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Research Article

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Genetic basis and origin of resistance to acetolactate synthase inhibitors in *Amaranthus palmeri* from Spain and Italy

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Abstract

BACKGROUND: ***Amaranthus palmeri*** **is an aggressive annual weed native to the United States, which has become invasive in some European countries. Populations resistant to acetolactate synthase (ALS) inhibitors have been recorded in Spain and Italy, but the evolutionary origin of the resistance traits remains unknown. Bioassays were conducted to identify cross-resistance to ALS inhibitors and a haplotype-based genetic approach was used to elucidate the origin and distribution of resistance in both countries.**

RESULTS: ***Amaranthus palmeri*** **populations were resistant to thifensulfuron-methyl and imazamox, and the 574-Leu mutant** ***ALS*** **allele was found to be the main cause of resistance among them. In two Spanish populations, 376-Glu and 197-Thr mutant** ***ALS*** **alleles were also found. The haplotype analyses revealed the presence of two and four distinct 574-Leu mutant haplotypes in the Italian and Spanish populations, respectively. None was common to both countries, but some mutant haplotypes were shared between geographically close populations or between populations more than 100 km apart. Wide genetic diversity was found in two very close Spanish populations.**

CONCLUSION: ALS-resistant ***A. palmeri*** **populations were introduced to Italy and Spain from outside Europe. Populations from both countries have different evolutionary histories and originate from independent introduction events. ALS resistance then spread over short and long distances by seed dispersal. The higher number and genetic diversity among mutant haplotypes from the Spanish populations indicated recurrent invasions. The implementation of control tactics to limit seed dispersal and the establishment of** ***A. palmeri*** **is recommended in both countries.**
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Supporting information may be found in the online version of this article.

Keywords: European Palmer amaranth; invasive weed; acetolactate synthase inhibitors; haplotype analysis; resistance spread; point mutations

1 INTRODUCTION

Weeds are unwanted plants in agroecosystems, and some have become invasive outside their native range. This is viewed as a growing threat to global sustainability.[1] Invasive weed species exhibit specific adaptive traits that facilitate their establishment, such as rapid growth from seedlings to maturity, phenotypic plasticity, and high adaptability to disturbed habitats.[2] Successful weed establishment may have negative effects on the environment, resulting in a decrease in biodiversity in the invaded area, and can also cause a decrease in crop yield due to competition.[3] Thus, early discovery, monitoring, and removal are fundamental to limit the spread of invasive plant species.

Herbicides are widely used to control weeds because of their low cost and ease of use. However, their repeated application exerts strong selection pressure on weed populations, allowing individuals with rare resistance-endowing traits to survive. If selection pressure persists, rare traits increase in frequency within

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the population, and herbicide efficacy decreases after a few generations.[4]

One of the most problematic weed to control is *Amaranthus palmeri* S. Wats, a diploid summer species belonging to the Amaranthaceae family. It is native to the Sonora Desert, the southern United States, and northern Mexico.[5] Its size, seed production, and rapid growth have made it one of the most harmful plagues for summer crops, reducing the production of maize (*Zea mays*), cotton (*Gossypium hirsutum*), and soybean (*Glycine max*) in South and North America.[6] *Amaranthus palmeri* is highly tolerant to abiotic stresses such as extreme heat and irregular rainfall and, to make matters worse, its ability to develop resistance to many herbicide modes of action makes most commercial products inefficient for its control.[6] Acetolactate synthase (ALS) inhibitors are herbicides commonly used as post-emergence treatments to control broadleaf weeds in maize and soybean, including weedy amaranths. The inactivation of this enzyme hinders the synthesis of the branched-chain amino acids valine, leucine (Leu), and isoleucine, resulting in plant death.[7] Resistance to ALS-inhibiting herbicides is most frequently caused by non-synonymous single nucleotide polymorphisms (SNPs) resulting in amino acid substitutions. These changes have been found at nine different codon sites of the *ALS* gene in several species,[8] to which they confer resistance to a few or many subclasses of ALS inhibitors, depending on the mutation.[9]

Since the first reported case in the mid-1990s,[10] *A. palmeri* populations resistant to ALS inhibitors have spread in both North and South America.[11] In Mediterranean countries, *A. palmeri* has been historically reported as a casual alien plant, or very rarely, naturalized in specific localities.[12] However, herbicide-resistant biotypes have recently been reported in Israel,[13] Spain,[14] Italy,[15] Greece,[16] and Turkey.[17] In Spain, the first three *A. palmeri* populations were collected along field edges and roadsides and were confirmed to be resistant to nicosulfuron-methyl (ALS-inhibitor) owing to mutations at positions 197, 574 and 653 (referred to as the *ALS* sequence of *Arabidopsis thaliana*).[14] In Italy, instead, the first three *A. palmeri* populations were found within soybean fields and yet confirmed to be cross-resistant to thifensulfuron-methyl and imazamox due to the point mutation Trp-574-Leu.[15] *Amaranthus palmeri* being a recently introduced alien weed in Europe, there is no history of herbicide selection acting on it, therefore the introduction of yet-resistant biotypes (through still unknown ways) is a reasonable suspicion.[18]

Understanding the invasion dynamics and spread of *ALS*-resistant alleles within agricultural habitats is important when considering the recent arrival of ALS-resistant *A. palmeri* populations in Italy and Spain. To investigate the evolutionary history of populations, haplotypes (a group of physically linked genetic variants that are inherited together) are powerful markers. Through haplotype analysis, it is possible to establish whether a point mutation shared among multiple populations emerged during a single selection process and then spread or if the resistance trait appeared via independent mutation events. This approach has been used to elucidate the evolution of resistance in several organisms (reviewed by Hawkins *et al.*),[19] but only few studies are related to weeds,[20,21] and none on *A. palmeri*. Among the possible genetic variants, SNPs are normally preferred for population genetics and evolutionary studies.[22] Haplotypes of diploid organisms can be obtained directly by cloning and Sanger sequencing or reconstructed *in silico* (phasing) after next-generation sequencing (NGS). Other methods rely on NGS to sequence allele-specific amplicons,[23] or allele-specific primers for Sanger sequencing.[24] For small sample sizes and targets, Sanger sequencing remains the most appropriate method.[25]

In this study, we investigated: (i) the resistance patterns to thifensulfuron-methyl and imazamox of *A. palmeri* populations collected in Italy and Spain; (ii) the main resistance mechanism involved and possible *ALS* alleles associated with resistance; and (iii) the evolutionary history of populations carrying the Trp-574-Leu mutation in *ALS* gene. To determine the *ALS* haplotypes, a cheap and easy approach was adopted, based on the Sanger sequencing of allele-specific polymerase chain reaction (AS-PCR) amplicons obtained from heterozygous plants.

2 MATERIALS AND METHODS

2.1 Plant material

Twelve *A. palmeri* populations were included in this study: four from Italy, collected from 2018 to 2021; seven from Spain, collected from 2016 and 2020 (Table 1); and one from North Carolina (USA), which was used as a susceptibility check.[26] For each population, seeds were collected from at least 30 mature female plants, randomly selected across the sampling site, cleaned, and stored at room temperature until the experiments began.

Table 1. *Amaranthus palmeri* populations sampled in Italy and Spain in different years and sites

Year of collection	Identifier	Country	Municipality	Sampling site
2018	IT-1[†]	Italy	Camposampiero	Soybean
2019	IT-2[†]	Italy	Curtarolo	Soybean
2019	IT-3[†]	Italy	Mira	Soybean
2021	IT-4	Italy	Lonato del Garda	Maize
2019	ES-1	Spain	La Portella	Maize
2019	ES-2	Spain	Torrefesneda	Maize
2019	ES-3	Spain	Torrefesneda	MAIZE
2017	ES-4	Spain	Benavent de Segrià	Maize
2020	ES-5	Spain	Ulldecona	Roadside
2016	ES-6[‡]	Spain	Lleida	Roadside
2016	ES-7[‡]	Spain	Binéfar	Roadside

[†] Populations IT-1, IT-2 and IT-3 were previously proven to be cross-resistant to thifensulfuron-methyl and imazamox.[15]
[‡] Populations ES-6 and ES-7 were previously proven to be resistant to nicosulfuron.[14]

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2.2 Resistance pattern to ALS inhibitors

Bioassays were conducted in a glasshouse located in north-eastern Italy (45°21′ N, 11°58′ E) to determine the sensitivity to thifensulfuron-methyl (sulfonylurea) and imazamox (imidazolinone), except for IT-1, IT-2, and IT-3, which were studied previously (therein called 18-100, 19-174 and 19-177).[15] The seedlings were grown as previously described.[27] Glasshouse temperatures ranged from 15 to 25 °C and from 25 to 35 °C during the night and day, respectively. When seedlings reached the 4–5 leaves stage, they were treated with thifensulfuron-methyl [Harmony 50 SX, DuPont™, 500 g active ingredient (a.i.) kg^{-1}] and imazamox (Tuareg®, DuPont™, 40 g a.i. L^{-1}) at recommended field doses of 6 g a.i. ha^{-1} and 40 g a.i. ha^{-1}, respectively. Herbicides were applied using a precision bench sprayer with a boom equipped with three flat-fan (extended range) hydraulic nozzles (Teejet®, 11002), which delivered 300 L ha^{-1} at a pressure of 215 kPa and a speed of approximately 0.75 m s^{-1}. The experimental design was completely randomized with two replicates (one replicate, one tray, and 20 plants per tray). Four weeks after herbicide application, the number of surviving plants was recorded, and the percentage of resistant plants was determined for each population. The experiment was conducted twice, and the results represent the average of the data obtained from two identical experiments to determine the overall mean value. To confirm the similarity between the two sets of results, their standard errors were compared, and no significant differences were found. The mean and standard error were the statistical values used to express the data.

2.3 Detection of target-site mutations

2.3.1 *DNA extraction*

Fresh leaf samples were collected from herbicide-surviving plants for each population. All samples were stored at −20 °C until genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) protocol[28] adapted for centrifuge tubes.[15] The DNA concentration and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham. MA, USA) and the concentration was adjusted to 20 ng μL^{-1}.

2.3.2 *Cleaved amplified polymorphic sequence (CAPS) assay*

To detect the point mutation 574-Leu in *ALS*, a cleaved amplified polymorphic sequence (CAPS) assay was set up.[29] DNA was extracted from plants that survived the imazamox treatment. PCR products of 1,394 bp were obtained using the primers Caps_F1 (5′-GGGAAGAATAAGCAACCTCATGTG-3′) and 3UTR_R1 (5′-TGGCTGATGAAAGGCAACAC-3′). PCRs were performed using GoTaq® G2 Hot Start Polymerase (Promega, Madison, WI, USA) in 15 μL mixture, including 3 μL of 5× Green GoTaq Flexi Buffer, dNTPs mix 0.2 mM, magnesium chloride ($MgCl_2$) 1.5 μM, forward and reverse primers 0.2 μM each, 0.005 μL GoTaq DNA Polymerase (5 U μL^{-1}), and 1.5 μL DNA. Amplification conditions: 95 °C for 2 min; 35 cycles 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min 30 s, and 72 °C for 5 min. After PCR, 5 μL of unpurified amplicons were incubated at 37 °C for 1 h with 0.1 μL of MunI (*MfeI*) (10 U μL^{-1}) (Thermo Fisher Scientific), 9 μL of water and 1 μL of 10X Buffer G. Digested samples were run on a 1% agarose gel: uncut samples were ascribed as homozygous wild type (WT), while double-cut samples (two bands of about 514 and 880 bp, respectively) were ascribed as homozygous mutants, and one-cut (three bands of 1.394, 880 and 514 bp, respectively) as 574-Leu/Trp heterozygous mutants.

2.3.3 *ALS partial amplification*

Primers 3F and 4R (5′-GCGATGTTCTCGTTGAAGCTC-3′; 5′-GCACAATCTTAGCCCGGCTAGC-3′) were used to amplify the *ALS* region encompassing codons Ala-122 to Arg-377. PCR was conducted as described in Section 2.3.2, except for the amplification time that was set to 30 s and the reagents adjusted to a volume of 30 μL. The PCR products were purified using a PCR Clean-up kit (Macherey-Nagel GmbH & Co., Duren, Germany) following the manufacturer's instructions. Once purified, PCR products were Sanger-sequenced by BMR Genomics (Padova, Italy), visualized with FinchTV 1.4.0 and aligned with MEGA X.[30]

2.4 ALS haplotyping with allele-specific polymerase chain reaction (AS-PCR)

A method to directly obtain the ALS haplotype of *A. palmeri* by Sanger-sequencing of allele-specific amplicons is here proposed. The set-up and validation of the AS-PCR protocol for haplotype analysis was firstly carried out on *Amaranthus tuberculatus* (Moq.) J.D.Sauer DNA samples whose haplotypes were estimated computationally after targeted NGS in a previous work.[31] *Amaranthus tuberculatus* was chosen as validation because it is biologically and genetically similar to *A. palmeri* and because both the DNA samples and the NGS dataset were available.

2.4.1 *Set-up and validation*

In the previous work on *A. tuberculatus*,[31] two 574-Leu (mutant) *ALS* haplotypes were found in populations A, B, D, F and H. Eleven heterozygous samples (namely A4, A5, A12, B3, B12, D11, F1, F3, F5, H6, H7), retrieved from the original ones, were used herein to validate the haplotyping method based on the AS-PCR. Heterozygous samples were preferred over the homozygous to avoid any possible double peak and to resolve the haplotype with one PCR only. The amplification of haplotypes containing the 574-Leu substitution was obtained by using the leucine-specific forward primer AS-Leu (5′-ACATTTAGGTATGGTTGTTCACTT-3′) and the reverse primer 3UTR_R1 (5′-TGGCTGATGAAAGGCAACAC-3′), previously developed for *Amaranthus hybridus*.[32] PCR was conducted as described in Section 2.3.3, but the annealing temperature had risen to 60 °C and the elongation time to 60 s. Amplicons (900 bp) were purified and Sanger-sequenced (as in Section 2.3.3). Traces (electropherograms) obtained by sequencing the AS-PCR product were visually checked for possible SNPs with novoSNP[33] and trimmed at the 5′ and 3′ ends by approximately 100 bases. Each variable position was checked and manually edited in case of doubtful or incorrect base calling. The edited sequences were aligned with MEGA X and the variable sites were extracted to be re-aligned with the phased sequences of the same 11 samples obtained previously using the NGS pipeline.

2.4.2 *Analysis of* A. palmeri *allele-specific haplotypes*

Once the robustness of the haplotyping method had been assessed, it was used to determine the *ALS* haplotypes of Italian and Spanish populations of *A. palmeri*. Four heterozygous plants per population (as defined by CAPS in Section 2.3.2) were analyzed to determine the WT haplotype, whereas for the mutant haplotypes 9–10 heterozygous plants were analyzed. When possible, both the mutant and WT haplotypes were obtained from the same plant. The mutant 574-Leu allele was obtained as described in Section 2.4.1, whereas for the WT allele 574-Trp the forward primer AS-Trp (5′-ACATTTAGGTATGGTTGTTCACTG-3′) was used.[32] Sequences were analyzed and edited as in Section 2.4.1 and

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aligned in MEGA X to obtain a neighbor-joining (NJ) tree, which was exported into the NEXUS format to be graphically represented with the Interactive Tree of Life (ITOL) v5.[34] Identical sequences of the alignment were identified with POPART[35] (version 1.7) and renamed as 'IT-letter' or 'ES-letter' to represent haplotypes; those with numerosity equal or lower than 2 were excluded from the analyses. The NEXUS files were formatted to include the haplotype sequences and their new names, their distribution across populations, and the geographical position of each population. POPART was used to infer the TCS (Templeton, Crandall, Sing) network[36] of haplotypes[37] and draw georeferenced haplotype diversity maps.

3 RESULTS

3.1 Resistance pattern to ALS inhibiting herbicides

Thifensulfuron-methyl and imazamox applications completely controlled the susceptible WT population. All tested populations had individuals that survived the two treatments (Fig. 1). Only for population ES-7, a lower number of plants (ten for each replicate) were treated because of scarce seed germination at both experiment replications. The survival rates of thifensulfuron-methyl were generally higher than those of imazamox, ranging from 35% to 97% and 22% to 73%, respectively. Most populations showed similar survival rates to both herbicides, suggesting that survivors had, at least, an allele carrying a cross-resistance-endowing mutation. Populations ES-1, ES-4, and ES-7 had a 36–52% greater survival rate to thifensulfuron-methyl than to imazamox, indicating that a consistent number of plants were not cross-resistant, but resistant to thifensulfuron-methyl only. The survival rate to both herbicides of population ITA-4 is similar to the results previously reported for populations ITA-1, ITA-2 and ITA-3.[15]

3.2 Mechanism of resistance

The CAPS assay was used to investigate the presence of the cross-resistance-endowing Trp-574-Leu mutation in populations with more than eight imazamox-survived plants. The CAPS assay revealed the presence of the Trp-574-Leu mutation in most imazamox-survivors of all the tested populations (Table 2) (ES-7 was excluded because only five plants survived the imazamox treatment). The number of heterozygous individuals, with a copy of the mutant allele and a copy of the WT allele, ranged from 61% to 100% among the Italian populations, and from 59% to 85% among the Spanish ones. Homozygous resistant plants were also found in populations (ITA-2, ITA-4, ES-3, and ES-4). Since populations ES-1, ES-4 and ES-7 had a survival rate to thifensulfuron-methyl far greater than that to imazamox (Section 3.1), most plants survived to thifensulfuron-methyl were expected not to carry mutations at position 574 or 654, therefore only position 122 to 376 were checked by sequencing. The Asp-376-Glu mutation was found in most plants of populations ES-1 and ES-4, whereas the Pro-197-Thr mutation was found in population ES-7. Other non-synonymous mutations (not linked to resistance) were identified in the same region: Phe-177-Leu, Phe-282-Tyr, Ala-284-Asp, Lys-323-Glu, and Gln-348-His, with only Ala-284-Asp absent in ES-1 (Supporting Information Table S1).

3.3 ALS haplotype analyses

3.3.1 *Validation of the AS-PCR haplotyping method*

As shown in Fig. 2, the proposed AS-PCR haplotyping method correctly detected ten SNPs found by the NGS pipeline in this sequence range for all the tested *A. tuberculatus* mutants. The ten SNPs were found across a sequence of 900 bp (Section 2.4.1), and aligned against the 46 SNPs that were found across a sequence of about 4 kbp in total, spanning the whole *A. tuberculatus ALS* coding sequence (2 kbp) plus 1 kbp upstream and 1 kbp downstream non-coding sequences.

3.3.2 *Analysis of allele-specific haplotypes*

After performing AS-PCR, 130 sequences were obtained from 94 heterozygous plants and 36 WT. Out of these, 116 were of good quality (refer to Table S2 for details). The alignment comprised 697 nucleotides, with 55 variable sites, of which 231 nucleotides were located within the coding region and had 11 variable sites, while 466 nucleotides were found in the 3′ untranslated region and had 44 variable sites. Based on the NJ tree (Fig. 3), most of the WT haplotypes were grouped separately from mutant haplotypes. Identical sequences were not detected in either Italy or Spain; hence, the data were analyzed separately. To construct the TCS network, sequences that occurred only once

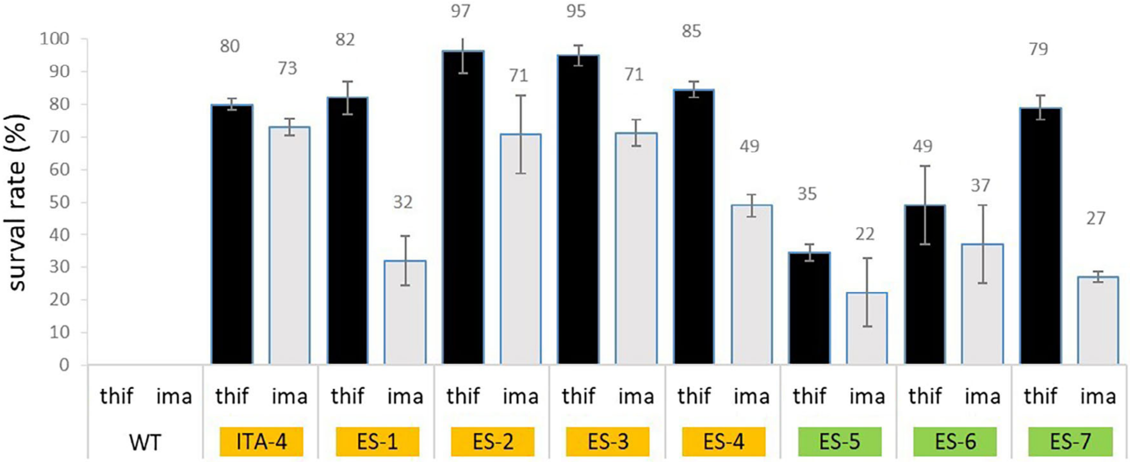

Figure 1. Percentage of *Amaranthus palmeri* plants that survived treatments with thifensulfuron-methyl (thif, black columns) and imazamox (ima, gray columns); the mean values of the two experiments (replicates) are shown; vertical bars represent the standard error. 'WT' is the susceptible check population. Populations collected in maize fields are labeled in orange, while those collected at the roadside in green.

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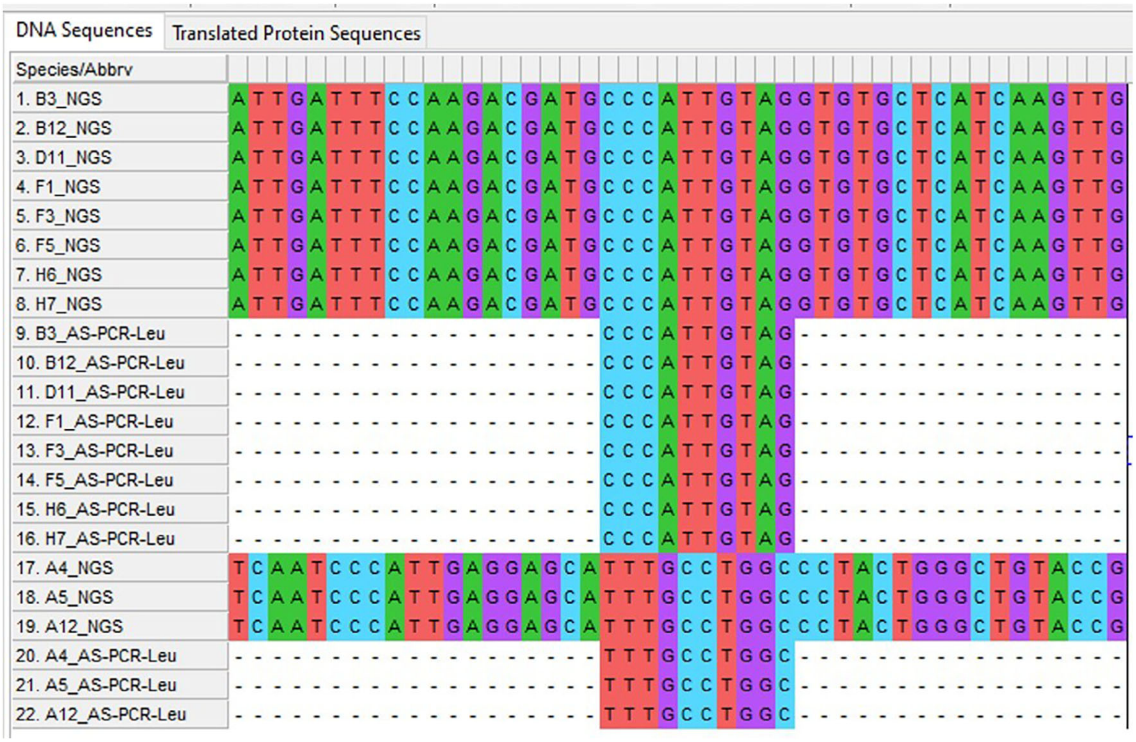
Table 2. Percentage of different genotypes at position 574 of *ALS* resulting from CAPS assay. LL means homozygous Leu-574-Leu, WL heterozygous Trp-574-Leu, and WW homozygous wild-type Trp-574-Trp

Identifier	Number of plants	Number of genotyped plants			Percentage of heterozygous plants
		LL	WL	WW	
ITA-1	14	—	11	3	78
ITA-2	24	7	11	6	45
ITA-3	12	—	12	—	100
ITA-4	13	5	8	—	61
ES-1	13	—	11	2	85
ES-2	17	—	10	7	59
ES-3	17	2	10	5	59
ES-4	12	1	10	1	83
ES-5	14	—	10	4	71
ES-6	11	—	9	2	82

(singletons = unshared haplotypes) or twice were excluded. In total, 107 of the 116 good quality sequences were retained for further analyses.

For the Italian sequences, 40 haplotypes (eight WT and 32 mutants; see Table S3 for details) were retained for further analyses, resulting in a change in the number of variable sites from 29 to 27. Three unique sequences were identified and named as haplotypes IT-A and IT-B, both mutant and differing by 20 nucleotides, and IT-C, WT, differing by 16 nucleotides from both IT-A and IT-B (refer to Fig. 4(a)). Mutant haplotypes IT-A and IT-B were equally frequent (16/40; Table S3), with IT-A found in populations IT-1, IT-2, IT-4, and IT-B in populations IT-2 and IT-3 (Fig. 4(b)). The WT haplotype IT-C was shared among three populations, IT-1, IT-2, and IT-3, whereas three WT sequences originating from IT-4 were excluded from the analyses due to their rarity. Considering the Spanish sequences, 67 haplotypes (15 WT and 52 mutants; see Table S4 for details) were retained for further analyses after removing rare haplotypes (4/6 WT), which caused the number of variable sites to change from 50 to 37. Seven unique sequences were found and renamed as haplotypes ES-A, ES-B, ES-C, ES-D, all mutants, and ES-E, ES-F, ES-G, all WT (Fig. 5(a)). The mutant haplotypes differed from each other by 15 nucleotides (ES-A and ES-B) to 25 nucleotides (ES-B and ES-D), with the most common haplotype being ES-D (20/67, Table S4). ES-A was found in ES-1, ES-3, and ES-4, whereas ES-B



Species/Abbrv	
1. B3_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
2. B12_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
3. D11_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
4. F1_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
5. F3_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
6. F5_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
7. H6_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
8. H7_NGS	ATTGATTTCCAAGACGATGCCCATTGTAGGTGTGCTCATCAAGTTG
9. B3_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
10. B12_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
11. D11_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
12. F1_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
13. F3_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
14. F5_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
15. H6_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
16. H7_AS-PCR-Leu	-------------------CCCATTGTAG-----------------
17. A4_NGS	TCAATCCCATTGAGGAGCATTTGCCTGGCCCTACTGGGCTGTACCG
18. A5_NGS	TCAATCCCATTGAGGAGCATTTGCCTGGCCCTACTGGGCTGTACCG
19. A12_NGS	TCAATCCCATTGAGGAGCATTTGCCTGGCCCTACTGGGCTGTACCG
20. A4_AS-PCR-Leu	-------------------TTTGCCTGGC-----------------
21. A5_AS-PCR-Leu	-------------------TTTGCCTGGC-----------------
22. A12_AS-PCR-Leu	-------------------TTTGCCTGGC-----------------

Figure 2. Alignment of haplotypes obtained from the same *Amaranthus tuberculatus* DNA samples using two different techniques: NGS (longer sequences, named as 'sample#_NGS') and AS-PCR (shorter sequences, named as 'sample#_AS-PCR-Leu'). Only variable sites are reported for shortness. Sequences 1 to 16 refer to 'haplotype 1' described in the *A. tuberculatus* work,[31] while sequences 17 to 22 refer to haplotype 2.

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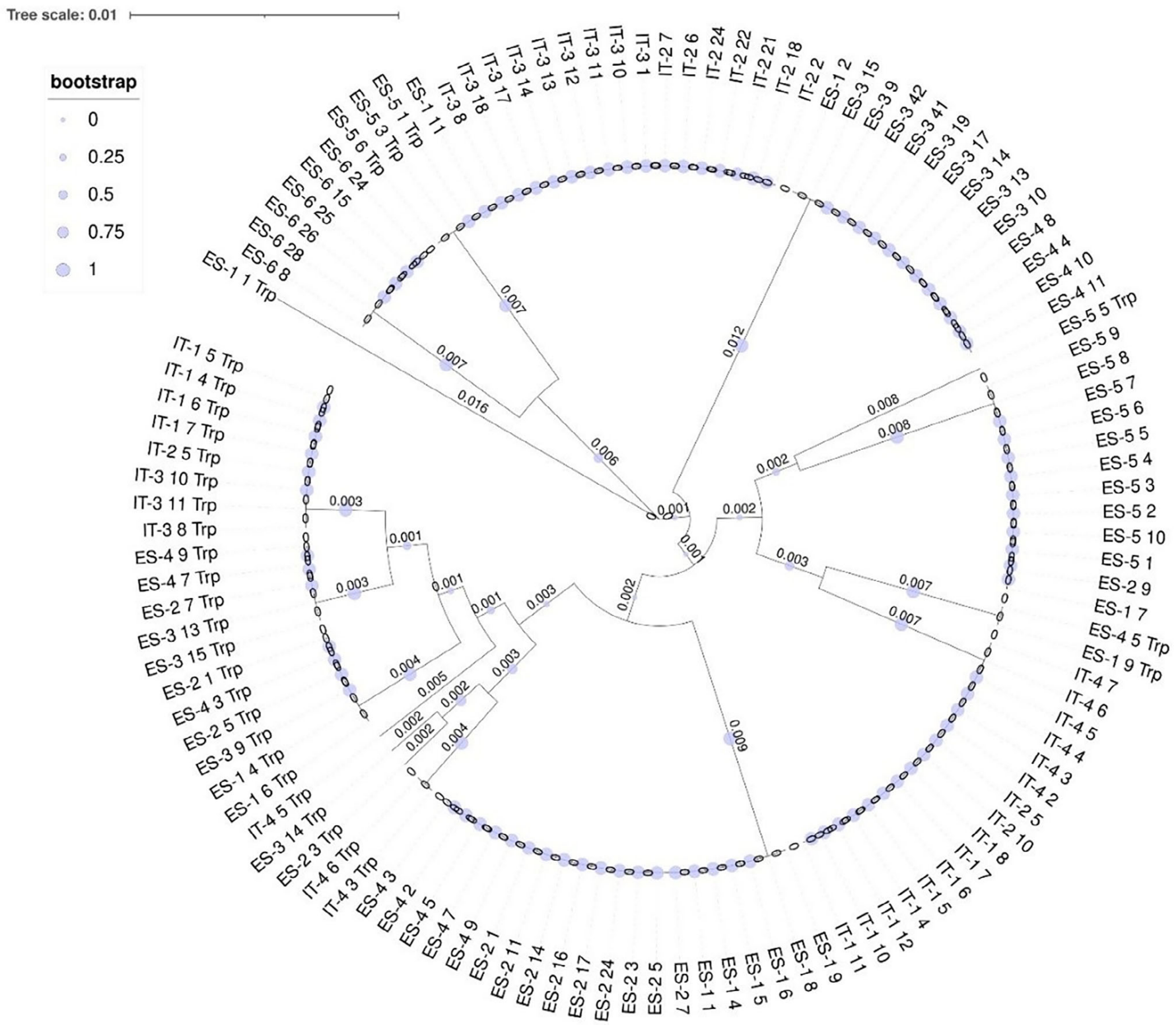

Figure 3. Neighbor-joining tree of *ALS* haplotypes (including singletons and rare haplotypes; 116 sequences in total). Wild-type sequences are indicated as '_Trp,' whereas all others carry the point mutation Trp-574-Leu. Branch lengths are reported along with bootstrap values.

was found in ES-1, ES-2 and ES-5, and ES-C only in population ES-6 and ES-D in ES-1, ES-2 and ES-4 (Fig. 5(b)). The WT haplotype ES-E was shared among the three populations, namely ES-2, ES-3 and ES-4, whereas ES-F was shared among ES-1 and ES-5, and ES-G was shared among ES-1, ES-2, ES-3 and ES-4.

4 DISCUSSION

4.1 Pattern of resistance to ALS inhibitors and resistance mechanism

Whole-plant bioassays combined with *ALS* genotyping (CAPS) and sequencing confirmed that eight *A. palmeri* populations, seven from Spain and one from Italy, have evolved resistance to ALS inhibitors (Fig. 1). These resistant *A. palmeri* populations, collected on roadsides and in crop fields, augment the cases previously found in both countries,[14,15] demonstrating the spread of resistance.

Our results showed that the resistance pattern varied with the population, and that resistance to thifensulfuron-methyl and imazamox was not necessarily associated. Five populations were cross-resistant to both herbicides, and among them, those collected from cropped fields (ES-2, ES-3 and ITA-4) had higher survival rates than those collected from roadsides (ES-5 and ES-6), suggesting that the selection pressure imposed by frequent herbicide use on the crop favors an increase in the frequency of *A. palmeri* resistant plants. The other three resistant populations (ES-1, ES-4 and ES-7) were characterized by a high survival rate to thifensulfuron-methyl and low to imazamox, indicating the presence of thifensulfuron-methyl resistant plants in addition to cross-resistant plants.

The different resistance profiles observed among the populations is in accordance with the detected target-site mutations. The amino acid substitution Trp-574-Leu was found in almost all populations except for ES-7. This mutant *ALS* allele is well known to confer broad cross-resistance to ALS inhibitors and has been reported to confer resistance to both thifensulfuron and imazamox in *A. palmeri*.[9] Notably, in all the investigated Italian populations (IT-1, IT-2, IT-3 and IT-4), the 574-Leu *ALS* allele was the unique target-site

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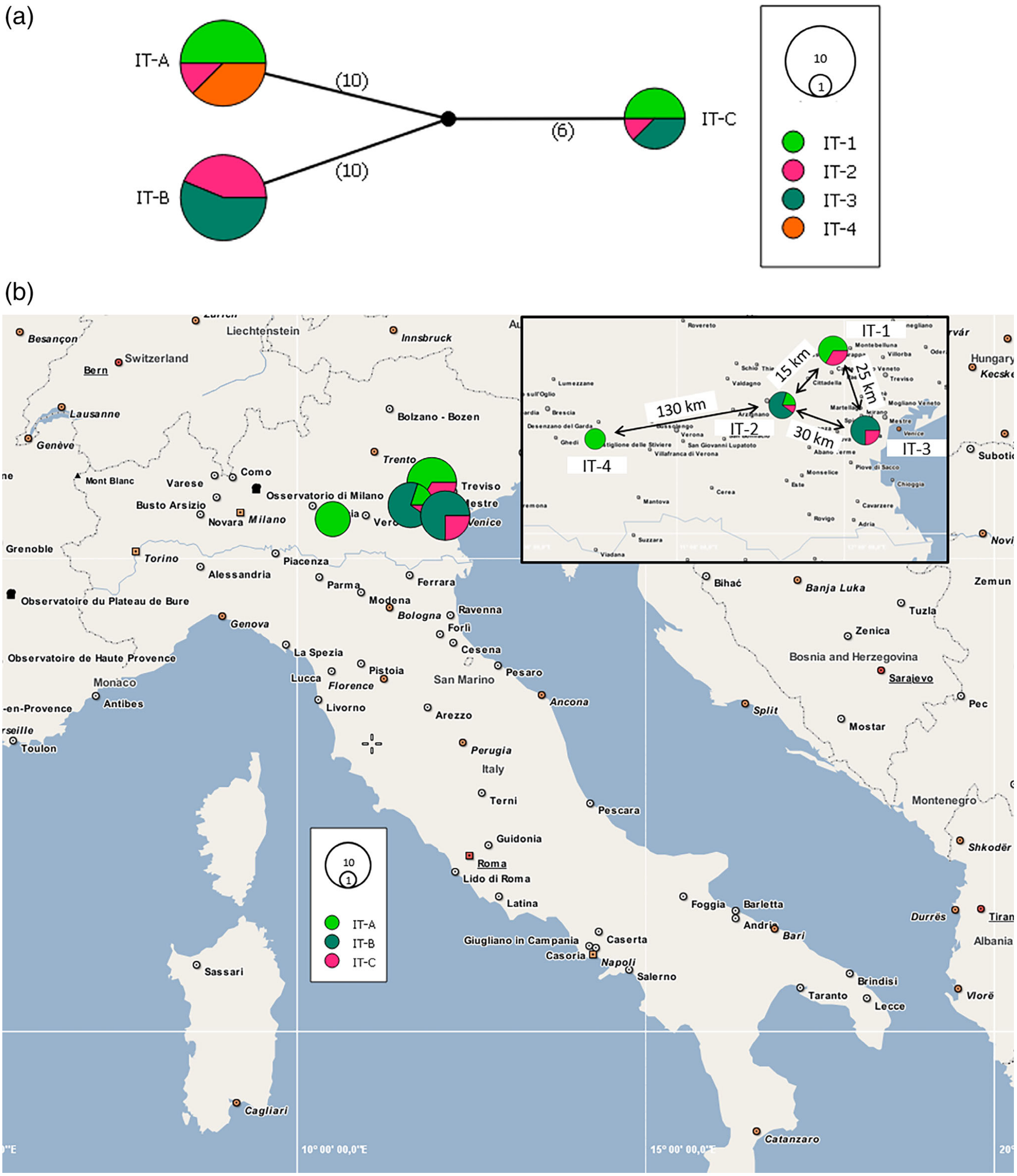

Figure 4. (a) TCS *ALS* haplotype network obtained after analysis of Italian samples. Each circle represents a different haplotype, and the circle size represents the proportion of individuals carrying the same haplotype. Colors refer to different populations. Black circles indicate missing (unobserved) intermediate haplotypes. Mutational steps are reported in parentheses. (b) Georeferenced map of haplotype diversity obtained after the analysis of Italian samples. Each color corresponds to a different haplotype. The position of close populations was approximated to avoid overlapping pie charts; distances across populations are indicated in kilometers.

mutation found. The frequent use of thifensulfuron-methyl and imazamox in soybean fields has led to the selection of *A. palmeri* individuals carrying this mutant *ALS* allele. In contrast, additional resistant *ALS* alleles were found in the Spanish populations. Population ES-7, previously found to be resistant to nicosulfuron,[14] also had a high survival rate to thifensulfuron-methyl and a low one to imazamox

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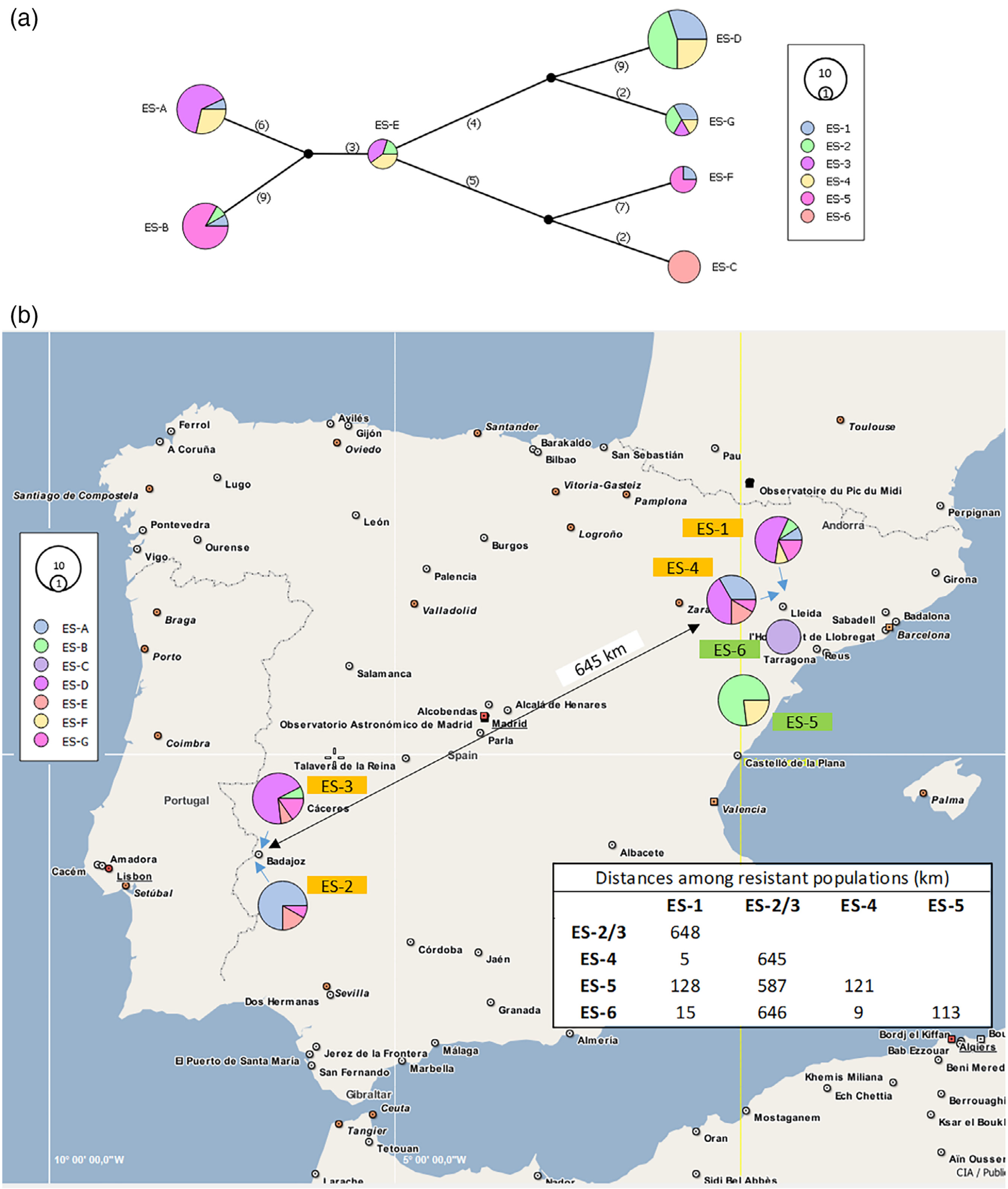

Distances among resistant populations (km)

	ES-1	ES-2/3	ES-4	ES-5
ES-2/3	648			
ES-4	5	645		
ES-5	128	587	121	
ES-6	15	646	9	113

Figure 5. (a) The TCS *ALS* haplotype network was obtained after analysis of Spanish samples. Each circle represents a different haplotype, and the circle size represents the proportion of individuals carrying the same haplotype. Colors refer to different populations. Black circles indicate missing (unobserved) intermediate haplotypes. Mutational steps are reported in parentheses. (b) Georeferenced map of haplotype diversity obtained after analysis of Spanish samples. Each color corresponds to a different haplotype. The position of close populations was approximated to avoid overlapping pie charts; distances among resistant populations are indicated in kilometers. Populations ES-2 and ES-3 were collected from different fields within a radius of less than 5 km. The orange label indicates populations sampled in maize fields, whereas green labels refer to populations sampled on roadsides.

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(Fig. 1). All plants that survived the sulfonylurea herbicide had the point mutation Pro-197-Thr, suggesting that it might confer resistance to sulfonylureas but not to imidazolinones. Similar to what was observed in population ES-7, the Pro-197-Thr amino acid substitution has been reported to confer resistance to chlorsulfuron and chloransulam-methyl (two sulfonylureas) but not to imazethapyr in *Raphanus raphanistrum*.[38]

The third target-site mutation found in Spanish populations (ES-1 and ES-4) was the Asp-376-Glu, which is reported for the first time in European *A. palmeri* populations. This mutation was first discovered in an Argentinean *A. palmeri* population resistant to bispyribac-Na, flucarbazone-Na and chlorimuron-ethyl, and with low resistance to imazethapyr.[39] Our results suggest that this mutation confers resistance to thifensulfuron-methyl but not to imazamox, as observed in other species.[38] The presence of target-site mutations not clearly associated with resistance to imazamox in populations ES-1, ES-4 and ES-7 is in accordance with the lower resistance to this herbicide observed in the other populations studied.

The selection of *A. palmeri* populations in Spain that harbor different mutant *ALS* alleles must be linked to the various environmental contexts in which they were gathered. Although plants from maize fields were subjected to agricultural practices involving the use of sulfonylurea herbicides, those from roadsides did not face ALS inhibitor selection pressure. As a result, populations from roadsides display distinct and lower resistance profiles than other populations. Notably, the roadside population ES-7 had a resistance pattern similar to that of maize-collected populations, with a high survival rate to thifensulfuron-methyl and a low survival rate to imazamox. It is plausible that this population was dispersed by trucks transporting feed from a grain bin dryer that is located nearby the sampling site. Another finding of this study was the presence of a large number of imazamox-resistant plants without the point mutation 574. The other point mutations that clearly confer resistance to imazamox, namely Ala-122, Ser-653 and Gly-654, were not investigated because they were out of the focus of the article. The presence of such mutations or non-target-site-resistance (NTSR) based mechanisms cannot be excluded in these individuals. Previous studies have demonstrated the presence of distinct NTSR mechanisms in Palmer amaranth.[40] However, further investigations are required to confirm the presence of NTSR mechanisms in these populations.

4.2 Origin and spread of 574-Leu haplotypes

The AS-PCR and its variants are widely adopted techniques for screening weeds for resistance-endowing mutations because they are cheap and easy to interpret. However, their application has typically been limited to obtaining a clear-cut result, namely, the presence or absence of an allele-specific amplification band.[32,41] To the best of our knowledge, there are no examples of direct haplotyping of AS-PCR products using Sanger sequencing. A similar method used to obtain allele-specific haplotypes employed a nested allele-specific primer only to sequence standard PCR amplicons of heterozygous samples.[24] However, this approach is expected to have a lower level of specificity compared to the method we proposed here. Indeed, the validation performed using the dataset obtained by NGS highlighted that our method is very reliable, and the results are robust, despite the use of a non-proofreading *Taq* polymerase. We did not use a high-fidelity (HF) DNA polymerase because proofreading polymerases correct mismatches of allele-specific primers, thus eliminating primer specificity.[42] We opted for using an allele-specific forward primer designed on the 574 allelic variants in place of 197 and 376 for two reasons. Firstly, the 574 mutation was the most prevalent among the populations examined. Secondly, the resulting haplotypes spanned a longer non-coding sequence, thus ensuring a higher genetic variability, as indicated by the number of SNPs. Although NGS techniques have dramatically improved SNP discovery, data analysis requires trained personnel. Moreover, if the number of samples and targets to be analyzed is relatively small, single-gene Sanger sequencing is a more cost-effective option.[25] While interpreting chromatograms is a common skill among weed scientists, doing so for a dataset much larger than the one examined here seems impractical because it is not automated. Although numerous NGS analysis tools exist,[43] including graphical user interface (GUI)-based tools,[44] there are only a few examples of similar tools for Sanger trace analyses that are only partially GUI-based.[45] We interpreted the data with the following assumptions: (a) haplotypes differing by several SNPs (> 10%) originated from independent selection events; and (b) the presence of the same mutant haplotype within different populations indicates gene flow and non-independent selection of the same mutation in identical WT *ALS* haplotypes. The Italian and Spanish populations had no identical haplotypes, suggesting that the selection events that originated them were independent, and no gene flow occurred between the two groups. In *A. tuberculatus*, a dioecious species similar to *A. palmeri*, gene flow had been estimated to decay by 90% within 100 m from the pollen source.[46] Similarly, pollen-mediated gene flow is expected to play a very marginal role in the evolution of resistance in *A. palmeri*. Therefore, it is plausible that seed dispersal is the main mechanism responsible for resistance spreading in *A. palmeri*. Two dispersal patterns have been observed in the haplotype diversity maps of both countries: a short-range and a long-range dispersal pattern. The presence of the same haplotype within populations localized within a short-range distance of about 30 km can be explained by seed dispersal mediated by farm machinery. This is the case for the Italian populations IT-1, IT-2 and IT-3, or the Spanish populations ES-1 and ES-4. Seed dispersal mediated by farm machinery is not feasible for the long-range dispersal observed for the Italian populations IT-1 and IT-4, and for the Spanish populations ES-1 and ES-3, or ES-2 and ES-4. In contrast to what was hypothesized for the evolution of ACCase mutant haplotypes in *Alopecurus myosuroides*,[20] it is unlikely that the same mutation evolved independently in identical WT haplotypes, at least for the *A. palmeri* European populations described here. This is because *A. palmeri* is not native to Europe and is mainly reported as a casual alien species outside the agricultural habitat,[11] with populations that are normally very small compared to their native range. Additionally, there is no history of herbicide selection pressure on this species in the continent.

It is therefore more probable that the evolution of mutant haplotypes occurred in their native range and that they were dispersed within Europe. A similar situation was observed for ALS-resistant *A. tuberculatus*[31] and, more recently, with glyphosate-resistant *A. palmeri* in Spain.[17] The specific pathway of introduction into Europe is not yet known,[47] but it is possible that infestations of *A. palmeri* in Italian soybean fields may be linked to the use of non-certified soybean grain contaminated with *A. palmeri* seeds. Non-certified soybean grains are often used by farmers because they ensure decent yields despite the low production cost. However, non-certified maize seeds are less commonly used because most maize cultivars are hybrids, and their progeny is sterile. Therefore, the repeated introduction of herbicide-resistant *A. palmeri* seeds into Spanish maize fields

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may be due to contaminated cattle feed, as reported in Minnesota in 2018.[48] The high diversity observed between very close populations supports the hypothesis of an independent and repeated introduction of resistant biotypes from outside Spain. For instance, ES-2 and ES-3 collected in the same area (less than a 5 km radius) showed different haplotypes, whereas ES-5 and ES-6 share a unique mutant haplotype. Since *A. palmeri* is a cross-pollinated species, the sequences obtained by AS-PCR of 574-Trp were expected to be very genetically different. Instead, they resulted to be quite conserved (see the NJ tree in Fig. 3). This might be due to the presence of additional upstream mutations along the chromosome carrying the WT tryptophan at position 574: the presence of such mutations would have caused the downstream sequence to be more conserved than expected.

5 CONCLUSIONS

For the first time, the genetic basis and evolutionary history of resistance to ALS-inhibiting herbicides is studied in *A. palmeri* from Europe. Our data revealed that resistance is linked to a single mutant *ALS* allele in Italy (574-Leu), and three distinct mutant alleles in Spain (197-Thr, 376-Glu, 574-Leu). However, it is possible that other mechanisms of resistance exist, and further investigations are necessary.

The genetic analyses revealed that ALS-resistant *A. palmeri* populations were introduced independently to Italy and Spain, resulting in different haplotypes in both countries. Agricultural machinery and contaminated feedstock were supposed to cause seed dispersal at a short- and long-range, respectively. Given the high survival rate of *Amaranthus* seeds to animal digestion,[49] careful evaluation of low-cost animal feed sources is recommended if the source crop is susceptible to infestations. The haplotyping method proposed in this study offers a cost-effective approach for determining potential genetic connections between newly discovered populations and previously known ones.

As *A. palmeri* is highly invasive, it is crucial to implement effective management strategies in both countries to limit its establishment. This includes taking special care to prevent the introduction of *A. palmeri* through contaminated grain or animal feed, and conducting accurate monitoring to quickly eradicate plants and prevent proliferation. The recent discovery of glyphosate-resistant biotypes on Spain's roadsides emphasizes the importance of adopting integrated weed tactics to reduce herbicide selection pressure.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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