GENETIC DIVERSITY OF *TAMARIX* LINEAGES IN SOUTH AFRICA AND HEAVY METAL TOLERANCE OF THE INDIGENOUS SPECIES

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

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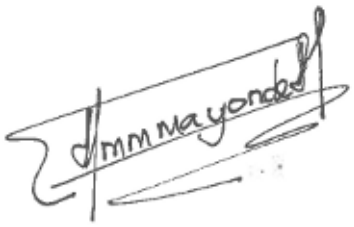
JOHANNESBURG

SEPTEMBER, 2018

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



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ABSTRACT

Three species of saltcedar (*Tamarix* L.; Tamaricaceae) occur in South Africa, although only *T. usneoides* is indigenous and is effectively used in southern Africa mines for phytoremediation. *Tamarix ramosissima* and *T. chinensis* were reportedly introduced into South Africa from Eurasia also for phytoremediation, but have since invaded riparian zones. *Tamarix* species are morphologically similar and hybridization adds to their taxonomic confusion. Biological invasions of *Tamarix* species have serious negative impacts in most riparian zones from changing the ecosystem function to altering the river flow system, to depositing salt crystals on the top soil and displacing native biodiversity. Mechanical and chemical control of the invasive *Tamarix* has been reported ineffective, leaving biocontrol as an alternative option to suppress *Tamarix* infestations thereby reducing its negative impacts. However, for the biocontrol programme of *Tamarix* in South Africa, hybridization and introgression events, and the presence of the indigenous species poses a challenge of host shifts for non-target effects to the indigenous species. Assessing the levels of genetic diversity and differentiation in invasive species and their closely related indigenous species is essential to better understand the invasion process and population genetic relations for an effective biocontrol programme. Molecular techniques are widely used during the initial phase of biocontrol programmes before the release of agents to accurately identify the invading species, assess the genetic diversity and compare the genetic structure between and within species.

In South Africa, the indigenous *Tamarix usneoides* is used for phytoremediation in rehabilitation efforts of mine lands contaminated with acid mine drainage. Although the plant has been planted on tailing storage facilities, its ability to tolerate the highly acidic conditions and heavy metals has not been fully investigated. Plants suitable for phytoremediation should have the ability to tolerate high concentrations of heavy metals and accumulate them in their above ground biomass. Therefore, this study investigated the physiological responses of *T. usneoides* to metal toxicity by assessing growth parameters. Profiles of gene expression patterns of *T. usneoides* for heavy metal tolerance were also investigated to assess the ability of the plant in activating its genome for tolerance against environmental stress conditions.

Nine microsatellite loci were used to assess the genetic diversity and differentiation of 150 *Tamarix* samples (117 individuals from South Africa and 33 from the U.S.). In South Africa, samples were collected from four *Tamarix* taxa viz. *T. usneoides*, *T. chinensis*, *T. ramosissima* and *Tamarix* hybrids with 30 samples per taxon; while from the U.S. we collected *Tamarix* hybrids to compare against those from South Africa. This study showed that the indigenous *T. usneoides* is genetically more diverse than the invasive *T. chinensis* but is less diverse than *T. ramosissima*, another invasive species. There was great genetic differentiation between the indigenous and the invasive *Tamarix* species in South Africa. In addition, private alleles unique to *T. usneoides* were obtained in some remote places in the north-western part of the Northern Cape Province suggesting unpolluted populations of the indigenous *T. usneoides* germplasm. The low genetic diversity in *T. chinensis* seems to be as a result of its autogamous nature other than caused by founder effects. Higher genetic diversity was observed in the South Africa *Tamarix* hybrids compared to their U.S. counterparts and there is substantial genetic differentiation between the two hybridization incidences. The high genetic differentiation suggests that there might be minimal or no non-target effects on the indigenous species from biocontrol control agents against the invasive genotypes. This study also suggests that exploration of biocontrol agents should be done in the place of origin of the invasive plants other than in the U.S. where species of the *Diorhabda* beetles are effectively controlling *Tamarix* infestations.

Three different concentrations of cadmium (Cd) – 6 ppm, 12 ppm, 18 ppm, were applied to 240 individuals of indigenous *T. usneoides* propagated from 20 trees from different localities. The plants were exposed to Cd for eight weeks where plant height, shoot growth and chlorophyll content were measured weekly to assess the physiological responses against Cd toxicity. Of the 240 individuals, 80 samples were randomly selected for gene expression profiling using cDNA-AFLP analysis. Height, shoot growth and chlorophyll content of *T. usneoides* were all found to decrease with an increase in Cd concentrations over time as there were significant differences between the Cd treated groups and the controls. Chlorophyll content was found to be the most sensitive to Cd toxicity followed by

shoot growth with plant height the least affected parameter. Although Cd toxicity affected the physiology and growth of *T. usneoides*, the plants were observed to be tolerant to the low (6 ppm) and medium (12 ppm) Cd concentrations as the measured parameters were not significantly reduced compared to the controls. Additionally, no visible signs of metal toxicity were observed at low and high concentrations. However, phenotypic signs such as leaf chlorosis, drying of branch bottoms, even the death of some plants were observed at the high (18 ppm) Cd concentration. The high Cd concentration level resulted in a significant reduction in the growth of *T. usneoides*. cDNA-AFLP analysis of *T. usneoides* showed different profiles in expression patterns where high numbers of AFLP bands were obtained with an increase in Cd concentrations over time. The medium concentration was observed to generate the highest transcript derived fragments (TDFs) associated with heavy metal tolerance. The ability of *T. usneoides* to tolerate heavy metal concentrations < 12 ppm and the wide regions of the genome being expressed with an increase in metal concentration suggest that *T. usneoides* is a good candidate for phytoremediation.

To sum up, the indigenous *Tamarix usneoides* is moderately diverse genetically and is greatly differentiated from the exotic and invasive *T. chinensis*, *T. ramosissima* and their hybrids. The invasive *Tamarix* genotypes can be considered for biocontrol with suggested minimum non-target effects on the native species which is considered for conservation and presents a good candidate for use in phytoremediation because it can tolerate more than usual concentrations of heavy metals in contaminated soils.

Key words: Biocontrol agents, Biotic and abiotic pressure, Genetic variation, Gene expression, Habitat fragmentation, Metal tolerance, Reproductive systems, Tamaricaceae.

DEDICATION

This work is dedicated to my lovely mother Bernadette Musenga Musumba and my beautiful bride Carolyne Donga.

ACKNOWLEDGEMENTS

My doctorate research was funded by the National Research Foundation (NRF) of South Africa, the Agriculture Research Council (ARC) awarded to Prof. Marcus Byrne through the Plant Protection Research Institute (PPRI) and the department of Trade and Industries (DIT) and National Research Foundation (NRF) "THRIP" awards to Ms. Isabel Weiersbye and Prof. Edward Witkowski (University of the Witwatersrand, Johannesburg). My research would not have been possible without the financial support of these organizations.

I am indebted to my co-supervisors as none of this would have been remotely possible without Prof. Marcus Byrne, Prof. Glynis Goodman-Cron and Dr. Kelsey Glennon. Thank you for sharing your knowledge, guidance, encouragement and criticism that have greatly contributed to my professional development and personal growth. I would like to thank all my friends and colleagues in the school of Animal Plant and Environmental Sciences, Wits University for making it such a welcoming, helpful and warm place to conduct research. Special thanks for the members of the biocontrol lab, especially Tshuxekani Maluleke "AKA CHUCHI" for his assistance with my field work, Peter Kgampe for helping with planting. To my lab assistance Lungile Masinga, thanks for assisting me with the metal experiment and data collection. To my brother, Naweji Katembo "Dr. Blood" thanks for making this journey FUN and enjoyable even during stressful moments. To my comrades, Dr. Solomon Newete – Dr. Lutendo Mugwedi – Archibold Sasa Tapfuma thank you for soldiering this journey by showing me the way or by walking side by side with me.

I would like to extend special thanks to staff, students and interns from the CE Moss Herbarium in the School of Animal Plant and Environmental Sciences, Wits University for unwavering support throughout my PhD time.

I am grateful to my friend and collaborator Dr. John F. Gaskin for providing me with some technical advice on the microsatellite amplification and for providing me with the U.S. *Tamarix* hybrids samples. Thanks for devoting your time to edit

the early version of my genetic diversity chapter and sharing your knowledge and honest advice on how molecular ecology can aid biological control.

Last but not least, I am eternally indebted to my family, my fiancé Carolyne Donga and my son Simphiwe Ashley Mayonde for continued support, encouragement and patience. They haven't read a word of anything I have written, but they provided constant support and sincere attempts and interest along the way. To my dad Mr. Mukazu Mayonde and my kinfolks thank you for awakening the vision within me, without you guys I wouldn't even started with my PhD. To my friend Roselyne Phakati, thanks your advice on how KISS (Keep It Simple and Short) my thesis. In Rund we say "**mwajidishan nawosh**" meaning THANK YOU ALL.

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ABBREVIATIONS

AFLP = Amplified Fragment Length Polymorphism

ANOVA = Analysis of variance

AMD = Acid Mine Drainage

BSA = Bovin serum albumin

Cd = cadmium

DNA = Deoxyribonucleic acid

cDNA = complementary deoxyribonucleic acid

DW = Deionized water

GPS = Global positioning system

GSH = Glutathine

H_E = Expected heterozygosity

H_O = Observed heterozygosity

HWE = Hardy–Weinberg Equilibrium

LSD = Least significant difference

MRDs = Mine Residue Deposits

PCoA = Principle coordinates analysis

PCs = Phytochelatins

PCR = Polymerase Chain Reaction

RT-qPCR = Quantitative reverse transcription polymerase Chain Reaction

RNA = Ribonucleic acid

ROS = Reactive oxygen species

RFU = Relative fluorescent units

SA = South Africa

SSRs = Simple Sequence Repeats

TDF = Transcript derived fragments

TSFs = Tailing Storage Facilities

U.S. = United States

VNTRs = Variable Number of Tandem Repeats

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. RATIONALE OF THE STUDY

Mining is one of the main economic activities in South Africa, and a major polluter and contaminator of the environment. It is therefore essential to look for an inexpensive, reliable and environmentally friendly approach to remediate the sterile landscapes of tailing storage facilities (TSFs) around the country. Phytoremediation using effective phreatophytes (plants with a deep tap root that reaches groundwater) is encouraged as it is both environmentally friendly and inexpensive to implement and manage (Vithanage et al., 2012).

Tamarix L. is a genus comprising diploid deciduous or evergreen shrubs or trees and is one of the four genera in the Tamaricaceae (Baum, 1978). *Tamarix* species are among the preferred phreatophytes for phytoremediation as they are resistant to abiotic stresses, are known to tolerate high salt conditions (DiTomaso, 1998), can accumulate a considerable amount of heavy metals and have a high above ground biomass (Manousaki et al., 2009). Given the potential importance of *Tamarix* in phytoremediation, further studies are needed to investigate genotypes of the southern African *T. usneoides* E Mey ex. Bunge that are the most metal tolerant for phytoremediation. Within *T. usneoides* (the species of interest for phytoremediation in South Africa), there is variation in the performance of the trees in the field with some plants unable to survive highly stressful conditions while others thrive (I. Weiersbye pers. comm.). As a result, we hypothesized that there are differences in metal tolerance exhibited by the various genotypes of *T. usneoides*. In addition, some level of genetic differentiation has been observed within *T. usneoides* (Mayonde, 2013). Therefore, we investigated potential differences in gene expression profiles for heavy metal tolerance in *T. usneoides* individuals. Genotypes with the most consistent gene expression patterns for high levels of heavy metal tolerance will be preferred for cultivation and planting in phytoremediation trials for rehabilitation of mine residue deposits (MRDs).

Among the *Tamarix* species in South Africa, the native *T. usneoides* is preferred for phytoremediation because it is indigenous even though the exotic species are equally effective (I. Weiersbye pers comm.). In addition to the indigenous *T. usneoides*, two exotic species, *T. chinensis* Lour. and *T.*

ramosissima Ledeb., and their hybrid genotypes are present on South African gold and uranium mines (Mayonde et al., 2015, 2016). These exotic *Tamarix* species and their hybrids have escaped their regions of introduction and have invaded riparian zones and water channels in South Africa (Mayonde et al., 2016; Marlin et al., 2017). They have also hybridised with the native species (Mayonde et al., 2015, 2016). Among the possible control measures, classical biological control (biocontrol), a "green" and inexpensive way to suppress alien invasive plants, is envisaged as a means to curtail the invasive *Tamarix* genotypes in the country. However, hybrids between the alien invasive *Tamarix* species and the indigenous species pose a challenge for biocontrol. Any biocontrol programme developed to remove the invasive *Tamarix* taxa in South Africa will need to ensure that introduced agents do not attack the related indigenous species, which is important for phytoremediation and should be conserved. Therefore, investigations into the genetic diversity and population genetics of *Tamarix* species and their hybrids using polymorphic DNA markers are needed to provide the foundation for useful recommendations to be considered during host-specificity testing of potential biocontrol agents.

This research aims to identify the best *Tamarix* genotype(s) for phytoremediation, provide additional insight into the genetic diversity and differentiation among *Tamarix* species, and will compare the genetic profile of invasive *Tamarix* hybrid genotypes from South Africa to those from the United States of America (U.S.) for a biocontrol programme using host-specific agents. The invasive *Tamarix* hybrids are the dominant genotypes in both South Africa and the U.S., and South Africa is considering implementing the biocontrol model currently running in the U.S. using beetles of the genus *Diorhabda* Weise that have successfully defoliated and thereby reduced *Tamarix* infestations.

1.2. INTRODUCTION

Gold and uranium mining is an important contributor to the South African economy but has the potential for significant negative environmental impacts. In 1886 George Harrison, an Australian prospector, discovered gold in the Main Reef conglomerates on the farm Langlaagte, which now falls within the present day city of Johannesburg (Sutton et al., 2006). Since then hundreds of mine residue deposits (MRDs) have accumulated across the Witwatersrand Basin with an estimated total footprint area of between 400 and 500 km^2, comprising some six billion tonnes of gold and uranium wastes (Chevrel et al., 2003). Tailings storage facilities (TSFs), commonly known as 'mine dumps' (Fig. 1.1), across the Witwatersrand basin contain ± 30 million tonnes of sulphur (Witkowski & Weiersbye, 1998) and 430 000 tons of low grade uranium (Winde, 2001). The volumes of TSFs increase by ±105 million tonnes per annum, or at an estimate of 200 000 tonnes of tailings per tonne of gold (Chamber of Mines South Africa, 2004). Although the TSFs are contained in localized areas, they pollute larger areas because of wind- and water-borne emissions termed acid mine drainage (AMD) (Fig. 1.2).

Acid mine drainage is a pollutant produced from exposing metal sulphide minerals such as pyrite (FeS_2) to oxygen and water and is associated with the Witwatersrand gold mines and MRDs (Maree et al., 1996). This contamination from gold mining has been identified many kilometres downstream from the original source (Naicker et al., 2003). It is unfortunate that historically, mining companies in South Africa often located MRDs in sensitive areas and employed no pollution control (Sutton et al., 2006). The right to an environment that is not harmful to human health and wellbeing and the right to protection of the environment are now basic constitutional rights in South Africa (Sutton & Weiersbye, 2007). However, in some instances there is a demand to use lands previously covered by MRDs for residential, agricultural and industrial purposes (Sutton et al., 2006). Therefore, there are several reasons to remediate the impacts from the curtailing of mining activities and clearing of lands previously covered by MRDs containing AMD.

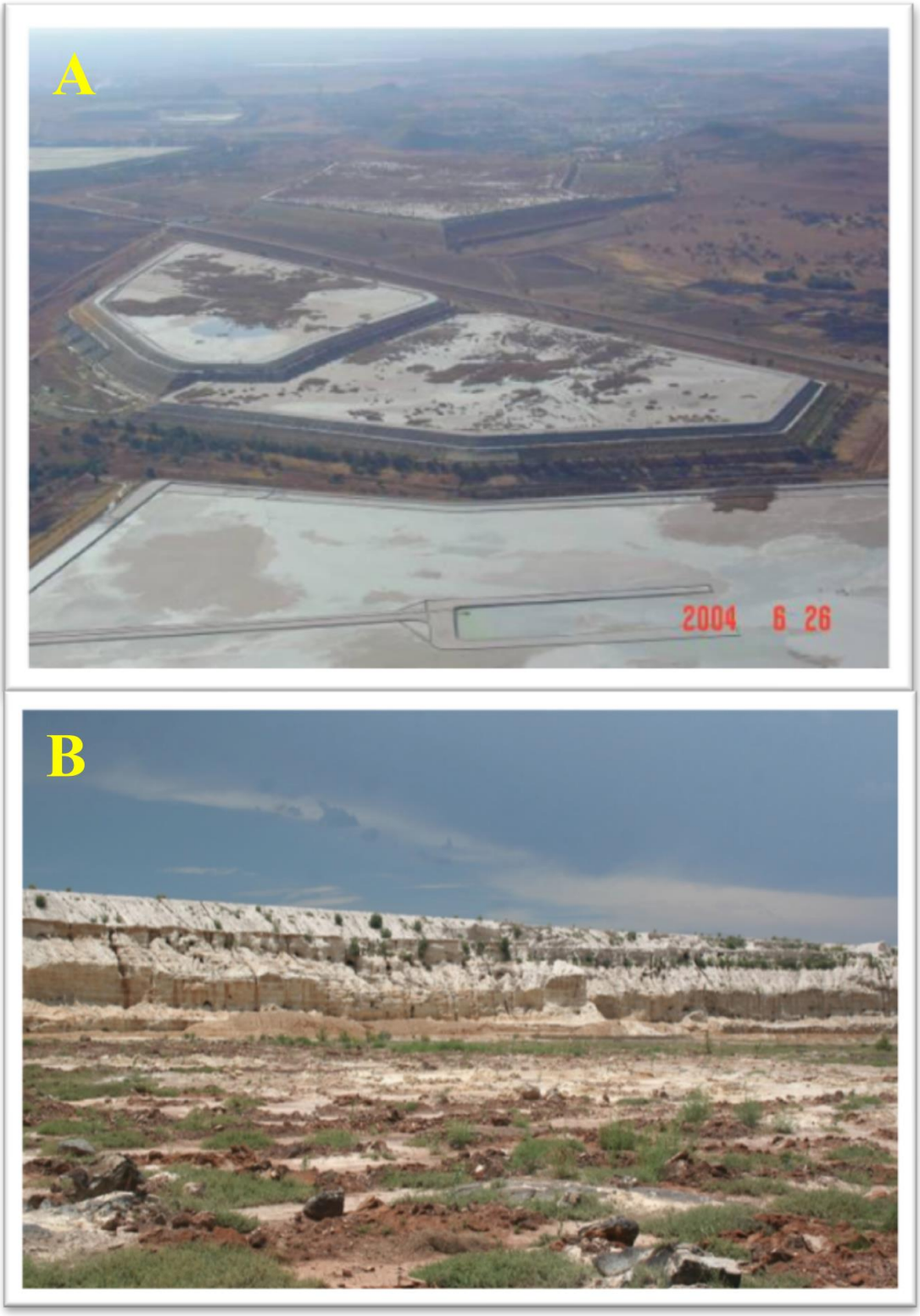

FIGURE 1.1. Gold tailing storage facilities (TSFs)/slimes dams occur across the Witwatersrand basin. (A) The aerial view of a TSF at Vaal River West Wits. (B) Gold mine residue deposit at Vaal River sulphur and East PayDam, Orkney in the North West Province, South Africa. (Photographs courtesy of I.M. Weiersbye).

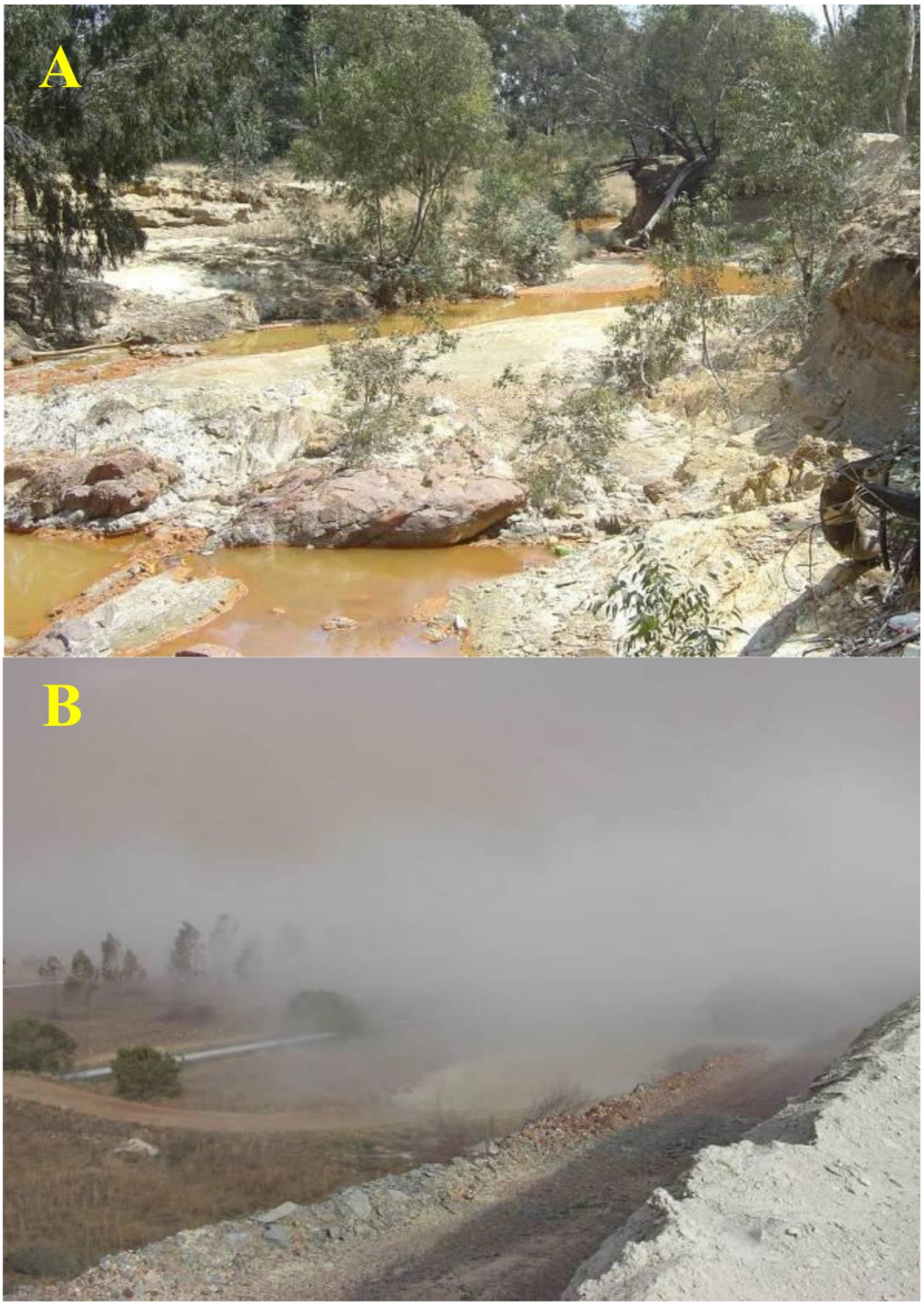

FIGURE 1.2. Air and water pollution impacts from gold and uranium TSFs in the Vaal River, North West Province, South Africa. A) Iron hydroxides precipitated from gold mine residue seepage. B) Dust generated by wind from an uncovered mine residue deposit. (Photographs courtesy of MW Sutton).

1.3. REHABILITATION OF LAND MINES

More than 120 years of gold and uranium mining in the Witwatersrand basin has resulted in mine residue deposits (Robb & Robb, 1998; Tutu et al., 2008). Mine dumps and their footprints (areas where TSFs have been removed) are known to stock concentrations of sulphur, chloride and some metals which are transported via water into soils, watercourses and groundwater, creating heavy metal-enriched sink areas (Mphephu et al., 2004). There is a substantial volume of literature associated with deleterious impacts of AMD on freshwater ecosystems and the environment globally (Akcil & Koldas, 2006). However, concerns in South Africa focus in particular on the impacts of AMD on soils, groundwater and water channels (Naicker et al., 2003; Sutton et al., 2006), and the bioaccumulation of hazardous contaminants such as metals in plants, animals and humans (Weiersbye et al., 1999; Tutu et al., 2005). Therefore, legislation has been put in place to enforce rehabilitation of land mines in South Africa (Sutton & Weiersbye, 2007). From the mining point of view the term 'rehabilitation' means restoring the land disturbed and polluted by mining activities back to a more sustainable and usable state (Salt et al., 1998; Ali et al., 2013). Mine rehabilitation can be a long term process and is now recommended to begin during the planning phase of a mine's development (Sutton & Weiersbye, 2007). Metal contaminated soils can be remediated in various ways using chemicals, mechanical approaches, and/or plants (Salt et al., 1998; Ali et al., 2013). However, chemical and mechanical techniques are expensive to implement and generally environmentally unfriendly. Therefore, implementation of a phytotechnological technique such as phytoremediation (using plants) is often preferred. A range of phytotechnological techniques is available for the extraction, immobilization or degradation of contaminants such as salts, metals, cyanides and sediments.

1.3.1. Phytoremediation of heavy metals

Phytoremediation technique is seen as a "green" resolution to manage heavy metal pollution problems across the globe and is known as the process of using plants and related microbes to remove heavy metals and other pollutants from the environment or to reduce them to harmless levels (Singh, 2012; Ali et al., 2013). The phytoremediation approach is said to be a low cost *in situ*

technique for the remediation of polluted sites, is efficient, environmentally and eco-friendly (Salt et al., 1998; Vithanage et al., 2012). The phytoremediation approach uses plants to stabilise heavy metals in the soil, uptake, transport, and store them into their above ground biomass (Fig. 1.3) (Salt et al., 1998). By doing so, the plants reduce concentrations of heavy metals in the soil, thereby improving its fertility without affecting its texture and structure (Mench et al., 2009; Greipsson, 2011). Vegetation established on contaminated soils also prevents soil erosion and the heavy metal pollutants from leaching in the ground and underground water tables (Weiersbye et al., 2006). Techniques such as phytostabilization, phytoextraction, phytofiltration, phytovolatilization, and phytodegradation correspond with the practice of phytoremediation (Alkorta et al., 2004).

1.3.1.1.Phytostabilization, phytodegradation and phytoextraction

Phytostabilization, also known as phytoimmobilization, uses specialized plants for stabilizing heavy metals present in the soils and render them immobile (Singh, 2012). During phytostabilization, plants reduce the mobility and bioavailability of heavy metals present in the soil through root absorption, precipitation and reduction in metal valence in the rhizosphere (Wuana & Okieimen, 2011). Phytostabilization, as a technique, only stabilizes toxic contaminants to minimize chances of the metals leaching into ground water (Vangronsveld et al., 2009). Therefore, phytostabilization is considered to be a non-permanent solution to metal reduction as it reduces the chance of heavy metals being taken up by plants by reducing their mobility (Greipsson, 2011).

In contrast, phytodegradation technique involves the use of plants to degrade organic pollutants, using enzymes such as oxygenase and dehalogenase (Vishnoi & Srivastava, 2008). During phytodegradation, plants can detoxify organic xenobiotics through their metabolic activities and accumulate them from polluted lands (Mench et al., 2009). Although, phytodegradation is said to be the "Green liver" for the biosphere (Ali et al., 2013), it is restricted to striping only organic pollutants from the soil since heavy metals are non-biodegradable by nature.

FIGURE 1.3. Phytoremediation operations against acid mine drainage (AMD) pollution using *Tamarix usneoides* and other vegetation. A) A young *T. usneoides* (±2 years old) planted at the foot of a gold tailing storage facility (TSF) at Sulfur and East PayDam, Vaal River, North West Province, South Africa. B) ±7 years old *T. usneoides* occurring at Sulfur and East PayDam, Orkney, Vaal River. (Photographs courtesy of I.M. Weiersbye).

Otherwise known as phytoaccumulation, phytoextraction as technique of removing heavy metals from polluted lands is central to phytoremediation (Milic et al., 2012). By definition, phytoextraction is the uptake of heavy metals by plants from contaminated soil or water through the roots, followed by translocation and accumulation of these metals in the above ground biomass such as shoots (Yoon et al., 2006; Rafati et al., 2011). Soil properties, type of heavy metal and their bioavailability in the soil, as well as the plant species concerned, are important aspects for the efficiency of phytoextraction (Mench et al., 2009; Ali et al., 2013). It is said to be an important phytoremediation application with the potential for commercialization (Sun et al., 2011), however the choice of suitable plants as "heavy metal accumulators" for phytoextraction is the most critical aspect for its success.

1.3.2. Characteristics of suitable plants for phytoremediation

Plants used in the phytoremediation practice are those that are capable of taking up contaminants from water or soil and accumulating them in the shoots. Plants such as willow, poplar, *Jatropha* spp., eucalyptus, *Searsia lancea* (Black Karree) and *Tamarix* spp. (salt cedars, Fig. 1.4) are preferred for phytoremediation because they are fast-growing and have high above ground biomass (Abhilash et al., 2012). In addition, appropriate candidate plants for phytoremediation should also possess characteristics such as: a deep tap root and adventitious root system, high rate of metal accumulation , translocation and storage of the accumulated metals into shoots, be easy to cultivate and harvest, and resistant to pathogens and pests (Adesodun et al., 2010; Shabani & Sayadi, 2012). Although plants possessing all these characteristics are hard to find, phytoremediation programmes have been applied using several plant species for their different specific abilities. For example, the following plants are known to be favoured in phytoremediation for their specific characteristics: *Brassica* spp. (hyperaccumulators), *Trifolium* spp. (multiple branched and fast growing), *Searsia* spp. and *Tamarix* spp. (deep and multi branch rooted), and *Salix* spp. (high above-ground biomass) and some can be used simultaneously for an efficient phytoremediation application (Manousaki et al., 2009; Nonnie et al., 2012; Yadav & Srivastava, 2014; Ullah et al., 2015). Alternatively, genetic

engineering is being investigated as a way to create plants that contain genes with the desired characteristics for phytoremediation (Yang et al., 2005). Although genetically engineering of suitable plants for phytoremediation is desirable and seems like a simple process, it requires a considerable number of laboratory and field trials as well as financial resources, which makes it difficult to implement (Ali et al., 2013).

Plants that have evolved to occur and thrive in heavy metal rich soils are called metallophytes (Sheoran et al., 2011). Metallophytes evolved as a result of being exposed to metals in their natural habitats. Paradoxally, mining and other anthropogenic activities, which need metallophytes for pollution control, are known to remove metal rich plant habitats during the establishment of these activities, thereby destroying metallophytes' niches (Alkorta et al., 2004). Consequently these plants, which are botanical curiosities, should be conserved because they are currently needed for phytoremediation in lands disturbed by mining activities. Phytoremediation is an exciting technique that has public support, because after phytoextraction of the metals, the contaminated plant biomass can be harvested and disposed of, or undergoes a process called phytomining for the recovery of desirable metals (Sheoran et al., 2011; Ali et al., 2013). During the phytomining practice, the metal contaminated plant biomass will be combusted to extract energy and the metal rich left over ash known as "bio-ore" can be treated to recover the metals of interest (Sheoran et al., 2011).

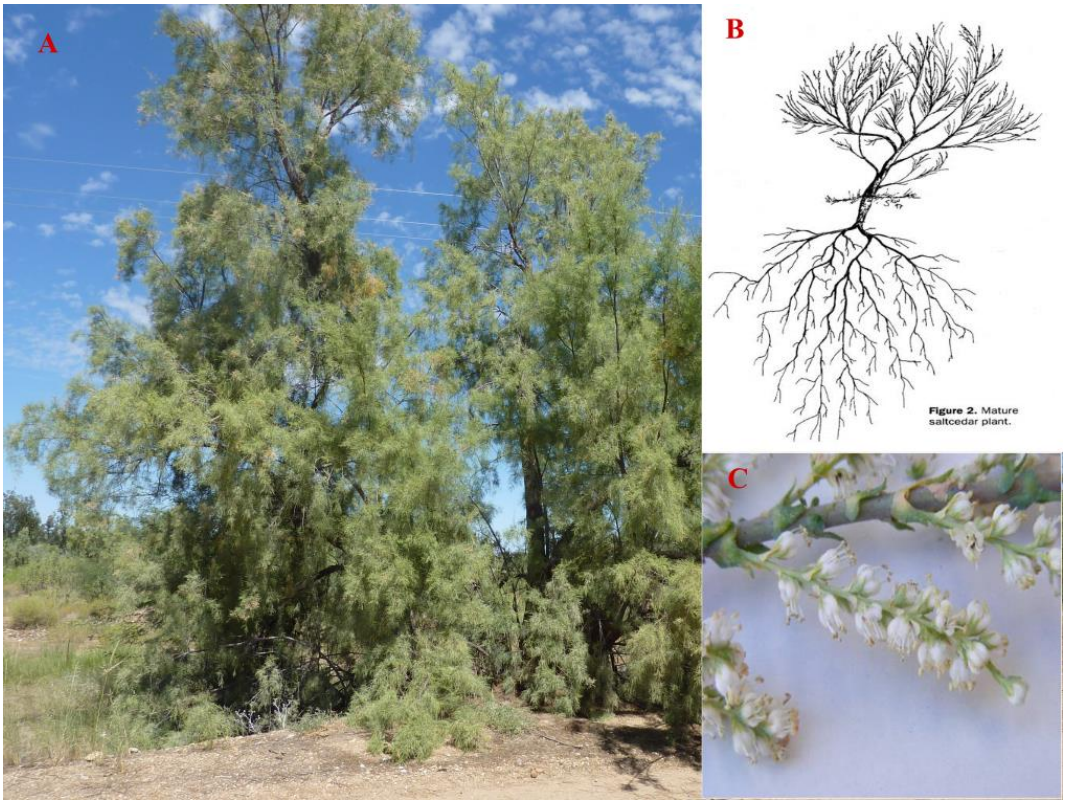

FIGURE 1.4. Morphological appearance of a *Tamarix* tree. A) The indigenous *Tamarix usneoides* tree of approximately 20 m in height growing alongside a road (S28°37.38; E20°20.849) near the Augrabies Falls National Park in the Northern Cape, South Africa. B) A sketch of a mature saltcedar shows its extensive tap root. The illustration was obtained from Obermeyer, (1976). C) The image showing the white flowers of *T. usneoides* (Photographs by S.G. Mayonde).

1.4. STUDY SYSTEM: *TAMARIX* SPECIES IN SOUTH AFRICA

In South Africa, several plant species such as *Phragmites australis* (Cav.) Steud., *Vachellia/Senegalia* (previously *Acacia*), *Atriplex* spp., and also *Tamarix* spp., are used in the mines for contaminant remediation from mining activities (Weiersbye et al., 2006). Five morphologically characterized *Tamarix* L. (Tamaricaceae) species were thought to occur in South Africa, viz. *T. aphylla* (L.) Karst, *T. chinensis*, *T. parviflora* DC, *T. ramosissima*. and *T. usneoides* (Obermeyer, 1976; Bredenkamp & Phepho, 2008), with the latter being the only species indigenous to south-western Africa (Obermeyer, 1976). However, genetic analyses have confirmed the presence of only three species: *T. chinensis*, *T. ramosissima* and *T. usneoides* and their hybrids (Mayonde et al., 2015, 2016).

The genus *Tamarix* (Tamaricaceae) is from the Old World and comprises 55 species (Baum, 1978). The Tamaricaceae (Tamarisk family) is mostly

distributed in temperate and sub-tropical regions and comprises three large genera: *Tamarix*; *Myricaria* Desv. (10 species), *Reaumuria* L. (12 species), and one small genus: *Myrtama* Ovcz. (Baum, 1978; Gaskin, 2003). *Tamarix* species are native to the Mediterranean countries, southern Europe, China, India, Mongolia, North Africa and southwestern Africa (Baum, 1967; Heywood et al., 2007). Morphologically, *Tamarix* trees grow as shrubs, semi-shrubs and tall trees that can reach up to 18 m in height (Baum, 1978; Gaskin, 2003). They are classified as halophytic or xerophytic plants that have multiple stems and slender branches (Brotherson & Winkel, 1986). Leaves of *Tamarix* are scale-like, approximately 3 mm in length (Baum, 1978), and usually contain salt glands (Bredenkamp & Phepho, 2008) which gave rise to their common name 'saltcedar'.

1.4.1. Inflorescence and seed ecology of *Tamarix*

Tamarix produces flowers that are often bisexual, rarely unisexual, and the species are either monoecious or dioecious (Baum, 1978). The indigenous species in South Africa, *T. usneoides*, is dioecious, while the exotic species are both monoecious and dioecious (Obermeyer, 1976; Baum, 1978). The flowers usually have five or four sepals and a corresponding number of petals (Baum, 1978; Gaskin, 2003). Male flowers have five or numerous stamens which are free or rarely fused and are inserted into a fleshy, glandular, hypogynous disc (Obermeyer, 1976). *Tamarix* species can outcross or self-pollinate, and the small white (indigenous *T. usneoides*) to pink (exotic *T. chinensis* and *T. ramosissima*) flowers are pollinated by many different species of insects and/or by wind (Brotherson & Field, 1987). *Tamarix* produces fruits that are three- to five-valved capsules (Brotherson & Field, 1987), containing about half a million light small viable seeds in each capsule (0.1 mg each; DiTomaso, 1998), containing a tuft of hairs to aid dispersal by wind; they are also easily deposited along sand banks and riverbeds by water (Brotherson & Field, 1987).

1.4.2. Root system and suitable habitat

Tamarix trees are classified as facultative phreatophytes because of their extensive, deep root system that reaches underground water tables (Kerpez & Smith, 1987). The *Tamarix* root system, which is desirable for phytoremediation,

gives them an advantage over other plant species once they invade a new habitat (DiTomaso, 1998) and under certain circumstances, they grow where no ground water is available (Brotherson & Field, 1987). The root system of *Tamarix* gives them an advantage to grow and multiply as they vigorously resprout into new plants if the top growth is removed or damaged (Everitt, 1980). This gives *Tamarix* trees a weedy characteristic. *Tamarix* grows on a wide variety of soils (silt loams and silt clay loams) that have high organic matter content, with intermediate moisture and high water levels, soils high or low in mineral and drought stress conditions (Brotherson & Field, 1987), however very saline soils are best for their growth (DiTomaso, 1998). Although alkaline conditions (pH 7.5) are optimal conditions for growth, *Tamarix* trees are also found on more acidic soils (Brotherson & Winkel, 1986).

1.4.2.1 Physiological responses to saline and metal toxicity in **Tamarix**

Most *Tamarix* species tolerate salt and metal concentrations beyond the normal threshold (~800 ppm for NaCl and ~8 ppm for Cd) (Manousaki et al. 2009). It should be noted that thresholds of metal concentrations vary depending on the nature of the metal and the compound formed with other metals. For example, *T. aphylla* and *T. smyrnensis* Bunge. (both from the Mediterranean region), tolerate up to 100 ppm and 16 ppm of lead (Pb) and Cd respectively, and accumulate them in their shoots (Manousaki et al., 2009). They also tolerate and accumulate as much as 200 mmol/L of NaCl and in the process excrete the metal ions through specialized salt glands (Hagemeyer & Waisel, 1988; Manousaki et al., 2009). Although, Manousaki et al., (2009) concluded that *T. smyrnensis* is not considered to be a Cd hyperaccumulator as it accumulated less than 100 ppm in its shoots, they recommend this plant for phytoremediation based on the relative considerable amounts of metals accumulated and the ability to tolerate stress conditions.

High levels of salt and metal toxicity can negatively affect plant health. *Tamarix* species respond differently to the presence of high levels of salt and metal toxicity depending on where they occur naturally (DiTomaso, 1998). Greenhouse experiments have shown that signs of saline toxicity in *Tamarix* include the wilting of the branches, which weakens the branches and reduces their

overall size (Kadukova & Kalogeraski, 2007), while high metal toxicity causes growth inhibition and can result in the death of the plant because of reduced enzymatic activities, photosynthetic rate, respiration and transpiration as well as soil nutrient uptake (Cho & Seo, 2005; Kadukova et al., 2008, Manousaki et al., 2009). High metal toxicity in *Tamarix*, known to make the roots fragile, is thought to be due to metal accumulation in the cell walls (Xiong, 1997). Shoot length and plant biomass is reduced in *Tamarix* by saline and metal toxicity (Kadukova & Kalogeraski, 2007; Kadukova et al., 2008). It is only normal that *Tamarix* trees have responded negatively to extreme levels of salt and metal concentrations. Nonetheless, they remain possible candidates for phytoremediation because they are tolerant to levels of salt and metal concentrations found in polluted soils (Kadukova et al., 2008; Manousaki et al., 2009). Additionally, because *Tamarix* trees can accumulate considerable amounts of heavy metals in their shoots, together with their high biomass production, they are considered suitable candidates for phytoremediation application (Manousaki et al., 2009).

1.5. CHARACTERIZATION OF *TAMARIX* SPECIES IN SOUTH AFRICA

Three diploid species of *Tamarix* have been confirmed to occur in South Africa (Mayonde et al., 2016). *Tamarix usneoides* is the only indigenous species and can be found in sandy river banks, riverbeds and desert palaeochannels (Baum, 1978; Obermeyer, 1976). *T. usneoides* is native to southern Africa with its distribution ranging from Angola, to Namibia and western South Africa (Fig 1.5). The two exotic species, *T. chinensis* and *T. ramosissima,* have been declared invasive in South Africa by the Alien Invasive Species regulations of the National Environmental Management: Biodiversity Act 2014 (NEMBA). The two invasive species originated from Eurasia (their native ranges) where they have a wide distribution which overlaps for about 4200 km across China to Korea (Gaskin & Schaal, 2002). *Tamarix ramosissima*, native to temperate Asia, is distributed from eastern Turkey to Korea, and *T. chinensis*' distribution ranges from China, Mongolia, to Korea and Japan (Baum, 1978; Gaskin & Schaal, 2003). In South Africa, the exotic *Tamarix* species hybridize with each other and with the indigenous species (Mayonde et al., 2016, 2015). Hybrids between the two exotic

species (*T. ramosissima* × *T. chinensis*) are the dominant genotype in the invasion as they constitute over 64.7% of all exotic and invasive *Tamarix* sampled across South Africa (Mayonde et al., 2016).

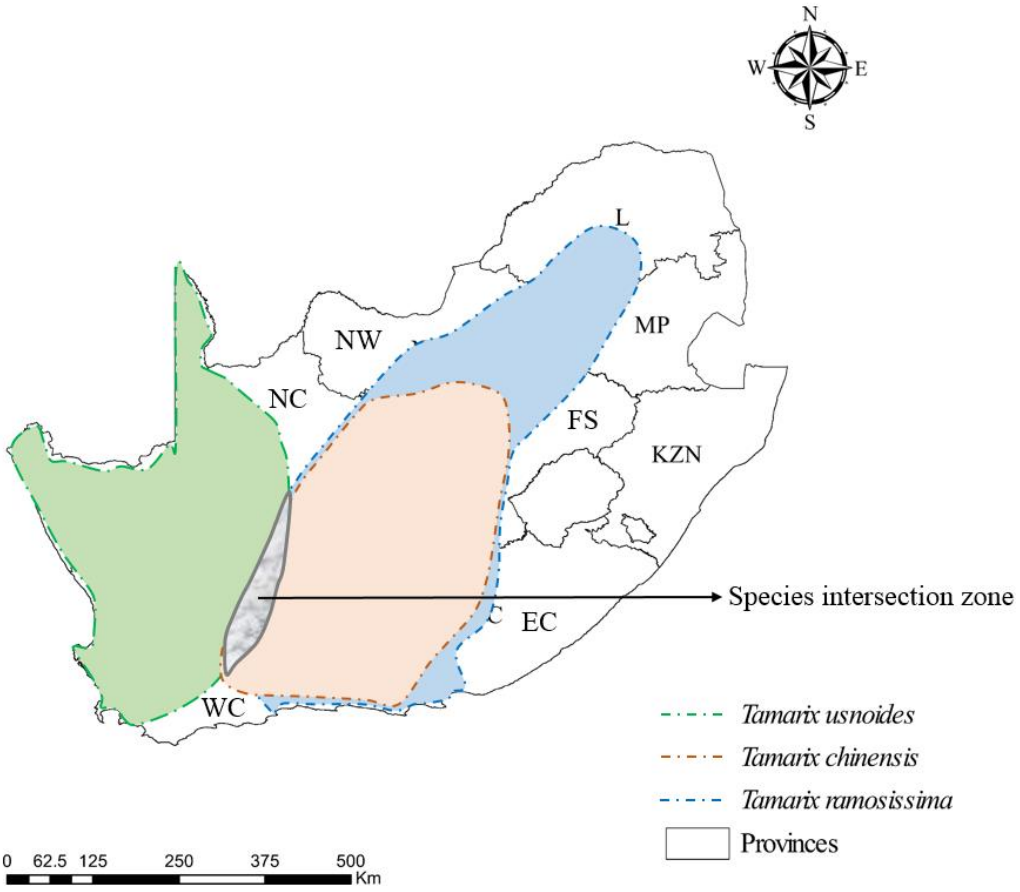

FIGURE 1.5. Approximate distribution map of *Tamarix* taxa in South Africa with the indigenous *T. usneoides* mostly occupying the western side of the country while the invasive *T. chinensis* and *T. ramosissima* are mostly distributed in the inner lands of the country. Dashed circles of the distribution ranges of species sensu Baum, (1978). The species interaction zone represents the localities where all three species have been seen to co-exist, see Fig 2.1; Mayonde et al., (2016).

1.5.1. Molecular genetics of *Tamarix* in South Africa and its application for species identification for optimal phytoremediation and potential biocontrol program

Molecular data are useful to clarify taxonomic issues, assess evolutionary relationships, separate cryptic species that cannot be distinguished morphologically, and also to elucidate evidences of hybridization and introgression (Le Roux & Wieczorek, 2009; Gaskin et al., 2011). Molecular

markers, such as microsatellites, may be used to investigate the identity of different plant species by comparing allelic patterns and identifying private alleles that are specific to one species (Sunnucks, 2000). They are also essential to infer genetic variation and investigate genetic diversity within and between species (Ellegren, 2004; Vieira et al., 2016). In the case of biological invasions, molecular markers provide a tool to better understand the population dynamics and the origins of target weeds (Gaskin et al., 2011). Molecular data shed light on prevention, management, and restoration strategies of alien invasive plants (Le Roux & Wieczorek, 2009). The use of molecular markers is essential for species' identification, invasion biology, and biocontrol of *Tamarix* in South Africa. Molecular DNA markers are different from one another by the amount of polymorphism displayed caused by essential processes that happen at the DNA level, e.g. recombination and mutation (Sunnucks, 2000). Therefore, an appropriate marker choice is crucial to ensure the necessary amount of variation in the invading species is attainable to answer the research question (Beebee & Rowe, 2004; Freeland et al., 2011).

1.5.2. Microsatellite markers: a tool to investigate genetic diversity, differentiation and structure among *Tamarix* genotypes in South Africa

In this study, microsatellite markers developed by Gaskin et al., (2006) were used to investigate the genetic variation, genetic diversity, genetic differentiation and structure in *Tamarix* species in South Africa. Simple sequence repeats (SSRs) termed microsatellites, are sequences of DNA that belong to a class of genome sequence known as Variable Number of Tandem Repeats (VNTRs; Ellegren, 2004). They are tandemly repeated sequence motifs that are short in length containing iterations of approximately six nucleotides (e.g. CGCGCG or CTACTACTA; Ellegren, 2004; Vieira et al., 2016). Microsatellites markers are highly polymorphic because of their distinct loci and co-dominant alleles (two alleles that are fully expressed in heterozygotes; Ellegren, 2004) and often reveal genetic discrepancy between closely related species (Balloux & Lugon-Moulin, 2002; Selkoe & Toonen, 2006). Microsatellites are amplified using a polymerase chain reaction (PCR) and have a number of applications in

molecular biology, population and conservation genetics, plant breeding and forensic and medical fingerprinting (Ellegren, 2004; Selkoe & Toonen, 2006; Vieira et al., 2016).

1.5.2.1.Genetic variation and diversity inferred by microsatellite markers

Genetic variation and genetic diversity are two closely related terms, but with some slight differences between them, and are both important in molecular ecology studies. By definition, *genetic variation* is simply the difference in allele composition in the genome of organisms. On the other hand, *genetic diversity* is concerned with the whole genetic makeup of a species and refers to the variety and variability of genes present in an organism. As such, genetic diversity is an important attribute of any population and is considered the baseline level of biodiversity (Freeland et al., 2011). Assessing the genetic diversity of plants is essential in population genetics studies, and has significant implications in conservation and evolutionary biology. For example, low genetic diversity in invasive species resulting from low propagule pressure and inbreeding can reduce both individual and population fitness (Freeland et al. 2011). Genetic diversity of organisms is estimated based on either allele frequencies or genotype frequencies such as allelic diversity (a), observed (H_O) and expected (H_E) heterozygosities, and gene diversity (h) (Beebee & Rowe, 2004). The Hardy–Weinberg Equilibrium (HWE) principle is an essential foundation of population genetics studies as it first explains the simple relationship existing between allele and genotype frequencies within a population, and secondly, hypothesises that genotype frequencies within a population will not change between generations if allele frequencies remain constant (Wright, 1943; Balloux & Lugon-Moulin, 2002). Estimating HWE proportions determines whether a population is stable with respect to gene and genotype frequencies. Populations under HWE are assumed to have these properties: the organisms are diploids, reproduce sexually with equal gene frequencies between males and females, mate randomly, with no migration, no mutation, and no natural selection occurring (Rodriguez et al., 2009). The populations should also be infinitely large with non-overlapping generations. Deviation from HWE signifies that a population has violated one or more of these assumptions. From an ecological point of view, assessing genetic variation and

genetic diversity is essential as they can ultimately impact ecosystems by influencing the survival of certain species and populations (Kwak et al., 2017, Hoeltgebaum & dos Reis, 2017).

1.5.2.2.Genetic differentiation and population genetic structure

Natural populations of most plant species display a certain degree of genetic structure as a result of interactions between their biology, geography, climate and the environment (Loveness & Hamrick, 1984; Alvares-Carvalho et al., 2016). Genetic structure is influenced by the number of alleles exchanged between distinct populations, which create genetic differentiation between them (Pritchard et al., 2000; Balloux & Lugon-Moulin, 2002). Biological factors such as breeding systems, seed dispersal and pollination mechanisms can influence genetic structure of a population (Vergara et al., 2014). Environmental barriers, historical processes and geographical range may to some degree also affect the genetic structure of populations (Wright, 1943; Balloux & Lugon-Moulin, 2002; Vergara et al., 2014). The extent of the geographic range shaping the genetic composition of a population leads to the concept of isolation by distance (IBD; Wright, 1943) and is known to influence the genetic sub-structure within the species. Understanding genetic structuring and sub-structuring and subsequent effects on a population is central to the management and conservation of indigenous *Tamarix usneoides*, and for application in a biological control programme of alien invasive *Tamarix* genotypes in South Africa.

1.5.3. Genetic diversity of invasive *Tamarix* in South Africa: implications for biocontrol implementation

Biocontrol practitioners are mandated to minimize the risk of non-target effects, especially on indigenous species, by introducing more specific and more effective agents (Balciunas, 2004; Gaskin et al., 2011). Alien invasive *Tamarix* species are considered to have tremendous negative side effects on the ecosystem because of their high water consumption, deposition of salts on underlying soils, and modification of hydrologic regimes (Bailey et al., 2001). *Tamarix* invasions have caused considerable alterations in flooding and erosion patterns of river channels, fire frequency, and affect biodiversity in the U.S. (DiTomaso, 1998).

Mechanical and chemical control measures have not been effective at suppressing the *Tamarix* infestation in South Africa (Holmes et al., 2005). Given the lack of effectiveness of mechanical and chemical controls, biocontrol measures may be a viable option for controlling the *Tamarix* invasions in South Africa. In the U.S., four species of *Diorhabda* Weise (1883) beetles (Coleoptera: Chrysomelidae: Galerucinae: Galerucini) have been released as biocontrol agents and effectively control the invasive *Tamarix* (DeLoach et al., 2011; Knutson et al., 2012). A biocontrol programme is only now beginning to be implemented in South Africa (Marlin et al., 2017). *Diorhabda carinata* (Faldermann, 1837), originally from Uzbekistan, has been imported into South Africa from the U.S. and is currently under quarantine for host specific testing for release against *T. ramosissima*, *T. chinensis* and their hybrids (Marlin et al. unpublished). One of the concerns for *Tamarix* biocontrol in South Africa is the non-target effect on the indigenous *T. usneoides.* The current research builds on my previous work by assessing the genetic diversity of the alien invasive *Tamarix* genotypes and investigating the genetic differentiation between the indigenous and the invasive *Tamarix* species. Lastly, my work aims to investigate the genetic diversity and differentiation between *Tamarix* hybrids from South Africa and those from the U.S. The abundance of hybrids in the *Tamarix* invasions is a common factor between South Africa and the U.S. Therefore, comparing the allelic composition and evaluating variation found among invasive populations in the two countries will assist in predicting the potential of suitable biocontrol agents to establish in South Africa.

1.5.4. Accurate identification of metal tolerant *Tamarix usneoides* genotypes in South Africa: implications for phytoremediation in land mine rehabilitation.

Tamarix usneoides is often used for phytoremediation in land mine rehabilitation (Fig. 1.3). Phytoremediation is a technique employed in South Africa to control AMD. AMD is considered as one of serious environmental pollutants produced by mining industries. Although, there are many sources of AMD, heavy metals produced from mining activities are a major contributor. *Tamarix* species are used for various reasons across the globe depending on where they occur naturally. In southern Africa, the indigenous *T. usneoides* is essential

for removing pollutants in gold and uranium mines and is currently used for phytoremediation (Weiersbye et al., 2006; Marlin et al., 2017). The identities of most *Tamarix* on the mines appear to be a mixture of *T. usneoides*, *T. ramosissima*, *T. chinensis* and their hybrids (Mayonde et al., 2015, 2016). However, *T. usneoides*, being indigenous, is preferred for use on mines for phytoremediation of AMD in South Africa as opposed to the exotic species that have been declared invasive (I. Weiersbye, pers com.). Principal coordinates analysis (a distance metric analysis) using amplified fragment length polymorphisms (AFLPs) markers showed some degree of sub-clustering within *T. usneoides* species in South Africa, and was geographically structured (Mayonde, 2013), which leads to the hypothesis that there is genetic differentiation within the South African *T. usneoides*. *Tamarix usneoides* trees have also been reported to respond differently on the mine tailing dams depending on their provenance, with some genotypes of *T. usneoides* unable to cope with the high stress levels of AMD (I. Weiersbye pers comm; S. Mayonde pers obs). Consequently, this research aims to identify genotypes of *T. usneoides* most tolerant against heavy metal as they will be the most suitable for phytoremediation.

1.5.5. Gene expression patterns of heavy metal tolerance in *Tamarix usneoides* using cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP)

cDNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) is an AFLP-based method for genome-wide transcript profiling for gene expression pattern analysis of an organism (Cappeli et al., 2005; Gabriels et al., 2006; Wang et al., 2009). cDNA-AFLP is preferred to northern blotting and RT-qPCR among others, because it deals with a transcript profiling of the whole genome for expression analysis without sequencing the organism and is also inexpensive relative to other techniques for investigating gene expression (Kim et al., 2012). Identifying genotypes of *Tamarix usneoides* with gene expression patterns that may be linked to tolerance and resistance to extreme levels of heavy metals is crucial for phytoremediation programmes as these individuals will be preferred for propagation and planting on the mines.

While AFLPs are conducted with genomic DNA, cDNA-AFLPs involve the reverse transcription of total mRNA into double stranded cDNA, which is then processed using a standard AFLP protocol (Bachem et al., 1996; Vuylsteke et al., 2007). cDNA-AFLP analysis is expected to reveal gene expression patterns in *Tamarix usneoides* that may correspond with tolerance responses prompted by experimental treatments of low (6 ppm), medium (12 ppm) and high (18 ppm) concentration levels of heavy metal (Cadmium). Additionally, physiological and growth characteristics such as chlorophyll content, shoot counts and height may be assessed as indicators of *T. usneoides*' ability to tolerate heavy metals. Individuals of *T. usneoides* that exhibit strong physiological tolerance responses against heavy metals, and whose physiological responses relate to their gene expression patterns, will be most suitable for planting in the mines for phytoremediation.

1.6. RESEARCH AIMS

Broadly, the main aims of this study were: 1) to investigate genetic variation within *T. usneoides* in South Africa using microsatellites, 2) to estimate the genetic diversity among *Tamarix* species and their hybrids to compare genotypes of invasive South African *Tamarix* hybrids with those in the U.S. and thereby assist in developing an effective biocontrol programme, 3) to assess the physiological responses of *T. usneoides* for tolerance of cadmium (Cd), and 4) to test whether gene expression patterns correspond with heavy metal tolerance in *T. usneoides* as characterized by the physiological data using cDNA-AFLPs.

1.7. RESEARCH OBJECTIVES

Objective 1. To identify the most genetically diverse *Tamarix* species and to determine the level of genetic differentiation between the indigenous and the invasive genotypes.

Objective 2. To compare microsatellite profiles between invasive alien *Tamarix* hybrids in South Africa and their U.S. counterparts and to evaluate their genetic diversity.

Objective 3. To investigate the genetic variation within the single indigenous species *Tamarix usneoides* using microsatellite markers.

Objective 4. To subject *T. usneoides* genotypes to different levels of heavy metal concentrations and then genotype these plants using cDNA-AFLP to identify if there are changes in expression patterns that correspond to individuals that are the most metal tolerant.

Objective 5. To evaluate the physiological responses of heavy metal tolerance in various *T. usneoides* individuals from different geographic localities across South Africa.

1.8. STUDY APPROACH AND THESIS STRUCTURE

This section gives an overview of the study approach, highlighting the relevant questions asked and how they are linked to the different objectives to address the main research aims. However, detailed descriptions of the study designs and methods are presented in each of the data chapters (Chapters 2–4). This research accomplished its aim by using microsatellite markers to investigate the genetic diversity, genetic differentiation and structure of *Tamarix* species in South Africa for implications in the conservation of the indigenous species and biocontrol of the invasive species. Furthermore, an experimental approach was used to examine the effects of heavy metal (cadmium) in *T. usneoides* and investigate expression patterns associated with heavy metal tolerance. The data chapters of my thesis are written in the format of scientific papers to be submitted to *Molecular Ecology*, an internationally accredited journal, for consistency. As such, there will be some inevitable repetition, especially in the introduction and method sections. Following the introductory chapter is the genetic diversity chapter, then the metal tolerance chapter, and gene expression chapter. Finally, a general discussion and concluding chapter integrates and combines findings from each of the data chapters, describing the implications of the overall study for conservation, biocontrol and phytoremediation.

Chapter 2: Genetic diversity assessment of *Tamarix* species using microsatellite markers

This data chapter investigates which of the three *Tamarix* species and their hybrids in South Africa is the most diverse genetically, and the extent of genetic differentiation between the indigenous and invasive *Tamarix* taxa. Furthermore, it estimates how genetically similar the alien invasive *Tamarix* hybrids in South Africa are relative to their counterparts in the U.S. The outcomes from this study are used to draw recommendations for conservation of the indigenous *T. usneoides* and for biocontrol of the alien taxa.

Chapter 3: Physiological responses of *Tamarix usneoides* to heavy metal stress

This chapter focuses on assessing the effects of cadmium (Cd) 'heavy metal' on the growth and physiology of the indigenous *T. usneoides* by investigating how tolerant the genotypes of *T. usneoides* are when exposed to extreme concentrations of this heavy metal. Here I sought to reveal the most tolerant *T. usneoides* genotypes suitable for propagation on the TSFs for phytoremediation.

Chapter 4: Gene expression patterns for heavy metal tolerance in *T. usneoides*

In this chapter, the emphasis shifts from examining the physiological responses of *Tamarix usneoides* when subjected to different concentrations of heavy metals to investigating the gene expression patterns of *T. usneoides* genotypes in response to heavy metal toxicity. This investigation addresses the question, how does the gene expression pattern of *T. usneoides* change when subjected to different levels of heavy metal concentrations, and is there any significant difference in the gene expression patterns between the various heavy metal concentration levels. This study aims to identify genotypes of *T. usneoides* with the highest gene expression responses against heavy metal toxicity indicating its ability to tolerate extreme level of heavy metals.

Chapter 5: General discussion and conclusion

The concluding chapter is a synthesis and general discussion of the research with particular reference to the implications of genetic diversity and differentiation in the biocontrol efforts of the invasive *Tamarix* genotypes, thereby insuring non-target effects on the indigenous species. My research also has implications for phytoremediation efforts of acid mine drainage by revealing the most heavy metal tolerant genotypes of the indigenous *T. usneoides*.

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CHAPTER 2

Assessment of genetic diversity of *Tamarix* in South Africa – Biocontrol and Conservation implications

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(Submitted to the *South African Journal of Botany*)

ABSTRACT

Genetic diversity information can be valuable for establishing successful management strategies for indigenous and invasive species. Here we conduct a genetic assessment of two invasive alien and one native *Tamarix* species in South Africa, where all species are known to hybridize. Hybridization can encourage biological invasion by creating unique allele combinations that facilitate the invasiveness of plants. Using nine microsatellite markers, we genotyped 150 individuals from four *Tamarix* taxa, viz. *T. usneoides, T. chinensis, T. ramosissima,* and *Tamarix* hybrids. We investigated the genetic diversity in the indigenous and invasive *Tamarix* species, and their genetic differentiation in South Africa, and compared the genetic diversity between South African *Tamarix* hybrids to hybrids from the United States. We aimed to elucidate information useful in the biocontrol efforts against invasive genotypes and the conservation of the indigenous species. Our results show that there is clear genetic differentiation between the indigenous and invasive species of *Tamarix* in South Africa. The indigenous *T. usneoides* was found to have greater genetic diversity than the exotic *T. chinensis*, but lower than *T. ramosissima*. Higher genetic diversity was detected in South African *Tamarix* hybrids compared to hybrids from the United States and there is substantial genetic differentiation between *Tamarix* hybrids from the two countries. Additionally, remote places in the northwest of South Africa contain private alleles suggesting non-polluted indigenous *T. usneoides* germplasm which should be preserved. These results suggest that there would be minimal risk of non-target effects on the indigenous species should a biocontrol programme be implemented.

Key words: Autogamous species; Biocontrol; Genetic differentiation; Hybridization; Microsatellites; Non-target effects; Tamaricaceae.

2.1. INTRODUCTION

Genetic structuring is characterized by the amount of alleles exchanged by distinct populations and can have important implications for the genetic make-up of native and invasive species (Balloux & Lugon-Moulin, 2002; Mori et al., 2016). Gene flow and genetic diversity are key factors in the invasion dynamics of plants, largely because they inherently affect population growth rates and the adaptive potential of invasive species (McCormick et al., 2016). In invasion biology, genetic structure analyses are widely used to investigate origins, spread, genetic variation, and admixture of invasive populations (Fitzpatrick et al., 2012; Rius & Darling, 2014). Additionally, for classical biocontrol applications, population genetic structure is used to understand the introductions, address agent selection prior to release (Goolsby et al., 2006), predict agents establishment (Hopper et al., 1993), and assess post-release environmental risks as well as non-target effects (Sethuraman et al., 2015) of agents. In natural populations, gene flow generates novel polymorphisms, increases population size, and produced novel gene recombinations upon which natural selection can act, thereby counteracting random genetic drift (Balloux & Lugon-Moulin, 2002; McCormick et al., 2016). All of these effects influence the genetic structure of a species to some degree (Sexton et al., 2014). Investigating population genetic structure of an indigenous species may help implement proper *in situ* species conservation and habitat management actions plans at both regional and global scales (Su et al., 2017).

The role of gene flow in natural and introduced plants is a key topic in both conservation and invasion biology because of its opposing effects —it may increase genetic variation and population size and yet reduce specific adaptive genetic combinations which can reduce population fitness (Balloux & Lugon-Moulin, 2002; Hughes et al., 2008). The critical objective of scientists undertaking biological control of invasive species is to minimize the risk of non-target effects (Gaskin et al., 2011), especially to indigenous species closely related to the target species (Briese, 2005). In recent years, molecular techniques have assisted in the management of invasive species using a biological control approach by clarifying their taxonomy, distinguishing the identity of closely

related species (cryptic species), revealing their origins, while investigating the genetic diversity in both their place of origin and the introduced region, and elucidating hybridization involving invasive species (Gaskin et al., 2011). In addition, genetic data are also applied to manage and develop conservation strategies for indigenous species that are threatened by their closely related alien species through hybridization events (Alvares-Carvalho et al., 2016; Lucek, 2016) by shedding light on their genetic variation, genetic differentiation and population structure (Neel & Ellstrand, 2003).

Genetic diversity and population structure have also been used to aid the conservation of native species in various countries (Kwak et al., 2017, Hoeltgebaum & dos Reis, 2017) as much as they have been used to create management programmes for various invasive species (Williams et al., 2005; Gaskin & Kazmer, 2009; Zenni et al., 2014; Lucek, 2016). Consequently, estimates of genetic diversity across *Tamarix* taxa in South Africa using polymorphic DNA markers such as microsatellites may be useful to develop conservation strategies for the native species (*T. usneoides* E. Mey. ex Bunge), a valued entity of South African biodiversity due to the roles it plays in both riverine ecosystems and phytoremediation of land mines. In addition, these data can also be used to manage the invasive genotypes (*Tamarix chinensis* Lour., *T. ramosissima* Lebed.) and their hybrids.

The indigenous *Tamarix usneoides* is a gregarious and copiously branched shrub or tree which can grow up to 5 m high. *Tamarix usneoides* is mostly found in sandy and salty dunes and flats, rocky deserts and river beds (Obermeyer, 1976). In South Africa, *Tamarix usneoides* is commonly distributed in the western part of the country along the banks of the Orange River from near Upington in the Northern Cape to its mouth and also along its tributaries and around saline depressions in Namaqualand and further south east in the Western Cape Province (Fig. 1.5). *Tamarix usneoides* is dioecious tree that usually flowers from July to October, but sometimes between January or February and May. It has vaginate leaves and white flowers, which distinctively differentiate it from the exotic and invasive *T. ramosissima* and *T. chinensis* (Obermeyer, 1976; Baum, 1978;

Mayonde et al., 2015). The native *T. usneoides*, which has been shown to hybridize with the invasive species (Mayonde et al., 2015, 2016), could be under threat of homogenization and as a result succumb to non-target effects from potential biocontrol agents introduced against invasive genotypes. Hybridization might also reduce the genetic diversity within the indigenous *T. usneoides* and could lead to its extinction through introgression. New trait interactions derived from hybridization between indigenous species and their exotic counterparts can lead to the extinction of one or both parental species through introgression (Rieseberg et al., 2007; Abbott et al., 2013; Goulet et al., 2017). For example, a rapid decline was shown in *Juglans cinerea* L. (Hoban et al., 2009), *Platanus racemosa* Nutt. (Johnson et al., 2016), and also *Populus nigra* L. (Van den-Broeck et al., 2012) due to hybridization and introgression with introduced counterparts. *Tamarix usneoides* in South Africa presents another example of a native species hybridizing with non-native species where these hybrids are part of the invasion (Mayonde et al., 2016). Moreover, novel hybrid taxa can also add a level of complexity in biological control efforts of invasion genotypes (Ellstrand & Schierenbeck, 2000; Lee, 2002) by altering the phenotype of the target plant such that the biocontrol agent may not establish. Therefore, a genetic assessment of *Tamarix* taxa is one of the first steps necessary to establish strategic management plans for biological control against the invasive genotypes and pro-active conservation of the indigenous germplasm.

Eurasian *Tamarix ramosissima* and *T. chinensis* and their hybrids have been declared invasive in South Africa by the Alien Invasive Species regulations of the National Environmental Management: Biodiversity Act 2014 (NEMBA) and are classified as category 1b, which means that these species require immediate control. It is not known when or how alien *Tamarix* species entered South Africa, however they were most likely imported from Eurasia in the early 1900s for ornamental purposes because of their attractive pink flowers and or for remediation of mining pollutants (Henderson, 2001; Weiersbye et al., 2006). The introduced *Tamarix* species have since escaped their point of introductions and now occur in most of the nine provinces in South Africa (Henderson, 2011; Mayonde et al., 2015, 2016). The alien *T. ramosissima*, *T. chinensis* and their

hybrids have invaded most riparian zones in the Eastern Cape, Western Cape Provinces and part of the eastern Northern Cape Province and the Free State Province (Fig. 1.5) (Mayonde et al., 2016; Newete et al., 2018).

Mechanical and chemical control measures of invasive *Tamarix* in South Africa have proven to be ineffective (Holmes et al., 2005), which leaves classical biological control as the best option (Marlin et al., 2017). Classical biological control of invasive *Tamarix* has been successfully implemented in the U.S. using mainly four species of the *Diorhabda* Weise (1882) beetles (Coleoptera: Chrysomelidae) (DeLoach et al., 2011; Knutson et al., 2012). The *Diorhabda* beetles have supressed infestations of *Tamarix*, especially the dominant *T. ramosissima*, *T. chinensis* and their hybrids (Gaskin and Kazmer, 2009; Knutson et al., 2012), which are also the dominant invasive genotypes in South Africa (Mayonde et al., 2016). Therefore, the biocontrol of *Tamarix* in South Africa looks at importing *Diorhabda* beetles from the US and test them against the invasive genotypes in South Africa. Taxonomic clarification and population genetics of the target weed using highly polymorphic DNA markers is an important aspect for biocontrol (Gaskin et al. 2011).

Microsatellite markers are known to be highly polymorphic, with multiple co-dominant alleles that may differ even between closely related individuals (Vieira et al., 2016). Our previous studies using DNA markers (viz. AFLPs, ITS and *trn*S-*trn*G sequences) and morphological characters (Mayonde et al., 2015; 2016) clarified the identities of *Tamarix* taxa in South Africa and revealed hybridization between the various *Tamarix* species. In contrast, the current study represents a first assessment of the genetic diversity within and differentiation between native and introduced *Tamarix* taxa in South Africa using microsatellite markers thereby assessing aspects such as gene flow, inbreeding and quantifying genetic diversity (heterozygosity). The co-dominance of microsatellite markers improves the data analyses to adequately address any introgression and hybridization. The microsatellite markers used in our study were developed on *T. ramosissima* but cross species amplification was done on *T. chinensis* and their

hybrids (Gaskin et al., 2006). However, our study represents the first time these markers are used to cross amplify *T. usneoides*.

We evaluated the genetic diversity and structure of *Tamarix* species and differentiation between the indigenous and invasive *Tamarix* taxa in South Africa. In addition, we compared the *Tamarix* hybrids from South Africa to those in the US in terms of genetic diversity and differentiation. The data from this research will indicate whether biological control agents currently utilized in the US are likely to establish on the invasive hybrids in South Africa. Nine microsatellite loci were used to achieve these aims, investigating and assessing (in this order): 1) the genetic diversity of the various *Tamarix* taxa in South Africa, 2) the genetic differentiation between the indigenous and invasive *Tamarix*, and 3) the similarity in genetic diversity and differentiation between *Tamarix* hybrids from South Africa and those from the US. Results from this research will reveal the genetic dynamics of the invasive *Tamarix* species that will inform a planned biological control programme as to the likelihood of any non-target impact on the indigenous species. In addition, our findings will indicate if the *Diorhabda* beetle species currently used in the US as biocontrol agents are likely to establish on *Tamarix* in South Africa.

2.2. MATERIAL AND METHODS

2.2.1. Plant material collection

Young twigs (stems with leaves) from 120 *Tamarix* individuals were collected from four different taxa across six provinces in South Africa (Table 2.1; Fig. 2.1). Samples included in the genetic diversity analysis were selected based on their known identity and the different localities of provenance were chosen from previously sampled localities by Mayonde et al., (2016). Approximately, three *Tamarix* individuals per locality were selected. Although, some cultivated *Tamarix* (mine and garden planted) were included in the analysis, samples were mainly selected from naturally occurring populations for an accurate estimation of gene flow. An additional 33 *Tamarix* hybrid individuals were obtained from six different localities (Butler Rapids-Pool, Mineral Bottom-Boat Ramp, Mineral Bottom-Excavation Trench, Sand Knolls Rapids-Gravel Bar, Short Canyon

Rapids-Pool, Stone Cabin Rapids-Pool and Stone Cabin Rapids-Eddy) along the Green River in Utah, U.S. with four to seven individuals per locality. The identities of the U.S. *Tamarix* hybrid individuals were previously confirmed by Gaskin et al., (2012) using AFLP markers. For the purpose of this study a 'population' represents a specific taxon and 30 individuals per taxon were collected in South Africa (Table 2.1). We considered the different taxa as populations because we are investigating the genetic diversity and differentiation within and between the different *Tamarix* taxa in South Africa. The four different *Tamarix* taxa include *T. usneoides*, *T. ramosissima*, *T. chinensis* and their hybrids (constituting of all hybrid forms from the three parent species). The preliminary identification of the South African *Tamarix* was based on morphology as per Mayonde et al. (2015) and AFLP data (Mayonde et al., 2016).

TABLE 2. 1 Provenance information of the 120 individuals from four *Tamarix* taxa collected from six provinces in South Africa for the genetic diversity assessment of the taxa using microsatellite markers.

Species name	Northern Cape	Western Cape	Eastern cape	Gauteng	Free State	North West	Total
Tamarix usneoides	17	5	1	2	0	5	30
Tamarix chinensis	7	11	9	0	3	0	30
Tamarix ramosissima	8	7	9	3	3	0	30
***Tamarix* hybrids**	10	9	3	4	2	2	30
Totals	42	32	22	9	8	7	120

2.2.2. DNA extraction and microsatellite analysis

Total genomic DNA was extracted from 20 mg of silica-dried tissue material using the Qiagen DNeasy Plant Mini Kit following the manufacturer's protocol (Qiagen®, Hilden, Germany). All 153 individuals were tested for amplification success using ten polymorphic microsatellite loci developed by Gaskin et al. (2006) with only nine resulting in successful amplification (Appendix 2A).

Polymerase chain reaction (PCR) amplification for the microsatellite loci was performed in 10 µl reaction volumes consisting of 5 µl of Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., UK), 20 µM of the forward and reverse primers, and 1mg/mL of Bovine Serum Albumin (BSA). Loci were amplified with the following cycling parameters: initial denaturation at 98 °C for 30 s, then 30 cycles of denaturing at 98 °C for 10 s, annealing at 54–63 °C (see Appendix 2A for locus-specific temperatures) for 30 s, extension at 72 °C for 30 s; followed by eight cycles at 98 °C for 10 s, 51 °C for 30 s, 72 °C for 120 s; and one step at 72 °C for 10 min. Amplification success of PCR products was screened by gel electrophoresis using a 4% high-resolution agarose gel (Sigma-Aldrich Inc, Germany). Forward primers were fluorescently labelled (FAM, NED, VIC; Appendix 2A) to allow for pooling of PCR products for fragments analysis. Fragment analysis was conducted using an ABI PRISM 3730xl automated Genetic Analyser (Applied Biosystems, Foster City, California, USA) using GeneScan 500LIZ® size standards at the Central Analytical Facility at Stellenbosch University (CAF). Microsatellite alleles were scored manually using GeneMarker v 2.6.7 (Soft Genetics®) following allele size ranges shown in Appendix 2A as guidelines. To avoid scoring errors three samples from each of the four taxa (*T. usneoides*, *T. ramosissima*, *T. chinensis* and *Tamarix* hybrids) had their PCR repeated three times and their peaks were scored manually to check for peaks. After scoring all150 samples at nine loci, heterozygote deficiencies as an indicative of genotyping errors as suggested by Gomes et al., (1999) was calculated. Furthermore, null alleles were detected using GenAlEx v.6.5 (Peakall & Smouse, 2012).

2.2.3. Data analyses

We analysed the data first by estimating the genetic diversity within, and differentiation between *Tamarix* taxa in South Africa, and secondly by comparing the genetic diversity of *Tamarix* hybrids from South Africa to that of the U.S. hybrids.

2.2.3.1. Genetic diversity analysis within **Tamarix** *taxa*

The genetic diversity was determined using standard calculations: number of alleles (Na), observed (H_O) and expected (H_E) heterozygosities and tests for linkage disequilibrium between pairs of loci and for deviation from Hardy-Weinberg equilibrium (HWE) were calculated using GenAlEx v.6.5 (Peakall & Smouse, 2012). The percentages of polymorphic loci across each taxon were also determined using GenAlEx. Allelic richness (Ar) and the number of private alleles were calculated using FSTAT v.2.9.3 (Goudet, 2001).

2.2.3.2. Population genetic structure analysis

Population structure analyses were conducted in STRUCTURE v.2.3.4 (Pritchard et al., 2000) to probabilistically assign individuals to clusters. This program implements the Bayesian–Markov chain Monte Carlo (MCMC) model-based method and we used the admixture and correlated allele frequencies options to assign individuals into genetic clusters. The model was run with ten replicate chains of 1,000,000 MCMC iterations with 100,000 burn-in iterations for each K (i.e. the number of genetically distinct clusters). The number of clusters (K) ranged from one to ten, to test for any potential cryptic species or indications of sub-structuring within species. The most likely number of clusters was estimated using Structure Harvester (Earl & von Holdt, 2012) that implements an approach to estimate deltaK as described by Evanno et al. (2005). Individuals were assigned to the different clusters based on their Q-matrix from STRUCTURE. An individual belonged to a particular cluster if the admixture was less than 10%; otherwise it was classified as "admixed" (as per Pritchard et al., 2000 and Blair & Hufbauer, 2009). The average posterior probability values of samples assigned to each of the clusters (across the 10 replicate runs of the selected K value of three) were calculated using Microsoft Excel 2010 and the same programme was used to

generate the STRUCTURE barplot using posterior probability values from the additional K = 3 run done in STRUCTURE.

2.2.3.3. Genetic differentiation and distance metric analyses

Genetic differentiation among *Tamarix* species was measured by calculating pairwise F_{ST} (Nei, 1978). *F*-statistics values (F_{IS}, F_{IT} and F_{ST}) were estimated using FSTAT v.2.9.3. Significance levels of inbreeding F_{IS} values for each taxa were determined with 95 % confidence intervals using FSTAT. For the principal coordinates analysis (PCoA), the pairwise differentiation measure (F_{ST}) for each individual was calculated as the variance component (Wright, 1965) using GenAlEx (Peakall & Smouse, 2012).

2.3. RESULTS

2.3.1. Genetic diversity of *Tamarix* taxa

For the genetic diversity analyses of *Tamarix* taxa in South Africa and the U.S., one locus failed to amplify across 114 samples and three samples from South Africa did not amplify at more than five loci. Therefore the three individuals and the one locus were removed from the dataset. In South African *Tamarix* taxa, a total of 71 alleles were detected among nine microsatellite loci from 117 *Tamarix* individuals. All loci were found to be polymorphic across the four *Tamarix* taxa. Overall, the mean number of alleles per locus was 7.8, ranging from 5 at locus T1G9 to 11 at locus T1E1. The mean number of alleles per locus per taxon was 5.2, ranging from 4 in *T. chinensis* to 6 in the *Tamarix* hybrids. Most loci showed moderate to high levels of genetic diversity, with a mean expected heterozygosity (H_E) of 0.631 and a mean observed heterozygosity (H_O) of 0.583 (Table 2.2). We detected no significant linkage disequilibrium between the different *Tamarix* taxa in South Africa, and there was no linkage disequilibrium among loci. Most of the loci deviated significantly from HWE in the three main species, while in the *Tamarix* hybrids taxon, none of the 9 loci deviated from HWE (Table 2.2).

TABLE 2. 2 Summary of genetic diversity indicators over nine polymorphic microsatellite loci across four *Tamarix* populations in South Africa showing the number of different alleles (Na), the observed (H_O), expected (H_E) and unbiased expected (uH_E) heterozygosities, and the exact test of Hardy-Weinberg Equilibrium (HWE). Values in bold indicate loci in HWE. The total number of samples for each taxon included in the genetic diversity were obtained after STRUCTURE analysis, which have changed from the initial 30 samples per taxon.

Summary of statistics for nine microsatellites loci in four *Tamarix* taxa in South Africa																				
Loci	Native					Introduced										Admixed				
	Tamarix usneoides (n = 27)					*Tamarix chinensis* (n = 28)					*Tamarix ramosissima* (n = 34)					*Tamarix* hybrids (n = 28)				
	Na	H_O	H_E	uH_E	HWE	Na	H_O	H_E	uH_E	HWE	Na	H_O	H_E	uH_E	HWE	Na	H_O	H_E	uH_E	HWE
T1C1	5	0.444	0.567	0.577	0.000	5	0.500	0.722	0.735	0.000	8	0.688	0.749	0.761	0.000	6	0.667	0.691	0.706	**0.435**
T1G6	5	0.360	0.446	0.455	0.003	3	0.192	0.297	0.302	0.000	7	0.667	0.662	0.672	0.000	7	0.792	0.698	0.713	**0.795**
T1C7	5	0.654	0.524	0.535	0.000	5	0.607	0.576	0.586	0.000	6	0.839	0.637	0.647	0.000	6	0.667	0.682	0.695	**0.185**
T1G9	3	0.667	0.640	0.652	**0.719**	4	0.680	0.671	0.685	**0.139**	4	0.500	0.596	0.605	0.004	4	0.714	0.662	0.674	**0.650**
T1G11	5	0.593	0.622	0.634	**0.749**	6	0.393	0.638	0.649	0.000	6	0.469	0.647	0.657	**0.456**	7	0.667	0.717	0.731	**0.857**
T1B8	4	0.720	0.700	0.714	0.001	5	0.593	0.691	0.704	0.000	6	0.788	0.650	0.660	0.000	7	0.571	0.684	0.697	**0.021**
T1B9	7	0.538	0.682	0.695	0.001	3	0.458	0.478	0.488	**0.631**	7	0.414	0.716	0.728	0.000	8	0.750	0.717	0.731	**0.031**
T1E1	5	0.481	0.523	0.533	**0.393**	3	0.357	0.482	0.491	0.000	9	0.588	0.627	0.637	**0.854**	8	0.786	0.777	0.791	**0.014**
T1C10	5	0.462	0.703	0.717	**0.105**	2	0.357	0.436	0.444	**0.337**	6	0.529	0.671	0.681	**0.013**	6	0.852	0.740	0.754	**0.500**

Tamarix chinensis had the lowest heterozygosity (mean H_O = 0.460 and mean H_E = 0.555), while the most heterozygous were *Tamarix* hybrids (mean H_O = 0.718 and mean H_E = 0.708; Table 2.3). Although the genetic diversity across all *Tamarix* taxa was relatively diverse, there was no significant evidence of inbreeding (mean F_{IS} = 0.093, P = 0.156) among the *Tamarix* species in South Africa. Only *Tamarix* hybrids exhibited out-crossing (F_{IS} = −0.014; Table 2.3). The native *T. usneoides* had the highest proportion of loci harbouring private alleles (11 private alleles), with private alleles present in loci T1C1, T1G9 and T1E1. Four (36.3 %) of these private alleles were unique to individuals from Goodhouse, Henkries and Vioosldrif in Namaqualand, in the Northern Cape Province (Fig. 2.1). The invasive species had a low number of private alleles (3 private alleles each).

The nine microsatellite loci exhibited high genetic variability in the *Tamarix* hybrids, both those from South Africa and the U.S., relative to the parent species. Genetic diversity indices for all 61 *Tamarix* hybrid samples (33 hybrid individuals from the U.S. and 28 from South Africa) resulted in 86 private alleles separating the two hybrid populations (47 alleles in South Africa and 39 alleles in the US). The expected and observed heterozygosities (H_E = 0.795 and H_O = 0.636 respectively) were higher than those for *T. usneoides*, *T. ramosissima* and *T. chinensis*. A fixation index of (F_{IS} = 0.201) was observed in *Tamarix* hybrid genotypes indicating inbreeding. At country level, the observed (H_O) and expected (H_E) heterozygosity values were higher in the South African individuals than in the U.S. *Tamarix* hybrids (Table 2.4), which shows that the South African *Tamarix* hybrid population is more diverse than the US population. Some evidence of outcrossing supports the higher level of diversity in South Africa (mean F_{IS} = −0.018; P < 0.001), whereas the US population exhibited considerable inbreeding (mean F_{IS} = 0.330; P < 0.001, Table 2.4). A slightly higher number of private alleles (25 alleles) were found in South African *Tamarix* hybrids population compared to the 21 found among the U.S. hybrids.

TABLE 2. 3 Calculated mean values for genetic diversity estimates of each *Tamarix* taxon based on nine microsatellite loci. Genotype number (N), number of different alleles (Na), number of effective alleles (Ne), observed (H_O), expected (H_E) and unbiased expected (uH_E) heterozygosities, and fixation index (F_{IS}). The number below the genetic diversity index represents the standard error (SE) value calculated in GenAlEx v. 6.5 (Peakall and Smouse 2012).

Taxa		N	Na	Ne	H_O	H_E	uH_E	F_{IS}
Tamarix usneoides	Mean	26.222	4.889	2.620	0.547	0.601	0.612	0.086
	SE	0.278	0.351	0.193	0.040	0.030	0.031	0.059
Tamarix chinensis	Mean	26.889	4.000	2.448	0.460	0.555	0.565	0.178
	SE	0.512	0.441	0.249	0.051	0.047	0.048	0.054
Tamarix ramosissima	Mean	32.444	6.556	3.011	0.609	0.662	0.672	0.075
	SE	0.556	0.475	0.155	0.049	0.015	0.016	0.077
***Tamarix* hybrids**	Mean	26.778	6.556	3.469	0.718	0.708	0.721	-0.014
	SE	0.547	0.412	0.154	0.028	0.012	0.012	0.034
Mean		28.083	5.5		0.583	0.642		0.093

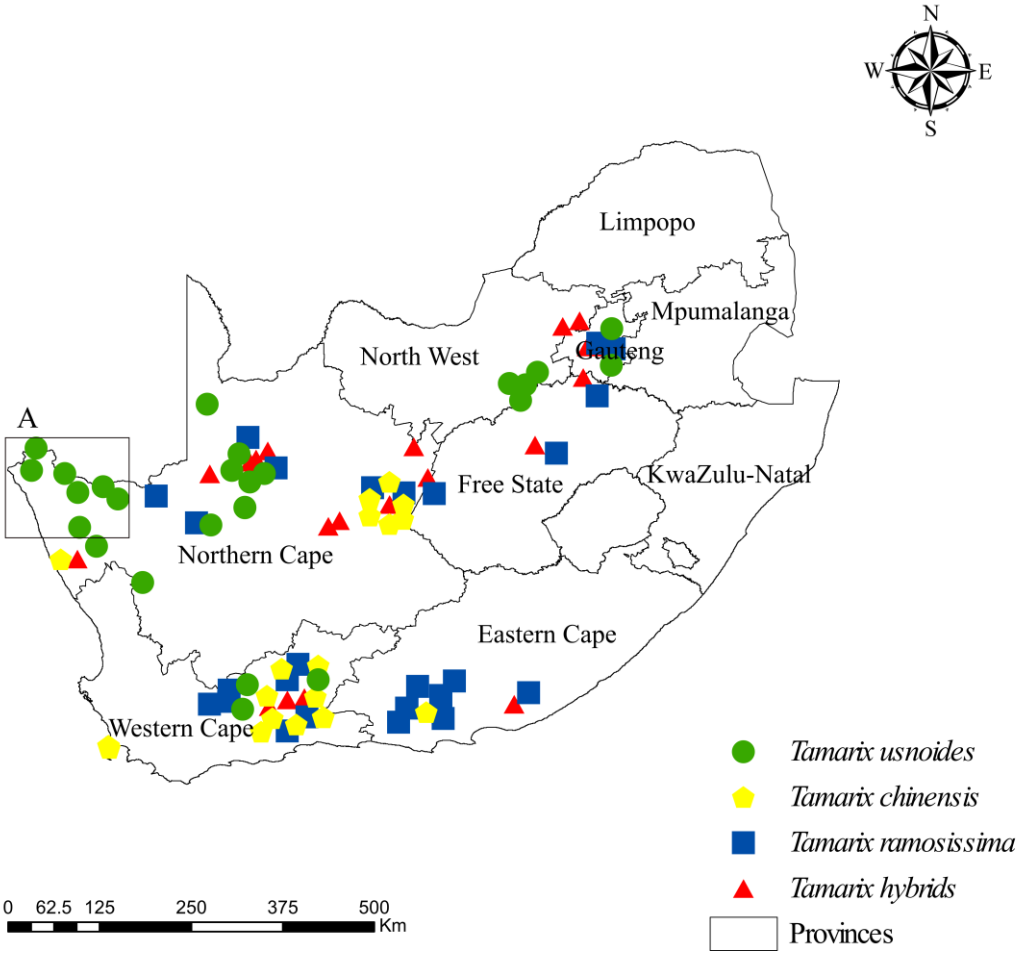

FIGURE 2. 1 Map showing locations of *Tamarix* specimens across six different provinces of South Africa. Locations are based on GPS coordinates. The identities of the populations were verified using microsatellite markers. 'A' indicates the region where individuals carrying distinctive indigenous *T. usneoides* private alleles are concentrated.

TABLE 2. 4 Comparison of genetic diversity between *Tamarix* hybrids from South Africa and US populations based on nine polymorphic nuclear microsatellite loci, using the following estimators: Genotype number (N), number of different alleles (Na), number of effective alleles (Ne), expected (H_E), observed (H_O), and unbiased expected (uH_E) heterozygosities, and fixation index (F_{IS}). The number below the genetic diversity index represents the calculated standard error (SE) value in GenAlEx v. 6.5 (Peakall and Smouse 2012).

Populations		**N**	**Na**	**Ne**	**H_O**	**H_E**	**uH_E**	**F_{IS}**
South African *Tamarix* hybrids	Mean	46.222	7.000	4.289	0.774	0.760	0.769	−0.018
	SE	0.760	0.441	0.258	0.017	0.014	0.014	0.018
U.S. *Tamarix* hybrids	Mean	31.333	7.000	3.717	0.435	0.685	0.696	0.390
	SE	0.624	0.408	0.451	0.057	0.051	0.052	0.057

2.3.2. Population structure and admixture analyses

STRUCTURE analyses inferred four genetically distinct clusters ($\Delta K = 4$; Appendix 2B) representing the three distinct species (*Tamarix chinensis, T. ramosissima* and *T. usneoides*) in South Africa and the US *Tamarix* hybrid genotypes as indicated by the average mean posterior probabilities (Fig. 2.2). Twenty three percent of individuals were assigned to the *T. usneoides* taxon with an average posterior probability of 0.967. Individuals assigned to the *T. chinensis* cluster constituted 23% of total genotypes, but with the highest relative average posterior probability (0.9735). The third cluster constituting of *T. ramosissima* genotypes represented 29% of the South African samples and had an average posterior probability of 0.966. The South African *Tamarix* hybrids were found to be intermediate between the three parent species and the US *Tamarix* hybrid genotypes and comprised 24% of the genotypes in South Africa (Fig. 2.2). The mean posterior probabilities of the South African *Tamarix* hybrids across the four clusters were 0.260 from *T. chinensis*, 0.477 from *T. ramosissima*, 0.187 from the US *Tamarix* hybrids and a low 0.073 from the indigenous *T. usneoides*. The fourth genetic cluster grouped all the U.S. *Tamarix* hybrid genotypes with an average posterior probability of 0.933 and had 30 individuals assigned with 90% or more of the genotypic composition unique to the U.S. *Tamarix* hybrids while the remaining three showed admixture with the South African taxa.

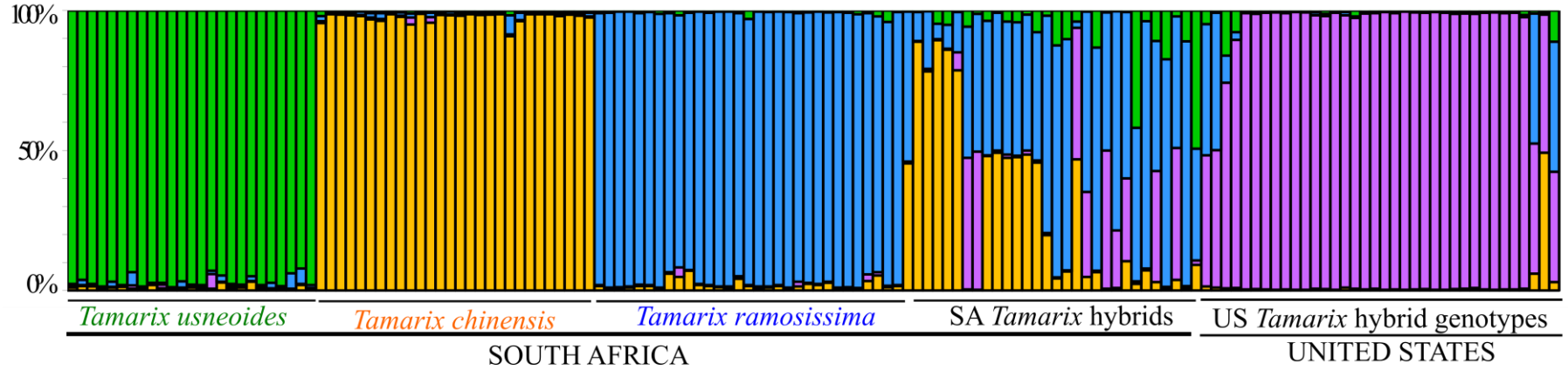

FIGURE 2. 2 Graphical summary of population structure analysis for 117 *Tamarix* samples. Bayesian estimates of *Tamarix* populations as inferred by Evanno et al. (2005) method, K= 4 (*T. usneoides* n = 27; *T. chinensis* n = 28; *T. ramosissima* n = 34; SA *Tamarix* hybrids n = 28; US *Tamarix* hybrid genotypes n = 33) based on nine microsatellite loci. Each vertical bar represents an individual. Different colours represent the proportion of each genotype comprised by each of three genetic clusters. Individuals were grouped by taxon.

2.3.3. Genetic differentiation among *Tamarix* taxa and distance analyses

The lowest genetic differentiation was between *Tamarix ramosissima* and *Tamarix* hybrids (Nei's pairwise F_{ST} = 0.048). Species with the highest genetic differentiation were *T. usneoides* and *T. chinensis* (F_{ST} = 0.197; Table 2.5). The results show that all taxon pairs were significantly genetically differentiated (Table 2.5). The principal coordinate analysis (PCoA) based on Nei's genetic distance among *Tamarix* species provides a graphical representation of the genetic clustering in South Africa (Fig. 2.3). Our results show that 95.61% of the variation was explained by the first two components (57.76% and 37.85%, respectively). The PCoA confirms that the invasive *Tamarix* species are genetically closely related, but are genetically distinct from the indigenous *T. usneoides*. *Tamarix* hybrids were found to overlap with both the invasive and the indigenous species and form a continuum between them (Fig. 2.3). The genetic differentiation results signal high gene flow between the exotic *Tamarix* taxa in South Africa.

TABLE 2. 5 Genetic differentiation estimates pairwise F_{ST} values of different *Tamarix* taxa in South Africa and significance levels of genetic differentiation between each pair. Lower diagonal is the pairwise F_{ST} values, upper diagonal is the significance of the population differentiation. Nei's F_{ST} values were calculated using GenAlEx v.6.5 software (Peakall & Smouse, 2012).

	T. usneoides	***T. chinensis***	***T. ramosissima***	***Tamarix* hybrids**
T. usneoides	0.000	***	***	***
T. chinensis	0.197	0.000	***	***
T. ramosissima	0.176	0.139	0.000	***
***Tamarix* hybrids**	0.065	0.099	0.048	0.000

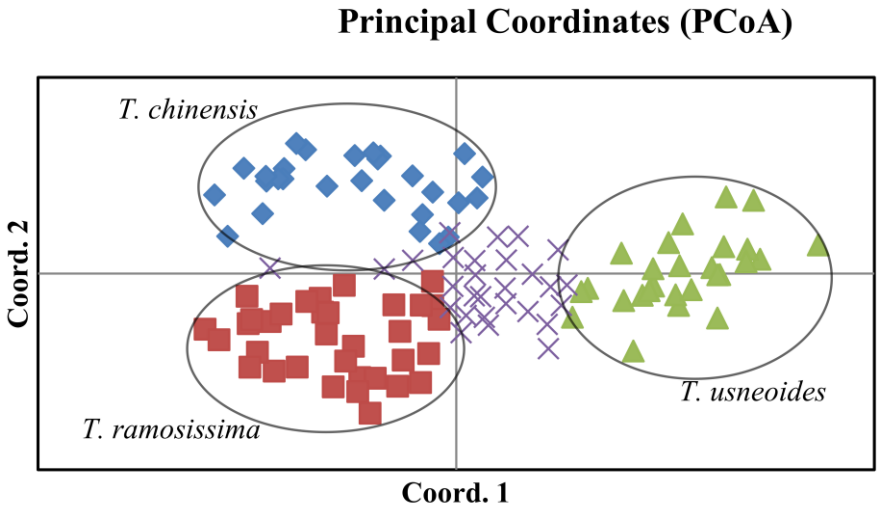

FIGURE 2. 3 Principle coordinate analysis (PCoA) of *Tamarix* species (genetic clusters) according to the Nei's standard genetic distance estimated using GenAlEx 6.503 (Peakall & Smouse, 2012). Genetically pure species (parent species) are circled and the South African admixed (hybrid) individuals (crosses) form a continuum between the parent species.

2.4. DISCUSSION

2.4.1. Genetic diversity among *Tamarix* taxa in South Africa and US hybrid genotypes

Genetic diversity is fundamental to biodiversity and can either directly or indirectly affect a wide range of introduced and indigenous populations and ecosystem processes (Hughes et al., 2008). The moderate levels of genetic diversity found in *Tamarix* taxa in South Africa is similar to that observed in the United States of America (U.S.) (Friedman et al., 2008) using the same DNA markers. Unlike in South Africa, there are no native *Tamarix* species so the genetic assessment in the U.S. was conducted only on the invasive *T. chinensis*, *T. ramosissima* and their hybrids. In contrast, low genetic diversity was reported for *T. taklamanensis* from the Taklamakan Desert in China, where it is indigenous (Su et al., 2017). In comparison to other vascular tree species, the low to moderate genetic diversity in *Tamarix* taxa is similar to *Vouacapoua americana* Aubl. (Dutech et al., 2004) in France; and *Populus* (Wu et al., 2008) in Eurasia. However, most vascular trees such as the common ash (*Fraxinus excelsior* L, Ferrazzini et al., 2007) and the Scotts pine (*Pinus sylvestris* L., Scalfi et al., 2009) have high genetic diversity influenced by life history events such as high seed dispersal. The genetic diversity of plant populations is often influenced by

breeding systems and life forms, as well as local environmental conditions (Loveness & Hamrick, 1984). It is hard to predict the major factors determining the moderate to high genetic diversity in *Tamarix* taxa in South Africa given that we assessed an indigenous and two introduced species.

Introduction of plants into a new environment is usually accompanied by low genetic diversity (Londo & Schaal, 2007) caused by founder effects. This study shows that the introduced invasive *Tamarix chinensis* species has the lowest genetic diversity in South Africa (H_E = 0.555 and H_O = 0.460). This estimate is comparable with genetic diversity measured in the U.S. (Friedman et al., 2008) and surprisingly even with its native range in China (H_E = 0.553 and Ho = 0.420) where 800 individuals from 26 populations covering an area of approximately 6622 km^2 were analyzed using microsatellite markers (Zhu et al., 2016). Our observed low genetic diversity within *T. chinensis* could be explained by the fact that the species is facultatively autogamous and is mostly pollinated by insects (Baum 1978). Although autogamous plants can out-cross, they may retain a low genetic diversity due to selfing (Bateman et al., 2015). Therefore, the low genetic diversity in the introduced invasive *T. chinensis* might not be caused by founder effects due to isolation from source populations, which usually results in reduced genetic variation (Allendorf & Lundquist, 2003; Dlugosch & Parker, 2008). Instead, the low genetic diversity in *T. chinensis* seems to be a natural occurrence as a result of its autogamous mode of breeding. Despite *T. chinensis* having the lowest level of heterozygosity in both the South African and U.S., it remains one of the key species in the *Tamarix* invasion in both countries (Gaskin & Schaal, 2003; Gaskin & Kazmer, 2009; Mayonde et al., 2016). Unlike *Tamarix* hybrids, the genetically depauperate *T. chinensis* appears to possess other mechanisms that make it a good invader.

The indigenous *T. usneoides* shows a higher level of genetic diversity (H_E = 0.601; H_O = 0.547) than the exotic *T. chinensis* but slightly lower than *T. ramosissima* (H_E = 0.662; H_O = 0.609), the other introduced invasive species. This finding is encouraging as it suggests that hybridization between the native and non-native invasive *Tamarix* species has not affected the genetic diversity of the

indigenous species. Hybridization could lead to the loss of the native genetic diversity through introgression (Abbott et al., 2013), as evidenced with the California sycamore (*Platanus racemosa*) in the U.S. (Johnson et al., 2016) and the European black poplar (*Populus nigra*; Vanden-Broeck et al., 2012). Under certain circumstances, such introgression can lead to extinction of the native species (Milne & Abbott, 2000; Ellstrand & Rieseberg, 2016). From a biocontrol point of view, hybrids from a cross between native and non-native parents need to be assessed for the extent of native genetic assimilation before they should be tolerated and not controlled (Gaskin, 2017). If the admixed *Tamarix* individuals can alter the phenotypic or biochemical composition of the plant through genetic recombination, then the inclusion of *T. usneoides*' germplasm in hybrids could be considered a concern for biocontrol as these hybrids could act as a 'bridge' for introduced biocontrol agents to shift hosts to the native species. Despite the continuum of hybrids observed (in the PCoA), our study obtained more private alleles present in the native species, than the few (three private alleles) found in each of the invasive species. Individuals carrying these private alleles are located in remote areas in north-western South Africa at Goodhouse, Henkries and Vioosldrif in Namaqualand, in the Northern Cape Province. These remote sites with unpolluted *T. usneoides* germplasm, which could have undergone local adaptation, are geographically distant from any of the invasive species and hybrid populations, therefore should be preferred for propagation if any management plan for conservation of the pure indigenous *T. usneoides* species is instigated. This study found an interesting and encouraging results concerning the genetic purity of the native *T. usneoides* populations based on the private alleles information. Despite the hybridization with the invasive species (Mayonde et al., 2016), introgression (back-crossing) does not seem to occur. This is a positive outcome for conservation efforts of the native species (preservation of biodiversity) and biocontrol of invasive genotypes.

Hybridization is known to affect the diversity of a population by increasing the frequency of heterozygotes or enabling the expression of recessive gene traits (Abbott et al., 2013). *Tamarix* hybrids in South Africa are the most genetically diverse relative to the other three taxa (H_E = 0.708 and H_O = 0.718).

High genetic diversity caused by hybridization could be accompanied by the presence of novel genotypes not found in parental populations (Schierenbeck and Ellstrand, 2009; Ellstrand and Rieseberg, 2016). In South Africa, all three species of *Tamarix* are known to hybridize, with some hybrids carrying alleles from all parent species (Mayonde et al., 2016). Whether hybridization helps *Tamarix* colonize new habitats is not known, however *Tamarix* hybrid genotypes dominate in the invasive populations – both in the U.S. (Gaskin & Kazmer, 2009) and in South Africa (Mayonde et al., 2016). More intriguingly, the difference in genetic diversity and composition among hybrids formed by two or more introduced species in different environments provide evidence of different mechanisms such as genetic drift, inbreeding, environmental conditions and adaption which might influence gene flow and genetic structure in invasive pants. Our results suggest that high genetic diversity could be responsible for the hybrid dominance in *Tamarix* invasion. Hybrid individuals might also have higher fitness than their parent taxa in colonizing populations, which makes them better invaders (Ellstrand & Schierenbeck, 2000). Adversely, hybridization could be problematic in biocontrol programmes as it increases variation and potentially alters the phenotypes of the target species (Ellstrand and Rieseberg, 2016). For example, the invasion of *Lantana* L. across the globe is driven by hybrid cultivar genotypes, which are inadequately controlled by their biocontrol agents (Urban et al., 2011).

2.4.2. Characterization and genetic differentiation between the indigenous and invasive *Tamarix* species

Accurate identification of plant species is one of the first crucial steps usually required in any ecological, evolution and conservation studies (Avise, 2010); and also in biocontrol of alien invasive plants (Briese, 2005; Gaskin et al., 2011). The presence of the indigenous *Tamarix usneoides*, and the exotics *T. chinensis*, *T. ramosissima* and *Tamarix* hybrid taxa previously identified in South Africa using AFLP markers (Mayonde et al., 2016) have been confirmed by our microsatellite data.

Our microsatellite analyses show high genetic differentiation between the indigenous *Tamarix usneoides* and the invasive *T. chinensis* (F_{ST} = 0.197) and *T. ramosissima* (F_{ST} = 0.176) species in South Africa. This high genetic

differentiation between the indigenous *T. usneoides* and *T. ramosissima* refutes the close relationship inferred by Baum (1978) who placed both species in Section *Tamarix*. Furthermore, the high genetic differentiation between the indigenous and invasive *Tamarix* could be one of the many factors assumed to be the driving force in the invasive potential of *T. chinensis* and *T. ramosissima* in South Africa. Genetic differentiation between the invasive species and their closely related native species should be beneficial to the planned biocontrol programme as it could influence morphological differences in plants (Muller-Scharer et al., 2004). These, in turn, might influence the control agent's host preference, e.g. shrubby *T. chinensis* and *T. ramosissima* vs. tall *T. usneoides*; sessile leaf morphology and attachment in the exotic *T. chinensis* and *T. ramosissima* vs. vaginate in the indigenous *T. usneoides* (Obermeyer, 1976; Baum, 1978; Mayonde et al., 2015).

The low genetic differentiation between the two invasive *Tamarix* populations in South Africa suggests a close relationship between the two alien species, as hypothesised by their sister species status in the phylogenetic analyses of Gaskin & Schaal (2003), thereby contradicting Baum's (1978) placement of *T. ramosissima* in Sect. *Tamarix* and *T. chinensis* in Sect. *Oligadenia*, two very distinct sections in the Tamaricaceae. Our results suggest that *T. chinensis* and *T. ramosissima* species very likely shared a recent evolutionary history and could have similar natural enemies. Hence, these two species can very likely share biocontrol agents (Tracy and Robbins, 2009; DeLoach et al., 2011), as the natural ranges of *T. chinensis* and *T. ramosissima* are known to overlap for about 4,200 km from China to Korea (Gaskin & Schaal, 2002).

2.4.3. Implications for conservation of the indigenous species and biocontrol of the invasive populations

Genetic diversity, genetic differentiation and population genetic structure investigations are tools upon which biocontrollers frequently draw recommendations for host specificity testing of agents for a biological control programme (Muller-Sharer et al., 2004). Therefore, this study provides valuable information on which decisions for a biocontrol programme of alien invasive *Tamarix* genotypes could be based. The low genetic diversity within the invasive *T. chinensis* in South Africa, which seems to be a natural fact caused by its

autogamous nature, suggests they might have originated from similar Eurasian populations. This suggests that exploration for host specific biocontrol agents against *T. chinensis* in its native range for introduction in South Africa can be achieved. In addition, similar levels of genetic diversity between invasive *Tamarix* in South Africa and the U.S. suggest that the *Diorhabda* beetles successfully controlling *Tamarix* invasions in the U.S. should be tested for introduction into South Africa. However, the high population genetic differentiation between the South African *Tamarix* hybrids and those in the U.S. suggests that biocontrol agents from the U.S. might not successfully establish in South Africa to biologically control the invasive *Tamarix* hybrids. The high genetic differentiation between *Tamarix* hybrids in South Africa and those in the U.S. could be explained by the fact that 12 putative *Tamarix* species and their hybrids are found in the U.S. [viz. *T. africana* Poir, *T. aralensis* Bge., *T. aphylla* (L.) Karst., *T. canariensis* Willd, *T. chinensis*, *T. gallica* L., *T. juniperina* Bge., *T. parviflora* DC., *T. pentandra* Pall., *T. ramosissima*, *T. tetramdra* Pall ex M.B. ehrenb. Willd, *T. tetragyna*, identifying four invasive *Tamarix* entities constituting of *T. aphylla*, *T. parviflora*, *T. canariensis*/*T. gallica* and *T. chinensis*/*T. ramosissima*] (Gaskin and Schaal, 2002), as opposed to only three *Tamarix* species in South Africa (i.e. *T. usneoides*, *T. chinensis* and *T. ramosissima*; Mayonde et al., 2016). We recommend that South Africa focuses its exploration efforts for potential biocontrol agents in Eurasia (i.e., the place of origin). The low genetic differentiation between the two invasive populations suggests that they share a relatively recent common ancestor. Since both *T. chinensis* and *T. ramosissima* originate from Eurasia, they might share the same natural enemies, with the implication that these species could be effectively controlled by the same biocontrol agents. Despite, the two Eurasian *Tamarix* species overlapping for approximately 4200 km in their native range (Gaskin and Schall, 2002), they share similar habitats and are known to contain similar entomofauna composition, with insects; i.e. *Diorhabda* beetles, *Coniatus tamarisci*, *Ornativalva* spp., *Corimalia tamarisci* etc. (DeLoach et al., 1996). Furthermore, the genetic differentiation with their indigenous counterpart, *T. usneoides*, supports the likelihood of finding host-specific agents against the invasive populations with

minimal non-target effects on the indigenous species. In addition, the *T. usneoides* from remote localities in the Northern Cape should be propagated for planting on the mines (for phytoremediation), and nurseries should be set up for *in situ* conservation to preserve the unpolluted indigenous genome which will mitigate loss of genetic diversity due to hybridization. In conclusion, our assessment of the genetic diversity and differentiation among the various *Tamarix* taxa in South Africa has revealed a strong likelihood for a successful biocontrol programme of the invasive *Tamarix* populations, which is an encouraging outcome for biocontrol practitioners. Furthermore, this study emphasised the need for conservation of the pure genomes of the indigenous species, which is hybridizing with the invasive species, and is needed for phytoremediation on the country's mines. It is not evident that intentional propagation of the native *T. usneoides* would promote spread of hybrids and or introgressed genotypes if they are collected from the isolated remote places with pure germplasm. Biocontrol of introduced *Tamarix* and their hybrid populations can be initiated concurrently with the planting of the indigenous species provided that propagation is done using individuals from the isolated localities in the Northern Cape.

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CHAPTER 3

Effects of heavy metal (cadmium) toxicity on physiology and growth of *Tamarix usneoides* E Mey ex. Bunge (Tamaricaceae)

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(In preparation for submission to the *Journal of Plant Physiology*)

ABSTRACT

Phytoremediation is an option for the restoration of contaminated soils and it is usually achieved by using suitable plants that can tolerate and accumulate metals. High concentrations of heavy metals are known to affect a plant's health negatively. However, suitable candidates for phytoremediation have the ability to withstand high concentrations of heavy metals through detoxification mechanisms known as chelation and subcellular compartmentalization of the metals. In this study, we experimentally evaluated the effects of cadmium (Cd) on the growth and chlorophyll content of *Tamarix usneoides*, a halophyte used in South African mines for phytoremediation of acid mine drainage. In a greenhouse experiment, *Tamarix usneoides* clones were artificially polluted with a once off 100 mmol.L^{-1} NaCl and exposed to three different Cd concentrations (6, 12 and 18 ppm) for eight weeks. The specific aim of the study was to evaluate the physiological responses of heavy metal tolerance in *T. usneoides*. Plant height, shoot growth and chlorophyll content were found to decrease with an increase in Cd concentration. There were significant differences in plant height and shoot growth between the controls and Cd treated plants. The shoot growth in *T. usneoides* was found to be more sensitive to Cd than plant height as it took six weeks of the experiment to observe a significant difference in height while shoot growth was significantly affected by Cd toxicity after four weeks. No correlation between the effects of Cd toxicity on shoot growth and height increase was found, suggesting that *T. usneoides* tends to grow more in height than in diameter under extreme heavy metal concentrations. The chlorophyll content of *T. usneoides* was significantly reduced by Cd toxicity, suggesting that the plant would likely have a reduced photosynthetic rate when exposed to heavy metals. *Tamarix usneoides* was found to be tolerant to Cd at concentrations of 6 ppm where the height and shoot counts and leaf chlorophyll content were not significantly reduced compared to their controls and no external signs of metal toxicity were observed. At a higher concentration of 18 ppm, however, the plants succumbed to metal toxicity, resulting in the death of 21% of individuals in that treatment group. According to our results, *T. usneoides* should be regarded as a heavy metal tolerant plant, an attribute which together with its naturally high biomass growth, make it a suitable candidate for phytoremediation. In addition, we recommend further studies to

investigate the ability of *T. usneoides* to extract (phyto-extraction) and accumulate (phyto-accumulate) heavy metals, two important aspects required by plants used for phytoremediation.

Key words: Acid Mine Drainage, Chlorophyll content, Halophytes, Phytoremediation, Photosynthesis, Salinity

3.1. INTRODUCTION

The mining of minerals such as gold, uranium, diamond and copper, is linked with acid mine drainage (AMD), a substance that can affect long-term destruction to waterways and influence metal contamination in the soil and ground water biodiversity (Azapagic, 2004; Akcil & Koldas, 2006). Some wastes produced by the metal mining industry contain high concentrations of toxic substances such as cyanides and heavy metals (Azapagic, 2004). Elevated concentrations of heavy metals pose a substantial threat to life when they exceed limits as they impact negatively on the health of living organisms (Xiong et al., 2006; Burkhead et al., 2009). Therefore, to reduce the impacts of heavy metals and other toxic substances, such as cyanides, on human health and other ecological implications, AMD needs to be remediated.

Cadmium (Cd) is one of the heavy metals associated with AMD in South African uranium and gold mines (Naicker et al., 2003; Tutu et al., 2008) and is regarded as one of the most problematic pollutants because it is highly soluble in water and has tremendous negative effects (e.g., carcinogenic, mutagenic, and teratogenic) in the food chain (Das et al., 1997; Pál et al., 2006). Phytotoxicity caused by heavy metals, especially Cd, may be as a result of many physiological processes alterations at the cellular and/or molecular level —from inactivating enzymes and blocking functional groups of important molecules for metabolism, to displacing or replacing essential elements, and unsettling membrane integrity (Rascio & Navari-Izzo, 2011). Cd in plants has been proven to decrease carbon assimilation, create oxidative stress, induce stomatal closure and affects plant water relations, inhibit chlorophyll synthesis which impair photosynthesis, damage root tips which impacts nutrient uptake and subsequently slows down growth (Zhou & Qiu, 2005). The fact that Cd is present in AMD (Naicker et al., 2003; Akcil & Koldas, 2006; Tutu et al., 2008) and exhibits strong toxicity to plants even at low concentrations, as well as its high toxicity to animals and health risks to humans (Garbisu et al., 2001; Zhou & Qiu, 2005), make it the metal of interest in this study.

Heavy metals such as copper (Cu), iron (Fe) and zinc (Zn) are essential micronutrients required for various cellular processes in plants, but they might become cytotoxic at high concentrations. However, heavy metals such as

cadmium (Cd), mercury (Hg) and lead (Pb) can be toxic to plants even at very low concentrations, because they are non-essential trace elements for plants' cellular metabolic activities (DalCorso, 2012). Metallophytes are plants known to contain possible cellular mechanisms that help avoid accumulation of toxic concentrations at sensitive organelles within the cell (Yang et al. 2005). Strategies used by plants to tolerate heavy metals can include the chelation of metals by organic acids, amino acids, or peptide sequestration in the vacuole, as well as repairing damaged proteins caused by heavy metal toxicity (Craciun et al., 2006). The main mechanism of Cd tolerance in plants' cells is through chelation by ligands such as glutathione, phytochelatins, and metallothioneins (Hall, 2002; Clemens & Simm, 2003). Other than tolerating high levels of heavy metals, certain plants (hyper-accumulators) have adapted to accumulating high amounts of heavy metal contaminant in their above ground biomass (Yang et al. 2005). Such plants that tolerate and take up heavy metals from the soil can be used in phytoremediation, an environmentally friendly and low cost method to strip heavy metals directly from the soil (Yanai et al. 2006; Yang et al. 2005; Paz-Alberto & Sigua, 2013).

About 450 angiosperm species are known to hyper-accumulate heavy metals such as As, Cd, Cu, Zn, Magnesium etc. (Rascio & Navari-Izzo, 2011). Plants such as *Salix*, *Poplar*, *Sersia*, and *Tamarix* spp., are known to be preferred for phytoremediation because they are fast-growing and have a high-biomass (Clemens et al., 2002; McIntyre, 2003). *Tamarix usneoides* E Mey. ex Bunge, is a phreatophyte plant indigenous to southern Africa. The native distribution of *T. usneoides* ranges from western South Africa (Northern and Western Cape Provinces), Namibia and part of Angola, and occurs mostly in riverbeds, desert paleochannels and salt rich habitats (Obermeyer, 1976; Baum, 1978). It is one of the woody plants used for phytoremediation in southern African gold and uranium mines (Weiersbye et al., 2006), and together with *T. chinensis* Lour. and *T. ramosissima* Lebed., has been confirmed to occur at various mines across South Africa (Mayonde et al., 2015, 2016). It is one of 55 species in the *Tamarix* (Baum, 1978), which are salt tolerant and seem to be adapted to also take up and accumulate certain metals. For instance, *T. aphylla* (L.) Karst., and *T. smyrnensis* Bunge., both from the Mediterranean region, are known to take-up and

accumulate lead (Pb) and Cd, and excrete the metal ions through specialized salt glands on their leaves (Hagemeyer & Waisel, 1988; Manousaki et al., 2009). Manousaki et al. (2009) investigated the characteristics of *T. smyrnensis*' metal tolerance and its ability to accumulate Pb and Cd under different salinity levels in order to evaluate the plant's effectiveness in heavy metal cleanup for phytoremediation. Growth responses such as tree height, leaf size, shoot growth, root growth, and physiological mechanisms such as chlorophyll contents, gas exchange, as well as metal accumulation capacity have been used to investigate heavy metal tolerance and hyperaccumalation by plants needed for phytoremediation (Clemens et al., 2002; McIntyre, 2003; Zhou & Qiu, 2005; Manousaki et al., 2009; Liu et al., 2009). *Tamarix usneoides* is known to be able to accumulate sulphate and other metals from AMD and in the process excrete gypsum (Weiersbye et al., 2006). Although *T. usneoides* is known to tolerate and accumulate heavy metals, little is known about its physiological responses when doing so. In this study we investigated if different genotypes of *T. usneoides* exhibited different depressed physiological status under extreme heavy metal (Cd) conditions while testing for tolerance to heavy metal toxicity by measuring leaf parameters such as chlorophyll content, as well as the shoot growth rate and height increase. These results can help to determine the effects of metal toxicity in *T. usneoides* and evaluate its metal tolerance ability when used for phytoremediation programmes.

3.2. MATERIAL AND METHODS

3.2.1. Plant material and experimental design

Cuttings were collected from 20 naturally occurring *Tamarix usneoides* individuals across three provinces in South Africa with 17 individuals collected in the Northern Cape Province (most *Tamarix usneoides* widely distributed province), with two and one collected from the Western and Eastern Cape Provinces respectively (Appendix 3A). Plants were collected between March and April of 2016 across a broad geographic range to represent a large portion of the genetic diversity within *T. usneoides*. *Tamarix usneoides* trees were cloned as cuttings to yield twelve replicates per plant, making the total sample size n = 240.

Tamarix usneoides cuttings (15 cm long) were propagated in silica sand for 6 weeks (between May and June) until substantial shoot and root systems developed. Dynaroot1 rooting hormone (PBR Trading International CC) was applied to enhance development of the root system. Rooted cuttings were transplanted into individual plastic pots (16.5 cm in height and 20 cm diameter) filled with the same amount (1.5 kg in dry weight) of substrate containing a mixture of ¼ organic compost, ¼ silica sand and ½ top soil. Individually transplanted cuttings were left to grow for another five weeks (between June and July 2016) to allow establishment of the roots and were fertilized with 100g constituting of 95g/kg of N, 14g/kg of P, 41g/kg of K, and 80g/kg of C (Wonder Vitaliser Lawn and Leaf, 7:1:3 fertilizer) before treating them with Cd.

For the metal tolerance trial, the plants were divided into four independent treatments constituting of the control (0 ppm), low (6 ppm), medium (12 ppm) and high (18 ppm) Cd concentrations. The 12 cloned individuals from each plant were then divided amongst the four groups (treatments and control) with three independent replicates per group. This resulted in a total of n = 60 individuals in each group. The maximum Cd concentration (18 ppm) chosen in this study is twice the concentration used by Manousaki et al. (2009) and more than twice the known upper limit of Cd metal concentration in toxic soils (Orcutt & Nilsen, 2000). Concentrations of Cd in contaminated soils range from 3 to 8 ppm dry weight and these levels are considered to be toxic to most plants (Orcutt & Nilsen, 2000; DalCorso, 2012).

A once-off amount of 200 ml of salt water (100 mmol.L^{-1} NaCl) was applied to all *T. usneoides* plants three days before Cd treatment. Salinity is said to be a key factor in increasing the bioavailability of metals in the soil because it reduces soil metal sorption (Bingham et al., 1983; Weggler et al., 2004; Wahla & Kirkham, 2008). Sodium chloride also plays an important role in the translocation of metals from roots to the above ground plant biomass (Fitzgerald et al., 2003; Weggler et al., 2004). All Cd treated plants were supplied with 200 ml of cadmium nitrate tetrahydrate [$Cd(NO_3)_2.4H_2O$] to the roots via the soil in liquid form at the different concentrations and deionized water (DW) was used for the control plants. The experiment was done in a glass house and plants were treated

twice per day and three days a week (Mondays, Wednesdays and Fridays) for eight weeks (between August and September 2016). Collecting trays were placed at the base of the pots to avoid loss of metal contaminated water. During the course of the experiment morphological symptoms of metal toxicity exhibited by the plants and any form of phenotypic changes were noted on a weekly basis.

3.2.2. Measurements of growth parameters

Growth indicators in the different Cd treatments were recorded weekly using digital callipers to measure plant height and fully developed new twigs/shoots were counted. Plant heights were measured from the base of the pot to the highest growing shoot, to eliminate bias in case the level of soil in the pots was not even.

3.2.3. Chlorophyll content measurement

As an indicator of a physiological response to the metal stress, chlorophyll content measurements were taken on the last day of the weekly Cd treatment. The plant's relative chlorophyll content (mg/m^2) was measured on the second shoot from the top of each branch (newest growing twig) using a CCM-300 Chlorophyll meter (OPTI-SCIENCES, INC. Hudson, NH, USA). Three branches were randomly selected on each plant as measurement points and the average was recorded.

3.2.4. Statistical analyses

Tree height, shoot counts and chlorophyll content data measured over time were compared between treatments using one way repeated measures analysis of variance (ANOVA) with Fisher LSD post hoc (StatSoft, 2014) and least significant difference (LSD) tests were assumed to compare significance differences (P <0.05). The statistical tests in tree height, shoot counts and leaf chlorophyll content at weeks three and eight were done using a one way ANOVA with the significance level set at ($P < 0.05$). Data were tested for normality and homogeneity and graphical representation were done using GRAPHPAD PRISM (ver. 704 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). A two- tailed Pearson's correlation with 95% confidence intervals (C.I) was used to test the relationship between the height and shoot growth of *T. usneoides* as a result of metal toxicity.

3.3. RESULTS

Even though Cd is not an essential metal element for plants, its presence usually affects plant health negatively, especially if in high quantity. This study showed that *Tamarix usneoides* developed visible signs of metal and salt toxicity such as leaf chlorosis, growth inhibition and dry bottom branches at the high Cd concentration (18 ppm). In addition, the high Cd concentration toxicity resulted in 13 deaths (representing over 21% of individuals in that group) by the end of our experiment. Additionally, the bottom branches of plants grown at high Cd concentration dried off, a sign of saline toxicity (Weggler et al., 2004; Terrones et al., 2016). This suggests that our *T. usneoides* might have accumulated the salt we applied to increase the metal bioavailability, as it has been observed in *T. smyrnensis* (Kadusaki et al. 2008; Manousaki et al. 2009) and other plants that there is an increase in salt concentration in the shoots with the increase of metal concentration.

TABLE 3. 1 The effect of Cd exposure on *T. usneoides* growth and physiology after eight weeks of treatment. Cd induced changes showed reductions in growth parameters and physiological responses with increased concentration over time. The data are presented as Means±Standard Errors for three replicates (n = 240).

Treatment groups	Height (cm)	Shoot counts	Chlorophyll content (mg/m^2)
Control	54.644±2.613	14.206±2.419	320.147±5.699
Low (6 ppm)	53.385±2.017	11.970±2.761	302.893±13.580
Medium (12 ppm)	52.328±1.958	11.212±2.868	273.862±16.167
High (18 ppm)	50.015±1.710	10.191±3.177	254.498±16.369

3.3.1. Effects of Cd stress on plant growth

Plant height and shoot number were used as growth indicators to assess Cd stress tolerance by *T. usneoides*. Reduced plant height and low new shoot generation were observed in *T. usneoides* with increased metal concentration (Table 3.1). The ANOVA showed a significance difference in heights when comparing the three Cd concentrations to the control ($F_{3,236}$ = 13.927; P < 0.0001; Fig. 3.1). The Post hoc Fisher LSD tests showed that when the concentration of

Cd was at 18 ppm, the cumulative height (height measurements accumulated with time) of *T. usneoides* was significantly reduced when compared with the other two treatment groups and the control. However, no significance difference was observed between the 12 ppm and 6 ppm Cd concentrations, and between 6 ppm and the control. A significant difference in tree heights induced by Cd toxicity was obtained between the medium Cd concentration (12 ppm) and the control (Fig. 3.1). Although, heavy metal stress began to have an effect on *T. usneoides*' height after week 3 as the mean heights of the trees in all three Cd treatment groups remained below the controls (Table 3.1; Fig. 3.1), a one way ANOVA showed no significant difference in height reduction at week three ($F_{3,236} = 0.883$; $P = 0.45$) . Our results showed that the heavy metal toxicity started showing a significant reduction in the heights of *T. usneoides* individuals after six weeks of Cd treatment (Fig. 3.1).

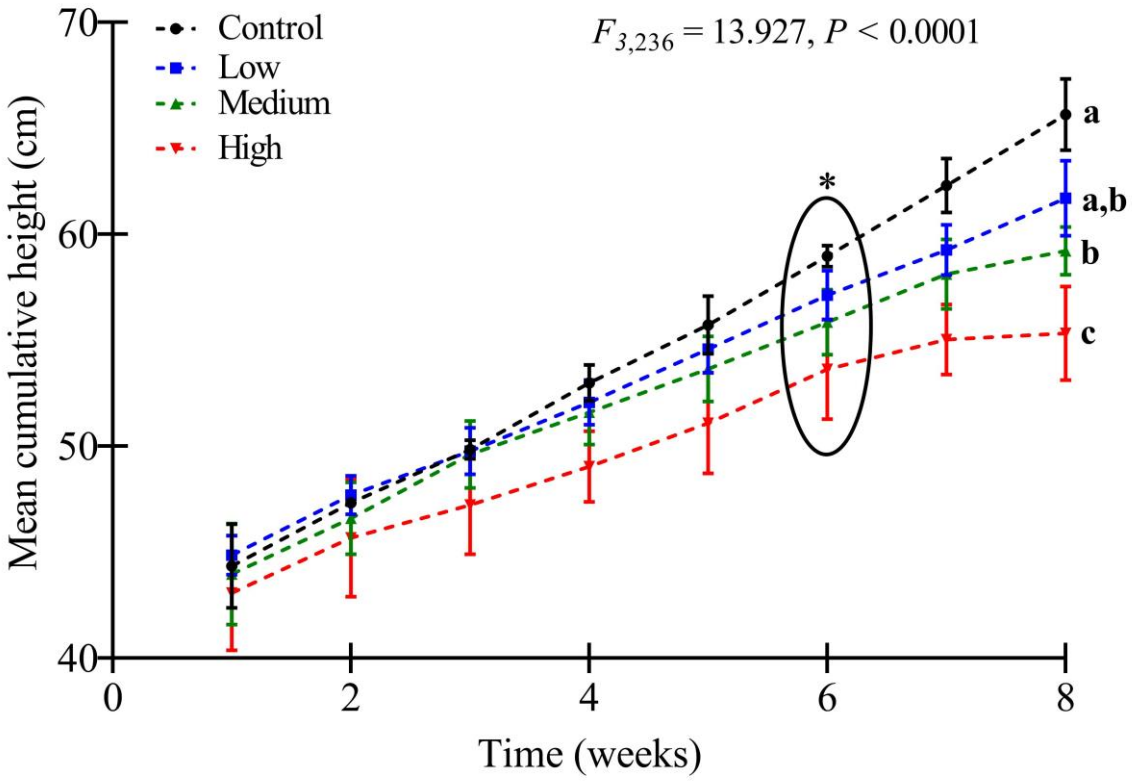

FIGURE 3. 1 The effect of Cd exposure on the heights of 240 *Tamarix usneoides* individuals. ANOVA test between the means of the three treatment groups showing a significance difference ($P < 0.0001$) with the controls (n = 20). All plants were treated with 200 mL of 100 mmol. L^{-1} NaCl three days and then treated with 200 mL Cd three times per week for eight weeks. The Cd concentrations were Low = 6 ppm, Medium = 12 ppm, High = 18 ppm. (Letters a,b,c and d indicate the significance among treatments). * indicates when the significant differences were first observed ($F_{3,236} = 5.636$; $P < 0.0001$).

The number of shoots generated by *T. usneoides* was shown to decrease with an increase in Cd concentration (Table 3.1; Fig. 3.2). Similarly to the effect of Cd on height, there was a significant difference ($F_{3,236} = 93.153$, $P < 0.0001$) in shoot growth between Cd-treated *T. usneoides* and the control. Unlike the effect of Cd on *T. usneoides*' height, the Post hoc Fisher LSD tests showed that there was significant difference between the low (6 ppm) and the medium (12 ppm) treatment groups. However, no significant difference in shoot growth was observed between the low and the control groups (Fig. 3.2). Contrary to the effect of Cd on height, the one way ANOVA showed that the effect of Cd toxicity significantly ($F_{3,236} = 40.754$; $P < 0.0001$) reduced shoot growth regeneration after

week 4 during our experiment, with the significant difference between the control and the high concentration treatment (18 ppm). This indicates that shoot growth was more sensitive to Cd toxicity than height of *T. usneoides*. The differences observed in the effects of Cd on *T. usneoides* growth parameters measured (shoots growth and height) was supported by the correlation relationship test between the effect of Cd on tree height and shoot growth which showed a weak but significant correlation between plant height and shoot numbers at week 8 (Fig. 3.3).

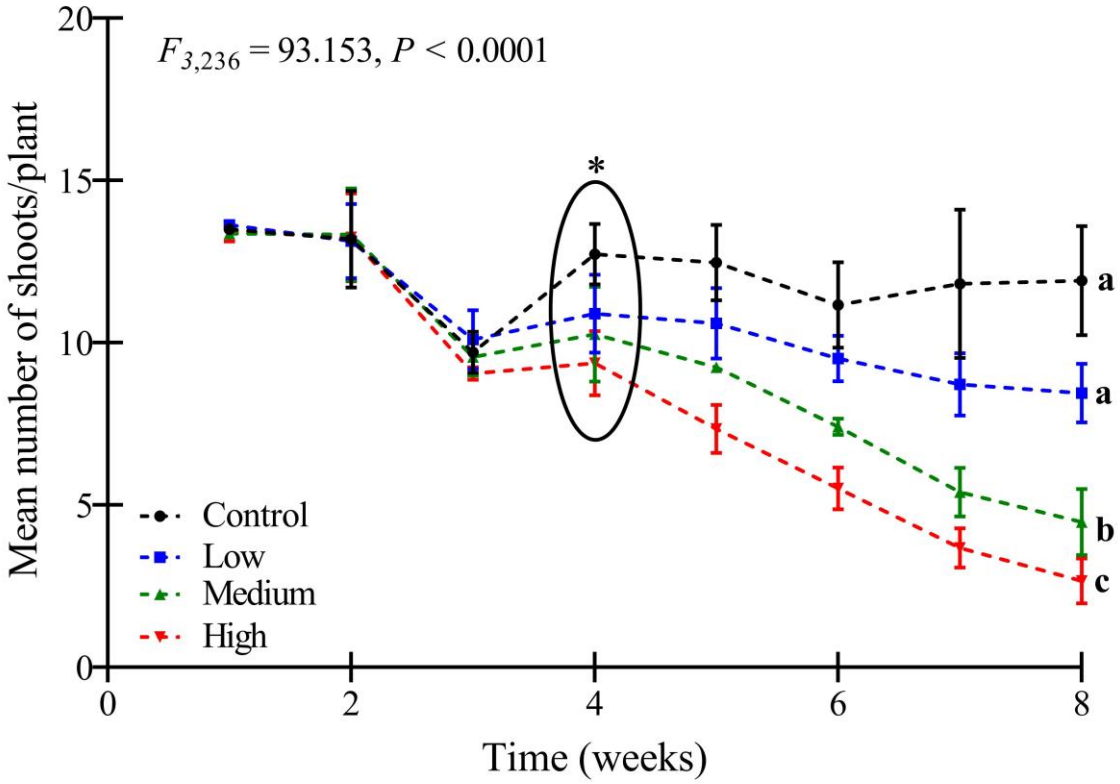

FIGURE 3. 2. The effect of Cd on shoot growth of *Tamarix usneoides* (n = 240). The data presented are the means of three replicates showing a significance reduction in shoot growth induced by metal toxicity over time ($F_{3,236}$ = 93.153, P < 0.0001). All plants were treated with 200 mL of 100 mmol. L^{-1} NaCl three days and then treated with 200 mL Cd three times per week. (Letters a,b,c and d indicate the significance between). * indicates when the significant difference was first observed ($F_{3,236}$ = 40.754; P < 0.0001). The Cd concentrations were Low = 6 ppm, Medium = 12 ppm, High = 18 ppm.

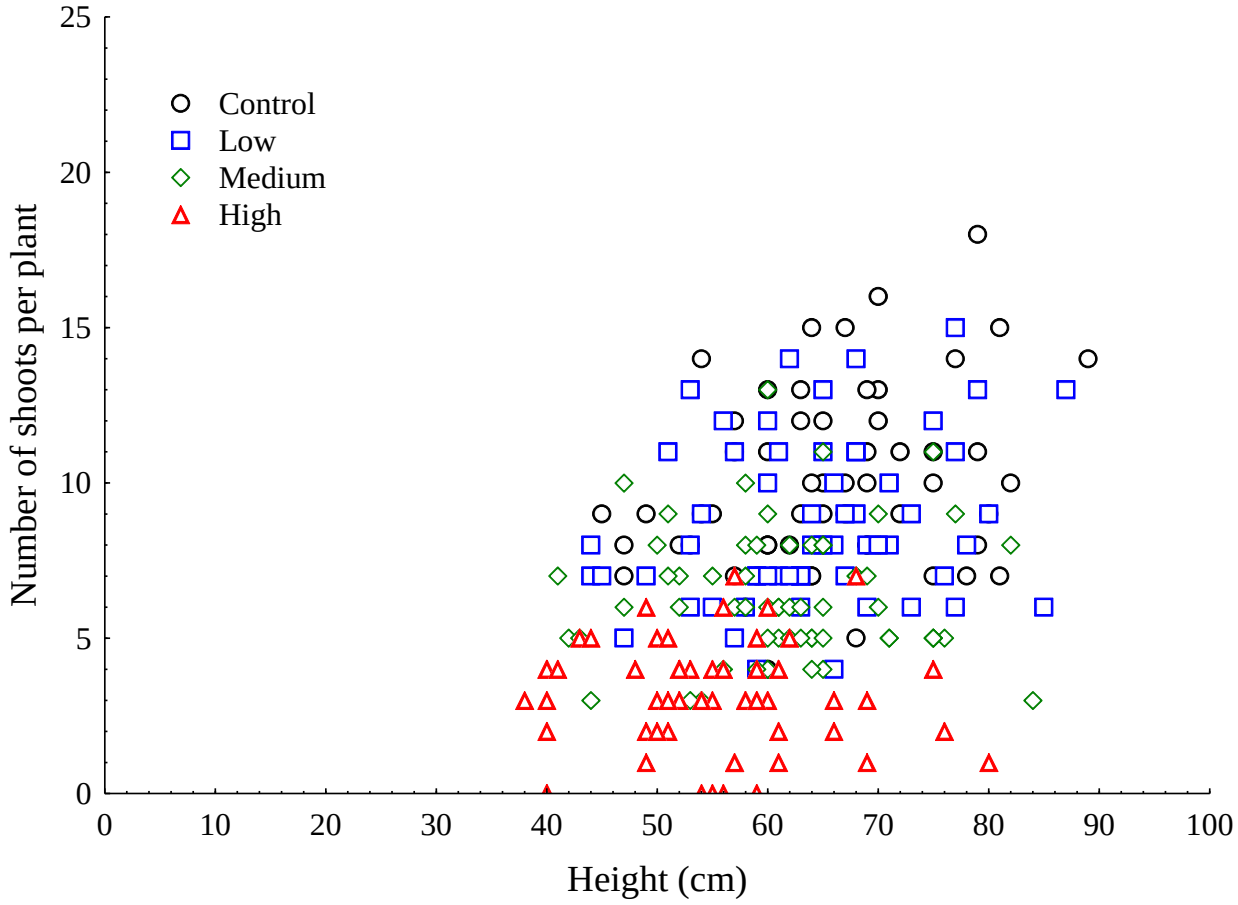

FIGURE 3. 3. Effect of Cd concentrations on the relationship between the growth parameters (shoot growth and height increase) of *Tamarix usneoides* after eight weeks showing no correlation between the two parameters (Control: y = 4.1833 + 0.0873*x; r = 0.2933, p = 0.0230; r^2 = 0.0860; Low: y = 4.4773 + 0.0656*x; r = 0.2405, p = 0.0641; r^2 = 0.0579; Medium: y = 6.0656 + 0.0054*x; r = 0.0243, p = 0.8537; r^2 = 0.0006; High: y = 3.9497 - 0.0145*x; r = -0.0770, p = 0.5589; r^2 = 0.0059) . All plants were treated with 200 mL of 100 mmol. L^{-1} NaCl three days and then treated with 200 mL Cd three times per week. The Cd concentrations were Low = 6 ppm, Medium = 12 ppm, High = 18 ppm.

3.3.2. Effects of Cd stress on *T. usneoides* chlorophyll content

Chlorophyll levels in the leaves of *Tamarix usneoides* dropped as a result of Cd toxicity, as our results showed a significant difference ($F_{3,236}$ = 138.00; P < 0.0001) between the control and the treatments, indicating a decrease in *T. usneoides* chlorophyll content with the increase in Cd concentration over time. The Post hoc Fisher LSD tests showed that the medium (12 ppm) and high (12 ppm) Cd concentrations significantly reduced the chlorophyll levels when compared to the control. However, there was no significant difference between the

low Cd concentration and the control (Fig. 3.4). One way ANOVA test showed that leaf chlorophyll content in *T. usneoides* individuals was significantly reduced ($F_{3,236}$ = 2.972; P < 0.05) after just three weeks of Cd treatment with the significant difference seen between the high concentration (18 ppm) and the control. However, no significant difference was observed between the low or medium Cd concentrations and the control group at week 3.

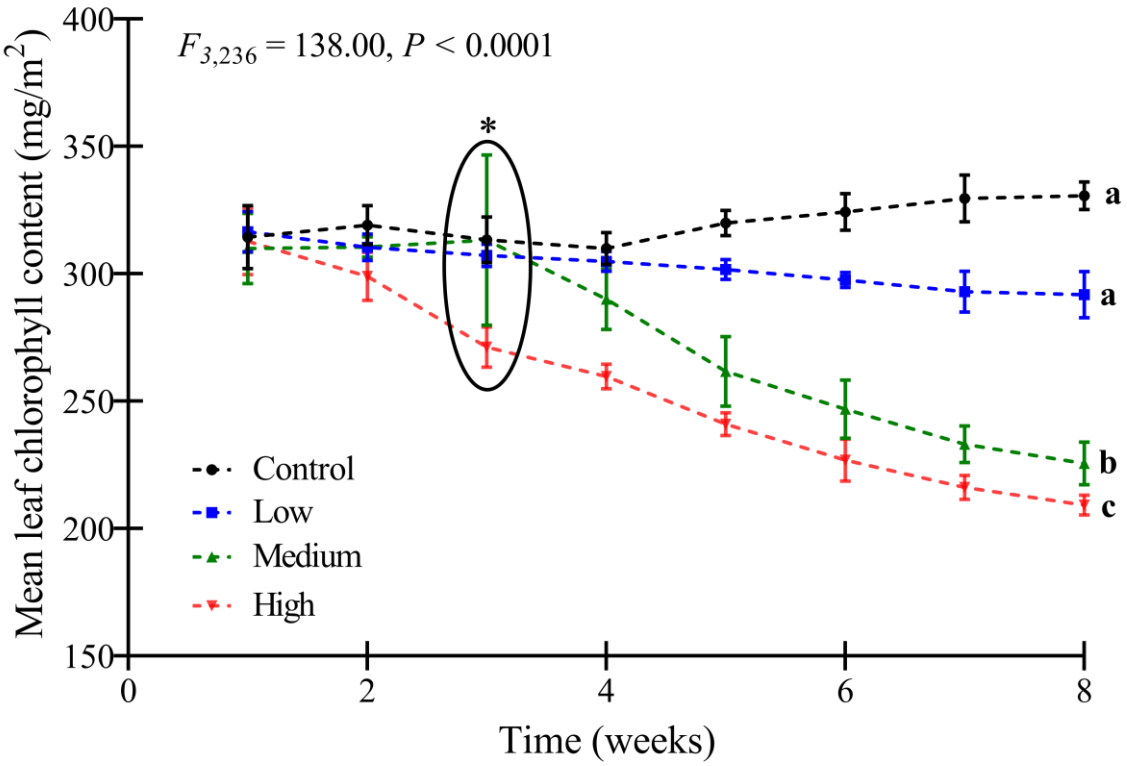

FIGURE 3. 4. Effects of Cd on leaf chlorophyll content (mg/m^2) in *Tamarix usneoides* individuals over time (n = 240). The data are presented as mean values of three replicates with error bars. All plants were treated with 200 mL of 100 mmol. L^{-1} NaCl for three days and then treated with 200 mL Cd three times per week. The Cd concentrations were Low = 6 ppm, Medium = 12 ppm, High = 18 ppm. An ANOVA test between the means of the different independent groups showed significant difference ($F_{3,236}$ = 138.00; P < 0.0001) between the controls and the treatments (n = 20). (Letters a,b,c and d indicate the significance levels among treatments). * indicates when significant difference was first observed ($F_{3,236}$ = 2.972; P < 0.05).

3.4. DISCUSSION

3.4.1. Effect of Cd toxicity on the growth of *Tamarix usneoides*

High concentrations of heavy metals usually affect plant health negatively (Yadav, 2010) with frequently observed effects being growth inhibition and even plant death caused by the reduction in enzymatic activity, photosynthetic rate, respiration and transpiration, as well as nutrient uptake (Pal et al., 2006; DalCorso et al., 2008; DalCorso, 2012). Root length, shoot length, tree height and biomass are parameters currently used to evaluate tolerance of plants against heavy metals (DalCorso et al., 2008; Liu et al., 2009). In this study, the cumulative *Tamarix usneoides* height and shoot growth were significantly reduced with an increase in Cd concentrations over time, showing a significant reduction in growth. Similarly, Cd was found to reduce shoot length and plant biomass in *T. smyrnensis* Bunge. (Kadukova et al., 2008). In a similar study, Cd was also found to reduce leaf size (by changing the leaf shape) in *Pennisetum purpureum* K. Schumach × *P. thyphoideum* Rich., an energy crop, and the leaf was found to be the more sensitive to Cd than root length and biomass (Zhang et al., 2014). In *Tamarix*, the leaf shape could not be easily measured during our experiments because of their scale like shape (Baum, 1978; Bredenkamp and Phepho, 2008), but our study showed a similar observation that the shoot growth was more sensitive to Cd toxicity than the plant height. Unlike Cd, Pb another toxic heavy metal did not affect shoot growth and height in *T. smyrnensis* (Kadukova et al., 2008). In contrast, the growth of *Lonicera japonica* Thunb. was not affected by Cd concentrations of up to ~12 ppm, as the height and the dry biomass of the leaves and roots showed no significant differences compared with the controls (Liu et al. 2009). Over time, our analysis showed that Cd toxicity caused a significant reduction in shoot growth from the third week of our experiments, as the high treatment group (18 ppm) was found to be significantly different from the control. However, our results showed that Cd did not cause any significant reduction in plant height at week three, with the significant difference seen after six weeks of metal treatment ($F_{3,236} = 5.636$; $P < 0.0001$). This suggests that shoot growth is more sensitive to Cd than height addition. In addition, our observation of the differences in the plants' response to the effects of Cd on shoots and height was

supported by the lack of relationship between the two with a weak correlation obtained in our analysis. Our observations suggest that *T. usneoides* is likely to grow more in height than in diameter under heavy metal induced conditions, a growth form in *T. usneoides* seen in propagated plants at mining polluted sites at Vaal River Mine, Orkney, in the North West Province, South Africa (Mayonde pers. obs.). It is suggested that the proportion between height : diameter growth ratio of a plant is known to be influenced by several factors including nutrients assimilation and allocation to either axillary or apical shoots by the plant (Thornley, 1999). Cadmium is known to antagonize the uptake and transport of essential elements such as Cu and Zn, which can affect the addition of plant biomass (Orcutt & Nilsen, 2000). Although the basis of Cd toxicity in plants is still not well documented, Cd toxicity mechanisms in *T. usneoides* could be due to Cd^{2+} ions interfering with homeostatic pathways for essential metal ions, which are important for enzymatic activities in plants (Hall, 2002; Yadav, 2010). It is suggested that the chemical similarity between Cd^{2+} ions and functionally active ions situated in active sites for enzymes and signalling components (Roth et al., 2006) increases Cd toxicity in plants by displacing cations such as Zn and Fe from the protein (Yadav, 2010). The visual signs of Cd metal toxicity in *T. usneoides* during our esperiment, could be evidence of the deficiency of important trace elements such as Fe, Cl, Mn, Zn and Cu that are essential for plant growth. Cd toxicity in *T. usneoides* could have also resulted in the displacement of divalent cations from proteins, which is known to cause oxidative injuries in plants (Hall, 2002).

3.4.2. Effects of Cd toxicity on chlorophyll content of *Tamarix usneoides*

Leaf chlorosis and damage to the root tips as well as a change in leaf color are the most commonly observed consequences of Cd toxicity in plants, as pollution by this heavy metal can depress plant chlorophyll fluorescence, thereby negatively impacting its photosynthetic rates (Ralph & Burchett, 1998; Ekmekci et al., 2008). Our study showed that chlorophyll content in *T. usneoides* was significantly reduced by Cd toxicity. At 18 ppm concentration of Cd, *T. usneoides* show a significant reduction in leaf chlorophyll content in the third week of our experiment, as there was a significant difference between the high concentration

and the control. The low and medium concentration treatments did not show any significant effect on *T. usneoides* chlorophyll content until week four. Zaman & Zereen. (1998) observed a significant decrease in the content of chlorophylls a and b, and the total amount of chlorophyll in radish plants due to exposure to Cd applied in liquid form through the soil. In contrast, Kadukova et al., (2008), found that Cd toxicity did not significantly reduce chlorophyll content in *T. smyrnensis*, albeit that their experiment was a mixture of Cd and Pb metals, and salt. Similarly, Cd did not significantly inhibit chlorophyll content in *Lonicera japonica* leaves, even when plants were exposed to ~ 8 ppm and ~12 ppm concentrations. In fact, there was improvement in the chlorophyll content when exposing *L. japonica* to ~5 ppm of Cd, which suggests an improvement in the physiological functioning of the plant (Liu et al., 2009). Similarly, aluminium (Al^{3+}), another heavy metal element, is known to enhance growth in *Triticum aestivum* L. by increasing Fe solubility, promoting P uptake and protecting against Cu and Mn toxicity (Kinraide, 1993). The decrease in *T. usneoides* chlorophyll content relating to a higher Cd concentration in the leaves could possibly be a mechanism used by the plant to monitor Cd-induced damage by giving the plant the ability to synthesize Cd-phytochelatin complexes or bind the Cd^{2+} ions to the cell wall structures, a phenomenon explained by de la Rosa et al. (2004) — a known mechanism used by plants to tolerate heavy metals.

3.5. CONCLUSION

The data obtained from our study suggest that *Tamarix usneoides* has the ability to tolerate Cd at 12 ppm, which is greater than the maximum concentration found in natural soils (> 8 ppm, Orcutt and Nilsen, 2000). However at Cd concentration of 18 ppm (44.4% more than in natural soils), the heavy metal had been found to reduce growth and chlorophyll contents in *T. usneoides* resulting in the death of some plants. Shoot generation was found to be more sensitive to Cd toxicity than increase in plant height in *T. usneoides*, suggesting that increased concentrations of Cd will not stunt the growth of the plant by significantly reducing its above ground biomass. *T. usneoides* did not exhibit visible signs of metal toxicity at low and medium concentrations of Cd. The properties observed in this study therefore confirm the ability of *T. usneoides* to tolerate heavy metals,

and suggest that it is should be considered for planting on the mines for phytoremediation. However, we recommend metal translocation and accumulation (phytoextraction and phytoaccumulation analyses) studies should be conducted on *T. usneoides* to confirm whether it is a metal hyper-accumulator or not. Since *T. usneoides* has been documented to be a metal tolerant plant and is known to have a high biomass, if found to be a metal hyper-accumulator, *Tamarix usneoides* will be nothing less but a biological pump for heavy metals and could be a crucial component in phytoremediation of polluted soils.

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CHAPTER 4

Gene expression patterns from cDNA-AFLPs differ among heavy metal treatments in *Tamarix usneoides* E. Mey. Ex Bunge. (Tamaricaceae)

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(Intented for submission to the *Plant Physiology and Biotechnology*)

ABSTRACT

Tamarix usneoides is a good candidate for phytoremediation programmes because of its rapid growth, extensive root system, high above ground biomass, tolerance of high saline conditions and excretion of salts through specialized salt glands; however its tolerance to heavy metals has not as yet been fully investigated. Cadmium (Cd) was used in this study to investigate metal tolerant expression patterns in *T. usneoides* because the ability to tolerate Cd is regarded as a constitutive species-level trait investigated in plants used for phytoremediation. In order to characterize differentially expressed genotypes, 80 *T. usneoides* individuals were treated with three different Cd concentrations (6 ppm; 12 ppm; 18 ppm). Cd was applied in aqueous form via the soil and the plants were exposed for eight weeks. cDNA-AFLP analysis, a genome-wide transcriptome profiling technique, was used to investigate genotypes of *T. usneoides* with a high number of differentially expressed bands for heavy metal tolerance. cDNA-AFLP profiles of the treatment groups and controls (untreated plants) were generated and compared using three primer combinations. A total of 36 unambiguous loci were successfully amplified across three primer pairs in 80 samples. There was a difference in gene expression patterns associated with Cd tolerance and it was observed that expression patterns increased with an increase in Cd concentration. The medium Cd concentration produced more differently expressed bands than the low and high concentrations. Ten transcript derived fragments were found to be shared among *T. usneoides* individuals from the three treatment groups, where four (40 %) of those were consistent across all Cd treatment groups. Interestingly, two private brands associated with individuals from the 12 ppm Cd treatment group were obtained. This study shows that there are molecular mechanisms associated with heavy metals tolerance in *T. usneoides*. Further studies are needed to investigate and characterize candidate genes responsible for metal tolerance in *T. usneoides*. Information from this study can be used to potentially identify genes that are useful for phytoremediation programmes.

Key words: Differential gene expression, Heavy metal stress, Leaf chlorosis, Phytoremediation, RNA, Transgenic plants

4.1. INTRODUCTION

Undesirable environmental conditions caused by salt, drought, and heavy metal pollutants are known to limit plant growth and development. Although heavy metals are naturally present in soils, anthropogenic activities such as mining, energy production and agriculture increase their occurrence and concentrations (Manousaki et al., 2009). Most heavy metals are harmless to most living organisms but can result in high health risks if they occur beyond critical levels (Xiong et al., 2006; Burkhead et al., 2009). The mining of minerals such as gold, uranium and copper is known to generate acid mine drainage (AMD) that can cause the dissolution of heavy metals in surrounding areas because of its high acidity. The presence of AMD in the soil can cause impairment to waterways and biodiversity over time (Akcil and Koldas 2006).

Elevated concentrations of micronutrients, which convert them into heavy metals, renders them cytotoxic, and in plants, heavy metals are directly involved in the generation of reactive oxygen species (ROS) through Haber-Weiss reactions (Mithofer et al., 2004; Yadav, 2010). Indirect effects of heavy metals include disruption of the electron transport chain and essential elements metabolism (Qadir et al., 2004; Dong et al., 2006), disturbs antioxidant systems (Srivastava et al., 2004), and lipid peroxidation (Dermiral and Turkan, 2005) — all of which could result in the death of the plant. However, plants adapted to high concentrations of heavy metals have developed mechanisms such as the production of thiols [e.g. glutathione (GSH) and cysteine], low molecular weight substances with high affinity for toxic metals (Bricker et al., 2001), to combat toxicity problems.

Micronutrients such as copper (Cu), iron (Fe), zinc (Zn), manganese (Mn) and nickel (Ni) are essential metals required for various cellular processes in plants such as gene expression, stress tolerance, and the biosynthesis of proteins, nucleic acids, growth substances, chlorophylls, and secondary metabolites (Rengel, 2004). Unlike micronutrients, non-essential metals such as cadmium (Cd), mercury (Hg) and lead (Pb) can be toxic at very minimal concentrations (DalCorso, 2012). Cd is one of the most problematic heavy metals because of its high soluble nature in water and the negative effects such as carcinogenic, mutagenic, and teratogenic it has in the food chain (Das et al., 1997; Pál et al.,

2006). Phytotoxicity caused by Cd results from changing several physiological processes at the cellular level thereby affecting enzyme activities, reducing functions of important metabolically molecules, replacing essential elements and disrupting membrane integrity (Das et al., 1997; Pál et al., 2006; Rascio & Navari-Izzo, 2011). However, GHS — a sulphur containing tri-peptide thiol, is a substrate for phytochelating synthesis and important in the detoxification process of heavy metals such as Cd and Ni (Freeman et al., 2004). Phytochelatins (PCs) are small cysteine-rich polypeptides that form complexes with toxic metal ions in the cytoplasm of the cell and then transport them into the vacuole (Salt & Rauser, 1995); therefore, protect plants against cytotoxic effects of heavy metals.

Phytoremediation is an alternative to the traditional method of restoration or strategy for the cleaning of heavy metal contaminated soils. It uses plants to strip pollutants such as heavy metals from the environment and render them harmless (Yanai et al., 2006; Paz-Alberto & Sigua, 2013). Since the identification of plants accumulating certain levels of heavy metals in the early 19th century, a considerable amount of research has been done investigating the physiology and biochemistry of metal hyper-accumulation in plants (Salt & Rauser, 1995; Yang et al., 2002). Identifying the subsets of differentially expressed genes is a fundamental requirement in clarifying the molecular regulation of physiological events (Cappelli et al., 2005). Most research on heavy metal tolerant and hyper-accumulating plants has focused on the physiological effects of heavy metal uptake, their transport from roots to shoots and their sequestration in the tissues (Long et al., 2002; McIntyre, 2003), while not much about the genetic basis of metal tolerance and accumulation has been done. Identifying any variability in the genetic expression of plants capable of tolerating and accumulating heavy metals is important because such variation is fundamental in the evolution of metal tolerant and hyperaccumulating plants (Yang et al., 2005). Our study aims at genotyping differentially expressed individuals of *Tamarix usneoides* E Mey. ex Bunge associated with Cd tolerance.

Tamarix usneoides is one of the 55 species of the genus *Tamarix* L. in the Tamaricaceae and is indigenous to southern Africa (Obermeyer, 1976; Baum, 1978). Together with species of *Vachellia*, *Senegalia* (both previously *Acacia*),

and *Searsia*, *Tamarix* spp. are used for phytoremediation in southern African gold and uranium mines (Weiersbye et al. 2006). The indigenous *Tamarix usneoides*, together with the two exotic species, *T. chinensis* and *T. ramosissima*, and their hybrids have all been confirmed to occur on tailing storage facilities across South Africa (Mayonde et al., 2015, 2016) where they are used for phytoremediation. The indigenous *T. usneoides* is preferred for phytoremediation in South Africa over the exotic species (Obermeyer, 1976). The exotic species have been declared invasive by the Alien Invasive Species regulations of the National Environmental Management: Biodiversity Act 2014 (NEMBA) and are currently being investigated for biocontrol (e.g., Marlin et al., 2017).

Tamarix species are halophytes and may also be adapted to taking up and accumulating certain metals (Kadukova et al., 2008; Manousaki et al., 2008, 2009). Although *T. usneoides* is known to tolerate and accumulate heavy metals including Cd, little is known about its genetic responses when doing so. In this study we identified genotypes with differential gene expression patterns for metal (Cd) tolerance using cDNA-AFLPs. These results may be useful in functional gene characterization and in the propagation of *T. usneoides* genotypes that are most tolerant against heavy metals toxicity for effective phytoremediation programmes (Yadav, 2010). There is interest in breeding stress tolerant transgenic plants for phytoremediation of heavy metal pollutions, as well as for salt, drought and cold stresses. Therefore, studying the functions of stress tolerant genes in plants and cloning them present an opportunity to discover novel genes and provide valuable information for improving stress tolerance through genetic engineering (Yadav, 2010).

Amplified fragment length polymorphisms (AFLPs) of complementary DNA (cDNA), commonly known as cDNA-AFLPs, have emerged as a valuable and inexpensive alternative to hybridization-based microarray techniques in the field of functional genomics and genetics (Vuylsteke et al. 2007; Kim et al. 2012). cDNA-AFLP is a gel-based transcript profiling method to investigate expression patterns of any organism at genome-wide level (Vuylsteke et al. 2007). The cDNA-AFLP technique follows the principle of AFLPs as outlined by Vos et al. (1995). While AFLPs use genomic DNA, cDNA-AFLPs involve the reverse

transcription of mRNA into double stranded cDNA followed by the standard AFLP protocol (Bachem et al. 1998; Vuylsteke et al. 2007). The cDNA-AFLP technique is based on selective PCR amplification of restriction endonuclease fragments from a total digest of complementary DNA (Vos et al., 1995). The AFLP process involves the following steps: restriction and ligation of the DNA, pre-selective amplification, selective amplification and electrophoretic separation of the amplified fragments (Vos et al., 1995; Vuylsteke et al. 2007).

Although cDNA-AFLPs quantifies the relative differences in the gene expression levels by observing the differences in band intensities (brightness), this study focused on assessing the gene expression patterns in *T. usneoides* for different Cd concentrations. We hypothesized that patterns of gene expression will change when exposed to higher Cd concentrations signalling the plant's gene expression patterns for tolerance of metal toxicity. Plant response to heavy metal toxicity is a complex trait controlled by multiple genes (Yang et al., 2005; Craniun et al., 2006) giving the plant the ability to reduce the toxic level of metal so that it can be tolerated once absorbed. cDNA-AFLP analysis is a technique that has successfully been used to profile expression patterns, and subsequently, isolate novel genes in under different experimental conditions in several organisms (Bachem et al., 1998, 2001; Durrant et al., 2000; Craniun et al., 2006; Vuylsteke et al., 2006; Ambrosone et al., 2013). Generating transcript profiles of organisms has numerous applications in plant biology, from the identification of up- and down-regulated genes responsible for certain metabolic processes, to the identification of biotic and abiotic stressed-induced transcripts (Yan et al., 2005).

4.2. MATERIALS AND METHODS

4.2.1. Heavy metal treatment experiment

For this study, four clones per tree were collected from 20 different *Tamarix usneoides* trees making a total of 80 individuals. Plants were collected across 20 different localities in three provinces (Appendix 3A) to represent the genetic diversity within *T. usneoides* in South Africa. Samples were mainly collected from the Northern Cape Province, which is the native range of *T. usneoides,* with a few samples coming from natural populations in the Western and Eastern Cape Provinces. *Tamarix usneoides* cuttings (15 cm long) were

propagated in silica sand for 6 weeks until substantial shoot and root systems developed. Dynaroot1 rooting hormone (PBR Trading International CC) was applied to enhance development of the root system. Rooted cuttings were transplanted into individual plastic pots (16.5 cm in height and 20 cm diameter) filled with the same amount (1.5 kg in dry weight) of substrate containing a mixture of ¼ organic compost, ¼ silica sand and ½ top soil. Individually transplanted plants were left to grow for another four weeks to allow establishment of roots before treatments began.

The four clones per tree were divided into four treatment groups with each group containing 20 independent replicates (Table 4.1). *T. usneoides* trees were cloned to ensure that any observed differences in gene expression patterns are due to the Cd treatments and not the genetic variability of individuals. The maximum Cd concentration (18 ppm) chosen in this study is more than twice the known upper limit of Cd metal concentration in toxic soils, which range between 3 to 8 ppm dry weight (Orcutt & Nilsen, 2000). A once-off amount of 200 ml of salt water (100 mmol.L^{-1} NaCl) was applied to all *T. usneoides* plants three days before Cd treatment to increase the bioavailability of metals in the soil (due to reduced soil metal sorption; Bingham et al., 1983; Weggler et al., 2004; Wahla & Kirkham, 2008). NaCl also plays a significant role in the movement of metals from roots to the above ground plant biomass (Greger et al. 1995; Salt & Rauser, 1995; Fitzgerald et al. 2003). An amount of 200 ml of cadmium nitrate tetrahydrate [$Cd(NO_3)_2.4H_2O$] was applied to the roots via the soil in liquid form with the concentrations shown in Table (4.1) and deionized water (DW) was used for the control plants. Plants were watered twice per day and three days per week (in the order of Monday, Wednesday and Friday) for eight weeks. Collecting trays were placed at the base of the pots to avoid loss of metal contaminated water.

TABLE 4. 1 Experimental design showing the different Cd concentrations exposed to *Tamarix usneoides* clones for gene expression patterns investigation using cDNA-AFLP analysis.

Treatment groups	Treatment Codes	Cd concentration (ppm)	No. of samples per group
1	Control	0	20
2	Low	6	20
3	Medium	12	20
4	High	18	20

4.2.2. cDNA-AFLP analysis and isolation of transcript derived fragments

4.2.2.1. RNA extraction and cDNA synthesis

For cDNA-AFLP analysis, 80 individuals were analysed. Fresh, young shoots of *Tamarix usneoides* were collected, submerged in RNA Later™ (Sigma, Germany) in 2 mL eppendorf tubes and immediately stored at −40°C until analyses began. Total RNA was isolated from liquid nitrogen frozen shoot tissue using InviTrap® Spin Plant RNA Mini Kit (Stratec Molecular, Berlin/Germany) according to the manufacturer's instructions. To obtain high RNA concentrations (> 1000 ng/µL) in *Tamarix* samples, the Lysis Solution RP of the Invitrap kit was used (as opposed to Lysis solution DCT), and generally yielded < 500 ng/µL of RNA. Total RNA concentration and quality (260/280 ratio) was checked using a NanoDrop Spectrophotometer (Thermo SCIENTIFIC, USA) and stored at −40°C. For the first strand cDNA synthesis, 4 µL (~1000 ng) of total RNA was reverse transcribed using ReadyScript™ cDNA Synthesis Mix (Sigma-Aldrich, Germany) following the manufacturer's instructions. The double stranded cDNA was obtained with RNase H (New England Biolabs Inc., UK), DNA polymerase I and dNTPs (New England Biolabs Inc., UK) following manufacturer's instructions.

4.2.2.2. cDNA-Amplified Fragment Length Polymorphisms analysis

Using the double stranded cDNA template, digestion, ligation, pre-selective and selective amplifications were performed following the standard protocol by Vos et al. (1995) with the following modifications: 2 µl (~500 ng) of double stranded cDNA was digested in a 11 µl reaction during restriction-ligation

(done in a single step) containing 0.1 µl of 10 U/µl *Mse*I enzyme, 0.25 µl of 20 U/µl *Eco*RI enzyme, 1.10 µl of 10X T4 DNA ligase buffer, 0.15 µl of 400 U/µl T4 DNA ligase, 1.10 µl of 0.05 M NaCl, 0.55 µl of 10X BSA (1 mg/ml), 1 µl of 50 uM *Mse*I adapter pair, 1 µl of 5 uM of *Eco*RI adapter pair and water to a final volume of 11 µl. The restriction-ligation mix was incubated overnight at room temperature. The restriction-ligation product was diluted using sterile DNA free water at 1:5 ratio. The diluted digestion-ligation product was used as template for the pre-selective amplification.

The pre-selective polymerase chain reaction (PCR) was performed in a 15 µL reaction containing 2.5 µL of the diluted digestion-ligation reaction mix, 1 µM *Mse*I+0 forward and 1 µM *EcoR*I+0 reverse primers (Appendix I), 7.5 µl of Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., UK) and 2 µl PCR graded water. Pre-selective amplification cycling parameters were done as per Blignau et al. (2013). Successful pre-selective amplification was confirmed by running 5 µL of the product on a 1% agarose gel and smear was observed between 100 and 500 bp. The successful pre-selective product was diluted (1:19) with nuclease free water.

The selective amplification was performed in a 20 µL volume containing 5 µL of the diluted pre-selective product as the template. Each 20 µL selective PCR reaction contained 10 µl of Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., UK), 2.5 µl of PCR water, 0.25 µM of fluorescently labelled *EcoR*I+CAT and *EcoR*I+ATG selective primers were paired with 1 µM of unlabelled *Mse*I+CTT and *Mse*I+CTA selective primers (Table 4.2). All four selective primer combinations of *EcoR*I+CAT, *Eco*RI+ATG, and *Mse*I+CTT, *MseI*+CTA were pre-screened for 12 individuals and the three primer pairs that successfully amplified were chosen (*EcoR*I+CAT/*Mse*I+CTT , *EcoR*I+ATG/*Mse*I+CTT and *EcoR*I+ATG/*Mse*I+CTA). The successful *EcoR*I+CAT/*Mse*I+CTT , *EcoR*I+ATG/*Mse*I+CTT primer combinations were selected from Blignau et al. (2013) while *EcoR*I+ATG/*Mse*I+CTA primer combination was taken from Mayonde et al. (2009) based on the high total number of selective fragments they produced. The selective PCR cycling parameters followed that of pre-selective PCR but with 30 cycles. The selective

PCR was done as per Blignaut et al. (2013), without the step-down PCR step. After selective amplification, 5 µL of each fluorescent labelled PCR product was pooled for electrophoresis analysis. Fragment analysis was done in the Central Analytical Facility (CAF), University of Stellenbosch where one microliter of the pooled selective PCR product was mixed with 0.3 µL of 600 bp size standard and 28.7 µL of de-ionized formamide and loaded into an Applied Biosystems (Foster City, CA, USA) 3130 Genetic Analyser for electrophoresis fragment analyses of AFLP bands.

TABLE 4. 2 Sequences for all the primers required during restriction-ligation, pre-selection and selective amplifications (standardized AFLP protocol) including the source of the used sequences.

Primer name	Sequence (5′–3′)	Length (bp)	Label	Source reference
*EcoR*I- adaptor forward	CTCGTAGACTGCGTACC	17	None	Blignau et al. (2013)
*EcoR*I- adaptor reverse	AATTGGTACGCAGTCTAC	18	None	Blignau et al. (2013)
*Mse*I- adaptor forward	GACGATGAGTCCTGAG	16	None	Blignau et al. (2013)
*Mse*I- adaptor reverse	CTACTCAGGACTCAT	15	None	Blignau et al. (2013)
*EcoR*I+0	GACTGCGTACCAATTC	16	None	Blignau et al. (2013)
*Mse*I+0	GATGAGTCCTGAGTAA	16	None	Blignau et al. (2013)
*EcoR*I +CAT	GACTGCGTACCAATTCCAT	19	FAM™	Gaskin & Kazmer, (2009)
*EcoR*I+ATG	GACTGCGTACCAATTCATG	19	5-HEX™	Gaskin & Kazmer, (2009)
*Mse*I+CTT	GATGAGTCCTGAGTAACTT	19	None	Gaskin & Kazmer, (2009)
*Mse*I+CTA	GATGAGTCCTGAGTAACTA	19	None	Gaskin & Kazmer, (2009)

The AFLP loci (alleles) were manually scored as dominant alleles using GeneMarker ver. 2.6.7 (Soft Genetics®) software. Despite the automated fragment size calling and bin settings, the presence and absence of all fragments was confirmed manually, as shorter and smaller picks in some individuals might be considered as absence through the automated allele scoring. An initial scoring with high threshold set for peak height of 250 relative fluorescent units (RFU) and two nucleotides width (bin size) was done. To check for false amplification and scoring errors, three samples from each treatment group were repeated three times from the selective PCR stage and the peaks were cross compared to comfirm alleles. All *T. usneoides* individuals were scored at the same time to avoid manual scoring of artefacts and errors. For individuals with fragments of less than 250 RFU, a rerun of the scoring analysis was done using a lower threshold of 100 RFU with a width of one nucleotide.

4.2.3. Genetic diversity

Differentially expressed genetic diversity generated by *T. usneoides* individuals from the four treatment groups was estimated using GenAlEx ver. 6.5 (Peakall and Smouse 2012). In GenAlEx the dominant AFLP data were treated as a binary presence-absence matrix to estimate the total number of alleles generated per primer pair and calculate the percentage of polymorphic loci obtained from each treatment group. Nei's gene diversity (*H*j) and estimates of allele frequencies at all AFLP amplified loci were calculated using AFLP-SURV (Vekemans et al. 2002). The Bayesian method with non-uniform prior distribution of alleles option assuming Hardy–Weinberg equilibrium was used for estimation of allele frequencies.

In order to identify TDFs associated with Cd tolerance in *T. usneoides*, expression profiles of the control and the treatment groups were compared. Fragments not associated with Cd tolerance were eliminated by discarding bands observed from the Cd treated individuals that were present in the control group as well. Fragments present in the Cd treated plants that were not observed in the controls, were therefore considered to be associated with heavy metal tolerance, with a focus on bands which were consistently observed in the low, medium and high Cd treatment groups.

4.2.4. Principal coordinate analysis

To investigate whether the various expression patterns for heavy metal tolerance were common among the individuals of a treatment group, a principal coordinate analysis (PCoA) was conducted for both the treated and untreated data sets in NTSYSpc v2.2 (ROhlf, 2005). The Jaccard similarity coefficient was used in the analyses (e.g., Sesli & Yegenoglu, 2010), which is uses the same equation as the Nei and Li (1978) similarity coefficient: $2a/(2a + b + c)$ where a = number of bands present in samples, b and c = number of bands present in only one or the other sample. The Jaccard similarity coefficient was preferred here because it excludes negative co-occurrences (Jaccard, 1901). The DCENTER and EIGEN options of NTSYS were then used to complete the ordination analysis.

4.3. RESULTS

The cDNA-AFLP markers used to analyze transcripts of heavy metal tolerance in 80 *Tamarix usneoides* individuals at three different Cd concentrations and the control group yielded 36 successfully amplified loci from three primer combinations. The number of transcript-derived fragments (TDFs) obtained per primer combination ranged from 6 in the 6 ppm treatment to 15 in the 12 ppm treatment group (Table 4.3). *Tamarix usneoides* individuals exposed to 12 ppm of Cd concentration yielded the most expressed bands, producing a high of 35 TDFs (Table 4.3) and had the highest mean genetic diversity of $Hj = 0.292$. Individuals in the medium and high Cd concentration treatment groups produced five shared bands that were found in 50% or fewer individuals in those groups with a 5% frequency of occurrence. The low Cd concentration (6 ppm) had the lowest genetic diversity index ($Hj = 0.088$) and produced the fewest polymorphic loci (n= 21, Table 4.3).

Our cDNA-AFLP analysis identified a total of 10 TDFs shared among the Cd treatment groups, which could be categorized as Cd tolerance-related TDFs. Of the 10 shared Cd TDFs, four (40%) were observed to be common to all treatment groups. The remaining six had four TDFs shared between the low (6 ppm) and the medium (12 ppm) concentrations, and of the remaining two, one was shared between the high (18 ppm) and the low and the other one between the

high and medium Cd concentrations. Two differentially expressed bands were found to be unique to the 12 ppm Cd treated group.

TABLE 4. 3 Summary of the number of cDNA-AFLP polymorphic bands from three primer pairs amplified across 80 *Tamarix usneoides* individuals generated in response to three concentrations of Cd (low = 6 ppm, medium = 12 ppm and high = 18 ppm). All plants were treated with a once off 200 mL of 100 mmol. L^{-1} NaCl three days and then treated with 200 mL Cd three times per week for eight weeks. Band pattern analysis was done using GenAlEx treating the data as binary diploid.

	Eco*RI*+ATG/ Mes*I*+CTT (9 loci)	Eco*RI*+CAT/ Mes*I*+CTT (12 loci)	Eco*RI*+CAT/ Mes*I*+CTT (15 loci)	Total number of bands	% polymorphic loci
Control	7	9	9	25	58.33
Low	6	7	8	21	27.78
Medium	9	11	15	35	91.67
High	9	11	14	34	83.33

The PCoA showed that the first two principal coordinates account for 30.45% and 12% of the total variation, respectively (Fig. 4.1). There is clear separation of the treatment groups based on their expression patterns, resulting in about five distinct clusters spreading across our scatter plot graph (Fig. 4.1). Eighteen individuals from the control group cluster together, however one of the control individuals clusters with the low (6 ppm) Cd treatment group, while another one groups together with the high (18 ppm) Cd treated individuals (Fig 4.1.). All the individuals treated with 12 ppm Cd cluster together with two individuals treated with 18 ppm Cd in a group that appears to be genetically distant from the other groups (Fig. 4.1). The *Tamarix usneoides* individuals exposed to 18 ppm Cd appear to be more dispersed across the ordination space, possibly showing a variety of genetic responses to the extremely high level of metal toxicity. In contrast, individuals treated with the 6 ppm Cd form a tight cluster showing the similarities in their responses to the low concentration of Cd. (Fig. 4.1).

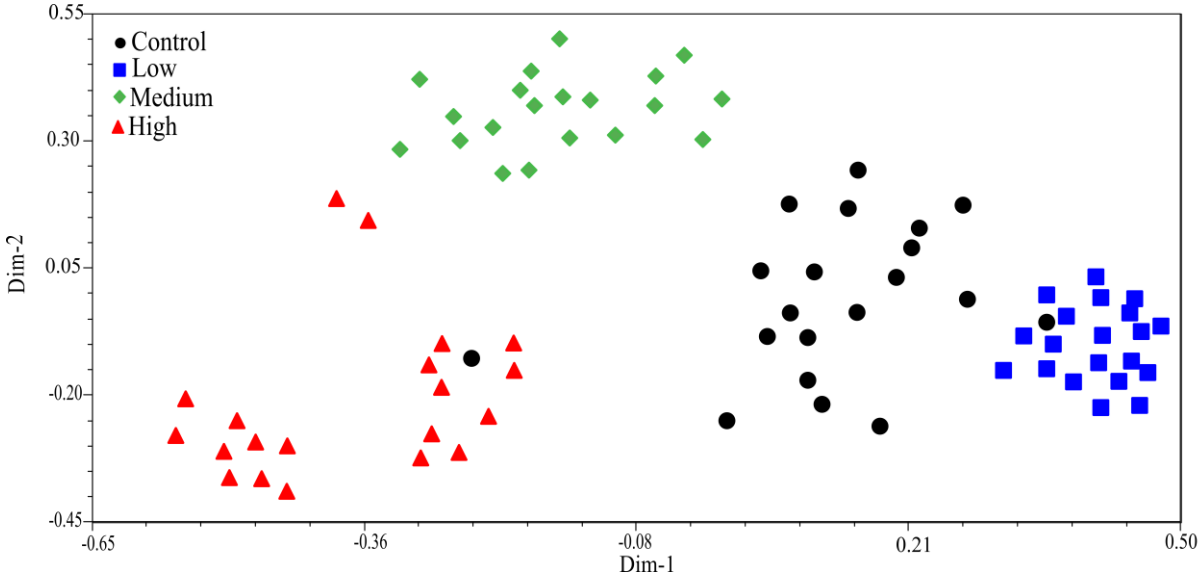

FIGURE 4. 1. Clustering of *Tamarix usneoides* individuals based on their cDNA-AFLP gene expression patterns for heavy metal (Cd) tolerance using principal coordinates analysis of Jaccard similarity coefficients. The first two principal coordinates accounted for 30.42 and 12 % of the total variation among the individuals. *T. usneoides* individuals were subjected to one of three concentrations of Cd (low = 6 ppm, medium = 12 ppm and high = 18 ppm) over a period of eight weeks prior to the cDNA-AFLP analysis.

4.4. DISCUSSION

This study is the first endeavour to identify genotypes of *Tamarix usneoides* with varying expression patterns that may be related to heavy metal tolerance. The cDNA-AFLP data obtained in this study resulted in ten bands (~27.7 %) which seemed to be associated with Cd tolerance in *T. usneoides* (i.e. bands shared only among the Cd treatment groups). A similarly low number of bands was evident based on cDNA-AFLP analysis when testing gene expression of *Brachypodium distachyon* L. for salt tolerance responses using a similar number of primer combinations (Kim et al., 2012). It seems therefore that generally a low number of expressed cDNA fragments have been observed in other organisms because the cDNA-AFLP approach focuses the expressed part of the genome (e.g., Brachem et al., 2001; Yao et al., 2007; Church & Spaulding, 2009). However, a similar study investigating gene expression pattern of *Suillus luteus* L., a fungus exposed to Cd and tested for metal tolerance comparing "sensitive and tolerant" strains using cDNA-AFLP data yielded a high number of 125–175 AFLP bands (Ruytinx et al., 2011). The low number of cDNA-AFLP

expressed bands obtained in this study could be due to the selective primers used, since the genomic information obtained using AFLP analysis can differ between organisms depending on the selective primers and the thermocycling parameter such as the number of cycles (Brachem et al., 2001; Vuylsteke et al. 2007).

A trend of increased expression pattern (number of bands) with increased Cd concentration was evident when comparing the 6 ppm and 12 ppm Cd treatments. Our investigation showed that *T. usneoides* produced the highest number of differentially expressed bands (n = 35) when exposed to 12 ppm Cd as compared to when subjected to 6 ppm (n = 21) and 18 ppm (n = 34) Cd concentrations. The cDNA-AFLP data highlight that *T. useneoides* is able to alter gene expression, in that it increases expressed regions of DNA, when exposed to increased levels of Cd. Our results therefore indicate that there is the possibility of identifying genes associated with heavy metal tolerance in *T. usneoides*, since genes encoding enzymes such as γ-glutamylcysteine synthetase (*GSH1*), serine acetyltransferase (*SAT*) and phytochelatin synthase (*PCS*) are found to be crucial for the efficient production of phytochelatins, a substance associated with Cd tolerance in plants (Wawrzynski et al., 2006). Similarly, overexpression of a bacterial *GSH1* gene in the cytosol or chloroplast of grey poplar (*Populus* × *canescens*) increased GSH level resulting in tolerance of heavy metals (Bittsanszkya et al., 2005). It was shown that over-expression of enzymatic genes associated with glutathione and cysteine in plants has been associated with higher tolerance and accumulation of heavy metals (Yadav, 2010).

The slightly lower gene expression evident in the 18 ppm Cd treatment group (n = 34) compared to the 12 ppm group (n = 35) could be an indication that at this extreme level of toxicity, functions within the plant cells are starting to shut down, and/or that the capacity for coping with the toxicity levels in the cells has been maximised in terms of gene expression. Such a conclusion is possibly corroborated by the fact that plants treated with 18 ppm Cd became chlorotic and started to die after seven weeks of Cd treatment.

Since concentrations of Cd in contaminated soils range from three to eight ppm (dry weight) and these levels are considered to be toxic to most plants

(Orcutt and Nilsen, 2000; DalCorso 2012), these results and those shown in Chapter III therefore suggest that *T. usneoides* has the ability to tolerate up to 12 ppm concentrations of Cd. Furthermore, no signs of metal toxicity were observed at 6 and 12 ppm Cd treatment levels (e.g., visible signs of leaf chlorosis and dry bottom branches), whereas 13 individuals died as a result of the high (18 ppm) Cd treatment. *Tamarix* species are widely considered for phytoremediation efforts to control domestic and industrial environmental pollutions.

Some of the heterogeneity in the gene expression pattern profiles observed here is somewhat surprising, notably that two of the control individuals also express certain loci apparently associated with metal tolerance. It is possible that some of the differentially expressed fragments obtained in this study which are also observed in these two control individuals could be attributed to other environmental stresses such as salt. Although 100 mmol.L^{-1} NaCl was applied to all the *T. usneoides* individuals to increase the bioavailability of Cd in the soil (Weggler et al. 2004; Wahla and Kirkham 2008), it has been evidenced that there is an increase in salt crystals excreted by the leaves of *Tamarix smyrnensis* in the presence of heavy metals (Manousaki et al., 2008). Therefore, the TDFs observed in the control group could be linked to saline stress since cDNA-AFLP analysis does not reveal the actual genes involved in the expression patterns process. However, since the sodium chloride was a once off application at the beginning of the experiment, not many salt related TDFs should have been generated to confuse our findings.

Previous work has shown that *Tamarix smyrnensis* has the ability to tolerate, extract and accumulate heavy metals (e.g., lead [Pb], and Cd) in its roots and shoots (Manousaki et al., 2008, 2009). However, studies have not been conducted to investigate expression profiles involved in metal tolerance, extraction and accumulation in *Tamarix*. To the best of our knowledge, this study is the first to investigate the genomic expression profiles behind the ability of *Tamarix* to tolerate heavy metals. Gene expression patterns for heavy metal tolerance and accumulation have been investigated in several other metal tolerant plants (Craciun et al., 2006). For example, cDNA-AFLP analysis has revealed

gene expression patterns and identified genes corresponding to manganese tolerance in *Citrus sinensis* and *Citrus grandis* (Zhou et al., 2013). Similarly, gene expression relating to Cd tolerance was investigated in *Arabidopsis halleri* and *A. lyrata* spp. *petraea* using cDNA-AFLP (Craciun et al., 2006). Our cDNA-AFLP analysis of Cd tolerance in *T. usneoides* shows that more candidate genes responsible for heavy metal tolerance could be identified at Cd concentrations ≥ 12 ppm. Moreover, the private bands obtained at 12 ppm Cd, together with the shared bands, suggest that a more complex experiment to identify differentially expressed genes is needed to investigate which genes are up- or down-regulated with increased metal concentrations. The private bands observed among the individuals treated with 12 ppm Cd should be sequenced and compared with the nucleotide sequences with NCBI and TAIR databases using Blastn, Blastx and tBlastx sequence programmes (Altschul et al., 1997) to identify whether the expressed sequence tag coincides with a gene that has been shown to be expressed in the presence of heavy metals.

Contrary to the lack of studies involving gene expression patterns of heavy metal tolerance, facultative halophytes within the genus *Tamarix* have been extensively investigated for their ability to tolerate high saline and drought conditions (Wang et al., 2006; Gao et al., 2008; Yang et al., 2017). To investigate the gene expression profiles in response to saline stress, cDNA libraries have been constructed for *T. hispida* Willd, where over 9400 expressed sequence tags (ESTs) representing about 3945 unigenes (including 986 contigs and 2959 singlets) have been identified (Gao et al., 2008). Novel genes such as *ThbZIP1*and *ThDREB* isolated in *T. hispida* have been proven to mediate salt and drought stress in transgenic tobacco plants and *T. hispida* (Wang et al., 210; Yang et al., 2017). Our study suggests that once the observed bands have been sequenced, differentially expressed *T. usneoides* genotypes could be used to isolate the *ThbZIP1*and *ThDREB* genes. In addition, genes such as *LEA* and *TaMnSOD* from *Tamarix androssowii* Livt. have enhanced drought, salt and manganese tolerance in transgenic tobacco and *Populus davidiana* × *P. bolleana*, respectively (Wang et al., 2006; Wang et al., 2010). A vacuolar membrane H^+-ATPase c subunit gene (*ThVHAc1*) from *T. hispida* confers tolerance to abiotic stress including against

Cd metal in *Saccharomyces cerevisiae* Meyen ex. E.C. Hassen, where real-time RT-PCR demonstrated that over expression of *ThVHAc1* gene was induced by NaCl, $NaHCO_3$ and $CdCl_2$ (Gao et al., 2011). This shows that there is a possibility of identifying novel genes such as *ThVHAc1*, which enhance heavy metal tolerance in *T. usneoides* once the plant is exposed to high heavy metal stress conditions. However, the bands obtained in our study could also be associated with metal accumulation, since cDNA-AFLP does not characterize the actual genes (Bachem et al., 1998; Vuylsteke et al., 2006). Subsequent work to characterize the expressed loci will be essential to develop transgenic *T. usneoides* plants capable of tolerating or accumulating high concentrations of heavy metals. Such work would be essential for improving the utility of *Tamarix* in phytoremediation of acid mine drainage from tailing storage facilities.

4.5. CONCLUSION

Using cDNA-AFLP, we were able to identify and compare expression profiles of *Tamarix usneoides* genotypes across three Cd treatments groups and the control. This study showed that more transcript derived fragments are produced by *T. usneoides* with an increase in Cd concentration. Differences in gene expression patterns between the 6, 12 and 18 ppm Cd concentrations were observed with greatest number of differentially expressed bands identified in the treatment individuals exposed to 12 ppm Cd. There were two bands identified in this medium treatment group (12 ppm Cd) that were not shared with the low and high Cd treatment groups. These bands would be ideal targets for sequencing in order to characterize potentially novel genes that enhance heavy metal tolerance in *T. usneoides*. Our study shows that there is potential to develop transgenic *Tamarix* plants capable of tolerating extreme concentrations of heavy metals and salts that could be considered for use in effective phytoremediation programmes. Generally, a heterogeneous in expression patterns with some observed bands possibly influenced by the salt application at the beginning of the experiment or other environmentally induced stresses.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

5.1. Background

Impacts of biological invasions can cause environmental changes such as ecosystem degradation, affect ecosystem functioning by reducing biodiversity and can cause problems to agriculture and human health (Richardson & Pysek, 2006). Therefore, understanding pathways of biological invasions successes is necessary for prevention and management strategies against alien invasive species. Control of invasive species is becoming an integral part of natural ecosystem management. In most instances, mechanical and chemical control strategies of alien invasive plants are labour-intensive, ineffective and expensive, and are usually considered environmentally unfriendly because of their secondary effects (Ruiz & Carlton, 2003), leaving classical biological control as an alternative management option (Gaskin et al., 2011). Biocontrol is declared to be a safer, cheaper and environmentally friendlier method of suppressing plant infestations (Muller-Sharer et al., 2004). Molecular analyses have the ability to critically enhance our understanding of invasive species, thereby providing necessary information for biocontrol efforts and contributing to the protection of native biodiversity (Allendorf & Lundquist, 2003; Gaskin et al., 2011).

Population genetic theories suggest that the movement of plants species into a new environment might reduce their levels of genetic variation, which would reduce their genetic diversity, compared to the natural population due to the founder effects and high inbreeding (Allendorf & Lundquist, 2003; Jensen et al., 2013). It is also predicted that introduced populations derived from low propagule pressure usually result in a significant reduction in genetic diversity (Hufbauer & Sforza, 2008; Krzeminska et al., 2016). In contrast, those established by a large number of individuals from different localities in their native range tend to have a high genetic diversity (due to intra-specific hybridization) and usually result in increased genetic variability and adaptive traits that may influence invasion (Allendorf & Lundquist, 2003; Bock et al., 2015). Molecular techniques are used to determine the relationships between the native species and their alien invasive counterparts, which enable more accurate assessments of biological control host shift (non-target effects) (Le Roux & Wieczorek, 2008; Gaskin et al., 2011). At the species level in this study, our data from the nuclear genome-wide

microsatellite markers revealed the genetic diversity of the native *Tamarix usneoides* and the invasive *T. chinensis* and *T. ramosissima* and established the genetic differentiation among them. Although our microsatellite data confirmed the identity of each species and that hybrids between the two alien species and between them and the native species exist, these aspects were addressed by my previous investigations using nuclear ITS, chloroplast *trn*S-*trn*G DNA sequences and AFLP markers (Mayonde et al., 2015, 2016). It was useful to confirm the identities of the various taxa using another (microsatellites) DNA marker because *Tamarix* is known to be taxonomically problematic, especially when using morphological characters (Gaskin & Schaal, 2003; Mayonde et al., 2015). Moreover, in the present study we investigated the levels of inbreeding within each species, to investigate whether the alien invasive species originated from a single or multiple introductions.

Hybridization is a widespread and evolutionarily important phenomenon in plants, which can have devastating effects in encouraging biological invasion by creating unique allele combinations that can facilitate the evolution of invasiveness (Ellstrand & Schierenbeck, 2000; Abbott et al., 2013). Hybridization and introgression between the invasive and their closely related native species may reduce the genetic composition of species, which might subsequently result in extinction of the indigenous species (Rhymer & Simberloff, 1996; Todesco et al., 2016). More importantly, this study also established whether the indigenous *T. usneoides* has undergone gene erosion through hybridization with the exotic species, since it was previously shown that there is high incidence of hybridization among *Tamarix* species, with hybrids between *T. chinensis* and *T. ramosissima* being the most abundant invasive genotype in South Africa (Mayonde et al., 2016). It is essential to preserve the pure indigenous *T. usneoides* germplasm because of its ecological importance in natural riparian ecosystems and for its use in phytoremediation of acid mine drainage from tailing storage facilities.

Pollutants such as heavy metals are one of the most serious environmental problems that limit plant productivity, threaten biodiversity and human health, and

require immediate attention. Phytoremediation is a restoration technique that uses plants' natural processes such as water and chemical uptake, metabolism, and the physical and biochemical impacts of the roots to remediate metal-polluted lands by taking up pollutant metals (McIntyre, 2003). The presence of heavy metals, including cadmium (Cd), in soils is on the rise because of human activities such as mining, energy production and agriculture (Clemens, 2001). Cd soil contamination is a major concern because it is readily absorbed by plants and other life forms, and is easily transferred to the human food chain (DalCorso, 2012). In addition, Cd^{2+} ions cause oxidative stresses in plants by displacing Ca^{2+} or Zn^{2+} in protein (Goyer, 1997), which makes it difficult to be tolerated.

Plants used for phytoremediation should have the ability to tolerate high concentrations of heavy metals and accumulate them in their above ground biomass. *Tamarix usneoides* is one of the preferred phreatophytes for phytoremediation because it can withstand high salt and acid concentrations, and it has a high above ground biomass (Manousaki et al., 2008). However, the ability of *T. usneoides* to tolerate heavy metals has not been investigated. Therefore, my study assessed metal toxicity, looking at shoot growth and height increase and leaf chlorophyll content, and revealed the physiological responses of *T. usneoides* growing under extreme Cd concentrations. Since Cd is not an essential element for plant growth, it can cause phytotoxicity symptoms such as leaf chlorosis, root putrescence, growth inhibition and even the death of a plant at low concentrations (Arduini et al., 1996; Das et al., 1997). In order to minimize the detrimental effects of high concentrations of heavy metals, plants, like all other organisms, possess a complex network of homeostatic mechanisms to control the uptake, accumulation and detoxification of heavy metals (DalCorso, 2012). In plants, toxic ions appear to be removed from the cytosol by chelation and sequestration (Yang et al., 2005). Metal tolerance in plants is said to be ubiquitous and there are regulated activities at the cellular level which ensure proper distribution of metal ions, resulting in a basic level of metal tolerance (Clemens, 2001; Yang et al., 2005; Yadav, 2010). A number of molecular components including phytochelatin genes, as well as some chaperone genes associated with metal tolerance in plants, have been identified in recent years (Clemens, 2001; Yadav, 2010). My study

using cDNA-AFLP expression profiling has revealed genotypes of *Tamarix usneoides* containing differentially expressed bands associated with Cd metal tolerance.

5.2. Genetic diversity assessment of invasive and indigenous *Tamarix*: implications for biocontrol and conservation

The molecular investigation in this study using nine microsatellite markers showed that indigenous *Tamarix usneoides* is genetically more diverse than the exotic and invasive *T. chinensis*. This finding supports the population genetic theory which suggests that translocated populations usually show reduced levels of genetic diversity due to founder effects and inbreeding depression (Hufbauer & Sforza, 2008). In contrast, natural populations usually have a high level of genetic variation caused by intra-specific outcrossing (Ellstrand & Rieseberg, 2016). Our observed low genetic diversity in *T. chinensis* is similar to that noted by Zhu et al. (2016), who investigated the genetic diversity in its native range (China). Therefore, we refuted the likelihood of a reduced genetic diversity due to the founder effect, but attributed it rather to the breeding system of the species (viz., facultative autogamy; Baum, 1978). Although some autogamous plants have low genetic diversity due to selfing, most can out-cross and thereby increase the genetic variation (Zhang et al., 2014). For instance, a high level of genetic diversity was observed in an autogamous monocarp *Lobelia inflata* L. (Campanulaceae) using microsatellite markers and was associated with phenotypic traits such as flower colour and size (Hughes & Simons, 2015). Self-fertilization in plants usually results in low genetic diversity caused by genetic drift, because the genetic variation is shared within rather than between species. *Tamarix chinensis* is known to be a notorious weed that has invaded riparian systems in South Africa and the U.S. (Gaskin & Kazmer, 2009; Mayonde et al., 2016), but its invasive traits have not been investigated at all. Self-fertilization could enhance invasiveness when the plant fitness is simulated from reproductive mechanism through the transmission of advantageous, and isolation from maladaptive genes (Goodwillie et al., 2005; Zhang et al., 2014). Therefore, selfing in *T. chinensis* could be advantageous during colonization of new areas and therefore associated with its invasive status.

Although the indigenous *Tamarix usneoides* was genetically more diverse than *T. chinensis*, it was however found to have less genetic diversity than *T. ramosissima*. The moderately high genetic diversity in the invasive *T. ramosissima* suggests that it might have originated from different localities in the native range. Contrary to introductions from low propagule pressure, invasive species established from individuals originating from a variety of areas in the native range show increased genetic variability (Bock et al., 2015; Krzeminska et al., 2016). The low genetic diversity in the indigenous species, *T. usneoides*, is surprising and does not compare with other species such as *Pinus sylvestris* L. (Scalfi et al., 2009) and *Abies koreana* E.H.Wilson (Kwak et al., 2017) which have high genetic diversity in their native ranges. The moderate level of genetic diversity obtained in the indigenous *T. usneoides* could be caused by population fragmentation. It was observed during sample collection that populations of *T. usneoides* are localized in specific areas which are 50 or more km from one another (Mayonde, pers. observ.). For instance, there is an isolated population near Noenieput, an area in the Kalahari Desert, which is approximately ~175 km away from the Upington population in the Northern Cape Province. Depending on the life history traits of a plant species, habitat fragmentation can reduce the population size, increase the distance between isolated populations, and alter seed and pollen dispersal; all of which may cause loss of genetic diversity through genetic drift (England et al., 2002; Frankham et al., 2002; Yao et al., 2007). Another possible reason for a relatively low genetic diversity observed in the indigenous *T. usneoides* could be attributed to the small sample size (~2 samples per locality) included in our analysis collected from the already isolated populations.

Our measured F_{ST} values showed that there is high genetic differentiation between the indigenous and the two invasive *Tamarix* species despite evidence of hybridization among them. When non-native, genetically distinct species are introduced into a new habitat, hybridization and introgression with their indigenous congeners may erode and homogenize the genetic composition of the parent species (Abbott, 1992; Rhymer & Simberloff, 1996; Todesco et al., 2016). However, this study showed that the highest genetic differentiation was between

the indigenous *T. usneoides* and the invasive *T. chinensis* (F_{ST} = 0.197), followed by *T. usneoides* and *T. ramosissima* (F_{TS} = 0.176). There was also a fair genetic differentiation between the two invasive species (F_{ST} = 1.39). The substantial genetic differentiation among the *Tamarix* species in South Africa was supported by the clear genetic structure obtained by our STRUCTURE analyses, which resulted in three distinct clusters. The high genetic differentiation and clear genetic structure suggests no genetic homogenization of the parent species has occurred. Furthermore, the genetic differentiation findings suggest that no or limited non-target effects of the biocontrol agents against invasive genotypes should be observed on the indigenous species. However, further tests such as host-specificity of potential biocontrol against invasive *Tamarix* in South Africa needs to be done including all genotypes to check for non-target effects on the indigenous *T. usneoides*.

5.3. Hybridization in invasive *Tamarix* species: implications for biocontrol

Hybridization and introgression can potentially facilitate the evolution of invasiveness in plants, primarily by increasing fitness relative to the parents through heterozygote advantage (over-dominance of beneficial traits; Edmands, 2002; Lee et al., 2016). Hybridization may also aid invasiveness by masking deleterious alleles (heterosis) and reducing inbreeding depression, thereby increasing genetic adaptation and facilitating adaptive evolution (Fisher, 1930; Goulet et al., 2017). Several species of *Tamarix* (have been declared noxious weeds (Gaskin & Schaal, 2003, Marlin et al., 2017), with their hybrid combinations being the dominant taxa in the U.S. (Gaskin & Kazmer, 2009) and South African (Mayonde et al., 2016) infestations. In this study, we compared the genetic diversity of two different hybridization events, the South African and the U.S. *Tamarix* invasions. We compared the evolutionary patterns of the two hybridization events (viz., creation of new genotypes, increased genetic variation, and genetic structure and differentiation) and used these patterns to infer the possibility of *Diorhabda* beetles, currently controlling invasive *Tamarix* in the U.S., establishing successfully in South Africa as a biocontrol agent.

Our study showed that the South African *Tamarix* hybrid population is genetically more diverse than the U.S. hybrids. The low genetic diversity in the

U.S. is justified by the fact that most *Tamarix* genotypes around the Green River in Utah, U.S. (our sampled area) are *Tamarix ramosissima* and *T. chinensis* and their hybrids (Gaskin et al., 2012). However in South Africa, *Tamarix* hybrids have been shown to originate from three species (Mayonde et al., 2016). In addition, our F_{IS} results indicated the U.S. *Tamarix* hybrid population might be under inbreeding pressure. High levels of inbreeding can increase the number of homozygote alleles thereby reducing the genetic diversity (Londo & Schaal, 2007; Goulet et al., 2017). In contrast, South African *Tamarix* hybrids showed evidence of out-crossing, suggesting high levels of heterozygosity, which can lead to higher genetic diversity (Ellstrand and Rieseberg, 2016; Hirsch et al., 2017). Increased genetic diversity through hybridization events has been demonstrated to promote establishment and dispersal of colonising populations and produce populations with high growth rates. The effects of this increased genetic diversity caused by hybridization in *Tamarix* have not been investigated to conclusively associate invasive characteristics such as high growth rate, establishment success and dispersal with hybridization events. In contrast, the invasiveness attribute associated with *Tamarix* hybrids, both in South Africa and in the U.S. invasions, is their abundance (Gaskin and Kazmer, 2009; Mayonde et al., 2016).

This study showed a clear genetic differentiation between *Tamarix* hybrids in South Africa and those in the U.S. as evidenced by the F_{ST} value, which suggests distinct populations with different mating patterns. The clear genetic differentiation between *Tamarix* hybrids in South Africa and those in the U.S. is further supported by a distinct genetic structure. In the Green River, Utah, U.S., hybridization was quantified over different time scales using data generated from amplified fragments length polymorphisms (AFLPs), and it was found that *Tamarix* hybrids established as early as the 1900s (Gaskin et al. 2012). The finding by Gaskin et al. (2012) suggesting that the invasive *Tamarix* taxa in Utah, U.S. might initially have been introduced as hybrids is further supported by this study through the low genetic diversity and the positive F_{IS} values, which suggest that *Tamarix* hybrids in the Green River region have experienced inbreeding depression. In contrast, *Tamarix* hybridization events in South Africa appear to have occurred post-introduction (Mayonde et al. 2016), and the high genetic

diversity may be caused by out-breeding depression (negative F_{IS} value) - as observed here. Consequently the high population genetic differentiation between the South African hybrid population and the U.S. suggests that biocontrol agents from the U.S. might not successfully establish in South Africa to biologically control the invasive *Tamarix* hybrid populations. Although *Tamarix* is not indigenous to the U.S., South Africa is investigating biocontrol agents already released in the U.S (Marlin et al., 2017). However, with the high genetic differentiation between *Tamarix* hybrids in South Africa and those in the U.S., the biocontrol efforts in South Africa would need to focus its attention for exploration of biocontrol agents to Eurasia (native range of the invasive species) because in biocontrol practice it is suggested that matching the genetics of invasive plants to those in the place of origin increases the chances of agents' establishment (Muller-Scharer et al., 2004).

It is intriguing that the Eurasian *Tamarix chinensis* and *T. ramosissima* are known to hybridize in their introduced ranges, yet no hybridization events are known in their place of origin (Gaskin & Schaal, 2002). These contrasting findings lead to the question: do they really not hybridize in their native ranges and what could be the reasons for their not hybridizing? The fact that *T. chinensis* and *T. ramosissima* overlap for about ~ 4200 km across from China to Korea in their native ranges should geographically provide the opportunity for them to interbreed. The autogamous nature of *T. chinensis* could be a possible reason for the lack of hybridization, but then again in the presence of presumably shared natural pollinators 'insects' in their native ranges, transmission of pollen from one species to another remains a possibility. Furthermore, the flowering periods of the two species overlap between March and August (Baum, 1978), giving them enough time to interbreed. There seems not to be any good reason why *T. chinensis* would not hybridize with its close relative *T. ramosissima* where they co-occur in their native ranges. Finding evidence of *Tamarix* hybrids in Eurasia and investigating their natural enemies could add a new dimension for biocontrol efforts against the most dominant genotype.

5.4. Heavy metal tolerance in *Tamarix usneoides*: implications for phytoremediation

Once exposed to heavy metal stress conditions, the plant's cells are usually able to perceive metal stress signals and convert them into appropriate responses, which in turn give plants signals to tolerate stressful conditions (Yang et al., 2005). In order to tolerate heavy metal stress, a combination of plant tolerance mechanisms such as physiological and biochemical processes, including change in gene expression patterns, protein modification, and primary and secondary metabolites compositions are required (Yang et al., 2005; Manousaki et al., 2008; DalCorso, 2012). Heavy metal pollution, especially cadmium (Cd), is of major environmental concern because of the negative effects on biodiversity and human (Arduini et al., 1996). Cd is a non essential elements to plants, thus is known to cause phytotoxicity even at low concentrations (DalCorso, 2012). Roots are known to be the primary point through which heavy metals access the plant; and an electron microscope analysis has shown that Cd and copper (Cu) are localized more in the root cell walls than in cytoplasm (Arduini et al., 1996). This is expected given that the cell wall has a negative charge which gives it the capacity for heavy metal binding and retention. Cd toxicity affects root tips which affect root growth (DalCorso et al. 2008; Liu et al. 2009). After entering the plant through the roots, heavy metals are translocated to the above ground biomass and affect the health of the plant due to various interactions at the molecular level, e.g. by inactivating enzymes, displacing essential elements and disrupting membrane integrity (Rascio & Navari-Izzo 2011).

This study showed that Cd concentration between 12–18 ppm inhibit the physiology of *T. usneoides* as it significantly reduced the height, shoot growth and leaf chlorophyll content. Generally, the physiological parameters assessed in this study were observed to be negatively affected with an increase in Cd concentrations from 6 ppm (low) to 12 ppm (medium) to our high of 18 ppm. Over time, chlorophyll appeared to be the most affected parameter as a significant difference was observed between the high Cd concentration (18 ppm) and the control (0 ppm) by the third week. However, no significant difference in chlorophyll content was observed between the low, medium Cd concentrations

and the control until week 5 of our experiment. Shoot growth appeared to be the second most affected character with a significant reduction shown after week 4. Similarly to chlorophyll content, the difference was only observed between the high Cd concentration and the control. The plant height was seen to be the parameter least affected by Cd toxicity in *T. usneoides* over time with the significant difference in height increase observed after six weeks of Cd exposure. The negative effect of Cd seen on leaf chlorophyll content could be deleteriously affecting photosynthesis of *T. usneoides*. It is observed that negative impacts of Cd on photosynthesis have been mainly attributed to the inhibition of chlorophyll biosynthesis (Padmaja et al., 1990; Iqbal et al., 2010). Furthermore, the reduction in *T. usneoides* leaf chlorophyll content is a major concern for the general health of the plant given that *Tamarix* have scale-like leaves which have a small surface area over which they need to achieve a high photosynthetic rate. In conclusion, the observed reduction in *T. usneoides* growth (shoot growth and height) under Cd stress conditions may be a direct consequence of the inhibition in photosynthesis. This finding supports our field observations that the canopy cover structure of *T. usneoides* growing on contaminated soils (mine dumps) is reduced in size with increased spaces in between branches which seem to reduce light interception and as a result impact on photosynthesis and growth. This observation is contrary to the canopy morphology of *T. usneoides* trees occurring in natural habitats. Cd was found to reduce the leaf size in two mustards cultivars (*Brassica juncea* L. Czern and Coss) which subsequently affected light interception and together with the effect of Cd on chlorophyll content, the rate of photosynthesis was found to be reduced in the process (Iqbal et al., 2010). Although the leaves of *T. usneoides* are vaginate (sheathing around the stem), the reduction in shoot growth could result in less light interception and together with the decrease in chlorophyll fluorescence (Fv/Fm) could affect photosynthesis. Despite Cd affecting the physiology of the plant, this study proves that *T. usneoides* to be Cd tolerant, it did not show signs of metal toxicity when exposed to Cd concentrations of up to 12 ppm over eight weeks. This is an encouraging finding for phytoremediation as Cd concentrations in contaminated soils range from 3 to 8 ppm (DalCorso, 2012).

5.5. Gene expression patterns in *Tamarix usneoides*: importance in functional genomics

To reduce Cd-induced stress, plants avoid building up high concentrations of Cd in sensitive sites by binding ions in cell wall, thereby removing metal ions from the symplasm, forming metal-peptide ligand complexes in the vacuole or by forming metal-resistant enzymes as a response mechanism (Hall, 2002; Zhang et al., 2006). Since, plants are said to undergo some change in their gene expression and protein modification during the metal tolerance process (Yang et al., 2005; Yadav, 2010), cDNA-AFLP profiles were used to assess the gene expression patterns of *T. usneoides* for Cd tolerance. Our gene expression analysis revealed some differentially expressed bands in plants exposed to the low, medium and high Cd concentrations which were different from those observed in the controls. Despite the heterogeneous expression patterns observed in this study, about ten transcripts derived fragments were associated with heavy metal tolerance of which 40% of those were shared among the 6 ppm, 12 ppm and 18 ppm Cd concentration levels. Interestingly, *T. usneoides* yielded a much higher gene expression pattern at a Cd concentration of 12 ppm compared to when exposed to 18 ppm. This observation corresponds with the physiological observations, as at 12 ppm concentration *T. usneoides* seems to show heavy metal tolerance vigour as opposed to when exposed to 18 ppm, where heavy metal toxicity symptoms were observed and resulted in the death of up to 21 % of individuals in the high concentration treatment group. Moreover, no variation among individuals was observed in the *T. usneoides* expression patterns for heavy metals suggesting that profiles scanned were due to the plant's response to heavy metal toxicity. This study suggests that *T. usneoides* is Cd tolerant and at once subjected to a concentrations of 12 ppm the plant has the ability to change their gene express patterns. Therefore, our study presents a possibility of identifying heavy metal tolerant genes for use in genetic engineering of transgenic plants which could then be used for an effective phytoremediation practice.

5.6. Suggestions for future research arising from this study

- The unexpected moderate genetic diversity in the indigenous *T. usneoides*, which could be due to population fragmentation, resulting in reduced

intra-specific out-crosses and increased inbreeding–should be investigated further. Using microsatellite markers, I recommend investigating the genetic diversity and structure within *T. usneoides* by sampling at least 30 individuals per locality in as many locations across South Africa. More rigorous and systematic sampling should done in the Northern Cape Province, where the species is widely distributed naturally and appears to be uncontaminated by hybridization. This investigation will have further implications for conservation of the indigenous *T. usneoides*.

- The low genetic diversity observed in the invasive *T. chinensis*, which is similar to that observed in its native range, seems to be associated with its facultative autogamous breeding strategy. Therefore, I recommend investigating autogamy in *T. chinensis* and the invasive traits that might be retained within the same species through self-fertilization.
- The lack of hybridization between the notoriously invasive *T. chinensis* and *T. ramosissima* in their Eurasian native ranges raises interesting questions such as reproductive compatibility, identity and abundance of pollinators, and geographic isolation in order to understand the ease with which they hybridize once introduced into new environments. This investigation could elucidate further on factors influencing *Tamarix* invasiveness and aid future biocontrol efforts.
- Since the metal tolerance trials undertaken here (for the first time) reveal that the indigenous *T. usneoides* appears to be Cd tolerant, I recommend investigating the phyto-extraction and metal accumulation abilities of *T. usneoides*. This could be done on single metals such Cd, lead (Pb), Zinc (Zn), etc. separately or on combinations of the heavy metals. Metal accumulation could be assessed between the roots, stem, and shoots/leaves to see which parts of the plant store the metals the most.
- Furthermore, the TDFs associated with heavy metal tolerance obtained in this study should be cloned and sequenced to verify the identity of the expressed genes using RT-PCR techniques. This information will be used if transgenic *Tamarix* trees are desired to be used in phytoremediation efforts.

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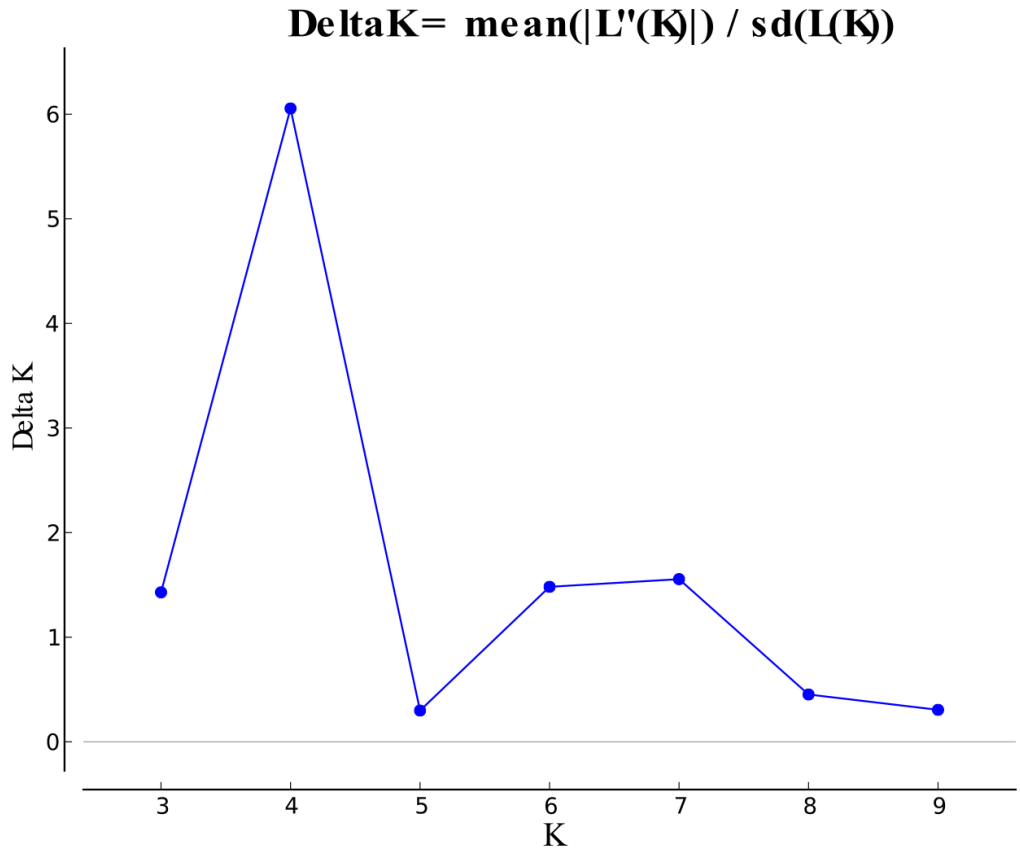
APPENDICES

Appendix 2A. Information for the nine microsatellite markers used to characterize *Tamarix* species and investigate their genetic diversity and differentiation in South Africa. (Ta) the annealing temperature. (di) dinucleotide repeats. (tri) trinucleotide repeats. The dye label was tagged only to the forward primers.

Locus	Dye	Ta (°C)	Size range (bp)	repeats	motifs	Primer sequence (3′–5′)	
						Forward	Reverse
T1C1	VIC	54	219–280	di	(AC)11	GAGGCAAGCCTCTTGAAATG	TGTGCTGCCGTCTATTTCTC
T1G6	NED	56	294–380	di	(GT)3T(GT)8	CAACCCAACGTGTCACAGTC+	TCCAAACATAAATCGGGTCAA
T1C7	FAM	58	284–345	tri	(CAA)3(CAT)2(CAA)5	TAAGGGTGGGAATGTCTTGG+	TTGTTTGGGATAATTTTTGGA
T1G9	VIC	58	349–434	tri	(ATC)9	CCATAAGTGCCCCATCAAAG+	AAAAGCTTTCCCCAAATACCA
T1G11	FAM	58	129–193	di	(AG)10AA(AG)4AT(AG)9	AAGCTCCATGCTTGCTTCAT+	GACCATTGATATGCCCCAAT
T1B8	NED	60	313–424	di	(AC)6(GC)2(AC)12	CGTTAGCAGGTTGGACATGA+	TTTGAGTGTCAGTCGATGGTG
T1B9	FAM	60	253–311	tri	(CTT)7	TCCGCCTCTCTTTCTGTCAT+	CGCCAAGAACCACAATTTTT
T1E1	VIC	60	197–265	di	(GT)17	ATTACGACCTGCAAGCATCC+	AATCGAATGCCTCGTGACTT
T1C10	NED	63	297–363	di	(AC)10	AACGAGGATCATGAAAAGGA+	GACACATGTCCCTACCATTGAA



Appendix 2B. Graph of the estimated deltaK (estimate of the maximum number of clusters) obtained from 150 *Tamarix* individuals from South Africa and the U.S. based on nine microsatellite loci using STRUCTURE HARVESTER as determined by the method of Evanno et al. (2005). The population genetic structure was estimated using STRUCTURE ver 2.3.4 (Pritchard et al., 2000) using 1000000 MCMC with 100000 burn-in with K ranging from 1–10

Appendix 3A. Twenty selected *Tamarix usneoides* trees collected from 20 different localities across three provinces in South Africa for heavy metal experiment. NC = Northern Cape, WC = Western Cape and EC = Eastern Cape Provinces. The metal treated individuals were used to assess the physiological responses in *T. usneoides* for metal toxicity and the treated samples were used to generate profiles of gene expression pattern using cDNA-AFLP data.

Sample ID	**Origin**		**GPS coordinates**	
	Province	**Locality**	**Latitude**	**Longitude**
GM231	NC	Kakamas	S28°51.398′	E20°38.534′
GM031	NC	Upington	S28°27.782′	E21°15.193′
GM050	NC	Kenhardt	S29°21.292′	E21°.86.200′
GM052	NC	Augrabies	S28°37.263′	E20°20.883′
GM080	NC	Marchand	S28°41.627′	E20°30.466′
GM090	NC	Witbank	S28°53.757′	E18°30.724′
GM092	NC	Goodhouse	S28°51.263′	E18°38.125′
GM099	NC	Steinkopf	S29°03.709′	E17°50.786′
GM109	NC	Springbok	S29°38.190′	E17°52.855′
GM111	NC	Kamieskroon	S30°01.095′	E17°52.815′
GM116	NC	Loerifontein	S30°50.701′	E19°07.810′
GM120	NC	Kanonieland	S28°37.382′	E21°06.568′
GM122	NC	Noenieput	S27°18.981′	E20°07.296′
GM095	NC	Henkries	S28°54.546′	E18°07.444′
GM113	NC	Garies	S30°35.671′	E18°00.606′
GM114	NC	Kliprand	S30°42.559′	E18°25.655′
GM117	NC	Brandkop	S31°17.119′	E19°22.576′
GM141	WC	Swart River	S33°09.896′	E21°58.884′
GM143	WC	Dwyka River	S33°05.217′	E21°04.783′
GM151	EC	Waterford	S33°04.678′	E25°00.962′