Molecular characterization of herbicide resistance in Palmer amaranth (*Amaranthus palmeri* S. Wats) populations in South Africa

By

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DECLARATION

I, Nondunduzo Adelaide Simelane declare that the dissertation, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own work and has not been submitted by me for a degree at this or any other tertiary institution.

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Nondunduzo Adelaide Simelane

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Date

TABLE OF CONTENTS

DECLARATION i
TABLE OF CONTENTS ii
ACKNOWLEDGEMENTS v
LIST OF FIGURES vi
LIST OF TABLES viii
ABBREVIATIONS AND SYMBOLS ix
SUMMARY xi
COMPOSITION OF DISSERTATION xii
CHAPTER ONE 1
LITERATURE REVIEW 1
1.1 Introduction 2
1.2 *Amaranthus palmeri* S.Watson 3
1.3 *Amaranthus tuberculatus (=A. rudis)* 5
1.4 *Amaranthus hybridus* (syn:quitensis) 7
1.5 *Amaranthus retroflexus* L. 8
1.6 Impact on agriculture 9
1.7 Development of herbicide resistance in *Amaranthus* weeds 12
1.8 Mechanisms of herbicide resistance to different sites of action 13
1.8.1 Resistance to EPSPS inhibitors 13
1.8.2 Resistance to ALS inhibitors 14
1.8.3 Resistance to PPO inhibitors 15
1.8.4 Resistance to PSII inhibitors 16
1.8.5 Resistance to HPPD inhibitors 17
1.8.6 Resistance to Auxin mimics inhibitors 17
1.8.7 Resistance to VLCFA inhibitors 18
1.8.8 Microtubule assembly inhibitors resistance 18
1.9 Multiple resistance 19
1.10 Management strategies of herbicide resistant *Amaranthus* species 20
1.11 Population and genetic diversity studies 21
1.11.1 Interspecies hybridization 21
1.11.2 Molecular markers in *Amaranthus* population genetic studies 21
1.11.3 Microsatellite markers 22
1.12 Problem statement 23
1.13 Aim and objectives 23

CHAPTER II..24
MOLECULAR IDENTIFICATION AND TARGET SITE CHARACTERIZATION OF *AMARANTHUS PALMERI* POPULATIONS IN SOUTH AFRICA 24
2.1 Introduction..25
2.2 Materials and Methods..27
2.2.1 Plant material 27
2.2.2 DNA extraction..27
2.2.3 Confirmation of Amaranthus Identity...27
2.2.4 PCR amplification and sequencing of the *ALS* and *EPSPS* genes 28
2.2.4 Determination of *EPSPS* gene copy number by qPCR..29
2.2.5 *PPO* gene characterization..29
2.2.5.1 dCAPS assay 29
2.2.5.2 Investigating the presence of G210 deletion 30
2.3 Results..31
2.3.1 Confirmation of Amaranthus identity...31
2.3.2 *ALS* gene Sequencing..33
2.3.3 Partial *EPSPS* gene sequencing 36
2.3.4 *EPSPS* gene copy number..37
2.3.5 *PPO* gene characterization..37
2.3.6 Multiple herbicide resistance 39
2.4 Discussion..39
2.4.1 Confirmation of Amaranthus identity...39
2.4.2 Sequencing of the *ALS* gene...40
2.4.3 *EPSPS* gene sequencing and copy number 41
2.4.4 Characterization of the *PPO* gene..42
2.4.5 Multiple herbicide resistant Palmer amaranth..43
2.5 Conclusion 43
CHAPTER III 45
GENETIC DIVERSITY OF THE INTRODUCED PALMER AMARANTH POPULATION REVEALED BY SIMPLE SEQUENCE REPEATS (SSR) MARKERS: PRELIMINARY STUDY. 45
3.1 Introduction..46
3.2 Materials and Methods..47
3.2.1 Plant material 47
3.2.2 SSR genotyping 47
3.2.3 Genetic data analysis..49
3.3 Results..49

3.3.1 Microsatellite marker genotyping 49
3.3.2 Microsatellite marker diversity 51
3.3.3 Genetic variation revealed by SSR markers 51
3.4 Discussion 52
3.5 Conclusion 53
CHAPTER IV 54
GENERAL DISCUSSION AND FUTURE RECOMMENDATIONS 54
SUPPLEMENTARY DATA 57
REFERENCES 69

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LIST OF FIGURES

Figure 1.1. (**A**) Global distribution of Palmer amaranth (gbif.org) (**B**) Palmer amaranth plant growing in a field in South Africa. (**C**) The petiole: leaf blade comparison (Vorster B. J, University of Pretoria) (**D**) Comparison of the inflorescences (left to right, smooth pigweed (*A. hybridus*), Palmer amaranth (female) and Palmer amaranth (male))……………………...4

Figure 1.2. A. The current global distribution of common waterhemp (gbif.org) **B**. Young vegetative plant **C**. Inflorescence (Ackley B., invasive.org)…………………………………...6

Figure 1.3. A. Current global distribution map (gbif.org) **B**. Vegetative stage (Ackley B., invasive.org) **C**. Inflorescence (Vorster B.J, University of Pretoria) …………………………7

Figure 1.4. (**A**) Current global distribution of *A. retroflexus* (gbif.org) (**B**) Broad leaf form (**C**) Inflorescence (Videki R. Doronicum., Bugwood.org)……………………………………....9

Figure 1.5. Weed species that have developed resistance to multiple herbicide Modes of action globally. The four *Amaranthus* species are among the top 15 weeds to develop multiple resistance (Heap, 2021)……………………………………………………………………11

Figure 1.6. Schematic representation of the ALS gene. Common mutations are found in five conserved domains (A-E), [Adapted from Tranel and Wright (2002)]………………………..15

Figure 2.1. Phylogenetic tree of *Amaranthus* genus using ITS region. The species from the two populations used in this study are in bold. Coloured in green is the *Amaranthus palmeri* clade. Strong bootstrap values (>70%) support the branching on the external nodes……….33

Figure 2.2. *ALS* gene BE domain amino acid alignment of all accessions used in this study. Polymorphisms are marked in red. The top panel shows the Trp-574-Leu mutation and the bottom panel shows the Ser-653-Asn mutation. The sequence KT833339.1, an ALS sensitive Palmer amaranth sample from GenBank was used as a reference……………………………35

Figure 2. 3. Amino acid alignment of the partial *EPSPS* gene for all accessions used in the study**.** Polymorphisms are shown by different colour amino acid. The glyphosate sensitive Palmer amaranth accession (KC169785.1) and glyphosate resistant smooth pigweed accession (MG595170.1) were used as references…………………………………………………36

Figure 2.4.Variablity in relative *EPSPS:ALS* gene copy number in all *Amaranthus* accessions used in this study. Gene copy number is presented in descending order……………………....37

Figure 2.5. Gel image of dCAPS assay for Northern Cape population. Accession labels were shortened for clear presentation of results (N1=NC1_A. Pal, N2= NC2_A. Pal and so on). Both undigested (-) and digested (+) PCR products are shown. (A) represents Arg-98-Met and B represents Arg-98-Gly assay. A 100bp DNA ladder (MW) (Thermo Fischer Scientific, RSA) was used and the PCR products fall between ~400 and 350 bp fragments…………………....38

Figure 2.6. Venn diagrams depicting the linkage of the accessions with multiple resistance. All 36 accession from both populations (KZN and NC) were investigated for the TSR to glyphosate, ALS, and PPO inhibitors……………………………………………………...39

Figure 3.1. Multiplexed amplification of the SSR marker sets on the three Amaranthus species used in this study. Three accessions from each species *A. palmeri* (A. P), *A. hybridus* (A.H) and *A. standleyanus* (A.S) were amplifies and used for polymerase chain reaction optimization…………………………………………………………………………….49

Figure 3.2. Allele frequencies observed in all four loci during SSR marker analysis………51

LIST OF TABLES

Table 1.1. Maize, soybean, and cotton yield losses as affected by the four *Amaranthus* species…………………………………………………………………………………..10

Table 1.2. Summary of the reported mechanisms of resistance to glyphosate in the investigated *Amaranthus* species………………………………………………………………………14

Table 1.3. A summary of multiple herbicide resistant populations of the four investigated Amaranthus species. (www.weedscience.org) 19

Table 2.1.Primers used in polymerase chain reaction and sequencing………………………31

Table 2.2 Species identity of all 36 accessions used in this study as determined by ITS region sequencing…………………………………………………………………………………32

Table 2. 3. A summary of the accessions with mutations in the BE domain of the *ALS* gene……………………………………………………………………………………34

Table 3.1. Microsatellite marker primers and their properties used for the analysis of genetic variation in this study. Markers were developed by Erika Viljoen (2018)……………………48

Table 3.2. Alleles scored at each locus for each accession of *Amaranthus* species. The zero (0) indicates that these alleles could not be scored…………………………………………50

Table 3.3. Genetic diversity parameters observed in the Palmer amaranth population…….52

ABBREVIATIONS AND SYMBOLS

A	Alanine
Ala	Alanine
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
cm	Centimetre
D	Aspartic acid
dCAPs	Derived cleaved polymorphism sequences
ddNTPs	Dideoxy nucleotides phosphates
dH_2O	Distilled water
DNA	Deoxy ribonucleic acid
E	Glutamic acid
G	Glycine
gDNA	Genomic deoxyribonucleic acid
Gly	Glycine
Ille	Isoleucine
L	Leucine
Leu	Leucine
M	Methionine
m^{-1}	Per metre
ml	Millimetre
Met	Methionine
Min	Minute

NaCl	Sodium chloride
ng	Nanogram
nM	Nanomolar
P	Proline
PCR	Polymerase chain reaction
Pro	Proline
R	Arginine
S	Serine
Sec	Second
Ser	Serine
Thr	Threonine
Trp	Tryptophan
V	Valine
Val	Valine
W	Tryptophan
w/v	Weight over volume
α	Alpha
β	Beta
Δ	Change in
µl	Microlitre
µg	Microgram
°C	Degree Celsius
%	Percentage

SUMMARY

Weeds such as Palmer amaranth (*Amaranthus Palmeri* S.Wats.) have over the years become problematic in the agricultural industry due to the proclivity to easily develop resistance to multiple herbicides. Reports of herbicide resistant Palmer amaranth invading most parts of North America and some parts of South America, Europe, Asia, and Africa are becoming more frequent. In 2018, A population of Palmer amaranth was found in a cotton field in the Northern Cape province of South Africa. A second population was also reported in 2019 in a field in the KwaZulu Natal Province of South Africa. Using molecular techniques, we set out to confirm the identity of the populations by sequencing the ITS region and to characterize the target site resistance mechanisms conferring resistance to Acetolactate (ALS), 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS) and protoporphyrinogen oxidase (PPO) inhibitors. Preliminary genetic diversity studies were also carried out using microsatellite markers. Single nucleotide polymorphisms (SNP's) were able to differentiate Palmer amaranth from smooth pigweed and *Amaranthus standleyanus* accessions. Resistance to ALS inhibitors was due to the amino acid change S653N in 9/29 accessions from the NC population and the presence of both the W574L (1/7) and S653N (7/7) amino acid changes in the KZN population. The two populations also differed in the EPSPS inhibitor resistance profiles as the NC population had the *EPSPS* gene duplication and the KZN population however had the rare amino acid change P106S but no gene duplication. Moreover, smooth pigweed accessions from the KZN population had the triple amino acid change TIPS-IVS. No target site resistance was observed in the *PPO* gene in both populations. Immediate genetic diversity was revealed by the microsatellite markers.

Findings of this study confirmed the introduction of two different herbicide resistant Palmer amaranth populations in South Africa based on their resistance profiles. This study therefore serves as reference for the South African Herbicide Resistance Initiative when devising management strategies for the introduced population of Palmer amaranth.

COMPOSITION OF DISSERTATION

Chapter 1 of this dissertation comprises of a literature review where we elaborately discuss the biology, distribution, and agricultural impact of the four most problematic Amaranthus species worldwide. The known mechanisms of resistance to commonly used herbicides which have evolved in the four species is also dealt with. Finally, we summarize on the genetic diversity studies that have been carried out on Amaranthus species.

In **chapter 2**, we employ molecular techniques to confirm the identity of two introduced populations of *Amaranthus palmeri* S. Wats. We further investigate the presence of target site mutations known to confer resistance to ALS, EPSPS and PPO inhibitors which these populations might possess. This is especially important in a suspected introduction as it gives direction on the types of management strategies to be employed to try to curb this weed.

Chapter 3 is a preliminary study with the aim of investigating the genetic diversity of the introduced populations using microsatellite markers developed by a previous student.

Chapter 4 gives the general discussions of the whole study. We look at how the study can be improved and how questions that arose during the study can be addressed in future studies. Literature consulted while working on this dissertation is listed in **references.**

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

The status of global food insecurity is alarming as the world population is currently 7.7 billion (www.worldometer.info) and is anticipated to reach 10 billion by 2050 (www.fao.org). This means greater demand for food and agricultural outputs, as well as increased pressure on other natural resources. In the 20th century, much attention has been directed into improving crop yield and less directed into crop protection. The greatest causes of row crop losses are biotic and abiotic stressors such as lack of water, extreme temperatures, lack of nutrients as well as pests, pathogens, and weeds (Savary et al., 2012).With the expansion of agriculture and the ease of movement worldwide, weeds have invaded new territories and have become difficult to control. Weed species from the Amaranthaceae family are among the most problematic in the United States of America (USA) and most parts of the world (Heap, 2021). This family consists of about 75 species of Amaranths commonly known as pigweeds divided into vegetable, grain, and weedy species. Species in this family are found in tropical, subtropical, and temperate regions worldwide (Trucco and Tranel, 2011). According to Heap (2021), numerous weed species in the *Amaranthus* genus have over the past years increased in invasiveness and severity, and are currently among the top 15 most problematic weeds worldwide. These weeds outgrow and compete with crops for sunlight, water, nutrients, and space. Moreover, weeds also harbour insects and pathogens and destroy native habitats (Chauhan, 2020). The *Amaranthus* species biology and ecological plasticity such as morphology, high growth rate, prolific seed production, extended seed emergence, drought tolerance and adaptability greatly contributes to their success as weeds (Steckel, 2007). Of more concern is that this genus has the ability to easily develop herbicide resistance. To date, several *Amaranthus* species, Palmer amaranth (*Amaranthus palmeri* S.Watson), common waterhemp (*A. tuberculatus* (=*A.rudis*)), smooth pigweed (*A. hybridus* (syn:quintensis)), redroot pigweed (*A. retroflexus*), spiny amaranth (*A. spinosus*), slender amaranth (*A. viridus*), Powell amaranth (*A .powellii*), livid amaranthu (*A .blitum (ssp.oleraceus*)), prostate pigweed (*A. blitoides*), tumble pigweed (*A.albus*) and red amaranth (*A. cruentus*) have developed herbicide resistances to commonly used herbicides. The first four above mentioned *Amaranthus* species have further developed multiple herbicide resistances and have spread, established, and require alternative control strategies (Heap, 2021). Control of these weed species using herbicides has been difficult and this is due, in part, to their congenital genetic variability.

This review will look at the biological, physiological, and reproductive traits of the four most problematic *Amaranthus* weeds. Global distribution, distinguishing features and herbicide resistance profiles will be also considered. Lastly, research that has been done on their genetic variability and propensity for hybridization with other Amaranthus species will be reviewed.

1.2 *Amaranthus palmeri* S.Watson

Palmer amaranth is a dioecious species in the *Amaranthus* genus. It originated from the Southwestern USA and Northern Mexico where it has a long history of its leaves and seeds used as a food source (Ward et al., 2013). It is currently considered the most aggressive *Amaranthus* weed in the world (Heap, 2021) and has spread and naturalized in counties such as Egypt, Israel, Madeira Island, Turkey, Cyprus and South Africa (Sukhorukov et al., 2021). Casual sightings of this weed in Japan, Luxembourg, Belgium, Germany, the Netherlands, Norway, the United Kingdom, and Sweden were also recorded (Figure 1.1A) (GBIF,2020; (Kistner and Hatfield, 2018). Herbicide resistant Palmer amaranth has been confirmed in over 28 USA states as well as in Israel, Mexico, Argentina, and Brazil (Berger et al., 2016; Küpper et al., 2017; Heap, 2021) in the past decade. Kistner and Hatfield (2018) estimated the potential global distribution of Palmer amaranth based on current and future climatic conditions. The authors concluded that the major maize production regions of Australia and Africa were suitable for its growth and therefore at high risk of Palmer amaranth establishment.

Palmer amaranths biology, physiology and reproduction gives it superiority over other weeds thus dubbed the name “superweed”. Being a summer annual species, it is characterized by aggressive growth as a single mature plant can reach a height of about three metres and accumulate a dry biomass of about five kilograms in seven months under favourable conditions (Bond and Oliver, 2006). The plant has a central reddish-green stem with several lateral branches. Young leaves (Figure 1.1B) of this species are lanceolate sometimes with a white V shaped variegation on the adaxial side, and usually become more ovate as they mature (Horak et al., 1994). The leaves are alternate, with petioles that are longer than the leaf blade (Assad et al., 2017). The petiole leaf blade length ratio is one of the main distinguishing features of Palmer amaranth.

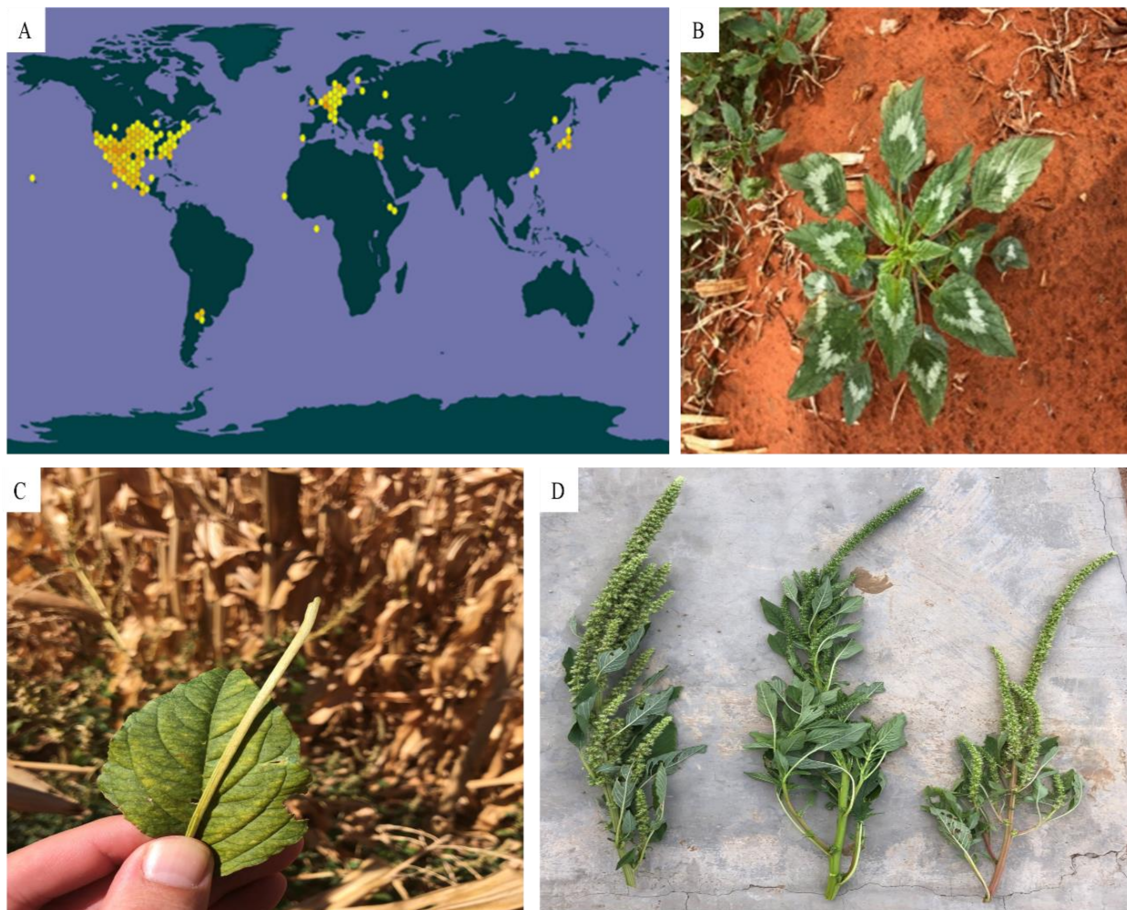

Figure 1.1. (**A**) Global distribution of Palmer amaranth (gbif.org) (**B**) Palmer amaranth plant growing in a field in South Africa. (**C**) The petiole: leaf blade comparison (Vorster B. J, University of Pretoria) (**D**) Comparison of the inflorescences (left to right, smooth pigweed (*A. hybridus*), Palmer amaranth (female) and Palmer amaranth (male))

The aggressive and highly competitive growth of this weed is partially due to its C4 photosynthetic pathway. This weed has the highest rates of photosynthesis (81 μmol $m^{-2}s^{-1}$) when compared to other *Amaranthus* species at very high temperatures of 42°C (Ward et al., 2013; Steckel, 2007). Palmer amaranths diaheliotropic leaves allows it to maximize photosynthesis even under unfavourable light conditions (Wright et al., 1999). This enables the weed to grow and outcompete row crops and other *Amaranthus* species. In addition, Palmer amaranth has a high-water use efficiency and this, together with the high photosynthesis rate enable the weed to be adaptable to a wide range of environmental conditions (Assad et al., 2017).

Palmer amaranth's invasiveness is aided mostly by the reproductive biology of the weed. This weed species is an obligate out-crosser, having male and female inflorescences on separate plants (Figure 1.1C). These inflorescences can be used as a distinguishing feature between male

and female plants (Assad et al., 2017; Ward et al., 2013). The male inflorescences are softer to the touch whilst the female ones feel stiffer and pricklier. Female plants produce between 200-600 thousand small viable seeds under favourable conditions which have an extended period of germination (Keeley et al., 1987). Large amounts of pollen are also produced by the male plants, and pollination is facilitated predominately by wind which allows the pollen to be transported longer distances from the source plant (Chahal et al.,2015). Dispersal of Palmer amaranths seeds is facilitated by strong winds, irrigation water, birds, as well as agricultural machinery (Norsworthy et al., 2014) . These dispersal methods allow this weed to invade places where it was previously not found. The spread and establishment of such a noxious weed should be highly monitored and Palmer amaranth should be put under zero tolerance threshold.

1.3 *Amaranthus tuberculatus (=A. rudis)*

Common waterhemp is another troublesome dioceous species in the *Amaranthus* weeds. Native to the Midwest USA, this species was originally found west of the Mississippi river ranging from Nebraska to Texas (Costea et al., 2005). Current distribution expands into 19 states in the USA and Canada (Figure 1.2A) where it is found in maize, soybean, cotton, sorghum, and pastures (Heap I, 2020).

This species is also a summer annual plant that can reach a height of about two metres and has smooth erect stems usually green or pinkish red in colour. The stems are branched and have terminal inflorescences that are in the form of linear spikes to panicles. The leaves are also smooth, long, and narrow with shorter petioles and this feature is used in morphological identification of this species (Costea et al., 2005; Horak et al., 1994) (Figure 1.2B).

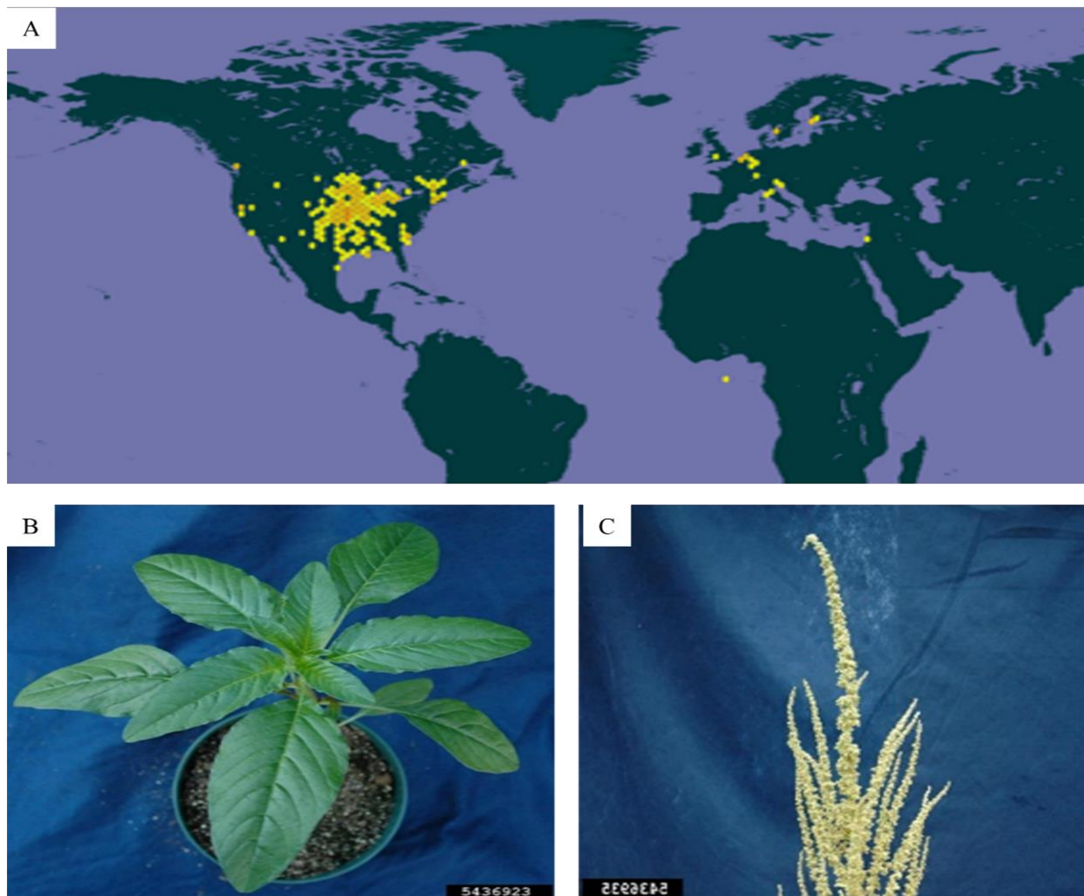

Figure 1.2. A. The current global distribution of common waterhemp (gbif.org) **B**. Young vegetative plant **C**. Inflorescence (Ackley B., invasive.org)

Like Palmer amaranth, this species also utilizes the C4 photosynthetic pathway and is characterized by rapid growth under favourable conditions. In terms of growth rate per day, photosynthetic capacity, and biomass accumulation, common waterhemp comes second after Palmer amaranth (Sellers et al., 2003).

Common waterhemp grows in a wide range of climatic conditions given that it is highly adaptable. Like most successful invasive *Amaranthus* weeds, common waterhemp produces copious amounts of seeds (>500 000) that contribute to a persistent seed bank (Sellers et al., 2003). The seeds are highly viable, and have sporadic germination periods (Ward et al., 2013). According to Heap (2021), common waterhemp is the second most troublesome *Amaranthus* weed species in the USA.

1.4 *Amaranthus hybridus* (syn:quitensis)

This weed falls under the *Amaranthus hybridus* species complex which consists of *A. hypochondriacus, A. cruentus, A. caudatus* and *A. quitensis* (Adhikary and Pratt, 2015). Weed species in this complex are difficult to distinguish morphologically and genetically because of their high hybridization status. The hybridus complex is cosmopolitan (Figure 1.3A). Costea et al. (2004) mapped the origin of smooth pigweed to eastern North America, Mexico, Central and South America. Currently, smooth pigweed is distributed worldwide where it has naturalised and considered as a weed or used as a leafy vegetable.

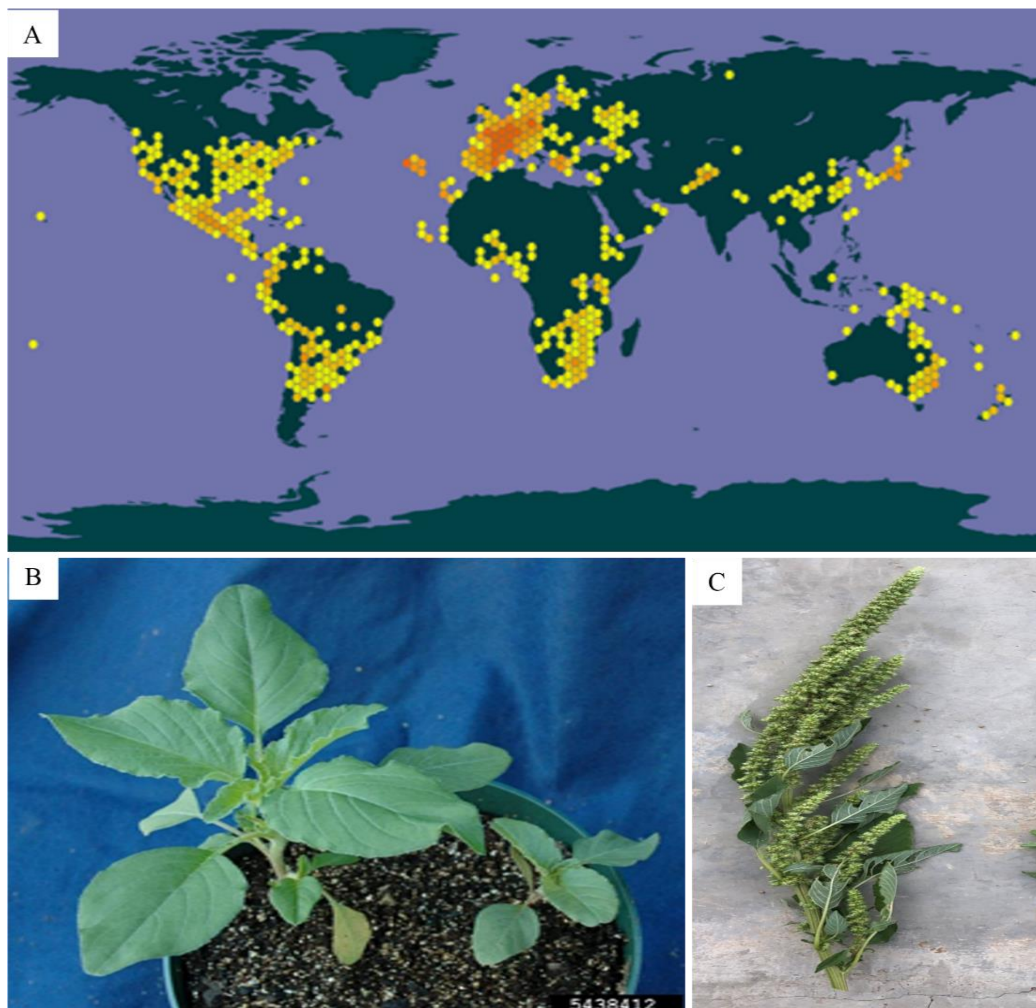

Figure 1.3. **A**. Current global distribution map (gbif.org) **B**. Vegetative stage (Ackley B., invasive.org) **C**. Inflorescence (Vorster B.J, University of Pretoria)

This erect summer annual herb can grow up to a height of about two metres. Stems of smooth pigweeds are often green in colour, branched and ribbed. Leaves (Figure 1.3B) are alternate and broadly ovate, rhombic ovate or lanceolate (Costea et al., 2004; Horak et al., 1994). Flowers are often numerous and green and are arranged in terminal inflorescences that are often

long and dense (Figure1.3C). This monoecious species is primarily self-pollinated, and wind is the main pollinator. Cross pollination leading to hybridization with other monoecious or dioecious species is also frequent (Trucco and Tranel, 2011). Seeds produced by one inflorescence under favourable conditions are about 100 000 and can remain viable for extended periods of time when buried in the soil (Sellers et al., 2003). Smooth pigweed utilizes the C4 photosynthetic pathway which allows it to be highly adaptable in diverse environmental conditions (Assad et al., 2017).

1.5 *Amaranthus retroflexus* L.

Redroot pigweed is currently the fourth most troublesome *Amaranthus* weed in agronomic crops in the USA and other parts of the world (Heap, 2021). Originally found in Central and Eastern North America this weed has now spread to most parts of the world (Figure 1.4A).

This edible herbaceous plant can grow up to two metres tall and is characterized by a pinkish taproot and a stem which is either light green or pinkish which can be branched or unbranched (Iamonico, 2010). Longitudinal ridges and white fine hairs are found on the main stem. Leaf shape (Figure 1.4B) varies from cordate, ovate to rhomboidal with entire or undulate margins (Costea et al., 2004; Horak et al., 1994). Leaf hairs are found along the purplish veins on the abaxial side. This monoecious species has a main terminal panicle with whitish green inflorescences (Figure 1.4C). Smaller inflorescences are sometimes found on the axils of the middle to upper leaves (Assad et al., 2017). Pollination is aided by wind and like other weedy *Amaranthus* species, it is a prolific seed producer and is highly adaptable (Sellers et al., 2003).

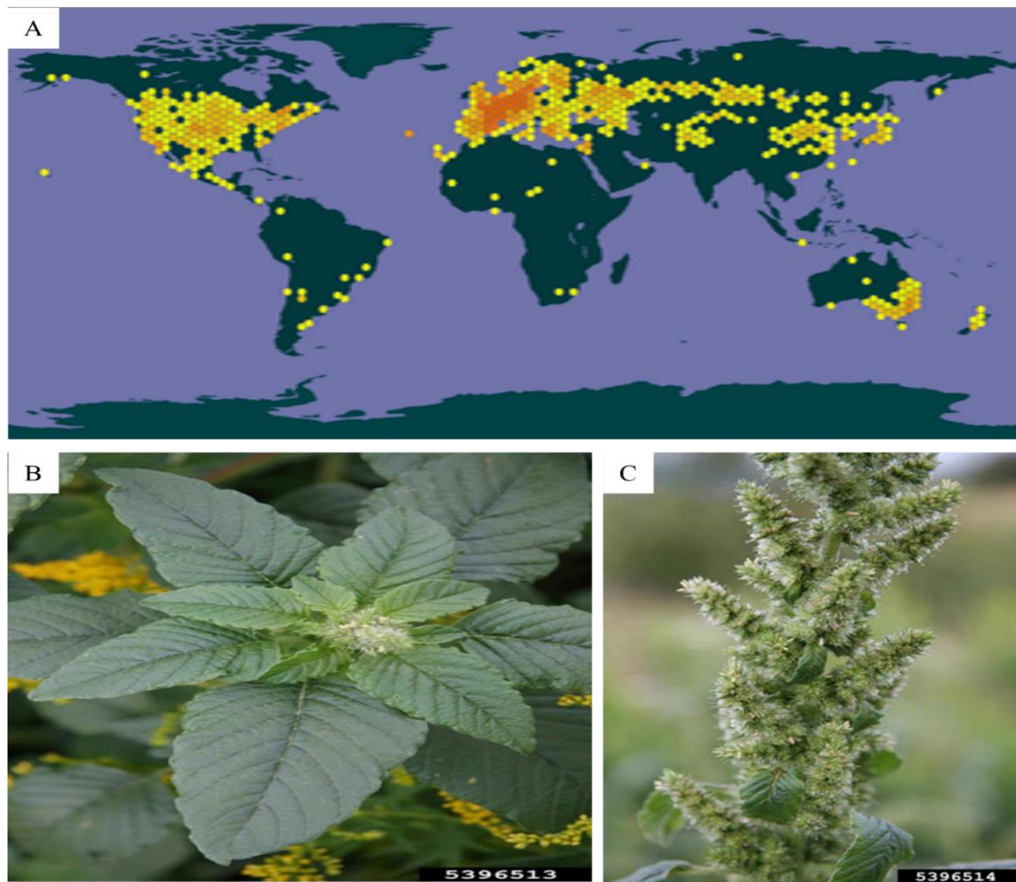

Figure 1.4 (**A**) Current global distribution of *A. retroflexus* (gbif.org) (**B**) Broad leaf form (**C**) Inflorescence (Videki R. Doronicum., Bugwood.org)

1.6 Impact on agriculture

Amaranthus weeds have been confirmed to reduce yields quality and quantity in crops such as sorghum (*Sorghum bicolor L.*), maize (*Zea mays* L.), cotton (*Gossypium hirsutum L.)*, soybean (*Glycine max L.*), and sweet potato (*Ipomoe*a *batatas L*) (Massinga and Currie, 2002; Morgan et al., 2001). These highly competitive weeds have been shown to cause major crop losses directly and indirectly. Direct yield losses as a result of crop-weed competition are dependent on the weed emergence period, weed density and environmental variations (Steckel and Sprague, 2004). Bensch et al. (2003), reported maximum yield losses when *Amaranthus* weeds emerged with the crop. This is because *Amaranthus* weeds have a high daily growth rate, therefore outcompete the crop, accumulate more biomass and shade the crop. Table 1.1 summarizes yield losses caused by the four *Amaranthus* species in major crops in the world.

Table 1.1. Maize, soybean, and cotton yield losses as affected by the four *Amaranthus* species

Crop	*Amaranthus* species	Time of weed emergence	Weed density	Yield losses (%)	References
maize	*A. palmeri*	Along with crop	0.5-8 plants m^{-1}	11 to 91	(Massinga and Currie, 2002)
	A. tuberculatus	V4	_	13 to 59	(Steckel and Sprague, 2004)
	A. retroflexus	2-4 leaf stage	0.5-8 plants m^{-1}	5 to 34	(Knezevic et al., 1994)
	A. hybridus	Along with crop	10-15 cm band	39	(Moolani et al., 1964)
Soybean	*A. palmeri*	Along with crop	8 plants m^{-1}	79	(Bensch et al., 2003)
	A. tuberculatus	Along with crop	8 plants m^{-1}	56	(Bensch et al., 2003)
	A. retroflexus	Along with crop	8 plants m^{-1}	38	(Bensch et al., 2003)
	A. hybridus	Along with crop	10-15 cm band	55	(Moolani et al., 1964)
Cotton	*A. palmeri*	Along with crop	1 -10 plants m^{-1}	13-54	(Morgan et al., 2001)
	A. tuberculatus	_	_	_	_
	A. retroflexus	_	_	_	_

Yield reductions are not the only losses recorded in *Amaranthus* infested fields. Since these weeds grow aggressively up to three metres in height, the bushy weeds also interfere with harvesting. Mechanical losses have been reported in fields with high densities of *A. palmeri* (Morgan et al., 2001). According to Smith et al. (2000), the weed stems become stuck between the rotating brushes of the stripper heads delaying the harvesting process in cotton. At higher weed densities, extraneous plant material harvested with the crop can reduce the quality of the harvest, which then affects marketability. Complete crop failure can also result in highly infested fields, causing severe economic losses (Norsworthy et al., 2014).

Another competitive ability of Palmer amaranth and other *Amaranthus* weeds is allelopathy. The weeds exude secondary chemicals usually produced for their own defence that influence the growth and development of neighbouring plants. Both above and below plant parts of *Amaranthus* weeds contain these chemicals and the extent of competition is dependent on the concentration of the secondary compounds in the ground (Chahal et al., 2015). For example, residues from one big plant or a lot of small plants in one area will exert more allelopathic effects than small or sparsely spaced plants. Inhibitory effects on seed germination and growth of vegetables and maize caused by allelopathic chemicals from *Amaranthus* weeds have been well documented (Menges, 1988; Mlakar et al., 2012).

Even with their aggressive growth rate, competitive nature, high seed production and high adaptability, these weeds could not be regarded as the most troublesome if they were controllable by herbicides. The main reason for these noxious weeds to be regarded as 'super weeds' is their ability to easily develop herbicide resistance to several herbicide compounds commonly used in agronomic fields in the USA and most parts of the world.

Extensive cultivation of crops genetically engineered to be herbicide (mostly glyphosate) resistant has led to the overuse and heavy reliance on the same kinds of herbicides for weed control, leading to the evolution of herbicide resistant weeds. Weedy amaranths have evolved resistance to multiple herbicides and are currently resistant to eight sites of action (SOA) in total (Figure 1.6). They have been confirmed to be resistant to herbicides targeting; 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS), acetolactate synthase (ALS), Photosystem II (PSII), hydroxyphenylpyruvate dioxygenase (HPPD), protoporphyrinogen oxidase (PPO), microtubule assembly, synthetic auxins, and very long fatty acid synthesis (VLFA) inhibitors (Heap, 2021; Berger et al., 2016; Nandula et al., 2012) . The mechanisms by which these *Amaranthus* species has developed resistance to some of these classes of herbicides will be discussed more in detail.

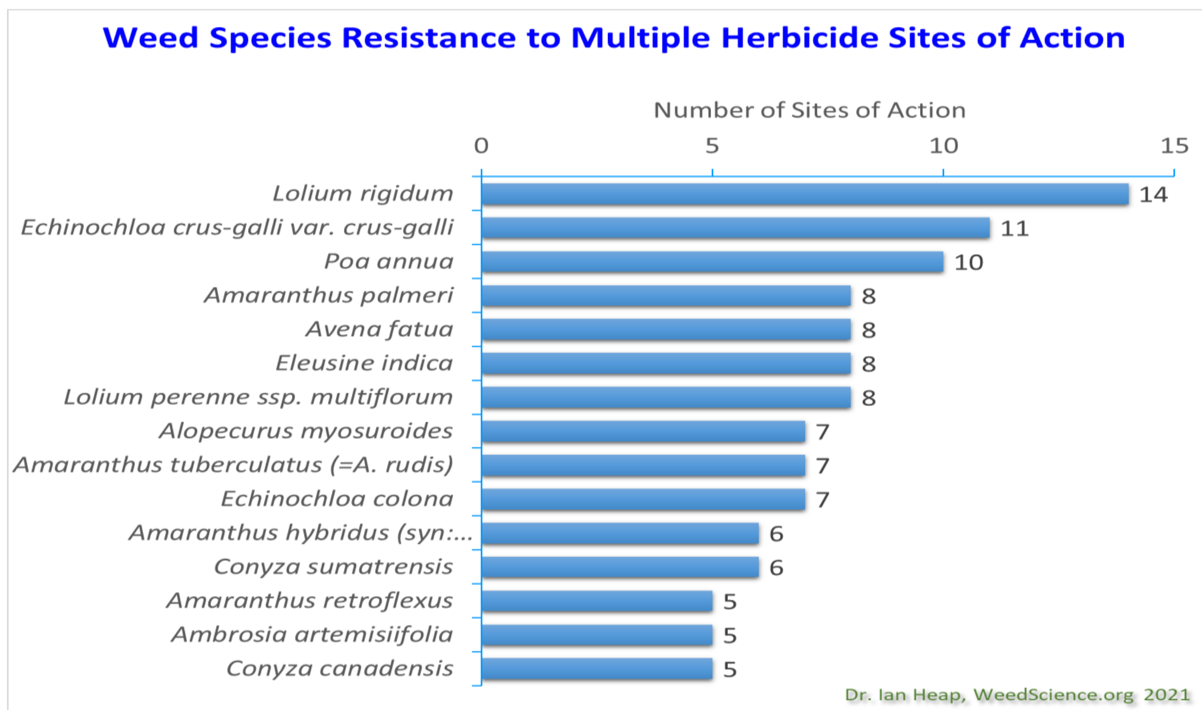

Figure 1.5. Weed species that have developed resistance to multiple herbicide Modes of action globally. The four *Amaranthus* species are among the top 15 weeds to develop multiple resistance (Heap, 2021)

1.7 Development of herbicide resistance in *Amaranthus* weeds

Weeds growing at certain places and or time where they were not wanted have been controlled by synthetic herbicides for more than 70 years (Heap, 2021). Herbicides kill plants by inhibiting certain essential metabolic or bioenergetic pathways by interacting with a crucial target enzyme (Böger, 2003). The adoption and use of herbicides reduced the cost of production, increased yields and allowed for the introduction of more environmentally friendly tillage systems. With the over reliance and incorrect use of herbicides, weeds were subjected to selective pressure and developed herbicide resistance to most of the used inhibitors (Heap and Duke, 2017). Resistance to herbicides is mainly because of two types of mechanisms, target site resistance (TSR) and non-target site resistance (NTSR). Target site resistances are due to a single or several mutations in the DNA sequence of the gene encoding the herbicide target enzyme which causes amino acid changes and subsequently changes in the protein structure of the target enzyme (Tranel and Wright, 2002; Murphy and Tranel, 2019). This in turn decreases the ability of the herbicide binding to the active site of the enzyme allowing the protein to continue to function in the presence of the herbicide. Most recently another type of TSR was discovered in glyphosate resistant Palmer amaranth and this is gene copy number proliferation (Gaines et al., 2010). This mechanism causes resistance by amplifying the copies of the target enzyme thus outnumbering and diluting the molecules of the herbicide absorbed leading to the normal functioning of the uninhibited enzyme and survival of the weed.

Non target site resistance is a mechanism that lessens the amount of herbicide before it reaches the enzyme target site (Jugulam and Shyam, 2019). Weeds achieve this by reducing absorption (penetration) and translocation, increasing herbicide metabolism (detoxification) and increasing herbicide sequestration compounds (Nakka et al., 2017b; Singh et al., 2018). Herbicide metabolism involves the rapid degradation of the herbicide actives by the plant's natural enzymes. Four key enzyme families have been identified to play this role namely, cytochrome P450 monooxygenases (P450), glutathione S-transferase (GSTs), ABC transporters and glucosyltransferases (GTs) (Suzukawa et al., 2021). NTSR are complex and their genetic basis and inheritance are currently not well understood. This resistance mechanism can endow cross-resistance to multiple herbicides with different modes of action (Jugulam and Shyam, 2019).

The two resistance mechanisms can sometimes co-exist within the same plant or same population. This is most often due to successive selection by herbicides and cross pollination between two populations with different mechanisms (Gaines et al., 2020). Co-existence of TSR

and NTSR mechanisms to EPSPS inhibitors have been observed in common waterhemp (Nandula et al., 2013). Palmer amaranth populations from Kansas were found to have rapid detoxification coupled with increased gene expression in response to HPPD inhibitors (Nakka et al., 2017c). Multiple resistance and co-existence of resistance mechanism within a weed population/species or individual plant greatly narrows the choices of herbicides that farmers can use (Jugulam and Shyam, 2019; Gaines et al., 2020). This poses very serious weed control and management concerns.

1.8 Mechanisms of herbicide resistance to different sites of action

1.8.1 Resistance to EPSPS inhibitors

Glyphosate is the only active ingredient that inhibits the EPSP synthase to have been commercialized. It is a broad spectrum systemic and non-selective pesticide widely used to control weeds (Duke, 2017). This herbicide has been adopted to control weeds in Roundup Ready maize, cotton and soybean in 25 countries including the USA, Brazil, Argentina and South Africa (Green and Owen, 2011). Its effectiveness, flexibility and ease of use attracted more growers into adopting glyphosate resistant crops (Gage et al., 2019). Glyphosate works by inhibiting the EPSP enzyme synthesis in plants. This enzyme is required in the shikimate pathway which provides precursors for the synthesis of the aromatic amino acids, phenylalanine, tryptophan, and tyrosine. Glyphosate blocks this pathway resulting in the lack of the amino acids needed for protein synthesis which eventually leads to plant death (Chahal et al., 2017).

Resistance to glyphosate in these weeds is prevalently because of TSR (amino acid substitutions) (Dominguez-Valenzuela et al., 2017b; García et al., 2019) and most recently *EPSPS* gene duplication (Table 1.2). First recorded in a resistant population in Georgia USA (Gaines et al 2010), the *EPSPS* gene duplication mechanism was novel in Palmer amaranth but has since spread to other *Amaranthus* weeds. Glyphosate resistant plants with an increased copy number of the *EPSPS* gene are able to synthesize these amino acids even when the herbicide has been applied as there is a surplus of copies uninhibited by the herbicide (Fernández-Escalada et al., 2017; Nandula et al., 2014; Chahal et al., 2017). These copies are spread throughout the genome in large (~400 kbp) heritable nuclear vehicles called extrachromosomal circular DNA (eccDNA)(Koo et al., 2018; Molin et al., 2020).

The minimum number of *EPSPS* gene copies needed to confer resistance to the recommended field application rate of glyphosate is still unknown. Different biotypes of glyphosate resistant

Palmer amaranth from different states in the USA have different copy numbers ranging from 8 -160 (Mohseni-Moghadam et al., 2013; Chahal et al., 2017; Gaines et al., 2010). A study by Singh et al. (2018), investigated whether gene copy number is correlated with resistance level to glyphosate reported that highly resistant biotypes had more *EPSPS* gene copies, and that the injury declined by 4% with each additional gene copy. These differences in the *EPSPS* gene copy number needed to confer resistance to field level applications have not yet been thoroughly investigated, though it has been hypothesized that genetic, environmental and or plant related factors as well as time of exposure to glyphosate may contribute to the varying copy numbers (Singh et al., 2018; Mohseni-Moghadam et al., 2013).

In addition to these TSR mechanisms, reduced translocation and rapid metabolism of glyphosate have been reported in Palmer amaranth (Palma-Bautista et al., 2019) and common waterhemp (Nandula et al., 2013).

Table 1.2. Summary of the reported mechanisms of resistance to glyphosate in the investigated *Amaranthus* species

***Amaranthus* species**	**Resistance mechanism(s)**					**References**
	TSR				**NTSR**	
	Thr102Ile	Ala103Val	Pro106Ser	EPSPS duplication		
A. palmeri	-	-	√	√	Reduced translocation	(Gaines et al., 2010; Dominguez-Valenzuela et al., 2017a; Palma-Bautista et al., 2019)
A. tuberculatus	-	-	√	√	Reduced translocation	(Nandula et al., 2013; Bell et al., 2013)
A. hybridus	√	√	√	-	-	(García et al., 2019; Perotti et al., 2019)

√ Mutation is present in the species.
-Mutation is absent in the species.

1.8.2 Resistance to ALS inhibitors

The *ALS* gene is the target site to over 50 commercial herbicides active ingredients Tranel and Wright (2002) grouped into five classes; imidazolinones (IMIs), triazolopyrimides (TPs), sulfonylureas (SUs), sulfonylaminocarbonyltriazolinones (SCTs) and pyrimidinylthiobenzoates (PTBs) (Nakka et al., 2017c). These groups of herbicides are mostly used because they control a broad spectrum of weeds, are low cost and they are applied at low use rates which makes them fairly safe for mammals and the environment (Nakka et al., 2017c).

Over the years, more weeds have developed resistance to this class of herbicides than any other class including glyphosate (Tranel and Wright, 2002; Heap, 2021).

Acetolactate synthase enzymes are needed in the synthesis of the branched amino acids leucine, isoleucine, and valine. Acetolactate synthase inhibitors starve the plant of these amino acids leading to the plant's death (Tranel and Wright, 2002). Resistant weeds have the TSR mechanism where mutations in the *ALS* gene modify the binding site. This makes it difficult for the inhibitor to fit into the active site, thus allowing the weed to continue synthesizing the branched amino acids. Resistance levels varying from 30 - 3200-fold have been reported depending on the type of substitution (Molin et al., 2016b; Patzoldt and Tranel, 2007; Nakka et al., 2017c). Six common amino acid changes in the *ALS* gene are known to confer resistance to ALS inhibitors in *Amaranthus* species (Figure 1.6). Each amino acid mutation confers resistance to a certain class of ALS inhibitors for example, the trypophan-574-leucine have been shown to confer cross resistance to most herbicides across all five classes (Molin et al., 2016b). Nakka et al. (2017c), also reported NTSR in the form of enhanced metabolism via the cytochrome P450 in Palmer amaranth from Kansas, USA.

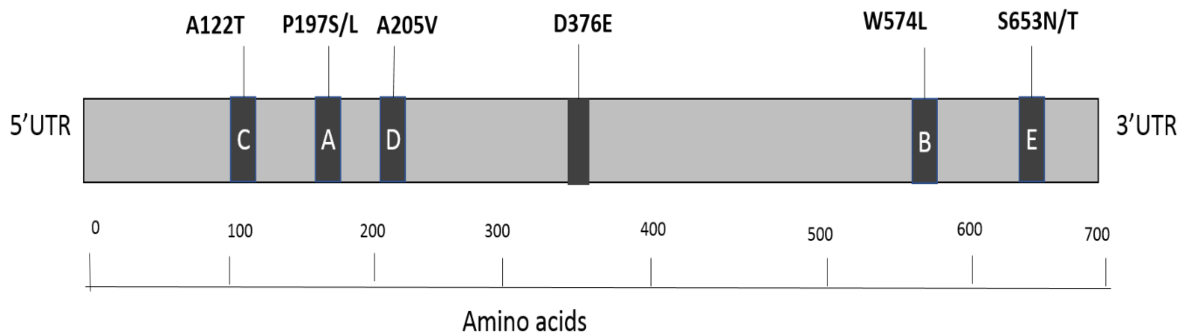

Figure 1.6. Schematic representation of the ALS gene. Common mutations are found in five conserved domains (A-E), [Adapted from Tranel and Wright (2002)]

1.8.3 Resistance to PPO inhibitors

Protoporphyrinogen oxidase inhibitors such as fomesafen, saflofenacil, flumioxazin and sulfentrazone are long residual, broad spectrum, pre- and post- emergence herbicides that have been sparingly used for decades in row crops (Salas-Perez et al., 2017). However, over the past years as more weeds evolved resistance to ALS inhibitors and glyphosate, more farmers have turned to PPO inhibitors as an alternative (Salas et al., 2016). The PPO enzyme catalyses the conversion of protoporphyrinogen 1X to protoporphyrin 1X which is essential in the biosynthesis of heme and chlorophyll. Inhibition of the PPO enzyme results in the generation

of singlet oxygen species that degrade the lipid and protein membranes leading to plant death (Nie et al., 2019).

In *Amaranthus* weeds, the most prevalent mechanism known to confer resistance to PPO inhibitors is a TSR which involves a deletion of a glycine codon at amino acid 210 (ΔG210). This codon deletion was first identified in common waterhemp (Patzoldt et al., 2006) and subsequently in Palmer amaranth from Arkansas where it conferred up to 19 fold resistance levels to the PPO inhibitor fomesafen (Salas et al., 2016). Giacomini et al. (2017a) identified two new mutations in the *PPX2* gene that were also responsible for resistance to this class of inhibitors in Palmer amaranth. The Arg-98-Gly and Arg-98-Met mutations were identified in resistant populations from Tennessee and Arkansas which did not have the previous codon deletion. Redroot pigweed from China also possesses the Arg-98-Gly mutation (Wang et al., 2020). Most recently, a novel single site mutation G399A was discovered in a resistant Palmer amaranth population that did not have either the G210 nor either of the R98 mutations (Rangani et al., 2019). Though this novel mutation was found to be rare, it still showed that PPO resistance in Palmer amaranth plants is under high selective pressure. Non target site resistance mechanisms were also confirmed in Palmer amaranth populations from Arkansas where rapid detoxification via the cytochrome P450 monooxygenases (P450s) and glutathione S-transferases (GSTs) was observed (Varanasi et al., 2018).

1.8.4 Resistance to PSII inhibitors

Photosystem II inhibiting herbicides consists of different chemical classes including triazines, triazinones and ureas. Photosystem II complexes are embedded within the thylakoid membranes of chloroplasts where they are involved in the electron transport chain. Once the PSII inhibitors are applied pre-and/ or post-emergence in the field, they disrupt the photosynthetic electron transfer from photosystem II to photosystem I by competitively binding to the plastoquinone binding site on the D1 protein in the PSII (Nakka et al., 2017b). This blocks the electron transport chain and leads to the production and accumulation of reactive oxygen species which damage cell integrity and kill the weeds.

This class of herbicides is among the oldest to be commercialized and a number of weed species have evolved resistance to most PSII inhibitors (Dayan et al., 2019; Heap, 2021). According to Heap (2021), PSII inhibitor resistance, primarily atrazine resistant weeds dominated the USA and Europe maize fields as early as 1970-1980's and has since increased in the number of unique cases. Both TSR and NTSR were discovered in *Amaranthus* weeds. An amino acid

substitution Ser264Gly in the *psbA* gene (which encodes the D1 protein) was reported to cause high levels of resistance to atrazine in smooth pigweed and redroot pigweed (Nakka et al., 2017b). Rapid metabolism of atrazine or simazine via the GST and cytochrome P450 was discovered in waterhemp (Ma et al., 2013) and Palmer amaranth (Nakka et al., 2017b). It is interesting to note that all the populations of redroot pigweed from different locations (USA, Brazil, Argentina, Canada, Italy etc.,) are all reported to be resistant PSII inhibitors (Heap 2021). This raises the questions on whether this species is more prone to developing resistance to this group of herbicides or the herbicide resistant biotype originated from one place and spread all over.

1.8.5 Resistance to HPPD inhibitors

This group of inhibitors such as mesotrione, tembotrione and topramezone work by inhibiting the 4-hydroxyphenylpyruvate dioxygenase enzyme thus disrupting catabolism of tyrosine leading to failure to provide plastoquinone, tocopherols and carotenoid biosynthesis (Nakka et al., 2017a). Plastoquinone is essential for the photosynthetic electron transfer in the process of generating ATP. Carotenoids are light harvesting molecules and plants lacking them cannot protect themselves from the radicals generated by the light activation of chlorophyll leading to bleaching, necrosis, and death (Nakka et al., 2017b). HPPD inhibiting herbicides just like PSII herbicides are mostly used as premixes to control multiple herbicide resistant *Amaranthus* weeds, though some populations of Palmer amaranth and common waterhemp have evolved resistance. The first populations of these *Amaranthus* weeds resistant to HPPD inhibitors were reported in 2009 in Kansas, Illinois, and Iowa (USA). Resistant populations in other states which include Nebraska, North Carolina and Wisconsin have been subsequently reported (Heap, 2021). The mechanism conferring resistance in these populations were reported as NTSR in the form of rapid metabolization via the cytochrome P450 enzymes in both species (Nakka et al., 2017a; Ma et al., 2013; Küpper et al., 2018b). Kaundun et al. (2017), reported no TSR mechanism in the form of mutations or *HPPD* gene duplications in common waterhemp.

1.8.6 Resistance to Auxin mimics inhibitors

Synthetic auxins have the longest history of use to selectively control broadleaf weeds in a variety of crop and non-crop (roadside, pastures) fields (Bernards et al., 2012). Auxinic herbicides such as 2,4-D and dicamba mimic the endogenous plant hormone indole-3-acetic acid (IAA) when applied at low concentrations. Interestingly, there are still questions as to how they exactly kill weeds upon application (Gaines, 2020). Introduced in the mid-1940s, synthetic

auxins have been in use longer than any other herbicide site of action, yet they have the lowest rate of weeds evolving resistance to them (Heap I 2021). In the *Amaranthus* genus, only three species have been reported to have developed resistance to this herbicide site of action. In Argentina, a smooth pigweed biotype was reported to be resistant to dicamba and 2,4-D and the in the USA (Illinois, Nebraska, Tennessee and Kansas) waterhemp and palmer amaranth are also resistant to these herbicides (Kumar et al., 2019; Heap, 2021). Rapid metabolism was suggested to be the mechanism contributing to 2.4-D resistance in waterhemp from Nebraska (Figueiredo et al., 2018).

1.8.7 Resistance to VLCFA inhibitors

These inhibiting herbicides are pre-emergence herbicides used in corn, wheat, rice, and soybean fields to control mostly monocotyledonous weeds and some small seeded broadleaved weeds. They work by interfering with elongases in the endoplasmic reticulum which catalyzes a series of biochemical reactions to form >18C fatty acids. The lack of very long chain fatty acids (VLCFA) disrupts the synthesis of sphingolipids, cutins and waxes which are crucial components of membrane function and are barriers against environmental stresses. These disruptions happen in the roots and shoots of germinating plants, causing plant emergence failure and eventually death (Busi, 2014). Although this class of herbicides have been used for over 60 years (Ouml and Ger, 2003; Böger, 2003), only twelve grasses in five species have evolved resistance around the world. These are, *Lolium rigidum, Alopecurus myosuroides, Avena fatua, Echinochloa crus-galli* var. *crus-galli*, and *Lolium perenn*e ssp. *multiflorum* (Heap, 2021*)*. Palmer amaranth and waterhemp are the only broadleaved weeds to have recently developed resistance to VLCFA inhibitors in the Midwest United states (Brabham et al., 2019; Strom et al., 2019). This is of course because of farmers reverting to these herbicides as they are faced with multiple herbicide resistant biotypes of weeds (Brabham et al., 2019; Jones and Owen, 2021). The mechanism of resistance to VLCFA as seen in *L. rigidum* and Palmer amaranth is an increase of GSTs genes expression in the roots which leads to rapid detoxification of the inhibitors (Busi et al., 2018; Rangani et al., 2021).

1.8.8 Microtubule assembly inhibitors resistance

Microtubules are encoded by the *α* and *β tubulin* genes and are vital components of the cytoskeleton and function at different stages of cellular division (Chahal et al., 2015). Microtubules assist cell wall synthesis in plants which helps support cell shape. Microtubule inhibitors disrupt microtubule formation and or elongation during cell division and results in swollen and stunted roots that cease to emerge or grow. To date, only Palmer amaranth in three

USA states (South Carolina, Tennessee, and Arkansas) has evolved resistance to this group of herbicides. The mechanism of resistance is still unknown.

1.9 Multiple resistance

Most populations of Amaranthus weeds in the USA have developed resistance to more than one herbicide site of action limiting the chemical control choices that farmers can use to manage this troublesome weed (Heap, 2021). The weedscience.org website keeps record of all reported herbicide resistant weeds worldwide. The table below (Table1.3) was compiled from data from this website and it summarizes *Amaranthus* populations with multiple resistances to at least two herbicide sites of action. As mentioned earlier, most of these weed populations have already evolved resistance to at least two groups of herbicides. This serves as an indicator about the seriousness of herbicide resistance evolution in *Amaranthus* species and in other weeds as well.

Table 1.3. A summary of multiple herbicide resistant populations of the four investigated Amaranthus species. (www.weedscience.org)

***Amaranthus* species**	**Location**	**Sites of Action /Inhibitors)**
A. palmeri	USA(Arkansas)	ALS, EPSPS, Microtubule assembly, PPO, VLCFA
	USA(Kansas)	ALS, Auxin mimics, EPSPS, HPPD, PSII
	USA (Georgia)	ALS, EPSPS, PSII
	USA(Illinois)	ALS, EPSPS, PPO
	USA(Nebraska)	HPPD, EPSPS, PSII
	USA (Tennessee)	Microtubule assembly, EPSPS, ALS, PPO, Auxin mimics
	Brazil	ALS, EPSPS
A. tuberculatus	Canada (Ontario)	ALS, PSII, EPSPS, PPO
	USA(Illinois)	ALS, PSII, EPSPS, PPO, HPPD, Auxin mimics, VLCFA
	USA(Iowa))	ALS, EPSPS, PSII, HPPD
	USA(Kansas)	ALS, PPO, EPSPS, PSII
	USA(Missouri)	ALS, PPO, EPSPS, PSII
	USA(Nebraska)	ALS, Auxin mimics, PSII, HPPD, PPO
	USA (North Carolina)	ALS, EPSPS, PSII, HPPD, PPO
A. hybridus	Argentina	ALS, EPSPS, Auxin mimics
	Brazil	ALS, EPSPS
A. retroflexus	Brazil	ALS, PSII, PPO
	Canada (Ontario)	PSII, ALS
	China	PSII, ALS, PPO
	Germany	PSII, ALS
	USA(Pennsylvania)	PSII, ALS

1.10 Management strategies of herbicide resistant *Amaranthus* species

Since *Amaranthus* weeds have evolved resistance to multiple herbicides, management requires careful planning. Management of these weeds calls for understanding the weed before planning any control strategies (Chauhan, 2020). Most farmers assume that increasing the dose and the number of applications of a herbicide controls the weed but this may in fact lead to a more resistant weed over time (Peterson et al., 2018).

A more integrated weed management system is required for weeds with such aggressiveness (Jason et al., 2012). The basic step is to start clean, which means adopting a post-harvest weed control system where all weeds are pulled out and burned or buried to reduce the seed bank and residues in the fields (Owen, 2017). Since they have small seeds, deep ploughing according to Bell et al. (2016) can reduce their seed bank in the top soil.

In terms of chemical management, overlapping residual herbicides with multiple SOA can effectively control Amaranthus weeds. Kohrt and Sprague (2017), reported the management strategy that provided most Palmer amaranth control to be PRE- followed by POST- herbicides. Both PRE- and POST- herbicides used in their study contained at least two effective herbicide SOA and had a residual herbicide. The herbicide application timing also plays a major role in the control of such aggressive growing weeds (Peterson et al., 2018).Though some PRE- and POST- herbicides can control these weeds, they are not one hundred percent effective and some herbicides have been shown to cause crop injury.

Alternatives to herbicides have to be incorporated into the management strategies. Wiggins M.S et al. (2017), evaluated the use of cover crops in Palmer amaranth management and reported that winter wheat and cereal rye provided the greatest amount of this species suppression in a cotton field. Scouting and hand weeding before the plants reach reproductive stage is also another effective strategy. However, human labor is expensive and this might not be feasible for most commercial production farms (Peterson et al., 2018; Sosnoskie and Culpepper, 2014). Field edges have also been reported to harbor weeds that spread into the field during planting season, therefore such areas should also be weeded or sprayed with herbicides (Sosnoskie and Culpepper, 2014).

Chemical management strategies towards these weeds seem to be ineffective unless they are integrated. Farmers need to pay attention not to spread this weed to uninfected farms through farm machinery and irrigation equipment or furrows (Owen, 2017). Harvesting infested fields

last can also be one way to minimize the spread of weeds. More work still needs to be done to come up with the best and effective control strategies.

1.11 Population and genetic diversity studies

1.11.1 Interspecies hybridization

The reproductive biology of Palmer amaranth and common waterhemp permits for outcrossing to easily occur via pollen movement (Mohseni-Moghadan.,2013). This allows for interspecies hybridization between the *Amaranthus* species. Hybridization between weedy amaranths such as Palmer amaranth X spiny amaranth, Palmer amaranth X smooth pigweed, Palmer amaranth X common waterhemp and common waterhemp X smooth pigweed does happen naturally if the species co-exist in a field (Denise et al., 1999b; Nandula et al., 2014; Gaines et al., 2012). Crosses between these species produce fertile hybrids at varying frequencies. The frequency of producing viable and fertile hybrids is high when the parental species are genetically related and/or have the same chromosome numbers as seen in Palmer amaranth X spiny amaranth (Trucco et al., 2005) as compared to the other *Amaranthus* species. The hybrids are usually morphologically different from the parents which adds to the problem of *Amaranthus* morphological identification difficulty reported by scientists (Franssen et al., 2001; Molin and Nandula, 2017). The introgression of parental herbicide resistance traits to the hybrids have been reported for glyphosate in spiny amaranth X Palmer amaranth hybrids (Nandula et al., 2014; Gaines et al., 2012) and for ALS resistance in Palmer amaranth X spiny amaranth hybrids (Molin et al., 2016a) and Palmer amaranth X common waterhemp (Franssen et al., 2001). Though the fitness of these hybrids over many generations still needs to be investigated, their presence poses a great problem in the control and management strategies (Tranel and Wright, 2002).

1.11.2 Molecular markers in *Amaranthus* population genetic studies

The *Amaranthus* family is considered difficult to genotype because it is highly variable. Differing amounts of outcrossing, occasional interspecific and intervarietal hybridization and adaptability to a wide range of geographic distributions all contribute to *Amaranthus* high genetic variability (Suresh et al., 2014). Several molecular markers have been developed and utilized in correctly identifying *Amaranthus* species and in investigating the population structure and diversity of *Amaranthus* germplasm. Rapid amplification of polymorphic DNAs (RAPDs) has been used to identify and cluster three species of *Amaranthus* (Lymanskaya, 2012) also to study the genetic diversity of crop and wild species of Amaranthus (Chan and

Sun, 1997). Restriction fragment length polymorphisms (RFLPs) were used to differentiate between 10 common weedy Amaranths (Denise et al., 1999a) and lately genotype by sequencing (GBS) which is an single nucleotide polymorphism (SNP) based fingerprinting method was utilized to investigate the population genetic structure in sensitive and resistant Palmer amaranths (Küpper et al., 2018a). Inter simple sequence repeats (ISSRs) have also gained popularity in *Amaranthus* genetic diversity studies (Gelotar et al., 2019). Lee et al. (2008), developed 14 single sequence repeats (SSR) markers that have widely used to interrogate intra- and inter- species diversity in *Amaranthus* populations. The choice of markers to use in a specific study is influenced by their ease of use, level of polymorphism, genomic abundance, available budget and most importantly by the research questions to be answered (Gelotar et al., 2019).

1.11.3 Microsatellite markers

Microsatellite markers also known as SSRs, are widely used in plant genetic diversity studies. These markers consist of short tandem repeating motifs of 1-6 nucleotides widely distributed within a gene or intergenic at a known locus in a chromosome and are found throughout the genomes of all prokaryotic and eukaryotic organisms (Zane et al., 2002).These markers have several characteristics that make them attractive in genetic diversity studies. They are heritable, highly polymorphic, codominant, multi allelic, transferable between closely related species and experimentally reproducible (Vieira et al., 2016). Their use in investigating and addressing questions of genetic relationships among closely related species in a population, mechanisms involved in population divergence and occurrences of hybridization in populations makes them important in molecular studies especially in a highly variable family such the Amaranthaceae family (Suresh et al., 2014). They are valuable genomic tools in *Amaranthus* as they can be used to study geographically diverse germplasm and identify informative traits which could be used in varietal improvements of these species which are widely utilized as food in most parts of the world (Erika Viljoen PhD theses, University of Pretoria).

1.12 Problem statement

Herbicide resistant Palmer amaranth has over the years spread and invaded new geographical territories mostly aided by human practices. In South Africa, the documented most common *Amaranthus* species indigenous or naturalized to South Africa are *Amaranthus hybridus*, *A. cruentus*, *A. spinosus, A. caudatus*, *A. thunbergii, A graecizans, A.viridus, A. deflexus and A. muricatus* (Department of Agriculture Forestry and Fisheries, 2010 (Gerrano et al., 2015). Previously, there had not been any current record of *A. palmeri* until 2018 when the South African Herbicide Research initiative (SAHRI) reported the first putative population of this species. The first introduced population was detected in a cotton field in the Douglas region of the Northern Cape province. It was identified morphologically as Palmer amaranth by the South African National Biodiversity Institute (SANBI). A second population was detected in the KwaZulu Natal Province of South Africa in 2020.Two more populations were reported in the Kruger national park along the Limpopo river and in Botswana (Sukhorukov et al., 2021) Correct identification and characterization of Palmer amaranth is of paramount importance in devising management strategies. Molecular identification and herbicide resistance profiling of these populations was deemed necessary so South Africa could know what it was up against and to devise strategies to reduce this weed species as early as possible.

1.13 Aim and objectives

The aim of this study was to use molecular techniques to identify and confirm the presence of *Amaranthus palmeri* and to establish the population diversity of the introduced populations in South Africa. The study had the following objectives:

i. To confirm the identity of *Amaranthus palmeri* species by sequencing the nrDNA ITS region.
ii. To develop the herbicide resistance profile by investigating the presence of mutations in the *ALS*, *EPSPS* and *PPO* genes known to confer resistance these classes of herbicides.
iii. To investigate the population genetic diversity of Palmer amaranth using microsatellite markers.

CHAPTER II

MOLECULAR IDENTIFICATION AND TARGET SITE CHARACTERIZATION OF *AMARANTHUS PALMERI* POPULATIONS IN SOUTH AFRICA

2.1 Introduction

Worldwide, the evolution of herbicide resistance is increasing at an alarming rate thus posing challenges to agricultural production. Chemical weed control using herbicides was introduced in agriculture around the 1960's where inhibitors mostly of the auxin type were used. Following thereafter, was what can be referred to as the "herbicide discovery boom" from the 1970s to the early 1990's, where most of the current herbicides were discovered and commercialized (Kraehmer et al., 2014). Due to the success and high efficacy of the already introduced herbicides, the herbicide discovery industry became saturated, and no new herbicide SOA had been introduced since then (Duke, 2012). The introduction of herbicides came with many advantages, a major one being increases in crop yields. Time, money, and other resources were saved, consequently, making crop production more profitable. Many farmers therefore adopted chemical weed control thereby solemnly relying on them and abandoning the outdated mechanical/ traditional weed control strategies. The overreliance and incorrect use of these herbicides, especially ones with the same SOA (e.g., glyphosate) led to the evolution of herbicide resistant weeds (Gaines et al., 2020).

The current problem of herbicide resistance, more specifically multiple herbicide resistance that the agriculture industry is currently faced with is devastatingly serious. Heap (2021) estimates the number of herbicide resistant weeds to be around 263 globally, consisting of both monocots and dicots. He further mentions that weeds have already evolved resistance to 21 of the 32 known SOA. As highlighted earlier, multiple herbicide resistance in weeds limits the choices that farmers have in weed control.

Herbicide resistant Palmer amaranth (*Amaranthus palmeri* S. Watson) is the most problematic as it is highly invasive and difficult to control. Populations of this *Amaranthus* species with confirmed resistances to EPSPS, ALS, PPO, HPPD, PSII, VLCFA, auxin mimics and microtubule assembly inhibitors have been reported in a few continents. Having invaded most parts of the USA and caused major yield losses, palmer amaranth has over the past years invaded very distant geographic countries, consequent of the ease of transport between countries and continents (Torra et al., 2020). Kistner and Hatfield (2018), modelled the potential distribution of Palmer amaranth under current and future bioclimatic conditions and they concluded that the major crop producing areas of Africa, south of the Sahara, were suitable for establishment and proliferation of this noxious weed. In Africa, Palmer amaranth has been reported in Egypt, Ethiopia, and Botswana but there has not been any reports of herbicide resistance or disruptions in agricultural fields (EPPO,2021). In 2018, however, a herbicide

resistant population of this weed was reported in a cotton field in the Northern Cape province of South Africa (Villa crop Science). This population was growing among smooth pigweeds and was responding differently to chemical control. In March 2020, another population was reported and observed in a field in the KwaZulu Natal province some 806 km away from where the first population was reported. Observations of both populations showed that they had found hospitable environments and were quickly naturalizing which is a feature inherent of Palmer amaranth.

Morphological identification was carried out on the first reported population in Northern Cape province by the South African National Biodiversity Institute (SANBI) (Record number 871HB collected 09/02/2018). As part of this study, ITS sequencing was carried out on the initially collected plants to confirm their identity. Greenhouse experiments were also carried out from seeds collected from the first population. The Palmer amaranth population showed significant resistance to chlorimuron and glyphosate. Decreased efficacy of mesotrione, atrazine, saflufenacil, metolachlor and dicamba was also observed (Reinhardt et al.2021 to be published).

The introduction of a weed with such a reputation calls for a rapid response so to assess the impact potential on the country's biodiversity, ecosystem, and agricultural production. The first step calls for correctly identifying the weed species especially since *Amaranthus* species present great phenotypic plasticity and can be easily misidentified by botanical descriptions. Studies carrying out genetic analysis to correctly identify the weed species are therefore deemed necessary. The *internal transcribed spacer* (ITS) region has been shown to contain single nucleotide polymorphisms (SNPs) that can easily differentiate *Amaranthus* species (Murphy and Tranel, 2018b).

Characterizing the weed's herbicide resistance profile is of utmost importance so farmers know exactly what they are dealing with and can tailor effective management strategies based on that. The herbicide resistance profile can also be used to trace where the introduced weed might have come from (Torra et al., 2020). Target site resistances (TSR) are frequently reported as the common mechanism observed in Palmer amaranth populations resistant to ALS, PPO and EPSPS inhibitors although this does not mean non target site resistances (NTSR) are not also observed. Point mutations in one or more of the six amino acid position in the *ALS* gene have been reported, so has an amino acid deletion (G210) in the *PPO* gene and lastly amplification of the *EPSPS* gene and recently a point mutation (P106S) in the same gene. All the reported

TSR mechanism offer varying amounts of resistance to the three commonly used herbicide groups.

The objectives of this chapter were therefore to identify and confirm the presence of Palmer amaranth using the ITS barcode, to investigate the presence of known mutations in the *ALS, EPSPS* and *PPO* genes that have been shown to confer resistance to these groups of inhibitors and to use this information to create awareness to farmers regarding the presence of Palmer amaranth in South Africa.

2.2 Materials and Methods

2.2.1 Plant material

Following the confirmation of the presence of herbicide resistant Palmer amaranth in the Northern Cape province, an awareness with a manual for identification was published in South Africa (http://www.villacrop.co.za/wp/wp-content/uploads/2018/12/Wicked-US-weed-Sep-2018-Charlie-Reinhardt.pdf.). Scouting for more palmer amaranth plants was carried out in the Northern Cape and more young plants suspected to be this *Amaranthus* species were collected and sent to the SAHRI at University of Pretoria for identification. Most of the plants sent in were collected after herbicide application. A total of 36 young plants were collected for this study, 29 from the Northern Cape and seven from the KwaZulu Natal Province. Samples of leaves from these pants were stored at -20°C until further use. Accessions used in this study were named according to where they were collected, NC for Northern Cape and ZN for KwaZulu Natal province.

2.2.2 DNA extraction

Leaf tissues were frozen in liquid nitrogen and ground into a fine powder using sterile mortar and pestle. Genomic DNA was extracted from the ground leaf tissue using ZR plant/seed DNA kit™ (Zymo Research, Inqaba, RSA) by following the manufacturer's protocol. The extracted genomic DNA was quantified using Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, RSA) and the quality checked by 1% agarose gel electrophoresis.

2.2.3 Confirmation of Amaranthus Identity

Plant material received from the two provinces were from areas where more than one *Amaranthus* species co-occur. To confirm their identity, the nuclear ribosomal ITS1 and ITS2 gene region was amplified and sequenced for each accession. Each PCR reaction contained 1 X dream Taq PCR master mix (Thermo Fischer, RSA), 400 nM each of the forward (ITSF-TCCTCCGCTTATTGATATGC) and reverse primers (ITSR- GGAAGTAAAGTCGTAACAAGG)

(Denise K. Wetzel, 1999), 20-70 ng gDNA, and 10 µl dH_2O to a total volume of 25 µl. Thermoprofile conditions were; initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, elongation at 72°C for 1 min, final elongation at 72°C for 10 min. The PCR products were visualized on 1% (w/v) agarose gel electrophoresis to verify desired DNA bands and purified by ethanol precipitation.

Purified PCR products were sequenced using Sanger sequencing at the ACGT sequencing facility (University of Pretoria, South Africa). The Big DYE Terminator cycle sequencing ready kit (Applied Biosystems, Thermo Fischer Scientific, RSA) was used and sequencing products were run on the ABI Prism™ 3500xl automated DNA sequencer (Applied Biosystems, Thermo Fischer Scientific, RSA). Analysis and assembly of the sequences was carried out on CLC Bio Main Workbench 8.0.1 (CLC Bio, a QIAGEN company, Aarhus, Denmark). Generated sequences were submitted to GenBank (MT811920-MT811924). Reference sequences of *A. palmeri, A. hybridus, A. spinosus* and *A. hybridus* were obtained from GenBank and included in the dataset used for alignment and SNP identification and subsequently phylogenetic inference. Phylogenetic analysis was done on MEGAX (Kumar et al.,2018) where the maximum likelihood model was used. Branch support was calculated through 1000 bootstrap replicates.

2.2.4 PCR amplification and sequencing of the *ALS* and *EPSPS* genes

Primers used in the amplification and sequencing of the *ALS* and *EPSPS* genes were sourced from literature and their properties and references are presented in Table 2.1. The *ALS* gene's domain 1 (CAD) and domain 2 (BE) were amplified separately.

Amplification of the *ALS* and *EPSPS* genes for all 36 samples was carried out using the Boeco TC-Pro (Boeco, Germany) thermocycler. Each PCR reaction contained 1 X dream Taq PCR master mix (Thermo Fisher Scientific, RSA),400 nM each of the forward and reverse primers (Integrated DNA Technology, RSA), 20-70 ng gDNA, and 10 µl dH_2O to a total volume of 25 µl. Thermoprofile conditions were; initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 59°C and 60°C for *ALS* and *EPSPS* genes respectively for 30 sec, elongation at 72°C for 1 min, final elongation at 72°C for 10 min and hold at 4°C for 59 min. The PCR products were visualized on 1% (w/v) agarose gel electrophoresis to verify desired DNA bands and purified by ethanol precipitation.

Purified PCR products were sequenced in both directions by Sanger sequencing at the ACGT sequencing facility at the University of Pretoria, South Africa. Primers used for PCR were also

used for sequencing for all the respective genes. Analysis and alignment of the sequences was carried out using the programme CLC Genomic Workbench 8.0.1 (CLC Bio, a QIAGEN company, Aarhus, Denmark). The identity and similarity of all generated consensus sequences were verified through GenBank database comparisons using Blastn and Blastx (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). Consensus sequences generated are shown in supplementary 2.

2.2.4 Determination of *EPSPS* gene copy number by qPCR

To measure the EPSPS genomic copy number relative to the monogenetic *ALS* gene, quantitative real time PCR was carried out as described by (Gaines et al., 2010). Primer sets EPSPS-F (ATGTTGGACGCTCTCAGAACTCTTGGT)X EPSPS-R (TGAATTTCCTCCAGCAACGGCAA) which amplifies a 195 bp product and ALS-F (GCTGCTGAAGGCTACGCT) X ALS-R (GCGGGACTGAGTCAAGAAGTG) which amplifies a 118 bp product were used. Primer efficiency curves were carried out using 1x, 1/5x, 1/25x and 1/125x dilution series of all genomic DNA. Primer efficiency curves and slopes were 98.7% and -3.555 (R^2 = 0.994) for EPSPS and 92.5% and -3.515 (R^2=0.997) for ALS. The qPCR reactions were carried out in triplicates using 10 ng genomic DNA templates and Luna® Universal qPCR master mix (New England Biolabs, Inqaba biotec, RSA) to a total volume of 10 µl. Quantitative PCR was carried out using Bio-Rad CFX96 Touch™ and the PCR conditions were as follows: 95°C for 1 min, 40 cycles at 95°C for 15s and 60°C for 30sec then increasing the temperature by 0.5°C every 5 sec to assess the melt curve. Melting peaks for both primer sets were 83.5°C. No template controls were also included, and no amplification was seen in these wells. Threshold cycles (Ct) were calculated using Bio-Rad CFX maestro. The experiment was carried out twice to verify the results and the averages used in analysis.

Relative quantification was carried out as described by Gaines at al., (2013) using a modification of the $2^{\Delta\Delta Ct}$ method. Relative quantification was expressed as ΔCt = (Ct, ALS-Ct, EPSPS) and $2^{\Delta Ct}$ was calculated to get a relative EPSPS copy number count.

2.2.5 *PPO* gene characterization

2.2.5.1 dCAPS assay

The presence of the mutation Arg-98-Met/Gly in the *PPX2* gene shown to confer resistance to PPO inhibitors in *Amaranthus* species was investigated using a dCAPS assay developed by (Giacomini et al., 2017b). A nested PCR was carried out with the initial primers R98-F and G210-R to amplify a 1600 bp product. A second PCR was carried using the dCAPS primers R98-F and the reverse primers Arg-98-Met-R and Arg-98-Gly-R (Table 1) to amplify 500 bp

which contains the mutation site. The PCR reactions consisted of 1X dream Taq master mix (Thermo Fisher Scientific, RSA), 400 nM of each primer (Integrated DNA Technology), 9,5 µl dH_2O and 20-50 ng gDNA to a total volume of 20 µl. Thermoprofile conditions were: Initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 1 min, final elongation at 72°C for 10 min and hold at 4°C for 59 min using the Boeco TC–PRO thermocycler (Boeco, Germany). To detect the Arg-98-Met mutation the resulting PCR product was mixed with 1 unit of *Kpn*I restriction enzyme and 1X FastDigest buffer (Thermo Fischer Scientific, RSA) and *Hind*III plus 1X FastDigest buffer (Thermo Fisher Scientific, RSA) was used for the Arg-98-Gly. Negative controls containing the PCR products and 1X FastDigest Buffer without the restriction enzyme were also prepared for all samples. All reactions were incubated at 37°C for 2 hours for complete digestion. The digested reactions were analysed on 4% agarose gel electrophoresis. For analysing the gel electrophoresis results, the following criteria was used: fully digested products were scored as wildtype, partially digested were scored as heterozygous and undigested products were scored as homozygous for that mutation.

2.2.5.2 Investigating the presence of G210 deletion

To investigate the presence of the ΔG210 deletion known to confer resistance to PPO inhibitors in *Amaranthus* species, a 100 bp segment of the *PPO* gene was amplified, cloned, and sequenced. PCR and sequencing primers are presented in Table 1. The PCR reactions consisted of 1X dream Taq master mix (Thermo Fisher Scientific, RSA), 400 nM of each primer (Integrated DNA Technology), 9,5 µl dH_2O and 20-50ng gDNA to a total volume of 20 µl. Thermoprofile conditions were: Initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, elongation at 72°C for 1 min, final elongation at 72°C for 10 min and hold at 4°C for 59 min using the Boeco TC–PRO thermocycler (Boeco, Germany). The PCR products were confirmed by agarose gel electrophoresis and the desired DNA bands excised and purified using Zymoclean™ Gel DNA Recovery kit (Zymo research, Inqaba, RSA). Purified PCR products were ligated into the linearized pMiniT 2.0 vector using NEB PCR cloning kit (New England biolabs, Inqaba, RSA) and grown on the stable outgrowth medium provided with the kit at 37°C for 60 min with shaking at 250 rpm. The outgrowth was spread onto Luria Broth (LB) 100 µg/ml ampicillin plates (1% tryptone, 0,5 % w/v yeast extract, 0,5% NaCl and 1,5% agar; Sigma-Aldrich Corp. St. Louis, MO) and incubated at 37°C overnight. The insert DNA was screened by colony PCR and Sanger sequencing of the plasmid. Plasmid DNA was extracted using QIAGEN™ Miniplasmid purification Kit (QIAGEN,

Hilden, Germany). The sequencing reactions contained 1 µl BigDye, 1 µl of each cloning primer (forward or reverse),1 µl sequencing buffer,5 µl dH_2O and 40-200 ng plasmid DNA to 10 µl. Cycle sequencing thermoprofile was 94°C for 2 min, 30 cycles of denaturation at 94 °C for 15 sec, annealing at 53°C for 15 sec, elongation at 68°C for 60 sec, final elongation at 68°C for 5 min and hold at 4°C for 59 min using the Boeco TC–PRO thermocycler (Boeco, Germany). To remove unincorporated ddNTPs ethanol precipitation method was used. The purified sequencing reactions were sent to the ACGT DNA sequencing facility at the University of Pretoria.

Table 2.2.Primers used in polymerase chain reaction and sequencing

Target gene	Primer name	Nucleotide sequence (5'-3')	TM°C	Product size(bp)	Reference
ALS	CAD-F	CCAGAAAGGTTGCGATGTTC	59	420	Berger et al., 2016
	CAD-R	AATCAAACAGGTCCAGGTC			
	BE-F	GAGAATCTCCCGGTTAAATCATGC	59	340	Berger et al., 2016
	BE-R	GCCCTTCTTCCATCACCCTC			
EPSPS	EPSPS-F	ATGTTGGACGCTCTCAGAACTCTTGGT	60	195	
	EPSPS-R	TGAATTTCCTCCAGCAACGGCAA			
PPO	R98-F	CTTGGGATACGTGAGAAGCAACAGTTG	56	400	Giacomini et al., 2017
	Arg-98-Met-R	TAGCAACGGAAGACCATCTCTATCTAGGTAC			
	Arg-98-Gly-R	TAGCAACGGAAGACCATCTCTATCTATGAAGC			
	G210F	TGATTATGTTATTGACCCTTTTGTTGCG	56	100	Giacomini et al., 2017
	G210R	GAGGGAGTATAATTTATTTACAACCTCCAGAA			

2.3 Results

2.3.1 Confirmation of Amaranthus identity

Sequencing the nrDNA ITS region generated a 719 bp containing the 18S-ITS1-5.8S-ITS2-28S region. Alignment of the generated and reference sequences identified three *Amaranthus* species (Table 2.2); *A. palmeri, A. hybridus* and *A. standleyanus*. Phylogenetic analysis (Figure 2.1) grouped the three species in their respective clades together with their references. Of the 36 accessions used in this study 23 were confirmed to be Palmer amaranth. Complete alignment of the ITS region can be found in supplementary Figure S2.

Table 2.2 Species identity of all 36 accessions used in this study as determined by ITS region sequencing

Accession name	Location	Species identity
NC1	Northern Cape	*Amaranthus palmeri*
NC2	Northern Cape	*Amaranthus palmeri*
NC3	Northern Cape	*Amaranthus palmeri*
NC4	Northern Cape	*Amaranthus palmeri*
NC5	Northern Cape	*Amaranthus palmeri*
NC6	Northern Cape	*Amaranthus palmeri*
NC7	Northern Cape	*Amaranthus palmeri*
NC8	Northern Cape	*Amaranthus palmeri*
NC9	Northern Cape	*Amaranthus palmeri*
NC10	Northern Cape	*Amaranthus palmeri*
NC11	Northern Cape	*Amaranthus palmeri*
NC12	Northern Cape	*Amaranthus palmeri*
NC13	Northern Cape (GWK Pressie Bdy)	*Amaranthus palmeri*
NC14	Northern Cape	*Amaranthus hybridus*
NC15	Northern Cape (Prieska)	*Amaranthus standleyanus*
NC16	Northern Cape (Douglas)	*Amaranthus hybridus*
NC17	Northern Cape (Prieska1)	*Amaranthus standleyanus*
NC18	Northern Cape (Prieska2)	*Amaranthus hybridus*
NC19	Northern Cape (Prieska3)	*Amaranthus standleyanus*
NC20	Northern Cape	*Amaranthus palmeri*
NC21	Northern Cape	*Amaranthus palmeri*
NC22	Northern Cape (Riet river4)	*Amaranthus hybridus*
NC23	Northern Cape (Modder river1)	*Amaranthus hybridus*
NC24	Northern Cape (Modder river2)	*Amaranthus hybridus*
NC25	Northern Cape	*Amaranthus palmeri*
NC26	Northern Cape	*Amaranthus palmeri*
NC27	Northern Cape	*Amaranthus palmeri*
NC28	Northern Cape (Douglas)	*Amaranthus palmeri*
NC29	Northern Cape (Douglas)	*Amaranthus palmeri*
ZN30	KwaZulu Natal (A9)	*Amaranthus palmeri*
ZN31	KwaZulu Natal (A9)	*Amaranthus palmeri*
ZN32	KwaZulu Natal (A9)	*Amaranthus hybridus*
ZN33	KwaZulu Natal (A9)	*Amaranthus palmeri*
ZN34	KwaZulu Natal (Sojas L1)	*Amaranthus hybridus*
ZN35	KwaZulu Natal (Sojas L1)	*Amaranthus hybridus*
ZN36	KwaZulu Natal (S6)	*Amaranthus hybridus*

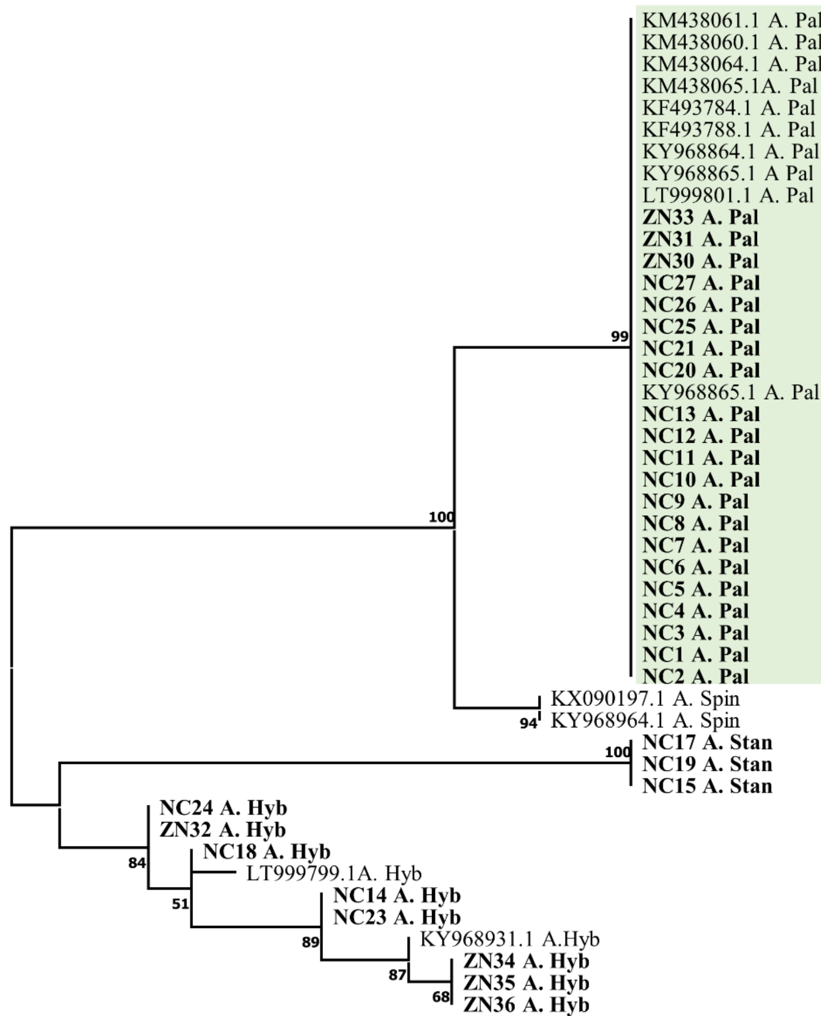

Figure 2.1. Phylogenetic tree of *Amaranthus* genus using ITS region. The species from the two populations used in this study are in bold. Coloured in green is the *Amaranthus palmeri* clade. Strong bootstrap values (>70%) support the branching on the external nodes.

2.3.2 *ALS* gene Sequencing

The CAD domain was sequenced and aligned separately as was the BE domain. No mutations were found in the CAD domain in all the accessions used in this study (Supplementary figure 3). A summary of the nature of the mutations found in this study is presented in Table 2.3. Accessions were labelled as heterozygous for a mutation if they had double peaks at that nucleotide position. Figure 2.2 shows the amino acid alignment for the BE domain. From the Northern Cape population, nine accessions possessed the Ser-653-Asn mutation, and they were all Palmer amaranth. No other ALS mutations were found in the Northern Cape population. All accessions from the KwaZulu Natal population *(A. palmeri* and *A. hybridus*) had the Trp-574-Leu mutation and one accession (ZN33_A. Pal) had both Trp-574-Leu and the Ser-653-Asn mutation.

Table 2. 3. A summary of the accessions with mutations in the BE domain of the *ALS* gene

	ALS mutations and genotype	
Accession name	**Trp-574-Leu**	**Ser-653-Asn**
NC1_A. Pal	No	Yes, HM
NC2_A. Pal	No	Yes, HT
NC3_A. Pal	No	Yes, HT
NC4_A. Pal	No	Yes, HM
NC6_A. Pal	No	Yes, HT
NC11_A. Pal	No	Yes, HM
NC13_A. Pal	No	Yes, HM
NC20_A. Pal	No	Yes, HM
NC25_A. Pal	No	Yes, HT
ZN30_A. Pal	Yes, HT	No
ZN31_A. Pal	Yes, HT	No
ZN32_A. Hyb	Yes, HT	No
ZN33_A. Pal	Yes, HT	Yes, HT
ZN34_A. Hyb	Yes, HM	No
ZN35_A. Hyb	Yes, HM	No
ZN36_A. Hyb	Yes, HT	No

Abbreviations: HT-heterozygous and HM-homozygous

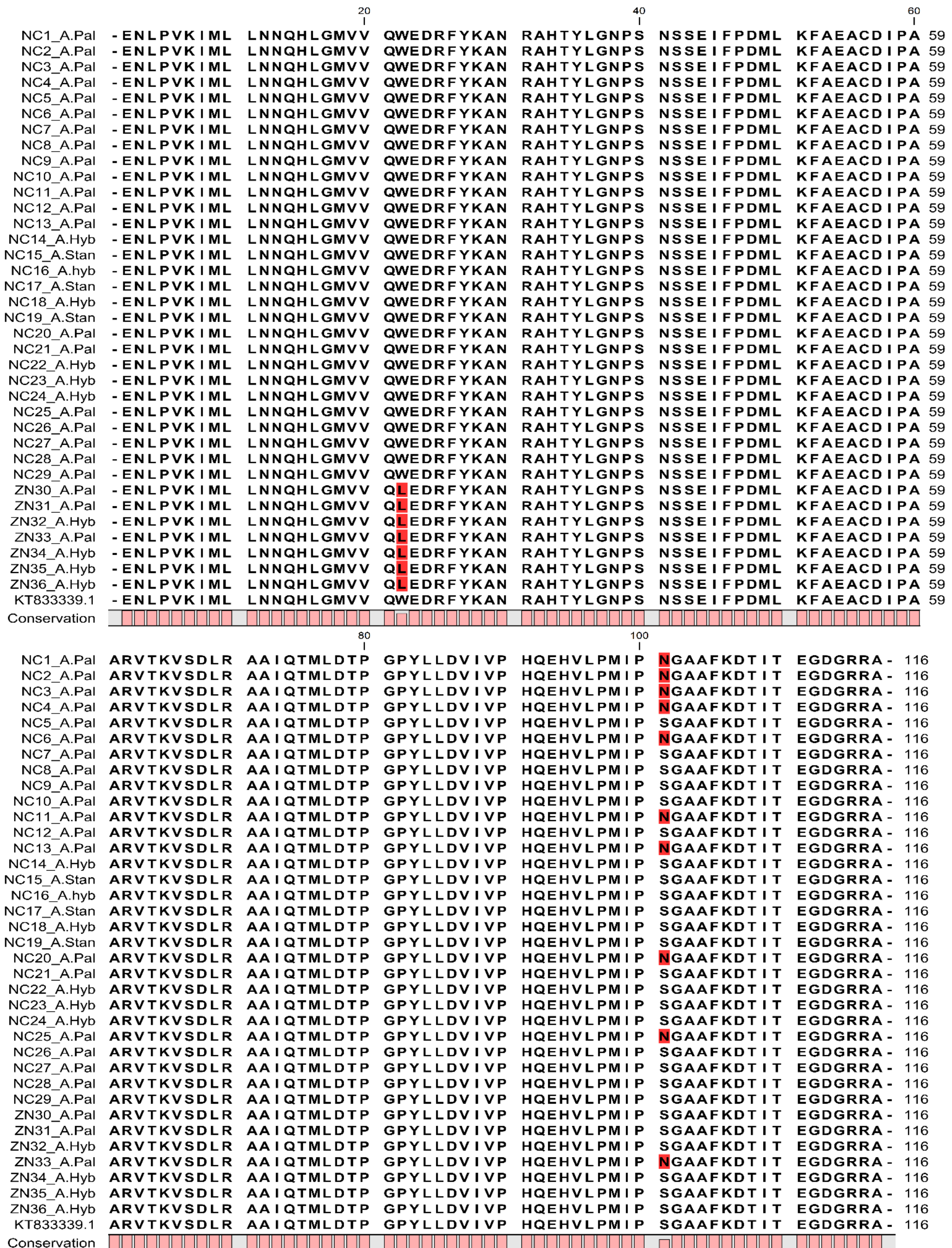

Figure 2.2. *ALS* gene BE domain amino acid alignment of all accessions used in this study. Polymorphisms are marked in red. The top panel shows the Trp-574-Leu mutation and the bottom panel shows the Ser-653-Asn mutation. The sequence KT833339.1, an ALS sensitive Palmer amaranth sample from GenBank was used as a reference

2.3.3 Partial *EPSPS* gene sequencing

The *EPSPS* gene in all three species used in this study was investigated for the presence of mutations known to confer resistance to glyphosate. None of the accessions from the Northern Cape population had any mutations (Figure 2.3). However, accessions from the KwaZulu natal population had mutations in the *EPSPS* gene. The three Palmer amaranth accessions (ZN30_A. Pal, ZN31_A. Pal and ZN33_A. Pal) had the Pro-106-Ser amino acid change whilst the rest of the accessions which were smooth pigweed (*Amaranthus hybridus*) had the triple amino acid change Thr-102-Ile, Ala-103-Val, Pro-106-Ser (TAP-IVS).

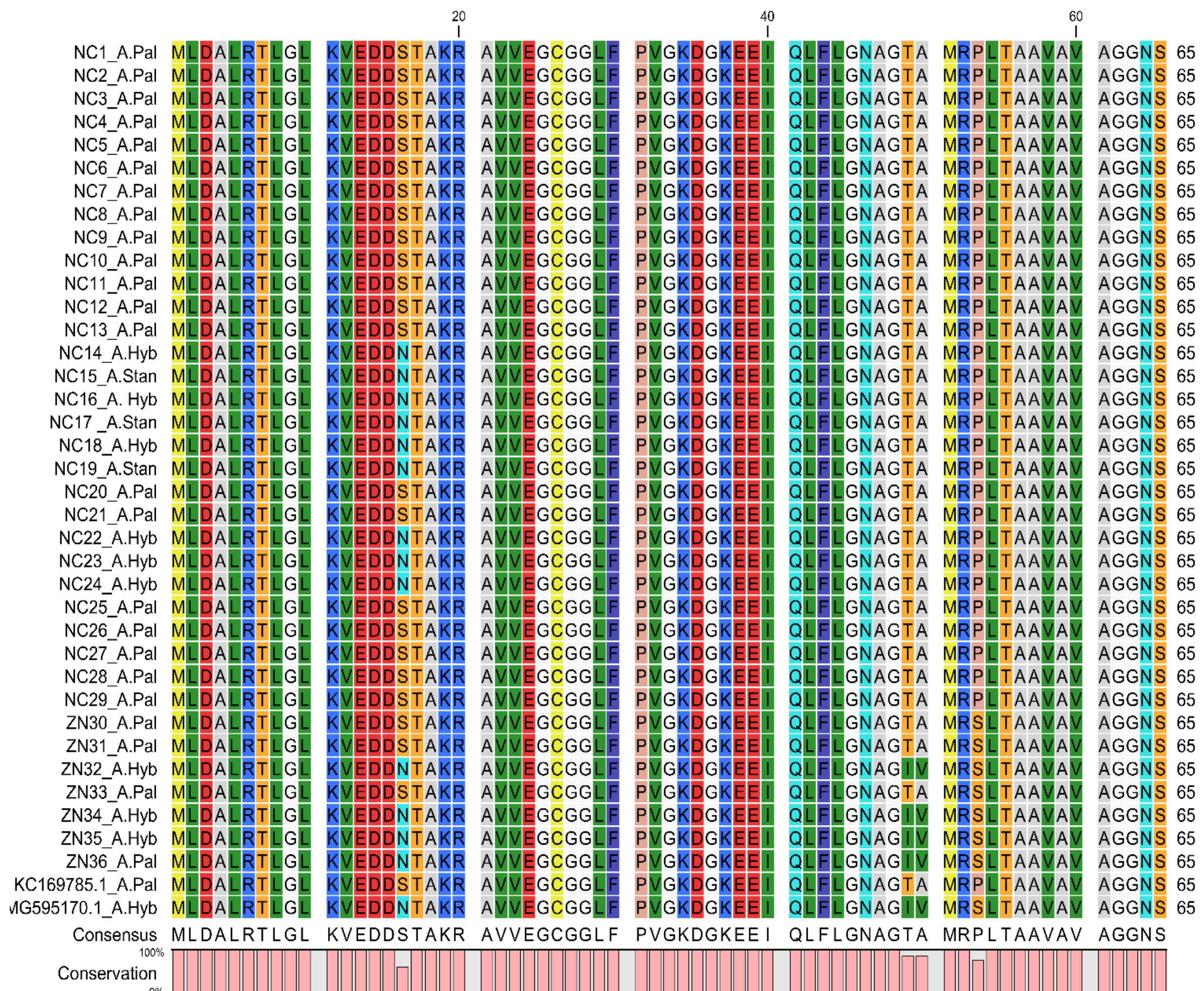

Figure 2. 3. Amino acid alignment of the partial *EPSPS* gene for all accessions used in the study**.** Polymorphisms are shown by different colour amino acid. The glyphosate sensitive Palmer amaranth accession (KC169785.1) and glyphosate resistant smooth pigweed accession (MG595170.1) were used as references

2.3.4 *EPSPS* gene copy number

Quantitative PCR was used to measure *EPSPS* gene genomic copy number relative to *ALS* gene. The results are presented in Figure 2.4. Palmer amaranth accessions from the Northern Cape population had EPSPS relative copies ranging from 2 to 140 with an average of 49 copies and the other species (*A. standleyanus* and *A. hybridus*) in this population had only one copy. Both *Amaranthus* species (*A. palmer*i and *A. hybridus*) from the KwaZulu Natal population had one *EPSPS* gene copy except for one accession (ZN36_A. Pal) which had two copies.

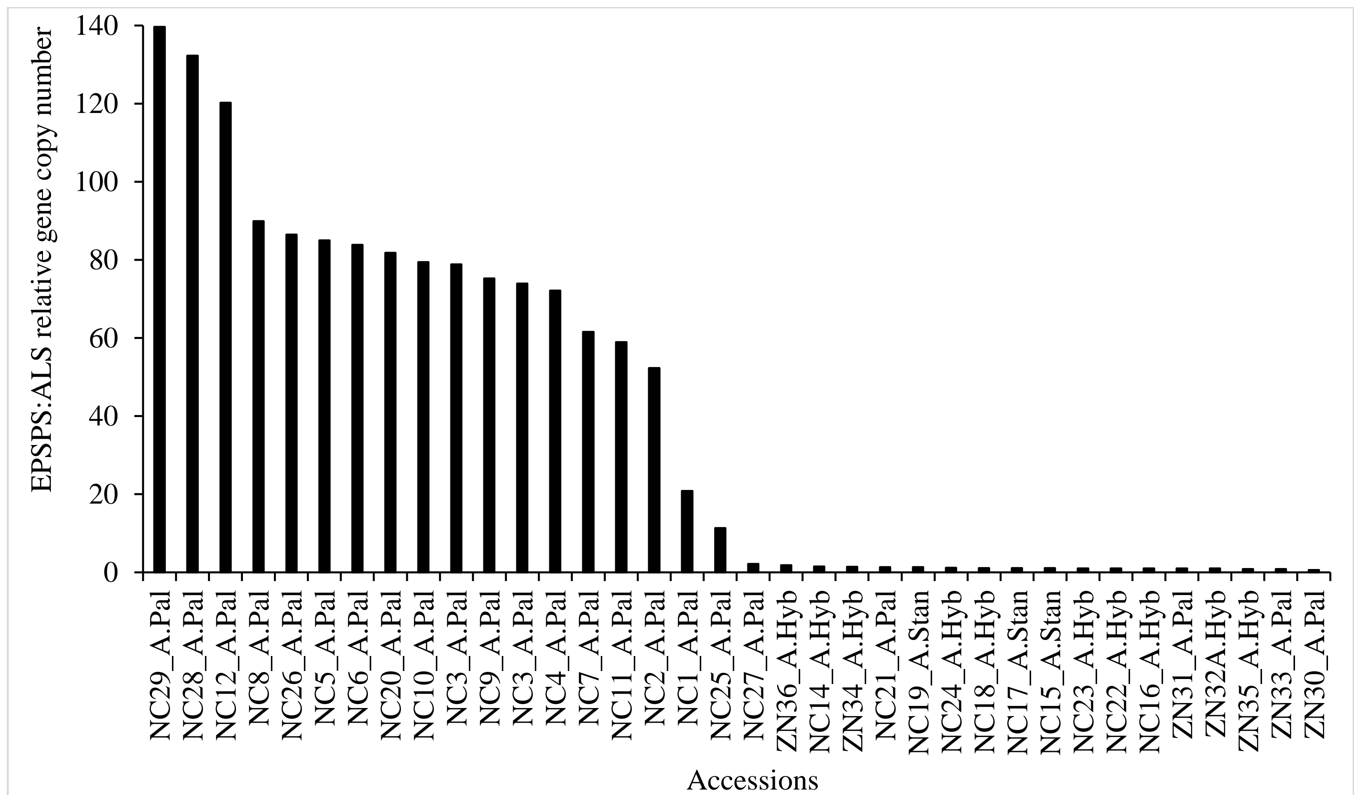

Figure 2.4.Variablity in relative *EPSPS:ALS* gene copy number in all *Amaranthus* accessions used in this study. Gene copy number is presented in descending order

2.3.5 *PPO* gene characterization

The *PPO* gene was genotyped for the herbicide resistant Arg-98-Met/Gly mutations by utilizing a dCAPS assay. Amplification of the desired PCR product (400 bp) was successful in all accessions which meant that the primers could bind perfectly at the modified 3' end. All PCR products were completely digested for both the Arg-98-Met (Figure 2.5A) and Arg-98-Gly (Figure 2.5B) assays meaning they did not contain the mutation. Cloning and sequencing of a part the *PPO* gene containing the G210 mutation site did not show the presence of the deletion in any of the investigated samples.

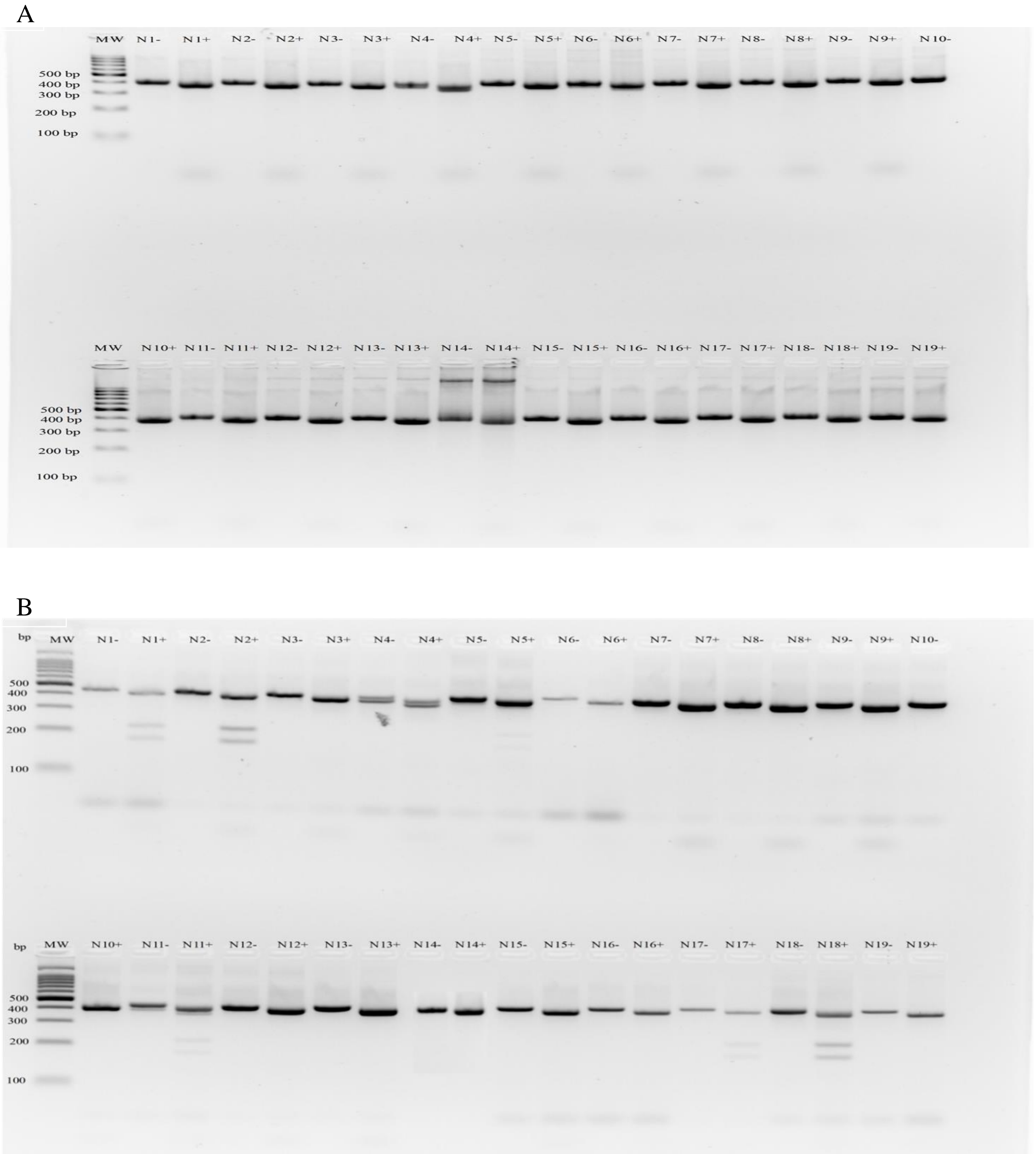

Figure 2.5. Gel image of dCAPS assay for Northern Cape population. Accession labels were shortened for clear presentation of results (N1=NC1_A. Pal, N2= NC2_A. Pal and so on). Both undigested (-) and digested (+) PCR products are shown. (A) represents Arg-98-Met and B represents Arg-98-Gly assay. A 100bp DNA ladder (MW) (Thermo Fischer Scientific, RSA) was used and the PCR products fall between ~400 and 350 bp fragments

2.3.6 Multiple herbicide resistance

Consequential to the discovery of the TSR mechanisms in some of the investigated accessions, the presence of multiple resistances was investigated. Out of all the 36 accessions used in this study, 12 were resistant to glyphosate alone, 15 were resistant to glyphosate and ALS inhibitors and none had mutations resulting to resistance to either PPO inhibitors/PPO + ALS/PPO + glyphosate or PPO +glyphosate + ALS.

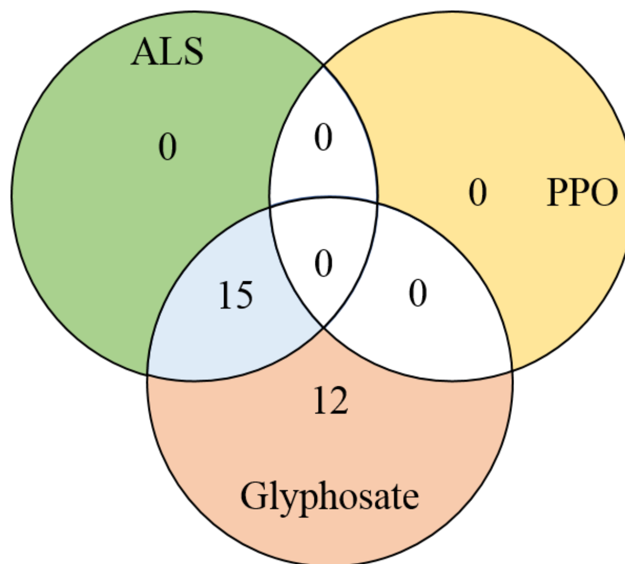

Figure 2.7. Venn diagrams depicting the linkage of the accessions with multiple resistance. All 36 accession from both populations (KZN and NC) were investigated for the TSR to glyphosate, ALS, and PPO inhibitors

2.4 Discussion

This study was undertaken to confirm the presence of *Amaranthus palmeri* in South Africa and to characterize the introduced populations by investigating their target site herbicide resistance profile. Herbicide resistant Palmer amaranth have been confirmed and characterized in the USA, Argentina, and Brazil, however, there have not been any record of this weed species in South Africa. The study has therefore reported and characterized the first introduced population of *Amaranthus palmeri* in Southern Africa.

2.4.1 Confirmation of Amaranthus identity

Due to limited morphological differences between species as well as intra-species variation, the genus Amaranthus is particularly difficult to correctly identify on a morphological basis, especially immature plants. Therefore, molecular characterization was done to correctly identify collected plant material. A two base pair nucleotide polymorphism at position 496 and

497 is one of the polymorphisms that differentiate Palmer amaranth from other *Amaranthus* species (Murphy and Tranel, 2018b). In this study, a total of seventeen SNPs were found between *A. palmeri* and *A. hybridus*, twelve between *A. palmeri* and *A. standleyanus* and five between *A. hybridus* and *A. standleyanus*. Within-species *ITS* SNPs were also observed in the *A. hybridus* cluster. In the hybridus cluster, accessions from the KwaZulu Natal population had different SNPs compared to the same species from the Northern Cape, but more similar to the reference sequence KY968931.1. This could have been as a result of geographic speciation (Murphy and Tranel, 2018a), or it can be an indication of the species origin if it was indeed introduced with the *A. palmeri* species. The ITS region is an informative barcoding system in plants and fungi as it has high inter-specific and intra-specific divergence, meaning it can distinguish different genus and species within the genus (Xu et al., 2018). This multicopy structure can be easily amplified by PCR even from herbarium specimen (Denise K. Wetzel, 1999). Murphy and Tranel (2018b), identified and developed markers for Palmer amaranth specific nucleotide polymorphisms (SNPs) in the internal transcribed spacer by aligning different *Amaranthus* ribosomal RNA sequences.

2.4.2 Sequencing of the *ALS* gene

More weeds have developed resistance to ALS inhibitors as compared to the other herbicide classes, and target site resistance has been shown as the primary mechanism conferring this resistance (Heap I.,2020). To investigate whether the two populations of Amaranthus in this study contained any of the known mutations, the *ALS* gene was sequenced. Nine accessions confirmed to be Palmer amaranth from the Northern Cape population were found to contain the Ser-563-Asn amino acid substitution. This mutation has been associated with high resistance to imidazolinones (IMIs) and intermediate resistance to sulfonylureas (SUs) in *Amaranthus* species (Tranel and Wright, 2002; Patzoldt and Tranel, 2007; Berger et al., 2016). No other mutations were found in the Northern Cape population. The presence of the Ser-653-Asn mutation explains the high level of resistance observed in plants from this population as evidenced by dose response assay (Reinhardt et al.,2021, to be published). All seven accessions (both Palmer amaranth and smooth pigweed) from the KwaZulu Natal population had the Trp-574-Leu amino acid change. High levels of resistance across most classes of ALS inhibitors (IMIs, SUs, and triazolopyrimides (TPs)) have been associated with the presence of the Trp-574-Leu amino acid change in *Amaranthus* weeds (Nakka et al., 2017c). In both populations, homozygous and heterozygous alleles were observed for the two amino acid changes. This could be an indication of ongoing intra- and/ inter-species hybridization where the resistant

allele is spread among co-existing plants. Molin et al. (2016b) reported hybridization with the introgression of ALS resistance alleles in Palmer amaranth X spiny amaranth hybrids and this was consistent with observations made earlier by Franssen et al. (2001) between Palmer amaranth X common waterhemp. In the field, herbicide resistance is mainly spread through seeds however it can also spread through pollen, more especially in genetically compatible plants growing in proximity (Jhala et al., 2020). Interestingly, the accession ZN33_A.Pal had both the Ser-653-Asn and the Trp-574-Leu amino acid changes which was also reported by (Singh et al., 2019) in accessions from Arkansas. The presence of ALS resistance mutations in both introduced Palmer amaranth populations and common waterhemp from the KZN province points to the seriousness of the issue of herbicide resistance faced by the South African agricultural industry.

2.4.3 *EPSPS* gene sequencing and copy number

The presence of target site mechanisms (EPSPS duplication and or mutations) conferring resistance to glyphosate in the introduced populations is of great concern in the South African agricultural industry as almost 80% of commercial farmers grow glyphosate resistant crops. Results obtained in this study revealed that all accessions of Palmer amaranth from the Northern Cape population had more than one *EPSPS* gene copy with the average being 49 relative copies. This is concerning as it means the NC Palmer amaranth population has high levels of resistance and cannot be controlled by the application of glyphosate. The other *Amaranthus* species from this population had one relative copy of this gene. Amplification of the *EPSPS* gene copy number has been confirmed as the main mechanism conferring high resistance to glyphosate in Palmer amaranth (Gaines et al., 2010). Relative genomic EPSPS copies of up to 150 in Georgia (Gaines et al., 2010), eight in New Mexico (Mohseni-Moghadam et al., 2013),105 in Nebraska (Chahal et al., 2017) and 150 in Arkansas (Singh et al., 2018) have been reported in glyphosate resistant Palmer amaranth. The minimum number of copies needed to confer resistance to the recommended field dosage is not known. However, all these studies concurred that resistance to glyphosate was additive, therefore populations with more genomic EPSPS copies were more resistant. Palmer amaranth accessions from the KwaZulu Natal population did not have any *EPSPS* gene amplification as did the other *Amaranthus* species identified in this study. This glyphosate resistance mechanism has only been recorded in two other Amaranthus species, Common waterhemp (Chatham et al., 2015) and spiny amaranth (Nandula et al., 2014) and they had lower numbers of this gene, 4-16 and 33-37, respectively. Interspecies hybridization has been confirmed to be one mechanism which

propagates gene duplication to the other Amaranthus species (Nandula et al., 2014; Jhala et al., 2020).

The presence of the proline-106-serine amino acid substitution in three Palmer amaranth accessions from KwaZulu Natal (ZN30_A. Pal, ZN31_A. Pal and ZN33_A. Pal) was observed. Mutations in the *EPSPS* gene in response to glyphosate in Palmer amaranth are rare thus only one mutation has been recorded so far. In Palmer amaranth, the P106S mutation has so far been reported in Mexico (Dominguez-Valenzuela et al., 2017a) and Argentina (Kaundun et al., 2019). The levels of resistance conferred by the amino acid change was lower compared to the levels resulting from *EPSPS* gene amplification or overexpression (Kaundun et al., 2019). Though the P106S mutation is not common in Palmer amaranth populations in the USA, it has however been reported in common waterhemp (Schultz et al., 2015). Smooth pigweeds accessions from the KwaZulu Natal population contained the triple amino acid mutation (TAP-IVS). These mutations have only been reported in smooth pigweeds populations from Argentina (Perotti et al., 2019; García et al., 2019) and were associated with high levels of glyphosate resistance. South Africa had no record of glyphosate resistant *Amaranthus* weeds before the introduction of Palmer amaranth. It is without doubt that the glyphosate resistant Palmer amaranth populations from the Northern Cape and KwaZulu Natal provinces are two distinct populations. The NC population has traits more like USA populations and the KZN population however has mechanisms that have been observed in Argentinian *Amaranthus* species. This raises questions whether the KZN population was introduced from Argentina or if just like the Argentinian population, was introduced from the USA but acquired the mutations because of the cropping systems and management practices in South Africa? More studies still need to be conducted to trace the origin of these populations, especially the KZN population as two different glyphosate resistant species were confirmed. These hypotheses still need to be tested before conclusions can be drawn regarding this population.

2.4.4 Characterization of the *PPO* gene

No target site mutations were found in the *PPX2* gene in all accessions investigated in this study using the dCAPS assay. Partial sequencing of the *PPX2* also did not how the presence of the glycine amino acid deletion at the 210th position. First discovered in common waterhemp (Patzoldt et al., 2006), the deletion also co-evolved independently in Palmer amaranth populations co-existing with common waterhemp (Salas et al., 2016; Lillie et al., 2019). Though the glycine deletion was prevalent in PPO inhibitor resistant population it did not fully account for all the observed resistance thus, Giacomini et al. (2017b) identified two new

infrequent mutations in the *PPX2* gene and these were Arg-98-Gly/ Met. Recently, Rangani et al. (2019) reported a new amino acid substitution from glycine to alanine at position 399 of Palmer amaranth *PPX2* gene. This new mutation was not investigated in the present study therefore it would still need to be investigated. Herbicide dose response assays carried out by Reinhardt et al.,2021 (to be published) showed the Northern Cape population had reduced efficacy to the PPO inhibiting herbicide saflufenacil yet no target site mechanism was observed in this population. This could indicate the presence of NTSR in the introduced population. Giacomini et al. (2017b) observed resistant plants which did not possess any of the three known mutations and so did Varanasi et al. (2018) and they both suggested the presence of NTSR in these plants.

2.4.5 Multiple herbicide resistant Palmer amaranth

The evolution of multiple herbicide resistance (MHR) in Amaranthus weeds, especially in Palmer amaranth is very common. This study reports the introduction of Palmer amaranth with a confirmed two-way resistance to glyphosate and ALS inhibitors and possibly to PPO inhibitors as well. Interestingly, the smooth pigweed accessions from the KZN province also possess MHR to the same two SOA. The discovery of TSR mechanisms in the (*ALS* and *EPSPS*) target genes of these inhibitors confirm the findings made by Reinhardt et.al., (2021) (to be published) through greenhouse dose response assay. They reported that the NC population showed high resistance to ALS and EPSPS inhibitors (chlorimuron and glyphosate) and decreased sensitivity to HPPD, PSII, PPO, VLCFA and dicamba. As more Palmer amaranth populations develop resistance to commonly used herbicides, farmers turn to other modes of action to control the weeds. Unfortunately, resistance continues to evolve over time and TSR plus NTSR mechanisms get stacked in those populations. In Kansas, USA ,Shyam et al. (2020) reported a six way resistance to ALS, EPSPS, 2,4-D, PPO, and HPPD inhibitors and Kumar et al. (2019) reported a population with high resistance to chlorsulfuron, atrazine, mesotrione and glyphosate and reduced efficacy to fomesafen. Resistance to ALS and EPSPS inhibiting herbicides have also been reported in Brazil in Palmer amaranth. The presence of MHR populations poses a serious threat to the agricultural industry as it limits the choices of herbicides modes of action that farmers can use.

2.5 Conclusion

The main aim of this chapter was to use molecular techniques to confirm the identity of Palmer amaranth and to further investigate the presence of known TSR mechanisms conferring resistance to ALS, EPSPS and PPO inhibitors in the introduced populations. The current study

confirmed the presence of two distinct herbicide resistant Palmer amaranth populations in the KZN and NC provinces of South Africa. Two-way resistance to glyphosate and ALS inhibitors was also confirmed in both populations by characterizing the TSR mechanisms. Resistance to glyphosate was due to two different TSR mechanisms with *EPSPS* gene duplication and amino acid mutation (P106S) in NC and KZN populations, respectively. The same was observed with TSR mechanisms conferring resistance to ALS inhibitors, the Trp574Leu mutation was prevalent in the KZN population and the Ser653Asn mutation was observed in the NC population. Characterizing and developing the resistance profile of these two populations showed that they were different. Interestingly, multiple herbicide resistant smooth pigweed co-existing with Palmer amaranth was also observed in the KZN province. Identical mutations as those observed in the *ALS* gene of the Palmer amaranth species were also found in this species. Resistance to glyphosate in the KZN smooth pigweed was conferred by triple amino acid substitutions (TAP-IVS) in the *EPSPS* gene active site. This is the first study to confirm and report the presence of a two-way herbicide resistance in *Amaranthus* species (Palmer amaranth and smooth pigweed) in South Africa. Characterizing these weeds have proven valuable as the information has already been used by CropLife SA as well as HRAC (Herbicide resistance Action Committee) SA as part of community engagement to devise an emergency eradication plan outlining herbicides that might still be effective and emphasizing the importance of integrated herbicide management systems. This work contributed to a larger study that was investigating this non-native species of *Amaranthus*. The broader study also did testing of dose responses of different herbicides in order to develop management plans. The outcome of the whole study will be published soon. Of more concern now is the spread of the herbicide resistant *Amaranthus* species into major grain producing areas of the country and the probable hybridization and introgression of herbicide resistant alleles into previously sensitive *Amaranthus* species. Considering this, it is thus important for the weed to be identified early. Since morphological identification is difficult in young plants, molecular identification by sequencing the ITS barcode is recommended and so is sequencing the herbicide target genes to quickly identify herbicide resistance alleles. Employing molecular techniques is also advantageous compared to greenhouse screening as it is quicker and limits the risks of spreading the weed to other parts of the country. Countrywide field survey for monitoring herbicide resistance in Amaranthus weeds is deemed necessary.

CHAPTER III

GENETIC DIVERSITY OF THE INTRODUCED PALMER AMARANTH POPULATION REVEALED BY SIMPLE SEQUENCE REPEATS (SSR) MARKERS: PRELIMINARY STUDY.

3.1 Introduction

Invasive plant species are increasingly becoming major threats to agricultural production, biological diversity, and human health (Lucardi et al., 2020). These plants are non-native/ alien where they are found and cause significant economic and environmental losses in industries such as agriculture, fisheries, wetlands, forests, and other natural areas (Paini et al., 2016). The plant species possess traits such as tolerance to a wide range of climatic and geographic conditions, short reproduction cycle and dispersal to name a few, which aids in invading, establishing, and exploiting new habitats (Lucardi et al., 2020). However, biological characteristics are not the only enablers of plant invasions as anthropogenic activities (e.g., global warming and climate change) and international trade perpetuate this (Smith et al., 2020). It is known that long distance transportation (international and domestic) because of trade and tourism accelerate plant invasions. Plant propagules are ferried across wide geographic borders in cargo shipments, as ornaments, and as hitchhikers in clothing and get introduced into new environments. Once introduced, invasive plant species must quickly adapt and overcome both biotic and abiotic factors in the alien environment for them to be successful invaders (Pulzatto et al., 2019). Though the introduced species usually does not have natural enemies in the new habitat, biological competition for resources with co-existing species exists and must be overcome. Physiological and genetic variation of the introduced species/population plays an important role in adapting, naturalizing and subsequently invading the new territories (Clements et al., 2004). Introduced populations usually have less genetic variation in the new environment as compared to the place of origin, being only a subset of the wider gene pool, thus face what is known as founders' effect which must be overcome for successful invasion, though this is not always the case (Frankham, 2005).

One of the many weed species which has mastered the art of invasions is Palmer amaranth (*Amaranthus palmeri* S. Wats. Originating from Southwestern USA, it has successfully invaded most parts of North America, and some countries in South America, Europe, Asia, and Africa (Heap, 2021). Previous work done on this species has shown contamination of grain shipments as the main mode of vectoring (Torra et al., 2020; Shimono et al., 2020). Consequent to introduction, the weeds' phenotypic plasticity, adaptability and reproductive biology helps in establishing and invading the new environments (Ward et al., 2013). Genetic bottlenecks are overcome by being an obligate out-crosser and the high rate of hybridization with other

Amaranthus species. This introduces more genetic diversity especially when more than one population has been introduced in proximity. Chandi et al. (2013) mentions that high inter and intra population genetic variation can affect weed management practices as selection acting on the population(s) can favour resistant genotypes, and this can result in lowered efficacies of chemical and biological control strategies. Knowledge of genetic variability is therefore an important tool in devising and adopting weed control methods and DNA based markers have been used to understand genetic variability in *Amaranthus* weeds.

Several studies utilizing different molecular markers have been undertaken in *Amaranthus* species to get important information needed to understand patterns of weed invasion, number of input events, gene flow (Chandi et al., 2013), diversity and taxonomic relatedness (Lee et al., 2008; Gelotar et al., 2019), heritability of traits (e.g. tracking herbicide resistant genotypes) (Torra et al., 2020) and points of origin (Küpper et al., 2018a). Single sequence repeats (SSRs)/microsatellite markers are used in genetic diversity studies of *Amaranthus* species because they are versatile, cost effective and highly polymorphic once developed though the process of their development is costly and labour intensive. Several SSR markers have been developed for leafy and grain amaranth but there are not many developed for weedy species specifically, nonetheless the cross-species versatility of SSR markers allows for them to be used to study weedy species. Recently, Erika Viljoen (University of Pretoria, 2018) developed six *Amaranthus* SSR markers based on *Amaranthus tricolor* that showed cross species amplification for her PhD study. This study, however, did not include Palmer amaranth samples. The developed markers were used to evaluate their suitability to investigate the genetic diversity of the introduced population of Palmer amaranth. This work will contribute into testing the cross-species transferability of the developed markers and evaluate their utility in genetic diversity studies of weedy *Amaranthus* species. This was thus a preliminary study into the diversity of Palmer amaranth in South Africa.

3.2 Materials and Methods

3.2.1 Plant material

All 36 accessions from the Northern Cape and KwaZulu Natal provinces were used as they appear in Chapter one.

3.2.2 SSR genotyping

Total genomic DNA extracted using the ZR™ plant seed kit in chapter two was used. Six pairs of polymorphic markers developed by Erika Viljoen (PhD thesis,2018) were used in a

multiplex PCR. Forward primers were labelled by fluorescent dyes manufactured by Thermo Fisher, Scientific, Applied biosystems, RSA. Marker information is presented in Table 3.1. Multiplex PCR reactions were first carried out on three individuals from each species and optimized. For genotyping, PCR amplification was carried out using Platinum™ multiplex kit (Applied biosystems, Thermo Fisher Scientific, RSA). Each PCR reaction contained three SSR primer pairs at a concentration of 1uM each, 50ng/ µl genomic DNA, 1X platinum™ multiplex master mix and nuclease free water to a final volume of 25 µl. Thermocycling conditions consisted of initial denaturation at 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C, extension at 72°C for 1 min and final extension at 72°C for 45 min and lastly a cooling period at 4°C for 10 min. The PCR products were visualized on a 2% (w/v) agarose gel and diluted using double distilled water. Diluted PCR products were resuspended in Hi-Di™ Formamide (Applied biosystems, Thermo Fisher Scientific, RSA) and 0.2 µl GeneScan™ Liz® 500 size standard (Applied biosystems, Thermo Fisher Scientific, RSA) was added. The samples were heated at 95°C for 5 min and immediately cooled on ice then separated on an ABI PRISM™ 3500 capillary sequencer (Applied biosystems, Thermo Fisher Scientific, RSA) at the DNA sequencing facility (University of Pretoria, SA). The Thermo Fisher Scientific online software tool Microsatellite Analysis (MSA) was used to resolve and score allele sizes.

Table 3.1. Microsatellite marker primers and their properties used for the analysis of genetic variation in this study. Markers were developed by Erika Viljoen (2018)

Marker	Forward and reverse primer (5'-3')	TM (°C)	Repeat motif	Fluorescent label
ATR8	GAAACCAACAAAGTAGTGGGAGTT	55	(GATAAA)7	6-FAM
	AGAACCCTCTTGTCCCTCTTTATC	56		
ATR12	GGACTAACTGAATAAAGCCAAGTCA	55	(ATT)12	VIC
	TGTATGAGTACGTACATGTGATAGTGC	56		
ATR19	ATACGCAGAAATCACATCTCTCTTG	55	(TAT)34	NED
	GAAGTCGATAGCGTGTGTTTGAC	56		
ATR28	TGAGGTCAATTGCCACAACTAC	55	(AGA)10	6-FAM
	GATTGAGGAAAGAGAAAGCGAAAG	54		
ATR32	GAACGGATCTCTGCTTGCTAAATA	55	(TTG)9	VIC
	GTAAAACACATCTGGGAGTTTGAG	54		
ATR62	TATGTAATGCCTGCACCTACT	53	(AGA)18	NED
	CACACAAAGGAGCTACTCAAC	53		

3.2.3 Genetic data analysis

Genetic analysis was performed only on *Amaranthus palmeri* accessions and the other *Amaranthus* species were excluded. For each microsatellite marker, the genetic statistics; number of alleles (Na) and their frequencies, number of effective alleles (Ne), observed (Ho) and expected (He) heterozygosity were calculated using GenAIEx 6.5 software (Peakall and Smouse, 2012). Marker polymorphism information content (PIC) was calculated using CERVUS software 3.0 (Kalinowski et al., 2007).

3.3 Results

3.3.1 Microsatellite marker genotyping

All 36 samples falling into three species, *A. palmeri*, *A. hybridus* and *A. standleyanus* were genotyped using the six microsatellite markers. Multiplex PCR was successful for all species (Figure 3.1). Each allele was visually inspected, called and scored for each locus and each accession (Table 3.2). Of the six markers used, two markers (ATR19 and ATR32) could not be genotyped for *A. palmeri* species as they have multiple alleles due to stuttering and could not be correctly scored. These markers were therefore scored as missing data and excluded in downstream data analysis.

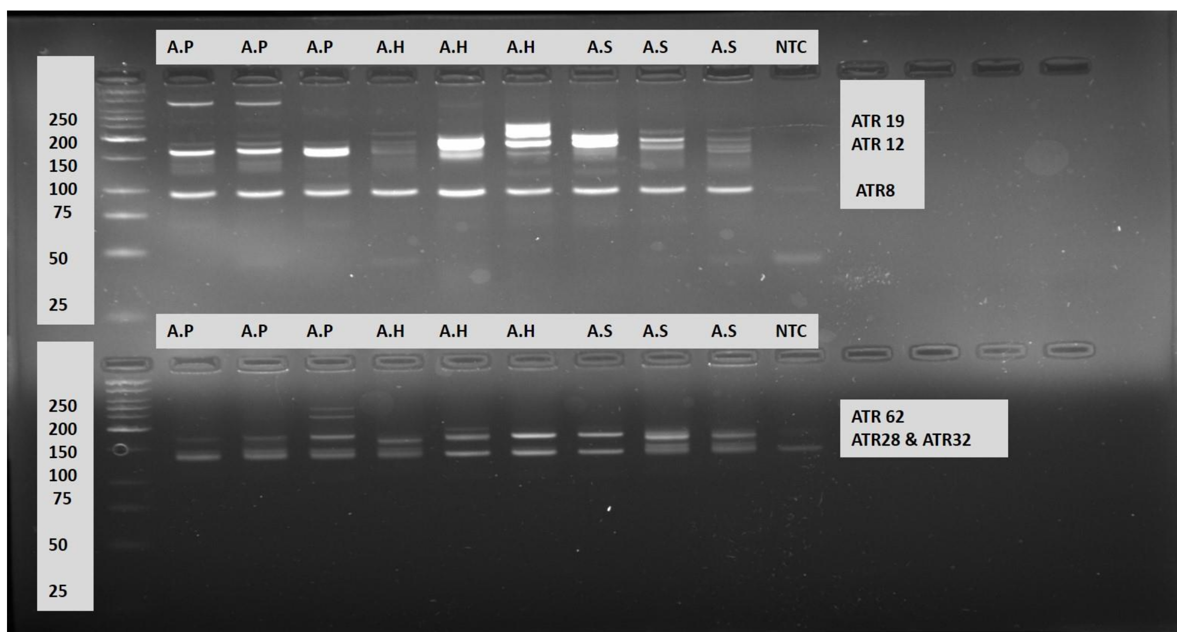

Figure 3.1. Multiplexed amplification of the SSR marker sets on the three Amaranthus species used in this study. Three accessions from each species *A. palmeri* (A. P), *A. hybridus* (A.H) and *A. standleyanus* (A.S) were amplifies and used for polymerase chain reaction optimization

Table 3.2. Alleles scored at each locus for each accession of *Amaranthus* species. The zero (0) indicates that these alleles could not be scored

Sample	Species ID	ATR8		ATR12		ATR19		ATR28		ATR32		ATR64	
NC1	*A. palmeri*	95	95	155	155	0	0	131	134	0	0	162	171
NC2	*A. palmeri*	95	95	164	164	0	0	131	134	0	0	168	171
NC3	*A. palmeri*	95	95	161	161	0	0	134	134	0	0	162	168
NC4	*A. palmeri*	95	95	155	161	0	0	134	137	0	0	162	171
NC5	*A. palmeri*	95	95	164	164	0	0	131	134	0	0	171	171
NC6	*A. palmeri*	95	95	164	164	0	0	134	137	0	0	171	171
NC7	*A. palmeri*	95	95	155	161	0	0	134	134	0	0	171	171
NC8	*A. palmeri*	95	95	155	161	0	0	134	134	0	0	171	171
NC9	*A. palmeri*	95	95	155	155	0	0	131	134	0	0	171	171
NC10	*A. palmeri*	95	95	157	163	0	0	131	134	0	0	171	171
NC11	*A. palmeri*	95	95	155	161	0	0	134	134	0	0	171	171
NC12	*A. palmeri*	95	95	161	161	0	0	134	137	0	0	171	171
NC13	*A. palmeri*	95	95	161	164	0	0	131	131	0	0	168	171
NC14	*A. hybridus*	95	95	172	172	186	208	137	137	130	130	160	163
NC15	*A. standleyanus*	95	95	177	177	195	195	134	134	123	123	168	168
NC16	*A. hybridus*	95	95	184	184	186	186	134	134	120	120	163	163
NC17	*A. standleyanus*	95	95	161	164	186	186	134	137	130	130	163	163
NC18	*A. hybridus*	95	95	181	181	186	186	134	134	120	123	162	168
NC19	*A. standleyanus*	95	95	155	164	195	195	131	134	120	130	160	163
NC20	*A. palmeri*	95	95	164	164	0	0	131	134	0	0	162	171
NC21	*A. palmeri*	95	95	160	160	0	0	131	134	0	0	162	171
NC22	*A. hybridus*	95	95	177	177	195	195	134	134	120	123	162	168
NC23	*A. hybridus*	95	95	177	177	195	195	134	134	120	120	162	168
NC24	*A. hybridus*	95	95	181	184	186	186	134	134	120	126	162	168
NC25	*A. palmeri*	95	95	157	160	0	0	134	134	0	0	162	168
NC26	*A. palmeri*	95	95	157	160	0	0	134	134	0	0	162	168
NC27	*A. palmeri*	95	95	161	161	0	0	134	134	0	0	171	171
NC28	*A. palmeri*	95	95	155	164	0	0	131	134	0	0	171	171
NC29	*A. palmeri*	95	95	155	155	0	0	134	134	0	0	171	171
ZN30	*A. palmeri*	95	95	155	161	186	208	131	134	0	0	162	171
ZN31	*A. palmeri*	95	95	161	161	184	184	131	134	0	0	168	171
ZN32	*A. hybridus*	95	95	180	184	186	186	134	134	130	130	162	168
ZN33	*A. palmeri*	95	95	155	158	186	186	134	137	0	0	171	171
ZN34	*A. hybridus*	95	95	180	180	184	184	134	134	120	126	162	168
ZN35	*A. hybridus*	95	95	180	180	211	211	134	134	120	120	162	168
ZN36	*A. hybridus*	95	95	180	180	208	214	134	134	120	120	168	168

3.3.2 Microsatellite marker diversity

Thirteen alleles were identified across the six SSR markers with an average of 3.25 alleles per locus (Figure 3.2). The least number of alleles was observed for ATR8 (1) with a frequency of 1.0. The SSR marker ATR12 had the greatest number of alleles (6) with allele frequencies ranging from 0.023 to 0.318 in this locus. ATR28 and ATR64 each had three alleles with one allele being more dominant in frequency than the other two in each locus.

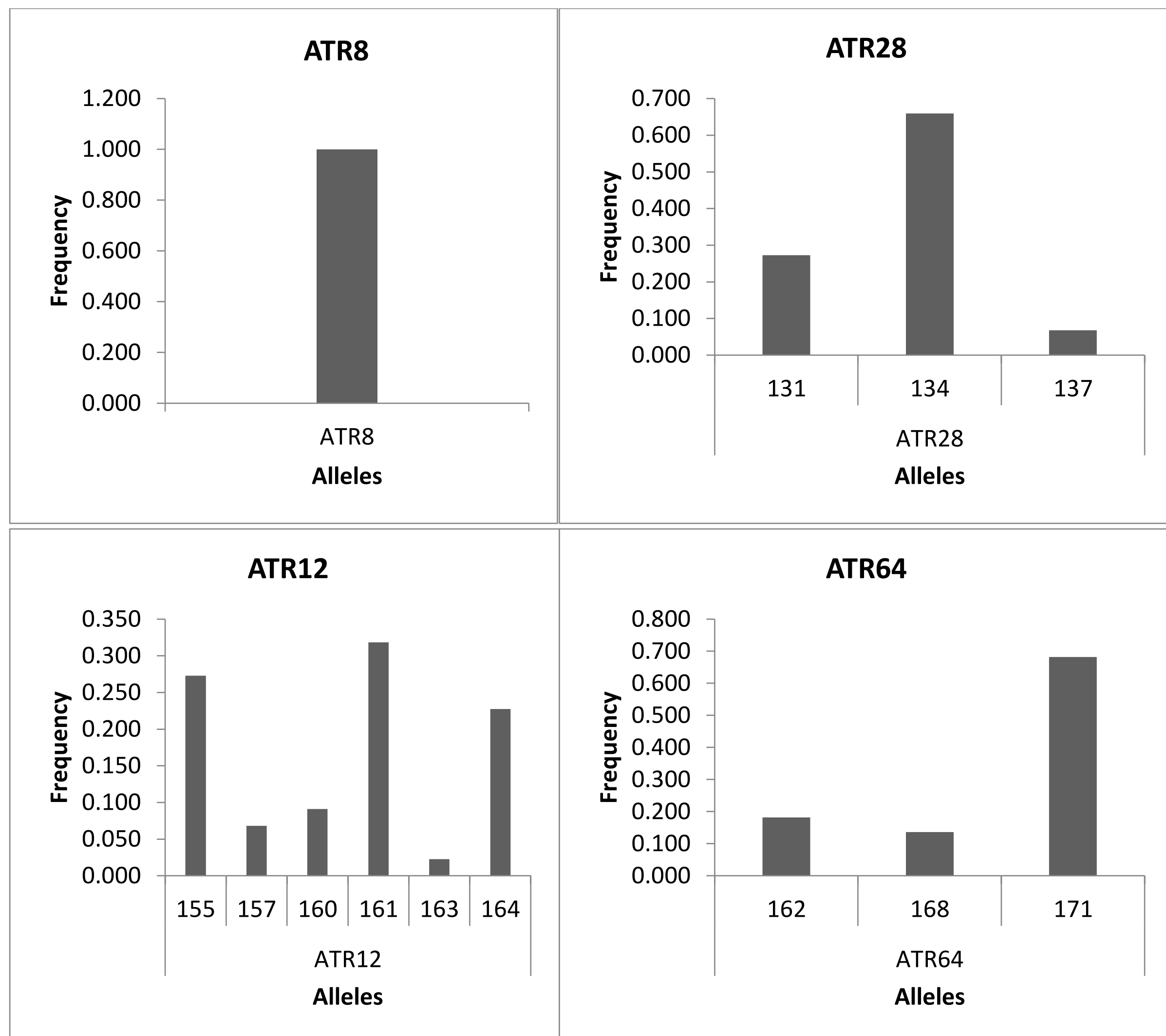

Figure 3.2. Allele frequencies observed in all four loci during SSR marker analysis

3.3.3 Genetic variation revealed by SSR markers

Three of the four loci were polymorphic and had more than one allele. The investigated genetic parameters are presented in Table 3.3. Across all loci the effective number of loci ranged from 1 (ATR8) to 4.1 (ATR12). Observed heterozygosity was greatest for ATR28 (0.591) and least for ATR8(0) with the other two markers having 0.455 (ATR12) and 0.500 (ATR64). The SSR marker ATR12 had the greatest expected heterozygosity (He) value (0.759) followed by both

ATR28 and ATR64 with almost equal values of 0.487 and 0.483 respectively. The mean He value across all loci was 0.432.

Table 3.3. Genetic diversity parameters observed in the Palmer amaranth population

Locus	N	Na	Ne	Ho	He	PIC	I
ATR8	22	1.000	1.000	0.000	0.000	0.000	0.000
ATR12	22	6.000	4.155	0.455	0.759	0.720	1.543
ATR28	22	3.000	1.948	0.591	0.487	0.417	0.812
ATR64	22	3.000	1.936	0.500	0.483	0.434	0.843
Mean	22	3.250	2.260	0.386	0.432	0.393	0.799

3.4 Discussion

Six microsatellite markers were initially chosen to genotype the population and two of these failed to genotype Palmer amaranth accessions but were successful in all other *Amaranthus* species used in this study. These failed markers were also unsuccessful in genotyping accession of *A. spinosus*, a *Amaranthus* species more genetically related to Palmer amaranth (Erika Viljoen, PhD study, 2018). This is an indication that these markers are not suitable to be employed in genetic diversity studies of these two weedy species.

Among the four remaining loci, ATR8 was monomorphic thus not informative at all. This was also observed by Erika PhD thesis, (2018) as only three alleles were recorded, and one allele had four-fold higher occurrence than the rest. The five remaining loci were informative as they had PIC values ranging from 0.417 – 0.720 with an average of 0.393 (Table 3.3). Weedy *Amaranthus* species have been observed to be more genetically diverse when compared to leafy and grain amaranths (Suresh et al., 2014). In this study, the mean expected heterozygosity which is a parameter used to estimate genetic diversity in a population was higher than the mean observed heterozygosity with values of 0.432 and 0.386 respectively. This was an indicator of moderate genetic diversity within the investigated Palmer amaranth population from the Northern Cape province. Chandi et al. (2013) observed high genetic diversity within palmer amaranth populations from North Carolina and Georgia as compared to between populations. Palmer amaranth's obligate outcrossing reproductive strategy greatly contributes to the observed genetic diversity and so does its propensity to hybridize with other co-existing *Amaranthus* species. The introduced population faces little or no constraints on establishment as South Africa's current and future climatic conditions are favourable for *Amaranthus* species (Kistner and Hatfield, 2018), this is evident by the already thriving and naturalized *Amaranthus*

species such as spiny amaranth and smooth amaranth. Palmer amaranth's ability to rapidly evolve novel traits and the propensity for genetic mutations will also greatly contribute into the introduced population's genetic diversity and eventually population structure. This will be in response to South Africa's cropping systems and weed management strategies which might exert selective pressure on the introduced population forcing fitness enhancing traits to be selected for.

3.5 Conclusion

This is the first study attempting to understand the extent of genetic variation within the introduced Palmer amaranth population in South Africa. Most of the developed SSR markers were informative and transferrable between *Amaranthus* species and revealed moderate genetic diversity in the Northern Cape population. The information derived from thus study will help in understanding the species more as it has successfully established and is reproducing and diversifying. A more broader scale population diversity study employing a larger sample size from all the geographic areas where Palmer amaranth was cited remains to be investigated to elucidate any relatedness between these populations and to see if the species introduction was a single or multiple events. It will, however, be necessary to use more SSR markers to ensure that the resulting genetic indices are more significant.

CHAPTER IV

GENERAL DISCUSSION AND FUTURE RECOMMENDATIONS

Globally, the agricultural industry is faced with a serious problem which is the increasing rate at which weeds are evolving resistance to most commercialized herbicides. Palmer amaranth has expanded beyond its original distribution range and has become a weed of economic importance in most places where it has been confirmed as invasive. Alternative herbicide management strategies are encouraged for a weed such as Palmer amaranth which has easily and quickly developed resistance to up to eight SOA. Of more importance immediately after an introduction is characterizing the herbicide resistance profile of the population to make informed recommendations on the type(s) of control strategies to be adopted.

In the present study, we identified and characterized the target site herbicide resistance profile of two populations of Palmer amaranth in South Africa. This was after the first report of this noxious weed in the country. Since this species was new in South Africa and was first reported in farms where there were already other morphologically similar *Amaranthus* species, every species suspected to be palmer amaranth was submitted for molecular identification. Sequencing the ITS region successfully identified palmer amaranth and two other species, *A. standleyanus* and *A. hybridus*. Resistance to ALS, EPSPS and PPO inhibitors was characterized in all three species. The two Palmer amaranth populations from the two provinces, Northern Cape and KwaZulu Natal had different profiles for the ALS and EPSPS inhibitors. This indicated that they were different populations and might have been introduced separately from different origins. Target site resistance was observed in both populations, and both had mutations conferring high resistance to both ALS and EPSPS inhibitors. No target site resistance was observed for PPO inhibitors though this does not mean that the populations were not resistant to this class of herbicide as only TSR was investigated. There is still a need therefore, for further studies to investigate the presence of NTSR that might exist in these populations. As mentioned earlier, the presence of NTSR in weeds is more worrying as one mechanism (e.g., rapid detoxification) can provide cross resistance to many herbicides SOA. Another interesting yet alarming finding of this study was the observation of TSR mechanisms to both ALS and EPSPS inhibitors in *A. hybridus* from the KwaZulu Natal province. The accessions were collected in the same field as the Palmer amaranth accessions which raised two main questions that will need to be investigated and answered. The first question being was the herbicide resistant *A. hybridus* introduced to KZN together with Palmer amaranth? and the second one being did herbicide resistance in the *A. hybridus* accessions evolve independently in SA because of herbicide management practices or is hybridization with the introgression of herbicide resistance genes already happening in these co-existing species?

Investigating and answering these questions will prove very valuable to the South African Herbicide Research Initiative (SAHRI) as it will give a clear indication of whether South Africa's herbicide resistance management practices are failing on their own regardless of the introduction of Palmer amaranth.

The preliminary study investigating the genetic population diversity of Palmer amaranth gave insights into the genotypic makeup of mostly the Northern Cape population. Moderate genetic diversity was observed based on three informative SSR loci in 22 accessions of Palmer amaranth. The sample size and number of loci used were not enough to provide significant results of the amount of genetic variation existing in the population. Since more populations of Palmer amaranth were discovered during this study, it would be beneficial to investigate within and between population diversity in all three populations (Northern Cape, KwaZulu Natal and the newly observed Limpopo population). A more in depth and full-scale study could give insights into the number of introduction events, the population structure and maybe even the origin of each population. While on the topic of more populations being discovered, it was noted that for the samples collected in the Northern Cape, their locations seemed to be situated along the main rivers flowing through that agricultural province with more samples being cited downstream. This is not necessarily new as irrigation channels are one of the major channels' weeds employ to spread. It would however be beneficial to investigate the extent of the contribution the irrigation channels have into the spread of palmer amaranth to other geographic areas downstream.

The aim of this study, to identify and characterize the resistance profile of Palmer amaranth using molecular techniques was achieved. Based on the findings of this study, more informed strategies on how to deal with this weed can be devised. It would be advisable for South African farmers to adopt more integrated weed management practices, which entails mixing herbicides with more than one SOA and mechanical control practices. They would also need to develop the habit of paying more attention to the behaviour of weeds especially after herbicide applications as this would enable early detection of the presence of herbicide resistant plant. A weed as invasive and devastating as palmer amaranth is a serious concern and every country with an introduction should be alert and swiftly put-up control and containment strategies before it gets out of control. Palmer amaranth should be put into every country's zero threshold preventative policy.

SUPPLEMENTARY DATA

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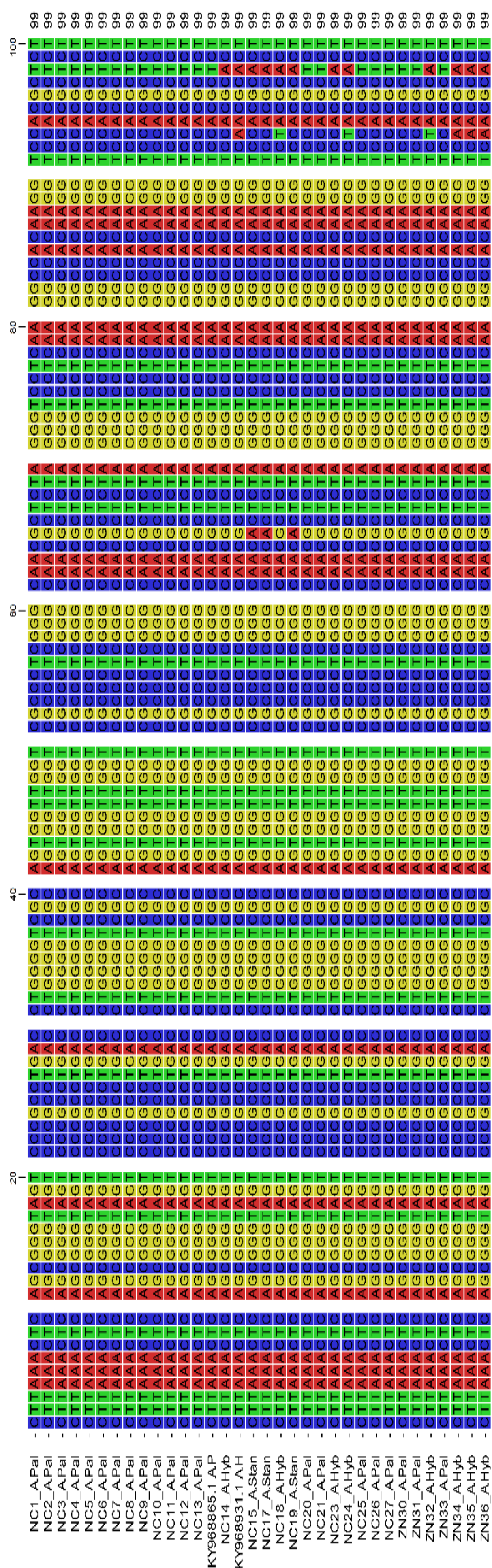
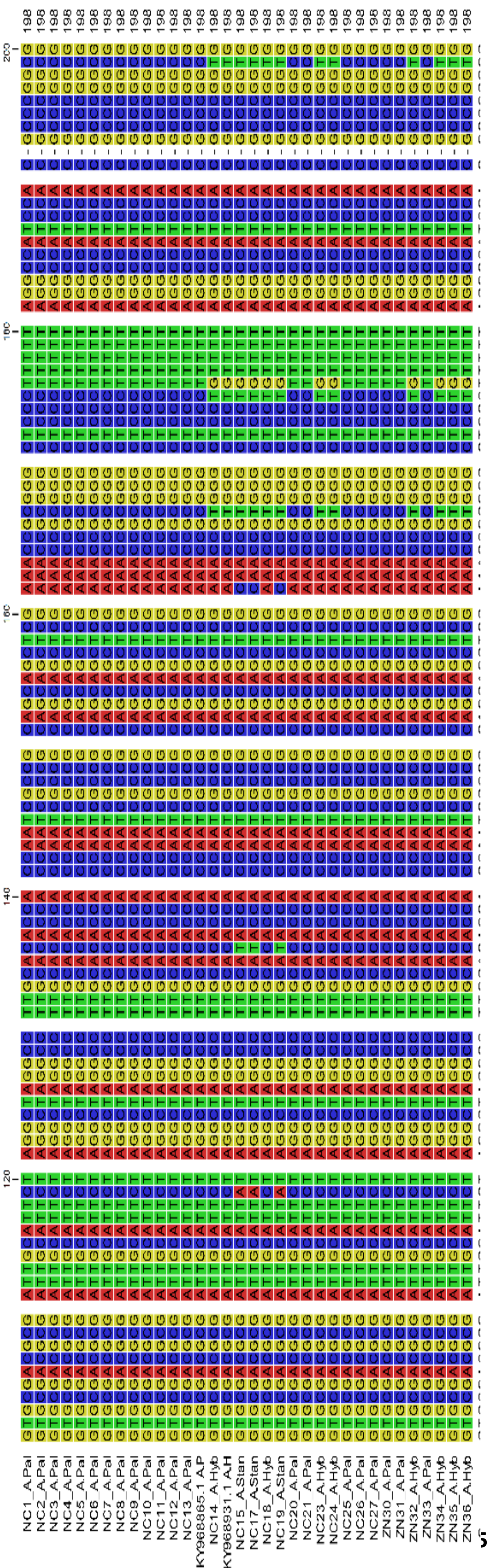
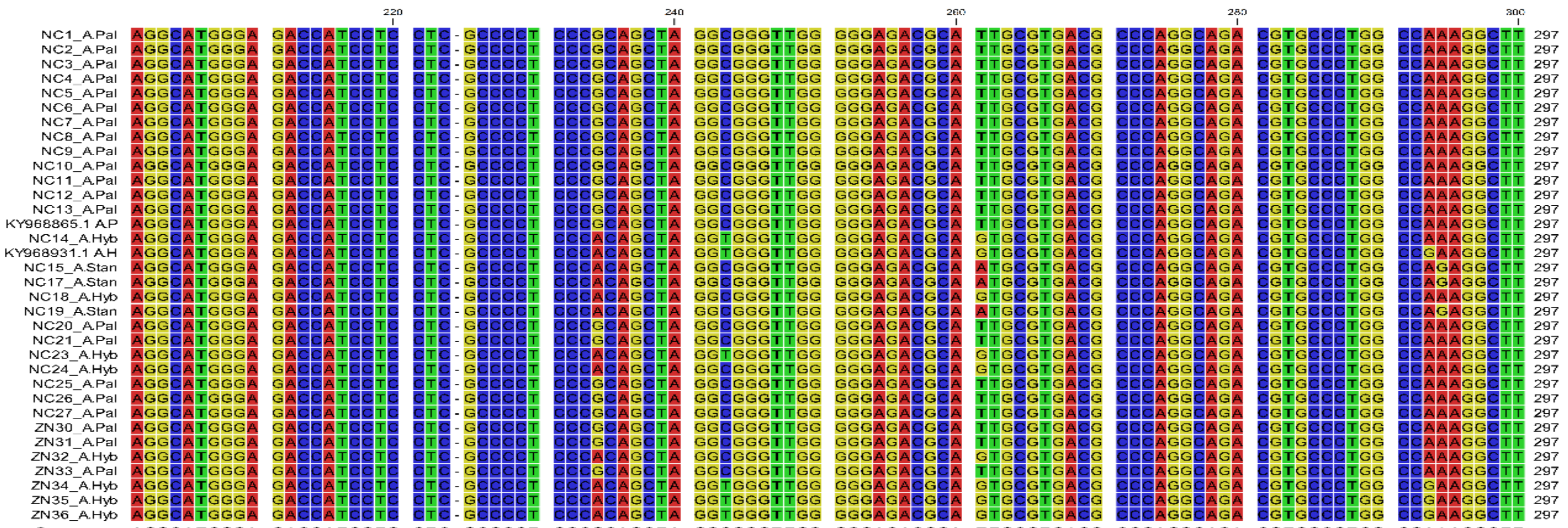
Supplementary data S1. All consensus sequences generated by sequencing a 708 bp fragment of the ITS region consisting of a partial sequence of the small ribosomal RNA unit; ITS1;15.8S rRNA; ITS2 and a partial sequence of the large ribosomal gene subunit. These sequences were submitted to GenBank.







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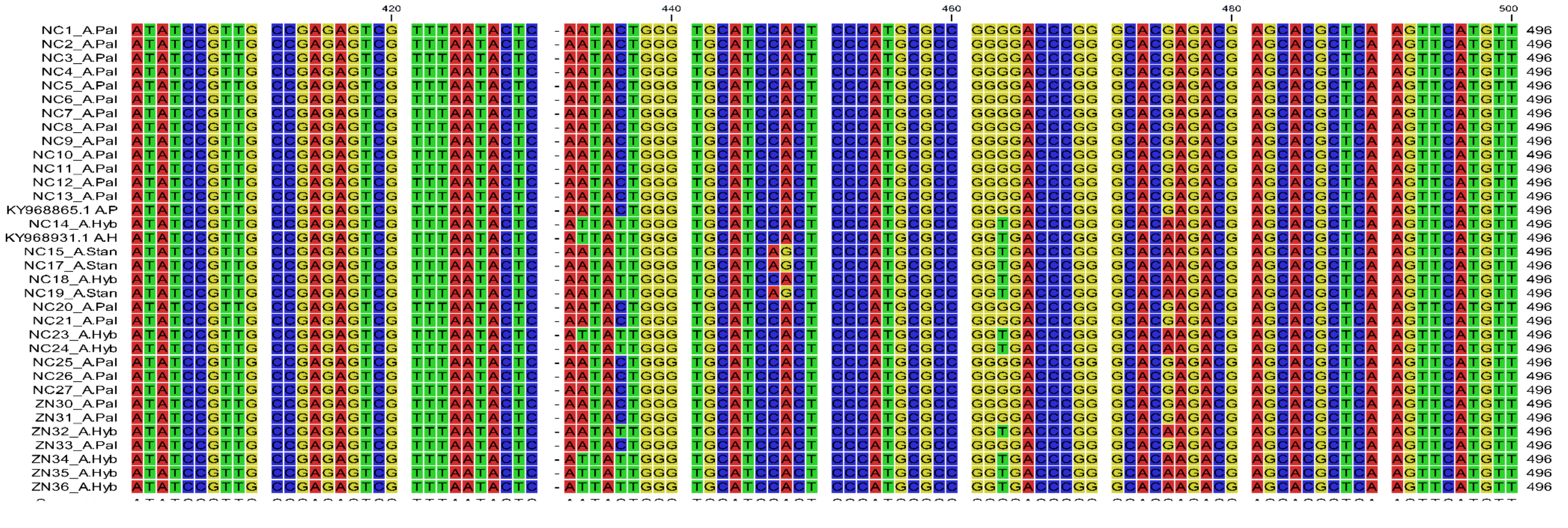

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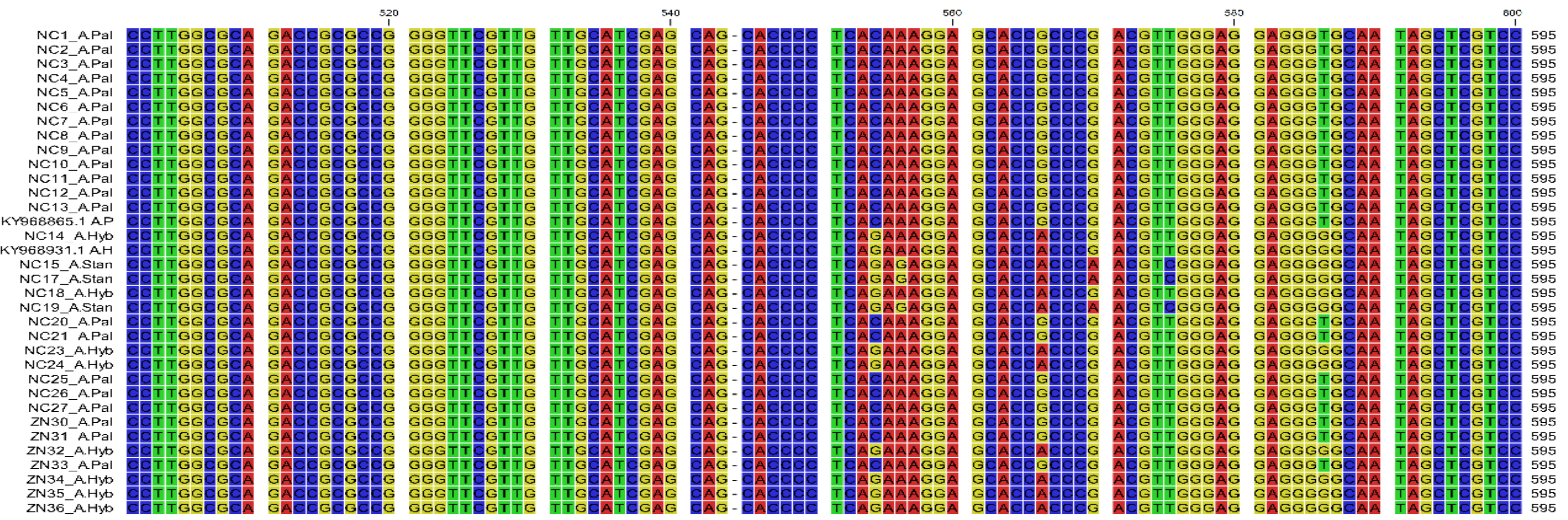

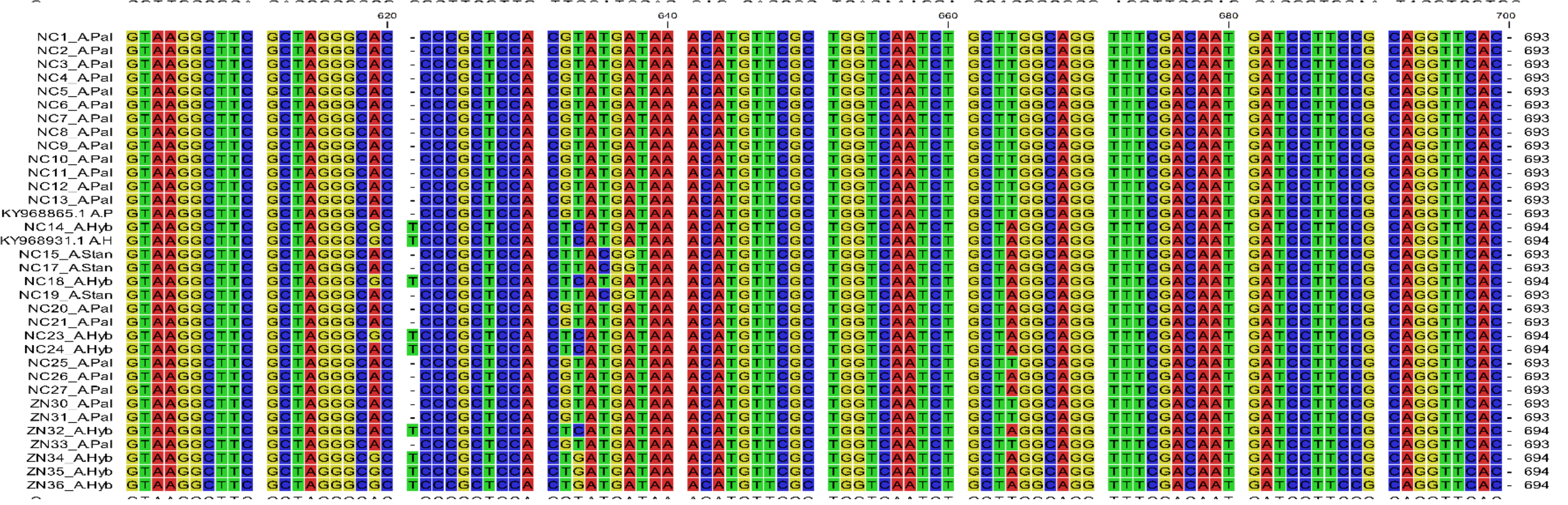

Supplementary figure S2. Nucleotide sequence alignment of the ITS region. SNP's differentiating the *Amaranthus s*pecies can be seen.

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NC21_A.Pal   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC22_A.Hyb   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC23_A.Hyb   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC24_A.Hyb   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC25_A.Pal   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC26_A.Pal   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC27_A.Hyb   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC28_A.Pal   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
NC29_A.Pal   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
KT833339.1   IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR 137
Consensus    IATSGPGATN LVSGLADALL DSVPLVAITG QVPRRMIGTD AFQETPIVEV TRSITKHNYL VLDVEDIPR
Conservation (100% / 0%)
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Supplementary figure S3. Amino acid alignment if the CAD domain of the *ALS* gene. No mutations were observed.

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