Initial invasion of glyphosate-resistant *Amaranthus palmeri* around grain-import ports in Japan

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Societal Impact Statement
The dispersal of alien species is tightly coupled to human activities such as trade and transport. Trade is known to spread troublesome weeds from countries exporting, to those importing, grain. Glyphosate resistant (GR) *Amaranthus palmeri* is one of the most problematic weeds in the US, which is the largest grain exporter to Japan. We demonstrate that GR *A. palmeri* has become established in a Japanese port in less than 10 years from the first report of GR *A. palmeri* in the US. The initial detection of alien species is critical to enable effective control measures to be undertaken, before problematic species are able to spread more widely.

Summary
- The US is the largest source to Japan of crops genetically modified to be glyphosate resistant (GR). The intensive use of glyphosate in the US has led to the evolution of GR *Amaranthus palmeri*, one of the most problematic weeds in the US. Here, we investigated the initial invasion and establishment of GR *A. palmeri* at grain-importing ports in Japan.
- The primary glyphosate resistance mechanism is a copy-number amplification of the 297-kb region containing the herbicide target site gene *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*). We used quantitative PCR to measure the *EPSPS* genomic copy number and used PCR to confirm the presence of the other amplified region. We used microsatellite marker analysis to compare the genetic similarities between Japanese populations and US accessions.
- We detected GR *A. palmeri* at three ports: although present as a casual plant at two of the three ports, GR populations were established at one of the ports investigated. The port populations were found to be genetically similar to the US accessions and showed no geographical genetic structure.
- This study shows that GR *A. palmeri* has naturalized in Japan in less than 10 years from the first report of GR *A. palmeri* in the US.

Keywords
- gene amplification, glyphosate-resistant, herbicide, introduced species, invasion, Palmer amaranth, seed contaminant
The land for global production of genetically modified (GM) crops increased from 1.7 million hectares to over 191.7 million hectares between 1996 and 2018. The US continues to be the largest producer of GM crops in the world (ISAAA, 2018). More than 80% of the area planted to GM crops has been planted with herbicide-resistant crops including stacked herbicide/insect-resistant crops (ISAAA, 2018). Among the herbicide-resistant crops, the glyphosate-resistant (GR) crops have been the most widely cultivated for the last 20 years (Bonny, 2016; Duke & Powles, 2008). The intensive use of glyphosate has resulted in the evolution of resistance to this herbicide in several problematic weeds (Heap & Duke, 2018). To date, resistance to glyphosate has been documented in 48 species (Heap, 2020).

Currently, one of the most problematic weeds is *Amaranthus palmeri* (Amaranthaceae), which has become a major GR weed of the US (Webster & Nichols, 2012). After the first report in 2004 in Georgia, US (Culpepper et al., 2006), GR *A. palmeri* was discovered in 29 states by 2018 (Heap, 2020). Despite the species never appearing as a problem until the early 1990s, *A. palmeri* is now ranked as the most troublesome weed among broadleaf crops in the US (Van Wychen, 2016) and the third most troublesome weed among graminaceous crops (Van Wychen, 2017). In particular, *A. palmeri* is listed among the most troublesome weeds of corn, sorghum, soybean, cotton, and peanuts. For corn, soybean, and cotton, herbicide-tolerant varieties account for more than 90% of the crops in the US (USDA, 2019). Exports from the US are the largest source of these crops to Japan. In 2017, Japan imported approximately 15.3, 3.2, and 0.1 million tonnes of corn, soybean, and cottonseed, respectively (Ministry of Finance, 2017), of which 78, 73, and 52%, respectively, was from the US (Ministry of Finance, 2017).

*Amaranthus palmeri* is an annual dioecious forb native to the area encompassing north-western Mexico and the south-western US (Ward, Webster, & Steckel, 2013). In the absence of competition, seed production for *A. palmeri* is over 600,000 seeds per female plant (Keeley, Carter, & Thullen, 1987). This species was reported in Japan for the first time in 1936 (Osada, 1972) and has spread throughout Japan except Hokkaido Prefecture after the 1960s according to records of herbarium specimens (GBIF; www.gbif.org). For example, the first records of the species are dated 1964 in Fukuoka Prefecture, southern Japan (Osada, 1972), and 1968 in Ibaraki and Chiba Prefectures, east-central Japan (Suzuki, 1981; Flora-Kanagawa Association, 2018). In Japan, the species has been relatively rare until now and there has been no report of any herbicide resistance or agricultural damage. However, because the weed has become prevalent in US croplands in recent years, GR *A. palmeri* introduction into Japan as a contaminant of imported GM commodities is inevitable.

Internationally traded grain commodities are recognized as a pathway for the introduction of weed seeds into new areas (Lehan, Murphy, Thorburn, & Bradley, 2013) because similarities in shape and size to crop seeds hinder removal of contaminant weed seeds (Michael, Owen, & Powles, 2010). Indeed, the seeds of major weeds in grain-exporting countries are found as contaminants in imported grain commodities (Asai, Kurokawa, Shimizu, & Enomoto, 2007; Norsworthy, Smith, Steckel, & Koger, 2009; Shimono & Konuma, 2008; Wilson, Castro, Thurston, & Sissons, 2016). The contaminants sometimes include herbicide-resistant seeds, which can later spill during the transport of grain commodities and become naturalized in importing countries (Shimono, Shimono, Oguma, Konuma, & Tominaga, 2015). In Japan, GM oilseed rape and GM soybean with glyphosate transgenes have been found at several major ports (Aono et al., 2006; MAFF, 2018; Saji et al., 2005). Because GM plants have not been commercially cultivated in Japan, their feral occurrence is evidence for spillage during transport of grain commodities (Saji et al., 2005). The recent appearance of GR *A. palmeri* in Brazil suggests the potential for intercontinental spread of GR individuals, mediated by seed transfer (Küpper et al., 2017).

In Japan, even though *A. palmeri* has been rare until now, GR *A. palmeri* has the potential to become a troublesome weed because glyphosate is one of the most widely used herbicides. Another concern is interspecific hybridization within the *Amaranthus* genus. In fact, the GR gene was transferred from *A. palmeri* to *A. spinousus* by pollen flow in the US (Gaines et al., 2012; Nandula et al., 2014). In Japan, plants of the *Amaranthus* genus (*A. spinousus, A. patulus, A. retroflexus* etc.) have been common problematic weeds of roadsides, cultivated ground, docks, and riverbanks (Ecological Society of Japan, 2002). These ruderal populations in roadside habitats may serve as conduits for further inter-population spread of the GR gene.

The copy-number amplification of the herbicide target site gene 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is the primary mechanism that confers glyphosate resistance to *A. palmeri*. The genomes of resistant plants contain from 5- to more than 100-fold more copies of the EPSPS gene than the genomes of susceptible plants (Gaines et al., 2010). The amplified region comprises not only the EPSPS locus but also genomic sequences corresponding to 71 putative genes, tandem repeats, and regulatory elements (Molin, Wright, Lawton-Rauh, & Saski, 2017). The “EPSPS cassette” is a 297 kb region and is found as extrachromosomal circular DNA (eccDNA) (Koo et al., 2018). The eccDNA in GR populations of *A. palmeri* from geographically distant locations within the US showed high sequence similarity with no structural variation and few single nucleotide polymorphisms (SNPs), supporting the hypothesis of a single origin of the EPSPS cassette in *A. palmeri* that then rapidly spread across the US (Gaines, Patterson, & Neve, 2019; Molin et al., 2018). On the other hand, Küpper et al. (2018) found distinct population genetic structure between GR *A. palmeri* populations from Georgia and Tennessee based on genome wide SNP analysis, suggesting multiple origins of GR populations. However, Gaines et al. (2019) speculated that the behavior of the eccDNA could be different from those of nuclear genomes because the eccDNA is not integrated with the genome. It would be unlikely for nearly identical EPSPS cassettes to independently evolve in any two individuals.

The EPSPS cassette can be a marker to detect initial plant invasions that occur through accidental introduction. Such a detection system is critical because there are substantial time lags between first arrival and subsequent spread of problematic alien species (Essl...
et al., 2011), during which time effective mitigation could be done. In this study, to elucidate the initial invasion of GR A. palmeri to Japan, we investigated whether GR A. palmeri was established in major grain-importing ports.

2 | MATERIALS AND METHODS

2.1 | Sampling of local populations in ports and plant materials

We visited 14 major Japanese ports of entry of international commodities in August–September of 2014 to 2017 (Figure 1) and censused mainly roadsides of about 10 km² around each port. At five of those ports, A. palmeri was growing. At Kashima Port, more than 10,000 individuals were growing thickly along 1.5 km of roadside and center divider. We randomly collected leaves from approximately 40–60 individuals per year at 2 points (Kashima 1 and 2) for 4 years (Table 1, Figure 1). Kashima 1 was near a grain-loading site, and Kashima 2 was approximately 500 m from Kashima 1. At the other ports, we collected leaves from all 1–7 individuals detected (Table 1, Figure 1).

Leaves of 13 individuals growing at an agricultural site in Kitsuki, Oita Prefecture, were provided by the Oita Prefectural Agriculture, Forestry and Fisheries Research Center, in 2018. A total of 433 samples were collected and stored at -20°C until DNA extraction.

Two accessions of A. palmeri seeds originated from the US (Ames 15,298: Arizona and PI 607,455: Kansas) and 1 accession from Mexico (PI 633,593: Colima) were provided by the United States Department of Agriculture-Agricultural Research Service (USDA-ARS). The Institute of Plant Science and Resources of Okayama University provided 1 accession (15682S) collected in 2003 in Okayama, Japan. Ten to fifteen seeds per accession were germinated and grown in pots containing commercial nursery soil. At the 2-3 leaf stage, the DNA was extracted from seedlings.

2.2 | DNA extraction

Total DNA was extracted from leaves by using a modified hexadezyltrimethyl ammonium bromide (CTAB) method (Murray & Thompson, 1980). The samples were ground to a fine powder and mixed with 800 µl of CTAB extraction buffer and incubated at 60°C for 20 min. Chloroform, 200 µl, was added and emulsified by shaking. The mixture was centrifuged at 10,000 g for 10 min, and the aqueous phase was collected. The DNA was then precipitated by adding two-thirds of a volume of isopropanol and washing once with 70% ethanol. The DNA was dried and suspended in 100 µl of TE.

2.3 | Quantitative PCR

Glyphosate resistance in A. palmeri is conferred by copy-number amplification of the herbicide target site gene 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Quantitative real-time PCR was used to measure the EPSPS genomic copy number relative to the acetolactate synthase (ALS) gene following the method of Gaines et al. (2010). The relative EPSPS copy number and glyphosate resistance are reported to be strongly associated and a threshold EPSPS copy number value for GR plants were set to 5 (Gaines et al., 2010). Primer efficiency curves were drawn for each primer set by using a 1x, 1/2x, 1/4x, 1/8x, and 1/16 × dilution series of susceptible genomic DNA. The primer sets EPSF1 and EPSR8 (195-bp product) for the EPSPS gene and ALSF2 and ALSR2 (118-bp product) for the ALS gene (Gaines et al., 2010) were used for quantitative PCR on genomic DNA.

The genomic DNA templates (10–50 ng) were run with each primer set in triplicate in 20 µl reaction volumes in the SYBR® Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan). The ABI Prism 7,000 Real-Time PCR Detection System (Thermo Fisher Scientific, Pittsburg, PA, US) was run with the following thermal profile: 2 min at 95°C; 40 cycles of 98°C for 10 s, 60°C for 10 s, and 68°C for 30 s; and finally a melt-curve analysis to check for primer-dimers.

2.4 | PCR analysis of EPSPS cassette

In a previous study, the length of the EPSPS amplicon was extended to 297 kb and termed the “EPSPS cassette” (Molin et al., 2017). The cassette comprises not only the EPSPS locus (10 kb) but also genomic sequences corresponding to 71 putative genes, tandem repeats, and regulatory elements, and 36% of the cassette region is estimated to be absent in susceptible plants (Molin et al., 2017). To clarify whether the entire EPSPS cassette was in resistant individuals, eight primer sets (AW550 × AW553, AW502 × AW293, AW426 × AW511, AW176 × AW211, AW301 × AW168, AW524 × AW129, AW156 × AW33, AW544 × AW259), which were designed to amplify regions of the EPSPS cassette (Molin et al., 2018), were chosen to cover the overall EPSPS cassette. The PCR was performed in a total volume of 10 µl, containing each primer at 0.1 µM, each dNTP at 0.2 mM, 0.2 U of KOD Plus Neo (TOYOBO), 1 × PCR buffer, and approximately 10–50 ng of template DNA. The PCR cycle conditions were 94°C for 2 min; 30 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 90 s; and a final extension step at 68°C for 5 min. The PCR products were detected by agarose gel electrophoresis. For the Kashima population, the eight primer sets were amplified for all individuals collected in 2017. Five primer sets (AW550 × AW553, AW502 × AW293, AW426 × AW511, AW301 × AW168, AW544 × AW259) were amplified for individuals collected in other years.

2.5 | Microsatellite marker analysis

Whole genome sequencing data for A. palmeri were downloaded from the Sequence Read Archive (accession number SRR5012825)
at NCBI (http://www.ncbi.nlm.nih.gov/sra). The reads were quality-trimmed and assembled de novo with CLC Genomics Workbench ver. 10.1.1 (Qiagen, Aarhus, Denmark) using default parameters to construct the contigs. All the contig sequences were used as input for the CD-HIT-EST, MISA, ipress, and BlastCLUST pipeline (Ueno et al., 2012) to obtain PCR primers for amplifying unique microsatellite sequences with the number of repeat units ≥ 9, 8, 7, 6, and 5 for di-, tri-, tetra-, penta-, and hexa-simple sequence repeats (SSRs) respectively. The contigs for which primer pairs were successfully designed were BLASTed against the NCBI nr database with an e-value of $1 \times 10^{-5}$. We selected 48 primer pairs for SSRs with the number of repeat units ≥ 10 and BLAST hits, and the primers with tail sequences for forward primers (Blacket, Robin, Good, Lee, & Miller, 2012) and reverse primers with a “PIG-tailing” modification (Brownstein, Carpten, & Smith, 1996) were synthesized by Eurofins Genomics. Of the 48 primer pairs, 10 were successfully amplified in the multiplex PCR and exhibited polymorphisms.

The PCR was performed in a 6-µl reaction volume containing $1 \times$ Multiplex PCR master mix (Qiagen), primer mix, and 10–50 ng of template DNA. The primer mix for each marker in the multiplex PCR contained 0.1 µM universal fluorescent primer, 0.1 µM tailed forward primer, and 0.2 µM reverse primer. The PCR cycle conditions were 94°C for 15 min; 30 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s; and a final extension step at 72°C for 7 min. The PCR products were analysed in a 3,500 Genetic Analyzer with GeneMapper software (Thermo Fisher Scientific). Samples that were genotyped at more than 9 of the 10 microsatellite loci were used for analysis.

Although A. palmeri were growing at five ports, less than 10 individuals were detected at all ports except Kashima. Therefore, genetic diversity parameters were estimated for the Kashima populations by using GenAlEx 6.5 (Peakall & Smouse, 2012). The number of alleles per locus ($A$), observed heterozygosity ($H_o$), expected heterozygosity ($H_e$), and the fixation index ($F_{IS}$) were calculated. The significance of the deviation from Hardy–Weinberg equilibrium (HWE) within each locus, as evidenced by the deviations of $F_{IS}$ from zero, was tested by 10,000 randomizations of alleles among individuals within the population, with Bonferroni correction. These calculations were performed with the software FSTAT 2.9.3.2 (Goudet, 1995). The null allele frequencies for each locus were estimated using the software FREENA (Chapuis & Estoup, 2007). Principal coordinates analysis (PCoA) was conducted

### Table 1

**EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene-amplified individuals detected at five ports in Japan and Gene bank accessions of *Amaranthus palmeri*. Number of investigated individuals (N), number of EPSPS gene-amplified individuals (GA), number of EPSPS cassette-present individuals (CP), and number of microsatellite analysed individuals (Nssr)**

| ID          | Origin          | Sampling date | N   | GA  | CP  | Nssr |
|-------------|-----------------|---------------|-----|-----|-----|------|
| **Individuals at Japanese ports** |                 |               |     |     |     |      |
| Kashima 1   | Ibaraki, Japan  | August 2014   | 57  | 7 (12%) | 15 (26%) |    |
|             |                 | August 2015   | 55  | 6 (11%) | 14 (25%) |    |
|             |                 | August 2016   | 49  | 8 (16%) | 12 (24%) |    |
|             |                 | August 2017   | 58  | 10 (17%) | 17 (29%) |   26 |
| Kashima 2   | Ibaraki, Japan  | August 2014   | 36  | 0   | 0   |      |
|             |                 | August 2015   | 50  | 0   | 0   |      |
|             |                 | August 2016   | 47  | 2 (4%) | 4 (9%)  |    |
|             |                 | August 2017   | 50  | 1 (2%) | 2 (2%)  | 28   |
| Nagoya      | Aichi, Japan    | September 2014| 4   | 0   | 0   |      |
|             |                 | September 2016| 7   | 0   | 0   |      |
| Himezi      | Hyogo, Japan    | September 2016| 1   | 0   | 0   |      |
| Mizushima   | Okayama, Japan  | September 2016| 2   | 1 (50%) | 1 (50%) | 1    |
| Hakata      | Fukuoka, Japan  | September 2014| 1   | 1 (100%) | 1 (100%) |    |
|             |                 | September 2015| 2   | 2 (100%) | 2 (100%) |    |
|             |                 | September 2016| 1   | 1 (100%) | 1 (100%) |    |
| **Individuals at Japanese rural area** |                 |               |     |     |     |      |
| Kitsuki     | Oita, Japan     | September 2018| 13  | 0   | 0   | 13   |
| **Gene bank accessions** |                 |               |     |     |     |      |
| 156825      | Okayama, Japan  | December 2003 | 7   | 0   | 0   | 7    |
| Ames15298   | Arizona, USA    | June 2014<sup>a</sup> | 8  | 0   | 0   | 8    |
| PI607455    | Kansas, USA     | June 2014<sup>a</sup> | 12 | 0   | 0   | 12   |
| PI633593    | Colima, Mexico  | June 2014<sup>a</sup> | 8  | 0   | 0   | 8    |

<sup>a</sup>Date received.
based on the pairwise genetic distance (Smouse & Peakall, 1999) by using the program GenAlEx version 6.5 (Peakall & Smouse, 2012).

3 | RESULTS

3.1 | Distribution of GR individuals

During 2014–2017, Amaranthus palmeri was distributed at 5 of the 14 ports investigated, and EPSPS gene-amplified individuals, which were assumed to be glyphosate resistant, were detected at three of the ports: Kashima, Hakata, and Mizushima (Figure 1). More than 10,000 individuals covered approximately 1.5 km of roadside at Kashima. The frequency of resistance was 12, 11, 16, and 17% at Kashima 1 in 2014, 2015, 2016, and 2017 respectively (Figure 1, Table 1). No resistant individuals were detected at Kashima 2 in 2014 and 2015, but 2 and 1 resistant plants were detected in 2016 and 2017 respectively. Although only a few individuals were growing at Hakata (2014; 1, 2015; 2, and 2016; 1), all of them were resistant. One of two individuals was resistant at Mizushima in 2016. At the three ports, compared with susceptible individuals, the EPSPS gene was amplified in resistant individuals from 5- to > 100-fold (Figure 2). For some individuals without EPSPS gene amplification, the regions of the EPSPS cassette were also detected. The frequencies of individuals with the EPSPS cassette were 26, 25, 24, and 29% at Kashima 1 in 2014, 2015, 2016, and 2017 respectively (Figure 1, Table 1).

3.2 | Genetic variation

We genotyped 112 A. palmeri individuals at 10 microsatellite loci (Table 1). No individuals had identical multilocus genotypes, indicating that sexual reproduction was dominant. Estimated genetic parameters were based on the genotypes of the 54 analysed individuals of the Kashima population (Table 2): the number of alleles per locus (Na) was 4–15, the observed heterozygosity (Ho) was 0.278–0.796, and the expected heterozygosity (He) was 0.453–0.905 (Table 2). Fixation index values (F<sub>IS</sub>), which measure deviation from Hardy–Weinberg equilibrium, deviated from 0 for 6 of the loci (Table 2). The frequencies of null alleles for these loci were estimated to be relatively high (Table 2).

The results of PCoA showed genetic similarity among individuals from Kashima 1 and 2, Mizushima, and Hakata, where EPSPS gene amplifications were detected (Figure 3). Except for the samples of...
### TABLE 2
Locus name, primer sequence, repeat motif, allele size range, number of alleles observed (Na), observed heterozygosity (H₀), expected heterozygosity (Hₑ), fixation index (Fis), and frequencies of the null allele (Null) for 10 microsatellite loci in *Amaranthus palmeri*. Fifty-four individuals of the Kashima population were analysed.

| Locus | Primer sequence (5ʹ−3ʹ) | Repeat | Size (bp) | Na | H₀   | Hₑ   | Fis | Null |
|-------|-------------------------|--------|-----------|----|------|------|-----|------|
| Po06  | F: GCCTCCCTCGCCCAAGTAAAGGATTTGCCGCTTTG | (AAC)₁₂ | 230–254   | 7  | 0.722 | 0.743 | 0.028 | 0.009 |
|       | R: GTTTCTTCTGCTTTGGGATTTGTGACG       |         |           |    |       |       |     |      |
| Po07  | F: GCCTCCCTCGCCACGAATTCCGAGATTTTCCG  | (TAT)₁₃ | 281–320   | 14 | 0.722 | 0.879 | 0.178* | 0.070 |
|       | R: GTTTCTTCTTAAAGGCAACCTCCGGAGC       |         |           |    |       |       |     |      |
| Po13  | F: GCCTTGACGACCGCTCGAGGCGAGACACACCACAAC | (AAT)₂₀ | 161–200   | 10 | 0.574 | 0.848 | 0.323*** | 0.146 |
|       | R: GTTTCTTCCCTCTTCTCCGATTTACACAGAC    |         |           |    |       |       |     |      |
| Po14  | F: GCCTTGACGAGCCGCTAGTGCGAGCTCCTCAAAC | (TAA)₁₀ | 219–230   | 5  | 0.648 | 0.739 | 0.123 | 0.057 |
|       | R: GTTTCTTGGGATTTGAGGAGAGATGGATGCAC   |         |           |    |       |       |     |      |
| Po19  | F: GCCTTGACGACCGCTACGAGCCTTCTGCTCCAC | (CT)₁₄  | 268–293   | 14 | 0.796 | 0.905 | 0.120 | 0.057 |
|       | R: GTTTCTTACGCTTAAATAGCCGACCTCCTTG    |         |           |    |       |       |     |      |
| Po24  | F: GCCTTGACGACCGGCAAGCTAGGTAGAGAGCAG | (TG)₁₀  | 293–301   | 4  | 0.516 | 0.538 | 0.036 | 0.000 |
|       | R: GTTTCTTGGGAGGAGATGGGAGAGAGACAC    |         |           |    |       |       |     |      |
| Po27  | F: CGAACAGAAGGCTCAGGACCGAGATTTTCGAGGGATGGATGGG       | (TG)₁₀  | 248–256   | 7  | 0.278 | 0.453 | 0.387** | 0.140 |
|       | R: GTTTCTTACCAGCATCATCAGAAGCAACCTGCG   |         |           |    |       |       |     |      |
| Po28  | F: CGGAGACGGGTGCTTGTCGAGGAGGCTTTCAAGTCAG | (TC)₁₅  | 238–267   | 12 | 0.389 | 0.863 | 0.549*** | 0.254 |
|       | R: GTTTCTTCAATGCGCTTGGGCATTTG          |         |           |    |       |       |     |      |
| Po37  | F: CGGAGACGACGAGAGGTTGCTGGAGAATTTGCTGACATCAG | (AC)₁₆  | 198–212   | 8  | 0.352 | 0.788 | 0.554*** | 0.236 |
|       | R: GTTTCTTGGATTTGCTGGACGGATGTCG       |         |           |    |       |       |     |      |
| Po38  | F: CGGAGACGGGTGAAGGTGAGTTGCGGGTCCCCCTCCCATTTACC  | (AG)₁₂  | 223–237   | 5  | 0.370 | 0.741 | 0.500*** | 0.207 |
|       | R: GTTTCTTACGAGGTATGTGAGCTTGGCG       |         |           |    |       |       |     |      |

* *, **, ***: Denotes significant departure from Hardy-Weinberg equilibrium at P < .01, < .001, < .0001.
the Mexican and Okayama gene bank accession, other individuals loosely clustered with overlap and were continuously distributed with the Kashima population; thus, there were no genetic discontinuities by geographical distance (Figure 3). The port individuals were genetically similar to the samples of the US accessions and the Japanese rural area (Figure 3). By contrast, the samples of the Japanese Okayama accession collected in 2003 and the Mexican accession were in separate cluster (Figure 3).

4 DISCUSSION

In this study, A. palmeri resistant to glyphosate were detected at Kashima, Hakata, and Mizushima ports (Figure 1). To date no GR A. palmeri has been found in any Japanese agricultural land, and the independent evolution of the same EPSPS cassette in multiple Japanese ports seems difficult and unlikely. These populations were most likely introduced via contamination in internationally traded grain commodities. This hypothesis is supported by the PCoA (Figure 3), which shows that the individuals collected from ports were genetically similar to the US accessions, with no genetic isolation by distance among Japanese and US individuals. However, the port populations were also genetically similar to individuals collected in the Japanese rural area. To exclude the possibility of independent evolution of GR in Japan, further study is needed, such as comparison of the genome sequence of the EPSPS cassette between Japanese and US GR individuals.

Glyphosate-resistant GM soybeans were detected at Kashima, Hakata, and Tomakomai ports in a survey on GM plants by the Ministry of Agriculture, Forestry and Fisheries (MAFF, 2018). We did not investigate the Tomakomai port in Hokkaido in northern Japan because A. palmeri was not reported in Hokkaido Prefecture (Hokkaido, 2010). At Hakata, GM soybeans were detected in eight consecutive years from 2010. Soybean is a highly domesticated crop and has lost some characteristics typical of weedy species (OECD, 2000). Therefore, because soybean cannot self-sustain without human interference, the detected GM soybeans were derived from spillage during transport of grain commodity rather than from seed produced by established plants. Thus, with the detection of GM GR soybeans at these ports, the evidence is strong that a species such as A. palmeri could be accidentally introduced as a seed contaminant with relatively high propagule pressure. Another study detected approximately 1,700 contaminant seeds in 10 kg of wheat imported from Canada to Japan (Shimono & Konuma, 2008).

In addition to Kashima, a few resistant individuals were growing at Hakata and Mizushima. In these areas, A. palmeri might be a casual plant. These alien plants may flourish and even reproduce occasionally in an area, but not form self-replacing populations. However, even if A. palmeri is currently a casual plant at these two ports, establishment in the near future is possible due to the high propagule pressure.

At Kashima, more than 10,000 individuals were growing, and the frequencies of individuals at Kashima 1 in which the EPSPS gene was amplified were stable (11%-17%) during 2014–2017. These GR individuals most likely succeeded in reproducing and sustaining populations without herbicide treatment. However at Kashima 2, the frequencies of resistant individuals was lower drastically than that of Kashima 1, despite this site being only 500 m away from Kashima 1. The distribution of EPSPS gene-amplified individuals may reflect the spillage pattern during transport of a grain commodity. The EPSPS gene did not diffuse and homogenize quickly during 4 years, despite the species being wind-pollinated. A similar pattern in ALS herbicide-resistant individuals may reflect the spillage pattern during transport of a grain commodity. The EPSPS gene did not diffuse and homogenize quickly during 4 years, despite the species being wind-pollinated. A similar pattern in ALS herbicide-resistant Lolium sp., a wind-pollinated outcrossing species, was detected in Kashima Port, and the frequencies of resistance differed significantly among sites within 1 km of Kashima Port (Shimono et al., 2015). A field study of pollen flow for A. palmeri demonstrated that the proportion of GR progeny decreased with increased distance from the pollen source; approximately 30% to 40% of the offspring were resistant at 100 to 200 m, whereas 20% of the offspring were resistant at 300 m (Sosnoskie et al., 2012). Thus, the probability of pollen dispersal generally decreases rapidly with distance from pollen sources and declines exponentially on a local scale, although the distance of pollen movement is affected by the aerodynamics of the pollen grain, the local atmospheric conditions, and the population density of the source plants.

The persistence of resistant individuals will highly depend on the fitness cost associated with the resistance, although attempts to determine the effect of EPSPS gene amplification on plant fitness traits are likely to be inconclusive (Vila-Aiub, Yu, & Powles, 2019). The amazing increase in EPSPS gene copy number and thus, the overproduction of EPSPS protein must incur additional energy and material expenses that should translate into a plant fitness cost in the absence of glyphosate selection (Vila-Aiub et al., 2018). However, in the EPSPS gene-amplified individuals of A. palmeri, no negative effects on plant growth and reproductive fitness traits are observed (Giacomini, Westra, & Ward, 2014; Vila-Aiub et al., 2014). Therefore, once resistance evolves in a population, the frequency of the resistance gene is expected to remain relatively constant over time, even though herbicide selection is absent.
The frequencies of individuals with the EPSPS cassette were approximately 24%-29% at Kashima 1 for 4 years. Some of these individuals had the cassette without EPSPS gene amplification. The EPSPS cassette is carried by eccDNA, as described earlier. The eccDNA is tethered to chromosomes by a structural protein, which enables transmission from cell to cell during mitosis and the copy number is variable among cells (Koo et al., 2018). Therefore, to explain the individuals without EPSPS gene amplification, the DNA may have been extracted from a leaf with low EPSPS gene amplification. Further study is needed to clarify whether individuals that possess the cassette without gene amplification are glyphosate resistant.

Glyphosate-resistant A. palmeri was first reported in Georgia in 2004 (Culpepper et al., 2006) and is now widespread in the southern US. Less than 10 years from that first report, GR A. palmeri has become established in Japan. Thus, this study provides evidence that plant invasions are being accelerated by global trade. Because the proportion of species introduced accidentally is expected to increase through time as trade increases (Lehan et al., 2013), we must expedite the process for initial detection of potentially problematic species.

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AUTHOR CONTRIBUTIONS
AS designed the research, conducted field sampling, analyzed the data, and wrote the paper. HK and SN conducted field sampling and genetic experiments. SU developed the microsatellite markers and extensively edited the paper. JY provided the seed materials. MA conducted field sampling and provided conceptual advice.

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