, and the early-season populations can produce more progenies than the late-season populations in the early rice-cultivation seasons.

In addition, we found evident advantages in plant height (Figure 2), the number of tillers per plant (Figure 3), and flowering time (Figure 5), reproductive traits (Figure 6) of all WR populations growing in the late rice-cultivation season (significance not shown), compared to that in the corresponding early season. These findings suggested that the late rice-cultivation seasons may be more suitable for weedy rice growth, probably due to its high temperature and short day length.

Evident local adaptation detected in the early-season WR populations

Evident local adaptation (LA) in the early-season WR populations was detected in the native early rice-cultivation season (Table 2), based on the method recommended by Hereford (2009). For example, the average level of LA was 0.15 for plant height and 0.14 for reproductive traits. Generally, the positive values of LA indicate local adaptation for traits, but the early flowering was regarded as an adaptive trait in this study. Therefore, the negative value of -0.04 also indicated local adaptation for flowering time in the early-season WR populations. However, no such evident local adaptation was detected in the late-season WR populations in the native late rice-cultivation season (Additional file 1: Table S7). These results indicated that ecological environment such as temperature and day length played an essential role in local adaptation of weedy rice.

In addition, the LA for plant height was stronger in population CDE (e.g., 0.32) and DCE (e.g., 0.21) than that in HJE (e.g., 0.14), as well as flowering time (Table 2). This result suggested a more pronounced local adaptation of the early-season WR populations in CDE/L and DCE/L population pairs, likely associated with population histories of WR infesting cultivated rice fields at different periods. Furthermore, the evident local adaptation for the reproductive traits, such as grains per plant and seed setting rate, were detected in all early-season WR populations. It indicated that the local adaptation in the early rice-cultivation season might be conducive to the reproductive growth of the early-season WR populations. Therefore, all of the abovementioned findings suggested that population differentiation may have

occurred between the sympatric early- and late-season weedy rice population, probably resulting from the local adaptation in the early seasons.

As expected, evident population differentiation, indicated by clear population clusters, was found between the two-season weedy rice populations in principal component analysis (PCA) (Figure 7) using nine phenotypical variables (A-I). Much clearer clusters can be clarified in the CDE/L and DCE/L population pairs (Figure 7a and c) than the HJE/L population pair (Figure 7b), which was consistent with their pronounced local adaptation. In addition, the first and second principal components (PC1 and PC2) can explain 62.7% and 57.5% variance in the CDE/L and DCE/L population pairs (Figure 7a and c), respectively. Noticeably, the same spatial patterns of variables were obtained from the principal component analysis for variables in the three population pairs, using the coordinates and correlation between variables. Clearly, the variables D, E, F, H (red) primarily contributed to PC1, A, B, C (gold) contributed to PC2, and I, G (blue) contributed to PC3. The contribution of variables was shown in Additional file 2: Figure S6. These results indicated that the factors causing population differentiation might be shared among different population pairs, and the differentiation between the two-season weedy rice populations probably follows a general pattern.

Discussion

The early rice-cultivation season is a stressful environment for weedy rice

Analytical results of 10-year climate data showed different variation patterns of air temperature and day length between the early and late rice-cultivation seasons in Leizhou. The 10-year average air temperature of the early season was significant 1.7 °C lower than that in the late season, particularly during the initial rice growth stages (4.6~8.2°C). In addition, the 10-year average day length of the early season was significant 0.45 hours longer than that in the late season, and it was up to 0.98~1.81 hours during the reproductive stages. Generally, low temperature and long day length are environmental restrictions on rice performance because rice is a short day and cold-sensitive plant [26, 27]. As for a conspecific weed that belongs to the same biological species of cultivated rice, weedy rice has the same responses to temperature and day length. Therefore, these findings indicate that the low temperature and long day length may be the primary stressful elements affecting weedy rice growth and development in the early-season environment.

The optimal temperature for rice cultivation is between 25 and 35 °C [6], and temperature beyond optimum is harmful to rice and negatively affects growth, development and ultimately reduces the grain yield. In this study, lower average air temperature was found during the seedling stage (20.9 °C) and the tillering stage (24.2 °C) in the early season, compared to the optimum. By contrast, the average air temperature was 29.1 and 28.8 °C, which fit the optimum, during the corresponding stages in the late season. Therefore, the early-season weedy rice had to experience the low temperature stress at the germination and seedling stages in the early season, and the low temperature may be an intense selective pressure to affect the growth and development of the early-season weedy rice in early stages. Altogether,

the above findings suggest that the early rice-cultivation season is stressful with lower ambient temperatures than the corresponding late season, especially during the initial growth stages.

Generally, rice growth at the early stage is essential for stable seedling establishment and subsequent vigorous vegetative growth [28], because seed germination, seedling emergence, stem elongation, and individual survival are typically temperature dependent [29]. During this period, the low-temperature stress inhibits seed germination, seedling establishment and causes the retardation of rice growth. For example, the apparent reduction in final seed germination ratio and the length of coleoptile and radical were observed, when rice seeds were germinated at a temperature below optimum [28], and reduced seedling growth and vigor of rice were also found under a low temperature condition [30]. In this study, a significant reduction in plant height and number of tillers was found in almost all weedy rice populations during the early stages in the early-season common garden, compared to the same stages in the late-season common garden. Therefore, these findings indicate that low-temperature stress directly negatively affects weedy rice growth, most likely caused by retardation in germination and seedling establishment during the initial stages. Low-temperature stress lengthens rice vegetative growth and causes lagging in reproductive growth by delaying flowering time [13]. Our common garden experiments provided solid evidence of a significant reduction in the number of seeds per plant, seed setting ratio, and 100-seeds weight in weedy rice populations during the early-season common garden, compared to the high-temperature late season. Therefore, the early rice-cultivation season is a relatively stressful environment for weedy rice growth and development because of its low ambient temperatures.

In addition, the day length was significantly longer in the early season than in the corresponding late season, especially during the reproductive growth stages (~2 hours), based on the 10-year day length data analyses in this study. Noticeably, the evident lagging in flowering time was found in almost all weedy rice populations in the early-season common garden. It was about ten days later in flowering time than that in the late-season common garden. Therefore, this finding supports the photoperiodical flowering response of rice in a long day environment because rice is known as a short-day plant and a long day length environment usually delays the flowering time of rice [27]. Therefore, long day length is another environmental constraint factor affecting weedy rice growth and development in the stressful early rice-cultivation season. Previous studies suggest frequent interactions between temperature and photoperiod in plant flowering time regulation, and low temperature and long day environments are fundamental causes of late flowering in rice [1, 13]. Altogether, we can conclude that the early rice cultivation season is stressful for weedy rice growth and development because of its low temperature and long day length.

Phenotypic differentiation between the sympatric weedy rice populations is associated with local adaptation

Substantial divergence in vegetative and reproductive growth traits, including plant height, number of tillers, flowering time, and reproductive traits, was detected between the early- and late-season weedy rice

populations during the early-season common garden. Furthermore, the early-season populations had evident advantages in growth and development, such as higher plant height, more tillers, and earlier flowering, compared to the corresponding late-season populations. However, no such divergence was detected in the late-season common garden experiment. These findings demonstrate considerable phenotypic divergence between the sympatric two-season weedy rice populations in the stressful early rice-cultivation season.

In addition, evident local adaptation (LA) for these divergent traits was only detected in the early-season weedy rice populations in the stressful early-season common garden, based on the method adopted from Hereford [31]. It demonstrates the occurrence of evident local adaptation in the early-season populations, which may be caused by the stressful environment in the early seasons. Noticeably, the principal component analysis (PCA) showed clear population clusters between the early- and late-season weedy rice populations using phenotypical data, and the same pattern of variables' contributions was found among all population pairs. Additionally, relative evident population clusters were also found in CDE/L and DCE/L population pairs, probably associated with their pronounced local adaptation. These findings also provide solid evidence to support population divergence between the sympatric early- and late-season weedy rice populations, and the local adaptation may play a major role in the process of population divergence.

The common garden experiment is generally used to test for local adaptation signals in traits of interest, such as life-history traits and physiology, because it enables to unraveling of the genetic basis of complex phenotypes across various populations without the confounding effects of the corresponding environment [22, 23]. For example, physiological evidence for local adaptation to both freezing and drought stress in closely related American live oaks species was obtained from the common garden experiments [32]. Additionally, van Boheemen et al. [33] found the rapid and repeated local adaptation to climate in an invasive plant by examining trait divergence in a common garden experiment. These studies demonstrate that local adaptation generally occurs in plant populations when the plants expand a new ecological niche or habitat and experience some environmental stresses, such as low temperature and drought. Therefore, the evident local adaptation detected in the early-season weedy rice populations suggested that the early-season weedy rice may expand from the late season and evolve in a rapid adaptive evolution process in the early season because of the stressful environment.

Plants often show differences in morphology within and between populations because of different local environments, which cause different selective pressures to shape adaptive genetic variation in plant individuals and lead to heritable differences in plant phenotype [25]. Generally, the phenotype of an individual is determined by the interactions between the environment and its genotype, which includes local adaptation [34]. A previous study detected considerable genetic divergence between the early- and late-season weedy rice populations in the same rice fields [21]. Therefore, the phenotypic divergence between the two-season weedy rice populations is closely associated with the genetic divergence, mainly caused by the local adaptation in the early-season populations. In addition, local adaptation is also

assumed to occur under limited gene flow [35, 36]. It means the barriers to gene flow between populations are conducive to maintaining adaptive traits or alleles within the populations.

Interestingly, the limited gene flow between the two-season weedy rice populations was also proposed by Kong et al. [21], which was explained to maintain genetic diversity within populations. This finding provided evidence of two cryptical populations in the same rice field and suggested that the weedy rice individuals, which adapted to low temperature and long day length conditions, can survive and only retain in the early rice-cultivation season. Therefore, through local adaptation in the stressful environment and limited gene flow between the two seasons, the genetic and phenotypical divergence both emerged in weedy rice populations occurring in the same rice fields, which is a typical event of sympatric divergence in plant species.

Differences in flowering time contribute to sympatric divergence in weedy rice populations

Differences in flowering time were observed between the early- and late-season weedy rice populations, and the early-season populations showed early flowering phenotype in the early rice-cultivation season. In addition, evident local adaptation for the early flowering time was also detected in the early-season weedy rice populations. These results suggested a divergent performance of flowering time between the sympatric two-season weedy rice populations, most likely caused by the local adaptation in the stressful early rice-cultivation season.

Generally, the shift in flowering time is usually accompanied by local adaptation in flowering plants [37], and can act as solid prezygotic reproductive barriers in plants. In some species, such as the grasses *Agrostis tenuis* and *Anthoxanthum odoratum*, heavy metals tolerant and intolerant races differ in seasonal time of flowering, and so are partially isolated reproductively at a pre-pollination stage [38]. In addition, flowering time divergence was also found in some compelling examples of sympatric divergence. For instance, the two palm species (*Howea*) in Lord Howe Island segregate according to the acidity of the soil, and obvious disjunctions in flowering time were found between the two palms [39]. In addition, two sister species of mountain rose (*Metrosideros*) endemic to Lord Howe Island also exhibited apparent divergence in flowering time because of their divergent ecological niches [40]. These studies provide reliable evidence for shifts in flowering time caused by local adaptation between sympatric plant populations and demonstrate its essential role in the sympatric divergence of flowering plants. Therefore, the findings of local adaptation associated disjunctions in flowering time indicate a possible rapid adaptive evolution in weedy rice populations occurring in the same rice fields and probably generate genetic and phenotypic divergence between the sympatric two-season weedy rice populations.

In addition, for agricultural weeds, the early flowering phenotype would likely result in the evolution of weed populations that display a shorter life cycle, allowing plants to set and shed seed before crop harvest [41]. Therefore, we propose two possible reasons why the early-season weedy rice evolved an early flowering phenotype, which caused divergent flowering time between the two weedy rice

populations. First, the genes that respond to the stressful environment in the early rice-cultivation season are closely linked to the genes that regulate flowering in weedy rice, or pleiotropy [42], most likely caused by genetic variations. Second, to ensure reproduction success in the new environment, weedy rice shortened its growth period to complete its life cycle as soon as possible, most likely caused by epigenetic modification [43]. In other words, flowering time is crucial in determining the adaptation of weedy rice in different ecological environments, and probably contribute to the reproductive isolation between sympatric plant populations because of genetic or epigenetic variations in specific adaptive genes. Therefore, it is necessary to identify the adaptive genes associated with divergent performances, such as flowering time, and investigate their mechanisms underlie local adaptation in the early-season weedy rice populations in future work.

Conclusion

Differences in temperature and day length were found between early and late rice-cultivation seasons in Leizhou, with a lower temperature and longer day length in the early seasons than those in the late seasons. These findings indicated that stressful ecological elements affecting weedy rice growth and development are probably present in the early rice-cultivation seasons. In addition, significant differences in vegetative and reproductive traits were detected between the sympatric early- and late-season weedy rice populations according to the early-season common garden experiment. The early-season populations showed evident growth and development advantages over the corresponding late-season populations. Noticeably, such phenotypical differentiation was associated with the evident local adaptation in the early-season weedy rice populations and PCAs also showed clear population clusters between the two-season weedy rice populations. These findings suggest that the adaptive evolution has occurred in weedy rice populations and has potential roles in causing differentiation between the sympatric two-season populations. Altogether, the findings of this study provided clear evidence of phenotypic differentiation between the sympatric early- and late-season weedy rice populations, probably associated with the local adaptation to their ambient temperature and photoperiod, and support the sympatric divergence theory in ecological speciation.

Materials And Methods

Plant materials

A total of six WR populations occurring in the early (code as WRE) and late (WRL) rice-cultivation seasons were collected from three rice fields in Leizhou, Guangdong Province of China (Table S1). The geographic distance between the sampled rice fields was >30 km. WR populations collected from the same field were treated as a population pair (e.g., CDE/L, DCE/L, or HJE/L pair). About 60 randomly selected samples were collected from each WR population of either the early- or late-season rice field at the spatial distance intervals of >10 m to avoid similar sampling genotypes. Matured panicles from a WR plant were collected as an independent sample. The average duration of the early rice-cultivation seasons (ES) was from March 1st to June 25th, whereas late rice-cultivation seasons (LS) were from July 20th to November 5th.

Historical climate data collection and analysis

The 10-year air temperature data (2011~2020) in Leizhou was collected from the Tianqi Database (<http://lishi.tianqi.com/leizhou/index.html>, in Chinese). The air temperature data included the daily minimum and maximum temperatures (Table S2). The averages of the minimum and maximum temperatures were defined as the daily average air temperature. Also, the 10-year day length data (2006~2015) was collected from an open website (https://richurimo.51240.com/leigaozhen_richurimo/, in Chinese). The day length data included the daily time of sunrise and sunset (Table S3). The differences between the time of sunrise and sunset were defined as the day lengths.

In addition, the WR growth period was artificially divided into four stages, corresponding to different rice growth phases: seedling (S), tillering (T), flowering (F), and ripening (R). The average values of each year in the ten years were used to estimate differences in air temperatures and day lengths between the early and late rice-cultivation seasons at different stages.

Design of common garden experiments

Common garden experiments were conducted in a rice field in Leizhou, Guangdong Province of China (Figure S1) in the early and late rice-cultivation seasons, respectively. In the experiments, 60 WR samples were included from each population, and 10 well-developed seeds from each sample were used to determine the ratios of seed germination. Seeds were germinated in black boxes placed in the experimental rice field (Figure S1b). Thirty-day-old seedlings (≥ 3 seedlings from the same sample) were transplanted to the experimental rice field. In the experiment, each plot containing 36 seedlings was arranged with a 6 × 6 grid (Figure S1c) and 30 cm between the hills and rows. Six plots (replicates) were included for the same treatments of each WR population. All plots were arranged in the experimental field with a completely randomized design with 60 cm spacing between the plots. About 80 days after seed germination, panicles were enclosed in mesh nylon bags to avoid accidental shattering of seeds (Figure S1d).

Measurements of fitness related traits

Vegetative growth traits, including plant height, number of tillers per plant, and leaf length/width, were measured in the common garden experiments. Plant height was defined as the distance from the ground to the tip of the longest leaf, and the number of tillers per plant referred to the total of branches that emerged from the main stem culm of a plant. Leaf length and width of the main stem culm were also involved, including the top-first leaf (the first leaf on the top of the main stem culm) and top-second leaf (the second leaf on the top of the main stem culm). The leaf length was defined as the distance from the leaf tip to the base, and the leaf width was measured at the middle of the leaf.

To detect the differences in vegetative growth between the early- and late-season WR populations at different stages, we measured these traits every 20 days after seed germination (DAG), including the 20, 40, 60, and 80 DAG. At the 20 DAG, considering the elder seedlings are too weak to avoid harm during the

measurement, the leaf length and width measurements were abandoned. Therefore, only plant height and number of tillers per plant were measured. After transplanting, the measurements of plant height, number of tillers, and leaf length/width for each plant individual were conducted in all planting plots at 40, 60, and 80 DAG. Experimental data obtained from the measurements and recording was mainly used to estimate differences in growth and development between the early- and late-season WR populations in the same rice-cultivation season and to compare the performance of the same population in different rice-cultivation seasons.

The flowering time of each plant from beginning to end was recorded, and a dynamic pattern of flowering time was constructed in each population. The beginning of flowering was defined as the date of the first flower emerging, and the rise of heavy panicles indicated the end of flowering. The proportion of flowering plants per day was marked and recorded in each population, which was used to construct the flowering time patterns of weedy rice populations. To estimate more vast differences in flowering time between the early- and late-season WR populations, the average flowering time in different phases, including 1%, 30%, 50%, and 80% plants flowered, were also calculated to make further comparisons between the two-season WR populations.

The reproductive traits were closely associated with the flowering time in rice. Therefore, the number of seeds per plant, seed setting rate, and 100-seeds weight were measured for each plot. After harvesting and threshing, the seed air cleaning instrument (CFY-2, Top Cloud-agri Technology Company, Zhejiang, China) was used to separate the full seeds. Seeds counting was conducted in an electronic seed counter (PME, Shanco Instruments, Shanghai, China), and 100-seeds weighting used an analytical balance.

Estimate magnitude of local adaptation

Fitness-related traits, such as plant height, number of tillers, and reproductive traits, were used to quantify the local adaptation (LA) of WR populations in the native rice-cultivation environment. In addition, the flowering time was also involved in this analysis based on the data of days to flowering, and early flowering was regarded as an adaptive trait. The quantitative measure of local adaptation was the relative fitness of the native population at a field site in a given year minus the relative fitness of a nonnative population at that site, following the equation from Hereford [42]:

$$LA = \frac{W_{\text{native}} - W_{\text{non-native}}}{\text{avg}(W)}$$

Where W represents the mean fitness of native and nonnative populations, and $\text{avg}(W)$ represents the mean fitness of all populations. In this study, the early-season WR populations were native in the early rice-cultivation season, and the late-season WR populations were native in the late rice-cultivation season. Generally, positive LA values indicate local adaptation in the native populations (Hereford, 2009). However, the negative LA values of flowering time also indicate local adaptation for early flowering in WR.

Principal component analysis (PCA)

Nine phenotypical data, including the number of tillers per plant at 40 (A), 60 (B), and 80 (C) DAG, plant height at 40 (D), 60 (E), and 80 (F) DAG, flowering time (G), seed setting rate (H) and 100-seeds weight (I), were used in the principal component analysis. The PCAs were conducted in R v.4.1.2 (<https://cran.r-project.org/bin/windows/base/>) with package “ggbiplot”, and the confidence intervals of the ellipse was set as 95%. Additionally, the principal component analysis for variables, including contributions, coordinates, and correlation, was conducted using the R package “FactoMineR”. The top three principal components (PC1, PC2, and PC3) were selected in the analyses according to their explained variance.

Statistical analyses of data

Two-way ANOVA (analysis of variance) was used to determine the factors, including the population (the corresponding early- and late-season WR populations), population pair (CDE/L, DCE/L, and HJE/L from different collecting sites), and transplant season (the EARLY and LATE rice-cultivation season), affecting the plant growth and development significantly. Four groups of ANOVAs conducted: (i) estimation of the effects of population and population pair in the EARLY rice-cultivation season (Table 1), (ii) estimation of the effects of population and population pair in the LATE rice-cultivation season (Table S4), (iii) estimation of the effect of transplant season and population pair in the early-season weedy rice populations (Table S5) and (iv) estimation of the effect of transplant season and population pair in the late-season weedy rice populations (Table S6). The first two groups of ANOVAs aimed to estimate the differences in growth and development traits, which were measured in common garden experiments, between the early- and late-season WR populations in the early and late rice-cultivation environments, respectively. The latter two groups of ANOVAs aimed to estimate the differences in growth and development of the same population in different rice-cultivation seasons.

In addition, difference analyses included in this study were tested based on the student t-test [44], and the method of two-tails and equal variance test of two samples were adopted. Two-way ANOVAs and student t-test were both performed using the software IBM SPSS Statistics ver. 22.0 for Windows (SPSS Inc., IBM Company Chicago, IL, USA, 2010).

Abbreviations

WR, Weedy Rice.

CDE/L, DCE/L, and HJE/L, the Early/Late-season weedy rice populations collected from Chi Dou, Dong Cun and He Jia villages.

ES/LS, the Early or Late rice-cultivation Season.

S, the seedling stage; T, the tillering stage; F, the flowering stage; R, the ripening stage.

LA, Local Adaptation.

PCA, principal component analysis

Declarations

Ethics approval and consent to participate

Not applicable. This manuscript does not report on or involve the use of any animal or human data or tissue.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare no conflict of interests.

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Authors' contributions

BRL conceived this study and acquired the funding. ZW analyzed the data and wrote the manuscript. ZW, XQJ, XC and QYX collected the plant materials. ZW and XQJ conducted the common garden experiments. QYX acquired the funding. All authors read and approved the final manuscript.

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Tables

Table 1. Two-way ANOVA for WR populations transplanted in the EARLY rice-cultivation season

Stage	Population			Population pair		
	df	F-value	<i>p</i> -value	df	F-value	<i>p</i> -value
20 days after germination						
Plant height	1	310.084	0.000	2	1.010	0.365
Number of tillers	1	0.105	0.746	2	0.833	0.435
40 days after germination						
Plant height	1	102.788	0.000	2	2.046	0.147
Number of tillers	1	72.346	0.000	2	1.661	0.207
Top-first leaf length	1	4.458	0.043	2	12.486	0.000
Top-first leaf width	1	14.070	0.001	2	0.465	0.633
Top-second leaf length	1	48.062	0.000	2	9.518	0.001
Top-second leaf width	1	5.127	0.031	2	0.759	0.477
60 days after germination						
Plant height	1	73.012	0.000	2	10.752	0.000
Number of tillers	1	8.282	0.007	2	19.784	0.000
Top-first leaf length	1	33.493	0.000	2	1.193	0.317
Top-first leaf width	1	8.889	0.006	2	3.472	0.044
Top-second leaf length	1	60.119	0.000	2	7.693	0.002
Top-second leaf width	1	5.444	0.027	2	2.111	0.139
80 days after germination						
Plant height	1	51.059	0.000	2	3.104	0.060
Number of tillers	1	3.729	0.063	2	30.440	0.000
Top-first leaf length	1	0.284	0.598	2	4.757	0.016
Top-first leaf width	1	0.495	0.487	2	2.857	0.073
Top-second leaf length	1	14.317	0.001	2	4.290	0.023
Top-second leaf width	1	0.542	0.467	2	21.145	0.000
Flowering time						
Days for 1% plants to flower	1	12.300	0.001	2	27.964	0.000
Days for 30% plants to flower	1	65.147	0.000	2	86.491	0.000
Days for 50% plants to flower	1	49.082	0.000	2	39.619	0.000

Days for 80% plants to flower	1	32.213	0.000	2	17.578	0.000
Days for 100% plants to flower	1	0.729	0.400	2	12.611	0.000
Fecundity						
Number of seeds per plant	1	19.841	0.000	2	30.944	0.000
Seed setting ratio	1	19.030	0.000	2	22.563	0.000
100-seeds weight	1	7.050	0.013	2	30.360	0.000

df, degree of freedom.

Table 2. Local adaptation estimation of the early-season WR populations in the early rice-cultivation season

Trait	Index	Local adaptation (LA)			
		CDE ³	HJE	DCE	Average
Plant height	20 DAG ¹	0.32	0.14	0.21	0.15
	40 DAG	0.15	0.09	0.13	
	60 DAG	0.16	0.05	0.16	
	80 DAG	0.15	0.02	0.17	
Tiller numbers	20 DAG	0.00	0.00	0.00	0.08
	40 DAG	0.27	0.18	0.18	
	60 DAG	0.11	0.10	0.03	
	80 DAG	0.07	0.04	-0.02	
Flowering time	1% ²	-0.06	-0.02	-0.09	-0.04
	30%	-0.09	-0.01	-0.05	
	50%	-0.07	-0.02	-0.04	
	80%	-0.04	-0.01	-0.03	
Reproductive traits	Grains per plant	0.23	0.32	0.25	0.14
	Seed setting rate	0.19	0.06	0.15	
	100-seed weight	-0.02	0.04	0.08	

¹ DAG, days after seed germination.

² %, proportion of flowering plants.

³ CDE, HJE, and DCE, the early-season weedy rice populations collected from Chi Dou (CD), He Jia (HJ) and Dong Cun (DC), respectively.

Figures

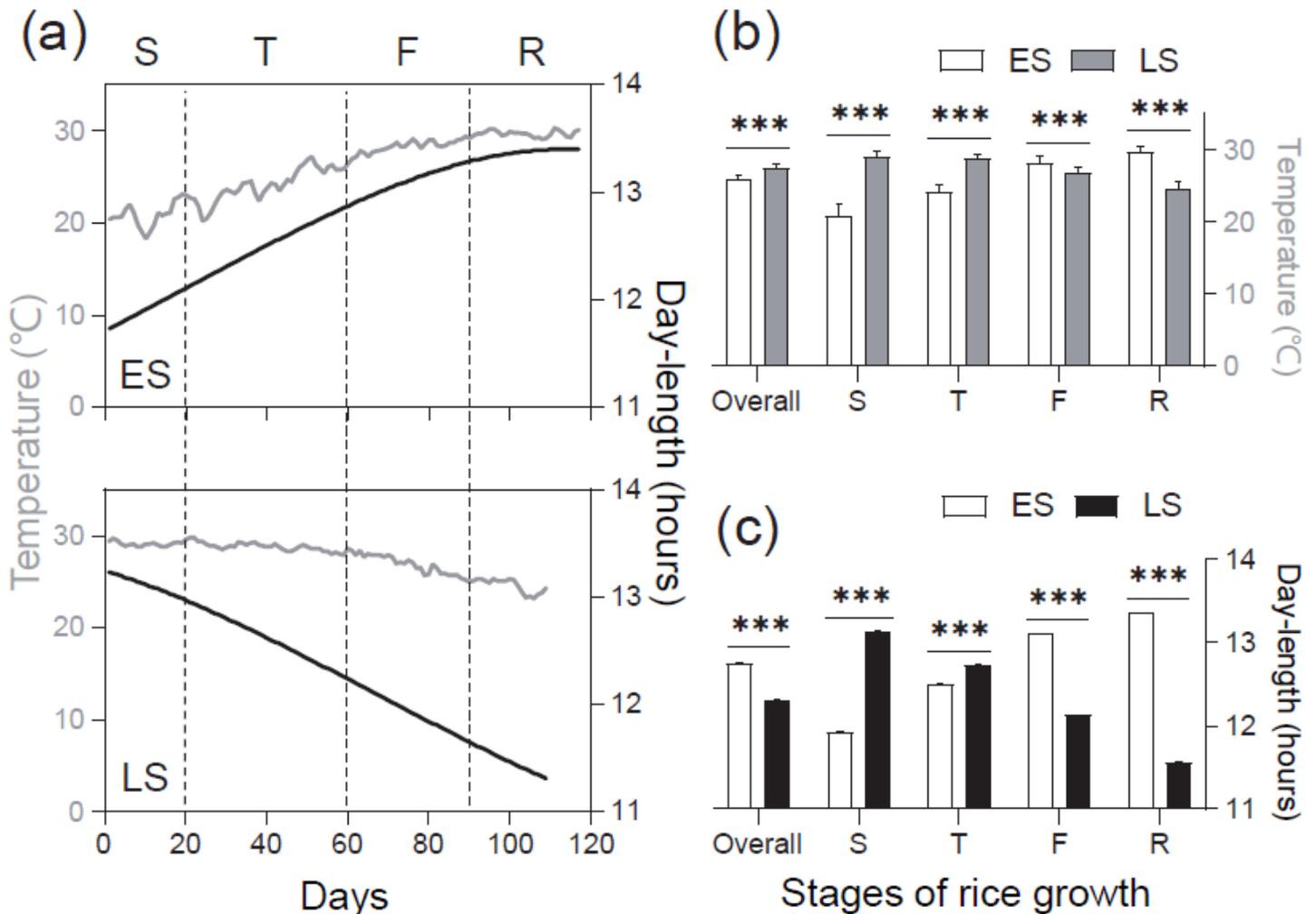


Figure 1

Differences in the average air temperature and day length between the EARLY and LATE seasons. (a) Patterns of 10-year average daily air temperature and day length variation in the EARLY (ES) and LATE (LS) rice-cultivation seasons, respectively. Grey lines represent the air temperature, and black lines for day length. (b) Air temperature differences between the two rice-cultivation seasons at different rice growth stages, and (c) Day length differences between the two rice-cultivation seasons at different rice growth stages. White bars represent the early rice-cultivation season, and black bars for the late season. S, seedling; T, tillering; F, flowering; R, ripening. ***, $p < 0.001$. Error bar, standard deviation.

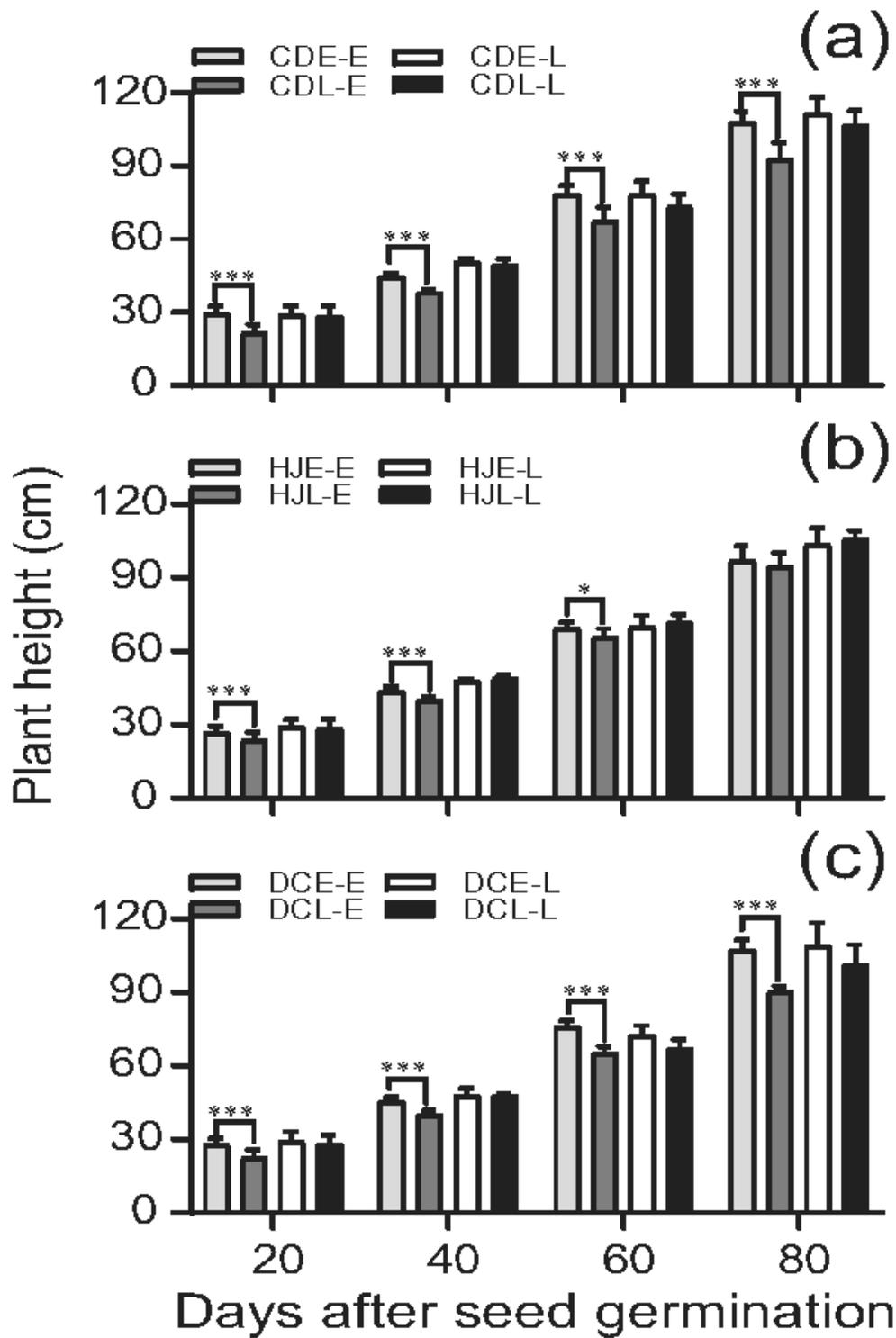


Figure 2

Plant height of weedy rice populations at different stages in the EARLY (-E) and LATE (-L) seasons. Bar plots show the plant height in (a) CDE/L, (b) HJE/L and (c) DCE/L population pairs at different stages, respectively. *, $p < 0.05$, ***, $p < 0.001$.

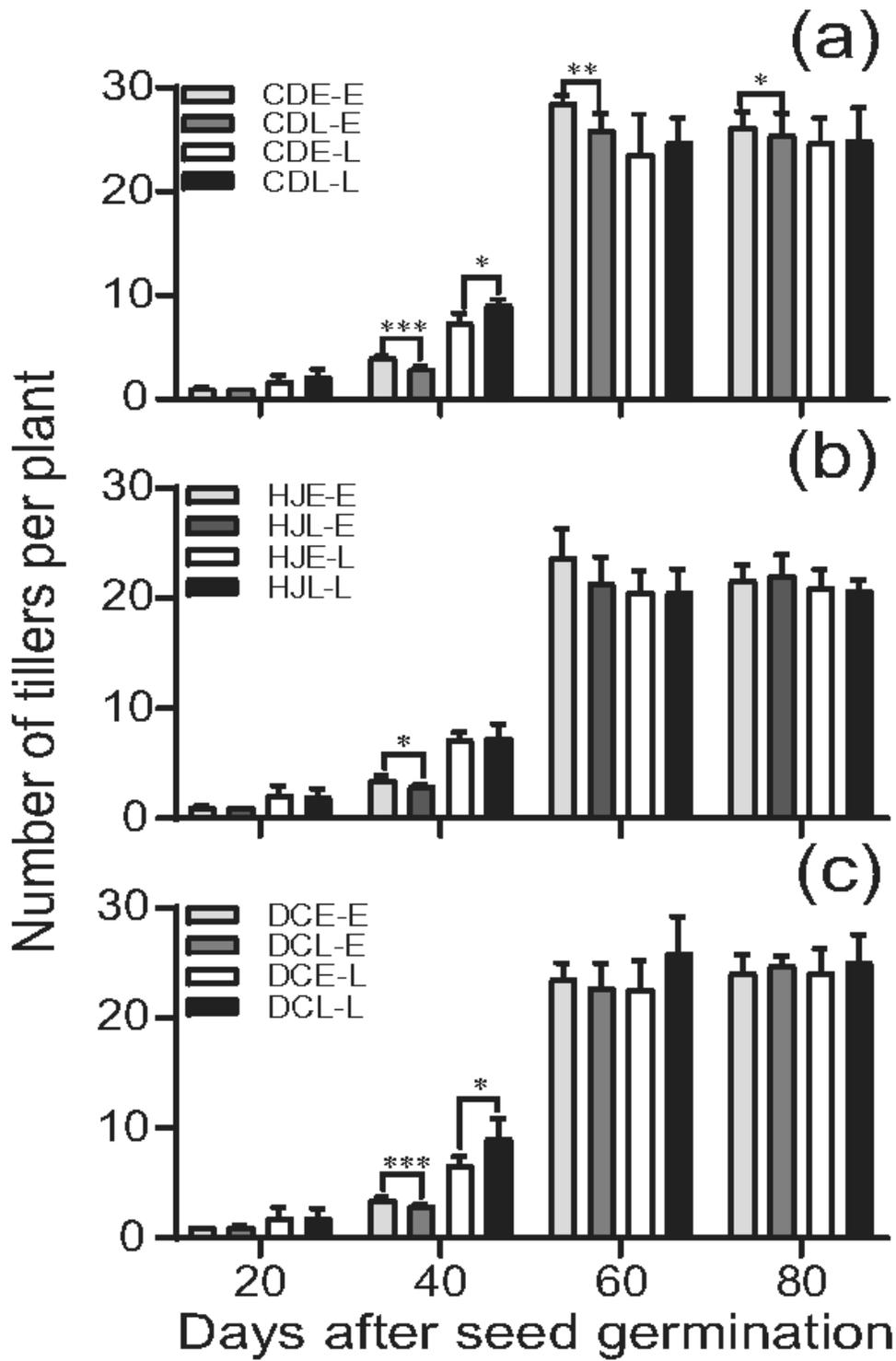


Figure 3

Tiller numbers of weedy rice populations at different stages in the EARLY (-E) and LATE (-L) seasons. Bar plots show the number of tillers per plant in (a) CDE/L, (b) HJE/L and (c) DCE/L population pairs at different stages, respectively. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

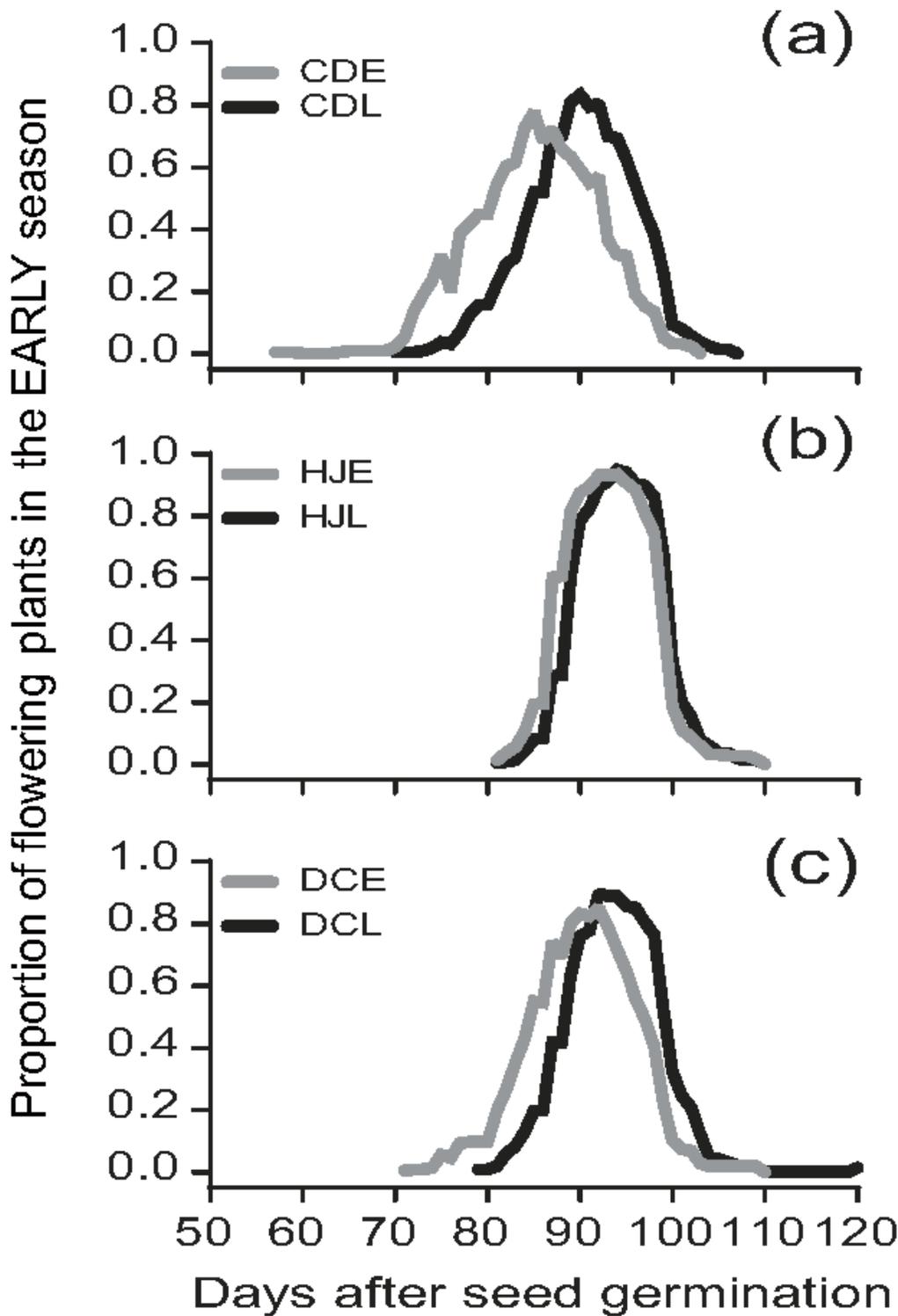


Figure 4

The flowering time patterns of weedy rice populations in the EARLY season. Flowering time patterns within population pair are showed in (a) CDE/L, (b) HJE/L and (c) DCE/L, respectively. Horizontal axes represent the date of flowering, indicated by days after seed germination (DAG), and vertical axes represent the proportion of flowering plants. Curves of weedy rice populations belong to the same pair are plotted together to show the disjunction in flowering time between the early- and late-season weedy rice

populations. Grey curves indicate the early-season weedy rice populations, and black curves indicate the late-season weedy rice populations.

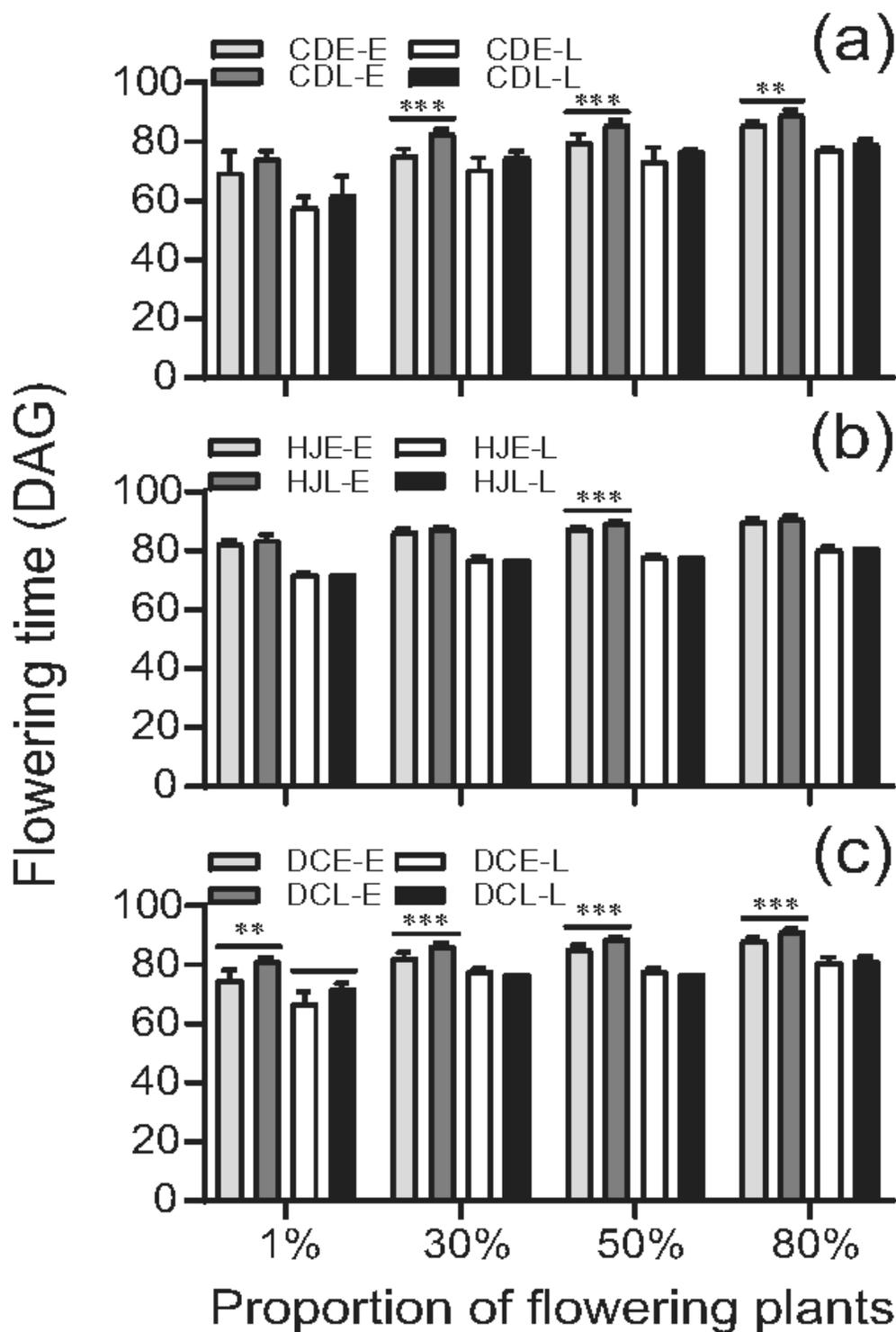


Figure 5

Flowering time of weedy rice populations at different stages in the EARLY (-E) and LATE (-L) seasons. Bar plots show the flowering time when the proportion of flowering plants is determined as 1%, 30%, 50% and

80% in (a) CDE/L, (b) HJE/L and (c) DCE/L population pairs, respectively. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

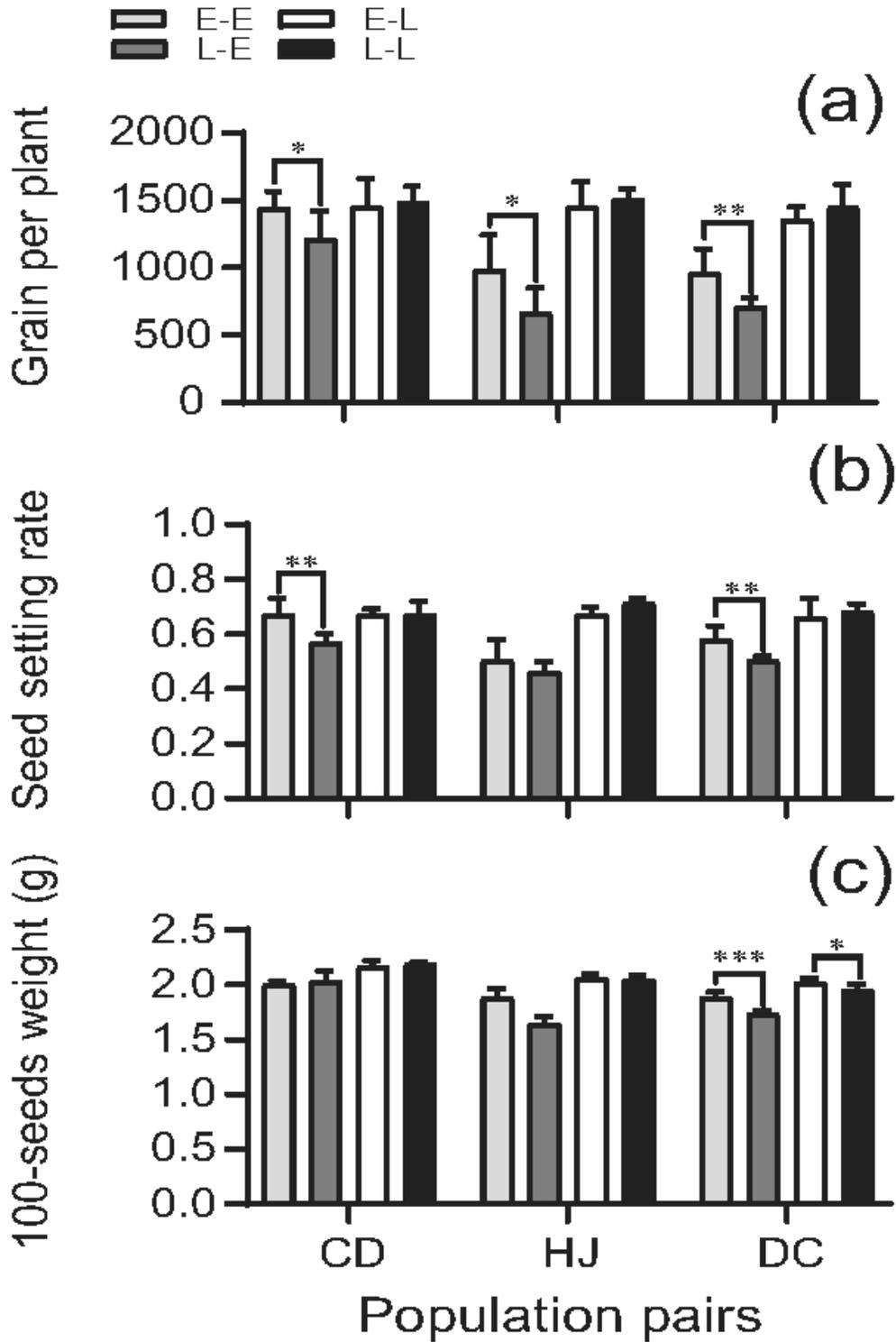
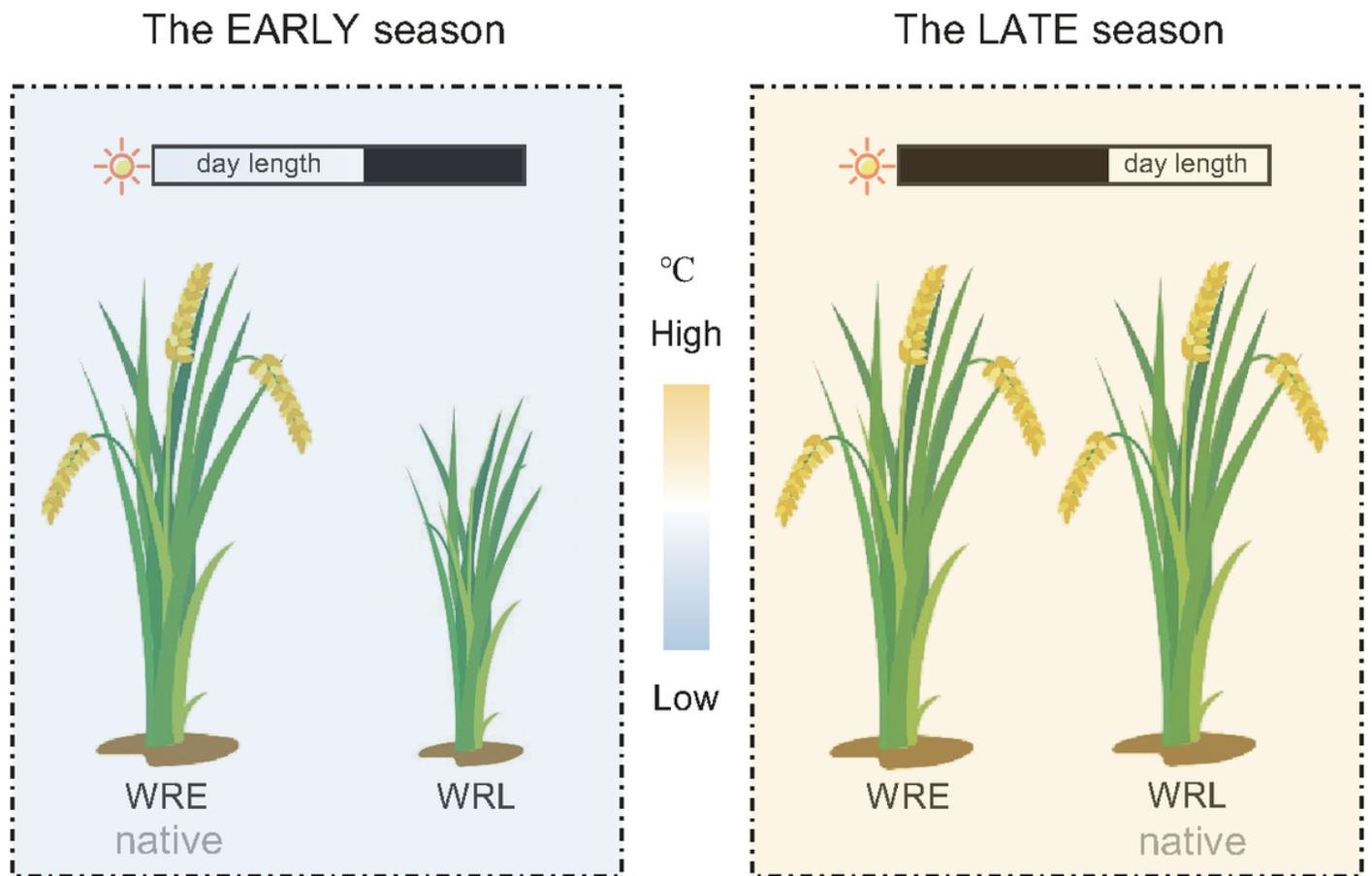


Figure 6

Reproductive traits of the two-season weedy rice populations planting in the EARLY (-E) and LATE (-L) seasons. (a) Grains per plant; (b) Seed setting rate; (c) 100-seeds weight. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

$p < 0.001$. CD, Chi Dou; HJ, He Jia; DC, Dong Cun. E/L-E, the early-/late-season populations planting in the EARLY season; E/L-L, the early-/late-season populations planting in the LATE season.



Local adaptation in the weedy rice populations

Figure 7

Principal component analysis (PCA) using phenotypical data obtained from the early season. The analyses were performed on 432 individuals and described by nine variables (A-I) in the three population pairs, respectively. (a) CDE/L population pair, (b) HJE/HJL population pair, and (c) DCE/L population pair. Each point represents one individual involved in the common garden experiment. Horizontal axes represent the first principal component (PC1), vertical axes represent the second principal component (PC2) and the explained variance was showed along the axes. Additionally, coordinates and correlation between variable were visualized based on results of variable analysis, and variables belonging to the same cluster were colored by the same color.

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