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SPECIES IDENTIFICATION OF *VACHELLIA PACHYCERAS* FROM KUWAIT AND ITS RELATIVES *VACHELLIA GERRARDII* AND *VACHELLIA TORTILIS*, BASED ON MULTILOCUS PLASTID GENE SEQUENCES

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The genus *Acacia* Miller is species-rich, and species discrimination is challenging owing to morphological similarities between closely related species. Naming of specimens is particularly difficult in the Middle East, where confusion in taxonomic identification exists within the context of a wider international debate on the generic systematics of *Acacia sensu lato*. At least five segregate genera for *Acacia s.l.* have been advocated: *Acacia sensu stricto*, *Vachellia*, *Senegalia*, *Acaciella* and *Mariosousa*. Furthermore, identification to species of the only remaining native *Acacia s.l.* tree in Kuwait is still a matter of controversy. The present study used multilocus chloroplast DNA sequence data analyses following maximum likelihood (ML) and Bayesian approaches to: 1) test the species concepts of *Vachellia pachyceras* (≡ *Acacia pachyceras* O.Schwartz) from the Middle East, and *Vachellia tortilis* (Forssk.) Galasso & Banfi (≡ *Acacia tortilis* (Forssk.) Hayne) and *Vachellia gerrardii* (Benth.) P.J.H.Hurter (≡ *Acacia gerrardii* Benth.) from Kenya, as well as to investigate species divergence times; and 2) identify the only remaining native *Acacia s.l.* tree in Kuwait (known as the Lonely Tree), as well as other unidentified *Acacia s.l.* specimens in cultivation. The Bayesian and ML topologies clearly differentiated *Vachellia pachyceras*, *V. tortilis* and *V. gerrardii*, and demonstrated that the three species are distinct. Divergence time estimates using the ML topology suggested that *Vachellia gerrardii* diverged from a common ancestor no later than the early Pliocene (3.3 Mya), whereas *V. pachyceras* originated at least 2.0 Mya (Pliocene). The unknown remaining native *Acacia s.l.* tree in Kuwait and other specimens collected from the nursery were identified as *Vachellia pachyceras*. These results stress the need to use plastid DNA barcodes complemented by population genetics approaches to address systematic issues in this complex of *Acacia s.l.* species in the Middle East and the Arabian Peninsula.

Keywords. *Acacia* complex, DNA barcoding, molecular phylogenetics, species identification, *Vachellia*.

Introduction

The genus *Acacia* Miller *sensu lato* (Fabaceae, Mimosoideae; hereafter, *Acacia s.l.*) is very diverse, comprising more than 1400 species (Maslin *et al.*, 2003a,b), which

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are distributed in the subtropics of Australia (c.1010 species), Asia (c.60 species), the Americas (c.185 species) and Africa (c.150 species). Owing to this rich diversity, *Acacia s.l.* has undergone multiple taxonomic revisions based mainly on cladistic analyses of chloroplast genes and internal or external transcribed spacer regions of DNA (Maslin *et al.*, 2003a,b; Murphy *et al.*, 2003; Orchard & Maslin, 2003; Maslin & Orchard, 2004; Walker & Simpson, 2004; Orchard & Maslin, 2005; Maslin, 2006; Seigler *et al.*, 2006; Smith *et al.*, 2006; Maslin & Orchard, 2009; Miller & Seigler, 2012; Kyalangalilwa *et al.*, 2013). Phenotypic similarities between closely related *Acacia* species make taxonomic distinction quite difficult, especially when species distributions overlap, as is the case in the Middle East and the Arabian Peninsula. Furthermore, there is a paucity of morphological characteristics available, these being subject to environmental modifications resulting in phenotypic plasticity, further complicating classification of related taxa (Hebert *et al.*, 2003). The use of genetic markers, which are not subject to environmental variations, can aid in the process of identification of cultivars or clones (Rahman & Rajora, 2002), subspecies (Fredua-Agyeman *et al.*, 2008) and species (Zietkiewicz *et al.*, 1994), as well as in parentage and kinship (Blouin, 2003) analyses.

Boulos (1995) described a complex of *Acacia* species that are distributed in the Middle East, including *A. johnwoodii* Boulos, *A. elatior* Brenan, *A. yemenensis* Boulos, *A. hockii* De Wild., *A. oerfota* (Forssk.) Schweinf., *A. pachyceras* O.Schwartz and *A. tortilis* (Forssk.) Hayne. Other *Acacia* species described from the region include *A. asak* (Forssk.) Willd., *A. ehrenbergiana* Hayne, *A. tortilis* subsp. *spirocarpa* (Hochst. ex A.Rich.) Brenan, and *A. iraqensis* Rech.f. (Abdulfatih, 1981). Chaudhary (1999) reported that *Acacia gerrardii* Benth. subsp. *negevensis* Zohary is found in Saudi Arabia, and mentioned that *A. iraqensis* and *A. pachyceras sensu* Boulos, 1995 non Schwartz, 1939, are synonyms of *A. gerrardii*. However, Boulos (1995) has named this species as *Acacia pachyceras*. Evidence from these studies indicates that the taxonomy of *Acacia tortilis*, *A. gerrardii* and *A. pachyceras* in the Middle East and the Arabian Peninsula is still controversial, and indeed the Plant List (no date) lists *Acacia pachyceras* as an unresolved name.

Confusion surrounding the naming of *Acacia* species in the Middle East is exacerbated by the ongoing debate on the generic taxonomy of *Acacia s.l.*, owing to the polyphyletic nature of the genus (Miller & Bayer, 2003; Miller *et al.*, 2003a,b; Luckow *et al.*, 2005; Murphy *et al.*, 2010; Moore *et al.*, 2011; Miller & Seigler, 2012; Kyalangalilwa *et al.*, 2013). Indeed, at least five segregate genera for *Acacia s.l.* have been advocated: *Acacia sensu stricto* (*s.s.* hereafter, formerly *Acacia* subgenus *Phyllodineae*), *Vachellia* Wight & Arn. (formerly *Acacia* subgenus *Acacia*), *Senegalia* Raf. (formerly *Acacia* subgenus *Aculeiferum* section *Aculeiferum*), *Acaciella* Britton & Rose (formerly *Acacia Aculeiferum* section *Filicinae*) and *Mariosousa* Seigler & Ebinger (including the species belonging to the *Acacia coulteri* group) (Kyalingalilwa *et al.*, 2013). Under this taxonomy, *Acacia gerrardii* and *A. tortilis* become *Vachellia gerrardii* (Benth.) P.J.H.Hurter and *Vachellia tortilis* (Forssk.) Galasso & Banfi, respectively.

Taxonomic identification of the only remaining native *Acacia s.l.* tree in Kuwait, known as the Lonely Tree (Fig. 1), has proven difficult. This tree survives in the Talha

FIG. 1. The Lonely Tree, the remaining native *Acacia s.l.* tree at the Sabah Al-Ahmed Natural Reserve (29°34.909′N, 47°47.734′E) in Kuwait in 2012.

area of Sabah Al-Ahmed Natural Reserve in Kuwait (29°34.909′N, 47°47.734′E). The age of the Lonely Tree was estimated at between 80 and 100 years in 2014. Moreover, it is considered to be the only native tree in Kuwait. Conservation and restoration of this emblematic tree is clearly of national importance. According to the extant literature, identification of the Lonely Tree is controversial (see, among others, Dickson, 1955; Boulos & Al-Dosari, 1994; Boulos, 1995; Shuaib, 1995; Chaudhary, 1999). Dickson (1955), Boulos & Al-Dosari (1994) and Dannin (2000) identified the tree as *Acacia pachyceras*, whereas Shuaib (1995) believed it to be *A. gerrardii*. Therefore, further research on the genetics of both *Vachellia gerrardii* (≡ *A. gerrardii*) and *V. pachyceras* (≡ *A. pachyceras*) collected from various sources is needed to delineate cryptic *Acacia* taxa in the Middle East, determine the identity of the Lonely Tree and standardise methods for its propagation. The distribution ranges of *Vachellia pachyceras*, *V. tortilis* (≡ *A. tortilis*) and *V. gerrardii* in the Middle East are illustrated in Figure 2.

DNA barcoding is a diagnostic technique for species identification that relies on a comparison of DNA sequences from a small fragment of the genome, and is particularly useful when traditional taxonomic characters lack discriminatory power. It has proven to be a powerful tool for delineation of cryptic species within *Acacia* (Newmaster & Ragupathy, 2009; Collins & Cruickshank, 2013; Ndlovu *et al.*, 2013; Nevill *et al.*, 2013), with both chloroplast DNA markers (hereafter cpDNA; Newmaster & Ragupathy, 2009; Ndlovu *et al.*, 2013; Nevill *et al.*, 2013) and nuclear

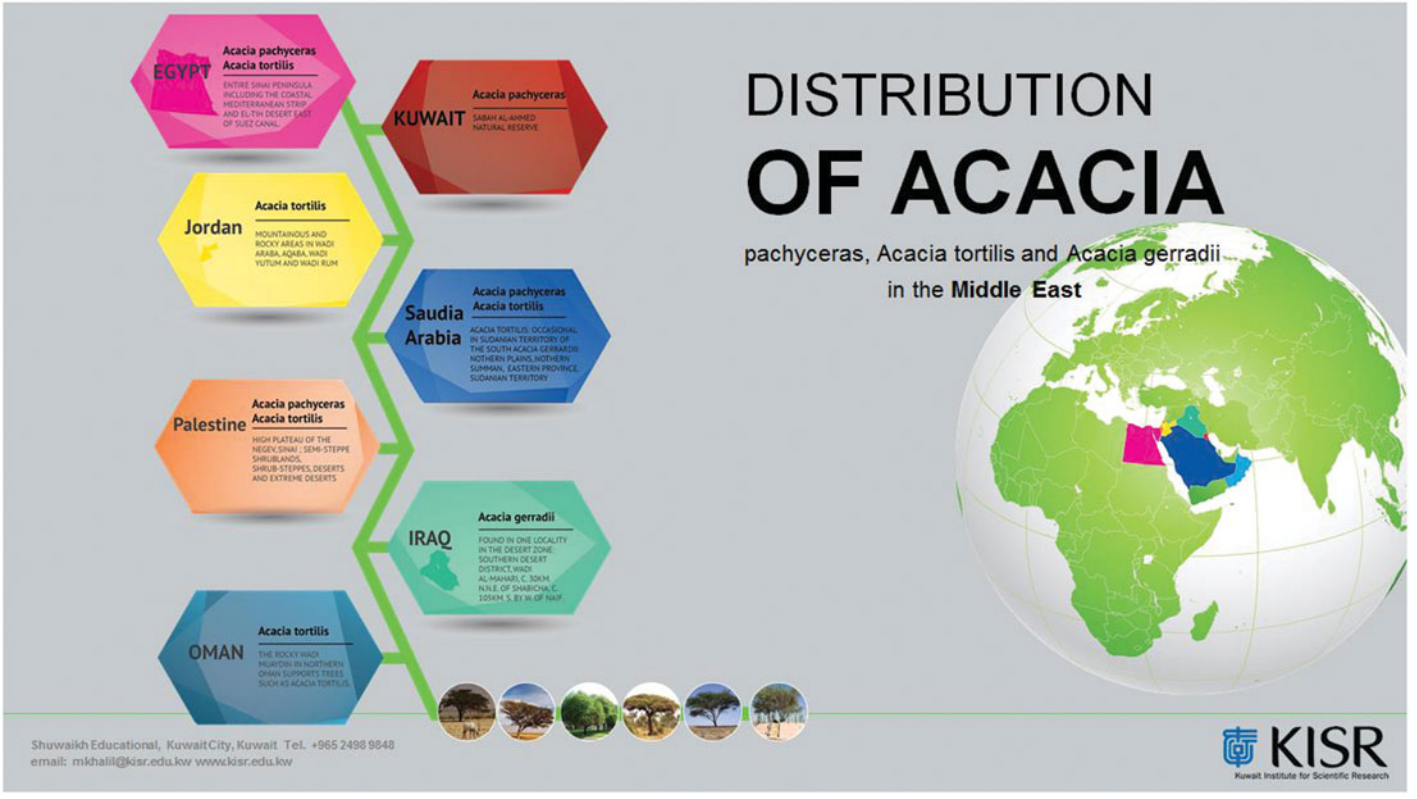

FIG. 2. Distribution of *Vachellia pachyceras*, *Vachellia tortilis* and *Vachellia gerrardii* in the Middle East.

DNA regions (Ndlovu *et al.*, 2013) having been used. The cpDNA regions have included complete or partial sequences of the *matK* and *rbcL* genes, the *psaB–rsp14* region, and non-coding regions associated with the *tRNA-Leu (trnL), tRNA-Phe (trnF)* and *tRNA-Lys (trnK)* genes, and statistical methods for discriminating taxa have included single- and multilocus analyses (Sites & Marshall, 2004; Dupuis *et al.*, 2012; Fujita *et al.*, 2012; Nevill *et al.*, 2013). However, single locus analyses for species discovery most often use broad sampling (Nevill *et al.*, 2013), and can be misleading for species identification (Dupuis *et al.*, 2012; Collins & Cruickshank, 2013).

Bouchenak-Khelladi *et al.* (2010) examined phylogenetic relationships among the five recognised genera of *Acacia*, using the most comprehensive sampling to date, with an emphasis on African species. The study reconstructed the evolutionary history of Mimosoideae in Africa, including *Acacia s.l.*, using sequences of the *trnL* intergenic spacer, *matK* and *trnH–psbA* from 152 Mimosoideae and nine Caesalpiniodeae species (Bouchenak-Khelladi *et al.*, 2010). Similarly, in the Hawaiian Islands, rich with diverse *Acacia* ecotypes, microsatellite markers were used for population differentiation of *A. koa* A.Gray individuals (Fredua-Agyeman *et al.*, 2008), and for discrimination of *A. koa*, *A. koaia* Hillibr. and their intermediate forms (Adamski *et al.*, 2012). The geographical disjunctions within *Acacia* remain unexplained (Sprent, 2007), therefore a study of phylogenetic relationships between *Acacia* species from different continents would shed light on the evolutionary history of the Mimosoideae. The present study focuses on using multilocus cpDNA sequence analyses to: 1) test the species concepts of *Vachellia pachyceras* from the Middle East and *V. tortilis* and *V. gerrardii* from Kenya, as well as to investigate species divergence times; and 2) identify and discriminate the only remaining native *Acacia s.l.* tree in Kuwait (the Lonely Tree) and other unidentified *Acacia s.l.* specimens in cultivation.

Materials and Methods

Plant material and DNA extraction

Twelve *Vachellia pachyceras* DNA samples from herbarium specimens were received from the Royal Botanic Gardens, Kew, and total DNA was extracted from leaf material of the Lonely Tree in Kuwait and from nine *V. tortilis* and ten *V. gerrardii* trees from the Kenyan Forest Research Institute (KEFRI, Nairobi, Kenya) (Table 1), using the DNeasy Plant Maxi Kit (Qiagen, Mississauga, Ontario, Canada). Total DNA was also extracted from three nursery-grown unknown *Acacia* seedlings from Kuwait (putative Lonely Tree Laila, and nursery-grown Lonely Tree), and one nursery-grown Saudi Arabian seedling (see Table 1).

Polymerase chain reactions

Three chloroplast regions (Table 2) were amplified using DNA samples extracted from 0.3 g of silica gel–dried *Vachellia* leaf material.

TABLE 1. DNA sample identification numbers, species names and voucher information for specimens used in this study, and GenBank accession numbers for the sequences obtained

No.	Sample identification no.	Species	Country, collector and year of collection	Voucher identification no(s).	GenBank accession no. for DNA sequence obtained		
					Chloroplastic *psaB–rps14* inter-genic/*rps14* partial sequence	Chloroplastic *rbcL* partial sequence	Chloroplastic *trnL*; *trnL–trnF* intergenic spacer partial sequence
1	SA39289[a]	*Vachellia pachyceras*	Saudi Arabia, Nassar, M., 2003	39289	MG386333	MG460705	MG460670
2	SA39288[a]	*V. pachyceras*	Saudi Arabia, Collenette, 1981	39288	MG386332	MG460704	MG460669
3	SA39290[a]	*V. pachyceras*	Saudi Arabia, Nassar, M., 2003	39290	MG386334	MG460706	MG460671
4	SA39404[a]	*V. pachyceras*	Saudi Arabia, Nassar, M., 2003	39404	MG386335	MG460707	MG460672
5	SA39405[a]	*V. pachyceras*	Saudi Arabia, Nassar, M., 2003	39405	MG386336	MG460708	MG460673
6	SA39406[a]	*V. pachyceras*	Saudi Arabia, Nassar, M., 2003	39406	MG386337	MG460709	MG460674
7	SA39411[a]	*V. pachyceras*	Saudi Arabia, Rillett & Rawi, 1947	39411	MG386340	MG460712	MG460677
8	I39416[a]	*V. pachyceras*	Iraq, Nassar, M., 2003	39416	MG386338	MG460710	MG460675
9	I39412[a]	*V. pachyceras*	Iraq, Hazim, 1961	39412	MG386341	MG460713	MG460678
10	I39282[a]	*V. pachyceras*	Iraq, Khatib & Khazi, 1964	39282	MG386339	MG460711	MG460676
11	K39421[a]	*V. pachyceras*	Kuwait, Boulos, L., Saleh, M. & Al-Mutaw'a, J., 1989	39421	MG386342	MG460714	MG460679
12	K39422[a]	*V. pachyceras*	Kuwait, Firmn, R., 1963	39422	MG386343	MG460715	MG460680
13	KA20	*Vachellia tortilis*	Kenya, Dr S. Omondi, 2014	AT1257020	MG386344	MG460716	MG460681
14	KA21	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT1257021	MG386345	MG460717	MG460682
15	KI22	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT12570122	MG386346	MG460718	MG460683
16	KA25	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT1257025	MG386347	MG460719	MG460684
17	KI23	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT12570123	MG386348	MG460720	MG460685
18	KI24	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT12570124	MG386349	MG460721	MG460686
19	KI25	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT12570125	MG386350	MG460722	MG460687
20	M19	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT12570219	MG386351	MG460723	MG460688
21	M25	*V. tortilis*	Kenya, Dr S. Omondi, 2014	AT12570225	MG386352	MG460724	MG460689
22	Ke1	*Vachellia gerrardii*	Kenya, Dr S. Omondi, 2014	AG453001	MG386353	MG460725	MG460690

TABLE 1. *(Continued)*

No.	Sample identification no.	Species	Country, collector and year of collection	Voucher identification no(s).	GenBank accession no. for DNA sequence obtained		
					Chloroplastic *psaB–rps14* intergenic/*rps14* partial sequence	Chloroplastic *rbcL* partial sequence	Chloroplastic *trnL*; *trnL–trnF* intergenic spacer partial sequence
23	Ke3	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453003	MG386354	MG460726	MG460691
24	Ke4	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453004	MG386355	MG460727	MG460692
25	Ke6	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453006	MG386356	MG460728	MG460693
26	Ke7	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453007	MG386357	MG460729	MG460694
27	Ke8	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453008	MG386358	MG460730	MG460695
28	Ke9	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453009	MG386359	MG460731	MG460696
29	Ke10	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453010	MG386360	MG460732	MG460697
30	Ke11	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453011	MG386361	MG460733	MG460698
31	Ke12	*V. gerrardii*	Kenya, Dr S. Omondi, 2014	AG453012	MG386362	MG460734	MG460699
32	LTL	Nursery-grown Lonely Tree Laila seedling	Kuwait, Dr A. Quoreshi, 2014	–	MG386329	MG460701	MG460666
33	NLT	Nursery-grown Lonely Tree seedling	Kuwait, Dr A. Quoreshi, 2014	–	MG386330	MG460702	MG460667
34	NSA	Nursery-grown Saudi Arabia seedling	Seeds from Saudi Arabia, grown in Kuwait, Dr A. Quoreshi, 2014	–	MG386331	MG460703	MG460668
35	LT	Unknown (Lonely Tree)	Sabah Al-Ahmed Natural Reserve Kuwait, Ms Majda Khalil 2013	#2701015C1, #2701015C2, #2701015C3[b]	MG386328	MG460700	MG460665

trnF, tRNA-Phe; trnL, tRNA-Leu.

[a] DNA samples (registered as *Acacia pachyceras*) from herbarium specimens were received from the Royal Botanic Gardens, Kew. Specimen vouchers for numbers 11–31 are deposited in the Kenya Forestry Research Institute (KEFRI) herbarium.

[b] Kuwait Institute for Scientific Research (KISR) herbarium specimen voucher identification numbers for the Lonely Tree. All DNA samples and sequences are available at the microbial and genomic collection of the Centre for Forest Research at Université Laval, Québec, Canada.

TABLE 2. Locus and primer sequences used in the present study

Chloroplast DNA region	Primer(s)	Amplicon size (base pairs)	Primer sequence(s) (5′–3′)	Reference
rbcL	rbcL–aF	670–720	ATGTCACCACAAACAGAGACTAAAGC	Newmaster & Ragupathy (2009)
	Ajf634R		GAAACGGTCTCTCCAACGCAT	
psaB–rps14	psaB–rps14-1	212–303	GCACGATTAGTTGGATTAGC	Gómez-Acevedo et al. (2010)
	psaB–rps14-2		CCATCTCACGGAGTATGTGT	
trnL–trnF	trnL–F/B49317	350–361	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)
	trnL–F/A50272		ATTTGAACTGGTGACACGAG	

The polymerase chain reaction (PCR) reaction mixture for amplifying the locus *psaB–rps14* using the primers psaB–rps14-1 and psaB–rps14-2 (Gómez-Acevedo *et al*., 2010; see Table 2) consisted of 3 µL of a 10 × PCR buffer, 1.8 µL of 25 mM magnesium chloride, 0.125 µL of a 10 mM dNTP solution in equimolar ratio, 0.3 µL for each primer at 25 µM, 1 U Taq polymerase (Sigma-Aldrich, St Louis, Missouri, USA), 65 ng of template DNA and additional double-distilled H_2O, for a total volume of 30 µl. Amplification was carried out with an initial denaturation step of 95°C for 10 min, followed by 40 cycles of a 94°C for 30 s, 55°C for 1 min and 72°C for 1 min, with a final extension period of 72°C for 10 min.

Amplification of the *trnL–trnF* locus was performed using the primers trnL–F and B49317 (Taberlet *et al*., 1991; see Table 2). The PCR reaction mixture consisted of 3 µl of a 10 × PCR buffer, 1.8 µL of 25 mM magnesium chloride, 0.125 µL of a 10 mM dNTP solution in equimolar ratio, 0.3 µL for each primer at 25 µM, 1.5 U Taq polymerase (Sigma-Aldrich) and 65 ng DNA in a total volume of 30 µL. Amplification was carried out with an initial denaturation step of 94°C for 5 min, followed by 36 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final extension period of 72°C for 10 min.

The PCR reaction mixture for amplifying the *rbcL* gene (partial sequence) using the primers rbcL–aF and Ajf634R (Newmaster & Ragupathy, 2009; see Table 2) consisted of 2 µL of a 10 × PCR buffer, 2 µL of 25 mM magnesium chloride, 0.1 µL of a 10 mM dNTP solution in equimolar ratio, 0.08 µL for each primer at 25 µM, 1 U Taq polymerase (Sigma-Aldrich) and 65 ng DNA in a total volume of 20 µL. The PCR cycling conditions were as follows: an initial denaturation step of 94°C for 5 min, followed by 31 cycles of a 94°C for 50 s, 52°C for 1 min and 72°C for 1 min, with a final extension period of 72°C for 10 min.

The PCR products were electrophoresed in 1% agarose gel buffer and stained with ethidium bromide (10 mg/mL).

Molecular systematics

Before alignment, each sequence was tested for correspondence with forward and reverse primer sequences in BioEdit, version 7.2.4 (Hall, 1999), using the Reverse Complement function. There were 924 positions in total in the data set of 36 sequences: positions 1–212, 213–574 and 575–924 corresponded to the *psaB–rsp14*, *trnL–trnF* and *rbcL* regions, respectively. Sequences were manually edited using BioEdit, and aligned using ClustalW multiple alignment in BioEdit. The multilocus sequence data set was analysed using maximum likelihood (ML) based on the HKY model, using MEGA6 (Tamura *et al*., 2012, 2013). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with the superior log-likelihood value. Support for internal nodes was assessed by bootstrap analysis using 1000 bootstrap replications.

Bayesian inference of phylogeny was performed on the multilocus data set with MrBayes, version 3.2.5 (Ronquist *et al.*, 2012; Huelsenbeck *et al.*, no date), assuming an HKY model and with the transition to transversion rate ratio fixed to 1.82. Analyses were based on two runs of four Markov chain Monte Carlo analyses, each of 5,000,000 generations, a burn-in fraction of 0.25 and sampling every 100 generations for a total of 50,001 generated trees.

The ML tree constructed in MEGA was then used to estimate relative times of divergence for branching points using the RelTime method in MEGA6 (Tamura *et al.*, 2012, 2013). The calibration constraint of approximately 10 Mya between *Vachellia tortilis* and *V. gerrardii* was taken from Bouchenak-Khelladi *et al.* (2010). Sequences of the three-locus combinations from all species were analysed using *Mariosousa centralis* (Britton & Rose) Seigler & Ebinger (≡ *Acacia centralis* (Britton & Rose) Lundell; HM020781), as an outgroup.

Results

The potential scale reduction factor (Gelman & Rubin, 1992) for the Bayesian analysis was equal to 1.000 for all the 36 branch lengths of the phylogeny (results not shown), and average and maximum standard deviations of split frequencies were equal to 0.003 and 0.01, respectively. Phylogenies estimated with MrBayes and MEGA were summarised in a single tree because their topologies were similar, although not identical (Fig. 3). The tree presented is the majority consensus from the Bayesian analysis, with bootstrap values from the ML analysis added next to posterior probability values. The Bayesian and ML tree topologies clearly differentiated *Vachellia pachyceras*, *V. gerrardii* and *V. tortilis*, with high probabilities and bootstrap values (see Fig. 3). Despite an unresolved polytomy between the species-level clades, most individual specimens were grouped with their expected species, and *Vachellia pachyceras* was resolved as distinct from both *V. tortilis* and *V. gerrardii*. By using the constraint of 10 Mya (Miocene) for the divergence time between *Vachellia tortilis* and *V. gerrardii* (Bouchenak-Khelladi *et al.*, 2010), we estimated divergence times using the RelTime analysis in MEGA (Fig. 4). According to the ML divergence time estimates, *Vachellia gerrardii* evolved from common ancestor no later than 3.3 Mya in the Pliocene, whereas *V. pachyceras* originated no later than 2.0 Mya (Pliocene; see Fig. 4).

The Bayesian and ML analyses also identified the Lonely Tree, the Lonely Tree seedlings (putative Lonely Tree Laila, and nursery-grown Lonely Tree) and the nursery-grown Saudi Arabian seedling as *Vachellia pachyceras* (0.992 posterior probability and 64% bootstrap support, respectively; see Fig. 3). As in the ML analysis, species clades were mostly well-resolved in the Bayesian analysis, except for the *Vachellia pachyceras* SA39405 sample, which had an unresolved position. *Vachellia pachyceras* SA39406 grouped with the other *V. pachyceras* specimens but seems quite distinct (see Fig. 3). Similarly, specimen *Vachellia gerrardii* Ke9 grouped with the *V. gerrardii* clade but is clearly distinct from the other exemplars of the species.

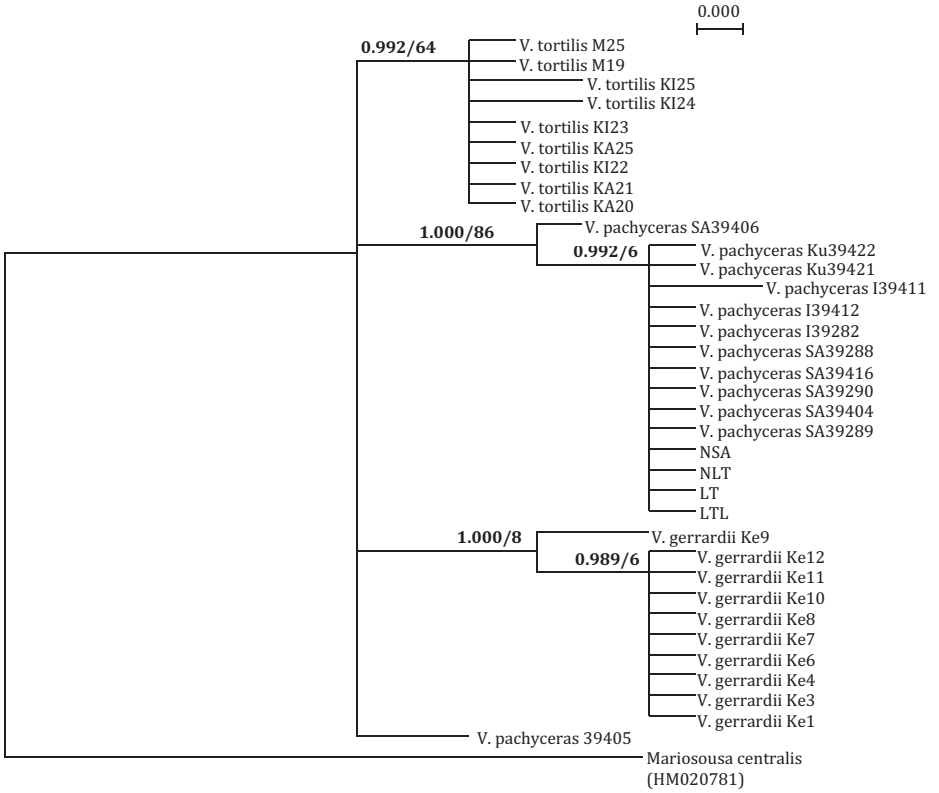

FIG. 3. Bayesian majority consensus tree based on analysis of the combined *psaB–rsp14*, *trnL–trnF* and *rbcL* sequences. Numbers on branches are Bayesian posterior probabilities (left) and ML bootstrap values (right). *Mariosousa centralis* was used as an outgroup.

DISCUSSION

Recent studies agree that *Acacia s.l.* is polyphyletic (Luckow *et al.*, 2005; Seigler *et al.*, 2006; Gómez-Acevedo *et al.*, 2010; Murphy *et al.*, 2010; Moore *et al.*, 2011; Kyalingalilwa *et al.*, 2013), and this has been used as the basis for segregation of the genus (Seigler *et al.*, 2006; Murphy *et al.*, 2010; Kyalangalilwa *et al.*, 2013). The new taxonomy is congruent with the grouping of *Acacia s.l.* species as described by Gómez-Acevedo *et al.* (2010) in the New World, Miller & Bayer (2001) in Australia, and Kyalangalilwa *et al.* (2013) in Africa, and supports pleas for generic splitting, with each clade being considered as a new genus (Kyalangalilwa *et al.*, 2013). Bouchenak-Khelladi *et al.* (2010) described the evolutionary history of Southern African *Acacia* and related species; however, the study did not cover Middle Eastern and Arabian species such as *Vachellia pachyceras*, or investigate intraspecific evolutionary relationships between subgroups of the studied species. Very little systematic work has

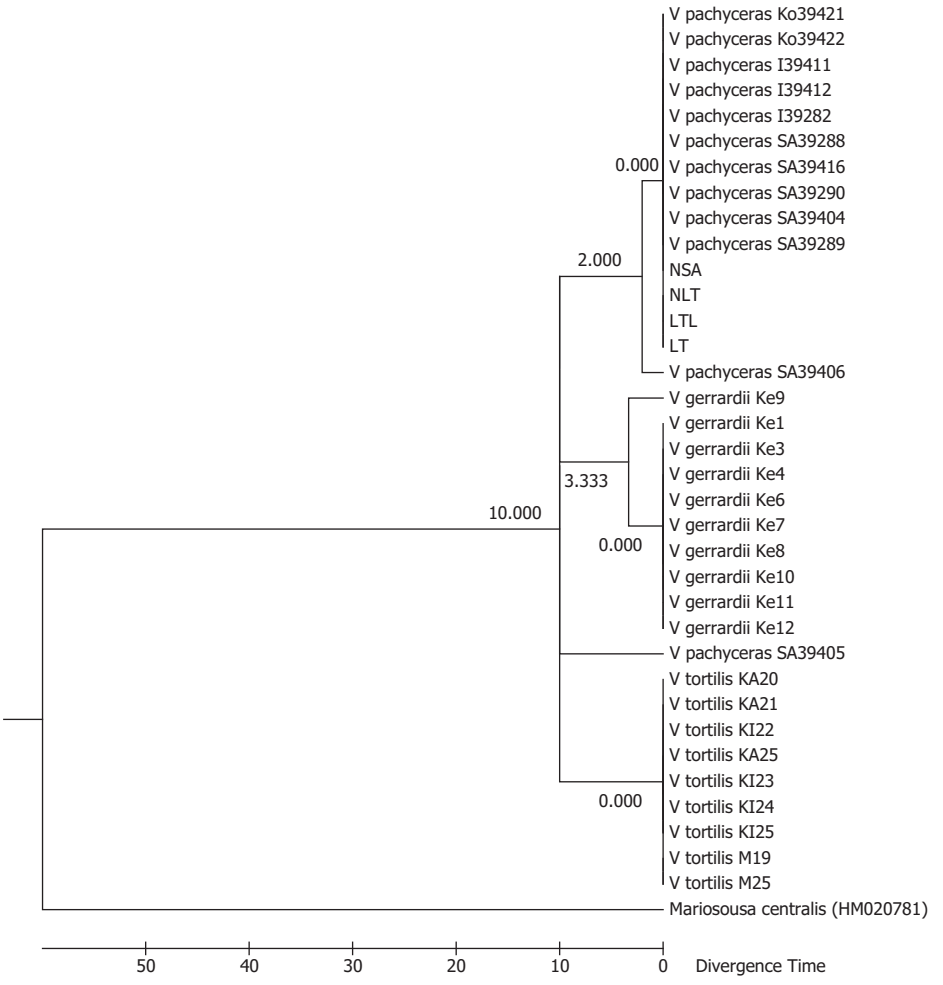

FIG. 4. Chronogram generated using the RelTime method. Divergence times were calculated using the maximum likelihood method based on the Hasegawa–Kishino–Yano model (Hasegawa *et al.,* 1985). Optimised relative times (shown next to branches) were based on a calibration constraint of 10 Mya for the divergence between *Vachellia tortilis* and *Vachellia gerrardii* (Bouchenak-Khelladi *et al*., 2010).

been done on the closely related species *Vachellia pachyceras*, *V. tortilis* and *V. gerrardii* in the Middle East and on the Arabian Peninsula (Boulos, 1995; Dannin, 2000). The results of our study show that *Vachellia pachyceras* is clearly distinct from both *V. tortilis* and *V. gerrardii*.

Results from Bouchenak-Khelladi *et al.* (2010) suggest that the lineages represented by the extant *Vachellia tortilis* and *V. gerrardii* diverged in the Miocene, around 10 Mya ago. Our results indicate a divergence time no later than 3.3 Mya (Pliocene) for

sampled representatives of *Vachellia gerrardii*, and no later than 2.0 Mya for our *V. pachyceras* exemplars. Odee *et al.* (2012), using internal transcribed spacer sequences, found that dispersal of *Acacia* to West Africa and across to the Arabian Peninsula and the Indian subcontinent from source populations located in the East African region, might have occurred during Plio-Pleistocene climate oscillations (from c.5 Mya), resulting in speciation (deMenocal, 1995; Byrne, 2008; Odee *et al*., 2012). A hypothesis of continuous speciation of plants since the late Eocene/early Oligocene has been suggested by Rull (2008). This was rooted in a review of literature on molecular DNA data of Neotropical species, and further studies using both molecular DNA and calibration times are needed to validate it, because molecular sequence data often fail to disentangle times from evolutionary trees (Sanderson *et al*., 2004). Because of the unresolved polytomy in our phylogenetic results, more comprehensive sampling of *Vachellia* species, as well as the use of different molecular-based approaches and calibration points to date divergence times, is required (Gómez-Acevedo *et al*., 2010; Perez *et al.,* 2013).

By definition, cryptic species are problematic to differentiate and identify using morphological characters. Barcoding techniques using cpDNA regions, including *rbcL*, have proven successful in discriminating sister species of *Acacia* (Newmaster & Ragupathy, 2009; Nevill *et al*., 2013). Also, molecular DNA barcoding has successfully confirmed the origin of ambiguously labelled seed collections (Nevill *et al*., 2013). The practical application of DNA barcoding using cpDNA regions is once more illustrated in this study, with the Lonely Tree and seedlings of unknown *Acacia* species having been successfully identified. The Bayesian and ML topologies were unable to group *Vachellia pachyceras* SA39405 within the clade containing the other exemplars of the species, and *V. pachyceras* SA39406 grouped with the *V. pachyceras* clade with high statistical support but distinct from it. This may suggest that more than one distinct group exists in the species, in conformity with Boulos (1995). Kyalangalilwa *et al*. (2013) described three subspecies of *Vachellia gerrardii* from Africa, including *V. gerrardii* var. *latisiliqua* (Brenan) Kyal. & Boatwr., found in Kenya. The *Vachellia gerrardii* trees that were sampled in this study formed a clade that is clearly distinct from *V. tortilis*, although a single *V. gerrardii* sample, Ke9, was clearly distinct from the other exemplars of the species. In our study, there is a single clade for *Vachellia tortilis* with no internal resolution. Considering that Kyalangalilwa *et al*. (2013) identified three subspecies, it is possible that all specimens in this study were from the same subspecies, either *Vachellia tortilis* subsp. *raddiana* (Savi) Kyal. & Boatwr. or *V. tortilis* subsp. *spirocarpa* (both are present in Kenya; Brenan, 1983).

Because subspecies are often subgroups naturally occurring in specific areas, a thorough and fine-scale phylogenetic study of *Vachellia pachyceras*, *V. tortilis* and *V. gerrardii* populations across the natural distribution range of the species is needed to clarify taxonomic delineation. Such a study should also target all *Acacia* species found in the Middle East and the Arabian Peninsula, to discriminate closely related species in the genus. Molecular barcoding techniques using *rbcL*, *psaB–rps14* and *trnL–trnF* markers could be used for such a study, because these have proven successful in

discriminating *Vachellia* species here. This is a significant contribution that addresses in part the recommendation of Bafeel *et al*. (2012) that further studies should be carried out to develop protocols to extend barcoding to cover a broader range of some arid plant species.

According to the Plant List (no date), *Acacia pachyceras* is an unresolved name, indicating that there is a need to determine whether it should be 'accepted' or regarded as a synonym. This could be done by delineating *Acacia pachyceras* using DNA-barcoding techniques (Newmaster & Ragupathy, 2009; Murphy *et al*., 2010; Nevill *et al*., 2013). Results from this study are consistent with *Vachellia pachyceras* being a distinct species rather than a synonym of *V. gerrardii* (Townsend, 1967; Boulos, 1995). Indeed, Boulos (1995), drawing heavily on Schwartz (1939) and on species' spatial distributions, described two varieties of *Vachellia pachyceras*, namely var. *pachyceras* and var. *najdensis*, and two subspecies, *V. iraqensis* and *V. gerrardii*. Our results suggest that *Vachellia pachyceras* could have more than one subspecies (see Fig. 4). Because the systematics of *Acacia s.l.* based on molecular sequence data is a 'hot topic', efforts are needed to describe phylogenetic relationships between all populations of *Vachellia pachyceras* in the Middle East and the Arabian Peninsula. To ensure maximum coverage of intraspecific diversity, a thorough sampling of *Vachellia pachyceras* populations in the Middle East and Arabian Peninsula, and investigation of their geographical structure using the cpDNA markers that were used in this study, would yield robust data useful for resolution of species (Wyler & Naciri, 2016).

Finally, the Bayesian and ML tree topologies in this study clearly indicate that the Lonely Tree, the nursery-grown Lonely Tree seedlings (putative Lonely Tree Laila, and nursery-grown Lonely Tree) and the nursery-grown Saudi Arabia seedling are all members of the *Vachellia pachyceras* clade, because their sequences were identical. These results confirm the identification of Dickson (1955) and Boulos & Al-Dosari (1994) of the Lonely Tree in the Talha area of Sabah Al-Ahmed Natural Reserve in Kuwait as *Vachellia pachyceras*. Further investigation would be needed, using highly variable genetic fingerprint markers such as microsatellites, if we are to determine whether the Lonely Tree, putative Lonely Tree Laila, nursery-grown Lonely Tree and nursery-grown Saudi Arabian seedling belong to the same genetic population, and if it is threatened (Khasa *et al*., 2006; Fredua-Agyeman *et al*., 2008).

In summary, these results represent a contribution to ongoing taxonomic studies of *Acacia s.l.* based on phylogenetics (Murphy *et al*., 2010) and DNA barcoding (Newmaster & Ragupathy, 2009). The application of DNA barcoding in this study has been effective in discriminating poorly differentiated *Acacia* groupings in the Middle East. Furthermore, molecular marker analyses have successfully identified the single remaining native representative of *Acacia s.l.* in Kuwait. The next step should be towards sampling native *Acacia* species in the Middle East, the Arabian Peninsula, and West, East and North Africa, and investigating their relationships using both morphometric and molecular characters. DNA barcoding and divergence time studies may help to resolve taxonomic uncertainties in complex species, but there is a need to reliably calibrate dating studies (e.g. with fossil evidence), to test the hypothesis

of continuous speciation (Rull, 2008). The discrimination of species in complex plant groups will gain precision if the origin of lineages in time can be quantified.

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