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RESEARCH ARTICLE

# Drastic shift in flowering phenology of $F_1$ hybrids causing rapid reproductive isolation in *Imperata cylindrica* in Japan

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## Abstract

1. Hybridization is a major source of phenotypic variation and a driving force for evolution. Although novel hybrid traits can often disrupt adaptive relationships between the parental phenotypes and their environments, how new hybrid traits disrupt local adaptation remains unclear. Here, we report how a new phenotype of hybrids between two *Imperata cylindrica* ecotypes contributes to rapid reproductive isolation from their parents and affects hybrid fitness.

2. We analysed 350 accessions of *I. cylindrica* collected from the 1980s to the 2010s throughout Japan to explore the genetic population structure of the hybrids. We surveyed the flowering periods, seed set, and germination of two ecotypes and their hybrids in both natural habitats and common gardens.

3. Genetic analyses of population structure revealed that the hybrid populations consisted of only $F_1$ individuals, without advanced generation hybrids. The flowering phenology of the $F_1$ plants was delayed until autumn, 5–6 months later than the parental ecotypes. The drastic shift in flowering phenology prevents $F_1$s from backcrossing. In addition, it changes their seed dispersal time to winter. Germination is inhibited by low temperatures, and the seeds likely decay before the next spring, resulting in the absence of an $F_2$ generation. We identified the environmental mismatch of the $F_1$ population as a specific mechanism for the maintenance of an only $F_1$ population.

4. *Synthesis.* We have demonstrated that this flowering phenology mismatch promotes reproductive isolation between the parents and $F_1$s and affects various temporal components of the hybrids, resulting in a unique hybrid population consisting only of $F_1$s. This system sheds light on the importance of hybrid traits in driving rapid reproductive isolation.

**KEYWORDS**
ecotype, $F_1$ hybrids dominated zone, flowering phenology shift, hybridization, *Imperata cylindrica*, instant reproductive isolation, phenological mismatch, population genetic structure

# 1 | INTRODUCTION

Hybridization is a major source of phenotypic variation (Grant & Grant, 1994) and a driving force for evolution (Coyne & Orr, 2004). Mating between individuals that have evolved independently can result in novel combinations of genomes or gene sets, which can generate a wide range of phenotypes, including over-dominant or transgressive phenotypes that can exceed or interpose the variation among the parental lineages (Johansen-Morris & Latta, 2006). The resultant hybrids are sometimes prevented from crossing with their parental lineages because of an altered trait related to reproduction (Kameyama & Ohara, 2006; Lo, 2010), which may ultimately contribute to speciation (Buerkle et al., 2000; Duenez-Guzman et al., 2009; Kagawa & Takimoto, 2018).

Previous studies have demonstrated that novel traits derived from hybridization directly contribute to reproductive isolation. For example, in plants, floral colour in *Iris* hybrids (Taylor et al., 2013) and floral odour in *Narcissus* hybrids (Marques et al., 2016) differ from those of their parents, resulting in the recruitment of novel pollinators for the hybrids and reproductive isolation between the hybrids and parents. Similarly, in animals, new hybrid phenotypes related to beak morphology and mating songs in Darwin's finches (Lamichhaney et al., 2018), wing colour patterns in *Heliconius* butterflies (Mavárez et al., 2006) and behavioural mate choice in cichlids (Selz et al., 2014) have caused reproductive isolation between hybrids and parents. Thus, novel hybrid traits can generate genetic and evolutionary changes.

However, such novel hybrid traits often disrupt adaptive relationships between the parental phenotypes and their environments, and reduced fitness often renders hybrids an evolutionary dead end (Arnold & Hodges, 1995; Burke & Arnold, 2001; Dobzhansky, 1934). Several studies have demonstrated that $F_1$ hybrid plants are widely distributed, but backcrossing and $F_2$ plants are rare (Kameyama et al., 2008; Kuehn et al., 1999; Milne & Abbott, 2008; Milne et al., 2003; Nagano et al., 2015; Nason et al., 1992; Zha et al., 2010). These studies imply that strong negative selection acts on the establishment of advanced generation hybrids or that sterile $F_1$ hybrids are repeatedly formed.

The mechanism of the mismatch between hybrid traits and parental environments is of great concern. Many theoretical and empirical studies illustrate that genomic incompatibility, or morphological or physiological mismatches in parental environments, cause low hybrid fitness (Dobzhansky, 1934; Mitsui et al., 2011). In contrast, knowledge regarding mismatches in the life-history events of hybrids is limited. The life-history traits of hybrids are unlikely to change because interfertile groups are expected to have similar life-history traits (e.g. breeding season) to their parental populations.

Changing life-history traits can cause wide range of adverse effects because many life-history events in plants (e.g. the timing of germination, leaf expansion and senescence, and flowering) are controlled by environmental cues to increase plant fitness (Hepworth & Dean, 2015; Penfield & MacGregor, 2017; Polgar & Primack, 2011). Among them, flowering time has a significant impact on mating partner availability in outcrossing species. If the synchrony of flowering time is disturbed, fitness can be greatly reduced. Furthermore, a shift in flowering time not only affects mating partner availability, but also the timing of seed set, seed dispersal, seed dormancy and germination after flowering, all of which also affect fitness.

Therefore, the effects of shifts and mismatches in life-history traits on hybrid fitness need to be investigated throughout the life cycle to understand the consequences of hybridization on population dynamics.

*Imperata cylindrica* (L.) Raeusch. (cogongrass) is a perennial rhizomatous grass with a self-incompatible and wind-pollinated reproduction system, that is native to tropical and subtropical areas of the Northern and Southern Hemispheres, including Japan (Holm et al., 1977). Asexual reproduction via rhizomes is the main means of reproduction in this species: rhizomes account for nearly 50% of the total biomass (Tominaga, 1993, 2003), seed-set percentages are low or even zero in some populations (Shilling et al., 1997; Tominaga et al., 1989a, 1989b), no seedbank germination is observed (Chikoye & Ekeleme, 2001) and seedling survival is generally low in open natural habitats (Shilling et al., 1997, Tominaga et al., 1989a, 1989b). Japanese cogongrass populations consist of two ecotypes: the common type (C-type) and early flowering type (E-type; Matumura & Yukimura, 1980, Tominaga et al., 1989c, Mizuguti et al., 2003). These ecotypes are typically distinguished by their morphology; the E-type has a glabrous culm, whereas the C-type has a hairy culm (Figure 1). They also differ in terms of habitat and flowering phenology. The C-type mainly lives in dry habitats (e.g. roadsides and the levees of paddy fields), whereas the E-type often lives in wet habitats (e.g. marshy areas and moist fallow fields). In addition, the E-type flowers approximately 1 month earlier than the C-type. These differences in habitat and flowering phenology isolate the two ecotypes, although the existence of hybrids between the two ecotypes was initially recognized through allozyme analysis using a single marker (Mizuguti et al., 2004; Tominaga et al., 2007). Since then, the detailed life-history traits of the hybrids, such as their flowering phenology, sexual reproduction, germination after seed dispersal and genetic population structure have remained unknown.

In this study, we report a nearly 6-month shift in the flowering phenology of $F_1$ hybrids between two *I. cylindrica* ecotypes, which has not been reported in previous studies on wild plants. We hypothesized that the dramatic shift in the flowering phenology of the $F_1$ hybrids would significantly alter the life-history strategy of the cogongrass and further affect the population genetic structure. Here, we analysed 350 accessions of cogongrass collected from the 1980s to the 2010s throughout Japan, using newly established molecular markers. The analysis of the population genetic structure revealed that there were only $F_1$ individuals in the hybrid population. By surveying natural populations throughout the year, we found that the flowering phenology of $F_1$ plants was delayed until autumn, 5–6 months later than that of the parental ecotypes. We performed common garden experiments and field surveys to determine the effects of the shift in the flowering period of the $F_1$ plants on their fitness and genetic population structure. Our findings provide insight into reproductive isolation resulting from hybridization.

## 2 | MATERIALS AND METHODS

### 2.1 | Accessions and experimental farm

In all, 350 accessions of cogongrass were collected throughout Japan, from the 1980s to the 2010s, and maintained at an experimental farm at Kyoto University, Kyoto, Japan (35°01′54.5″N, 135°46′59.5″E; Figure 2; Table S1).
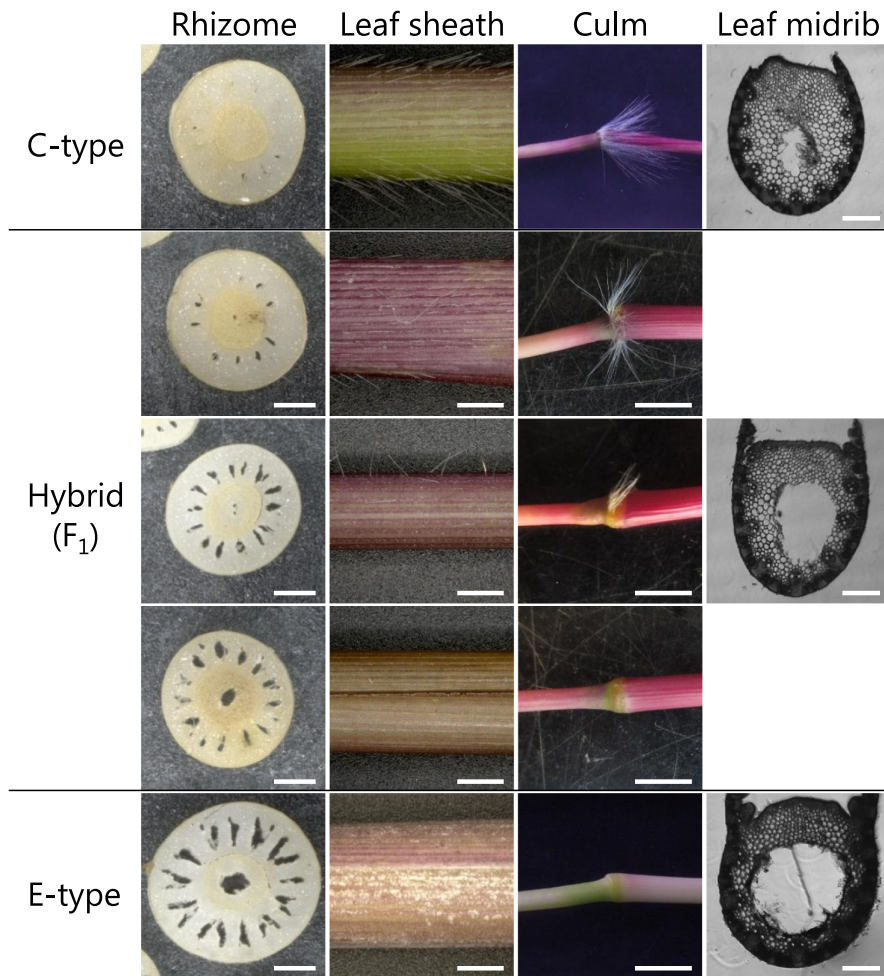
**FIGURE 1** Morphology of C-type, E-type and hybrids. Bars in the bottom right of the photographs are scale bars. Scale bars represent 1 cm, 1 cm, 0.5 cm and 250 μm in the photographs of the rhizomes, leaf sheaths, culms and leaf midribs, respectively
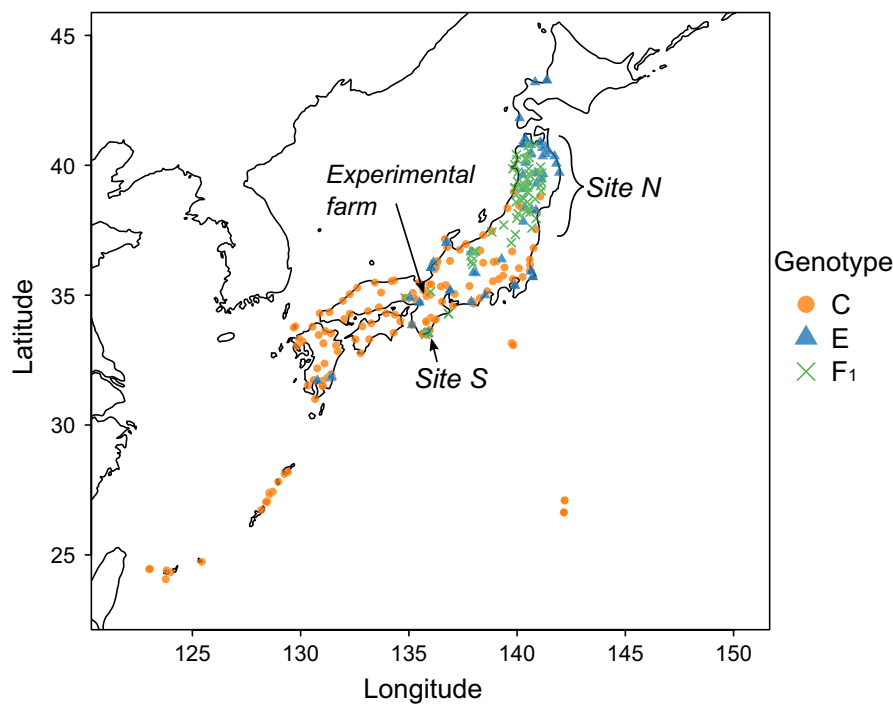


**FIGURE 2** Geographical distribution of each genotype. Genotypes were determined by the direct sequencing of 223 accessions and by using CAPS markers in the ITS region for 127 accessions

## 2.2 | Evaluation of morphological traits

The morphological traits of 46 accessions cultivated at the experimental farm were evaluated: 17 C-type accessions, 15 E-type accessions and 14 putative hybrid accessions (Table S1). The following morphological traits that commonly distinguish the ecotypes were examined: the presence of hairs on the culm nodes (hairless, scarce hairs, densely hairy), hairs on the leaf sheaths (hairless, scarce hairs, densely hairy) and wax on the leaf sheaths (no wax, waxy), and the ratio of aerenchyma diameter to midrib diameter and the ratio of aerenchyma diameter to rhizome diameter were recorded (Figure 1).

## 2.3 | Development of nuclear markers

Total RNA was extracted from the leaves of 22 E-type accessions collected throughout Japan using an RNeasy Plant Mini kit (Qiagen; Table S1) and used to construct a library using the TruSeq RNA Sample Preparation Kit v3 (Illumina, Inc.), according to the manufacturer's instructions. The library was sequenced using a MiSeq system (Illumina) from 300-bp of paired-end reads. Low-quality base (Phred scores < 20) and adapter sequence filtering, de novo transcriptome assembly and polymorphism detection were conducted using CLC Genomics Workbench software (version 8.0; CLC Bio Japan, Inc.).

In all, 10 primer sets were designed to amplify the 300–900 bp of DNA in which polymorphisms were detected in the above RNA sequencing analysis (Table S2). In addition to these 10 primer sets, two primer sets were designed based on the cogongrass sequence in GenBank: *ppc-C4* (AM690231) and its internal transcribed spacer (ITS) region (JN407507). The DNA was extracted from the leaves using the modified cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980) and PCR was performed using *Taq* DNA polymerase (New England Biolabs Japan Inc.). The PCR program consisted of initial denaturation at 95.0°C for 3 min; followed by 45 cycles of 95.0°C for 15 s, annealing at 55°C for 30 s, and extension at 68.0°C for 1 min; and final extension at 68.0°C for 3 min. Using the 12 abovementioned primer sets, 12 regions of nuclear DNA in 233 accessions were sequenced to investigate the population structure (Table S1).

## 2.4 | Analysis of population structure

Single nucleotide polymorphisms (SNPs) with a low frequency (<10% in all accessions) were excluded from the haplotype analysis using PHASE 2.1 (Stephens et al., 2001) because the subsequent NEWHYBRIDS analysis was not able to handle SNPs with frequencies below 10%. Principal coordinate analysis was conducted using GENALEX 6.502 (Peakall & Smouse, 2012). The allelic richness and gene diversity of both ecotypes and the hybrids were calculated by FSTAT 2.9.3.2 (Goudet, 2002). The population genetic structure was inferred using Markov chain Monte Carlo (MCMC) and Bayesian clustering algorithms implemented in STRUCTURE v. 2.3.1 (Pritchard et al., 2000) with 1,000,000 MCMC steps, following 100,000 burn-in MCMC steps. The number of clusters ($K$) was tested from 1 to 10, with 10 replicates each. The optimum $K$ was estimated by Evanno $\Delta K$ (Evanno et al., 2005).

Distinguishing hybrids from the parental ecotypes and identifying the generation of these hybrids were achieved using NEWHYBRIDS v. 1.1 (Anderson & Thompson, 2002). In all, 10 independent runs were conducted with 1,000,000 MCMC steps, following 100,000 burn-in MCMC steps, and the posterior probability was computed for each of the six classes: the two parental ecotypes, their $F_1$ and $F_2$ generations, and backcrosses to each parental class. The 10 independent results from STRUCTURE and NEWHYBRIDS were amalgamated using CLUMPP v. 1.1.2 (Jakobsson & Rosenberg, 2007).

## 2.5 | Development of CAPS markers to check for linkage disequilibrium

To confirm that there was no linkage disequilibrium among the four molecular markers used to distinguish the ecotypes, $F_2$ plants were artificially created and their genotypes determined using two cleaved amplified polymorphic sequence (CAPS) markers (ITS and EST104) and direct sequencing (*ppc-C4* and EST72). Seven $F_1$ plants were open-pollinated in a 15 × 15 m plot on an experimental farm in Kyoto when only $F_1$ plants were in bloom. The seeds collected from the plants were germinated on filter paper in a Petri dish incubated at 30/20°C light/dark with a 12-h photoperiod. The seedlings were transplanted into plastic pots (11.3-cm diameter × 14.0-cm height) and DNA was extracted from the leaves. The PCR products of the ITS and EST104 regions, amplified using the primer sets listed in Table S2 (ITS_CAPS and EST104_CAPS), were digested with *Dde*I and *Rsa*I (New England Biolabs Japan, Inc.), respectively (Figure S2).

The STRUCTURE and NEWHYBRIDS results indicated that the hybrids were likely only $F_1$s and we found that the ITS region alone was enough to discriminate between the F1s and their parental ecotypes. Therefore, we genotyped the remaining 127 of the 350 accessions using the CAPS marker in the ITS region to make a more detailed distribution map.

## 2.6 | Genotyping of chloroplast DNA to determine maternal ecotypes

To distinguish the maternal ecotypes, the chloroplast DNA (cpDNA) polymorphisms of 350 accessions were examined. A previous study demonstrated that polymorphisms in the cpDNA can be used to distinguish between the ecotypes (Nomura et al., 2015). Among the previously determined cpDNA regions, the *psbA-matK* region was amplified by PCR, using the primer set forward PSA-F and reverse MTK-R (Yasuda & Shibayama, 2006). The PCR products were digested with *Dra*I (TaKaRa Bio, Inc.).

## 2.7 | Investigation of flowering phenology and hand-pollination experiments in a common garden

The flowering phenologies of C-type, E-type and artificially crossed $F_1$ plants, cultivated from 1 April to November 30, 2018, were monitored at the experimental farm of Kyoto University. The survey included 141 C-type accessions from 122 populations, 83 E-type accessions from 54 populations and artificially generated $F_1$ hybrids. The artificial $F_1$s were generated by hand pollination between two ecotypes collected from the same prefecture in 2010 (Miyoshi & Tominaga, 2017) and 2017, resulting in populations from Miyagi, Ibaraki, Ishikawa, Fukui, Shizuoka, Aichi, Osaka and Wakayama. Hybridization success was evaluated using the CAPS marker in the ITS region. The plants were grown in plastic pots (15.9-cm diameter × 19.0-cm height) or clay pots (21.8-cm diameter × 17.5-cm height) containing paddy soil. The pot type used for cultivation was randomly assigned to each genotype (Table S3). The flowering date was defined as the date when the top of the first panicle emerged from the leaf sheath.

To estimate the fecundity of each ecotype, hand pollination was conducted between different accessions with the same ecotype: C-type × C-type, E-type × E-type and $F_1 × F_1$ (Table S4). Each accession pair was isolated in a mesh-covered cubic frame, and their panicles were rubbed together when their anthers and stigmas matured. The C-type and E-type pairs were crossed in April–May 2017 and the $F_1$ pairs in September–October 2018. Seed set was estimated as the percentage of filled spikelets to the total number of spikelets per panicle.

## 2.8 | Investigation of flowering phenology, seed set and hybridization between ecotypes in natural habitats

Flowering periods and seed set in natural habitats were investigated in spring (April–July) and autumn (October–December) during 2016–2018 in a northern site (hereafter referred to as Site N; 36°54′41″N–40°32′07″N, 139°48′34″E–141°40′10″E) and a southern site (hereafter referred to as Site S; 33°29′0″ N–33°39′39″ N, 135°46′39″ E–135°57′59″ E) where two ecotypes and their hybrids coexist (Figure 2; Figure S1). Permission for field work was not needed. A total of 1,343 ramets were investigated in spring and 1,677 in autumn from 64 populations. Each population consisted of 1–92 ramets. Ramets in the flowering and non-flowering phases were sampled at intervals of >1 m to minimize the possibility of repeatedly collecting the same genet. During the seed dispersal phase, panicles were collected from C-type (20 populations), E-type (22 populations) and $F_1$ (27 populations) plants. Seed set was estimated, as described above. The genotypes of seeds collected from sites where C- and E-type individuals occurred close together (1–20m) were determined to estimate the hybridization percentage between the ecotypes. In all, 599 seeds from 50 panicles in 10 C-type populations, and 481 seeds from 50 panicles in 14 E-type populations were

germinated at 30/20°C. The genotype of each ramet, panicle and seedling was evaluated using the CAPS marker in the ITS region, as described above.

## 2.9 | Germination tests

Seed germination tests were conducted under two conditions (outside and controlled), using the seeds collected for the seed-set survey (Figure S1) within 2 months of seed collection. The germination test under outside conditions was conducted to observe the germinability of the seeds of ecotypes and $F_1$s under wild environmental conditions after seed dispersal. The germination test under controlled conditions (temperature controlled at 30/20°C which is optimal for germination) was conducted to evaluate seed viability. The flowering time of the parental ecotypes was in spring; therefore, the C-type and E-type seeds were sown from late May to late August. The flowering time of the $F_1$s was in autumn, so the $F_1$ seeds were sown in late December. A total of 271, 433 and 58 seeds from six C-type populations, nine E-type populations and seven $F_1$ populations, respectively, were subjected to the germination tests under outside conditions. A total of 784, 535 and 41 seeds from 10 C-type populations, 13 E-type populations and 7 $F_1$ populations, respectively, were subjected to the germination tests under controlled condition. Because the seed set of $F_1$ plants in natural habitats is so low, only a small number of $F_1$ seeds were available for the germination tests.

Under outside conditions, the seeds were sown on the surface of potting soil in plastic pots at the experimental farm of Kyoto University, and the seedling emergence of the parental ecotypes was observed for 2 months, while that of the $F_1$s was observed for 7 months, until early summer of the following year. Under controlled conditions, the seed germination tests were conducted on filter paper in Petri dishes in a growth chamber (Biorton NC-220S, NK system) set to 12-/12-h light/dark and 30/20°C, which are the optimal germination conditions for the C- and E-types (Matumura et al., 1983; Mizuguti et al., 2002). In addition, seed germination tests were conducted using seeds produced by artificial crossing of the four abovementioned pairs of $F_1$ plants (Table S4). These seed germination tests were conducted on filter paper in Petri dishes under a 12-/12-h light/dark cycle at 30/20°C, 25/15°C, 20/10°C and 15/5°C.

To confirm the effect of seed dispersal season on germination, germination tests were conducted in early December 2017 using C-type seeds collected in June–July 2017. The seeds were stored at 4°C in a refrigerator and then sown under outside and controlled conditions, as described above.

## 2.10 | Statistical analysis

Hybridization (hybridized: 1/no: 0), flowering (flowered: 1/no: 0), seed set (set: 1/no: 0) and germination (germinated: 1/no: 0) in the

natural habitats were compared among genotypes (two ecotypes and $F_1$ hybrids) using generalized linear mixed models (GLMM) with a binomial distribution and a logit link function in the package LME4 in R 3.5.1 software (R Core Team, 2018). The data were illustrated as the percentage for each population or genotype. The genotypes and populations were treated as fixed and random effects, respectively. Pairwise significant differences were identified by post-hoc tests for GLMM (Tukey honestly significant difference test) using the package MULTCOMP in R 3.5.1.

## 3 | RESULTS

### 3.1 | Population genetic structure of cogongrass

In all, 53 SNPs were detected by direct sequencing of 12 nuclear regions from 223 cogongrass accessions. Haplotypes in four regions, *ppc-C4*, ITS, EST72 and EST104, were not shared by the C- and E-types in Japan (Table S5). These four regions showed low coefficients of linkage disequilibrium in an analysis of 28 putative $F_2$s, suggesting that they are located on different chromosomes (Table S6).

Principal coordinate analysis based on 12 nuclear markers separated the 223 accessions into three clusters along PCo1 (Figure 3a). The chloroplast CAPS marker, which is used to distinguish maternal ecotypes, revealed that the clusters on either side of PCo1 corresponded to the C- and E-types (Figure 3a). The cluster in the middle of PCo1 was considered to represent putative hybrids between the ecotypes. In the STRUCTURE analysis, the number of

optimum clusters was estimated to be $K = 2$ by Evanno $\Delta K$ (Evanno et al., 2005; Figure 3b), implying that Japanese cogongrass populations are composed of two genetic clusters. All of the putative hybrids showed $q$ values of approximately 0.5, suggesting that half of their genome was from the C-type and half from the E-type. The NEWHYBRIDS analysis assigned the two ecotypes and the putative hybrids to the parental classes and the $F_1$ hybrid class, respectively (Figure 3b). There were no putative $F_2$ hybrids or backcross individuals, except for one accession that belonged to the E-type cluster but carried the C-type haplotype of cpDNA. The allelic richness of the C-type, E-type and hybrids was 4.6 ($\pm 0.99$ SE), 4.3 ($\pm 1.1$ SE) and 5.0 ($\pm 0.90$ SE), respectively. Their gene diversity was 0.35 ($\pm 0.078$ SE), 0.44 ($\pm 0.069$ SE) and 0.62 ($\pm 0.026$ SE), respectively.

In addition to these 223 accessions, 127 accessions were genotyped using the CAPS marker in the ITS region. The geographical distribution of each genotype in the 350 accessions showed that $F_1$ hybrids were distributed across wide areas of Site N and limited areas of Site S (Figure 2).

### 3.2 | Hybridization percentages in natural habitats

The majority (85%) of the $F_1$ hybrids exhibited the E-type cpDNA haplotype (Figure 2b), indicating that the E-type was the maternal parent, as supported by the hybridization percentage in natural habitats (Figure 3c). The direction of hybridization was asymmetric: significantly more hybrids were observed among the seeds from E-type populations than those from C-type populations (GLMM, $p < 0.01$).



**FIGURE 3** (a) Biplot of principal coordinates 1 and 2 of 223 accessions, based on 12 markers in nuclear DNA. Each symbol represents a genotype of cpDNA. (b) Genotype of cpDNA and Bayesian clustering of 223 accessions, based on 12 markers in nuclear DNA (STRUCTURE and NEWHYBRIDS). (c) Hybridization percentage in natural habitats. Hybridization percentage was calculated as the number of germinated $F_1$ genotype seeds in all germinated seeds. Each point represents the hybridization percentage per population. Significant differences were evaluated by GLMM. (d) Biplot of principal components 1 and 2 of 46 accessions, based on five morphological traits
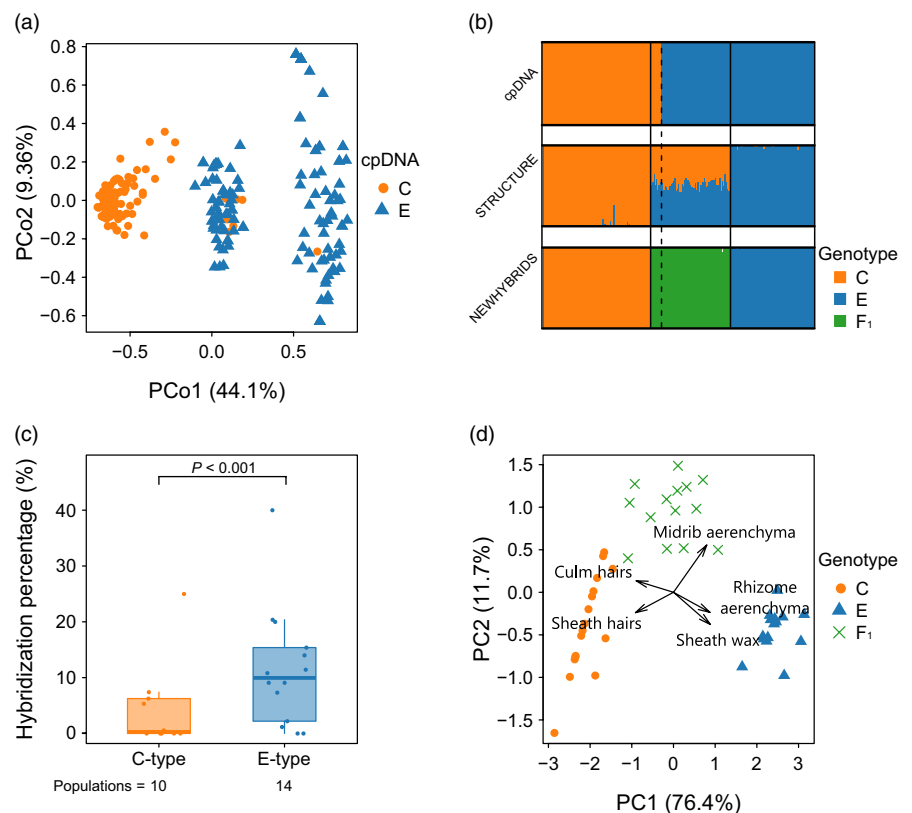
## 3.3 | Morphological traits of the ecotypes and F₁s

Each ecotype and their hybrids were clearly separated based on five morphological traits by PCA (Figure 3d). The C-type had hairs on the culm nodes and leaf sheaths and no wax on the leaf sheaths, whereas the E-type had no hairs on the culm nodes or leaf sheaths and wax on the leaf sheaths (Figure 1; Figure S3). The C-type also had smaller rhizome and midrib aerenchyma ratios than the E-type. The $F_1$s, located between the parental ecotypes along PC1 (Figure 3d), showed intermediate morphologies, with varying amounts of hair on the culm, no or few hairs and little wax on the sheaths, and intermediate aerenchyma ratios (Figure 1; Figure S3).
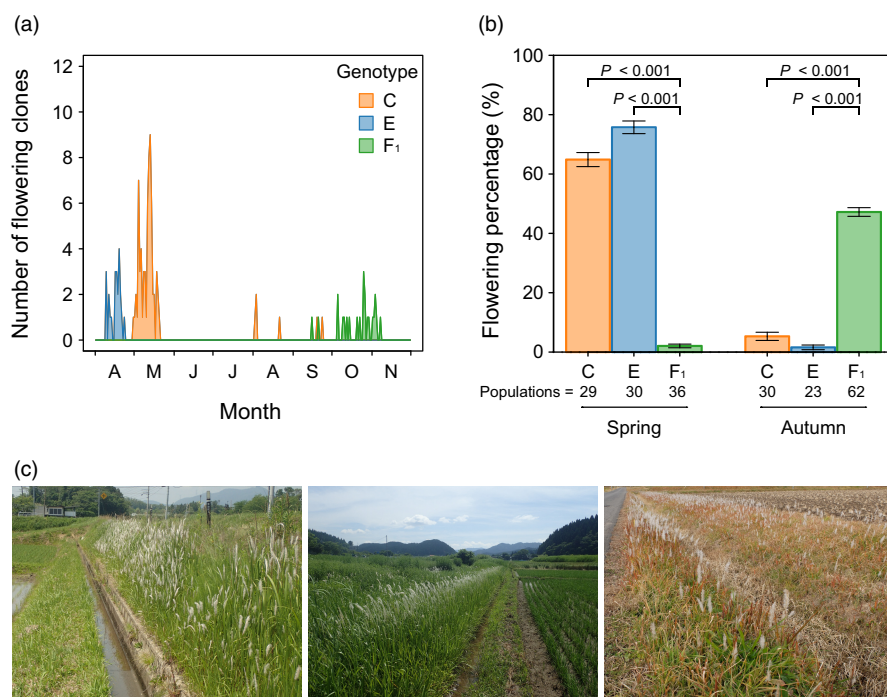
## 3.4 | Flowering phenology

The flowering phenology investigation at the experimental farm showed that the flowering of the E-type peaked during mid-April and the flowering of the C-type peaked approximately 1 month later (Figure 4a). During this period, none of the $F_1$ plants flowered; the $F_1$s flowered sporadically from September to November. The investigation of natural habitats also supported the results obtained from the experimental farm. On average, the number of genotyped ramets per population for the C-type, E-type and $F_1$s were 17.0, 15.0 and 19.0 in spring and 12.0, 16.8 and 27.0 in autumn, respectively (Figure S4). Sixty-seven percent of the C-type ramets and 78% of the E-type ramets were in the flowering stage in spring (Figure 4b). In contrast, only 2.0% of the $F_1$ ramets flowered in spring. In autumn, up to 48.0% of the $F_1$ ramets flowered, while only 4.8% of the C-type and 1.5% of the E-type ramets flowered. The flowering phenology of the $F_1$ plants was not affected by cpDNA type (Figure S5).

## 3.5 | Sexual reproduction

In natural habitats, the average seed set of $F_1$ was 0.12% (ranging from 0.0% to 2.5%); thus, most of the $F_1$ panicles in natural habitats did not carry mature seeds (Figure 5a). In contrast, the average seed sets of the C- and E-types in natural habitats were approximately 70-fold higher than that of the $F_1$ plants: 7.1% (range: 0.0%–70.6%) for the C-type and 9.0% (range: 0.0%–60.7%) for the E-type. However, a similar seed-set percentage as the parent ecotypes in natural habitats, 8.7% (range: 0.6%–24.7%), was observed in $F_1$ plants when they were artificially crossed (Figure 5b). This suggests that $F_1$ plants have an extremely low seed set in natural habitats, although they have fecundity similar to that of the parent ecotypes. The average numbers of spikelets per panicle of the C- and E-types were 359.1 and 246.1, respectively, in the natural habitats. $F_1$ plants produced 368.6 spikelets per panicle on average, suggesting that they have the same ability to produce flowers as the C-type.

## 3.6 | Germination under the natural and controlled conditions

Germination tests of seeds collected from natural habitats revealed that the germination percentage of the $F_1$ seeds was significantly lower than that of the C- and E-types under outside conditions, when the C- and E-type seeds were sown in May–June, and the $F_1$ seeds were sown in December. In contrast, the germination percentage of $F_1$ seeds was as high as that of parent seeds under controlled (30/20°C) conditions (Figure 5c). We further subjected the seeds derived from C-type plants to an overwinter experiment. While the C-type seeds germinated well under optimal conditions (83.1%), the seeds sown in outside conditions in December showed extremely



(a)

(b)

(c)

**FIGURE 4** (a) Flowering phenology at an experimental farm of Kyoto University. (b) Flowering percentages in natural habitats in spring and autumn. Flowering percentage was calculated as the number of ramets that had flowers of all the ramets surveyed. Values and error bars represent means ± SE significant differences were evaluated by Tukey HSD after GLMM. (c) Photographs during the seed dispersal stage of C-type (left), E-type (middle) and $F_1$ (right). The left-hand and middle photographs were taken during the rice cultivation season in June and July, respectively. The right-hand photograph was taken in November after the rice was harvested

**FIGURE 5** Seed reproduction in $F_1$ hybrids of C-type and E-type cogongrass. (a) Seed set in natural habitats. Symbols represent the survey year in natural habitats. Each point represents the seed set per population. (b) Seed set of hand-pollinated ecotypes and $F_1$s were cultivated on an experimental farm of Kyoto University. Each point represents the seed set per crossed pair. (c) Germination percentages of seeds from natural habitats under controlled conditions (30/20°C temperatures) and outside conditions. C- and E-type seeds were sown in summer (May–August), while $F_1$ seeds were sown in winter (December). Each point represents the germination percentage per population. (d) Germination percentages of seeds produced from $F_1 \times F_1$ crosses under four temperature conditions. Each point represents the seed set per crossed pair. Significant differences were evaluated by Tukey HSD after GLMM
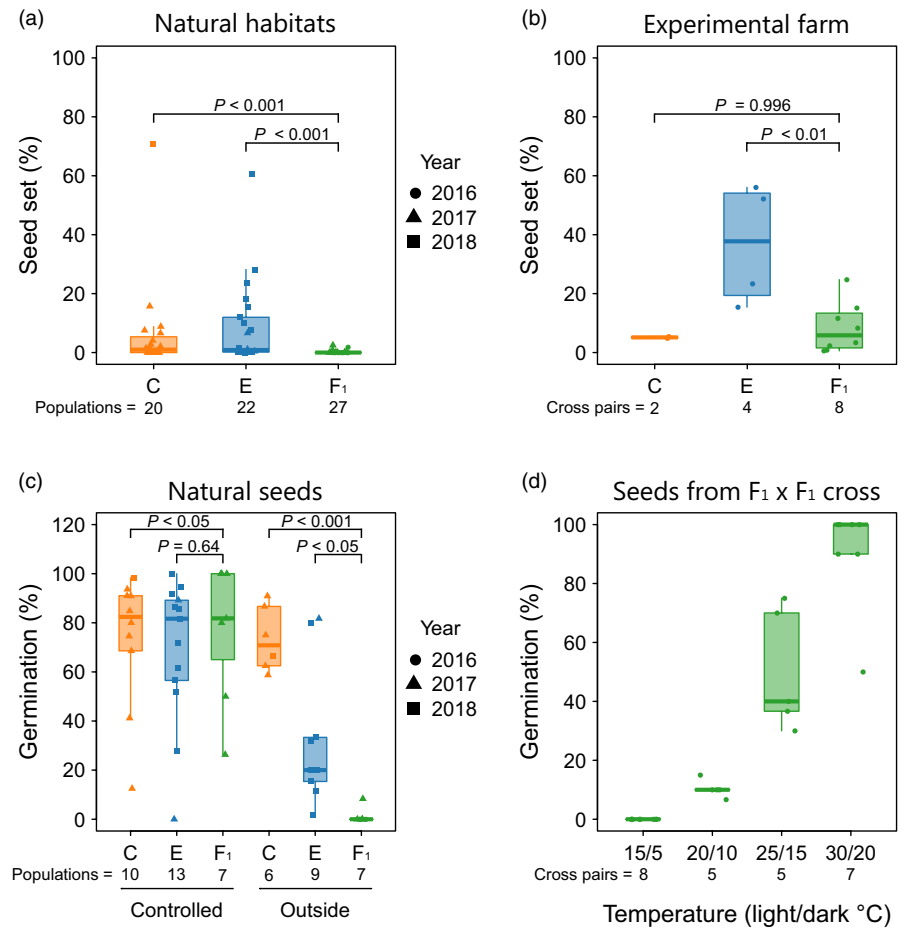


low germination (3.95%; Figure S6). As the above results suggest that the contrasting germination rates of the seeds of the $F_1$ populations resulted from the difference in temperature between the two experiments, seeds collected from artificial $F_1 \times F_1$ crosses were subjected to germination tests at various temperatures in growth chambers. The germination percentage was high at 30/20°C, but none of the seeds germinated at 15/5°C (Figure 5d). In natural habitats, the seeds of both ecotypes are dispersed in summer and exposed to temperatures above 25/15°C, which are suitable for germination. In contrast, the autumn-flowering $F_1$s disperse their seeds in winter when temperatures below 15/5°C prevent germination. Most of the seeds dispersed in winter did not germinate in early summer of the following year when the temperature increased to around 25/15°C.

## 4 | DISCUSSION

### 4.1 | Genetic population structure of cogongrass in Japan

We found clear genetic differentiation between the C- and E-types, not only in the chloroplasts but also in the nuclear genome (Figure 3a,b). This result was also supported by morphological data which showed clear differentiation between these ecotypes (Figure 3d). Hybrid populations consisted almost exclusively of

$F_1$ hybrids, with no $F_2$s or backcrosses. Both C- and E-types were found in the cpDNA of the $F_1$s, but the E-type was more strongly represented. This result is consistent with the fact that cogongrass is protandrous (Nomura et al., 2018; Tominaga et al., 1989a) so that the E-type, which flowers earlier, may be more likely to be the mother. The fact that the E-type panicles showed a higher percentage of hybridization supports this prediction (Figure 3c). Only one accession showed C-type cpDNA and E-type nDNA. We think that this was likely the result of incomplete linage sorting because the NEWHYBRIDS results indicated that this accession had a small probability of backcrossing, and the flowering phenology shift prevents backcrossing.

### 4.2 | Drastic shift in flowering phenology shapes population structure

We discovered that the flowering phenology of the $F_1$ plants was delayed until autumn, 5–6 months later than the parental ecotypes (Figure 4). This delay in flowering time in $F_1$ almost completely prevented further hybridizing with the two parent ecotypes. We further found that the seed set of $F_1$ plants in natural habitats was extremely low and that few mature seeds survived overwintering (Figure 5). These two factors can explain the absence of populations derived from $F_1 \times F_1$ crossing.

The first factor, the low seed set of the $F_1$ plants, may be caused by a lack of appropriate mating partners. Because of the self-incompatible and asexual reproductive nature of cogongrass, its seed-set percentages vary widely depending on the genetic diversity of the population (Shilling et al., 1997). If a new population founded by just a few seeds propagates through asexual reproduction, the population will consist of only one or at most a few genotypes, indicating low genetic diversity. In self-incompatible species, the lower the genetic diversity (or effective population size), the fewer partners there are available for mating and the lower the seed set (Brys et al., 2004; Luijten et al., 2000). Therefore, the low seed-set percentages of $F_1$ plants in natural habitats are likely owing to the low genetic diversity of the $F_1$ populations. This is supported by seed-set percentages in our artificial crossing of distinct $F_1$ populations which are likely to have different genotypes (Figure 5b). This result suggests that the $F_1$ plants are not sterile, but have low fertility owing to a lack of potential mates. In addition to the population structure, the non-synchronous flowering among $F_1$ plants may affect seed-set percentage. In contrast to the C- and E-types, the flowering of $F_1$ individuals occurred sporadically, and the flowering period was roughly 2 months, twice as long as those of the C- and E-types (Figure 4a). Further studies on the genetic population structures within/among $F_1$ populations and the flowering period under natural conditions are needed to assess the low seed-set percentage of the $F_1$ plants.

The second factor is the low seed survival in winter. We showed that the low temperatures in autumn and winter would prevent seed germination (Figures 5c,d), which may lead to seed decay in nature owing to the lack of seed dormancy in cogongrass (Dalling et al., 2011; Mizuguti et al., 2002; Shilling et al., 1997). This would prevent the establishment of $F_2$ plants. Cogongrass seed longevity in storage under dry and cold conditions is only about 10 months (Shilling et al., 1997) and it declines more rapidly in wetter conditions (Matumura et al., 1983), for example in moist soil. The germination and dormancy dynamics of this species, especially in nature, should be investigated to understand the mechanism that prevents successful $F_2$ establishment.

Although the detailed mechanisms behind the low seed set and seed survival rates remain unknown, the biotic and abiotic parameters, such as population structure and temperature, which $F_1$ plants confront because of the drastic shift in their flowering phenology, act as reproductive barriers. The reproductive isolation of $F_1$s from both ecotypes may also promote hybrid speciation. At present, the ploidy of the $F_1$s is the same as that of the two ecotypes (unpublished data), but if whole genome duplication occurs in the $F_1$s, as in other alloploid species (Soltis & Soltis, 2009), autumn flowering may be fixed and a new species may be born.

## 4.3 | Mechanisms of forming $F_1$-dominated hybrid zone

Hybrid populations comprising only the $F_1$ generation have been reported in other perennial plants, including herbaceous and woody plants (Kameyama et al., 2008; Kuehn et al., 1999; Milne & Abbott, 2008; Milne et al., 2003; Nagano et al., 2015; Nason et al., 1992; Zha et al., 2010). These previous studies have proposed two major hypotheses to explain the absence of $F_2$ and backcrossed hybrids: one involves an environmental mismatch and the other hybrid breakdown (Kameyama et al., 2008; Kameyama & Kudo, 2011; Kuehn et al., 1999; Milne & Abbott, 2008; Milne et al., 2003; Nason et al., 1992). The environmental mismatch hypothesis suggests that the parental environment is unsuitable for $F_2$ and backcrossed hybrids. In contrast, a decline in the fitness of post-$F_1$ generations owing to genetic incompatibility is known as hybrid breakdown. In this case, the normal development of $F_2$s would be disturbed even under optimal conditions. However, the abovementioned studies did not identify the specific mechanisms, environmental factors or genetic factors involved. In this study, $F_2$ plants, which were artificially created to check the linkage disequilibrium among the molecular markers, grew as well as parental ecotypes and $F_1$s under controlled conditions (30/20°C). In natural conditions, post-$F_1$s are dispersed as seeds in an environment unsuitable for growth, that is, in winter, which prevents the establishment of an $F_2$ generation. Therefore, the environmental mismatch hypothesis seems to be more applicable than the hybrid breakdown hypothesis to explain why the $F_2$ generation does not exist in cogongrass populations.

Hybrid populations are distributed widely in the northern part of Japan (Figure 2) and $F_1$ plants were found in materials collected 30 years ago (Table S1). On a large scale, $F_1$s have been formed recurrently (Prentis et al., 2007) because the $F_1$ genotypes vary between populations (Figure 3a) and are thought to result from multiple independent hybridization events. The hybridization percentage results also indicate that hybridization between ecotypes is still occurring (Figure 3c). At a patch scale, the $F_1$ populations are likely to be maintained by rhizome propagation for at least several years because a previous study demonstrated that some artificial $F_1$ accessions show higher performance in biomass production, that is, vigorous rhizome production, than their parental ecotypes under dry and wet conditions (Miyoshi & Tominaga, 2017). The reasons for this high performance in both dry and wet conditions are not fully understood, but the intermediate morphology of $F_1$s between the C- and E-types is likely a contributing factor (Figure 3d; Figure S3). In particular, differences in the rhizome aerenchyma would influence adaptation to differences in soil moisture content. A large aerenchyma in the rhizomes facilitates internal oxygen diffusion, and is therefore an adaptation to flooded conditions (Yamauchi et al., 2021). In contrast, a small aerenchyma in the rhizomes allows the maximum volume of the rhizome to be used as a sink for nutrients, which is adaptive in environments where the soil is well oxygenated. In support of this, the C-type, which lives in relatively dry habitats, and the E-type, which lives in wet habitats, have small and large rhizome aerenchymas, respectively (Figure 3d). $F_1$s show intermediate aerenchyma sizes with higher plasticity than the two ecotypes (unpublished data), which may be related to their high performance in a wide range of environments. Further studies on the role of rhizome aerenchyma in environmental preference may shed light on the distribution of

cogongrass in Japan. For these reasons, it is presumed that $F_1$, once established, can survive for an extended period and expands its distribution through asexual reproduction via rhizomes.

## 4.4 | Regulation of flowering phenology

To ensure reproductive success, plants must regulate their flowering to synchronize with the optimal environmental conditions for seed production and with suitable mates. Previous studies have suggested three hypotheses for the genetic mechanism of the flowering phenology shifts: (i) an epistatic genetic effect that directly affects flowering time; (ii) a pleiotropic genetic effect that causes the poor growth of the $F_1$ hybrids, resulting in delayed flowering time; and (iii) an additive genetic effect that controls bud development time (Lopez et al., 2000). In this study, the $F_1$ plants did not necessarily show poor growth (Miyoshi & Tominaga, 2017). It is therefore unlikely that the flowering phenology shift is a by-product of poor growth; it is more likely to be caused by an epistatic or additive genetic effect. A large number of genes are involved in the regulation of flowering in plants (Hill & Li, 2016), allowing plants to fine-tune their flowering periods. These players often function in an additive manner (Buckler et al., 2009; Martin et al., 2007; Martin & Willis, 2007); therefore, crossing between individuals with different flowering periods often results in an intermediate flowering period. In contrast, a marked delay in flowering has been observed in the $F_1$ hybrids of particular ecotypes/lines of *Arabidopsis thaliana* (L.) Heynh. (Henderson & Dean, 2004; Koornneef et al., 1994) and *Sorghum bicolor* (L.) Moench (Murphy et al., 2014; Yang et al., 2014). In both cases, the parental ecotypes/lines have a disrupted form of a floral repressor or its activator in their floral regulatory pathway, which lifts the repression of flowering. Hybridization of the two ecotypes/lines restores the flowering repression pathway because the hybrids carry functional alleles of each gene. The genes involved in the delayed phenology differed between *A. thaliana* and *S. bicolor*, although their basic relationships as floral repressors and activators are identical. The genes related to the delayed phenology of $F_1$ in cogongrass are unknown. Genomic and transcriptomic approaches will enhance our understanding of the dynamic shift in flowering time observed in cogongrass.

## 5 | CONCLUSIONS

A novel phenotype derived from the hybridization of two ecotypes has major effects on the generational structure of the hybrids in populations of cogongrass. The fact that the hybrid populations consist almost entirely of $F_1$ plants implies that the $F_1$ progeny of C- and E-type cogongrass has little chance of sexual reproduction, resulting in the dominance of asexual reproduction. Thus, the hybridization of the two ecotypes altered not only the flowering phenology, but also the reproductive strategy of cogongrass. Considering that similar delays in flowering phenology have been observed in other plant

lineages (e.g. *A. thaliana* and *S. bicolor*), it is reasonable to speculate that hybridization between independently evolved ecotypes may also cause drastic shifts in the flowering phenology of other plants. Our findings clarify the ecological role of flowering shifts caused by hybridizations in cogongrass.

## CONFLICT OF INTEREST

The authors declare no competing interests.

## AUTHORS' CONTRIBUTIONS

Y.N., Y.S., S.I. and T.T. designed the experiments; T.T. provided plant materials that he has maintained since the 1980s; Y.N., Y.S. and I.M. further collected the plant materials; Y.N. performed most of the experiments; Y.N, N.M. and K.S. and performed the RNA-Seq analysis. Y.N., Y.S., N.M., S.I., K.S. and T.T. wrote the paper.

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/1365-2745.13890.

## DATA AVAILABILITY STATEMENT

The RNA-Seq reported in this paper has been deposited in the DNA Data Bank of Japan: https://ddbj.nig.ac.jp/resource/bioproject/PRJDB6375 (Nomura, Shimono, Mizuno, et al., 2021). The Sanger sequence data reported in this paper have been deposited in the DNA Data Bank of Japan (Nomura, Shimono, Iwakami, & Tominaga, 2021): https://getentry.ddbj.nig.ac.jp/getentry/na/LC427254; https://getentry.ddbj.nig.ac.jp/getentry/na/LC427240; https://getentry.ddbj.nig.ac.jp/getentry/na/LC428387; https://getentry.ddbj.nig.ac.jp/getentry/na/LC428528; Data supporting the results, other than the sequence data and Supporting Information, have been archived in Dryad Digital Repository https://doi.org/10.5061/dryad.wm37pvmp6 (Nomura et al., 2022).

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