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Evidence of *Tamarix* hybrids in South Africa, as inferred by nuclear ITS and plastid *trn*S–*trn*G DNA sequences

S.G. Mayonde [a,*], G.V. Cron [a], J.F. Gaskin [b], M.J. Byrne [a]

[a] School of Animal, Plant and Environmental Sciences, University of Witwatersrand, Private Bag X3, Johannesburg 2050, South Africa
[b] USDA, Agricultural Research Service, 1500 N. Central Avenue, Sidney, MT 59270, USA

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ABSTRACT

Tamarix usneoides (Tamaricaceae) is a species native to southern Africa where it is currently being used in the mines for phytoremediation. *Tamarix aphylla*, *Tamarix ramosissima*, *Tamarix chinensis*, and *Tamarix parviflora* have been reported as exotic species in South Africa, with *T. ramosissima* declared invasive. The alien invasive *T. ramosissima* is hypothesized to be hybridizing with the indigenous *T. usneoides*. Accurate identification of *Tamarix* is of great importance in southern Africa because of the invasive potential of *T. ramosissima* and also the potential usefulness of *T. usneoides*. In this study, nuclear DNA sequence markers (ITS1 and ITS2 regions), together with the plastid marker *trn*S–*trn*G, are used to identify the genetic distinctiveness of *Tamarix* species and their putative hybrids. Phylogenies based on the ITS and *trn*S–*trn*G regions revealed that the indigenous *T. usneoides* is genetically distinct from the exotic species, which, however, could not clearly be separated from their closely related hybrids. The lack of congruence ($p > 0.0001$) between the ITS and *trn*S–*trn*G phylogenies suggests that there is high incidence of hybridization in *Tamarix* populations in South Africa. Importantly, molecular diagnosis of *Tamarix* was able to identify hybrids using polymorphisms and phylogenetic signals. Close to 45% of *Tamarix* genotypes were hybrids with more than 50% of them occurring on the mines. Spread of *Tamarix* hybrids in South Africa through phytoremediation could enhance invasiveness. Therefore, the outcome of this study will ensure that only pure indigenous *T. usneoides* is propagated for planting on the mines in South Africa and that a proper control measure for the alien invasive *Tamarix* is used. Interestingly, the molecular diagnosis of *Tamarix* species supported the preliminary morphological identification of the species using eight key characters. However, the molecular markers used were not informative enough to separate hybrids from their closely related parent species. Hybrids were more reliably identified using polymorphisms than morphological features.

1. Introduction

The Old World *Tamarix* L. is one of five genera in the family Tamaricaceae and is represented by 55 species (Heywood et al., 2007). *Tamarix* is native to the Mediterranean countries, former Soviet Union, China, India, North Africa, and southern Africa (Baum, 1978; Heywood et al., 2007). Various species of *Tamarix* have become naturalised and invaded the United States of America (USA), Australia, and other parts of the world (Brotherson and Field, 1987; Gaskin and Schaal, 2003), including South Africa (Henderson, 2001).

Tamarix usneoides E.Mey. ex Bunge is indigenous to southern Africa (Obermeyer, 1976; Baum, 1978; Henderson, 2001) but *Tamarix aphylla* (L.) Karst., *Tamarix ramosissima* Ledeb., *Tamarix chinensis* Lour., and *Tamarix parviflora* DC. are all exotic to South Africa (Bredenkamp, 2003). In South Africa, *T. ramosissima* has been declared as the main invader (Henderson, 2001) and is suspected to be hybridizing with the native *T. usneoides* (Hoffman et al., 1995; Weiersbye et al., 2006).

Tamarix plants can be used for erosion control and as ornamentals (Baum, 1978; DiTomaso, 1998). In South Africa, *T. usneoides* is useful in gold mines for phytoremediation (Weiersbye et al., 2006). *T. usneoides* is used to intercept multiple pollutants such as heavy metals in ground water (Salt et al., 1998; Dennis, 2008). *Tamarix* is known to lower the water level of acid mine drainage (AMD) from mine tailing storage facilities (TSFs), while hyper-accumulating sulphates, chlorides, and some heavy metals from polluted water and soils (Weiersbye et al., 2006; Weiersbye, 2007). Therefore, *Tamarix* plants are being cultivated for phytoremediation in South African mines. However, there is concern that hybridization between the useful indigenous *T. usneoides* and the alien invasive *T. ramosissima* has occurred and that pure *T. usneoides* stock is not being cloned for cultivation on the mines. It is therefore important to establish whether any of the exotic *Tamarix* species are hybridizing with the native *T. usneoides* in South Africa to avoid promulgation of new, potentially invasive genotypes in the form of hybrids through cultivation and planting on the mines.

* Corresponding author at: P. Bag X3, APES, Wits University, Johannesburg, 2050, South Africa.
E-mail address: mayondesam84@gmail.com (S.G. Mayonde).

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Tamarix remains one of the more taxonomically difficult genera among angiosperms (Baum, 1978) and when in the vegetative state, many taxa are almost indistinguishable (Crins, 1989). The high incidence of hybridization among *Tamarix* species also plays a role in the taxonomic confusion (Wilken, 1993). In this study, sequence data from the plastid intergenic spacer (*trn*S–*trn*G) and nuclear internal transcribed spacer (ITS) regions are evaluated as tools to identify *Tamarix* species and their hybrids in South Africa. Phylogenetic analyses of the two data sets are used to compare the evolutionary dynamics of two independent markers, one maternally and one bi-parentally inherited, to investigate hybrid status. The efficacy of their use is compared to that of morphological characters for identification purposes.

Nuclear DNA (nDNA) and plastid DNA (cpDNA) can both be used to address various ecological questions. While the nuclear DNA contains both unique single copy and repetitive regions (multiple copies), the chloroplast genome consists of coding segments such as ribosomal RNA genes or noncoding tandemly repeated units (Le Roux and Wieczorek, 2008). The internal transcribed spacer (ITS) regions between the nuclear ribosomal DNA (rDNA) genes are commonly used for detecting variability between species (Sun et al., 1994). In addition, it is also a widely used molecular marker for reconstructing angiosperm phylogenies at various taxonomic levels as they often provide the right level of variation at species level for well-resolved phylogenetic reconstruction (Baldwin et al., 1995). The *trn*S–*trn*G primers are used to infer phylogenetic comparisons. Moreover, chloroplast introns and intergenic spacer regions exhibit the highest levels of intraspecific polymorphism because they are less constrained by selection to maintain gene function (Hamilton, 1999).

2. Materials and methods

2.1. Sampling and morphological identification

Tamarix shoot tip samples were collected from cultivated plots at two mines: AngloGold Ashanti (Vaal River) gold mine in North West Province and Impala Platinum (East Rand) in Gauteng, as well as from wild and cultivated populations in the Northern Cape, Eastern Cape and Western Cape Provinces, South Africa. Twenty-nine *Tamarix* trees were sampled for morphological and molecular diagnosis. Samples were collected to represent the different species present in South Africa and from different habitats (viz. wild vs. garden/or mine planted, with wild plants being populations growing in a natural undisturbed environment, whereas cultivated plants are propagated plants planted either in gardens or on the mines). Voucher specimens of the *Tamarix* species and their putative hybrids were examined under a Zeiss stereo dissecting microscope and identified using the four floral and four vegetative characters in Table 1. Images of the characters distinguishing *T. usneoides* from *T. ramosissima* are shown in Fig. 1. To preliminarily identify the various *Tamarix* species, the following morphological characters were useful: leaf shape and attachment (vaginate, i.e., overlapping in *T. usneoides* versus not overlapping in *T. ramosissima*; Obermeyer, 1976; Henderson, 2001), petal shape and colour (Henderson, 2001) and the presence and/or absence of salt glands (Bredenkamp and Phepho, 2008), as summarised in Table 1 and visually displayed in Fig. 1. Note that the disc morphology of the indigenous *T. usneoides*, a dioecious species, was identified separately for male and female flowers. Dioeicy status was considered as one of the morphological characters for identification of *Tamarix* and was used in the field during plant collection as a preliminary discrimination tool to separate samples according to the two different species of study.

2.2. DNA isolation, PCR amplification, and sequencing

Genomic DNA was extracted from silica-dried shoot tip samples using a Qiagen DNeasy® Plant Mini Kit (Qiagen®). Polymerase chain reaction (PCR) amplification of the ITS regions and 5.8*S* gene region of the 18*S*–26*S* nuclear ribosomal DNA were achieved using primer pairs AB101 (5′-ACGAATTCATGGTCCGGTGAAGTGTTCG-3′) and AB102 (5′-TAAATT CCCCGGTTCGCTCGCCGTTAC-3′) of Sun et al. (1994) and the following cycling parameters: premelting at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 50 s; annealing at 54 °C for 45 s; extension by a TrueStart Taq DNA polymerase (Fermentas®) at 72 °C for 1.30 min, followed by a final extension at 72 °C for 7 min. The plastid region *trn*S–*trn*G was amplified using primer pair *trn*S (GCU) (5′-GCCGCTTTAGTCCACTCAGC-3′) and *trn*G (UCC) (5′-GAACGAATCACACTTTTACCAC-3′) from Hamilton (1999).

The PCR product was purified using a Zymo Clean and Concentrate Kit (Zymo Research Corporation®). The purified PCR product was sequenced following the standard DNA sequencing protocol for the BigDye® Terminator v3.0 cycle sequencing kit (Life Technologies) at the University of Stellenbosch, in the Central Analytical Facility (CAF) DNA Sequencing Unit. Sequences were cleaned using Princeton Separations Centri-sep clean-up plates and samples were run on a 3730xl Genetic Analyser following standard protocols (ABI Applied Biosystems®).

2.3. Phylogenetic analysis

The forward and reverse sequences were aligned and edited using Sequencher™ version 4.1 (Gene Codes Corporation®). The consensus sequences were aligned and compared at the species level and then at the population level in order to track hybridization events. The alignment was refined manually, and mutations were confirmed by checking them against the electropherograms. Gaps caused by insertion and/or deletion (indel) events were treated as missing data, and multiple states (polymorphisms) in the nuclear region were scored as polymorphisms which are effectively also treated as missing data as they do not contribute toward phylogenetic tree reconstruction. The polymorphisms in the nrDNA were however analyzed separately as they are a good indicator of hybridization (Bailey et al., 2003). Indels in the *trn*S–*trn*G regions (Table 2) were coded as a separate matrix at the end of the data set, as per Simmons and Ochoterena (2000), and analyses were run including and excluding coded indels.

Parsimony analysis of the nuclear (ITS) and chloroplast (*trn*S–*trn*G) DNA data sets was performed using PAUP* version 4.0b10 (Swofford, 2002). The phylogenetic trees were rooted using GenBank sequences of *Myricaria alopecuroides* Schrenk, a sister genus to *Tamarix* (Zhang et al., 2010). Heuristic searches comprising 10 random repetitions holding 20 trees at each step were performed with the maximum number of trees

Table 1
Important diagnostic morphological characters for the identification of southern African *Tamarix* species.

Character	*Tamarix usneoides*	*Tamarix ramosissima*	Reference
1. Salt gland	Present (abundant)	Absent	Bredenkamp and Phepho (2008)
2. Petal shape	Ovate elliptic	Obovate elliptic	Henderson (2001) and Baum (1978)
3. Insertion of filaments	Peridiscal	Hypodiscal	Baum (1978)
4. Petal color	White	Pink-purple	Henderson (2001)
5. Leaf shape and attachment	Vaginate	Sessile	Baum (1978)
6. Leaf shape	Vaginate	Sessile	Obermeyer (1976), Henderson (2001)
7. Bract shape and attachment	Vaginate	Sessile	Baum (1978)
8. Disc shape/gender	Hololophic to paralophic (Male)	Hololophic	Baum (1978)
	Synlophic to para-synlophic (Female)		Baum (1978)

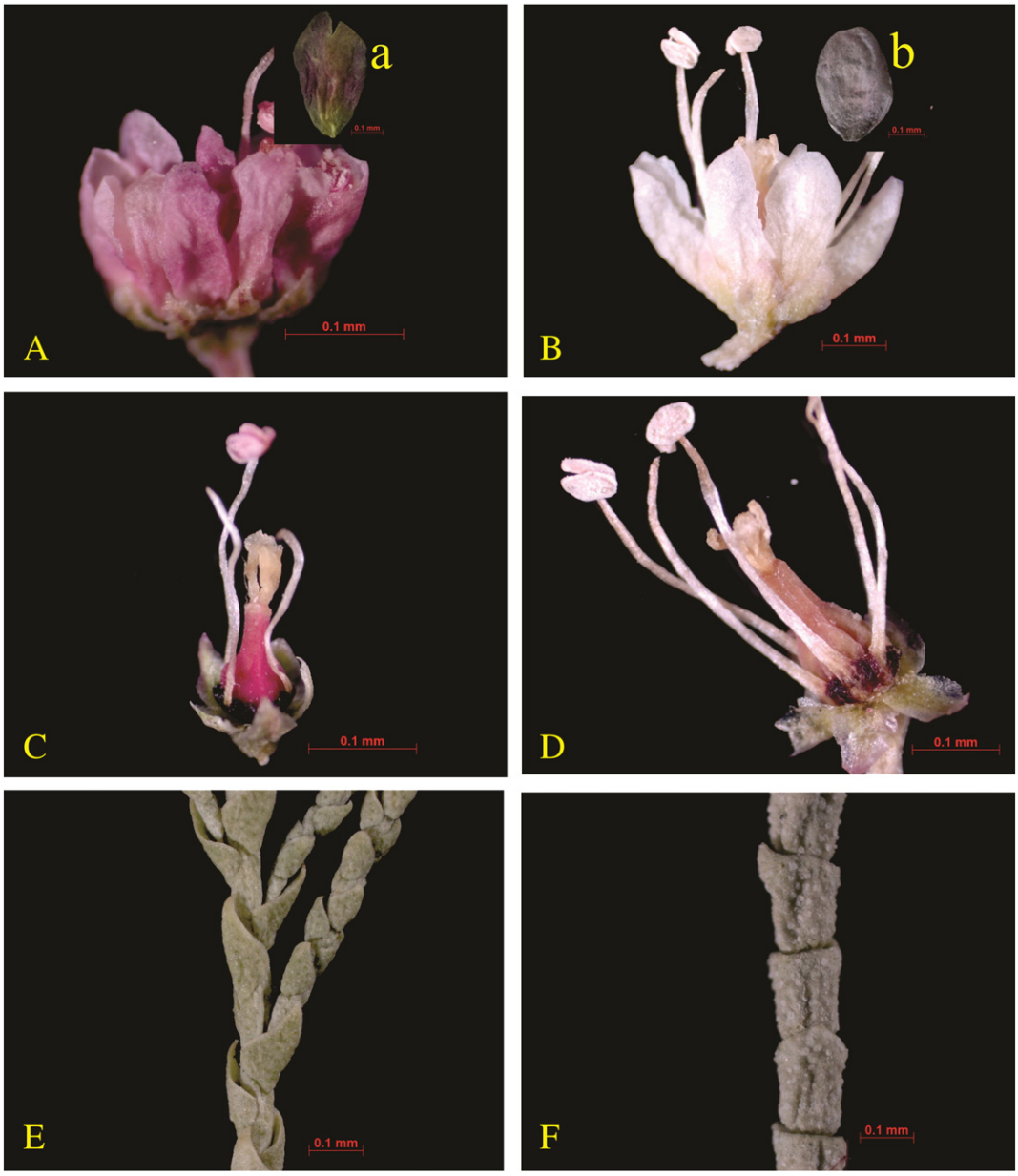

Fig. 1. Morphological features showing differences between *Tamarix usneoides* and *Tamarix ramosissima*: (A) *T. ramosissima* flower with pink petals and (a) showing an obovate petal shape. (B) *T. usneoides* flower with white petals and (b) showing an ovate petal shape. (C) Hypodiscal insertion of filaments. (D) Peridiscal insertion of filaments. (E) Sessile leaf shape and bracts with no presence of salt glands. (F) Vaginate leaf shape and bracts with abundant salt glands.

set at 10,000. Branch swapping on best trees only was used with tree bisection reconnection (TBR), saving multiple trees. Bootstrap analyses (excluding coded indels) were conducted to assess clade support (Felsenstein, 1985), using the same settings as above with 100 replicates. The in-group comprised 10 samples of *T. usneoides*, 9 *T. ramosissima*, and 10 *Tamarix* hybrids. The locality information and GenBank accession numbers for the various specimens are provided in Appendix A.

Table 2
Coded indels that are parsimony informative in the plastid (*trnS–trnG*) regions.

Base positions	Nucleotides	Presence in taxa
154–158	ATTAT	Deletion in all *T. ramosissima* and hybrids (in clade A)
163–168	TAAAAA	Insertion in *T. usneoides* hybrids (GM031 and GM054) and *T. usneoides* (GM021 and GM035)
189–190	TA	Deletion in *T. ramosissima* (GM125, GM126 and GM060)
191–196	TATATA	Deletion from all *T. ramosissima* and hybrids
548–554	TTTTTCA	Insertion in all *T. usneoides*

The partition homogeneity test of Farris et al. (1995) was performed in PAUP* v4.0b10 to test for congruence between the plastid and the nuclear sequence data sets. Phylogenetic trees resulting from the analyses of the plastid and nuclear DNA data sets were compared to trace the evolutionary dynamics of the two independent genome regions in *Tamarix* species. Based on the result, the two data sets (plastid and nuclear) were analyzed and discussed separately.

In addition to the phylogenetic analyses, variable (polymorphic) mutations were analyzed in the ITS regions to separate pure-breed species from their putative hybrids, as they were considered as missing data by the program (PAUP* ver4.0b10) during phylogenetic reconstruction (Fig. 2). Double base readings (polymorphisms) reflecting alleles from both parents were considered informative and used to assist in recognition of hybrids, while the analyses of variable characters was used to distinguish the parent species (Table 3). Parental polymorphisms (Table 3) occur due to heterozygosity at a locus and appear to be a good indicator of hybridization (Nickrent and Soltis, 1995). Polymorphisms having only one allele from either of the parents were not

used to identify hybrids or separate them from pure-breed specimens. Specimens were scored for the number of mutations that reflected either of the two putative parents. Polymorphisms (double base readings) having nucleotides present in both parents were counted in every individual in order to trace evidence of hybridization (Table 3). Any individual with more than 10% parental polymorphisms (artificial cut-off) was considered to be a hybrid (Table 3).

3. Results

3.1. Morphological characterization of Tamarix species and their putative hybrids

Among the 29 specimens diagnosed morphologically, 10 (34.5%) were identified as pure-breed *T. usneoides*, nine (31%) as pure *T. ramosissima*, eight (27.5%) as *T. usneoides* hybrids, and two (7%) as *T. ramosissima* hybrids (Appendix B). Hybrid status was judged based on the presence of morphological features intermediate between *T. usneoides* and *T. ramosissima* (Appendix B).

3.2. Molecular diagnosis of Tamarix species and their hybrids

The partition homogeneity test for the ITS and *trn*S-*trn*G data sets resulted in a $p > 0.0001$, rejecting the null hypothesis of congruence. This suggests that the phylogenetic signals in the ITS and *trn*S–*trn*G data sets were not sufficiently comparable to combine the data sets for analysis. Therefore, the two sequence data sets were analyzed separately.

3.2.1. Phylogenetic analysis of the nuclear ITS sequence data

The aligned matrix of the ITS sequence data set comprising 29 South African *Tamarix* specimens had 806 aligned bases with 138 variable

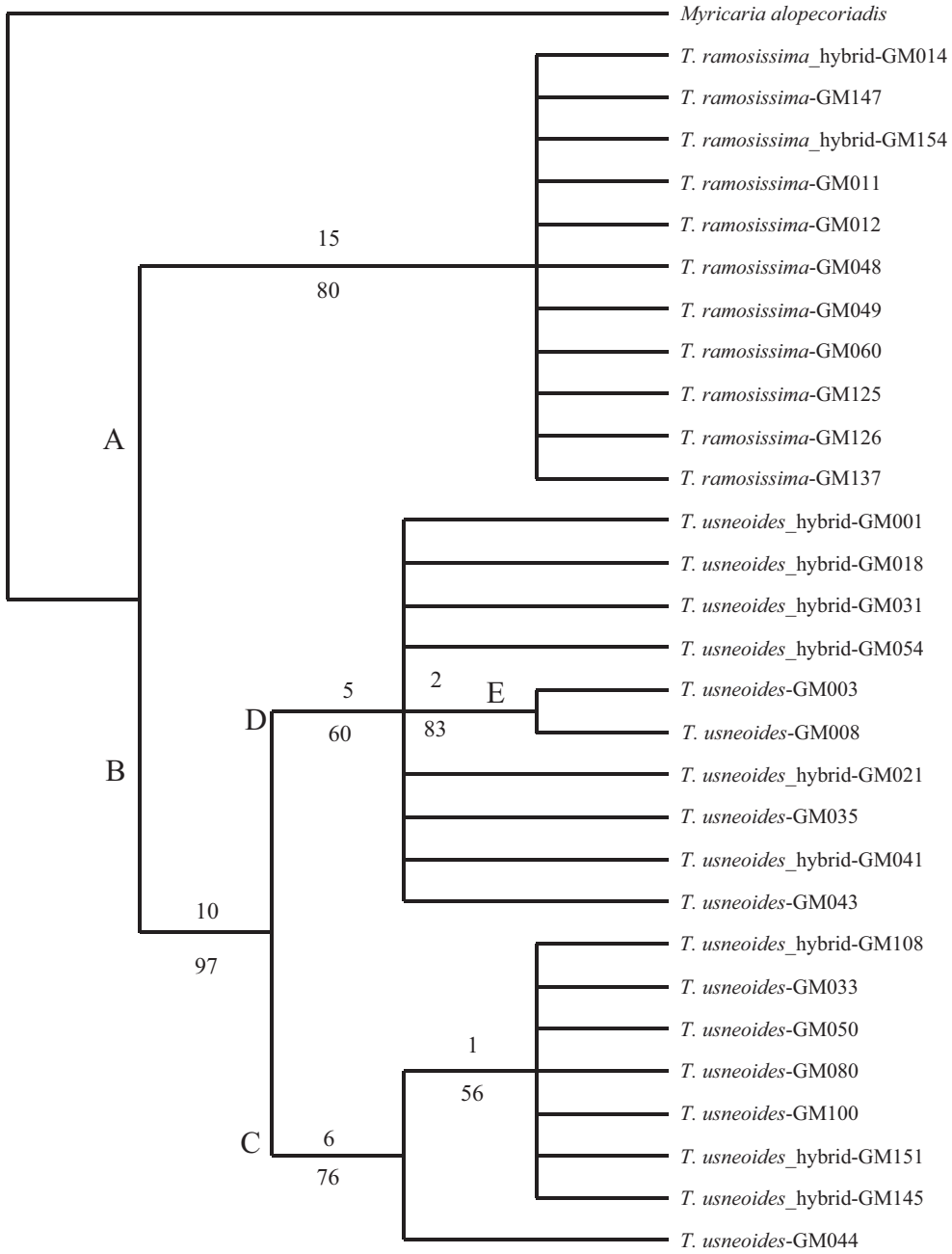

Fig. 2. Strict consensus of 10 000 EMP trees of a restricted ITS data set comprising 29 *Tamarix* specimens and the outgroup *Myricaria alopecuroides*. Bootstrap values are below the branches. Numbers above lines are minimum branch lengths. CI = 0.874; RI = 0.965. Species names at tips of tree branches were derived from morphological analysis of sequenced specimens.

Table 3
Summary of nucleotide variations and polymorphisms in the ITS region of Tamarix specimens from southern Africa and a comparison of identification based on morphological versus molecular features.

ID number	T. u. characters	T. r. characters	Parental polymorphisms	Independent polymorphisms	Species names based on molecular identification	Locations
GM031	42	7	3	0	*T. usneoides*	Upington
GM033	49	4	0	0	*T. usneoides*	Upington
GM035	38	13	2	0	*T. usneoides*	Upington
GM043	37	15	1	1	*T. usneoides*	Upington
GM050	50	3	1	1	*T. usneoides*	Kenhardt
GM080	50	3	0	0	*T. usneoides*	Marchand
GM100	50	3	0	0	*T. usneoides*	Kuboes
GM108	49	3	0	0	*T. usneoides*	Richtersveld
GM145	50	3	0	0	*T. usneoides*	Leeu-Gamka
GM151	50	3	0	0	*T. usneoides*	Waterford
GM011	20	31	2	0	*T. ramosissima*	Vaal River
GM012	20	30	1	0	*T. ramosissima*	Vaal River
GM125	21	31	1	0	*T. ramosissima*	Magaliesberg
GM126	21	31	1	0	*T. ramosissima*	Magaliesberg
GM154	20	31	1	0	*T. ramosissima*	Grahamstown
GM048	20	32	2	1	*T. ramosissima*	Upington
GM001	36	5	12	0	*T. usneoides* × *T. ramosissima*	Vaal River
GM003	39	2	13	1	*T. usneoides* × *T. ramosissima*	Vaal River
GM008	37	2	15	1	*T. usneoides* × *T. ramosissima*	Vaal River
GM018	36	5	12	0	*T. usneoides* × *T. ramosissima*	Vaal River
GM021	36	6	11	0	*T. usneoides* × *T. ramosissima*	Vaal River
GM041	39	8	6	0	*T. usneoides* × *T. ramosissima*	Upington
GM044	37	1	15	0	*T. usneoides* × *T. ramosissima*	Upington
GM054	37	5	11	0	*T. usneoides* × *T. ramosissima*	Impala Platinum
GM014	20	27	10	4	*T. ramosissima* × *T. usneoides*	Johannesburg
GM049	18	28	8	3	*T. ramosissima* × *T. usneoides*	Upington
GM060	19	31	6	3	*T. ramosissima* × *T. usneoides*	Impala Platinum
GM137	14	29	11	1	*T. ramosissima* × *T. usneoides*	Prince Albert
GM147	17	34	5	1	*T. ramosissima* × *T. usneoides*	Steytlerville

characters, of which 43 (31%) were phylogenetically informative. Parsimony analysis of the nuclear (ITS) sequence data set reached a consensus of 10 000 trees (maximum trees set) of 142 steps, excluding uninformative characters, with retention index (RI) = 0.97, consistency index (CI) = 0.87, and rescaled consistency index (RC) = 0.94. The strict consensus of the 10,000 most parsimonious trees is shown in Fig. 2.

The consensus tree (Fig. 2) has two clear clades separating the morphologically identified *T. usneoides* specimens from the *T. ramosissima* specimens and their respective hybrids. There are a minimum of 25 point mutations separating the two main clades (A and B). Clade A (Fig. 2) has 15 synapomorphies (shared derived characters) uniting all the *T. ramosissima* and *T. ramosissima* hybrids. This clade is well-supported with a bootstrap value of 80% and includes both mine and garden-planted *T. ramosissima* specimens and their hybrids (e.g., GM011 and GM014) together with the wild specimens (e.g., GM137). Clade B (Fig. 2) comprises all *T. usneoides*, including their hybrids with strong bootstrap support (97% BS). Contrary to Clade A, there is some branching within clade B: the weakly supported (60% BS) sub-clade D comprises all of the specimens from the mines as well as a few (four) from the wild. This sub-clade contains most of the morphologically identified *Tamarix* hybrids. Sub-clade C comprises only pure-breed *T. usneoides* from wild populations except for GM108, GM145, and GM151, which were identified morphologically as *T. usneoides* hybrids and are all from the wild.

3.2.2. Analysis of ITS polymorphisms for identification of Tamarix hybrids

Excluding the outgroup *M. alopecuroides*, the aligned ITS data matrix (806 characters) comprising the 29 specimens from South Africa has 66 parsimony informative characters of which 46 (69.7%) are variable across the species. Forty (60.6%) of these 66 characters are polymorphic, with 30 (75%) of these comprising alleles reflecting both *T. usneoides* and *T. ramosissima* (Table 3). Based on the analysis (count) of polymorphisms, 10 specimens (34.5%) were identified as pure-bred *T. usneoides*, as opposed to six (20.7%) *T. ramosissima*. Of the remaining samples, eight (27.6%) were identified as *T. usneoides* hybrids and five (17.2%) as *T. ramosissima* hybrids. Hybrids were judged based on the criterion of having more than 10% (4 or more) of their polymorphisms reflecting both putative parents (i.e., GM001, Table 3).

3.2.3. Phylogenetic analysis of the plastid trnS–trnG sequence data

The plastid DNA sequence data set of the same 29 *Tamarix* specimens consisted of 940 aligned base pairs, of which 98 (10.37%) were variable and of these 36 (36.73%) were parsimony informative. There were 17 indels across the plastid data set of which five (29.4%) indels (Table 2) were parsimony informative within *Tamarix*.

The parsimony analysis of the plastid region (*trn*S–*trn*G), including 30 specimens (29 *Tamarix* samples and *M. alopecuroides* as outgroup), resulted in 10,000 trees (maximum limit set) of 109 steps with an RI of 0.99. Excluding uninformative characters, a CI of = 0.94 and an RC of = 0.96 were obtained. The strict consensus tree is shown in Fig. 3.

Two strongly supported clades resulted, separating the exotic *T. ramosissima* from the indigenous *T. usneoides* specimens and their hybrids, with some further resolution within each clade (Fig. 3). Fourteen mutations distinguish the two main clades (A and B), of which nine synapomorphies are shared by *T. ramosissima* samples (Clade A) and five synapomorphies support the grouping of the *T. usneoides* samples and their hybrids in Clade B. The strongly supported (92% BS) Clade A (Fig. 3), like the ITS phylogeny (Fig. 2), groups all *T. ramosissima* specimens together with its hybrids. On the other hand, all specimens of *T. usneoides* and their hybrids are grouped in Clade B, united by four synapomorphic point mutations plus an indel (Table 2) with 93% BS (Fig. 3). Contrary to the ITS phylogeny (Fig. 2), both Clades A and B in the *trn*S–*trn*G exhibit further branching. However, hybrids are not clearly separated from pure-breed species in either clade.

3.2.4. Comparison of the phylogenies based on the nuclear (ITS) regions and plastid (trnS–trnG) regions

Comparing the *Tamarix* phylogenies generated based on the nuclear ITS (Fig. 2) and plastid *trn*S–*trn*G (Fig. 3) shows that there is no

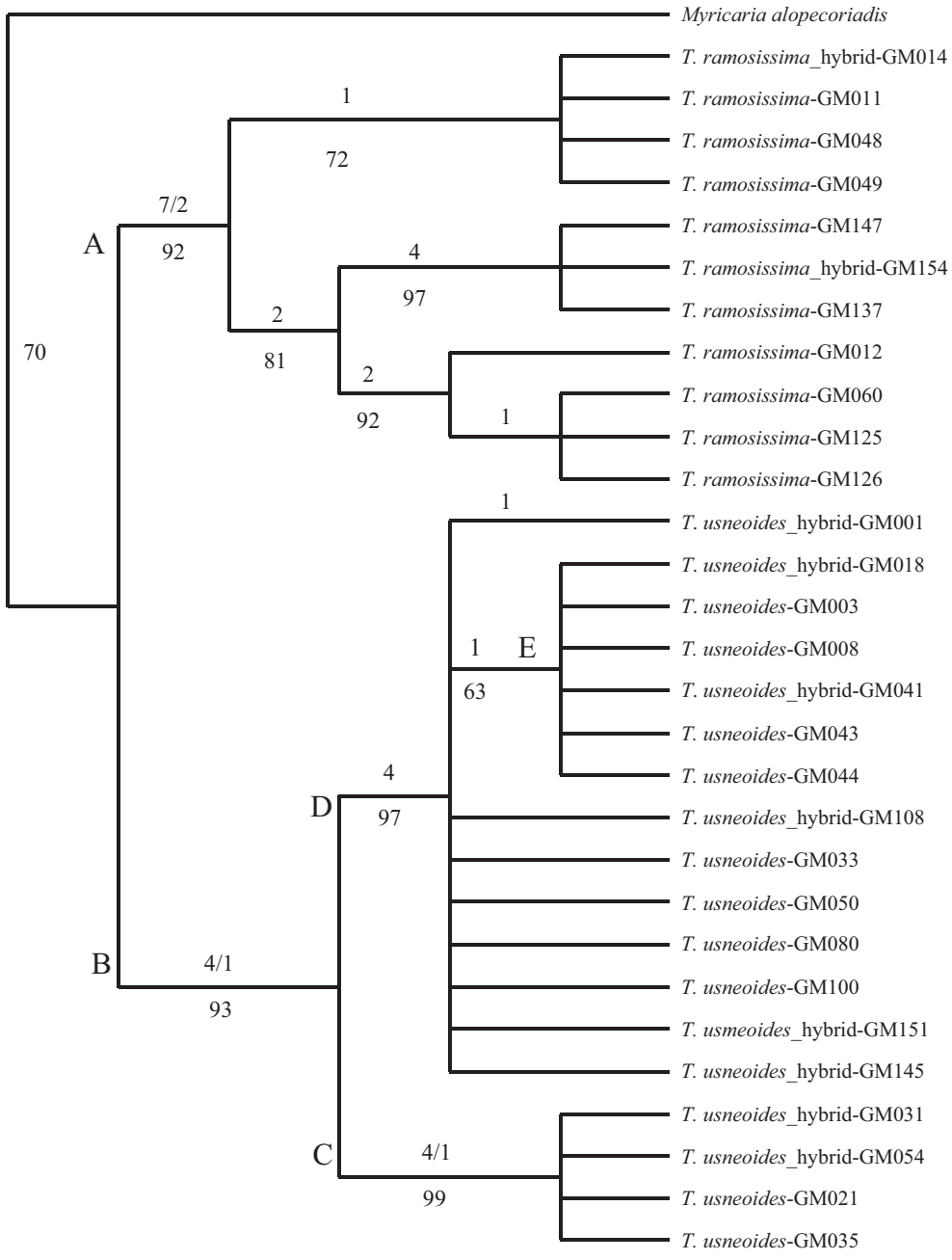

Fig. 3. Strict consensus of 10 000 EMP trees of 29 *Tamarix* specimens and the outgroup *M. alopecuroides* based on *trn*S–*trn*G plastid sequence data including coded indels. Bootstrap values (100 replicates with 20 trees per replicate) are below the branches. Numbers above lines are minimum branch lengths (point mutations/indels). (CI = 0.9348; RI = 0.9879). Species names at tips of tree branches were derived from morphological analysis of sequenced specimens.

consistent pattern that differentiates pure-breed species from their closely hybrid individuals. In addition, the different clades and sub-clades do not group individuals based on the location (viz. wild vs. cultivated) either. In the ITS phylogeny (Fig. 2), all exotic *T. ramosissima* and their hybrids group together in a monophyletic clade, while in the *trn*S–*trn*G phylogeny (Fig. 3), they further subdivide into three sub-clades. The lack of resolution in the clade comprising *T. ramosissima* specimens in the ITS analysis (clade A Fig. 2) means that it is not possible to compare the placement of specimens with those in the consensus tree of the plastid data set (Fig. 3). Clade B (Figs. 2 and 3) containing the indigenous *T. usneoides* specimens show an almost similar pattern in the two phylogenies. However, sub-clade C in the ITS region (Fig. 2) groups more pure *T. usneoides* than in Fig. 3 and contains none of the specimens found in sub-clade C of Fig. 3. On the other hand, sub-clade E in the *trn*S–*trn*G phylogeny (Fig. 3) contains more specimens than sub-clade E (Fig. 2, ITS phylogeny), including the only two specimens (GM003 and GM008) in sub-clade E (Fig. 2). Comparison of the *T. usneoides* clades (B) in both the ITS and *trn*S–*trn*G phylogenies (Figs. 2 and 3 respectively) shows that there are changes in placement of some of the specimens in the two main clades in each consensus tree. However, there is no consistent pattern that differentiates *T. usneoides* specimens from their hybrids.

4. Discussion and conclusion

4.1. Characterization of Tamarix species and their putative hybrids based on Molecular markers (ITS and TrnS–trnG regions)

The phylogenetic analyses of both the nuclear (Fig. 2) and plastid (Fig. 3) sequence data of *Tamarix* species in South Africa resulted in

two clear clades separating the indigenous *T. usneoides* from the exotic *T. ramosissima* with strong bootstrap support. However, there is no clear distinction between the parent species and their closely related hybrid individuals within each clade. Clade A (Figs. 2 and 3) comprises all exotic *T. ramosissima*, from both the wild and cultivated populations, together with their hybrids. In both phylogenies *T. ramosissima* and its hybrids form a polytomy, leaving hybrids grouping together with their closely related, morphologically similar parents in the same clade. The polytomy in Clade A (Figs. 2 and 3) seems to be as a result of the high level of hybridization (Whitney et al., 2010) in the *Tamarix* populations. In South Africa, the nuclear and plastid sequence data of *T. ramosissima* specimens and their hybrids are identical except for differences in the number of polymorphisms (Table 3). Similarly, their morphologies are almost indistinguishable, with some intermediate characters evident in hybrid individuals (Mayonde, 2010). Therefore, the presence of intermediate morphological characters and ITS polymorphisms appears to be good indicators of hybridization in the *Tamarix* invasion in South Africa.

T. ramosissima hybrids in South Africa seem to have escaped from gardens to invade the surrounding water streams. Invasive *Tamarix* hybrids were observed in Prince Albert, Western Cape (GM137) and Steytlerville, Eastern Cape (GM147) populations. It could be argued that garden-planted trees were propagated as hybrids. Alternately, it is possible that the severe infestation in these provinces is due to hybridization with the indigenous species and/or other alien species. A number of recent studies have documented that hybridization enhances invasiveness (Gaskin and Kazmer, 2009; Moody and Les, 2007). Hybridization is regarded as an extremely rapid mechanism for increasing genetic variation by producing novel gene combinations that can potentially enhance the evolution of invasiveness (Schierenbeck and Ellstrand, 2009).

All specimens identified as pure-bred *T. usneoides* and the "morphologically identified" *T. usneoides* hybrids group in Clade B of both the plastid and nrDNA phylogenies. Most of the pure *T. usneoides* specimens in this clade are from the wild populations, whereas most *T. usneoides* hybrids (75%) appear to be occurring on the mines (Table 4). The remaining 25% of hybrids are from wild populations, including some from around Upington in the Northern Cape Province, the source of specimens for propagation for planting on the mines (I. Weiersbye, personal communication). Both indigenous and exotic species and their hybrids occur in and around Upington. Thus, *T. usneoides* hybrids on the mines could have originated from wild populations in/near Upington. Therefore, it is likely that the phytoremediation program is propagating *Tamarix* hybrid clones for cultivation on South Africa mines.

The nuclear and plastid DNA sequence data of the indigenous *T. usneoides* and the exotic *T. ramosissima* in South Africa are distinct, suggesting they are distantly related species within the genus (Gaskin and Schaal, 2003), as indicated by their morphological distinction (Baum, 1978; Mayonde, 2010). It has been shown here that sequence data of hybrid individuals in the ITS DNA marker contain admixed nucleotides from both potential parents (*T. usneoides* and *T. ramosissima*) as well as polymorphisms, either reflecting one or both of the putative parents (Table 3). The admixed sequence data in *Tamarix* individuals in South Africa are observed in specimens that displayed intermediate morphological characters. Therefore, the morphological characterization of *Tamarix* species using the eight characters described in Table 1 is supported by the molecular diagnosis.

The lack of resolution within the species clades in the *Tamarix* phylogenies based on the nuclear and plastid DNA sequence data (Figs. 2 and 3, respectively) signifies that there is a high incidence of hybridization (Moody and Les, 2002) and not due to insufficient signal as previously thought. The lack of resolution in the phylogenies is explained by the high levels of homoplasy observed in the DNA markers investigated and by the high numbers of polymorphisms from the ITS regions which are treated as missing data. The lack of congruence ($p > 0.0001$; Farris et al., 1995) between the two data sets (cpDNA vs. nrDNA sequences) also supports the conclusion that there are many hybrids of *Tamarix* in the South African populations. Similarly, the null hypothesis of the nuclear and plastid *Tamarix* sequence data sets (ITS vs. *trn*S–*trn*G) being similar was rejected by Gaskin and Schaal (2003). When combined DNA sequences data from distantly related plant species (such as *T. usneoides* vs. *T. ramosissima*) are used to infer phylogenetic relationships, the phylogenetic tree reconstruction results in gene-tree species-tree conflicts (Nickrent and Soltis, 1995). This conflict in phylogenetic reconstruction has also been proven to be due to hybridization events (Whitney et al., 2010). Therefore, the lack of congruence evident in the consensus trees resulting from these two data sets is very likely due to the high incidence of hybridization between the species of study (the indigenous *T. usneoides* and the exotic *T. ramosissima*) or because of introgression with either one of the parent species.

Polymorphisms do not contribute toward phylogenetic reconstruction because of their heterozygous status, but they are a good indicator of hybridization (Nickrent and Soltis, 1995). Thus, the analysis of polymorphisms (Table 3) in the ITS regions provides additional evidence of hybridization between *T. usneoides* and *T. ramosissima*. Polymorphisms occur during DNA recombination or duplication in cell division (Solomon et al., 2008) or they could be due to hybridization (Nickrent and Soltis, 1995). The analysis of single nucleotides and polymorphisms (Table 3) gives much insight into hybridization events. Thus, the suspicion raised by observation of morphologically intermediate phenotypes is confirmed by the molecular DNA sequence data, in the form of lack of congruency between cpDNA and nrDNA and the presence of polymorphisms in the ITS sequence data.

It is thus seen that the ITS sequence data can distinguish between *Tamarix* species and their hybrids in South Africa through analysis of polymorphisms and presence of admixed nucleotides. Morphological and molecular modes of identification of *Tamarix* species and hybrids appear to be similarly effective in identifying pure-breed species, with only 10.3% difference between the two approaches (Appendix B and Table 3). However, the molecular method of identification was better able to distinguish hybrids from their parent species than the morphological approach (Table 3).

Certain morphological floral features were shown to be more reliable for identification of *Tamarix* species (e.g., insertion of filaments) compared to most vegetative characters. Intermediate morphological characters (e.g., pale pink petal colour) alone cannot be considered as evidence of hybridization since the *Tamarix* species may exhibit them phenotypically simply by growing in different climatic condition, or soil type (DiTomaso, 1998). In this case, molecular characters were better suited to identify hybrids. The analysis using nucleotide polymorphisms to identify hybrids shows that *Tamarix* populations on the mines are dominated by hybrids which were introduced through cuttings from mother trees from Upington, originally incorrectly identified as pure-breed indigenous *T. usneoides* based on morphological features. Future work using more informative DNA markers such as multilocus amplified fragment length polymorphisms

Table 4
Summary of Tamarix species composition per habitat (wild, mines, garden) based on molecular diagnosis.

Tamarix species composition	Sites/habitats			Total number of specimens
	Wild	Mines	Gardens	
T. usneoides	100%	–	–	10
T. ramosissima	50%	33.3%	16.6%	6
T. usneoides × *T. ramosissima*	25%	75%	–	8
T. ramosissima × *T. usneoides*	20%	60%	20%	5
Sub-total				29

(AFLPs) or simple sequence repeats (SSRs) should be done to further reveal the genetic composition of *Tamarix* populations in South Africa (Vos et al., 1995; Gaskin et al., 2006; Meudt and Clark, 2007) in order to confirm hybrids and indicate levels of introgression (Gaskin and Kazmer, 2009).

In conclusion, the nuclear ITS sequence data provide useful information in the form of polymorphisms and admixed nucleotides that enable distinction between "pure" *Tamarix* species and hybrids in South Africa. Accurate characterization of *Tamarix* species and their hybrids in South Africa is important because of the usefulness of the indigenous *T. usneoides* and the potential invasiveness of the exotic *T. ramosissima* and their hybrids. The finding of *Tamarix* hybrids in South Africa, especially those present on the mines, shows that phytoremediation program is not propagating pure indigenous *T. usneoides* for cultivation around mine tailings storage facilities. The propagation of hybrid individuals between the alien and indigenous *Tamarix* species could spread potentially invasive *Tamarix* in the form of hybrids. Recent studies have shown that hybridization could enhance invasiveness in both terrestrial and aquatic plant species (Gaskin and Kazmer, 2009; Moody and Les, 2007; Gaskin and Schaal, 2003; Gaskin and Schaal, 2002). More interestingly, infestation of *Tamarix* species in the USA is predominantly by hybrids (Gaskin and Kazmer, 2009). The presence of admixed individuals in the invasion of biological material can create difficulties for a classical biocontrol program (Gaskin et al., 2011). This is of great concern when the hybridization is between an indigenous and exotic organism as this poses a danger to conservation of the indigenous genetic material.

Abbreviations and symbols

AMD	acid mine drainage
TSFs	tailing storage facilities
ITS	internal transcribed spacer
nDNA	nuclear deoxyribonucleic acid
cpDNA	plastid deoxyribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
nrDNA	nuclear ribosomal deoxyribonucleic acid
PCR	polymerase chain reaction
TBR	tree bisection reconnection
RI	retention index
CI	consistency index
RC	rescaled consistency index
AFLPs	amplified fragment length polymorphisms
SSRs	simple sequence repeats
T. u.	*Tamarix usneoides*
T. r.	*Tamarix ramosissima*
(–)	dashes across floral features were samples without flowers.
(+++)	salt glands present and abundant
(++)	salt glands present
(+)	salt glands present and spare
(-)	salt glands absent

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Appendix A. List of *Tamarix* samples for molecular diagnosis including their localities, GPS coordinates, and GenBank accession numbers

Collecting number	Locality	Habitat	GPS coordinates		GenBank accession numbers/nuclear ITS; plastid *trnS–trnG*	Species names based on molecular identification
GM001	Vaal River	Mine cultivated	26°55.952 S	26°41.575 E	KM657155; KM657184	*T. usneoides* × *T. ramosissima*
GM003	Vaal River	Mine cultivated	26°55.952 S	26°41.590 E	KM657160; KM657189	*T. usneoides* × *T. ramosissima*
GM008	Vaal River	Mine cultivated	26°55.585 S	26°40.376 E	KM657161; KM657190	*T. usneoides* × *T. ramosissima*
GM011	Vaal River	Mine cultivated	26°55.432 S	26°40.534 E	KM657147; KM657176	*T. ramosissima*
GM012	Vaal River	Mine cultivated	26°55.431 S	26°40.513 E	KM657148; KM657177	*T. ramosissima*
GM014	Gauteng	Garden planted	26°11.368 S	28° 24.553 E	KM657144; KM647173	*T. ramosissima* × *T. usneoides*
GM018	Vaal River	Mine cultivated	26°54.540 S	26°45.800 E	KM657156; KM657185	*T. usneoides* × *T. ramosissima*
GM021	Vaal River	Mine cultivated	26°54.510 S	26°45.840 E	KM657162; KM657191	*T. usneoides* × *T. ramosissima*
GM031	Upington	Wild	28°27.782 S	21°15.193 E	KM657157; KM657186	*T. usneoides*
GM033	Upington	Wild	28°27.829 S	21°15.233 E	KM657163; KM657192	*T. usneoides*
GM035	Upington	Wild	28°27.851 S	21°15.910 E	KM657164; KM657193	*T. usneoides*
GM041	Upington	Wild	N/A	N/A	KM657165; KM657194	*T. usneoides* × *T. ramosissima*
GM043	Upington	Wild	28°27.946 S	21°15.863 E	KM657166; KM657195	*T. usneoides*
GM044	Upington	Wild	28°27.918 S	21°15.856 E	KM657167; KM657196	*T. usneoides* × *T. ramosissima*
GM048	Upington	Wild	28°28.379 S	21°15.709 E	KM657149; KM657178	*T. ramosissima*
GM049	Upington	Wild	28°28.379 S	21°15.709 E	KM657150; KM657179	*T. ramosissima* × *T. usneoides*
GM050	Kenhardt	Wild	29°21.292 S	21°08.862 E	KM657168; KM657197	*T. usneoides*
GM054	Implat	Mine cultivated	26°13.059 S	28°26.760 E	KM657158; KM657187	*T. usneoides* × *T. ramosissima*
GM060	Implat	Mine cultivated	26°13.133 S	28°26.801 E	KM657151; KM657180	*T. ramosissima* × *T. usneoides*
GM080	Marchand	Wild	28°41.627 S	20°30.466 E	KM657169; KM657198	*T. usneoides*
GM100	Kuboes	Wild	28°24.107 S	16°52.632 E	KM657170; KM657199	*T. usneoides*
GM108	Richtersveld	Wild	28°18.655 S	16°58.290 E	KM657159; KM657188	*T. usneoides*
GM125	Cape Town	Garden planted	34°04.345 S	18°26.828 E	KM657152; KM657181	*T. ramosissima*
GM126	Cape Town	Garden planted	34°04.432 S	18°26.808 E	KM657153; KM657182	*T. ramosissima*
GM137	Prince Albert	Wild	33°10.923 S	22°01.648 E	KM657154; KM657183	*T. ramosissima* × *T. usneoides*
GM145	Leeu-Gamka	Wild	32°46.067 S	21°58.780 E	KM657172; KM6571201	*T. usneoides*
GM147	Steytlerville	Garden planted	33°19.330 S	24°20.410 E	KM657145; KM657174	*T. ramosissima* × *T. usneoides*
GM151	Waterford	Wild	33°04.678 S	25°00.962 E	KM657171; KM6571200	*T. usneoides*
GM154	Grahamston	Garden planted	33°17.873 S	26°32.001 E	KM657146; KM657175	*T. ramosissima*

Appendix B. Identification of *Tamarix* species and their putative hybrids in South Africa based on morphological characters (*quantitative and qualitative characters which showed intermediacy in hybrids; [1] represent floral characters; *usn* = *usneoides*, *ram* = *ramosissima*)

Locality	Collect no.	Characters								Species names based on morphological characters
		Salt glands*	Petal shape[1]	Petal colour*[1]	Insertion of filaments[1]	Nectary disc shape[1]	Leaf shape	Leaf attachment	Bracts	
Vaal River	GM003	(++)	Ovate	White	Peridiscal	Synlophic	Vaginate	Overlapping	Vaginate	*T. usneoides*
Vaal River	GM008	(++)	Ovate	White	Peridiscal	Synlophic	Vaginate	Overlapping	Vaginate	*T. usneoides*
Vaal River	GM021	(+++)	–	–	–	–	Vaginate	Not overlapping	Vaginate	*T. usneoides*
Upington	GM033	(+++)	Ovate	White	Peridiscal	Synlophic	Vaginate	Overlapping	Vaginate	*T. usneoides*
Upington	GM035	(+++)	Ovate	White	Peridiscal	Synlophic	Vaginate	Overlapping	Vaginate	*T. usneoides*
Upington	GM043	(-)	Ovate	White	Peridiscal	Synlophic	Vaginate	Overlapping	Vaginate	*T. usneoides*
Upington	GM044	(+++)	Ovate	White	Peridiscal	–	Vaginate	Overlapping	Vaginate	*T. usneoides*
Kenhardt	GM050	(+++)	–	–	–	–	Vaginate	Overlapping	Vaginate	*T. usneoides*
Marchand	GM080	(+++)	–	–	–	–	Vaginate	Overlapping	Vaginate	*T. usneoides*
Kuboes	GM100	(+++)	–	–	–	–	Vaginate	Overlapping	Vaginate	*T. usneoides*
Vaal River	GM011	(+)	Obovate	Pink	Hypodiscal	–	Sessile	Overlapping	Sessile	*T. ramosissima*
Vaal River	GM012	(+)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Overlapping	Sessile	*T. ramosissima*
Upington	GM048	(-)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Upington	GM049	(-)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Impala Plats	GM060	(+)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Cape Town	GM125	(-)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Cape Town	GM126	(-)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Prince Albert	GM137	(-)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Steytlerville	GM147	(-)	Obovate	Pink	Hypodiscal	Hololophic	Sessile	Not overlapping	Sessile	*T. ramosissima*
Vaal River	GM001	(+++)	Obovate	White	Peridiscal	–	Vaginate	Not overlapping	Vaginate	T. u hybrid
Upington	GM031	(-)	–	–	–	–	Vaginate	Not overlapping	Vaginate	T. u hybrid
Upington	GM041	(++)	Ovate	White	Peridiscal	–	Vaginate	Not overlapping	Vaginate	T. u hybrid
Impala Plats	GM054	(-)	Ovate	White	Peridiscal	Hololophic	Vaginate	Overlapping	Sessile	T. u hybrid
Richtersveld	GM108	(++)	–	–	–	–	Vaginate	Not overlapping	Vaginate	T. u hybrid
Leeu-Gamka	GM145	(+)	–	–	–	–	Vaginate	Not overlapping	Vaginate	T. u hybrid
Waterford	GM151	(+)	–	–	–	–	Vaginate	Not overlapping	Vaginate	T. u hybrid
Gauteng	GM014	(-)	Elliptic ovate	Pink	Hypo-peridiscal	–	Sessile	Overlapping	Sessile	T. r hybrid
Vaal River	GM018	(+++)	Ovate	White	Hypodiscal	Hololophic	Vaginate	Not overlapping	Vaginate	T. r hybrid
Grahamston	GM154	(-)	–	–	–	–	Sessile	Not overlapping	Sessile	T. r. hybrid

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