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The Expansion Route of Ryegrasses (*Lolium* spp.) into Sandy Coasts in Japan

Yumiko Higuchi, Yoshiko Shimono, and Tohru Tominaga*

Although an increasing number of investigations have been made into the evolution of alien species once introduced, few studies have identified the invasion routes of these introduced species. Because multiple introductions are common in invasive species, failing to take into account the introduced lineages can be misleading when studying evolutionary change in alien species after they begin to extend their ranges. In Japan, diverse lineages of ryegrasses (*Lolium* spp.) were introduced as forage crops and contaminants in trading grain and have expanded to sandy coasts. We studied the expansion route of populations established along the coasts of three geographic regions within Japan by comparing variations in morphology and nuclear microsatellite and chloroplast DNA in the two habitats where ryegrasses were first introduced: croplands and international seaports. Chloroplast DNA haplotypes did not differ significantly among habitats and regions, but the coastal and seaport populations displayed similar microsatellite genetic compositions and morphological characteristics. Our results revealed that coastal populations originated from seaport populations derived from contaminants. Selective forces from the past, including domestication and naturalization, may have assisted the introduced lineages in colonizing new habitats.

Nomenclature: Italian ryegrass, *Lolium multiflorum* L.; rigid ryegrass, *Lolium rigidum* Gaudin.

Key words: Exotic, genetic diversity, intentional/unintentional introduction, introduction pathways, nSSR, postintroduction process.

Exotic species can sometimes become more problematic in their introduced range than in their native range due to rapid evolution (e.g., Colautti and Barrett 2013; Li et al. 2015; Maron et al. 2004; Ridley and Ellstrand 2010). At the same time, those species provide ideal opportunities to study evolutionary processes on contemporary timescales (Colautti and Lau 2015; Sax et al. 2007). Evolutionary studies of invasive species have often been conducted by comparing the genetic structure of populations living in their native and introduced habitats. These studies can detect the mechanisms of invasion, including hybridization, increasing/decreasing genetic diversity, or multiple introductions (Kolbe et al. 2004; Lavergne and Molofsky 2007; Ridley et al. 2008).

During invasion, however, alien species must undergo many processes to survive. These include transport, introduction, establishment, and range expansion (Blackburn et al. 2011). Because different types of selective pressure and stochastic events are at work, a large proportion of individuals fail to survive, and the demographic and genetic characteristics of populations are inevitably changed (Blackburn et al. 2011). Therefore, each invasion process should be considered separately to correctly understand its underlying mechanisms and develop tactics for management (Suarez and Tsutsui 2008).

The postintroduction process, from first establishment to range expansion, is especially important in ecological and evolutionary terms and can determine the success of an invasion by an alien species (Lambrinos 2004; Sakai et al. 2001). Colonizing the new habitat may activate selection pressures that differ from those of the first-established habitat, and different genotypes may be favored (Kawecki and Ebert 2004; Leimu and Fischer 2008). Genetic admixture between different lineages of the same species resulting from multiple introductions may stimulate range expansion by heterosis or by enhancing evolutionary potential (Chun et al. 2009; Keller and Taylor 2010).

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*First author: Graduate Student, Center for Ecological Research, Kyoto University, 509-3, 2-chome, Hirano, Otsu, Shiga, Japan, 520-2113; second and third authors: Assistant Professor and Professor, Laboratory of Weed Science, Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto, Kyoto, Japan, 606-8502. Corresponding author's E-mail: ymhiguchi@ecology.kyoto-u.ac.jp

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Management Implications

Identifying the route of invasion is the first and highly valuable step for biosecurity, because it is one of the most rewarding, cost-effective attempts to prevent invasion. Although studies about invasion routes have recently been in demand, few studies of invasion routes were published in a comprehensive way in Japan. *Lolium* species have been introduced to Japan by two routes, intentional introduction as forage and unintentional introduction as contaminants in grain commodities, and have naturalized along the sandy coasts. Because ryegrasses are an economically important exotic forage in Japan, their potential for invasion should be judged carefully. Our analysis revealed that preadaptation of seaport lineages derived from contaminants facilitated the invasion to coasts where cropland lineages derived from cultivars failed to colonize. Although the deliberate introduction previously seemed to be the major source of invasive *Lolium* populations in Japan, our results indicated that the relatively minor, unintentional pathway also had the potential to result in the colonization of new habitats and highlighted the importance of considering multiple pathways of invasion for successful weed management. One prevention measure against further introduction of new genotypes into coastal areas could be appropriately timed control of weeds before *Lolium* species mature in seaport areas.

In contrast, alien species may invade new habitats despite a shortage of genetic variation if they have a wide range of phenotypic plasticity (Geng et al. 2007), reproductive assurance (Hao et al. 2011), or preadaptation in a particular lineage (Le Roux et al. 2008).

Keller and Taylor (2008) have warned that unrepresentative samplings of dame/lineage may result in apparent adaptive evolution when studying the evolution of invasive species. Because multiple introductions are common during invasion events (Bossdorf et al. 2005; Dormontt et al. 2011), and different lineages may be introduced to distinct areas or habitats, failing to take their introduction histories into account may result in a misleading picture of their subsequent evolution. However, because of a lack of historical and observational records relating to the time and location of introductions (Estoup and Guillemaud 2010; Hulme et al. 2008), few studies have directly compared the genetic variation in alien plant populations before and after expansion in a new habitat.

In the present study, the annual outcrossing species *Lolium multiflorum* L. and *Lolium rigidum* Gaudin (family Poaceae) were used to investigate selective forces and demographic effects involved in the primary introduction of new species and their subsequent expansion in new habitats. Both species are self-incompatible, and the interspecific cross occurs easily (Naylor 1960; Terrell 1966). The species are native to the Mediterranean region but have spread across the world as both a forage crop and a weed (Humphreys et al. 2010; Terrell 1968). Because the interfertility and continuous morphological variation within these species has made their taxonomic identification difficult (Bennett 1997; Bennett et al. 2000; Terrell 1968), we treated them as a *Lolium* species complex throughout this study.

In Japan, *L. multiflorum* is widely recognized and listed as a problematic weed (National Institute for Environmental Studies 2015). Unlike most alien plants, *Lolium* species are known to have been introduced into Japan by two routes. First, they were introduced deliberately as forage or revegetation materials and have become a major agricultural weed in croplands (Figure S1a and b; Asai and Yogo 2005; Kurokawa et al. 2010). Second, it has recently been recognized that *Lolium* species were unintentionally introduced as contaminants of trading commodities (Figure S1c and d; Asai et al. 2007; Shimono et al. 2010).

Although *Lolium* species are usually found in anthropogenically disturbed habitats, including croplands, roadsides, and vacant lands, they are also common in the sandy coasts around Japan (Figure S1e and f). These coastal habitats are quite distinctive and subject to strong winds that create a challenging living environment characterized by sand burial and wind abrasion (Maun 1994). Moreover, coastal plants must contend with high soil and airborne salinity, and these stresses restrict the distribution of many species (Barbour 1978; Lowry et al. 2008). Because they were not introduced directly to these sandy coastal habitats, the *Lolium* plants must be derived from other locations.

In the present study, we investigated the invasion process of *Lolium* populations established in sandy coasts, where this invasion occurred via the two introduction routes discussed earlier. By comparing the morphological and genetic microsatellite variation between plants found as secondary invaders on the coast with those in the original areas of introduction, we addressed the following questions: Which lineages and introduction routes are responsible for the expansion into coastal habitats, or does an admixture of different lineages account for this? Have coastal populations undergone a reduction in genetic diversity during their expansion? Do *Lolium* species show different morphological traits according to habitats?

Materials and Methods

Study Sites and Sampling. Sampling was conducted in three geographic regions in Japan (Kansai, Kanto, and Kyushu) (Figure 1). In each region, three distinct habitats were chosen: cropland, seaport, and sandy coast. All of the selected seaports (Kobe, Kashima, and Hakata ports) are major international trading ports where more than 100 kt of imported wheat is unloaded every year (Ministry of Land, Infrastructure, Transport and Tourism 2013).

In each habitat, three transects were set and one flowering tiller from each of 10 *Lolium* plants was collected along each transect at intervals of at least 2 m. The sampled plants grew along the roadside at seaports, on paddy levees in the

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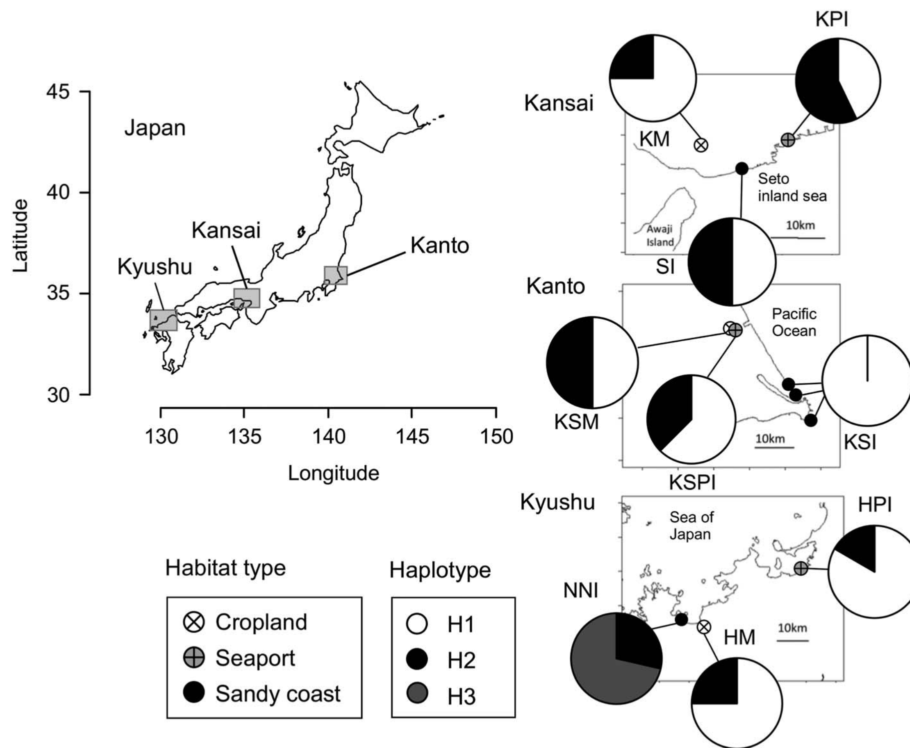

Figure 1. Sampling locations of *Lolium* species used in this study. Left: locations of three regions in Japan. Right: locations of populations in each region. The small points indicate habitat types. Pie charts shown in the right figure indicate geographic distribution of cpDNA haplotypes of *Lolium* species based on Table 3. The colors of the chart segments show the haplotype proportion of individuals within a population.

croplands, and in sandy areas within 200 m of the coastline along sandy coasts. Basically, we set transects parallel to levees, traffic roads, and shorelines, except when the number of plants was small and installation was difficult. In the Kanto region, *Lolium* plants were distributed sparsely, and the 30 samples in total were collected from three coasts (Figure 1). The distances between populations within each region ranged from 1 to 43 km. All samples were collected in 2014, except those from the Suma coast in the Kansai region, which were collected in 2013. Sample leaf tissue was stored at −80 C for DNA extraction, and the remaining samples were used for morphological measurements.

Measurement of Morphological Characteristics. Ten morphological characteristics were measured from each flowering tiller: rachis length, rachis width, number of spikelets per spike, spikelet width, spikelet length, glume length, number of florets per spikelet, floret length, anther length, and awn density. The measured characteristics were chosen for their usefulness in the identification of *Lolium* species (Bennett 1997; Terrell 1968). Awn density was graded on a scale of 1 to 3 depending on the number of awns on a spike (1 = no awn, 2 = some florets have awns, 3 = almost all florets have awns).

DNA Extraction and Molecular Analysis. Total DNA was extracted from leaves using a slightly modified cetyl trimethyl ammonium bromide method (Murray and Thompson 1980) and diluted to a concentration of 10 ng/μl. Nuclear microsatellite and chloroplast DNA (cpDNA) sequence analyses were performed. For nuclear microsatellite DNA analysis, seven loci (LMgSSR 02–05D, 02–11G, 03–04F, 08–12G, 11–06E, 16–04F, and 17–02F; described in Hirata et al. 2006) were used for genotyping. Polymerase chain reaction (PCR) was performed in a final reaction volume of 8 μl, containing 10 ng of genomic DNA, 0.2 μM fluorescently labeled (Beckman dye D2, D3, and D4) forward primer, 0.2 μM unlabeled reverse primer, and 0.25 U Taq DNA polymerase (New England BioLabs, Ipswich, UK) in a 1 × Thermopol reaction buffer. Amplification included an initial denaturation step at 95 C for 30 s followed by 30 cycles of denaturation at 95 C for 15 s, annealing at 60 C for 30 s, and extension at 68 C for 20 s, and then a final extension step at 68 C for 5 min. Because a universal fluorescent Tail A primer (Blacket et al. 2012) was used for loci 11–06E and 17–02F, two-step PCR was conducted for reliable amplification. First, we performed PCR containing 10 ng of genomic DNA and 0.2 μM unlabeled tailed (Tail A) forward primer; then a second PCR reaction

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was performed using 1 μl of the products from the first PCR and 0.2 μM universal Tail A primer with fluorophore (D2). The remaining reaction mixture and the amplification conditions were identical to those described above, with the exception of the number of cycles that included the annealing step; 15 cycles were used for the first PCR reaction, and 25 cycles were used for the second reaction. Determination of allele sizes in the amplified products was carried out automatically on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Krefeld, Germany) using the internal size standard 400, and then genotyped manually. Some individuals that apparently amplified more than two alleles or none at all were treated as missing data for that particular locus.

For the cpDNA sequencing, we randomly chose seven to eight individuals from each population and used PCR to amplify the two intergenic regions *psb*A–*mat*K and *trn*S–*trn*T, using previously described primers (Demesure et al. 1995; Yasuda and Shibayama 2006). The amplification conditions for each reaction included an initial denaturation step at 95 C for 30 s. For *trn*S–*trn*T, the initial denaturation step was followed by 28 cycles of denaturation at 95 C for 15 s, annealing at 68 C for 30 s, and extension at 68 C for 1 min, with the annealing temperature decreasing by 1 C per cycle for the first 8 cycles, down to 60 C. For *psb*A–*mat*K, the initial denaturation step was followed by 35 cycles at 95 C for 15 s, annealing at 68 C for 30 s, and extension at 68 C for 40 s, with the annealing temperature decreasing by 1 C per cycle for the first 10 cycles, down to 58 C. Both reactions included a final extension step at 68 C for 5 min. After amplification, these products were sequenced by the Fasmac DNA sequence service (Fasmac, Kanagawa, Japan).

Data Analysis. To summarize the variation in morphological characteristics, we conducted a principal component analysis (PCA) for the 10 morphological measurements. Morphological differences among habitats and regions were examined using a stepwise multiple regression analysis in which the first and second principal component scores (PC1 and PC2) were treated as dependent variables, and the habitat, region, and interactions were treated as fixed factors. The model with the lowest Akaike information criteria (AIC; Akaike 1973) was selected as the best fit.

The difference by habitat for each morphological characteristic and the PCs (PC1 and PC2) were examined in generalized linear mixed models (GLMMs) using population as a random factor and Tukey's honestly significant difference (HSD) test for pairwise habitat comparisons. For the two PC axes and all characteristics except the number of florets per spikelet, number of spikelets per spike, and awn density, a GLMM with Gaussian errors (identity link) was used. The number of florets per spikelet and the number of spikelets per spike were analyzed using a GLMM with Poisson errors (logarithmic link). For awn density, the Steel–Dwass test was used instead of Tukey's HSD test, because it is categorical data. All statistical tests were performed using R v. 3.0.2 (R Development Core Team 2013).

For each microsatellite locus and population, Hardy–Weinberg disequilibrium and linkage disequilibrium between pairs of loci were examined using Genepop on the Web v. 4.2. Null allele frequencies were estimated using CERVUS v. 3.0.7 (Marshall et al. 1998). Standard genetic statistics were calculated for each population. The number of private alleles (N_p), the observed heterozygosity (H_o), and the unbiased estimate of the expected heterozygosity (H_e) were estimated using GenAlEx v. 6.501 (Peakall and Smouse 2012). Allelic richness (A_r) and Weir and Cockerham's (1984) estimators of the inbreeding coefficient (F_{IS}) for each population were estimated using FSTAT v. 2.9.3 (Goudet 2001).

The genetic differentiation of each population was estimated with the global and pairwise fixation index (F_{ST}) using FSTAT. Significant differentiation was tested using the Bonferroni correlation. To assess the genetic variation between habitats and among populations within each region, we performed analysis of molecular variance (AMOVA) using GenAlEx. We also performed AMOVA for populations in each habitat separately. The probabilities of variance components were estimated from 9,999 random permutations. To compare the individual-based genetic structure among habitats and detect potential hybrids, we used a Bayesian approach implemented in Structure v. 2.3.4 (Pritchard et al. 2000) to cluster similar multilocus genotypes, allowing for population admixture and correlated with allelic frequency. The simulation was run with the number of clusters (K) from one to nine, and repeated ten times for each K to confirm the repeatability of the results. Each run comprised a burn-in period of 100,000 iterations, followed by 500,000 Markov-chain Monte Carlo steps. As recommended by Evanno et al. (2005), we calculated the ad hoc statistic ΔK on the basis of the rate of change in the log likelihood of data between consecutive K values. We identified the most likely number of clusters (K) that maximized ΔK.

Sequencing data were aligned using CodonCode Aligner v. 5.1.5 (CodonCode, Dedham, MA). We determined cpDNA haplotypes from nucleotide substitutions and indels from a combined data set including the two regions. All indel size variations at one location were treated as one insertion/deletion event.

Results

Morphological Characteristics. According to the PCA for 10 morphological characteristics, the second principal component axis (PC2) separates cropland populations from

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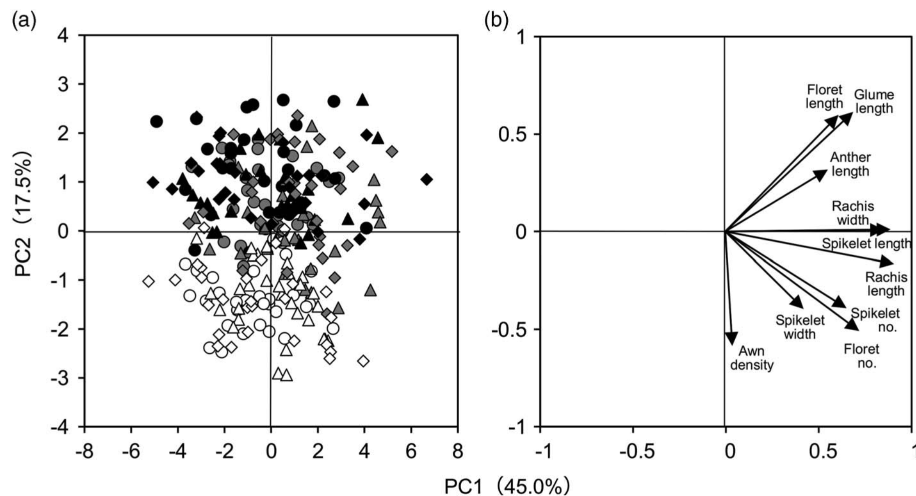

Figure 2. Principal component analysis of 10 morphological characteristics of *Lolium* species. (a) Distribution of measured samples in the PC1–PC2 score plane. Each symbol indicates a different region (triangle: Kanto; diamond: Kansai; circle: Kyushu), and each color indicates a different habitat (white: cropland; gray: seaport; black: sandy coast). (b) Display of the principal loading score of each of the morphological characteristics along PC1 and PC2.

the other habitat populations (Figure 2a). PC1 explains 45.0% of the total variation and shows a positive correlation with all traits, suggesting that PC1 is the parameter for plant size. PC2 explains 17.5% of the total variation and shows a positive correlation with glume length, floret length, and anther length, but a negative correlation with awn density, number of florets, number of spikelets, and spikelet width (Figure 2b). Stepwise multiple regression analysis indicated that models including habitat and geographic region as factors without interaction best fit both PCA axes (PC1, AIC = 378.28; PC2, AIC = −106.50). Although both habitat and region had a significant effect for those two PCA axes, habitat affected morphological variation more strongly ($F = 7.30$, df = 2, $P < 0.001$ for PC1; $F = 214.82$, df = 2, $P < 0.001$ for PC2) than did region ($F = 3.72$, df = 2, $P = 0.026$ for PC1; $F = 4.78$, df = 2, $P = 0.009$ for PC2), especially on the PC2 axis. In PC1, a significant difference ($P = 0.024$) was found only between seaport and cropland populations. In PC2, all pairwise habitat population comparisons were significantly different ($P < 0.05$).

Considering morphological characteristics separately, no significant difference was found among habitats for rachis length (Table S1). The mean glume length, floret length, and anther length in cropland populations were significantly lower than those in other habitats. Additionally, cropland populations usually had denser awns than other populations. Spikelet length was larger in seaport populations. Spikelet width, the number of spikelets, and the number of florets per spikelet were lowest in sandy coastal populations. The number of florets per spikelet was highest in cropland habitats, but a significant difference was not found between cropland and seaport populations (Table S1).

Microsatellite Genetic Diversity. In total, 269 individuals were genotyped. Overall, no linkage disequilibrium was detected among loci, but a few combinations of loci in almost every population showed some degree of linkage disequilibrium. Almost all loci in all populations were in Hardy–Weinberg disequilibrium. Because the level of Hardy–Weinberg disequilibrium decreased when reassessing samples divided by each transect, disequilibrium was partly due to subpopulation structure. Some null alleles were also found (null allele frequency, 0.05–0.66), especially at locus 17–02F (null allele frequency, 0.66). The other six microsatellite loci were highly polymorphic with a range of 12 to 32 (average, 20.5) alleles per locus. At locus 17–02F, only three alleles were found, and most individuals were homozygous. Because of the allelic distribution at locus 17–02F, we assumed that null alleles occurred widely across all populations. We conducted population genetic analysis both including and excluding locus 17–02F and found similar results. Therefore, we reported the results including all loci in the data analysis.

Genetic diversity was consistently high in all populations (Table 1). All populations had high values for expected and observed heterozygosity and for allelic richness. For most statistical evaluations, no consistent tendency was found throughout habitats or regions. F_{IS} values were significantly positive in all populations.

Population Differentiation. The global genetic differentiation was low, with $F_{ST} = 0.055$. Pairwise F_{ST} values ranged from 0.003 to 0.140 (Table S2). Significant differentiations were detected among all pairwise populations between cropland and other habitat populations and among

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Table 1. Genetic diversity of three habitat populations of *Lolium* species.[a]

Habitat	Population/region	ID	N	N_a	A_r	N_p	H_o	uH_e	F_{IS}
Croplands	Ikawadani/Kansai	KM	30	8.43	8.13	0.429	0.451	0.665	0.325
	Fukashiba/Kanto	KSM	30	7.57	7.37	0.429	0.514	0.627	0.183
	Hamasaki/Kyushu	HM	30	8.86	8.55	1.143	0.554	0.644	0.142
Seaports	Kobe Port/Kansai	KPI	30	8.14	7.80	0.286	0.500	0.633	0.213
	Kashima Port/Kanto	KSPI	30	9.29	8.93	0.714	0.544	0.685	0.208
	Hakata Port/Kyushu	HPI	30	8.57	8.32	0.286	0.494	0.740	0.337
Sandy coasts	Suma/Kansai	SI	30	9.00	8.67	0.714	0.517	0.654	0.213
	Kashima/Kanto	KSI	30	8.29	8.04	0.000	0.504	0.659	0.238
	Karatsu/Kyushu	NNI	29	8.86	8.58	0.286	0.560	0.675	0.172

[a] Abbreviations: N, number of samples; N_a, mean number of alleles per locus; A_r, mean allelic richness per locus; N_p, mean number of private alleles per locus; H_o, observed heterozygosity; uH_e, unbiased expected heterozygosity; F_{IS}, fixation index.

some other populations (Table S2). AMOVA indicated that 5.0% of the genetic variation could be attributed to differences between habitats, which was slightly greater than that among populations within habitats (4.0%; Table 2). The majority of genetic variation was found within populations (90%). Within each habitat, variation between populations was greatest in seaports (9.0%) and lowest in sandy coasts (1.0%).

In the Bayesian clustering analysis, the ΔK value was highest when $K = 2$ ($\Delta K = 334.26$) (Figure 3a). When $K = 2$, cluster 1 was dominant in cropland populations, whereas cluster 2 was dominant in port and coastal populations regardless of their geographic ranges (Figure 3b). In both seaport and sandy coastal populations, some high-proportion mixes were present. The average percentage of cluster 1 in each population was lower in seaport (10.5% to 22.6%) than in sandy coastal populations (21.1% to 30.0%), although both were much lower than in cropland populations (77.1% to 91.7%).

CpDNA Haplotype Diversity and Distributions. The total length of the aligned sequence of the two cpDNA regions was 1,653 bp. This included seven polymorphic sites comprising four substitutions, one indel, and two mononucleotide repeat variations. Excluding mononucleotide repeat variations, only 3 haplotypes were detected among 68 individuals (H1–H3; Table 3).

The geographic distribution of these three haplotypes is shown in Figure 1. Overall, the composition of haplotypes was similar throughout the regions. The H1 and H2 haplotypes were present in almost all populations, while H3 was detected only in the sandy coastal population (NNI) of the Kyushu region.

Discussion

Defining the Route of Expansion into Sandy Coasts. Morphological and nuclear microsatellite genetic variations of *Lolium* species were strongly correlated with the different introduction habitats (i.e., seaports and croplands) throughout all regions (Figures 2 and 3). These results strongly suggest that diverse lineages of *Lolium* were introduced into distinct habitats through different pathways.

Coastal populations were genetically and morphologically similar to seaport populations in all regions (Figures 2 and 3), suggesting that coastal populations originated from seaport populations derived from contaminants in trading grain. This relationship in genetic structure was maintained throughout the sampled locations, regardless of geographical distance between the sandy coasts and other introduced habitats (Figures 1 and 3b). Therefore, isolation by distance cannot explain relatedness between the seaport and sandy coastal populations.

Table 2. Results of hierarchical analysis of molecular variance (AMOVA) partitioning variance among habitats, populations in each habitat, and within a population

	All		Cropland		Seaport		Sandy coast	
	% Variance	P-value	% Variance	P-value	% Variance	P-value	% Variance	P-value
Among habitats	5	<0.0001	—	—	—	—	—	—
Among populations within habitats	4	<0.0001	2	0.009	9	<0.0001	1	0.034
Within population	90	<0.0001	98	—	91	—	99	—

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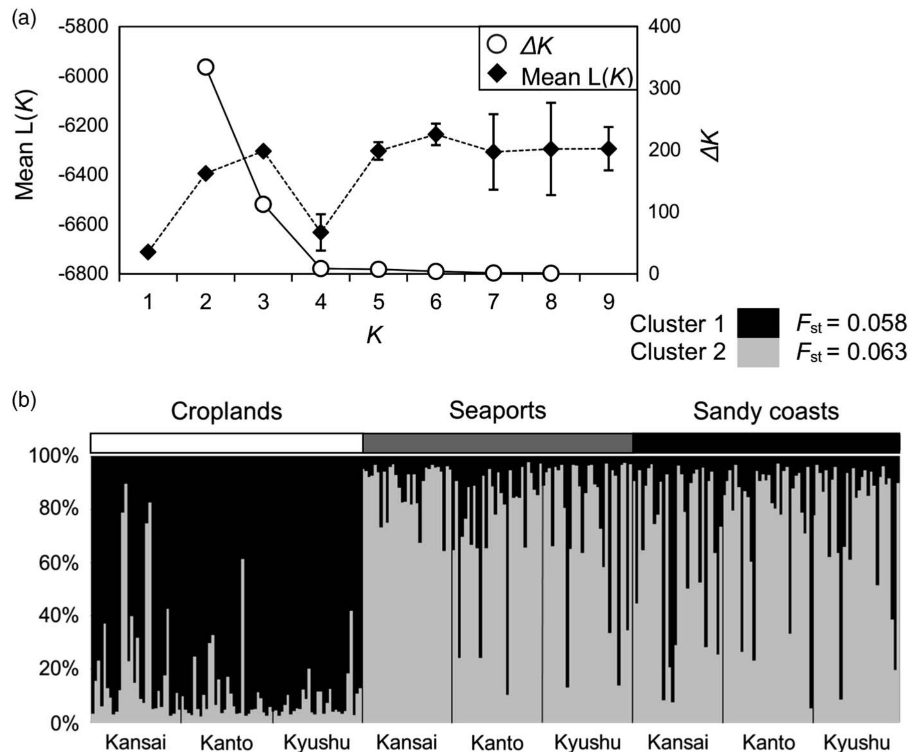

Figure 3. The result of Bayesian clustering analysis using Structure v. 2.3.4. (a) The logarithm of the probability of the data, mean L(*K*) (mean likelihood [filled diamond] ± SD) and the second-order rate of change in the probability between successive runs, Δ*K* (open circle), as a function of *K*, the number of clusters. (b) The results from *Lolium* samples used in this study, assuming two population clusters ($K = 2$). Each vertical line represents an individual, and each color represents a cluster. Individuals are grouped by populations of each habitat.

The level of mixing between two clusters was slightly higher in sandy coastal populations than in seaport populations, indicating a moderate amount of admixture during expansion. However, about 70% of the individuals from coastal populations were strongly associated with cluster 2 (containing >80% of cluster 2). Therefore, we concluded that the genetic admixture of the two introduced lineages was not necessary for the expansion of *Lolium* species into coastal environments.

In contrast to clear differences in *Lolium* species' morphology and nuclear microsatellite DNA, cpDNA sequences showed little differentiation among habitats. The different haplotypes were genetically similar, and most populations shared the same haplotypes (Figure 1; Table 3). Kurokawa et al. (2010) also reported less divergence in the cpDNA than nSSR of *Lolium* populations in Japan. Because cpDNA is more conserved than nuclear DNA (Wolfe et al. 1987) and nuclear microsatellites are especially variable (Sunnucks 2000), the lack of any association between cpDNA sequences and plant location/habitat may suggest a recent genetic differentiation of nuclear microsatellite loci along the species' introduction pathways. The history of *Lolium* species for agriculture may also have affected its genetic composition. Many hybridizations have been performed between cultivars and natural populations to develop new cultivars (Humphreys et al. 2010; Terrell 1966;

Table 3. Polymorphisms in the two cpDNA regions of *Lolium* species.

	*psb*A-*mat*K spacer				*trn*S-*trn*T spacer	Accession number	
Haplotype	341	481	487	493	1074–1077	*psb*A-*mat*K	*trn*S-*trn*T
H1	G	C	T	C	—	LC081321	LC081332
H2	T	T	G	T	—	LC081327	LC081332
H3	T	T	G	T	ATAG del	LC081328	LC081337

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Yamada et al. 2005). As a result, the identification of different *Lolium* species is difficult (Terrell 1968), and this may be the reason why the lineages cannot be separated by cpDNA sequences.

Considered by region, the proportion of haplotypes varies depending on the populations. At the moment, whether these differences are due to a limited number of samples or potential differences is not clear. Moreover, among all the populations, only the NNI coastal population in the Kyushu region was found to contain a unique haplotype (H3) (Figure 1). Because rare haplotypes may not be detected when the sample size is small (i.e., 8 samples per population), an uncommon haplotype in a seaport population could have become a major haplotype in the NNI coastal population as a result of genetic drift.

Comparison of Genetic Diversity before and after Expansion. In the present study, genetic diversity was high throughout all populations (Table 1). The mean expected heterozygosity of *Lolium* species in this study was similar to that in a previous study conducted in Japan (0.6015) (Kurokawa et al. 2010) and another outside Japan (0.62 to 0.67) (Peter-Schmid et al. 2008) using nuclear microsatellite loci.

No significant loss in genetic diversity was observed in sandy coastal populations compared with seaport populations. The allelic richness (A_r) and observed mean heterozygosity (H_o) in coastal populations were slightly greater than those in seaport populations, except in the Kanto region (Table 1). In the Kanto region, these diversity indices decreased in the coastal population (KSI) compared with the seaport population (KSPI), although the small size of the KSI population may have influenced this result. While these results indicate that there were no severe bottlenecks during the secondary invasion, the lower variation in coastal than in seaport populations found by AMOVA suggests that some alleles in each seaport population were lost by genetic drift during the expansions into the sandy coasts (Table 2). Studies comparing native and introduced populations have demonstrated that genetic diversity is not always reduced on invasion (Genton et al. 2005; Lavergne and Molofsky 2007; Shirk et al. 2014) and can actually increase when there is a high level of genetic variation in the source populations and several independent transportation and release events (Dlugosch and Parker 2008; Lockwood et al. 2013). Parallel arguments also apply to secondary invasions (Leger et al. 2009). Because the results in Structure showed high dissimilarity of sandy coastal populations from cropland populations (Figure 3), it is unlikely that gene flow from cropland to sandy coast occurs frequently. Therefore, we believe that sequential gene flow from seaport populations may mitigate bottlenecks in sandy coastal populations and assist in the colonization of new habitats.

Implications for the Process of Secondary Invasion. According to Dietz and Edwards (2006), alien plants first colonize habitats where preadaptation or propagule pressure (e.g., anthropogenic transport) act as a prominent factor, and thereafter usually invade habitats where species' traits (e.g., tolerance to environmental stress) are more important. In the case of *Lolium* species, croplands or roadsides near seaports would be the habitats initially colonized, and this would be followed by expansion into sandy coasts. Only the seaport lineages were successful invaders of these coastal areas. Documentary evidence shows that the introduction of commercial wheat grain into Japan began in the 1940s and became common in the 1960s (Yokoyama 2005), while the intentional introduction of *Lolium* species into croplands started at the beginning of the Meiji era (1868–1912), with the first specimen being collected in 1880 (Shimizu 2003). Despite cropland *Lolium* lineages establishing widely inland after being introduced at least 60 yr earlier than the seaport populations, the cropland genotypes have not invaded the sandy coasts. This suggests that the ecological differences between lineages may have affected their ability to become established in coastal environments.

Selective forces from the past, including domestication and naturalization, might explain differences between the two introduced lineages in their capacity for colonization. *Lolium* cultivars have been used by humans for a long time (Balfourier et al. 2000; Terrell 1968), and particular characteristics relating to their palatability and utility as forage, such as high yield, rich nutritional content, digestible leaves, and resilience to grazing, have been fostered agriculturally (Humphreys et al. 2010; Sampoux et al. 2011). Thus, human selection of those desirable characteristics in a fodder crop in cropland *Lolium* populations could have made them less fit to be potential invaders of the harsh sandy coastal environments.

The seaport lineages originate from naturalized weeds growing in agricultural fields abroad. Japan imports 99% of its wheat from the United States, Canada, and Australia (Ministry of Finance, Japan 2014). According to Shimono et al. (2015), Australian wheat contains greater than 30-fold the quantity of *Lolium* seeds compared with the same amount of wheat from other countries. Therefore, more seeds derived from Australian wheat fields will spill out around seaports. In addition, following the taxonomy of Terrell (1968), the morphology of the seaport lineage is similar to that of *L. rigidum* var. *rigidum*, whereas the cropland lineage suggested by PCA is more similar to *L. multiflorum*, although variations in their morphology are continuous. The morphology of the seaport lineages also resembled the *Lolium* species grown in Australia (Murrumbidgee Catchment Management Authorities, NSW, Australia 2008). In Australia, these *Lolium* species are becoming one of the most problematic weeds in wheat-dominated agricultural fields (Owen et al. 2014), partly because they

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can grow in harsh environments characterized by high soil-salt concentration or extreme drought (Chauhan et al. 2006; Rengasamy 2006). These environments would foster strong abiotic tolerance in *Lolium* species, and stress-tolerant traits in the seaport lineage could well assist its expansion into the sandy coasts of Japan.

Finally, although the genetic composition of seaport and sandy coastal populations was almost identical (Figure 3), several morphological traits differed significantly between the populations (Table S1). Because microsatellite loci are selectively neutral, these morphological differences probably suggest a rapid transition in traits during expansion. However, the possibility that the observed morphological differences result from phenotypic plasticity caused by environmental differences between the habitats cannot be excluded. Therefore, whether these characteristics directly reflect their ecological functions is not certain. Further study is necessary to conclude whether rapid evolution has taken place during *Lolium* species' expansion into sandy coasts.

In summary, we found that the seaport colonizers, not the cropland lineage, appear to be responsible for invading the sandy coasts. These coastal populations could be derived from preadapted Australian *L. rigidum* weedy biotypes tolerant of drought, sandy soils, and salinity that were imported accidentally as grain contaminants, suggesting the importance of the preadaptation to the past selective forces on invasion to new habitats.

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Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/inp.2017.1

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