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Was *Myriophyllum spicatum* L. (Haloragaceae) recently introduced to South Africa from Eurasia?

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Abstract

There is debate over the native or exotic status of *Myriophyllum spicatum* L. (Haloragaceae) in South Africa, which has important implications for developing and implementing management strategies. The aim of this study was to determine if *M. spicatum* was recently introduced from Eurasia by reconstructing the genetic relationships between South African and Eurasian *M. spicatum* using both a nuclear ribosomal (ITS1–5.8S–ITS2–26S) and a chloroplast intron (trnQ–rps16) sequence from 40 populations. For both these DNA markers, the South African populations were distinct from Eurasian populations, but always stemmed from a European origin. The data suggest that South African and European *M. spicatum* share a common ancestor, however the divergence of both markers are characteristic of a long period of isolation rather than a recent introduction from Europe. The genetic data from this study suggest that *M. spicatum* has not been introduced recently, but is most likely a native component of the South African flora.

1. Introduction

The geographic origin of exotic species is an important aspect of invasion biology (Goolsby et al., 2006; Le Roux and Wicczorek, 2009). Invasive species carry a genetic footprint into their introduced range that can often be traced back to the native geographic range (Petit et al., 2003; Taylor and Keller, 2007; Thum et al., 2011; Paterson et al., 2009; Paterson and Zachariades, 2013). Phylogeographic approaches using molecular markers are effective in determining invasion history (Schaal et al., 2003) and have been applied to several introduced plant species to determine their geographic origins and understand their introduction history (e.g. Madeira et al., 1997; Novak and Mack, 2001; Gaskin et al., 2005; Goolsby et al., 2006; Taylor and Keller, 2007; Paterson et al., 2009; Gaskin et al., 2011; Thum et al., 2011; Paterson and Zachariades, 2013).

There is debate over the native status of *Myriophyllum spicatum* L. (Haloragaceae) in South Africa. Van der Meijden and Caspers (1971) consider *M. spicatum* a native component of the aquatic flora in the highlands of East Africa, and according to their distribution map, the whole of southern and East Africa is part of the native distribution. The natural distribution of *M. spicatum* described by Cook (1985) did not include South Africa. However, Cook (2004) later treated *M. spicatum* as native to South Africa, Namibia and Botswana

because there was no evidence to suggest it was introduced and was not considered problematic (Cook personal communication). In addition to this, several ecological studies in South Africa have not made any reference to *M. spicatum* being introduced or problematic, and often refer to it as an important component of the aquatic flora of the region (Bolt, 1969; Bolt et al., 1969; Breen and Jones, 1971; Allanson et al., 1974; Jacot Guillarmod, 1976; Howard-Williams, 1979; Jacot Guillarmod, 1979).

Conversely, Mendes (1978) reports in Flora Zambesiaca that the genus *Myriophyllum* is represented by two exotic species south of the Sahara, namely *Myriophyllum aquaticum* (Vell.) Verdc. and *M. spicatum*. In South Africa, *M. spicatum* is listed under the National Environment Management: Biodiversity Act (NEMBA), 2004 and Conservation of Agricultural Resources Act (CARA), 1983 as a Category 1 weed, which is the highest category an exotic weed can attain. This status was applied as a precautionary measure due to the plant's weedy behavior in other parts of the world, especially the U.S.A. (Henderson personal communication). Despite being recorded in the country since 1829 (Weyl and Coetzee, 2014), it has only been reported as being problematic as recently as 2005 (Coetzee et al., 2011).

The debate over whether *M. spicatum* is native or introduced to South Africa has important implications for developing and implementing a control programme (Coetzee et al., 2011), especially whether or not biological control would be feasible. There are very few cases where biological control has been used on a native weed. Therefore, the aim of this study was to investigate the genetic relationships between populations of *M. spicatum* in South Africa versus those in the native Eurasian range to determine whether there is evidence for recent introduction(s) into South Africa. If *M. spicatum* was recently introduced from Eurasia, we would expect that South African populations will share haplotypes with Eurasian populations or have haplotypes that are nested within Eurasian haplotypes networks. In contrast, the identification of haplotypes that are unique to South Africa would provide evidence that the species was not recently introduced.

2. Materials and methods

A total of 40 populations of *M. spicatum* were sampled from throughout the native Eurasian range and South Africa (Table 1). At least 10 apical sections ~5–10 cm long were collected from each population, loosely wrapped in paper towels, and placed in a Ziplock bag with silica gel. All plants were cleaned before drying to minimize sources of DNA contamination from other organisms (e.g. periphyton). GPS records and in most cases, a photograph of the plants was taken for identification purposes. DNA was extracted from dried leaf material or apical meristems using the DNeasy Plant Mini Kits (Qiagen) following the manufacturers' protocol.

The internal transcribed spacer regions (ITS) of the nuclear ribosomal DNA and a chloroplast DNA (cpDNA) intron *trnQ-rps16* were used in this study. The ITS region has previously been shown to provide intraspecific variability among *Myriophyllum* (Moody and Les, 2010; Thum et al., 2011); and has been used to identify multiple genotypes in introduced *M. spicatum* populations (Zuellig and Thum, 2012). The cpDNA intron *trnQ-rps16* was chosen from a suite of introns for its high intraspecific variability in *M. spicatum*.

The nuclear ribosomal DNA Internal Transcribed Spacers 1 and 2 and the intervening 5.8S ribosomal subunit (hereafter ITS) was amplified using the universal primers ITS1 and ITS4 (Soltis and Kuzoff, 1995). The PCR reactions for the ITS region followed the standard protocol outlined in Thum et al. (2011) and contained the following: 1 µl of PCR buffer (Invitrogen), 2 mM MgCl₂, 0.2 M each primer, 0.2 M each dNTP, 1 unit of *Taq* DNA polymerase (Invitrogen), 1 µl template DNA and brought to a total volume of 10 µl with sterile, distilled and deionized water. Thermal cycling protocol was as follows: one cycle at 94 °C for 2 min followed by 25 cycles of 94 °C for 1 min, 53 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 8 min. 1X PCR buffer

The chloroplast DNA *trnQ-rps16* intron region (hereafter cpDNA) was amplified using the primers *trnQ*^(UUG) and *rps16* × 1 (Shaw et al., 2007) followed the protocol of Shaw et al. (2007). The PCR reactions for the cpDNA region contained the following: 1 µl of PCR buffer (Invitrogen), 2 mM MgCl₂, 2 µmol each primer, 0.2 mM each dNTP, 1 unit of *Taq* DNA polymerase (Invitrogen), 1 µl template DNA and brought to a total volume of 10 µl with sterile, distilled and deionized water. The thermal cycling protocol was as follows: one cycle at 80 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 50 °C for 1 min (followed by a ramp of 0.3 °C/s to 65 °C), 65 °C for 4 min and a final extension step of 5 min at 65 °C.

All PCR products were visualized on 1.5% agarose gel in TAE buffer stained with Ethidium Bromide or SYBR Safe (Life Technologies) to check for size and purity. The remaining DNA was treated with the enzymes Exonuclease I (New England Biolabs) and Antarctic Phosphatase (New England Biolabs) or ExoSAP-IT (Affymetrix) to eliminate unincorporated primers and dNTPs before sequencing.

The PCR products were sequenced using the same primers used for PCR and BigDye terminator chemistry (Applied Biosystems) on ABI 3130xl and 3730xl DNA sequencers. In cases where the ITS PCR products did not produce clean and unambiguous sequences, the PCR products were cloned using the TOPO TA cloning kit (Invitrogen) and 5–10 positive inserts were sequenced.

Chromatograms were assembled and edited in Sequencher 4.2 (GeneCodes Corp). ITS sequence alignments were proofread manually and aligned using ClustalW as implemented in Mega 3.1 (Kumar et al., 2004) with default parameters. The cpDNA was manually aligned using MacClade 4.08 (Maddison and Maddison, 2005) and indels were determined following Moody and Les (2010) and expressed as binary characters (T/A) at the end of the sequence. Where singletons were present in the ITS dataset for cloned individuals (one base change in a single sequence), these were replaced with a “?” to take a conservative approach and reduce the possibility of cloning error. When the ITS sequence was identical for different individuals from the same location, only one sequence was used for further analysis. There were a total of 18 indels identified in the cpDNA region for the *M. spicatum* samples that were used.

Haplotype networks were constructed for both ITS and cpDNA using statistical parsimony (Templeton et al., 1992), as implemented in the program TCS version 1.21 (Clement et al., 2000), with a 95% connection limit, with gaps treated as missing data. This methodology is especially useful for estimating genealogical relationships at the population level where ancestral haplotypes are present and traditional phylogenetic methods require larger numbers of variable characters than are not usually available at the population level. Haplotype networks have been successfully used in several studies to identify relationships at the population level with submerged macrophytes (Thum et al., 2011; Zuellig and Thum, 2012).

3. Results

The final ITS alignment was 671 bp. Eight unique ITS haplotypes were identified among the samples of *M. spicatum*, which was represented as a network (Fig. 1). One loop was identified in the ITS haplotype network between the H₆, H₇ and H₈ haplotypes, all of which occur in South Africa.

The ITS haplotypes found in South African populations (H₆, H₇ and H₈) were not found in any Eurasian populations, but geographically disparate South African populations shared haplotypes (Fig. 1; Table 2). For example, Lake Sibaya, Western Cape and Vaal River shared the ITS haplotype H₇, and the Hogsback and the Vaal River populations shared ITS haplotype H₆ (Fig. 1).

Several western European populations of *M. spicatum* (Belgium, France, Germany, Denmark, Scotland and Sweden) shared the same haplotype, H₁ (Fig. 1). There was little diversity at the ITS region for populations in Europe or Asia. The France population, FR03, shared the H₅ haplotype with the Scotland population, SC01, with both of these populations being one mutational step away from the H₁ haplotype (Fig. 1; Table 2). The populations collected in Asia were between two and three mutational steps from H₁ haplotype, separated by the H₂ haplotype, which is European dominated, between the Asian H₃ and H₄ haplotypes and the ancestral haplotype, H₁.

The final alignment of the cpDNA was 727 bp including the 18 indels. There were 14 unique haplotypes represented in the network (Fig. 2; Table 2). One loop was identified in the haplotype network involving the European and Asian lineages (Fig. 2). It was not possible to obtain cpDNA for all populations represented in the ITS alignment, however sequences from each of the major ITS haplotypes in South Africa, H₆ and H₇, Lake Sibaya/Western Cape/Vaal River and Vaal River/Hogsback, were obtained.

Table 1

The locations of the samples of *Myriophyllum spicatum* that were collected for the ITS and cpDNA analysis. The “Y” indicates whether the DNA sequencing was successful for each of the markers, ITS and cpDNA.

Code	Location	Latitude	Longitude	ITS	cpDNA
AF003	Vaalharts Weir, Vaal River, South Africa	−28.0916	24.9682	Y	
AF004	Barkley West, Vaal River, South Africa	−28.5503	24.5317	Y	
AF005	Lake Sibaya, Zululand, South Africa	−27.36	32.7132	Y	Y
AF007	D. Impson pond ex. Nuwejaars River, South Africa	−34.676	19.914	Y	Y
AF008	Vaalharts Weir, Vaal River, South Africa	−28.09164	24.96824	Y	Y
AF009	Klipplaat River, Hogsback, South Africa	−32.49387	26.94959	Y	Y
AF011	Prieska, Orange River, South Africa	−29.65969	22.74911		Y
Belg001	Dessel/SchötenChannel, Retie, Belgium	51.27733	5.15074	Y	
Belg002	Dessel/SchötenChannel, Turnhout, Belgium	51.32692	4.92699	Y	
Belg004	Dessel/SchötenChannel, Sint-Job-in-t'-Goor, Belgium	51.29949	4.56313	Y	
DEN001	Gudenå, Denmark	56.383561	9.727819	Y	Y
DEN004	Stilling Lk, Denmark	56.06448	10.02274	Y	
DEN005	Knudsø, Denmark	56.104162	9.783341	Y	
DEN007	Margrethe Kos, Denmark	54.937196	8.703486		Y
FR001	Yvonand, France	46.802845	6.732063	Y	Y
FR002	Camargue, France	43.68	4.63	Y	Y
FR003	Lac du Couze, France	45.098948	1.462235	Y	Y
FR004	Bandeville, France	48.57626	2.026683	Y	Y
FR005	Herepian, France	43.589261	3.102808	Y	
FR007	Lacoste, France	43.560184	3.302629	Y	
FR008	Gignac, France	43.652202	3.53083	Y	
GRM001	Were River, Lohne, Germany	52.18489	8.66707	Y	
GRM004	River Oker, Braunschweig-WatenbYttel in Lower Saxony, Germany	52.31056	10.46528	Y	Y
SCO001	Loch Leven, Scotland	56.206069	−3.37728	Y	Y
SWE001	Hudiksvall, Sweden	61.720232	17.13665	Y	Y
SWL001	River Treene, lpland (near Treia) in Schleswig-Holstein	54.53034	9.28958	Y	
IND001	Kasmir University	34.133122	74.84200	Y	
CH1825	Behai, TengChong, Yunnan province, China	25.1205	98.5586	Y	Y
CH1775	Laogao yu tang, Mjan ning, Sichuan province, China	28.538727	102.1569	Y	
CH3592	Daga Town, Qushui county, Tibet	29.336746	90.68417	Y	Y
CHN002	Wuhan Botanical Gardens, Wuhan City, Hubei Province, China	30.545264	114.4180		Y
CHN003	Kunming Lake of Summer Palace, Beijing, China	39.993662	116.2610		Y
CHN004	Nahu Park, Changchun City, Jilin Province, China	43.851125	125.2991		Y
CHN005	YanQi Lake, Yanqing District, Beijing City, China	40.396699	116.6768		Y
WU 40	Bosten Lake, Xinjiang, China	41.9067	86.7314	Y	
WU 49	Bosten Lake, Xinjiang, China	41.9067	86.7314		Y
WU 120	Fuyang, Zhejiang, China	29.9944	119.6944	Y	Y
WU 127	Tai Lake, Jiangsu, China	31.2228	120.4461		Y
WU 128	Tai Lake, Jiangsu, China	31.2228	120.4461		Y
KOR001	Shihwa Lake, Seonah Jeong, Jihee Kim, South Korea	37.2792	126.6242		Y

Fig. 1. Statistical parsimony haplotype network constructed in TCS version 1.21 (Clement et al. 2000) using the ITS sequence of *Myriophyllum spicatum*. The haplotypes are color coded by country, and each population that contains a haplotype is presented in Table 1. The small uncolored circles indicate an additional mutational step between haplotypes detected. The circle sizes are proportional to the number of populations from where that haplotype was found.

Fig. 2. Statistical parsimony haplotype network constructed in TCS version 1.21 (Clement et al. 2000) using the cpDNA sequence of *Myriophyllum spicatum*. The haplotypes are color coded by country, and each population that contains a haplotype is presented in Table 1. The small uncolored circles indicate an additional mutational step between haplotypes detected. The circle sizes are proportional to the number of populations from where that haplotype was found.

The South African populations consisted of two haplotypes, H_L and H_N , which is expected given their geographic isolation from each other. None of the South African haplotypes were found in any of the Eurasian populations, and are distinct from Eurasian haplotypes by a minimum of 4 mutational steps (Fig. 2).

The largest haplotype from the cpDNA network, H_A , included populations from France, Denmark and Scotland (Fig. 2; Table 2). There is a loop connecting the this haplotype and another haplotype found in populations from France and Denmark (H_J) to a group containing samples from a wide geographic area of China (H_B , H_C , H_D , H_E , H_F and H_G). These Chinese haplotypes are at least four mutational steps removed from all European haplotypes (Fig. 2; Table 2). The South Korean haplotype, H_M , stems from the European haplotype H_J differing by five mutational steps. The South African haplotypes also stem from haplotype H_J differing by 4 mutational steps. There was a relatively large amount of variability in the cpDNA region for all the biogeographical regions including the Asian, European and South African populations sampled (Fig. 2).

4. Discussion

The results from both the ITS and cpDNA analyses demonstrate that the South African populations of *M. spicatum* sampled in this study are unique not appearing in populations sampled throughout the European and Asian range. The South African populations do not share any ITS or cpDNA haplotypes with either the European or Asian populations sampled in our study. If the South African *M. spicatum* were introduced from Eurasia, it would be expected that populations would share ITS and/or cpDNA haplotypes with samples from the Eurasian range, or for South African haplotypes to be nested within the European and Asian accessions through the network analyses. Thus, the genetic data do not support the hypothesis that *M. spicatum* was recently introduced from Eurasia.

The data from this study were unable to identify a region or population of origin in Eurasia for *M. spicatum* in South Africa, however, the divergence stemming from the European lineage for both ITS and cpDNA suggests that the European and South African plants share a common ancestor. The important questions raised from this analysis, and which have important implications for

determining the status (native versus introduced) of *M. spicatum* in South Africa, are the time of divergence between the South African and European lineages, and the mechanism(s) of dispersal into South Africa (natural versus anthropogenic).

Many aquatic plant introductions have been linked to the aquarium plant trade (Madeira et al., 2007; Martin and Coetzee, 2011). The first record of *M. spicatum* in South Africa, in 1829, predates the aquarium trade (Weyl and Coetzee, 2014), but South Africa does have a long history of cultural and economic exchange with Europe (Les and Mehrhoff, 1999), and it is plausible that *M. spicatum* could have been introduced from Europe around that time. Given the genetic distances between South African and European haplotypes in our study however, it is unlikely that the common ancestor of South African and European *M. spicatum* coincides with the first record in South Africa. The divergent South African ITS and cpDNA alleles are more consistent with long-term geographic isolation rather than a recent introduction. Alternatively, it is possible that the South African samples originate from European ancestors that were either not sampled or have gone extinct. However, we find this unlikely because the divergence between South African and European haplotypes was greater than the divergence among European haplotypes. Analyses of additional molecular markers and additional geographic sampling, especially geographic regions that may be dispersal routes including North Africa and the Arabic Peninsula, could be used to further test this hypothesis.

It is common for aquatic plants to have wide geographical ranges, and in many cases to have intercontinental disjunct distributions (Santamaría, 2002; Les et al., 2003), which makes natural dispersal of *M. spicatum* to South Africa plausible. Many species found in both Eurasia and South Africa have long evolutionary histories, with divergence times dating back to between the Cretaceous and the Eocene, with many of these distributions linked to continental drift (Les et al., 2003; Mao et al., 2012; Baker and Couvreur, 2013). However, the divergence of *M. spicatum* and its sister species, *Myriophyllum sibiricum* Kom. was in the Quaternary approximately 5 million years ago (Chen et al., 2014) which is considered recent in geological time. This distribution across continents is relatively common in aquatic taxa, where many species with recent divergence times, 2.5 million years or less, have

Table 2

The haplotype allocations for both ITS and cpDNA for each *Myriophyllum spicatum* population identified by the statistical parsimony haplotype network. * Refers to the GenBank Accession numbers for the sequence relating to each haplotype.

Code	ITS	cpDNA
AF003	H ₆	–
AF004	H ₆	–
AF005	H ₇	H _L
AF007	H ₇	H _L
AF008	H ₆ , H ₇	H _L
AF009	H ₆ , H ₈	H _N
AF011	–	H _L
BELG001	H ₁	–
BELG002	H ₁	–
DEN001	H ₁	H _A
DEN004	H ₁	H _A
DEN005	H ₂	H _J
DEN007	–	H _J
FR001	H ₁	H _J
FR002	H ₁	H _A
FR003	H ₅	H _I
FR004	H ₁	H _J
FR005	H ₁	–
FR007	H ₁	–
FR008	H ₁	–
GRM001	H ₁ , H ₂	–
GRM004	H ₁	H _K
SCO001	H ₁ , H ₂	H _A
SWE001	H ₁ , H ₂	H _H
SWL001	H ₂	–
IND001	H ₃	–
CH1825	H ₃	H _D
CH1775	H ₃	–
CH3592	H ₃	H _G
CHN002	–	H _F
CHN003	–	H _C
CHN004	–	H _B
WU 40	H ₄	–
WU49	–	H _E
WU 120	H ₄	H _F
WU127	–	H _F
WU128	–	H _F
KOR001	–	H _M

*GenBank accession numbers for each haplotype: H₁: KT783507; H₂: KT783508; H₃: KT783509; H₄: KT783510; H₅: KT783511; H₆: KT783512; H₇: KT783513; H₈: KT783514; H_A: KT783493; H_B: KT783494; H_C: KT783495; H_D: KT783496; H_E: KT783497; H_F: KT783498; H_G: KT783499; H_H: KT783500; H_I: KT783501; H_J: KT783502; H_K: KT783503; H_L: KT783504; H_M: KT783505; H_N: KT783506

disjunct distributions between continents or major geographical barriers (Les et al., 2003). There has been a lot controversy over the mechanisms of long distance dispersal between the continents (Sculthorpe 1967; Clausen et al., 2002), however, natural dispersal from Europe to South Africa via historical water bodies connecting north of the Sahara to the rest of Africa (Drake et al., 2011) could be possible through bird dispersal (Clausen et al., 2002) in the last 5 million years.

The data presented thus far do not provide evidence for the recent anthropogenic introduction of *M. spicatum* from the Eurasian range, despite the extensive sampling across this region. The data are more consistent with geographically isolated populations that shared a common ancestor that predates the opportunities for anthropogenic introductions. It is, of course, possible that additional sampling and genetic analysis would provide evidence for a recent introduction of *M. spicatum*, but our data provide no evidence of such. Therefore, we conclude that *M. spicatum* is more likely a native component of the South African flora than a recent introduction from Eurasia.

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