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THE SOUTH AFRICAN AND NAMIBIAN POPULATIONS OF THE RESURRECTION PLANT *Myrothamnus flabellifolius* ARE GENETICALLY DISTINCT AND DISPLAY VARIATION IN THEIR GALLOYLQUINIC ACID COMPOSITION

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Abstract—The polyphenol contents and compositions in desiccated leaves of *Myrothamnus flabellifolius* plants collected in various locations in Namibia and South Africa were analyzed using UV spectroscopy and high-performance liquid chromatography–mass spectrometry. A study of the genetic relatedness of these populations was also performed by determination of the DNA sequence of the intergenic spacer region between the *psbA* and the *trnH* genes in the chloroplast genome. Namibian *M. flabellifolius* plants contained significantly more polyphenols than South African plants. Namibian plants essentially contained a single polyphenol, 3,4,5-tri-*O*-galloylquinic acid, whereas South African plants contained a variety of galloylquinic acids including 3,4,5-tri-*O*-galloylquinic acid together with higher molecular weight galloylquinic acids. Sequence analysis revealed a 1.4% divergence between Namibian and South African plants corresponding to the separation of these populations of approximately 4×10^6 years. The significance of the polyphenol content and composition to the desiccation tolerance of the two populations is discussed.

Key Words—Polyphenol, mass spectrometry, Myrothamnaceae.

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INTRODUCTION

Resurrection plants are a unique group of plants, the majority of which occur in southern Africa, capable of surviving dehydration to an air-dried state (Gaff, 1971, 1977). The resurrection plant, *Myrothamnus flabellifolius*, is a woody shrub with a widespread distribution throughout southern Africa (Kruger, 1998), where it grows in shallow, well-drained crevices in sandstone and in granite outcrops (Child, 1960; Mauve, 1966). Populations of *M. flabellifolius* occur along the west coast of Namibia, in central southern Zimbabwe, and in north-eastern South Africa (see Glen et al., 1999 for a general distribution map). This region experiences high summer temperatures and irradiance, with extremes of water availability (Mauve, 1966). Growing conditions on the rocky outcrops are such that the plants must sometimes survive for a year or more in a dry quiescent state, rehydrating only after summer rainfall (Farrant and Kruger, 2001). In attempting to elucidate the "resurrection" mechanism, we recently noted an extremely high polyphenol content in the leaves of this plant, with the main polyphenol present in plants collected in Namibia determined to be 3,4,5-tri-*O*-galloylquinic acid. In addition, we demonstrated that 3,4,5-tri-*O*-galloylquinic acid was able to protect membranes against desiccation and oxidation damage (Moore et al., 2005). The Namibian and the Zimbabwean/South African populations of *M. flabellifolius* are not only separated by the Kalahari Desert (Puff, 1978; Glen et al., 1999), but these two populations also occur in different biomes, with the Namibian population occurring in a dry Karoo biome and the Zimbabwean/South African population occurring in a moist savannah biome (Sinclair et al., 2002). In this report, we have investigated whether the Zimbabwean/South African population of *M. flabellifolius* also has high concentrations of 3,4,5-tri-*O*-galloylquinic acid present in the leaves. Because this population was found to have a different polyphenolic profile from plants collected in Namibia, we also investigated the genetic variation between the two populations based on nucleotide sequence comparisons of regions of the chloroplast genome.

METHODS AND MATERIALS

Plant Material. Desiccated plant material was obtained from populations in Namibia (N1–N5), namely, the Komas Hochland, near the Cunene River, and around Outjo, as well as from populations in South Africa (S1–S4), namely, Vaalwater in the Limpopo Province, near Lydenberg, and the Blouberg Mountains, Mpumalanga Province (see Figure 1 for a map of the region). The dry leaf material was stored at 20°C in the laboratory until use.

Polyphenol Extraction, HPLC Analysis, and Mass Spectrometry. Phenolic extraction was performed by homogenizing leaf material for 5 min in MeOH/

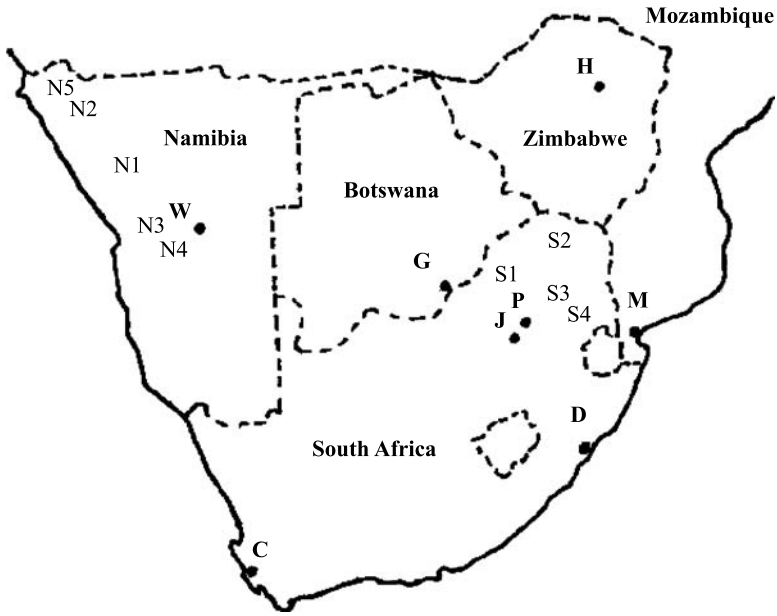

FIG. 1. Map of Southern Africa showing international borders. Major towns are shown as single letters—C: Cape Town; D: Durban; G: Gaberone; H: Harare; J: Johannesburg; M: Maputo; P: Pretoria; W: Windhoek. Collection sites in Namibia (N1–N5) were Outjo (N1), the Komas Hochland (N3 and N4), and near the Cunene River (N2 and N5). Collection sites in South Africa (S1–S4) were Vaalwater (S1), the Blouberg Mountains (S2), and near Lydenberg (S3 and S4).

H_2O (7:3, v/v) using a Polytron Homogenizer (Kinematica PT 2000). The homogenate was then subjected to sonication (Bandelin Sonorex bath sonicator) for 5 min, after which it was centrifuged (6000 × *g* for 5 min). The pellet was twice re-extracted using 3 volumes solvent per mass of sample. The supernatants were pooled, and the spectrum between 200 and 400 nm was determined. The polyphenol content was determined from the UV absorption at 280 nm using the previously measured molar extinction coefficient of 3,4,5-tri-*O*-galloylquinic acid of 21,000 (Altmann and Falk, 1995). Data reported are the mean ± standard deviation of three plants from each population analyzed in duplicate. Means were compared using a Student's *t* test with the level of significance set at $\alpha = 0.05$ (Microsoft Excel).

A 20-µl aliquot of the supernatant was subjected to analytical high-performance liquid chromatography (HPLC), performed on a Shimadzu LC-10A binary gradient system equipped with Photo-Diode Array detection (Shimadzu, Kyoto, Japan; Moore et al., 2005). Fractions were eluted from a

Jones chromatography RP-C18 (250 × 4 mm) column using a linear gradient between TFA/H_2O (1:1000, v/v) and TFA/acetonitrile (1:1000, v/v) over 40 min at a flow rate of 0.7 ml/min. MALDI-TOF mass spectra were obtained on a Perseptive Biosystems DE-PRO MALDI mass spectrometer equipped with a TOF analyzer (Perseptive Biosystems, Framingham, MA, USA). A 1-μl sample aliquot was dissolved in acetonitrile : water (1:1), mixed with 1 μl 2,5-dihydroxybenzoic acid matrix, and applied to a gold sample plate. The mixture was air-dried prior to use. The spectrometer was operated in positive and negative ion mode.

DNA Extraction, PCR Amplification, Purification, and Sequencing. Leaf material was frozen, lyophilized, and ground in a mortar using liquid nitrogen prior to DNA extraction. Total DNA was extracted using a modified CTAB DNA extraction protocol (Gawel and Jarret, 1991). The standard CTAB buffer was supplemented with 20 mg poly-(1-vinyl-pyrrolidine-2) (PVP; Merck, Darmstadt, Germany) and 1 ml of 2-mercaptoethanol : H_2O (1:1000, vol : vol; Gawel and Jarret, 1991). DNA purity was assessed using the A_{260}/A_{280} ratio and by agarose gel electrophoresis. The polymerase chain reaction (PCR) reaction mixture consisted of SuperTherm buffer, 0.025 U SuperTherm *Taq* polymerase, 5 mM $MgCl_2$, 0.1 mM dNTP, 0.33 μM each of the forward and reverse primers, and 3 μl template DNA in a total volume of 30 μl. The amplification program consisted of 95°C for 2 min, 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 2 min with a final extension of 72°C for 7 min. The forward and reverse primers were 5′GTTATGCATGAACGTAATGCTC3′ and 5′CGCGCATGGTGGATTCACAAATC3′, respectively. PCR reactions were supplemented with various amounts of soluble PVP (Merck) to reverse polyphenol inhibition (Koonjul et al., 1999). Amplified products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Product quantity and purity were estimated using agarose gel electrophoresis. Purified products were sequenced on a MegaBACE 1030 sequencer (Amersham BioSciences, Amersham, UK). DNA sequences were edited using Chromas 2.01 software (Technelysium PtyLtd., Helensvale, Queensland, AU). Alignment of sequences was performed using DNAMAN (Lynnon Corporation, Vaudreuil-Dorion, Quebec, CA) and MegAlign 3.08 software (DNASTAR, Madison, WI, USA).

RESULTS

We initially quantified the total leaf polyphenol content from plants collected in Namibia and in South Africa. Leaf material from each population was exhaustively extracted in MeOH/H_2O (7:3, vol : vol), and the polyphenol content was determined from the UV absorption at 280 nm. A significant difference in the total mean polyphenol content was observed between plants

TABLE 1. PERCENTAGE POLYPHENOL CONTENT IN DESICCATED LEAVES OF PLANTS COLLECTED FROM VARIOUS LOCATIONS IN NAMIBIA (N1–N5) AND SOUTH AFRICA (S1–S4)

Namibian locations	% Polyphenol content[a]	South African locations	% Polyphenol content
N1 Outjo	41.7 ± 6.7	S1 Limpopo	26.7 ± 0.7
N2 Cunene	30.1 ± 5.2	S2 Blouberg	21.7 ± 5.3
N3 Komas Hochland	28.3 ± 1.3	S3 Lydenberg	25.3 ± 4.6
N4 Komas Hochland	32.7 ± 6.7	S4 Lydenberg	22.9 ± 1.0
N5 Cunene	30.9 ± 0.1		
Average	32.7 ± 5.2	Average	24.4 ± 2.2

[a]Polyphenol content is expressed in 3,4,5-tri-*O*-galloylquinic acid equivalents in % g polyphenol per g dry weight. A minimum of three plants from each population was analyzed in duplicate. The data are expressed as the mean ± standard deviation; $P < 0.05$.

from Namibia and from South Africa. Plants collected in Namibia contained 32.7 ± 5.2% in contrast to plants collected in South Africa, which contained 24.4 ± 2.2% (Table 1, $P < 0.05$).

HPLC analysis of the extracted polyphenols was performed to determine whether South African *M. flabellifolius* plants contained the same polyphenol species as Namibian plants. The chromatograms obtained (Figure 2) revealed distinct compositional differences, the major difference being an abundance of a single polyphenol in Namibian plants (Moore et al., 2005). In contrast, South African plants contained a wide variety of different polyphenols. The main polyphenol present in leaves of Namibian plants (peak 1, Figure 2A) was ascribed to 3,4,5-tri-*O*-galloylquinic acid (Moore et al., 2005) and confirmed by mass spectrometry of the extract; this yielded a mass of 647 Da. The remaining phenolic peaks (peaks 2 and 3) in the chromatogram represented gallic and ellagic acid esters of 3,4,5-tri-*O*-galloylquinic acid. These assignments were made after mass spectrometry of the material eluting in these peaks yielded masses that varied from one another by an integral mass of either 152 Da, the mass addition of one extra galloyl group to the core molecule, or 298 Da, the mass addition of one extra ellagic acid moiety to the parent structure. A mass of 799 Da was found for the material eluting in peak 2 and a range of masses, namely, 799, 936, 953, 1570, and 1722 Da for that in peak 3. These latter masses were assigned to represent tetra-, penta-, nona-, and decagalloylquinate esters (Moore et al., 2005). In contrast, HPLC analysis of polyphenols extracted from leaves of South African *M. flabellifolius* showed that no single polyphenol was present in abundance. Instead, a variety of polyphenols were present, some of which were identical to those present in Namibian plants, but the majority of which eluted with longer retention times (Figure 2B). In general, there was a relationship between the molecular weight of the polyphenol and the HPLC retention time (Table 2). Fractions 1, 2, and 3 from South African plants had

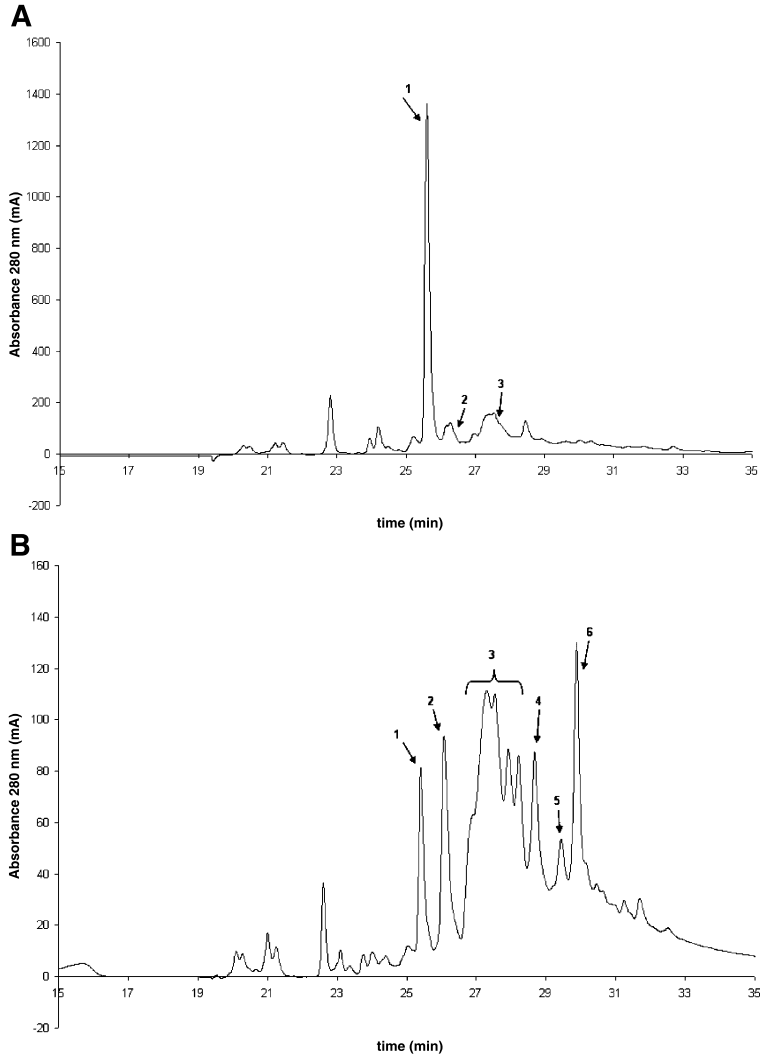

FIG. 2. High-performance liquid chromatography (HPLC) chromatograms of polyphenols extracted from leaves of *M. flabellifolius* collected in (A) Namibia and (B) South Africa and separated on a C_{18} column using a linear gradient between TFA/H_2O and TFA/acetonitrile as described in text. Fractions were pooled and lyophilized prior to analysis. The absorbance is shown in milliabsorbance units (mA). Note that the ordinate scale for (B) is 10^{-1} times of that shown in (A).

TABLE 2. ION SPECIES PRESENT IN VARIOUS HPLC FRACTIONS OF POLYPHENOLS EXTRACTED FROM LEAVES OF NAMIBIAN AND SOUTH AFRICAN PLANTS

Fraction number	R_t (min)	Galloyl groups	Quinic acid groups	M_r	Molecular ion[a]
1[b]	25.8	3	1	647[c]	$[M–H]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
2[b]	26	3	1	647[c]	$[M–H]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
		4	1	783[c]	$[M–OH]^-$
		5	1	936[c]	$[M–OH]^-$
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
3[b]	27	4	1	783[c]	$[M–OH]^-$
		4	1	799[c]	$[M–H]^-$
		5	1	936[c]	$[M–OH]^-$
		5	1	953[c]	$[M–H]^-$
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
4	29	4	1	767	$[M–2OH]^-$
		4	1	783[c]	$[M–OH]^-$
		5	1	936[c]	$[M–OH]^-$
		6	1	1171	2054 fragment
		7	1	1322	2203 fragment
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
		11	1	1874	$[M–H]^-$
5	29.7	4	0	636	$[M+CO–OH]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
				751	Unassigned
		6	0	912	$[M–OH]^-$
		5	1	936[c]	$[M–OH]^-$
		6	1	1088	$[M–OH]^-$
		7	1	1322	2203 fragment
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
		12	1	2054	$[M+K–2H]^-$
		13	1	2203	$[M+K–2H]^-$
6	30.1	4	0	636	$[M+CO–OH]^-$
		4	1	675[c]	$[M–C_6H_5O_3–H]^-$
				751	Unassigned
		5	0	882	2203 fragment
		5	1	936[c]	$[M–OH]^-$
		6	1	1088	$[M–OH]^-$
		7	1	1322	2203 fragment
		9	1	1570[c]	$[M–H]^-$
		10	1	1722[c]	$[M–H]^-$
		13	1	2203	$[M+K–2H]^-$

[a] $C_6H_5O_3$ refers to the pyrogallol fragment.
[b] Peak fractions common to both South African and Namibian plants.
[c] Molecular ions common to both South African and Namibian plants.

similar polyphenols as the same fractions from Namibian plants. Polyphenols that eluted in fractions 4–6 were unique to South African plants (Figure 3). As expected, the polyphenols in the later eluting fractions had higher masses, brought about by the addition of either gallic acid or ellagic acid moieties to the core molecule, 3,4,5-tri-*O*-galloylquinic acid (Table 2). A range of masses between *m*/*z* 636 and 2203 was evident; other masses present, e.g., 1322 Da, could be ascribed to the fragmentation of one of these parent molecules. Peaks displaying masses of 1874, 2054, and 2203 Da represent novel octa-, nona-, and decagalloylquinate ester molecules (assuming 3,4,5-tri-*O*-galloylquinic acid as the core molecule), respectively, each with the addition of a single ellagic acid moiety. These species were only found to be present in South African plants (Figure 2; Table 2). The final major peak eluting after approximately 30 min displayed masses of 882, 939, 1088, and 1322 Da. The mass of 1088 Da represents a hexagalloylquinic acid that was not found in plants collected in Namibia. We observed no change in galloylquinate ester composition between desiccated and hydrated plants (data not shown).

Because the two *M. flabellifolius* populations have distinct differences in their polyphenol content and composition, we investigated their genetic relatedness. The intergenic spacer region between *psbA* (photosynthetic subunit A)

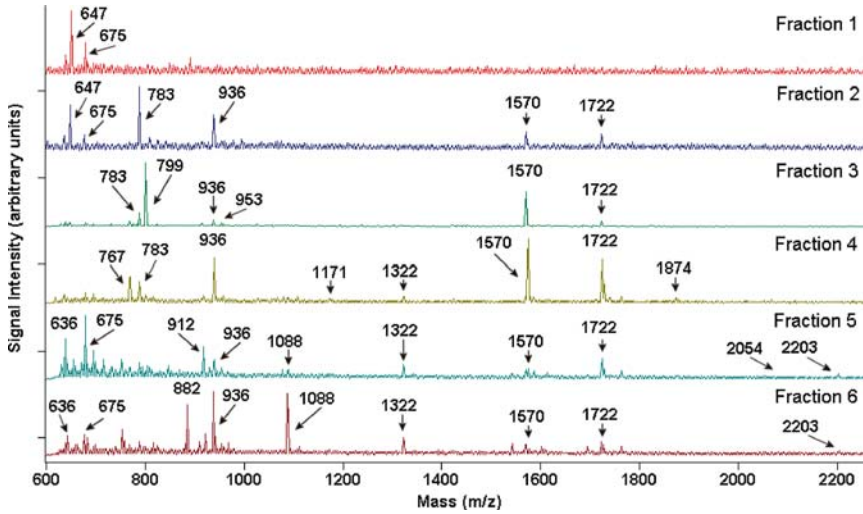

FIG. 3. Mass spectral analysis of HPLC fractions containing polyphenols extracted from leaves of *M. flabellifolius* collected in South Africa. Note that the ordinate scale is in arbitrary units and varies from fraction to fraction depending on the amount of material present.

and *trnH* (histidine amino acid biosynthesis) on the chloroplast genome was chosen. This region has been shown to evolve at a rate suitable for assaying population level differences (Aldrich et al., 1988; Sang et al., 1997). An approximately 400-bp fragment was consistently amplified from DNA extracts of both populations. Sequence analysis revealed that this fragment represented 288 bp of the intergenic spacer region with the remaining sequence derived from the 3′ and 5′ ends of *psbA* and *trnH*, respectively (Figure 4). The DNA

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S          CTAGCTGCTGTCGAAG.............CTCCATCTACAA      28
N          ----------------CCCCATCTACAA------------      40

S          ATGGATAAGACTTTGGTCTTAGTGTATACGAGTTTTTGAA      68
N          ----------------------------------------      80
                           3'↓
S          CTTAAAGGAGCAATAACCAATTTCTTGTTCTATCAAGAGG     108
N          ----------------------------------------     120

S          GTTGGTATTGCTCCTTTATTTAGTATTCTTTTATTTCTAT     148
N          ----------------------------------------     160

S          TTTCTTTTCATTTCTTTGCTTCAGCATAAGAAAAAAGAAA     188
N          ----------------------C-----------------     200

S          AAGTATTCTTATGGGTATTGAGTATCATACTTTTTTTCTG     228
N          ----------------------------------------     240

S          TACTAATCTCTAATTTTTCAAATAGAAAAATATTTTTCTG     268
N          ---------G----------------------C--------     280

S          ACTAATATTTGTATCTAAGAAGGAAGATAAGAAAGACTAA     308
N          ------------------------------------A---     320

S          ATGAAATAATTATAAATGGAATCCTTTCTAATTTGTAACT     348
N          ----------------------------------------     360
                                      ↓5'
S          TTCTAATTTGTAAATAGTATAGGGGCGGATGTAGCCAAGT     388
N          ----------------------------------------     400

S          GGATCAAGGCAGT                               401
N          -------------                               413
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FIG. 4. Chloroplast DNA sequence from leaves of *M. flabellifolius* representing the 3′ end of *psbA*, the intergenic spacer, and the 5′ end of *trnH* from plants collected in South Africa (S) and Namibia (N). Arrows denote the 3′ end of *psbA* and the 5′ end of *trnH*, respectively.

sequence of this region was identical for all plants collected from South Africa, but distinct from plants collected from Namibia, all of which displayed an identical sequence. Alignment of the 288-bp intergenic sequences obtained from all samples revealed four consistent differences between plants from South Africa and from Namibia. These differences occurred at positions 88, 154, 176, and 221 in the intergenic spacer, and represented a sequence divergence of 1.4% between the two populations. We also observed an insertion of 12 bp in *psbA* from all plants collected in Namibia (Figure 4).

The genus *Myrothamnus* is distantly related to the genus *Melianthus*, which has been proposed to have diverged from its sister group *Greyia* at a mutation rate of 0.0035 substitution per site per 10^6 years (Henning, 2003). Assuming an equal mutation rate, a divergence rate of 0.014 substitution per site yields a time of divergence for the Namibian and South African *M. flabellifolius* populations of approximately 4×10^6 years.

DISCUSSION

This work reports that the Namibian and South African populations of *M. flabellifolius* have distinct differences in their polyphenol content and composition. Whereas plants collected in Namibia essentially contained a single polyphenol, 3,4,5-tri-*O*-galloylquinic acid, South African plants contained a mixture of polyphenols that were derivatives of this molecule. Although both the Namibian and South African populations occur in similar geological environments, the rainfall patterns of the regions in which these populations occur are very different. South African plants grow in a region that experiences dry winters and regular annual summer rainfall. In contrast, Namibian plants grow in a more arid region of dry winters and irregular summer rainfall patterns. Farrant and Kruger (2001) reported that South African *M. flabellifolius* plants are only capable of surviving 9 months in a desiccated state. Whereas South African plants must only survive the dry winter months, Namibian plants must be capable of surviving not only a dry winter but also the ensuing dry summer and the following dry winter. In this instance, "dry" means that no rainfall whatsoever is experienced. Anecdotal evidence suggests that Namibian *M. flabellifolius* plants are capable of surviving for extended periods (3 or more years) in a quiescent state. We propose that the presence of the single polyphenol 3,4,5-tri-*O*-galloylquinic acid contributes toward this degree of desiccation tolerance. In support of this hypothesis, Moore et al. (2005) demonstrated that 3,4,5-tri-*O*-galloylquinic acid protects membranes against desiccation-induced damage as well as against free radical-induced oxidation.

M. flabellifolius in Africa south of the Sahara was originally divided on morphological grounds into three subspecies, namely, *M. flabellifolius sensu*

stricto, *elongata*, and *robusta*, the latter only occurring north of the Zambesi River in Zambia and Mozambique (Weimarck, 1936). A later revision resulted in the abandonment of *M. flabellifolius elongata* as a subspecies because of it being indistinguishable from *M. flabellifolius sensu stricto* (Puff, 1978). Because polyphenols do not display rapid turnover during plant growth and metabolism (Boudet et al., 1985; Haslam and Lilley, 1985), these molecules serve as useful chemotaxonomic markers for studies of plant systematics and evolution. Our data, therefore, suggest a new division of *M. flabellifolius* based on the phenolic compounds. This division would correlate with the biogeography of the species. We propose that there are two distinct populations on either side of the Kalahari Desert, respectively. This hypothesis is supported by the genetic data presented here that show that plants collected in Namibia were genetically identical and distinct from plants collected in South Africa, which were also genetically identical. The 1.4% genetic variation within the *psbA*/*trnH* intergenic region corresponds to a time of divergence of approximately 4 million years. This figure correlates with the climate change that occurred approximately 5 million years ago when the Karoo region along the western part of southern Africa changed from a wet to an arid region (Klak et al., 2004). Our hypothesis is that this climate change resulted in a requirement for Namibian population of *M. flabellifolius* to remain in a quiescent state for extended periods. The separation of the two populations by over 1000 km of desert presumably prevented cross-pollination and wind dispersal of the seeds (Child, 1960), resulting in two separate populations. Current research is focused on the relationship between the polyphenol content and the ability of Namibian plants to survive in a quiescent state.

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