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Population genetics of invasive and native *Nymphaea mexicana* Zuccarini: taking the first steps to initiate a biological control programme in South Africa.

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Abstract

Nymphaea mexicana Zuccarini (Nympheaceae) (Mexican waterlily) is a rooted floating-leaved aquatic plant native to southern USA and Mexico that has become a problematic invasive alien plant in South Africa. Biological control is considered a desirable management strategy for the plant in South Africa. A good understanding of the genetic structure of invasive populations has been useful in other biological control programmes because taxonomic uncertainty about the target plant can result in natural enemies that are not adapted to the invasive populations being considered as potential agents. For *N. mexicana*, hybrids exist in the wild and horticultural trade, but identification is difficult, so understanding the genetic structure of populations is required to ensure that potential agents are collected off plants similar to invasive populations in South Africa. ISSR (inter-simple sequence repeats)

analysis was used to determine whether invasive *N. mexicana* populations from South Africa were genetically similar to native range populations from USA or whether they were hybrids. Results from these analyses were matched with the morphotypes of each population based on petal colour, shape, and size. The genotypes suggested by the ISSR analyses corroborated the presence of both hybrid and pure forms of *N. mexicana* in South Africa. Populations of *N. mexicana* in the invaded range that are genetically similar to native range populations are more likely to be suitable for biological control, while other populations are likely to be hybrids formed by crossing of parents from the native range or within the horticultural trade, which may present difficulties for management using biocontrol.

Keywords: Mexican waterlily, yellow waterlily, hybrid, molecular markers, biological control

1. Introduction

Alien plant invasions cause many environmental, social, and economic problems, and are thus important to manage (de Lange and van Wilgen, 2010). Classical biological control (hereafter referred to as 'biological control') is an environmentally friendly, effective, and cost-efficient means of controlling invasive populations (de Lange and van Wilgen, 2010). This method employs host-specific natural enemies from the native range of the alien plant to manage invasive populations (Müller-Schärer and Schaffner, 2008). However, understanding how the genotypes of invasive plant populations compare to native populations is important because taxonomic uncertainty can hamper biological control efforts. For example, proper identification of the target plant is important to develop test plant lists, determine agent host specificity and compatibility with the target plant, and locate sites for exploratory surveys in the native range from where potential biological control agents could be collected (Gaskin et al., 2011).

The presence of hybrids of the target plant in the invaded range may inhibit the success of biological control programmes. Hybridisation introduces genetic variation in populations, and may result in the inheritance of traits that lead to higher fitness (Arnold et al., 2008; Latta et al., 2007) and thus potentially greater invasiveness. Insect herbivores may be adapted to feed on specific genotypes of a host plant species, and would thus be ineffective as biological control agents for multiple genotypes of a target plant (Goolsby et al., 2006a; Urban et al., 2011). Other insect herbivores may be able to optimally survive on multiple genotypes but

may also pose a risk to non-target species as a result of this broadened host range. Hence, it may be more difficult to find biological control agents that effectively manage hybrid plants with high genetic variability, without increasing the risk of non-target effects (Zalucki et al., 2007). Furthermore, hybrid plants may show varying levels of resistance to herbivory compared to their parent plants (Fritz et al., 1999; Whitham et al., 1994). While lowered resistance to herbivory would be beneficial for biological control programmes, heightened resistance could reduce the success of biological control. It is thus important to develop an understanding of the genetic structure of invasive alien plants and to determine whether invasive populations are hybrids or not during the early stages of biological control programmes.

Invasive populations will also be more efficiently managed by resolving taxonomic uncertainties so that biological control agents can be prioritised based on their adaptation to specific forms (Goolsby et al., 2006b). For example, the nomenclature and taxonomic status of *Lantana camara* Linneaus (Verbenaceae) is confused and unresolved as a result of genetic modification through hybridisation and horticultural selection (Urban et al., 2011). As a result of this and other factors, biological control of this plant is an ongoing challenge, as the varieties and/or hybrids of *L. camara* contain different compositions of allelochemicals that potential agents are not adapted to overcome, or if they are, are then not host specific enough to release (Urban et al., 2011).

Hybrids often combine characters from both parents, which creates forms with intermediate character states. Hence, it becomes difficult to distinguish hybrids based solely on morphological characters, so genetic analyses are necessary to discern species and hybrids. Molecular markers such as random amplified polymorphic DNA (RAPD) markers, amplified fragment length polymorphisms (AFLP), and inter-simple sequence repeats (ISSRs) are effective at distinguishing between genetically similar individuals and can be used to detect temporal and spatial patterns, modes of dispersal, sources of invasive species, and genotypes within clonal populations (Le Roux and Wieczorek, 2009). In addition, molecular techniques provide information about hybridisation (Vilà et al., 2000), population structure (Culley and Wolfe, 2001), and cryptic speciation (Canavan et al., 2020) that are not reflected in morphological characteristics.

Nymphaea mexicana Zuccarini (Nymphaeaceae) is a rooted floating-leaved aquatic plant with yellow petals, horizontal stolons, and vertical rhizomes (Jacobs and Hellquist, 2011), that is native to southern USA and Mexico (Figure 1). Through introduction via the horticultural trade, this plant has been introduced and become invasive in countries including Australia, New Zealand, India, Europe, Spain, and South Africa (Figure 1) (Gaertner et al., 2016; Garcia-Murillo, 1993; Henderson, 2010; Hussner, 2012; Johnstone, 1982; Newfield and Champion, 2010; Shah and Reshi, 2012). In the invaded range, *N. mexicana* infestations restrict water movement, decrease recreational value of water bodies, reduce water quality, and reduce gas exchange (Capperino and Schneider, 1985; Hofstra et al., 2013). *Nymphaea mexicana* has established in dams, ponds, and rivers in South Africa, and is listed as a Category 1b invasive plant according to the National Environmental Management: Biodiversity Act (No. 10 of 2004) (NEM:BA), which prohibits trade or planting, and legally requires that the species is managed. This species is recorded in seven out of the nine provinces in the country. Mechanical removal is difficult as the plant is able to resprout from rhizome fragments left in the soil, while chemical control results in depleted oxygen levels as the rhizomes die after being treated by herbicides, which in turn negatively impacts aquatic fauna (G-MW, 2009; Hofstra et al., 2013). Furthermore, mechanical and chemical control is not effective in the long term, as regrowth occurs within 8-12 months of treatment (G-MW, 2009; Hofstra et al., 2013). In contrast, biological control is environmentally friendly and cost-efficient, and is thus a desirable control strategy for *N. mexicana*. *Nymphaea mexicana* is a novel target for biological control, because no biological control programme has been developed against this invasive aquatic weed worldwide, so novel agents must be imported from the native distribution.

Figure 1: World distribution of *Nymphaea mexicana*. Circle icons indicate native range; diamond icons represent introduced range. Mapped in ArcMap (Environmental Systems Research Institute, 2014) using distribution data from GBIF (GBIF.org, 2021).

The genus *Nymphaea* comprises between 40 to 50 phenotypically diverse species, which also have high levels of morphological plasticity (Borsch et al., 2007). Hybridisation of *Nymphaea* species is not uncommon in the wild (Borsch et al., 2014), and the aesthetic appeal of the genus has resulted in the creation of numerous horticultural hybrids which are sold in nurseries around the world, including USA and South Africa. Hybrid forms of *N. mexicana* and its relatives have been recorded invading aquatic systems in many countries including South Africa (Borsch et al., 2014; Dana et al., 2017).

According to Verdcourt (1989), there are five varieties of *Nymphaea nouchali* Burm. f. native to Africa, namely var. *petersiana* (also treated as a synonym of *Nymphaea capensis* by Conard (1905)), var. *ovalifolia* (Conard) Verdc., var. *caerulea* (Savigny) Verdc., var. *mutandaensis* Verdc., and var. *zanzibariensis* (Casp.) Verdc. These, in addition to *Nymphaea lotus* L., are native to South Africa. Borsch et al. (2007) however, suggest that these varieties should instead be treated as separate species based on molecular evidence, and even recommend that *N. petersiana* be moved from the subgenus *Brachyceras*, where *N. nouchali* is classified, to subgenus *Lotos*. This recommendation is also supported by Löhne et al., (2007). Based on this more recent molecular analysis, four species may be considered indigenous to South Africa: *Nymphaea caerulea* Savigny, *Nymphaea capensis* Thunb., *Nymphaea lotus* L., and *Nymphaea petersiana* Klotzsch (USDA Agricultural Research

Service, 2021; John Wiersema pers. comm.). Two of these species, *N. capensis* and *N. caerulea*, belong to the subgenus *Brachyceras*, while *N. lotus* and *N. petersiana* (accepting the recommendation by Borsch et al. (2007)) are placed in the subgenus *Lotos*. Hence, there is some separation from *N. mexicana*, which is placed in subgenus *Nymphaea* (Borsch et al., 2007). Any biological control agents that are released against *N. mexicana* in South Africa must therefore be restricted in host range to the level of subgenus or lower in order to avoid non-target damage. The presence of hybrids in South Africa may be problematic for the biological control programme because they may not be suitable hosts for biological control agents of the 'pure' *N. mexicana,* and agents with a broad enough host range to feed on both hybrids and 'pure' forms of the plant may not have a sufficiently restricted host range to warrant release.

The aim of this study was to determine which *N. mexicana* populations in South Africa are hybrids, and which group are similar to 'pure' *N. mexicana* from the native range, in order to determine which populations are suitable targets for biological control. ISSR molecular markers and observations of floral morphology were used to achieve these goals by comparing samples from the invaded range in South Africa, and the native range in southern USA. This study forms part of the initial phases of a biological control programme for this plant in South Africa and will be useful to develop programmes in other countries where *N. mexicana* is invasive.

2. Material and methods

2.1. Sampling

Sampling was restricted to southern USA for this study. At each site, four to 17 healthy, whole leaves were collected at least 5 m apart from each other to avoid the resampling of clones and to include a representative sample of genetic variation at the sites. A greater number of samples were collected from sites that had greater areas (for example, in a large water body where there were multiple patches of *N. mexicana*). The leaf samples were rinsed with freshwater and wiped dry with paper towels to remove extraneous material and epiphytes. The samples were then wrapped in paper towelling and stored individually in clear plastic Ziploc bags containing approximately 30 g of silica gel or equivalent desiccant, which was changed as needed to desiccate the leaf material and ensure dry storage. Seventeen sites

were sampled in the invaded range in South Africa and 18 sites were sampled across south-eastern USA including Florida, Louisiana, and Texas (Table 1).

To obtain morphological data, photographs were taken of the flowers at selected sites in both the native and invaded range. These were used to classify selected populations as hybrids or 'pure' *N. mexicana* forms using morphological characteristics such as petal color and structure, in addition to the genetic groupings. 'Pure' forms were those that possessed the typical traits of *N. mexicana* according to the description by Capperino and Schneider (1985), and that looked more similar to populations samples from the native range.

171 Table 1: Details of invasive and native range sites of *Nymphaea mexicana* used for genetic matching using ISSR analysis. The number of
172 samples varied due to unequal sampling and removal of low-quality samples. Where large numbers of samples were used (more than four
173 samples for the invaded range), they were collected from multiple sites within the same area.

Province/State	**Locality**	**Latitude**	**Longitude**	**Number of DNA samples used**	**Sample code**
Invaded range: South Africa					
Western Cape	Muizenberg, Westlake	-34.0842	18.4438	17	WL
	Neil Ellis, Stellenbosch	-33.9249	18.8912	2	NE
	Century City	-33.8882	18.5138	4	CC
	Kluitjieskraal	-33.4282	19.1838	2	KK
	Maynardville Wynberg	-34.0059	18.4647	4	MAYN
	George	-33.9945	22.5262	3	GEO
	Dam 1, Plettenberg, Knysna	-34.0448	23.2919	2	KNY
	Yellowwood Dam, Somerset West	-34.0941	18.8651	2	SOM
	Bellevue Wine Estate, Stellenbosch	-33.8785	18.7642	4	BELL
	Cottage Farm Dam, Kromrivier	-32.5417	19.2811	4	KR
Eastern Cape	Boardwalk, Port Elizabeth	-33.9830	25.6574	3	BW
Gauteng	Benoni	-26.1705	28.2890	1	BE
	Moreleta Park, Pretoria	-25.8139	28.2848	2	PRET
	Emmarentia Dog Park, Randburg	-26.1602	28.0010	4	EMM

	Louw Geldenhuys Drive, Randburg	-26.1462	28.0036	4	LG
	Florida Lake	-26.1783	27.9065	3	FLL
North West	Potchefstroom NWU Botanical gardens	-26.6823	27.0950	3	POT
Native range: Southern USA					
Florida	Lake Kissimmee 1	27.9651	-81.3278	11	K
	Lake Kissimmee 2	27.9792	-81.2743	8	K
	Lake Lawne	28.5579	-81.4381	4	L
	Lake Apopka	28.6722	-81.6748	5	AP
	Lake George	29.2828	-81.5408	8	G
	Lake Okeechobee	26.9329	-81.0503	5	OKE
	Lake Seminole	27.8414	-82.7740	7	SEM
	Lake Maggiore	27.7373	-82.6475	6	M
	Pine Island lodge	29.3119	-81.5458	5	PI
	Esmeralda Marsh near Lake Griffin	28.9039	-81.8087	4	EM
	Orlando Wetlands Park	28.5824	-81.0022	8	OWP
	Everglades	26.3205	-80.3300	6	EV
Louisiana	Cote blanche Crossing	29.7774	-91.7155	5	CX
	Lake Boeuf	29.9111	-90.7117	8	B
	Salvador WMA	29.7657	-90.2930	13	S
Texas	Canal roadside Harlingen	26.1903	-97.6636	4	H
	Lewisville Research Facility	33.0524	-96.9373	7	TX

Big Lake, Welder Wildlife Refuge	28.1216	-97.3650	6	W
Quinta urban park	26.1767	-98.2298	4	Q

2.2. Plant DNA extraction

Total genomic DNA from dry leaf tissue (30–40 mg) was extracted using the QIAGEN Mini Plant Extraction kits (QIAGEN Inc.). Leaf tissue from individual plants was ground under liquid nitrogen using a mortar and pestle, and then the manufacturers' protocol was followed.

2.3. PCR protocol

Two primers were used in the analyses: the universal primer HB15 manufactured by Applied Biosystems Inc., U.K. (Wolfe et al., 1998) and UBC-852 manufactured by Integrated DNA Technologies, WhiteSci Whitehead Scientific (Pty) Ltd., RSA (Poczai et al., 2011). Both primers were labelled with 6-FAM fluorescent dye by the manufacturers. These primers were selected based on the number of peaks produced after conducting preliminary tests to identify useful primers and, in the case of UBC-852, based on the successful use of this primer for ISSR analyses conducted on *Nymphaea* (Poczai et al., 2011). The ISSR PCR reactions utilized the following concentrations and volumes to make up 20 μL per reaction for the HB15 primer: 0.8 μM of HB15 primer, 10 μL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad) (this supermix contains Taq DNA polymerase, dNTPs, MgCl2, enhancers, stabilizers, and dyes), 3 μL of plant DNA, and 6.2 μL denucleated water. The PCR amplification protocol for the HB15 primer followed Paterson et al. (2009). The same concentrations were used to make up the reaction volumes for the UBC-852 primer, except that half the volumes were utilized to make up a total of 10 μL per reaction. The PCR amplification protocol for the UBC-852 primer followed Poczai et al. (2011). PCR products were sent to Central Analytical Facilities (CAF) at Stellenbosch University, Stellenbosch, South Africa to visualize banding patterns. This was carried out by capillary electrophoresis using an ABI 3130 genetic analyzer. All samples had two replicates from the PCR amplification step to ensure reproducibility.

2.4. Analyses

Electropherograms were analyzed and sized using GeneMarker® ver. 2.7.4 (SoftGenetics LLC.) and then RawGeno ver. 2 (Arrigo et al., 2009) (an automated DNA fragment scoring application run through R ver. 3.5.3) (R Development Core Team, 2013) was used to score the datasets for each primer separately. As band scoring differs depending on the settings used in analytical software, a subset of the samples were used in preliminary tests to

determine the settings that produced low error rates (see Bonin et al., 2004; Holland et al., 2008; Pompanon et al., 2005). In GeneMarker, minimum intensity of peak detection threshold was set at 20, stutter peak filter and AFLP normalization was unchecked, smoothing was selected, minimum peak score default was set at "fail < 1 check < 1 pass", and all other settings were left at default (Holland et al. 2008). In RawGeno, all settings were left at default except for the bin widths, in which the minimum was set at 1 and the maximum was 1.5, as this bin width of 0.5 has elicited fewer errors and better resolutions with other plants (Holland et al., 2008). After binary matrices were generated, they were exported as tab-delimited text files and edited using Microsoft Excel®. Consolidated matrices were generated using BINMAT: For Fragment Analysis Data (Clarke van Steenderen - https://clarkevansteenderen.shinyapps.io/BINMAT/ or the R package can be downloaded at https://cran.r-project.org/web/packages/BinMat/) which combines the two replicates of each sample, only including peaks that were present in both replicates. This site was also used to generate error rates as well as data summaries and non-metric Multidimensional Scaling (nMDS) plots to test different settings and filtering parameters.

The first 80 base pairs were excluded from the analysis for both primers. These sections were chosen for exclusion as most of the samples shared the same peaks between these base pairs for each primer, and preliminary nMDS test plots did not show clear groupings without these exclusions. Thereafter, consolidated samples with fewer than 15 total peaks for HB15 and 5 total peaks for primer UBC-852 were removed from the analyses, as these samples appeared as outliers in preliminary nMDS plots and were considered to have failed to amplify. After data from each primer had been analyzed separately, the binary matrices for each primer were combined and analyzed together.

2.4.1. SplitsTree

A phylogenetic network was constructed for the ISSR data using the NeighbourNet construction and Jaccard's distances in SplitsTree4 ver. 4.12.3 with 1000 bootstrap replication for node support (Huson and Bryant, 2006). Unlike the traditional phylogenetic analyses such as NJ, MP and Bayesian analyses, the network analyses take intra–specific and population level phenomenon such as recombination into account (Posada and Crandall, 2001).

2.4.2 Genetic distances and AMOVA

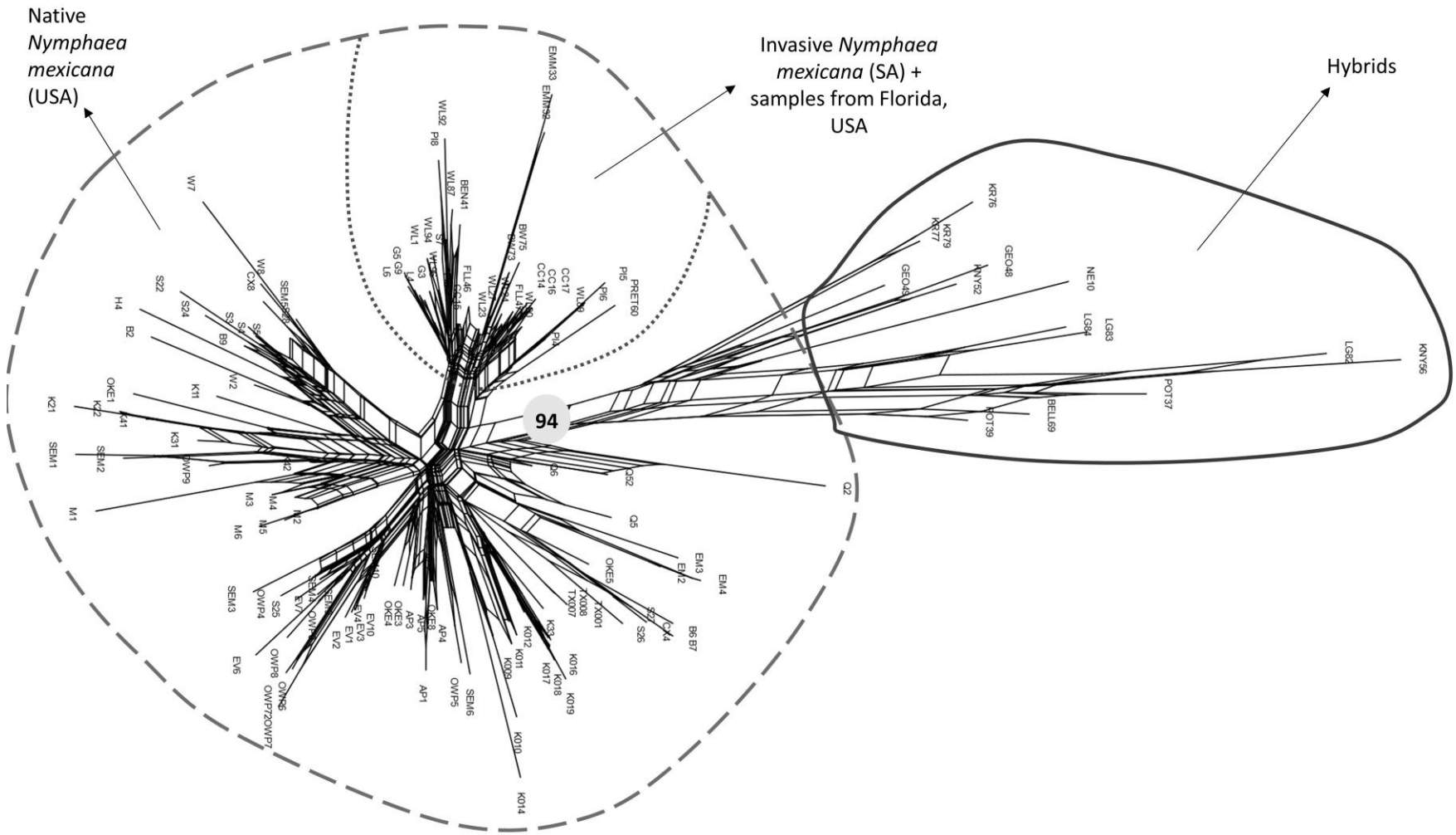
Pairwise binary genetic distances were calculated using the Gen-AlEx ver, 6 software package in Microsoft® Excel (Peakall and Smouse, 2006) by calculating values for all the samples collected from South Africa (invasive group) and the native samples from USA (native group), and using these to generate mean binary genetic distances for the invasive and native range populations. These genetic distances were used as a measure of genetic diversity. Genetic distances were also calculated for the samples from the native range (native group), the samples from the invaded range that grouped as 'pure' *N. mexicana* in the SplitsTree analysis (invasive *N. mexicana* group), and the remaining samples from the invaded range that grouped as hybrids (hybrid group), to generate means for each group separately. Significant differences between the populations were tested using a t-test when comparing the two main groups (native vs invasive), and a type II ANOVA when comparing the mean genetic distances of the three groups when the invasive population was separated (i.e. native group vs. invasive *N. mexicana* group vs. hybrid group) in R ver. 3.5.3 (R Development Core Team, 2013). Tukey post-hoc analyses were used to examine genetic differences between the groups.

AMOVAs (Analysis of Molecular variance) were conducted using Gen-AlEx ver, 6 to determine genetic variation between and among all the invasive and native samples, and the three groups identified in the SplitsTree analyses. Permutations were set at 999, and the population estimator PhiPT (ϕPT) was calculated from the amongst population variability determined in the AMOVA analysis. This population estimator is an analogous statistic of Fst, which measures population differentiation for binary data (Timm et al., 2010).

3. Results

The mean number of replicable peaks was 54.08, with 759 loci ranging in size from ~80 to 1190 bp. There was a minimum of 25 and maximum of 113 peaks. Overall, 644 sites were polymorphic (84.84%). The SplitsTree analysis indicated the presence of two major groups (Figure 2). The first major cluster consisted of invaded range samples from Knysna (KNY), Louw Geldenhuys (LG), Potchefstroom (POT), Bellevue Wine Estate (BEL), George (GEO), Krom Rivier (KR), and Neil Ellis (NE), all from South Africa. The second major grouping consisted of the remaining samples from South Africa and the samples from the native range. Within this group, one subset was formed by most of the native range samples, while the second subset consisted of the remaining invasive samples mixed with native samples from Lake George (G), Pine Island (PI), and Lake Lawne (L) (all in Florida), and some overlap with

a single sample from Salvador (S), Louisiana. Hence, the SplitsTree suggests that the invaded range samples from Westlake (WL), Florida Lake (FLL), Boardwalk (BW), Century City (CC), Pretoria (PRET), and Emmarentia (EMM) are more genetically similar to native populations (in particular populations from Florida) than the other invasive samples and indicates that the South African samples in this group are pure *N. mexicana*. The remaining invasive samples which formed a third distinct group are therefore considered hybrid forms of *N. mexicana*. The major split between the hybrid group and the native/invasive group was well supported (97.4%) (Figure 2).



274

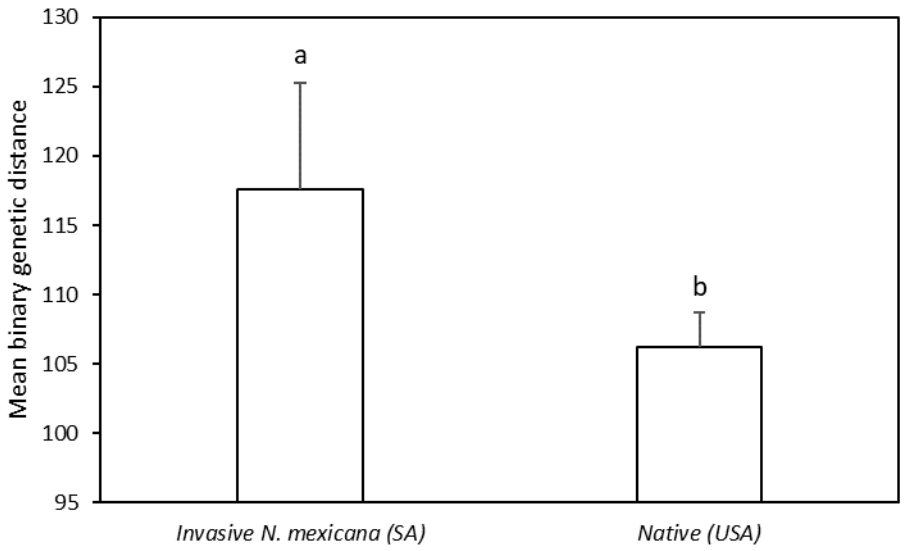
275 Figure 2: NeighbourNet tree for ISSR data using Jaccard's distance constructed in SplitsTree. The dashed outline represents native range samples,
276 with the smaller subgroup (dotted border) showing mixed invaded and native sites that group together, indicating that the South African samples in

277 this group are pure *Nymphaea mexicana*. The solid outline represents samples from South Africa that are hybrids. Bootstrap (1000 repetitions)
278 support for main split indicated as 94.

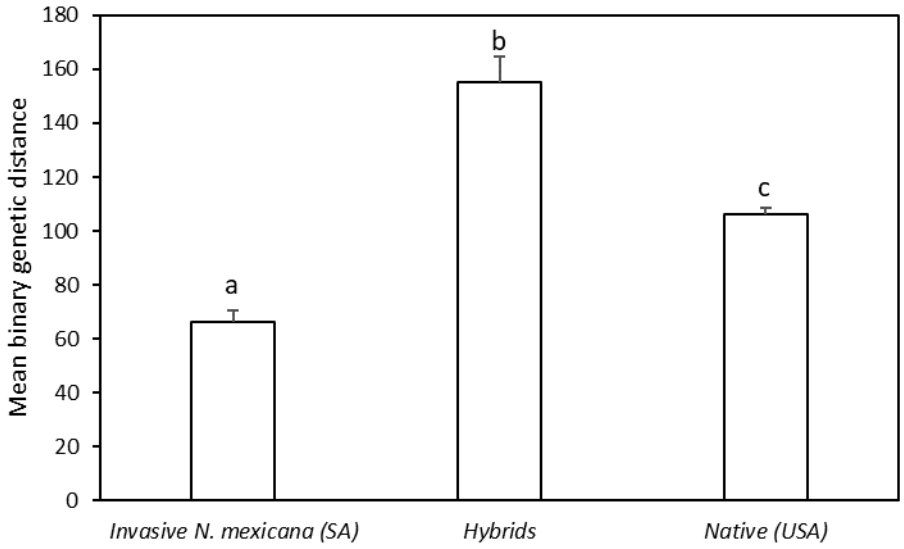
3.1. AMOVA and genetic diversity

Within the invaded distribution, 60% (P = 0.001) of the genetic variability could be attributed to within population variation and 40% was attributed to among population variation. Similarly, for the native range, 20% (P = 0.001) of the variation was significantly attributed to among population variation, and 80% to within population variation. There was moderate support for population differentiation between the invasive and native samples (ϕPT = 0.111, P = 0.001). When *N. mexicana* samples from the invaded range were separated from hybrids, there was support for differentiation between all three populations (ϕPT = 0.181, P = 0.001). Differentiation between hybrids and *N. mexicana* in the invaded range was strong (ϕPT = 0.261) and so was differentiation between hybrids and the native *N. mexicana* (ϕPT = 0.248). There was some support for differentiation between *N. mexicana* from South Africa and the native *N. mexicana* from USA (ϕPT = 0.112).

The mean (± S.D.) binary genetic differences of the invasive group (117.59 ± 46.29, n = 36) was higher than that of the native group (106.19 ± 24.35 n = 95), and this difference was statistically significant (t = -123.61, df = 5093, P < 0.05) (Figure 4a). When the invasive group was split and comparisons made between three groups, significant differences were observed between the native group (106.19 ± 24.35, n = 95), the hybrid group (154.98 ± 36.03, n = 14), and the invasive *N. mexicana* group (66.41 ± 18.75, n = 22) (F = 285.34, d.f. = 3, P < 0.05), as revealed by non-parametric post-hoc analysis (Figure 4b). The hybrid group had the highest mean genetic diversity and the invasive *N. mexicana* group had the lowest genetic diversity.



a



b

Figure 4: Mean (±S.E.) binary genetic distances of *Nymphaea mexicana* samples from the native and invaded range. Figure 4a shows the comparison of all the samples from the invaded range (n = 36) with all the samples from the native range (n = 95). Figure 4b shows the comparison of the hybrid group (n = 14) from the invaded range, the native group from the USA (n = 95), and the 'pure' *Nymphaea mexicana* group from the invaded range (these grouped with the native samples in the SplitsTree plot) (n = 22). The letters above the bars represent significant differences between the groups.

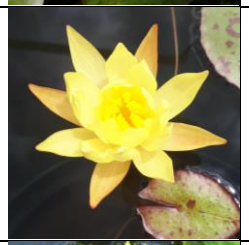
3.2 Morphological data

The morphotypes assigned to populations of *N. mexicana* at various sites based on flower morphology matched the genetic groupings determined by ISSR analyses (Table 1). Flowers with bright yellow petals and pointed tips were associated with 'pure' *N. mexicana* genotypes in the invaded and native range. Flowers with pale yellow petals, pink petals, or white petals were associated with hybrid genotypes. In some cases, the petals of these flowers had wider bases than the *N. mexicana* flower groupings, and/or the tips of the petals were rounded. The hybrid morphotype/genotype flowers had a more open lotus-like structure compared to the *N. mexicana* group, and sepals were light pink ventrally and deep red dorsally in some populations. In general, flowers with bright yellow lanceolate petals were associated with 'pure' *N. mexicana* genetic groupings for the ISSR analyses.

Flowers from the population sampled at the Boardwalk, Port Elizabeth, South Africa were grouped as hybrids based on morphology but were classified as *N. mexicana* according to ISSR

analyses. This population had pale yellow pointed petals that appeared wider and longer than native *N. mexicana*. Nevertheless, the petals had a similar shape to ‘pure’ *N. mexicana* and lacked the dorsally deep red sepals possessed by other hybrids.

Table 1: Morphotypes of *Nymphaea mexicana* populations and hybrids. Photographs of the flowers from selected sites are shown with the groupings based on morphological characteristics and ISSR analyses. H = hybrid, *Nm* = *Nymphaea mexicana.* Asterisks (*) indicate that the site has a morphotype that contradicts the genetic grouping.

MORPHOTYPE			**GENOTYPE**	**MORPHOTYPE**			**GENOTYPE**
Hybrid		**Site code**	**ISSRs**	***Nymphaea mexicana***		**Site code**	**ISSRs**
	Pink flowers with long petals pointed at the tips.	LG	Hybrids		Bright yellow flowers, short petals lanceolate.	WL	Invasive *N. mexicana* (SA)
	White broader based petals with slightly rounded tips.	LG	Hybrids		Bright yellow flowers, lanceolate petals.	EMM	Invasive *N. mexicana* (SA)
	White flowers. Petals are short and rounded at the tips.	POT	Hybrids		Bright yellow flowers, lanceolate petals, slight pink colouration on sepals.	K (Representative of all native range samples)	Native *N. mexicana* (USA)

	Large white/ pale yellow flowers. Petals have pointed tips.	BW	*** Invasive *N. mexicana* (SA)**		Light yellow lanceolate petals, pink tinged sepals.	PRET	Invasive *N. mexicana* (SA)
	Pale yellow flowers with pointed petal tips. Dorsal surface of sepals deep red, ventral surface lighter pink.	KNY (top) and GEO (bottom),	Hybrids		Bright yellow flowers, broad based petals with pointed tips.	BE	Invasive *N. mexicana* (SA)
					Bright yellow, lanceolate petals.	FLL	Invasive *N. mexicana* (SA)

4. Discussion

The ISSRs indicated that some *N. mexicana* populations in South Africa are more genetically similar to samples from the native range than others. The populations in South Africa that are more genetically similar to populations in USA may be more effectively managed using biological control agents collected from southern USA. In contrast, the South African populations that are less genetically similar to native range populations are likely to be hybrids with *N. mexicana* as one of the hybrid parents. The presence of these hybrid forms of *N. mexicana* in South Africa is reflected in the morphology of the flowers. Flowers with bright yellow lanceolate petals are more likely to be genetically similar to native populations, while flowers with pale yellow, cream/white, or pinkish coloured petals with or without rounded petal tips are more likely to be hybrid forms. These hybrid forms represent an intermediate state between *N. mexicana* and other parent plants and are more typical of highly aesthetic horticultural forms of the plants. Hybrids of *N. mexicana* and *N. odorata* are recorded in the wild where these species overlap (Borsch et al., 2014), while artificial waterlily hybrids created since the late 1800s are common in the horticultural trade (Sheldon, 2017). In order to identify the parent species of hybrids, further genetic analysis would be required. Putative parent species would need to be included in genetic analyses, so a much wider range of species would need to be included, given that there are multiple parent species of known hybrid varieties (Sheldon, 2017).

The ISSR analyses indicated that pure *N. mexicana* from South Africa grouped more closely with samples from sites in Florida, USA than other samples from the native range. It is possible that some of the invasive populations in South Africa thus originated from Florida, although this result is confounded by the fact that the majority of samples from the native distribution were from Florida. Indeed, the majority of the locality records of *N. mexicana* occur in Florida suggesting that it is where the plant is most abundant within the native distribution. Although samples from Mexico were not included, this should not conflict with our main goal, which was to determine which *N. mexicana* populations in South Africa were hybrids and which were 'pure' *N. mexicana*. The inclusion of samples from Mexico and from a greater range of southern USA would likely give us a more detailed insight into the genetic makeup of, as well as the origin of, the invasive *N. mexicana* populations in South Africa, and should be included in future genetic studies.

The genetic diversity of all the plants sampled from the invaded range was significantly higher than that of native range populations. This is unexpected for invasive plants, as introductions of small populations into a new location would likely induce a genetic bottleneck (Estoup et al., 2016) but can be explained by the presence of hybrids in the invaded range, or multiple introductions from populations in the native range that were not sampled in this study (Schierenbeck and Ellstrand, 2009). Significant differences in genetic diversity were observed when samples from South Africa were separated into two groups (hybrid and invasive *N. mexicana*) and compared with the native group. The hybrid group had the highest genetic diversity, followed by the native group and then the invasive *N. mexicana* group excluding hybrids. In other words, the inclusion of the hybrid group genetic diversity as part of the invasive group resulted in overall higher levels of genetic diversity than when the invasive group was separated into the hybrid and invasive *N. mexicana* groups.

The high genetic diversity seen in the hybrid group is expected as a result of genetic mixing between multiple parent species (Ward et al., 2008), while the high genetic diversity of the native group concurs with studies that have recorded higher levels of genetic diversity in the native compared to the introduced ranges of invasive alien plants (Li et al., 2006; Paterson et al., 2009). The lower genetic diversity of the invasive *N. mexicana* group (when the hybrids were separated as a second group) may be explained by single introductions of the plant and limited number of propagules in introductions (Burdon and Marshall, 1981), founder effects and bottlenecks, and the lack of plant sexual reproduction (Lawson Handley et al., 2011). Considering the aesthetic appeal of *N. mexicana* and other *Nymphaea* species, and the popularity of *Nymphaea* hybrids in the horticultural trade, it is unsurprising that these explanations for differences in genetic diversity would be true, especially considering that many of the sampled sites are located near major ports and highly populated cities.

Nymphaea mexicana and *Nymphaea* hybrids are becoming increasingly problematic around the world (Nierbauer et al., 2014). While biological control may be more likely to succeed in managing the populations in South Africa that matched genetically to samples from the native range, it is also possible that the origin of the plants would not make a difference to biological control efficacy (Paterson et al., 2012). Indeed, genotypes that are not locally adapted may be more effective as a result of the development of a new association (Hokkanen and Pimentel, 1989). Nevertheless, hybrid forms of *N. mexicana* may present challenges for biological control. For biological control to be successful, the agent should be suitably host specific while

effectively managing the target weeds. If the agent targets both the pure species and the hybrids, they are more likely to also target native *Nymphaea* species in the invaded range. The *Nymphaea* species native to South Africa are tropical waterlilies grouped in different subgenera compared to *Nymphaea mexicana*. Although there have been numerous reports of deliberate hybridisation of *Nymphaea* species, all known instances had involved crossing of species within the same subgenus until 2004 (Les et al., 2004). While the parentage of the hybrids remains unclear, if both parents occur within the same subgenus, perhaps a biological control agent specific to the subgenus level would be acceptable. Clarification of the parentage of the hybrids present in South Africa, and possibly more surveys directed at naturally occurring hybrid or parent populations in the native range, would be useful to better understand and develop ideas for the biological control of *N. mexicana* and its hybrids in South Africa.

Control of *N. mexicana* is warranted owing to the number of sites that are invaded, the extent of growth, and the risk of spread to other water bodies. Biological control is more likely to succeed for 'pure' *N. mexicana* than for both *N. mexicana* and the hybrids that are present in South Africa, but it is not impossible that an agent that is suitably host specific and also damaging to both 'pure' and hybrid plants could be found. If such an agent cannot be found, an integrated programme of chemical control of hybrids, and biological control of pure *N. mexicana* could be effective to manage these populations

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