Investigating the origins and evolution of a glyphosate-resistant weed invasion in South America

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Abstract

Amaranthus palmeri is a widespread glyphosate-resistant (GR) weed in the USA. Since 2015, GR populations of *A. palmeri* have been confirmed in South America, raising the prospect of an ongoing invasion. We used RAD-Seq genotyping to explore genetic differentiation amongst *A. palmeri* populations from Brazil, Argentina and Uruguay. We also quantified gene copy number amplification of the glyphosate target, 5-enolpyruvyl-3-shikimate phosphate synthase (*EPSPS*) and the presence of an extra-chromosomal circular DNA (eccDNA) replicon in these populations. Genetic analyses indicated that populations in Brazil, Argentina, and Uruguay were only weakly differentiated (pairwise FST ? 0.043) in comparison to USA populations. STRUCTURE analysis did, however, assign Argentinean populations to a discrete cluster to those from Brazil and Uruguay. Neither elevated *EPSPS* copy number, nor the eccDNA *EPSPS* replicon were present in Argentinean populations, this being consistent with recent observations of other GR mechanisms in Argentina, and an independent in situ evolution of glyphosate resistance. Elevated *EPSPS* copy number and the *EPSPS* replicon were identified in all populations from Brazil and Uruguay. The presence of this mechanism and the very high sequence similarity of the *EPSPS* replicon to that found in the USA are strongly suggestive of the recent invasion of GR into Brazil and Uruguay. Our results are consistent with a single introduction of *A. palmeri* into South America sometime before the 1980s, and subsequent local evolution of GR in Argentina but with a secondary invasion of GR *A. palmeri* from the USA into Brazil and Uruguay during the 2010's.

Introduction

Plant invasions to new habitats can be facilitated by seed movement in agricultural equipment and crop seeds. An agriculturally adaptive trait like herbicide resistance may facilitate the establishment success of weeds in new environments. To investigate the evolutionary history and invasion of a major agricultural weed into a new continent, we utilized population genomics tools for glyphosate-resistant *Amaranthus palmeri* (Palmer amaranth). This annual broadleaf species is native to the Sonoran Desert of the arid southwestern USA and Northern Mexico (Sauer, 1957) but has displayed a profound ability to adapt to colder and/or more humid climates. By 1915, *A. palmeri* is believed to have spread as far east in the USA as Virginia (Ward, Webster, & Steckel, 2013) and today can be found in 39 states (Briscoe Runquist, Lake, Tiffin, & Moeller, 2019). *A. palmeri* causes extensive yield loss and increases the cost of production for soybean (Klingaman & Oliver,

1994) and cotton (MacRae, Webster, Sosnoskie, Culpepper, & Kichler, 2013). In corn, *A. palmeri* can cause up to a 91% decrease in yield (Massinga, Currie, Horak, & Boyer Jr, 2001).

Being dioecious, the species exhibits a high degree of genetic variation (Küpper *et al* ., 2018), which has facilitated rapid adaptation in agricultural systems. Seeds may emerge from the seedbank throughout the growing season (Jha & Norsworthy, 2009) and subsequent rapid growth means that seedlings can surpass the recommended size range for herbicide treatment within 2-3 weeks of germination, meaning that frequent and timely herbicide applications may be required. Additionally, a single female of *A. palmeri* can produce upwards of 250,000 seeds (Sellers, Smeda, Johnson, Kendig, & Ellersieck, 2003) and male plants produce large quantities of low-density pollen that is slow to settle, resulting in gene flow of inherited traits such as herbicide resistance, up to at least 300 m from the male parent (Sosnoskie *et al* ., 2012). Other contributors to spatial migration of *A. palmeri* include movement on agricultural equipment, contamination of commercial crop seed (Oseland, Bish, Spinka, & Bradley, 2020), or dispersal by wildlife such as ducks and geese (Farmer, Webb, Pierce, & Bradley, 2017). The high fecundity and ease of dispersal of *A. palmeri* raise concerns about the evolution and geographic dispersal of herbicide resistance in this species.

A. palmeri has evolved resistance to eight different herbicide modes of action (Heap, 2020) and resistance to as many as five different modes of action have been observed within a single population (Kumar, Liu, Boyer, & Stahlman, 2019). Globally, glyphosate is the most widely used herbicide due in part to the introduction of glyphosate-resistant (GR) crops in the mid-1990's along with myriad uses in non-selective weed control; however, the evolution and dispersal of resistance in weeds is a major threat to the utility of glyphosate (Duke & Powles, 2008).

Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), a critical step in the synthesis of aromatic amino acids (Schönbrunn *et al* ., 2001). Glyphosate resistance in many *A. palmeri* populations is conferred by duplication of the *EPSPS*gene such that glyphosate treatment does not completely inhibit the over-expression of this enzyme (Gaines, Patterson, & Neve, 2019; Gaines*et al* ., 2010). Molin, Wright, Lawton-Rauh, and Saski (2017) assembled and sequenced BAC libraries from GR *A. palmeri* to investigate the *EPSPS* replication and flanking sequence, leading to the discovery that the *EPSPS* replicon is located within extra-chromosomal circular DNA (eccDNA) of over 400 kb (Molin, Yaguchi, Blenner, & Saski, 2020b) tethered to multiple chromosomes within the*A. palmeri* genome and transmissible at both mitosis and meiosis (Koo *et al* ., 2018). These eccDNA will be referred to here as the*EPSPS* replicon.

Portions of the *EPSPS* replicon have been used as markers to survey GR and glyphosate-susceptible (GS) populations of *A. palmeri* from six states in the USA, revealing that the *EPSPS*replicon was present in all GR populations and absent in all GS populations (Molin *et al* ., 2018). The ubiquity of this mechanism of resistance led to the hypothesis that GR populations in the USA shared a single origin and had subsequently migrated throughout the country, further supported by genomic resequencing of eccDNA from multiple GR populations showing very high similarity across the 400 kb replicon (Molin, Patterson, & Saski, 2020a). A population genomics approach using populations from distinct USA locations and genotyping by sequencing to compare relatedness of populations was unable to resolve whether independent glyphosate resistance evolution events occurred, showing divergence in population genetic structure between GR populations from Georgia and Tennessee (Küpper *et al* ., 2018).

A. palmeri was recorded as present in Argentina in La Pampa region in 1984 (Covas, 1984), possibly introduced as a contaminant of alfalfa seed (Covas, 1984; Michaud, Lehman, & Rumbaugh, 1988; Montoya, Garay, & Cervellini, 2015). GR *A. palmeri* was also reported in Brazil and Argentina in 2015 (Carvalho *et al* ., 2015; Heap, 2020). Kaundun *et al* . (2019) found that glyphosate resistance in a single *A. palmeri* population from Argentina was conferred by a proline 106 to serine mutation in the *EPSPS* gene, while Palma-Bautista *et al* . (2019) found a non-target-site glyphosate resistance mechanism in a different *A. palmeri* population from Argentina. These mechanisms have not been reported in *A. palmeri*from the USA, suggesting independent, local evolution of glyphosate resistance in Argentina. Sequencing of Argentinian *A. palmeri*populations indicated absence of an acetolactate synthase (ALS) target site mutation (Berger *et al* ., 2016) that was later characterized in populations from Brazil with multiple resistance to ALS herbicides and glyphosate (Küpper

et al ., 2017), establishing putative evidence for *A. palmeri* having independent introductions in Argentina and Brazil. *A. palmeri* was not recorded as present in Uruguay in a comprehensive weed survey conducted between 2005 and 2007 (Rios, Fernández, Collares, & García, 2007). Anecdotal evidence from the field suggests that GR *A. palmeri* was introduced on imported machinery from the USA between 2012 and 2015 in Uruguay (M. Alejandro Garcia *pers comm*) and in Brazil from 2011 and 2014 (Anderson Cavenaghi *pers comm*).

This study used RAD-seq genotyping (Baird *et al* ., 2008) analyses to compare patterns of genetic differentiation within and between populations of *A. palmeri* from Brazil, Argentina, Uruguay and the USA. We also conducted qPCR-based assays to measure *EPSPS* gene copy number and PCR assays to determine the presence of the *EPSPS*replicon in sampled populations. Together, these data were analyzed to infer if *A. palmeri* populations now present in three South American countries were likely recent introductions from the USA and whether there is evidence for a single introduction; multiple, independent introductions; or local evolution of glyphosate resistance in extant South American populations of the species.

Methods

Plant material

Leaf tissue was sampled from actively growing *A. palmeri* plants at field sites in Brazil (4 populations), Argentina (10 populations) and Uruguay (3 populations), where the species was known to be present. A population is defined as all plants collected at a discrete sampling location (Table 1). At each sampling location, a single newly emerged leaf was taken from up to 30 individual plants. Plants were selected to ensure that the geographical extent of the field populations was sampled at each location. Individual leaves were placed in sealable plastic bags and labelled with a population code and plant number. A small quantity of silica gel was placed inside each plastic bag to exclude moisture and bags were stored in darkness. After collection, all leaf material was shipped to the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil for sample processing and DNA extraction.

USA reference populations included KS-S, AZ-S, AZ-R, and AZ-S2 reported in Küpper *et al* . (2018); GA-R and GA-S reported in Culpepper*et al* . (2006); TN-R reported in Steckel, Main, Ellis, and Mueller (2008); NC-R reported in Culpepper, Whitaker, MacRae, and York (2008); and CO-R collected from 10 plants in a sugar beet field in 2015 in Colorado (40.14 N, -102.43 W). Plants were grown at Colorado State University from the collected seed and leaf tissue was sampled and immediately frozen in liquid nitrogen for DNA extraction.

DNA extraction

Samples were lyophilized and ground in the TissueLyser II (Qiagen, São Paulo, SP, Brazil). DNA isolation was performed following a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle & Doyle, 1987) and quantified on a Nanodrop spectrophotometer (Thermo Scientific) followed by normalization. DNA from the South American samples was lyophilized and shipped to Colorado State University for resuspension and quantification. DNA for the USA samples was extracted as described in Küpper *et al* . (2018). All samples were measured for DNA concentration using Qubit (Thermo Fisher Scientific) to normalize to 20 ng μL^{-1} in a total volume of 150 µL volume to provide 3 µg DNA for each sample. Samples were shipped to Floragenex in four 96-well plates with strip caps. Each plate contained 95 samples and one blank, for a total of 380 individual plant DNA samples.

RAD-seq genotyping SNP calling

RAD-sequencing was performed by Floragenex (Floragenex, Inc., Portland, OR, USA) using standard methodology (Slavov *et al* ., 2014). Libraries were created using the *PstI* restriction enzyme and all four plates were sequenced across all four runs of NextSeq 500 (Illumina, San Diego, CA, USA). A total of 347,799,399 good barcoded reads were generated, with each individual covered by an average of 905,728 reads.

The raw sequenced DNA reads were quality-checked and reviewed using FASTQC (Andrews, 2010). They were then used in the TASSEL-UNEAK v.3.0 network-based reference-free *de novo* SNP discovery pipeline (Lu*et al* ., 2013), following the published protocol (Glaubitz*et al* ., 2014) except where noted below.

Good reads with barcodes and cut site were demultiplexed, trimmed and truncated to 64bp as necessary, and then sorted into unique sequence tags by compiling exactly matching reads. Singleton or rare reads corresponding to 5 or fewer tags were discarded. Tag pairs were identified by pairwise alignment. Because one tag is usually involved in multiple tag pairs, a network filter was used to identify reciprocal tag pairs, using an error tolerance rate of 0.03 to discard repeats, paralogs and sequencing errors. Reciprocal tags pairs with 1bp mismatch were considered as SNPs. This leads to a HapMap file, providing a catalog of SNPs (haplotypes) by population sample, which was filtered to only retain SNPs with a minor allele frequency (MAF) of at least 0.025 and call rate of at least 80%. This resulted in a set of 4,659 SNPs which were used in all population genetic analyses.

RAD-seq data analysis

We used model-based clustering as implemented in the STRUCTURE programme (Falush, Stephens, & Pritchard, 2003; Falush, Stephens, & Pritchard, 2007; Pritchard, Stephens, & Donnelly, 2000) to detect genetic groups and attempt population assignment. The number of genetic groups (K) was varied between 1 and 10 and for each value of K. We ran the programme 10 times, with 1,000 burn-in and 10,000 data collection iterations. Runs were then summarized using CLUMPP (Jakobsson & Rosenberg, 2007) and plausible values of K were identified using the method of Evanno, Regnaut, and Goudet (2005) as implemented in STRUCTURE HARVESTER (Earl & vonHoldt, 2012). Results for these values were then illustrated using DISTRUCT (Rosenberg, 2004). To further assess the robustness of these results, we ran STRUCTURE assuming larger numbers of groups (up to K = 15) and after subsampling populations in Argentina, Brazil, and Uruguay, to avoid balances caused by unbalanced sampling (Meirmans, 2019). To quantify genetic differentiation between populations, we calculated pairwise F_{ST} values using the EIGENSOFT programme (Patterson, Price, & Reich, 2006) and an approach robust to the effects of rare alleles (Bhatia, Patterson, Sankararaman, & Price, 2013).

EPSPS copy number qPCR assay and EPSPS replicon specific marker PCR assay

DNA samples from Brazil, Uruguay, Argentina, and GA-R were used to measure *EPSPS* gene copy number and presence of the *EPSPS*replicon (Molin *et al* ., 2018). Relative *EPSPS* copy number was measured with 2X SYBRgreen master mix (Quantabio) using qPCR methods and primer sequences described by Gaines *et al* . (2010). Previously reported *EPSPS* cassette markers AW293xAW275, AW516xAW519, and AW216xAW541 (Molin *et al* ., 2018) are here referred to as the *EPSPS* replicon specific markers A (1757 bp), B (2352 bp), and C (1544 bp), respectively, while the qPCR primer set for the *EPSPS* gene from Gaines *et al* . (2010) was used as a positive control for amplification of the template DNA. The presence or absence of the three replicon markers in the purified *A. palmeri*DNA from Brazil, Uruguay, Argentina, and GA-R was used for a qualitative assessment of the *EPSPS* replicon in South America compared to the USA. 2X Econotaq master mix (Lucigen) was used along with the recommended cycling conditions of initial denaturing at 94 C for 4 min, followed by 30 cycles of 94 C for 30 s, annealing at 55 C for 30s, and an extension period of 72 C for 90s, and final extension at 72 C for 5 min.

Results

Population structure

Analyses of STRUCTURE results using the method of Evanno *et al* . (2005) strongly favored the assignment of three main genetic groups (K = 3). However, results for higher values of K were also informative and consistently revealed several patterns (Fig. 1). First, individuals from each South American country tended to cluster together, despite the fact that multiple populations were sampled in each country. Second, populations from Argentina consistently clustered in a separate group from those in Brazil and Uruguay, even when K = 3 was assumed for the entire data set. Populations from the latter two countries also clustered in

separate groups for higher values of K (K > 6). Finally, while identifying the exact geographic location of USA*A. palmeri* populations that were introduced to South America is not realistic, given the small number of USA populations we sampled, some sources (GA-R and NC-R for Brazil and Uruguay; CO-R and AZ-S for Argentina) appeared much more likely than others.

Pairwise F_{ST} values provided a further level of nuance to these patterns (Table 2). Levels of genetic differentiation between populations from the three South American countries were relatively low (pairwise F_{ST} [?] 0.043), suggesting that either gene flow between established populations is extensive or there was an introduction of *A. palmeri* to the continent from a common source.

A high degree of population structure was noted amongst the various sampled USA populations and the South American populations were less differentiated from populations from Arizona and Colorado than these populations were from the majority of other USA populations, suggesting that invasion may have occurred into South America from the south-western region of the USA.

EPSPS copy number qPCR assay and *EPSPS* replicon specific marker PCR assay

The GA-R population had high copy number of the *EPSPS* gene as expected (Table 3) and individuals from GA-S had the expected single copy of *EPSPS* . All three tested populations from each of Brazil and Uruguay had high *EPSPS* copy number (Table 3, fold increase of 56-103). The populations from Argentina had mean relative *EPSPS*copy number between one to two-fold higher than the reference (Table 3). The *EPSPS* replicon specific markers A, B, and C amplified in GA-R individuals but not in GA-S individuals (Table 3, Figure 2). Similar to GR populations in the USA, all three *EPSPS* replicon markers amplified in all three populations from each Brazil and Uruguay (Table 3, Figure 2). None of the *EPSPS* replicon specific markers amplified in any individual of the 10 populations from Argentina (Table 3, Figure 2), indicating that these populations do not contain the*EPSPS* replicon.

Discussion

Several converging agronomic factors have seen *A. palmeri* emerge as a major weed of cotton, corn and soybean production systems of the USA over the last 20-30 years (Ward *et al* ., 2013). Many of these agronomic trends, particularly the rapid and widespread adoption of glyphosate-tolerant crops, and the associated facilitation of more conservation tillage, have also been witnessed in cropping systems of South America, and it is notable that this has been accompanied by a recent increased incidence of *A. palmeri* in Argentina (Montoya*et al* ., 2015), Brazil (Gonçalves Netto *et al* ., 2019) and Uruguay (Kaspary *et al* ., 2020). In this study, we have attempted to address an obvious and significant question: has GR *A. palmeri*recently invaded South American cropping systems from the USA, or does the emergence of GR populations represent a similar phenomenon to that seen in the USA, where a relatively minor weed has risen to prominence with changing agronomic practices, high glyphosate selection pressure and *in situ* evolution of glyphosate resistance?

Using population and molecular genetic analyses, we have demonstrated relatively low genetic differentiation between *A. palmeri*populations from three South American countries (F_{ST}< 0.05) in comparison to a high degree of differentiation amongst sampled populations from the USA. On the other hand, STRUCTURE analyses have assigned populations from Brazil and Uruguay to a different genetic cluster than Argentinean populations. Finally, our analysis using quantitative *EPSPS* gene copy number and qualitative *EPSPS* replicon specific marker assays indicate that*EPSPS* gene copy is increased in populations from Brazil and Uruguay and associated with an eccDNA mechanism similar to the USA*A. palmeri* populations. However, populations from Argentina do not have notably elevated copy number for *EPSPS* .

The history and epidemiology of *A. palmeri* in Argentina shows that the species was recorded as present in La Pampa province in 1984 (Covas, 1984). Increasing *A. palmeri* population sizes were evident in a number of fields in Córdoba province by 2005 (Júlian Oliva,*pers comm*), and subsequently a growing number of glyphosate control failures were noted, culminating in the first confirmation of evolved glyphosate resistance in *A. palmeri* populations in Argentina (Kaundun *et al* ., 2019; Palma-Bautista *et al* ., 2019). Both studies

characterized populations from Córdoba, and whereas one of the reports identified the P106S mutation at the *EPSPS*target site as the main glyphosate resistance mechanism along with a 1.8 fold higher *EPSPS* expression (Kaundun *et al* ., 2019), the other established reduced foliar uptake and translocation as the glyphosate resistance mechanisms (Palma-Bautista *et al* ., 2019). While these studies only established the mechanism of glyphosate resistance in two populations, their findings are consistent with our results that confirm an absence of significant *EPSPS* gene copy number increase and *EPSPS* replicon markers amongst 10 sampled Argentinean populations. The P106S target site mutation and reduced glyphosate leaf absorption and translocation have never been documented in GR *A. palmeri* populations from the USA (Gaines *et al* ., 2020; Sammons & Gaines, 2014). Taken together, this observational and published data, alongside the new data presented by our study support a hypothesis that *A. palmeri* was introduced to Argentina sometime before the 1980's and that its subsequent spread and rise to prominence as an agricultural weed have been enabled by changing agronomic practices since the mid 1990's. Independent evolution of glyphosate resistance via a known target site mutation that is not present in the North American population has arisen in Argentina as a result of intense glyphosate selection in glyphosate-tolerant corn and soybean crops.

A. palmeri populations from Brazil and Uruguay all exhibited increased *EPSPS* gene copy number (>50 copies), as well as the presence of *EPSPS* replicon specific markers. The first confirmed identification of *A. palmeri* in Brazil was reported in cotton fields in 2015 in Mato Grosso Province (Andrade Júnior, Cavaenaghi, Guimarães, & Carvalho, 2015) with speculated introduction of seed with harvesters imported from the USA around this time. This explanation seems plausible given observations that 98% of*A. palmeri* seeds are retained on mature, surviving plants at the time of harvest (Schwartz-Lazaro, Green, & Norsworthy, 2017), facilitating passage through, and retention of seeds in harvesting machinery. This recent invasion route is also consistent with the confirmation of glyphosate resistance in the Brazilian populations (Carvalho *et al* ., 2015; Küpper *et al* ., 2017), and our observation that this resistance is conferred by amplified *EPSPS*copy number associated with the eccDNA replicon, the predominant mechanism amongst *A. palmeri* populations from the USA (Gaines*et al* ., 2019).

The epidemiology of Uruguayan populations seems generally similar with the species absent from Uruguay in 2007 (Rios *et al* ., 2007) and being first reported around 2015 likely as a recent introduction on harvesting machinery from the USA (Álvarez Luzardo, De Vries Carlotta, & Gabriel Long, 2017).

Considering the STRUCTURE analysis of the RAD-seq generated genomic marker data and the molecular genetic analysis of *EPSPS* replicon markers and copy number, there is good support for at least two introductions of *A. palmeri* into South America from the USA. The first is an earlier invasion into Argentina followed by local, independent evolution of a target site mutation that confers resistance to glyphosate (Kaundun *et al* ., 2019) as well as a separate local evolution in Argentina of a non-target-site resistance mechanism (Palma-Bautista *et al* ., 2019). The second more recent invasion(s) of GR (eccDNA mechanism) *A. palmeri* populations likely occurred via import of farm machinery into Brazil and Uruguay.

However, analyses of pairwise F_{ST} results are more equivocal, suggesting a high degree of relatedness amongst populations from Argentina, Brazil and Uruguay, and being putatively indicative of a single introduction. If this were the case, possibly arising from an initial introduction via contaminated alfalfa seed into Argentina and subsequent continental spread to Brazil and Uruguay, then we must account for the quite different mechanisms of glyphosate resistance that have been observed. One explanation is that the discrete glyphosate resistance mechanisms have all evolved *in situ* under intense glyphosate selection on the same genetic background that arose from a single introduction. However, this seems unlikely given the sequence similarity of the *EPSPS* replicon to that found in USA *A. palmeri* populations.

Another possibility is that there has been a more recent secondary invasion of *A. palmeri* populations from the USA into Brazil and Uruguay. These populations were glyphosate resistant, with that resistance being conferred by the eccDNA *EPSPS* replicon. If these newly invaded GR *A. palmeri* populations were indeed the first arrivals into those countries, it is difficult to account for the low levels of differentiation from the Argentinean populations. Therefore, an alternative explanation might be that the number of plants / propagules invading from the USA with eccDNA *EPSPS* replicon was very small and there has been a

widespread and rapid selective sweep of that mechanism on the genomic background of previously invaded populations from the USA.

A final intriguing, though highly speculative possibility is that the eccDNA replicon was recently introduced into Brazil and Uruguay from the USA and introgressed into the common South American genetic background for *A. palmeri* via some mechanism of horizontal gene transfer (HGT). Various mechanisms for HGT have been proposed for plants (Gao*et al* ., 2014) and HGT is well established as a mechanism for the evolution and spread of antimicrobial resistance (e.g., Bansal & Meyer, 2002). The eccDNA replicon is a potential candidate for HGT due to its incredibly high sequence homogeneity (fewer than 10 variants in 400 kb of eccDNA sequence) among multiple, geographically distant populations of *A. palmeri* in the USA (Molin *et al* ., 2020a) that in at least some cases show population genetic divergence (Küpper *et al* ., 2018). The probability of the identical 400 kb eccDNA sequence forming independently in multiple populations seems less likely than either 1) a small number of introduced plants with the eccDNA followed by a selective sweep for glyphosate resistance or 2) HGT that enables rapid spatial movement of the eccDNA replicon into new populations.

Our analyses have not been able to definitively answer questions about routes and modes of introduction of *A. palmeri* into South America. The recent rapid expansion of the species range in North America and the propensity for the evolution and spread of glyphosate resistance clearly demonstrate the extraordinary capacity of this species for rapid adaptation in agroecosystems. It seems highly likely that *A. palmeri* invaded into South America from the USA, though the evidence for a single versus multiple introductions is ambivalent. It certainly seems that both the P106S target site mutation in*EPSPS* and reduced glyphosate absorption and translocation have evolved locally in Argentina. However, the origin of the eccDNA based mechanism is less clear and could be via independent evolution of this mechanism in Brazil and Uruguay (although this seems unlikely given the presence of the same eccDNA found in USA populations) or more likely via the very recent introduction of this intriguing and rare genetic mechanism from the USA and its rapid selection and spread in those locations under selection.

Notwithstanding some unresolved questions around the precise origin of GR populations of *A. palmeri* in South America, it seems clear that the species is a recent introduction from the USA, probably with more than a single introduction event. The presence of the rare and unique *EPSPS* replicon in populations present in Brazil and Uruguay opens some intriguing lines of enquiry to establish how this mechanism of resistance could spread so quickly on genomic backgrounds in those two countries given relatively little genetic differentiation being present between populations with markedly different GR mechanisms in Argentina, Brazil and Uruguay.

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Data accessibility: We are in the process of making an upload to the Sequence Read Archive at the NCBI (BioProject: PRJNA672995).

Author contributions: The work was originally conceived by PN and TAG and funding was awarded to PN. The population sampling design and study objectives were finalized by PN, TAG, MVA and AMJ. Collection of plant material in South America was arranged and undertaken by JO, MVA, AMJ and MAG. DNA extraction for South American populations was completed by AMJ. DNA extraction for USA populations and DNA normalization for all samples was completed by AK. Quantification of*EPSPS* copy number and presence of *EPSPS* replicon markers was by CS and TAG. DH completed the data filtering and variant calling for RAD-Seq data and GS designed and completed all analyses of RAD-Seq data. The paper was written by TAG, PN and GS with editing and comments from all co-authors.

Table 1. Population identifiers and sampling locations for*Amaranthus palmeri* populations collected in South and North America.

Code	Country	# Plants	Location	Collection site	Year
ARG1	Argentina	11	West Rio Cuarto, Cordoba	Soybeans	2016
ARG2	Argentina	8	Sampacho, Cordoba	Soybeans	2016
ARG3	Argentina	15	Vizcacheras, San Luis	Roadside	2016
ARG4	Argentina	9	Justo Daract, San Luis	Corn	2016
ARG5	Argentina	12	Justo Daract, San Luis	Grain elevator	2016
ARG6	Argentina	13	Pizarro, Cordoba	Soybeans	2016
ARG7	Argentina	8	Pizarro/Valeria, Cordoba	Sorghum	2016
ARG8	Argentina	8	Las Lomas, Villa Valeria, Cordoba	Corn	2016
ARG9	Argentina	8	Melideo de La Serna, Cordoba	Soybeans	2016
ARG10	Argentina	18	Rio Quinto, Cordoba	Soybeans	2016
BRZ1	Brazil	21	Tapurah, Mato Grosso	Soybeans / cotton	2016
BRZ2	Brazil	18	Ipiranga do Norte, Mato Grosso	Soybeans / cotton	2016
BRZ3	Brazil	21	Ipiranga do Norte, Mato Grosso	Soybeans / cotton	2016
BRZ4	Brazil	28	Campos de Julio, Mato Grosso	Soybeans / cotton	2016
URU1	Uruguay	19	Colonia Valdense, Colonia	Corn	2017
URU2	Uruguay	17	Porvenir, Paysandú	Soybeans	2017
URU3	Uruguay	16	Colonia Tomas Berret, Rio Negro	Soybeans	2017
AZ-R	USA	17	Buckeye, Arizona	Cotton	2012

Code	Country	# Plants	Location	Collection site	Year
AZ-S	USA	17	Sahuarita, Arizona	Desert	2012
CO-R	USA	14	Yuma County, Colorado	Sugar beet	2015
GA-R	USA	16	Macon, Georgia	Cotton	2006
GA-S	USA	17	Worth County, Georgia	Cotton	2004
KS-S	USA	13	Ottawa, Kansas	Soybean	2005
NC-R	USA	2	North Carolina	Cotton	2006
TN-R	USA	17	Jackson, Tennessee	Soybean	2007
AZS-2	USA	17	Tucson, Arizona	Desert	1981

Table 2. Pairwise F_{ST} values for all*Amaranthus palmeri* populations (Argentina, Brazil and Uruguay samples considered as a single population in this analysis). Cells are colour-coded from light green through red to indicate progressively higher F_{ST} (i.e. increased genetic differentiation between populations).

Hosted file

`image1.emf` available at https://authorea.com/users/371999/articles/490450-investigating-the-origins-and-evolution-of-a-glyphosate-resistant-weed-invasion-in-south-america

Table 3. Mean relative *EPSPS* copy number in*Amaranthus palmeri* populations from the United States (GA-R and GA-S), Brazil, Uruguay, and Argentina, along with presence (+) or absence (-) of the *EPSPS* eccDNA replicon markers; SE, standard error of the mean.

Country	Population	n	Mean *EPSPS* Gene Copy Number	SE	*EPSPS* eccDNA Replicon Markers
USA	GA-R	6	125	4.1	+
	GA-S	6	1	0.0	-
Brazil	BRZ1	5	75	6.3	+
	BRZ2	6	56	5.4	+
	BRZ3	6	80	7.1	+
Uruguay	URU1	6	76	8.9	+
	URU2	6	75	4.9	+
	URU3	6	103	3.4	+
Argentina	ARG1	6	2	0.1	-
	ARG2	6	2	0.1	-
	ARG3	1	2		-
	ARG4	6	2	0.0	-
	ARG5	6	1	0.1	-
	ARG6	6	2	0.2	-
	ARG7	6	2	0.1	-
	ARG8	6	2	0.2	-
	ARG9	6	2	0.1	-
	ARG10	6	2	0.2	-

Figures

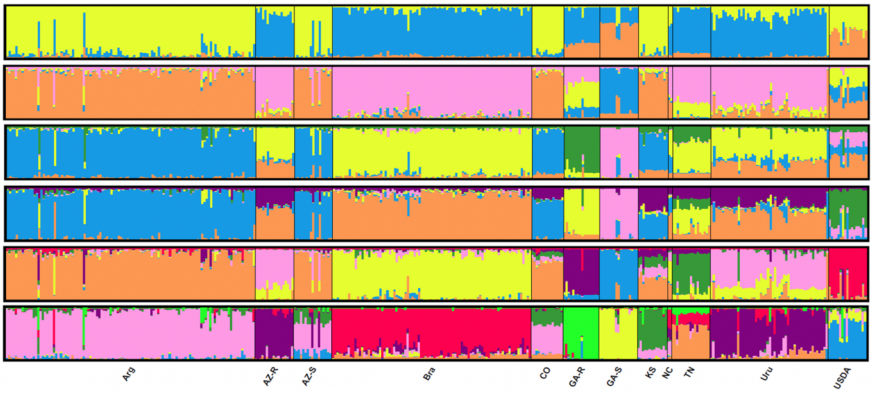

Figure 1. Results from model-based clustering using STRUCTURE, with the number of genetic groups varied between three and eight (K = 3-8).

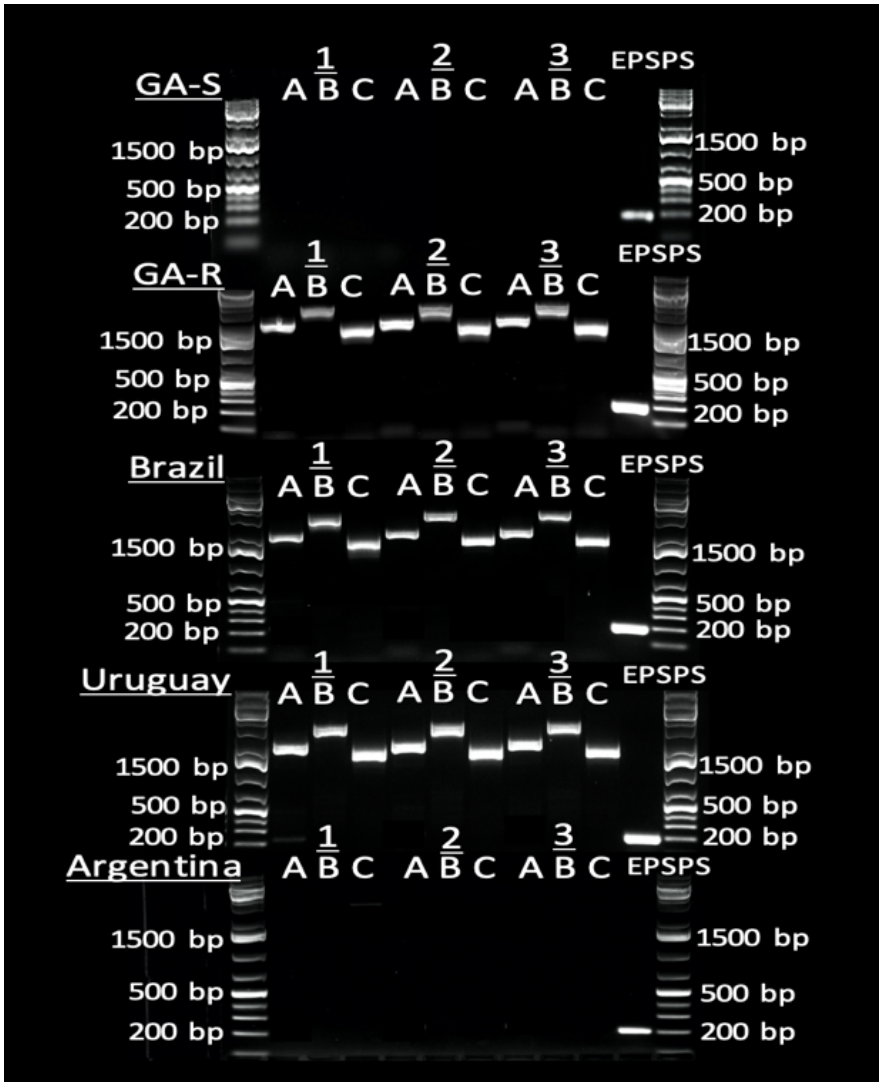

Figure 2. Agarose gel image depicting the qualitative analysis of the *EPSPS* eccDNA replicon markers A (1757 bp), B (2352 bp), and C (1544 bp), in three representative biological replicates of*Amaranthus palmeri* from glyphosate susceptible (S) and resistant (R) populations from Georgia, USA (GA), as well as Brazil, Uruguay, and Argentina. Individuals from all populations from Brazil and Uruguay display all three *EPSPS* replicon markers similar to GA-R individuals, while all tested individuals from the 10 populations from Argentina lacked the *EPSPS* replicon. The shorter *EPSPS*amplicon was included as a positive PCR control for the template DNA.