

## RESEARCH ARTICLE

# Introduction pathways and evolutionary mechanisms of alien species of *Lolium* spreading across sandy coasts in Japan

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## Abstract

1. Estimating the role of specific processes in the spread of alien species necessitates the determination of introduction pathways and source populations of invaded areas. Alien grasses in the genus *Lolium* that have extensively invaded Japan provide a unique opportunity to estimate the expansion process through direct comparison between source and naturalised populations because the introduction pathways, contaminants in grain commodities and commercial cultivars for fodder crops or revegetation materials are well-known. Therefore, by directly comparing source and naturalised populations, we estimated the introduction pathways and whether adaptive evolution occurred in *Lolium* species on sandy coasts in Japan.
2. *Lolium* individuals sampled from naturalised populations in croplands, seaports, and sandy coasts were compared with those from two introduction sources for morphological and genetic variations based on a genome-wide single nucleotide polymorphism analysis and a common garden experiment. Furthermore, we conducted a reciprocal transplant experiment between cropland and sandy coast.
3. Populations naturalised in croplands were closely related to the cultivars, whereas those naturalised in seaports and sandy coasts were associated with contaminants. These results indicate that the cropland and sandy coast populations are derived from cultivars and contaminants, respectively. In addition, asymmetric gene flow from cropland populations to sandy coast populations was observed. The reciprocal transplant experiment clearly demonstrated the home site advantage; populations derived from croplands yielded higher floret numbers than those derived from other habitats at the cropland site; sandy coast populations had higher survival rates than those from croplands at the coastal site. Port populations exhibited a similar tendency as sandy coast populations, indicating that contaminants may be originally adapted to salty and dry environments, such as that in sandy coasts. The flowering phenology in the sandy coast populations evolved in the late flowering; therefore, late flowering alleles may have been transferred from cropland populations to sandy coast populations.

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4. *Synthesis.* We demonstrated that two congeneric species with different ecological characteristics were introduced through multiple introduction pathways and spread across different habitats. A direct comparison between source and naturalised populations can considerably elucidate the patterns and processes of biological invasions.

#### KEYWORDS

admixture, home site advantage, intentional and unintentional introduction, local adaptation, MIG-seq, multiple introduction pathways, reciprocal transplant experiment

## 1 | INTRODUCTION

Human-driven translocation of organisms due to increasing global trade and transport has enormous impact on species distribution (van Kleunen, Dawson, et al., 2015). As invasive alien species threaten native ecosystems and cause economic losses (Diagne et al., 2021; Simberloff et al., 2013; Vilà et al., 2011), several studies have aimed to better understand the factors that lead to the successful establishment and adaptation of alien species (Catford et al., 2019; van Kleunen et al., 2018).

Several studies have shown that the genetic admixture of divergent lineages through multiple introductions is a common phenomenon in alien species (Dlugosch et al., 2015; Dlugosch & Parker, 2008; Rius & Darling, 2014). Such admixtures could play an important role as driving forces for the expansion of alien plant species into a broad range of habitats in their introduced ranges by increasing genetic diversity, which enhances colonisation success and evolutionary potential (Bock et al., 2018; Li et al., 2018; Qiao et al., 2019; Smith et al., 2020; van Kleunen, Röckle, & Stift, 2015). Contrastingly, other studies have shown that some invasive species succeed depending on rapid adaptation in a particular lineage without an admixture (multiple species: Schlaepfer et al., 2010; *Rubus fruticosus*: Clark et al., 2013; *Phragmites australis*: Guo et al., 2014; *Amaranthus tuberculatus*: Kreiner, Caballero, et al., 2022). Admixture and rapid adaptation are not mutually exclusive and sometimes both contribute to increased invasiveness. However, they are often analysed in isolation. Admixture is evaluated based on population genetic structure, whereas evaluating adaptation requires a reciprocal transplant experiment between the original and the new habitats (Colautti & Lau, 2015). Few studies have simultaneously used both approaches. Therefore, the understanding related to the relative importance of admixture and rapid adaptation in the range expansion of alien species is lacking.

Determining the introduction pathways and source populations of invaded areas to estimate the processes involved in the spread of alien species is particularly important (Estoup & Guillemaud, 2010; Keller & Taylor, 2008). However, historical and observational records of the introduction histories, such as the source populations in the native range or the time and location of the introductions, are often sparse (but see Castillo et al., 2021). Therefore, indirect methods based on population comparisons between native and introduced ranges have been widely conducted to estimate putative source populations and provide insights into the mechanisms related

to the invasion processes (Chown et al., 2015; Colautti & Lau, 2015). However, Keller and Taylor (2008) warned that the unrepresentative sampling of source populations could result in a misunderstanding of their invasion processes. In this study, a direct comparison of the morphological and genetic variation between introduced propagules and further established populations was conducted to understand the invasion processes of alien species in various habitats.

The genus *Lolium*, native to the Mediterranean region, includes three outcrossing species: the perennial *L. perenne* (perennial ryegrass), the annual *L. multiflorum* (Italian ryegrass), and *L. rigidum* (annual ryegrass; Terrell, 1968), which are interfertile and show a broad range of morphological variations (Naylor, 1960). Therefore, we collectively called them *Lolium* species. They are economically important and have been widely used worldwide as forage and turf for revegetation, including in Japan. Contrastingly, major agricultural weeds are found worldwide, especially in cereal crop fields (Matzrafi et al., 2021), and their seeds are introduced into grain-importing countries as contaminants in grain commodities (Ikeda et al., 2022; Shimono et al., 2015). Therefore, two sources of introduction into Japan exist for *Lolium* species, namely contaminants in grain commodities and commercial cultivars for fodder crops or revegetation materials.

*Lolium* species have naturalised in human-created open areas, such as croplands, abandoned land and roadsides throughout Japan. They are also distributed along the sandy coast of Japan (Higuchi et al., 2017). Abiotic stresses, such as high salinity, sand burial and wind abrasion (Lowry et al., 2008; Ogura & Yura, 2008; Wilson & Sykes, 1999), which are distinctively different from other habitats, are important selective forces on sandy coasts. Previous studies have suggested that individuals in croplands are derived from forage, whereas those on sandy coasts are derived from contaminants (Higuchi et al., 2017). Commercial cultivars of *Lolium* species are deliberately sown in pasturelands, riverbanks and bare ground after road repairs. This causes high propagule pressure at various sites, and individuals escaping from these introduction sites are widely established along roadsides or croplands (Kurokawa et al., 2010). Contrastingly, *Lolium* populations established at major grain-landing ports in Japan are derived from seeds that spill out from grain commodities (Shimono et al., 2015). The sandy coast populations are genetically and morphologically more similar to port populations than to cropland populations (Higuchi et al., 2017). However, a direct comparison of each introduction source and naturalised population has not yet been conducted, and whether this distribution difference is a common phenomenon throughout Japan is unknown.

Moreover, the life history traits that contribute to differences in distribution patterns remain unknown.

Here, we compared the genetic variation in *Lolium* individuals sampled from naturalised populations in croplands, seaports, and sandy coasts with those from two sources, contaminant seeds in imported wheat and commercial cultivars for fodder, using a genome-wide single nucleotide polymorphism (SNP) analysis technique. Furthermore, we conducted a reciprocal transplant experiment between cropland and sandy coast to evaluate the role of local adaptation in expanding the invasive range. We aimed to elucidate (1) the source of invasion of sandy coast populations, (2) whether genetic admixture of divergent lineages through multiple introductions occurs post-introduction, and (3) whether sandy coast populations adapted rapidly during expansion into coastal environments.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials

For contaminant seeds from imported grains, we obtained three 20-kg samples of five wheat classes imported between 2006 and 2007: three classes of US wheat (Hard Red Winter wheat: HRW, Western White wheat: WW and Dark Northern Spring wheat: DNS), one class of Canadian wheat (No. 1 Canada Western Red Spring wheat: 1CW) and one class of Australian wheat (Australian Standard White wheat: ASW; Table S1). Furthermore, we obtained three types of fodder crops imported from Australia between 2016 and 2017: ground barley, wheat straw and oat hay. The seeds of *Lolium* species were selected from each sample, according to the method described by Shimono et al. (2010).

For cultivated varieties, we purchased 1 kg bags each of Italian ryegrass seeds and perennial ryegrass seeds from Snow Brand Seed Co., Ltd. and Takii Seed Co., Ltd. in 2012. We selected the most currently available commodity, 'Common' (a generic term, not a cultivar name), for fodder crop or revegetation material (Japan Grassland Agriculture and Forage Seed Association, 2010). Additionally, eight Japanese cultivars of Italian ryegrass (Hanamiwase, Akatsuki, Waseyutaka, Inazuma, Tachimusya, Satsukibare, Mammos B and Jiant) were kindly provided by the Institute of Livestock and Grassland Science, National Agriculture and Food Research Organization in Japan. The following foreign cultivars were obtained from GenBank, National Agriculture and Food Research Organization (NARO): the United States cultivar of Italian ryegrass, Westerwold ryegrass (GULF); New Zealand cultivar of Italian ryegrass, Westerwold ryegrass (MOTO 191, TETRAPLOID); and Australian cultivar of annual ryegrass, Wimmera ryegrass (Table S2).

Additionally, seeds were collected from naturalised populations in three habitat types—cropland, seaport, and sandy coast—in each of the three geographic regions of Japan (Kanto, Kansai and Kyushu; Table S3; Figure S1). All the selected seaports (Kobe, Kashima and Hakata ports) are major international trading ports where more than 1000kt of imported grain is unloaded every year (Ministry of Land,

Infrastructure, Transport and Tourism, 2020). Mature seeds were individually collected from 10 plants at intervals of at least 2 m from each habitat from May to June, 2017. In the Kanto region, *Lolium* plants were sparsely distributed, and samples were collected from the two coasts (Table S3). The seeds were stored in an airtight container containing silica gel at room temperature (20–28°C) until further use.

### 2.2 | Morphological measurements

On October 2017, 10–30 seeds were randomly selected from each sample and germinated on two pieces of moistened filter papers placed in 9-cm plastic petri dishes in an incubator set at 25°C for a 12-h light period and at 15°C for a 12-h dark period. One week after germination, the seedlings were individually transplanted into pots (10.5 cm in diameter, 9 cm in depth) filled with potting soil (Tachikawa Heiwa Nouen Co.) and placed in a glasshouse under natural sunlight at the experimental farm of Kyoto University, Kyoto, Japan (35.032121°N, 135.783453°E). The plants were regularly watered.

To investigate whether phenological and morphological differences exist between individuals with different provenances and habitats, the heading (ear emergence from the flag leaf sheath) date and the following morphological characteristics at 3 weeks after heading were measured: number of tillers, length and width of the longest culm, inflorescence length and width of the longest culm, and number of spikelets of the longest culm (Figure S2). In addition, spikelet length, glume length, number of florets per spikelet, first floret length and awn length were measured using a spikelet placed in the middle of the inflorescence of the longest culm (Figure S2). Awn length was graded on a scale of 1 to 3 depending on the length (1: no awn, 2: <5 mm and 3: ≥5 mm). The ratio of glume to spikelet length was subsequently calculated. The measured characteristics were chosen for their usefulness in identifying *Lolium* species (Bennett, 1997; Terrell, 1968). A principal component analysis (PCA) using the `prcomp` function in the `stats` package in R v4.0.2 (R Core Team, 2020) was conducted to summarise the variation in morphological characteristics.

### 2.3 | Genome-wide SNP analysis

Approximately 100 mg of leaf tissue from each of the 4–10 individuals grown for morphological measurements was collected and stored at –80°C until DNA extraction. The total DNA for each individual was extracted from the stocked leaf tissue using the modified cetyltrimethylammonium bromide method (Murray & Thompson, 1980). The multiplexed inter-simple sequence repeat genotyping by sequencing (MIG-seq) library was prepared using the method described by Wagatsuma et al. (2022), which is a slightly modified version of the original protocol (Suyama & Matsuki, 2015). The library was sequenced on an Illumina MiSeq Sequencer (Illumina) using the MiSeq Reagent Kit v2 (300 cycles).

SNP selection was performed as described below. Quality checking of raw paired-end sequences with an average read length

of 157bp using FastQC (Andrews, 2010) showed that the quality score was quite low around after 80bp. Therefore, all reads were trimmed to the first 80bp and low-quality reads and primer regions were removed using Trimmomatic version 0.39 (Bolger et al., 2014). Quality-based trimming was conducted with a 5-bp sliding window and a minimum average quality score of 15. The trimmed reads were mapped with Bowtie2 version 2.5.1 (Langmead & Salzberg, 2012) under default parameters against the genome of *Lolium rigidum* (Paril et al., 2022). The sequence alignment map format files were converted to binary alignment map format files and sorted using Samtools version 1.9 (Li et al., 2009). To identify polymorphic loci, the 'ref\_map.pl' pipeline implemented in Stacks version 2.54 (Catchen et al., 2013) was used. The 'populations' program retrieved the SNPs by setting the minimum minor allele frequency (min\_maf) at 0.01, the maximum observed heterozygosity (max-obs-het) at 0.5 and the minimum percentage of a locus shared within a population ( $r$ ) at 50%, and all samples were regarded as a single population ( $p = 1$ ).

Summary statistics of within-population genetic diversity (effective number of alleles [ $N_e$ ], observed [ $H_o$ ] and expected heterozygosity [ $H_e$ ], and inbreeding coefficient [ $F_{is}$ ]) were calculated using GenoDive 3.04 (Meirmans, 2020). The individual-based genetic structures were inferred using ADMIXTURE ver. 1.3.0 (Alexander et al., 2009). The ancestral population number ( $K$ ) ranged from 1 to 9. The most likely  $K$  number was defined based on the lowest value of the cross-validation (CV) error for each  $K$  value. Additionally, we performed PCA using GenoDive 3.04.

Gene flow among naturalised populations, cultivars, and contaminants was estimated using the divMigrate-online program (<https://popgen.shinyapps.io/divMigrate-online/>; Sundqvist et al., 2016). The relative migration rates ( $N_m$ ) ranging from 0 to 1 between group pairs were calculated based on the genetic distance measure  $D$  (Sundqvist et al., 2016). The concept underlying this program is that for each pair of populations, a hypothetical pool of migrants is created with the geometrical means of the allelic frequencies in the corresponding two populations of interest. Then, as a measure of directional differentiation, genetic differentiation such as  $G_{st}$  and  $D$ , is estimated between the hypothetical pool and each pair of populations. The obtained directional differentiation is used to calculate the relative migration between the two populations (Sundqvist et al., 2016). Italian ryegrass Common (*L. multiflorum*) and contaminants from Australian ASW wheat were used as the representative cultivar and representative contaminant, respectively. In this case, the SNPs were retrieved with  $r$  at 50%, and each group was considered to be a distinct population ( $p = 11$ ).

## 2.4 | Sampling of naturalised populations at croplands and sandy coasts throughout Japan

To confirm that the difference in distribution was a common phenomenon throughout Japan, we visited 33 sandy coasts from May to July 2019 (Table S4). We evaluated the occurrence of *Lolium* species on each sandy coast at three levels: no distribution, low frequency

and high frequency (Figure S3). When *Lolium* species were distributed on the sandy coast at a high frequency, a leaf was collected from 10 individuals each at intervals of at least 3m. We also visited a cropland within 5 km of each sandy coast and collected samples as described above. Total DNA was extracted from the leaf tissue of each individual, and MIG-seq analysis was conducted as described above, except that a MiSeq Reagent kit v3 (150 cycles) was used. Eight of each of the ASW wheat and Common cultivar samples were also included in the analysis. Raw paired-end sequences with average read lengths of 78bp were obtained. SNP selection was performed as previously described.

The relationship between the mean proportion of each cluster for each population estimated by ADMIXTURE and within-population genetic diversity indices ( $N_e$  and  $H_e$ ) was tested using quadratic regression analysis to evaluate whether the admixture of different lineages increased genetic diversity in naturalised populations. The genetic diversity will be high at intermediate values of the cluster proportion if the admixture increases it. A quadratic curve was applied to the relationship using the nls function in the R stats package. Bootstrap parameter estimation with 1000 iterations was conducted to determine the confidence intervals for each parameter.

## 2.5 | Reciprocal transplant experiment

Habitat-specific adaptations were examined in a reciprocal transplantation experiment. The experimental farms of Kyoto University (35.032121°N, 135.783453°E) and Shimazaki Sandy Coast (34.7319463°N, 136.5287767°E) were selected as representatives of the cropland and sandy coastal habitats, respectively, and were used in a reciprocal transplant experiment (Figure S1). From May to June 2017, bulk seeds were harvested from more than 30 individuals in naturalised populations in three habitat types: cropland, seaport and sandy coast, in each of the three geographic regions of Japan (Kansai, Kanto and Kyushu). In total, nine populations (three habitats × three regions) were used for this experiment. The same populations were investigated for the genetic and morphological variations described above (Table S3). Seeds were stored in an airtight container containing silica gel at room temperature (20–28°C) until September 2017.

Fifty seeds from each population were sown in a plastic tray (length: 26.5cm, width: 18.3cm and depth: 7.7cm) filled with soil from bare land in each habitat. Eight trays were prepared for each population, three of which were set on croplands and five on the sandy coast on 29 September 2017, which is the typical germination period for *Lolium* species. The trays were covered with fine mesh nets to prevent further seed input and removal by wind or animal dispersal. The trays were buried at depths where the rims were at the same level as the soil surface. Seedling emergence, survival and flowering were recorded at 1-week intervals through July 2018, except for the period from December to March, during which observations were made every 2 weeks. Individuals were harvested 3 weeks after heading, and the numbers of culms, spikelets per culm and florets per spikelet in the middle of the inflorescence were measured. The total number of

florets produced by surviving individuals was calculated as follows: number of culms  $\times$  number of spikelets per culm  $\times$  number of florets per spikelet. Survival rate was defined as the ratio of the number of surviving individuals to the number of emerged seedlings.

Seedling emergence and survival rates were analysed using a generalised linear model with a quasi-binomial error and a logit link function, and the source habitat (cropland, seaport and sandy coast), the derived geographic region (Kanto, Kansai and Kyushu), and their interaction were incorporated as fixed factors. The number of florets was analysed using a generalised linear mixed model with a negative binomial error and a log link function, and the tray was integrated as a random factor using the `glmer.nb` function in the R `lme4` package version 1.1.27.1. The aforementioned fixed factors were applied. Days to heading were analysed using a general linear mixed model considering the tray as a random factor and using the `lmer` function in the R `lme4` package. The aforementioned fixed factors were applied. Tukey's honest significant difference test was used for pairwise comparisons. All statistical analyses were performed using R ver. 4.0.2 (R Core Team, 2020).

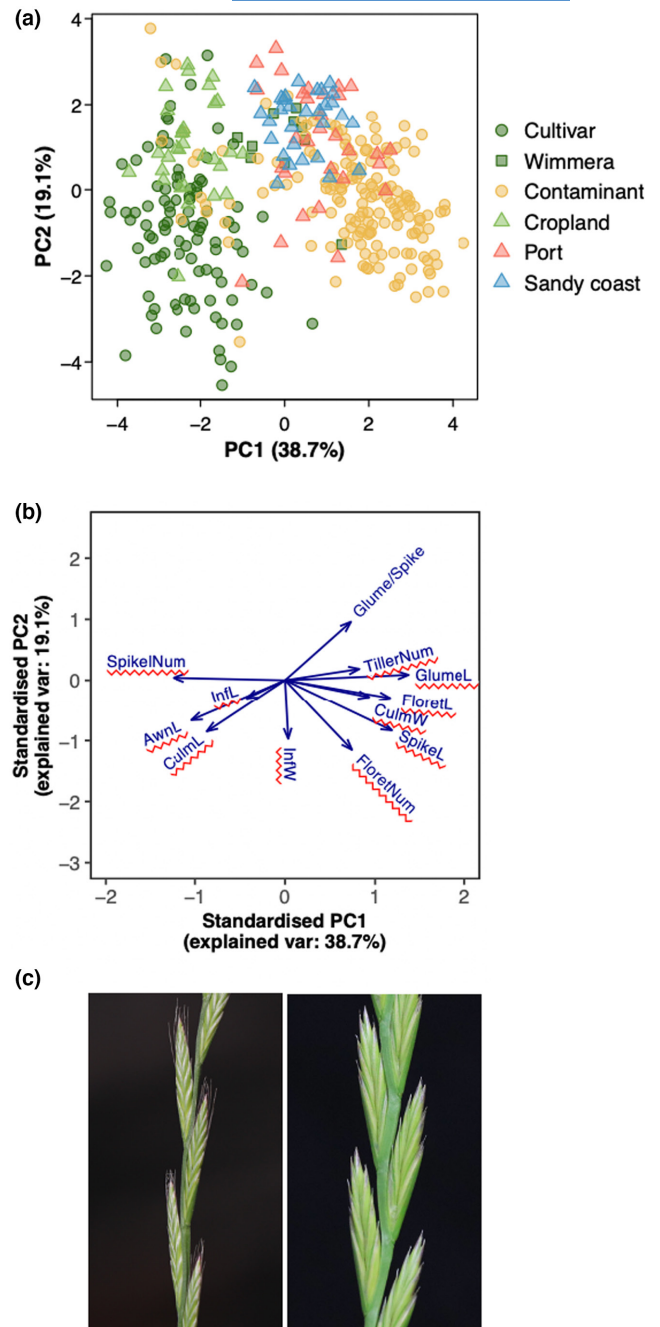
### 3 | RESULTS

#### 3.1 | Morphological variation

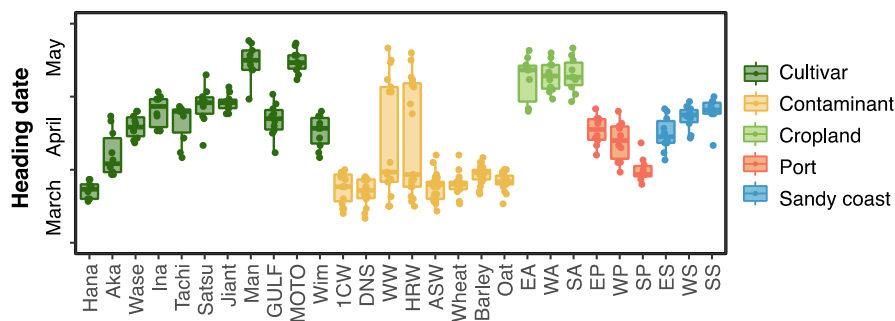
PCA, based on the morphological characteristics, separated two major groups along PC1, which explained 39.6% of the total variation (Figure 1). Most individuals of the contaminants, Australian cultivar Wimmera ryegrass, seaport and sandy coast, were positioned with positive scores along PC1, whereas the individuals of the Japanese cultivars, United States cultivar GULF, New Zealand cultivar MOTO and croplands were positioned with negative scores along PC1. Among the individuals with the contaminant, nine and seven individuals of HRW and WW, respectively, had negative scores.

PC1 was most positively correlated with glume length, followed by spikelet and floret lengths and was most negatively correlated with the number of spikelets, followed by awn length (Figure 1; Table S5).

The individuals headed continuously from late March to late May. The heading phenology of the Japanese cultivars differed by variety, with an extremely early maturing variety 'Hanami-wase' that headed earliest on March 22 and a late maturing variety 'Mammoth B' that headed latest around mid-May (Figure 2). Most contaminants headed in late May, but the contaminants in the HRW and WW included both early and late bloomers. Fifteen contaminants located in the negative direction of PC1 in the previous PCA were consistent with late-flowering individuals. Among the naturalised populations, the port and sandy coast populations headed earlier than the cropland populations.



**FIGURE 1** Principal component analysis (PCA) based on the 12 morphological characteristics of *Lolium* species. (a) PCA score plots with PC1 and PC2. (b) Biplot of the principal loading score of each of the morphological characteristics along PC1 and PC2. The morphological characteristics are abbreviated as follows: AwnL, awn length; CulmL, culm length; CulmW, culm width; FloretL, first floret length; FloretNum, number of florets per spikelet; Glume/Spike, The ratio of glume to spikelet length; GlumeL, glume length; InfL, inflorescence length; InfW, inflorescence width; SpikeL, spikelet length; SpikeNum, number of spikelets; TillerNum, number of tillers. (c) Typical inflorescence morphology of cultivars (left) and contaminants (right).



**FIGURE 2** Heading date of each group of *Lolium* species. Each boxplot shows median (line), interquartile range (box), and 1.5 times the interquartile range (whisker). Abbreviations of each group are defined in Tables S1–S3.

### 3.2 | Genetic variation

A total of 21,562,652 reads were sequenced using MIG-seq. After filtering these raw datasets, three samples with fewer than 10,000 reads were removed and an average of 73,118 reads per sample were obtained from the remaining 235 samples.

First, we identified the species to which the naturalised populations were genetically similar. For 56 individuals (eight individuals each from three cultivars, Italian ryegrass Common [*L. multiflorum*], perennial ryegrass Common [*L. perenne*] and annual ryegrass Wimmera [*L. rigidum*]) and contaminants from Australian ASW wheat and naturalised populations in three habitats (cropland, port and sandy coast in Kyushu), 1853 SNPs were obtained after assembling reads together and identifying polymorphic loci.

The results of the ADMIXTURE analysis showed that the lowest value of the CV error occurred when  $K=2$ ; however, the value was similarly low at  $K=3$  (Figure S4a). At  $K=3$ , *L. multiflorum*, *L. perenne* and *L. rigidum* were divided into different clusters (Figure 3a). Individuals growing in croplands were in the same cluster as *L. multiflorum*, whereas those growing in the seaport and on the sandy coast were in the same cluster as *L. rigidum* (Figure 3a). Contaminants from Australian ASW wheat were also divided into the same cluster as those of *L. rigidum*. *Lolium perenne* was assigned to an independent cluster. This result was consistent with that of the PCA (Figure 3b).

Next, all individuals except *L. perenne* were analysed in the same manner. ADMIXTURE analysis based on 2172 SNPs across 227 individuals identified  $K=2$  as the lowest CV error value (Figure S4b). This  $K$  value distinguished the two introduction sources; samples from all Japanese cultivars, the United State cultivar and New Zealand cultivar were assigned to the same gene pool (cluster 1), whereas samples from contaminant seeds and Australian cultivar were assigned to another pool (cluster 2), except for some contaminants derived from HRW and WW wheat (Figure 4a). Populations growing in croplands were in the same cluster as the cultivars, whereas those growing in seaports and on the sandy coasts were in another cluster, which included the most contaminated seeds (Figure 4a).

The PCA showed that most contaminants and the cultivars were situated at either end of PC1, which accounted for 17.6% of the genetic variance among the individuals, although some contaminant seeds derived from HRW and WW wheat were situated at the same end as the cultivars (Figure 4b). The cropland

populations were located at the same end as the cultivars, whereas the seaport and sandy coast populations were located at the other end of the cultivars (Figure 4b), corresponding to the results of the genetic structure analysis (Figure 4a).

The genetic diversity index,  $H_e$ , was significantly lower in the cultivars than in the other groups (Figure 4c). Naturalised populations and contaminants showed similar levels of diversity. The  $N_e$  values showed a trend similar to that of the  $H_e$  values (data not shown).

The relative gene flow estimated based on 491 SNPs indicated two strongly connected groups: cropland populations and cultivars; and port populations, sandy coast populations, and contaminants (Figure 4d; Table S6). In addition, asymmetric gene flow from cultivars and cropland populations to sandy coast populations was observed.

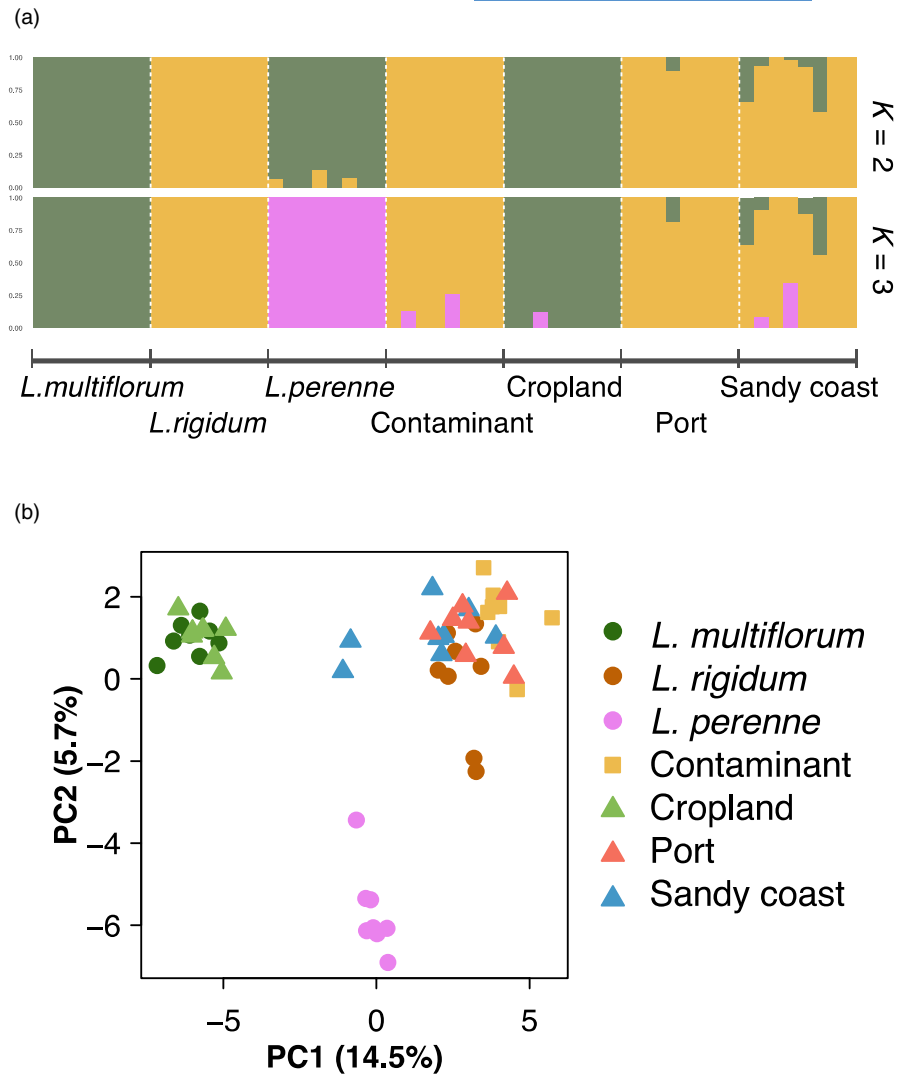
### 3.3 | Distribution pattern of *Lolium* species at croplands and sandy coasts throughout Japan

*Lolium* species were distributed at a high frequency in most croplands, except in the Tohoku region (Figure 5a). Contrastingly, the frequency of occurrence on sandy coasts was high in the western region and low or absent in the eastern region (Figure 5a). Therefore, *Lolium* species were sampled on 17 of the 33 surveyed sandy coasts. Samples were collected from 188 and 130 individuals from croplands and sandy coasts, respectively.

Using MIG-seq, 128,464,224 reads were sequenced. After assembling and identifying the polymorphic loci, 3149 SNPs across 334 individuals were obtained with an average of 87,238 reads per sample.

The population structure, estimated using ADMIXTURE, was best explained by two genetic clusters (Figure S4c), and the samples were classified according to the contaminants of ASW wheat or Common cultivars, which was consistent with the above results. The geographic distribution of genetic clusters in naturalised populations showed that most individuals collected from sandy coast populations were in the same cluster as the contaminants of ASW wheat, whereas those from cropland populations were in another cluster that included the Common cultivar (Figure 5a,b). However, most individuals from one sandy coast population were genetically close to the cultivar and two populations contained more than one-third of the cultivated genetic cluster. Three cropland populations contained more than one-third of the genetic contaminant clusters.

**FIGURE 3** Individual genetic structure of *Lolium* species. (a) Bar plot of Q values (proportion ancestry estimated using Admixture 1.3.0) in  $K=2$  and  $K=3$  for 8 individuals from each group. (b) Principal component analysis of individual genotypes. The individuals analysed here were the same as those in the admixture analysis.



The relationship between the mean proportions of cluster 1 for each population and  $H_e$  is shown in Figure 5c. Applying convex quadratic regression significantly explained 47.6% of the variance in  $H_e$  ( $p < 0.001$ ) and 50.6% in  $N_e$  ( $p < 0.001$ , data not shown). This indicated that the admixture of different lineages increased the genetic diversity in naturalised populations.

### 3.4 | Reciprocal transplant experiment

Seedling emergence began in late October and was completed within 1 month in both sowing habitats. Final germination percentages at the cropland site were 95% for the cropland and sandy coast populations and approximately 73% for the port population. These values were approximately 20%–30% lower at the coastal site (Figure 6a).

Almost all seedlings survived until flowering at the cropland site, irrespective of the population. Contrastingly, an average of only 30% seedlings survived in the coastal site, where cropland populations showed significantly lower survival (16%) than port (43%) and sandy coast populations (34%; Figure 6b).

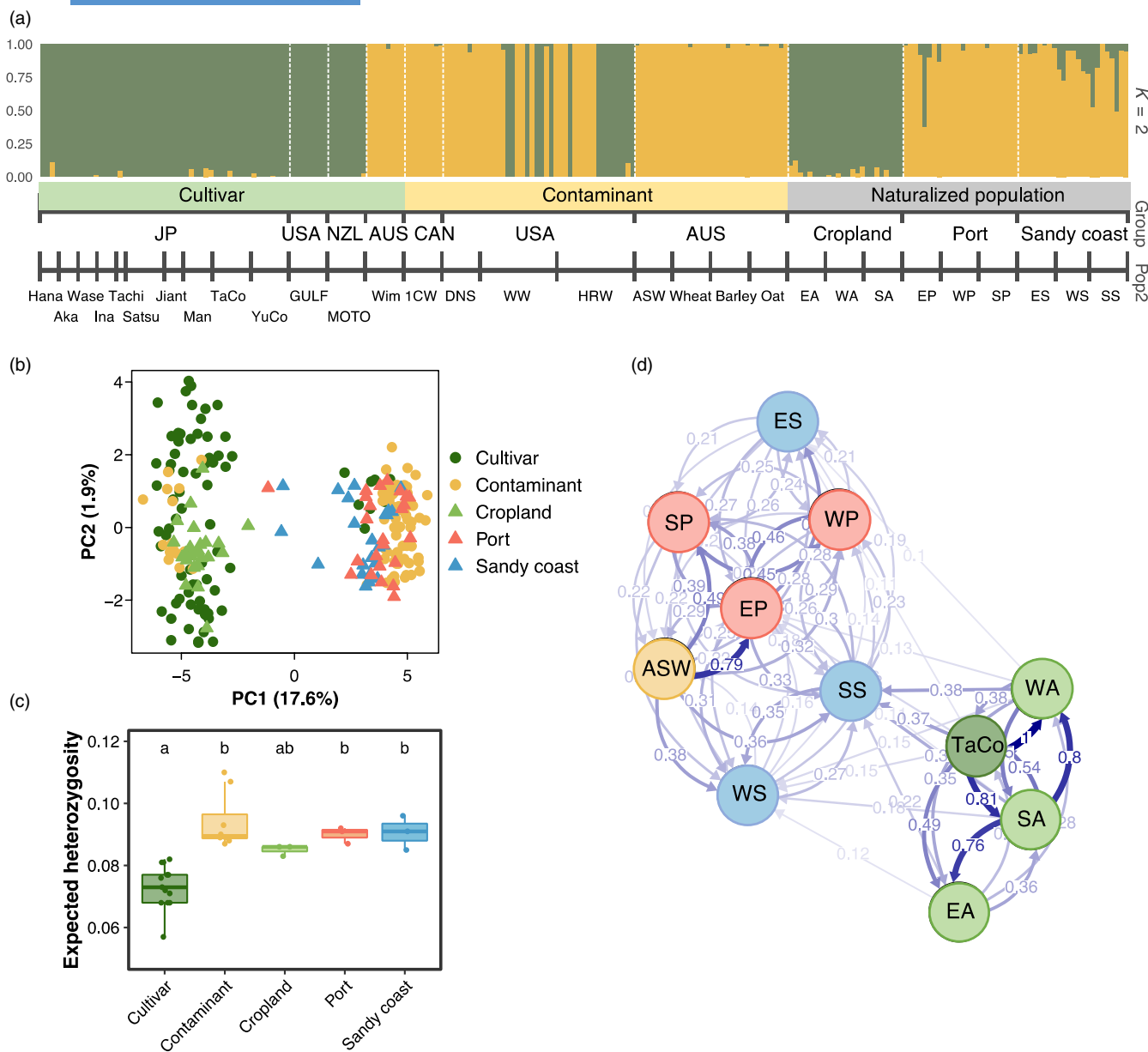
Populations derived from croplands yielded more than twice as many florets as those derived from other habitats at the cropland site. However, this advantage was not detected at the coastal site (Figure 6c), where all the plants produced a small number of florets, ranging from 17 to 38.

The timing of flowering differed among populations. Generally, populations from ports flowered the earliest followed by those from sandy coasts. Populations derived from the croplands flowered 2 and 3 weeks later than the other populations at cropland and coastal sites, respectively (Figure 6d).

## 4 | DISCUSSION

### 4.1 | Origin of the sandy coast populations

*Lolium* species have been introduced into Japan through two main pathways: as contaminants in grain commodities and as commercial cultivars for fodder crops or revegetation materials. In this study, a direct comparison of each introduction source and naturalised

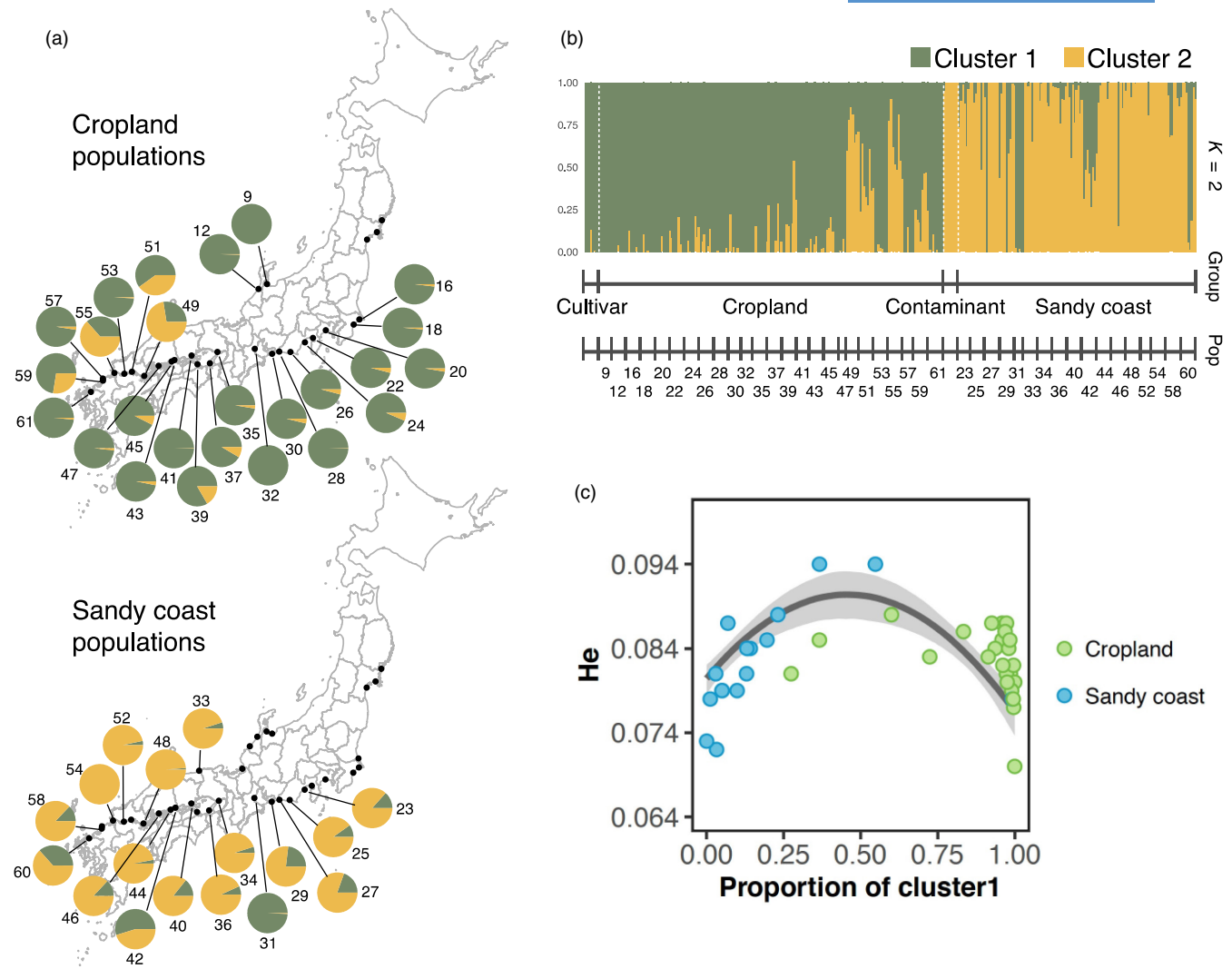


**FIGURE 4** Individual genetic structure, genetic diversity and gene flow of *Lolium* species. (a) Bar plot of Q values (proportion ancestry estimated using Admixture 1.3.0) in  $K=2$  for 76 individuals from cultivars, 88 individuals from contaminants and 71 individuals from the naturalised populations. (b) Principal component analysis of individual genotypes. The individuals analysed here were the same as those in the admixture analysis. (c) Expected heterozygosity of each population or lot from cultivar (blue), contaminants (orange), naturalised populations at croplands (greenish yellow), ports (red) and sandy coasts (sky blue). Different letters indicate significant differences among sample groups at  $p < 0.05$ . (d) Relative migration rates after filtering for the lowest 10% estimated using divMigrate-online. Populations are shown as circles and connected by arrows that become darker and thicker as gene flow increases. Abbreviations of each population are defined in Tables S1-S3.

populations revealed genetic and morphological differences between the Japanese cultivars and most contaminants, which are consistent with the differences between *L. multiflorum* and *L. rigidum*. The naturalised populations in croplands were closely related to the cultivars, whereas those in seaports and sandy coasts were closely related to the contaminants. These results indicate that naturalised populations in cropland are derived from cultivars and those in seaports and sandy coasts from contaminants.

A distribution survey across Japan showed that *Lolium* species invaded and thrived on most of the sandy coasts in western Japan but were extremely rare in eastern Japan. Cold winter temperatures may limit their distribution; however, other limiting factors are unknown. The ADMIXTURE analysis results show that 22.3% of individuals from sandy coast populations had  $>25\%$  of the genetic cluster of cultivars, indicating a moderate amount of gene flow. The divMigrate analysis showed asymmetric gene flow from cultivars and cropland

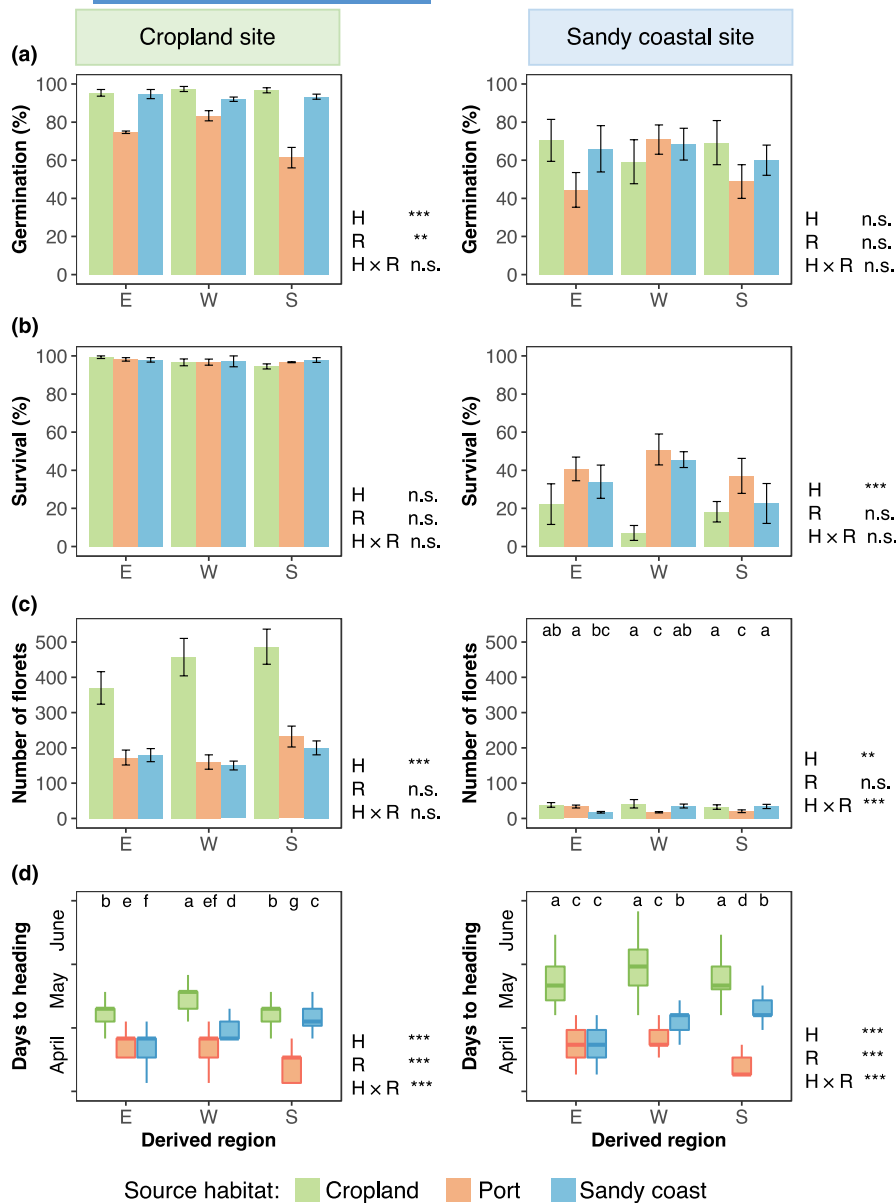




**FIGURE 5** (a) Geographic distribution of genetic clusters of naturalised populations in croplands and sandy coasts in Japan. Each population is represented by a pie chart showing proportions among two genetic clusters ( $K=2$ ) as determined by the analysis using Admixture 1.3.0. The black dots without pie charts indicate habitats where *Lolium* species was not distributed or distributed with low frequency. Abbreviations of each population are defined in Table S4. (b) Bar plot of Q values (proportion ancestry estimated using Admixture 1.3.0) in  $K=2$  for eight individuals from cultivars, eight individuals from contaminants, 188 individuals from the cropland populations and 130 individuals from the sandy coast populations. (c) A relationship between mean proportion of cluster 1 for each population and  $H_e$ . The line represents the estimated quadratic regression line. The grey zone represents 95% confidence interval.

populations to sandy coast populations, indicating hybridization post-introduction and hybrid survival. Moreover, the admixture of the two genetic clusters contributed to an increase in the genetic diversity of naturalised populations. *Lolium* species are likely to hybridise when they are geographically proximate because of their self-incompatible and wind-pollinated habits (Klaas et al., 2011). Hybridisation between populations with different genetic systems can aid adaptation to new environments and cause further range expansion (Dlugosch et al., 2015; Dlugosch & Parker, 2008; Ellstrand & Schierenbeck, 2000; Pfennig et al., 2016) but can reduce the fitness of hybrids (Rieseberg & Carney, 1998). Unravelling the impact of hybridisation on the invasive potential of these weed species is necessary to understand the risks of future range expansion.

All the cultivars of *L. multiflorum* from Japan, the United States, and New Zealand belonged to the same gene pool, whereas a cultivar of *L. rigidum* from Australia belonged to a separate gene pool that included samples of contaminants from Australian wheat and feed crops. *Lolium perenne* is genetically distinct from the other samples. Previous studies based on allozyme or chloroplast DNA variation have shown that *L. perenne* differed from outcrossing ryegrass (*L. multiflorum* and *L. rigidum*); however, *L. multiflorum* and *L. rigidum* are closely related and difficult to separate (Bennett et al., 2002; Charmet & Balfourier, 1994; Kurokawa et al., 2010). However, the high-resolution genome-wide SNP analysis method used in this study was effective in genetically distinguishing the *L. multiflorum* cultivars from the Australian *L. rigidum* cultivar.



**FIGURE 6** Differences in performance of cropland, port and sandy coast populations measured as (a) seedling emergence, (b) survival, (c) number of florets and (d) days to heading in reciprocal transplant experiments in cropland and sandy coastal sites. In (a–c), bars represent mean  $\pm$  standard error. In (d), each boxplot shows median (line), interquartile range (box), and 1.5 times the interquartile range (whisker). The significance of source habitat (H), derived geographic region (R) and their interaction (H  $\times$  R) are shown (\*\*\*:  $<0.001$ , \*\*:  $<0.01$ , \*:  $<0.05$ , n.s.: not significant). When a significant interaction was identified between the main effects, the results of pairwise comparisons were also shown using different letters indicating significant differences among populations at  $p < 0.05$ .

Moreover, applying high-throughput RAD-seq allowed for the identification of each Italian ryegrass variety (Yu et al., 2022).

Numerous *L. multiflorum* varieties were used throughout the temperate regions of the world, and 702 *L. multiflorum* varieties are listed on the OECD list of varieties eligible for seed certification (OECD, 2022). However, only three *L. rigidum* varieties are listed on the OECD list (OECD, 2022): two are registered in Australia and one in Italy. *L. rigidum* is widely used as a fodder crop (Humphreys et al., 2010) and has become the most problematic cereal crop weed in Australia (Matzrafi et al., 2021). Consequently, Australian wheat contains excessive *Lolium* contamination, approximately 4000 seeds per 20 kg of ASW wheat, whereas other classes of wheat imported from the United States and Canada contain 0–160 *Lolium* seeds (Shimono et al., 2015). Therefore, ASW wheat exhibited the highest propagule pressure for the introduction of *Lolium* species in seaports in Japan.

## 4.2 | Local adaptation of cropland and sandy coast populations

The reciprocal transplant experiment clearly demonstrated the home site advantage; populations derived from croplands yielded higher floret numbers than those derived from other habitats at the cropland site, but such an advantage was not detected at the coastal site. Populations derived from sandy coasts had higher survival rates than those derived from croplands at the coastal site. Port populations exhibited a tendency similar to that of sandy coastal populations, indicating that *L. rigidum* contaminants may have originally adapted to sandy coast environments more than *L. multiflorum* cultivars.

Environmental differences between sandy coasts and inland habitats cause strong divergent selection between populations, owing to salt stress, drought, sandblasting or sand burial (Lowry

et al., 2008; Maun & Perumal, 1999; Ogura & Yura, 2008). In the south-west agricultural region of Western Australia where ASW wheat is produced, the mean annual rainfall ranges from 300mm to 1200mm (Raper et al., 2014). The annual evaporation exceeds rainfall in most of the area, which is one of the factors cause the region to be prone to salt accumulation in the soil (Raper et al., 2014). These soil properties may have contributed to the acquisition of salt tolerance by *L. rigidum*. Wimmera ryegrass is more salt-tolerant than perennial and Italian ryegrass (Australian Herbage Plant Registration Authority, 1990).

Contrastingly, port and sandy coast populations produced fewer seeds than the cropland populations in the cropland site with low physical stress. According to Kloot (1983) who reviewed the taxonomy and ecology of *Lolium* species in Australia, *L. rigidum* is the most widespread ryegrass in the country, found in disturbed soils from the coast to the interior land, whereas *L. multiflorum* is restricted to high rainfall and irrigation areas in temperate Australia. In addition, *L. rigidum* has lower yields than *L. multiflorum* (Cooper & Saeed, 1949; Robbins & Faulkner, 1983). Therefore, trade-offs between the high yield under suitable conditions and the stress tolerance might explain the low performance of port and sandy coast populations in the cropland site.

A rapid adaptive evolution in response to changing environments has been demonstrated in agricultural weeds (Fukano et al., 2020; Kreiner et al., 2019; Kreiner, Latorre, et al., 2022; Quiroga et al., 2010). Likewise, *Lolium* species have evolved resistance to diverse herbicides, along with deep seed dormancy and early flowering phenology in response to agricultural management or growing season length (Matzrafi et al., 2021). In the present study, the flowering phenology of *Lolium* species showed interpopulation variability. While the genetic and morphological characteristics of sandy coast populations were similar to those of port populations, their flowering phenology exhibited notable differences from that of port populations. The flowering phenology in the sandy coast populations evolved in the late flowering; therefore, late flowering alleles may have been transferred from cropland populations to sandy coast populations.

Under the Mediterranean-type climate of Western Australia, annual grasses and herbs should complete their growth cycles and set seed before the dry summer season. Strong selection prevents the immigration of late flowering individuals. Conversely, Japan has a warm and humid climate, with relatively high precipitation throughout the year compared to the Mediterranean climate. The larger the plant size at reproduction, the higher the seed production (Donohue et al., 2010). The late flowering can prolong the vegetative growth period and increase seed production. This may cause the rapid evolution of late flowering time in response to the duration of the growing season.

## 5 | CONCLUSION

Through a population genetics approach and a reciprocal transplant experiment, we demonstrated that two congeneric species

with different ecological characteristics were introduced through multiple introduction pathways and spread across different habitats. Moderate gene flow from cropland populations may have contributed to the rapid evolution of the flowering phenology of sandy coast populations. Unravelling the impact of admixtures on the invasive potential of these weed species is required to understand the risk of future range expansion. A direct comparison between source and naturalised populations can greatly advance our understanding of the patterns and processes of biological invasions.

## AUTHOR CONTRIBUTIONS

Yoshiko Shimono conceived and designed the study. Yumiko Higuchi performed the pilot experiments. Momoko Hirata and Yoshiko Shimono performed the field work. Ayumi Matsuo, Yoshihisa Suyama, Mitsuhiko P. Sato and Momoko Hirata performed the genetic analyses. Takako Kiyoshi and Akihiro Konuma prepared sample materials. Momoko Hirata and Yoshiko Shimono wrote the manuscript, and Tohru Tominaga and Mitsuhiko P. Sato edited the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

## PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/1365-2745.14206>.

## DATA AVAILABILITY STATEMENT

Raw sequence data were deposited into the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (accession number: DRA013773, DRA015739). Data of the common garden and reciprocal transplant experiments are available on in Dryad Digital Repository <https://doi.org/10.5061/dryad.dr7sqvb39>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Figure S1.** (a) Locations of three regions (Kanto, Kansai and Kyushu) and sites for a reciprocal sowing experiment (Kyoto University and Shimazaki sandy coast) and sampling locations of naturalised *Lolium* populations in (b) Kanto, (c) Kansai, and (d) Kyushu.

**Figure S2.** Measured morphological characteristics of (a) whole plant, (b) inflorescence and (c) spikelet.

**Figure S3.** Examples of sandy coast sites where abundance of *Lolium* was (a) apparently zero, (b) low, and (c) high.

**Figure S4.** Values of cross-validation error for each K in the analysis conducted using Admixture 1.3.0.

**Table S1.** Number of contaminant seeds used for each experiment.

**Table S2.** Number of individuals from cultivated varieties used for each experiment.

**Table S3.** Number of individuals from naturalised populations used for each experiment.

**Table S4.** Sampling locations and number of individuals in the field populations used in each experiment.

**Table S5.** Summary of the results of principal component analysis of 13 morphological characteristics of *Lolium* species.

**Table S6.** Pairwise relative gene flow rates for the 11 populations.

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