# INITIATING BIOLOGICAL CONTROL FOR *NYMPHAEA MEXICANA* ZUCCARINI (NYMPHAEACEAE) IN SOUTH AFRICA

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# Abstract

*Nymphaea mexicana* Zuccarini (Nymphaeaceae) is an aquatic plant originating from south-eastern USA that is becoming increasingly invasive in South Africa as other invasive aquatic plants are being managed successfully through biological control. Mechanical and chemical control of aquatic weeds is expensive, damaging to the environment, and only effective in the short term, so biological control is more desirable as a management strategy for *N. mexicana*. The biological control of invasive alien plants requires that agents are host specific so that non-target risks are mitigated. For success to be achieved, it is important to ensure that the genetic structure of invasive populations is clarified so that agents can be collected from populations in the native range that match genetically to populations in the invasive range. This is especially important in cases where the morphology of invasive alien plants does not reflect genetic differences between populations. A previous study of the genetic structure of the invasive populations of *N. mexicana* in South Africa suggests the presence of hybrid forms of the plant in South Africa, with only one of these populations matching with samples from the native range. However, the study only used samples from two sites in the native range using amplified fragment length polymorphisms (AFLPs), so it was necessary to conduct further genetic analyses using samples from more sites in the native range. Hence, the first aim of this study was to develop a better understanding of the genetic structure of *N. mexicana* populations in the native and invaded range. Genetic samples were collected from sites in the native range during field surveys for potential biological control agents, and inter-simple sequence repeats (ISSRs) were used to compare the genetic structure of invasive and native populations of *N. mexicana* in South Africa. The results from these analyses suggest that seven of the 14 investigated invasive populations of *N. mexicana* in South Africa are genetically similar to populations in the native range, while the remaining seven populations are likely to be hybrid forms of the plant. This knowledge will be useful to target populations for biological control and highlights the need for further genetic analyses to determine the parentage of these hybrids so that biological control efforts are more likely to be successful.

The initiation of a biological control programme requires that a series of steps are taken in order to maximise the likelihood that this form of intervention will be successful. The first few steps include: identification of the target weed and its genetic structure; exploration in the native

range for potential biological control agents; and prioritisation of these agents based on factors such as climatic and genetic compatibility, feeding damage, abundance, and likely host range. Hence, the second aim of this study was to conduct surveys for potential biological control agents in the native range of *N. mexicana,* and to prioritise these agents. Field surveys were conducted between August and October in 2018 at 17 sites in Florida, Louisiana, and Texas, USA. Sites were selected based on climatic similarity of native sites compared to invasive sites by use of MaxEnt modelling. Native *N. mexicana* plants were searched for natural enemies, and these were prioritised based on feeding damage, abundance, incidence, and observations of field host range. Two species were prioritised: *Bagous americanus* LeConte (Coleoptera: Curculionidae) and *Megamelus toddi* Beamer (Hemiptera: Delphacidae). These species will be imported into quarantine facilities at Rhodes University for host specificity tests to be conducted.

Understanding the factors that contribute to the successful establishment of biological control agents is important to improve the efficiency and reduce the costs incurred during the initiation of biological control programmes. Acquiring knowledge of the factors that predict the efficacy of biological control agents is similarly important, and these factors are discussed in the last chapter of this study. The challenges of the biological control of hybrids are also considered, and recommendations are made for the control of *N. mexicana* and other plants in South Africa.

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# Chapter 1: General introduction

## 1.1 INVASIVE ALIEN PLANTS

Invasive alien plants (IAPs) threaten global biodiversity, natural resources, and the provision of ecosystem services (Kumar 2010; Reid et al. 2019). By outcompeting native species, exploiting water resources, and altering nutrient cycling and fire regimes, IAPs reduce native biodiversity and induce bottom-up effects on higher trophic levels (Richardson and Van Wilgen 2004; Vilà et al. 2011; Reid et al. 2019). In this way, IAPs are responsible for, or contribute to considerable ecosystem destabilisation, either by directly inducing environmental degradation (drivers), benefitting from existing disturbance (passengers), or a combination of the two (back-seat drivers) (Macdougall and Turkington 2005; Bauer 2012). Indeed, the negative impacts of invasive species are so extensive, that biological invasions are considered to be the greatest global threat to biodiversity after habitat destruction (Walker and Steffen 1997; Wilcove et al. 1998). This environmental destruction impacts human socio-economies and can have far reaching consequences for human well-being (Kumar 2010). The threats posed by invasive alien species are worsened by increased introductions of alien species over the past few decades, owing to increasing trade and ease of transport associated with globalisation, as well as increased habitat disturbance and land cover change (Walker and Steffen 1997; Hulme 2009). Furthermore, the ranges of invasive alien species are expected to broaden as a result of climate change (Richardson and Van Wilgen 2004; Tylianakis et al. 2008) and so, it is of utmost importance that existing invasions are controlled, and future introductions prevented.

When an alien plant species is introduced into a country, it invariably does not have the natural enemies that normally supress that species in its native range and thus the alien plant may become invasive (Keane and Crawley 2002). Without suppression by these natural enemies, invasive alien species are able to reallocate resources that would otherwise be used to produce defensive structures and chemicals to protect them against specialist predators (Joshi and Vrieling 2005). This resource reallocation allows the plant to grow and reproduce more rapidly, thus granting IAPs a competitive advantage over native species, allowing it to form dense populations and transform the landscape (Zimmermann et al. 2004; Joshi and Vrieling 2005).

For a plant to become invasive it must overcome barriers in a series of stages: transport, colonisation, establishment, and spread (Williamson 2006; Theoharides and Dukes 2007).

Various plant traits, such as rapid growth and vegetative reproduction, affect the ease and time through which each stage is progressed (Sakai et al. 2001; Bacher et al. 2011). Community susceptibility to invasion is elevated by eutrophication and disturbance (Davis et al. 2000), while ecosystems with fewer occupied niches may be more likely to be invaded by alien plant species that can occupy the vacancies (Shea and Chesson 2002). Hence, habitat characteristics as well as plant traits are important factors determining whether an introduced species becomes invasive or not. Indeed, only a proportion of species introduced into an area may become established in the invaded range, and of these only a few become invasive (Williamson & Brown 1986; but see Williamson & Fitter 1996).

Controlling invasive alien species becomes more difficult once an invader has established and spread in multiple regions of a country, and it is for this reason that preventative measures are desirable and economical when implemented in the early stages of invasion (Leung et al. 2002). Alien invasive plants often undergo a lag phase, during which few small plant populations have established but have not spread enough to cause concern (Hobbs and Humphries 1995; Aikio et al. 2010). Numerous factors may then contribute to the sudden increase in population size and distribution, and it is at this stage that the plant becomes problematic. Such factors are hypothesised to include: genetic adaptation, which lead to the presence of more invasive genotypes; change in dispersal mechanisms, environmental disturbances to allow increased range expansion, and overcoming Allee effects (Crooks et al. 1999).

South Africa has been subjected to introductions of thousands of alien plant species brought in for agriculture, forestry, ornamental gardening, and dune stabilisation, many of which have survived and spread unaided across the country (Lowe et al. 2000; Richardson and Van Wilgen 2004; Alston and Richardson 2006; Hill and Coetzee 2017). These taxa pose threats to natural ecosystems, and also impact economic productivity through the destruction of natural capital, hinderance of recreation, and costs of management strategies (Pimentel et al. 2000; van Wyk and van Wilgen 2002). In 2001 it was estimated that the cost to clear alien plant invasions in South Africa was about US$1.2 billion (van Wilgen et al. 2001). The environmental and economic impacts imposed by invasive species are especially problematic in South Africa, due to the high biodiversity and floral endemicity found in the region (Linder 2001; Barthlott et al. 2005) as well as the economic challenges faced by this developing country. With between 250 000 and 1 million species in the country, South Africa is ranked as the third most

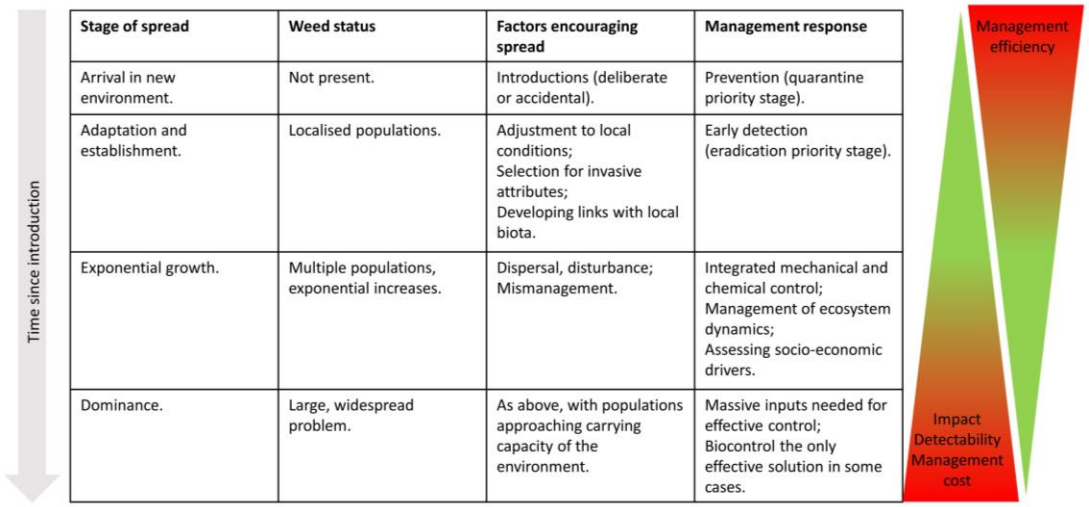
biologically diverse country in the world, and supports a considerable number of endemic species (Groombridge 1992). Hence, it is essential that this rich biodiversity is conserved, and invasive organisms controlled and/or eradicated. The problems posed by IAPs have motivated the development of organisations such as Working for Water of the Department of Environment, Forestry and Fisheries, which focus on IAP management to restore ecosystem stability, while also creating employment (van Wilgen et al. 1998). As of 2016, the Southern African Plant Invaders Atlas (SAPIA) recorded 773 naturalised alien plant taxa in the region, as well as range expansions for almost all recorded plants (Henderson and Wilson 2017), while Richardson et al. (2011) estimate that 750 exotic tree species and 8000 exotic shrubby and herbaceous species have been introduced into South Africa and could naturalise in the future. According to the National Environmental Management: Biodiversity (NEM:BA) Act 10 of 2004, 379 of these introduced species have been declared weeds. These records and estimates provide further motivation for continued efforts of IAP control in South Africa, and indeed in the world.

## 1.2 MANAGEMENT OF INVASIVE ALIEN PLANTS

The negative impacts that IAPs pose to their invaded environments have spurred numerous efforts to control alien species. It is necessary to understand the biology, extent of invasion, and impacts of the species in question through surveys before control measures can be taken (Scott 1996; McFadyen 1998). Once this information is acquired, scientists are able to research the best means of preventing further spread of the species, using an integration of mechanical, chemical, and biological control methods (Hobbs and Humphries 1995). Data on the impacts and extent of invasive alien plants also provides a baseline with which to compare post-control assessments, which will enable researchers to determine whether control measures are successfully reducing the range and effects of invasive plants, and whether further action is required.

Prevention of alien plant invasions is the most effective means of managing IAPs, and may be carried out by screening pathways and vectors of introduction (Hulme 2009; Martin and Coetzee 2011). Should this fail, early detection, rapid response, and eradication are the next steps taken if possible (Simberloff et al. 2013) (Figure 1.1). Depending on the extent of the invasion, and the ease at which plants may be removed, complete eradication of a species may not be possible, in which case efforts are focused on containing existing populations and reducing

population sizes wherever possible (Simberloff et al. 2013). As already mentioned, many invasive plants undergo lag phases before their populations explode, and it is especially important that these plants are eradicated or controlled before these population explosions occur. Control of IAP populations is typically exerted using either mechanical, chemical, or biological control, or a combination of these methods.




| Stage of spread | Weed status | Factors encouraging spread | Management response |
|---|---|---|---|
| Arrival in new environment. | Not present. | Introductions (deliberate or accidental). | Prevention (quarantine priority stage). |
| Adaptation and establishment. | Localised populations. | Adjustment to local conditions;<br>Selection for invasive attributes;<br>Developing links with local biota. | Early detection (eradication priority stage). |
| Exponential growth. | Multiple populations, exponential increases. | Dispersal, disturbance; Mismanagement. | Integrated mechanical and chemical control;<br>Management of ecosystem dynamics;<br>Assessing socio-economic drivers. |
| Dominance. | Large, widespread problem. | As above, with populations approaching carrying capacity of the environment. | Massive inputs needed for effective control;<br>Biocontrol the only effective solution in some cases. |

Figure 1.1: Invasive species management strategy. As the time since introduction progresses, the management efficiency decreases. Hence, prevention is the most efficient form of management, followed by early detection and management, at which time the invasive species is already established. Adapted from Simberloff et al. (2013) and van Wilgen et al. (2000).

Mechanical control

Mechanical control of IAPs involves manual and mechanical removal of the plants from invaded areas. Depending on the species in question, this may involve mowing, hand pulling, or burning (Working for Water 2007). Mechanical removal may be effective for some plants (e.g. *Acacia mearnsii* De Wild., Dye & Poulter 1995), but is ineffective for plants that grow more rapidly than they can be removed (e.g. *Pontederia crassipes* Mart. [≡ *Eichhornia crassipes* (Mart.) Solms] (Pontederiaceae) (Henderson 2010; Pellegrini et al. 2018). Furthermore, manual removal is labour intensive, and is inefficient over large areas (Hobbs and Humphries 1995).

Chemical control

Chemical control using herbicides is a common method of managing invasive plant populations, despite negative impacts to the environment (Hobbs and Humphries 1995). Some of the herbicides commonly used against IAPs in South Africa include glyphosate, 2,4-D amine, and diquat (Working for Water 2007). These, as well as the surfactants contained in herbicide formulations, have negative impacts on various organisms, and are thus not ideal for widespread use in natural ecosystems (Cooke 1977; Wang et al. 1994; Giesy et al. 2000; Wong 2000). For example, polyethoxylated tallow amine, a surfactant found in a herbicide formulation called Roundup, causes mortality in amphibians and shrimp by altering respiratory surfaces (Relyea 2005; Brausch and Smith 2007). While herbicides can be effective at controlling invasive alien species, they are not cost efficient for controlling large populations that require frequent reapplication, and are expensive and damaging to the environment (van Wyk and van Wilgen 2002).

Biological and integrated control

Biological control is a method that utilises host-specific predators in the form of insects, mites, or pathogens, to control IAPs in the invaded region. For classical biological control, the natural enemies of the invasive plant are imported from the plant's country of origin and undergo a rigorous series of tests to ensure that they are host specific and do not pose a threat to the native vegetation in the invaded country (Zimmermann et al. 2004). Once a successful biological control programme is initiated against an invasive weed, the damage caused by the biological control agents results in reduced population densities and constricts the distribution of the plant in question, thereby reducing expenses of other control methods (Zimmermann et al. 2004). Biological control provides a long term, self-sustaining, and relatively safe method of IAP management when compared to chemical and mechanical control, and provides significant benefits to natural ecosystems (Zimmermann et al. 2004; van Driesche et al. 2010).

Despite the benefits of using biological control agents to control invasive weed populations, nontarget effects can occur. *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) released to control *Opuntia* cacti in Australia is one such example whereby the moth moved accidentally into Florida and began to attack native cacti species (Cory and Myers 2000). However, for this and other examples, it is likely that these nontarget effects could have been

predicted from host range data (Cory and Myers 2000). This highlights the importance of ensuring host specificity before the release of biological control agents, and exercising caution when estimating possible impacts. Nevertheless, nontarget effects can occur even for host-specific biological control agents. For example, when a pest species integrates into the native community and replaces native species, other species may become dependent on the pest for certain physical and functional attributes (Pearson and Callaway 2003). Hence, control of the pest species will impact organisms that interact with it. Similarly, herbivory by biological control agents can result in compensatory growth and production of secondary compounds leading to increased competition with native species (Callaway et al. 1999; Ridenour and Callaway 2003). Furthermore, if an established biological control agent does not effectively reduce the density of its host plant, there is potential for biological control agent populations to become considerably abundant, which may in turn alter food-web interactions if native predators utilise the agents as an additional food source (Goeden and Louda 1976; Story et al. 1995; Pearson and Callaway 2003). These and other possible indirect effects are important to consider, but these risks can be greatly reduced when biological control agents effectively reduce populations of the target species, so that populations of the agents are regulated through density-dependent feedback loops (Pearson and Callaway 2005).

Although the risks associated with biological control are essential to consider and reduce, benefit cost ratios indicate that biological control is more cost effective than chemical or mechanical control alone, though costs may be high initially to carry out intensive host specificity tests to ensure that non-target plants are not affected (Zimmermann et al. 2004; Moran et al. 2005). For example, benefit-cost ratios for the successful control of skeleton weed *Chondrilla juncea* L. (Asteraceae) (Marsden et al. 1980) and tansy ragwort *Jacobaea vulgaris* Gaertner syn. *Senecio jacobaea* L.(Asteraceae) (Coombs et al. 1995) have been reported as 112 and 15 respectively. Other studies similarly demonstrate beneficial benefit-cost ratios for biological control (Chippendale 1995; McFayden 2000).

In,a case study that investigated the mean costs of each control method for water hyacinth, *P. crassipes*, biological control (at R309/ha) was almost five times more cost effective than herbicidal control (at R1 481/ha) (van Wyk and van Wilgen 2002). This is mainly because ongoing herbicidal applications are necessary in order to provide long term control, and lapses in management can result in rapid re-infestation. However, integrated control was the most cost

efficient, at R277/ha in the same study. Integrated control programmes utilise a combination of mechanical, chemical, and biological control to manage IAP populations, which allows invasive species to be targeted from multiple angles with the use of methods that are effective both in the short and the long term (Table 1.1). The use of integrated control requires that the methods utilised are compatible with one another – for example, if chemicals are used with biological control, it is important that the herbicides do not have negative impacts on the biological control agents, or that spraying occurs at lower doses, leaving sections of healthy plants to allow the agents to survive (for example Jadhav et al. 2007). Research into the compatibility of different control measures is vital for the continued use of these methods to establish cost-efficient control of invasive species in South Africa and elsewhere.

Table 1.1: Examples of invasive plant species in South Africa, including factors that contribute to their expansion, and strategies employed to control them. From van Wilgen et al. (2000).

| **Invasive alien species** | **Characteristics** | **Spread mechanisms** | **Ecosystem characteristics** | **Control strategy** |
|---|---|---|---|---|
| *Hakea sericea* (Proteaceae) | Fire-sensitive, non-sprouting shrub with short juvenile period and serotinous follicles that open after fire. | Winged seeds spread long distances by wind after fire. | Invades fire-prone shrublands; rugged inaccessible terrain. | Fell shrubs and burn within a year to kill resultant seedlings; biocontrol to reduce seed production. |
| *Acacia mearnsii* (Fabaceae) | Sprouting tree with hard-coated, soil-stored seeds. | Seeds spread down water courses and through the transport of soil. | Invades shrublands, grasslands and savannas, especially along water courses. | Fell and treat stumps with herbicide. Follow-up removal of seedlings essential. Biocontrol to reduce seed output. |
| *Opuntia stricta* (Cactaceae) | Succulent cactus with edible fruits. | Animals eat fruits and spread seeds; also vegetative reproduction. | Invades savanna ecosystems. | Injections of herbicides in isolated individuals; biological control effective in denser stands. |

The first biological control programme initiated in South Africa began in 1913, with the use of imported cochineal insects to control drooping prickly pear, *Opuntia monocantha* Haworth (Cactaceae) (Zimmermann et al. 2004). This programme was a success, and since then numerous biological control programmes have been initiated against various weeds in the country, including several Cactaceae, Australian *Acacia* species, and floating aquatic plants (Zimmermann et al. 2004; Moran et al. 2005; Coetzee et al. 2011a; Zachariades et al. 2017). Increased funding and international collaboration has improved biological control in South Africa in recent years, though further improvement is needed through investment in post-release assessments, research into control of new IAPs, and integration of control methods (Zachariades et al. 2017).

## 1.3 HISTORY OF AQUATIC INVASIONS IN SOUTH AFRICA

Since the end of the 1800s, South African aquatic water systems have been invaded by macrophytes that are introduced through horticulture and aquarium trade (Martin and Coetzee 2011). Eutrophication resulting from urban and agricultural pollution, along with the absence of natural enemies to control invasive plant populations, promotes rapid growth and proliferation of invasive species (Coetzee and Hill 2012). There are five aquatic weeds that were particularly problematic over large areas of South Africa, namely *P. crassipes* (water hyacinth); *Pistia stratiotes* L. (Araceae) (water lettuce); *Salvinia molesta* D.S. Mitch. (Salviniaceae) (salvinia/Kariba weed); *Myriophyllum aquaticum* (Vell. Conc.) Verd. (parrot's feather); and *Azolla filiculoides* Lam. (Azollaceae) (red water fern) (Hill 2003). Biological control programmes have been implemented against these species, with considerable success at reducing the negative impacts associated with their presence in South African ecosystems (Hill and Coetzee 2017) (Table 1.2).

Table 1.2: Impact scores of the generic impact scoring system (GISS) (Nentwig et al. 2016), which measures 12 categories for environmental and socio-economic impacts exerted by alien species. In each category, impact intensity is quantified on a scale of 0 (no impact detectable) to 5 (highest possible impact), so higher scores denote greater impacts. This table shows scores for the top five water weeds in South Africa, considering the worst-case scenario without biological

control in place, and the current situation in South Africa with biological control implemented. Adapted from Hill and Coetzee (2017).

| | **Socio-economic and environmental impact** | |
|---|---|---|
| **Weed** | **Before biological control** | **After biological control** |
| *Pontederia crassipes* | 43 | 23 |
| *Pistia stratiotes* | 38 | 6 |
| *Salvinia molesta* | 38 | 6 |
| *Azolla filiculoides* | 40 | 0 |
| *Myriophyllum aquaticum* | 38 | 15 |

Most of the plants that have invaded South African water systems are free-floating (all of the above mentioned, excluding *M. aquaticum*), and these have been targeted by researchers using integrated control methods which, for the most part, have been successful (Coetzee et al. 2011a). With the control of floating weeds, waterways are open to invasion by submerged weeds such as *Egeria densa* Planch. (Hydrocharitaceae) (Brazilian water weed) and *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae); as well as emergent species including *Sagittaria platyphylla* (Engelm.) J.G.Sm. and *S. latifolia* Willd. (Alismataceae); *Lythrum salicaria* L. (Lythraceae) (purple loosestrife), *Nasturtium officinale* W.T. Aiton. (Brassicaceae) (watercress); *Iris pseudacorus* L. (Iridaceae) (yellow flag); and *Hydrocleys nymphoides* (Humb. & Bonpl. ex Willd.) Buchenau (Alismataceae) (water poppy) (Coetzee et al. 2011a; Coetzee et al. 2011b). New floating weeds such as *Salvinia minima* Baker (Salviniaceae) and *Azolla cristata* Kaulf. (Azollaceae) (Mexican azolla) have also been recorded in their early stages of invasion (Hill and Coetzee 2017), as well as the rooted floating Mexican waterlily, *Nymphaea mexicana* Zucc. (Nymphaeceae) – the subject of this thesis. Targeting IAPs in their early stages of invasion increases the chances of successful control, and so it is vital that biological control programmes for these weeds are implemented before their ranges are allowed to expand further (Olckers 2004). Herein lies the motivation for this thesis.

## 1.4 *NYMPHAEA MEXICANA*

*Nymphaea mexicana* Zuccarini (Nymphaeaceae) (otherwise known as the Mexican waterlily or yellow waterlily) is a hardy waterlily (hardy lilies bloom during the day, while tropical lilies bloom during the day or night) that originates from south-eastern USA, and Mexico (Conard 1905 cited in Capperino and Schneider 1985) (Figures 1.2 and 1.3). The leaves of *N. mexicana* are green and purple with brown patterning, and float on the surface of still or gently flowing water bodies while the roots and rhizomes remain planted in the soil (Wiersema 1988). This biology allows *N. mexicana* and other species in the same genus to outcompete emergent vegetation in deep waters, as well as submerged plants when the floating leaves of the lilies block out sunlight (Wiersema 1988). A 'brood-body' – that is, a cluster of root tubers and buds – is also found in *N. mexicana*, and has been likened to bananas, thus giving rise to an alternative common name of banana waterlily (Johnstone 1982). New plants are produced from thick stolons or via the brood-bodies from thin stolons, and the rhizomes are erect (Johnstone 1982). In its native range, canvasback ducks *Aythya valisineria* Wilson and ring-necked ducks *Aythya collaris* Donovan feed on these tubers (Alexander 1987). *Nymphaea mexicana* also reproduces sexually by means of insect pollination. The yellow flowers have a diameter of 15-33 cm and a calyx with four elliptic to lance ovate sepals, and a stigmatic fluid is produced that facilitates pollination by washing pollen from visiting insects (Capperino and Schneider 1985). Once fertilisation has occurred, green berries holding the seeds grow underwater, and new plants grow from the seedlings.

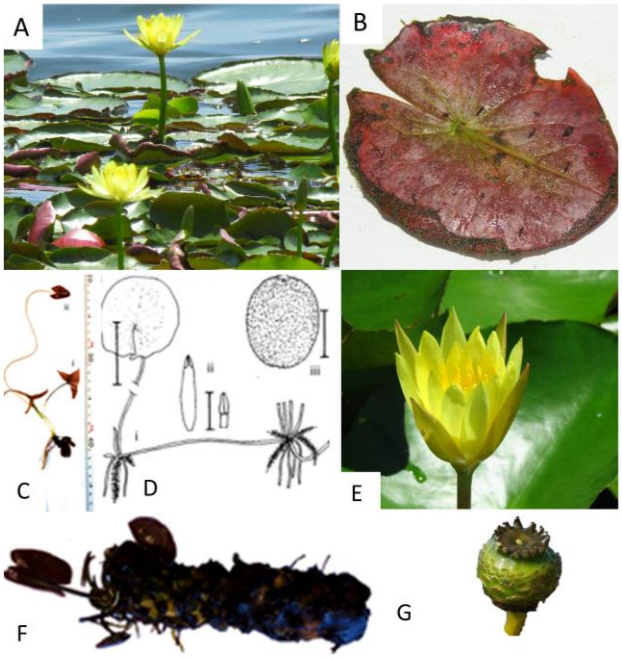



Figure 1.2: Anatomy of *Nymphaea mexicana.* A: Large flat leaves with purple/brown patterning float on the water surface. B: Leaves are often red on the underside. C: Two leaf forms found in *N. mexicana*; top: sagittate leaf, bottom: elliptic leaf (Photo: Prinavin Naidu). D: Components of *N. mexicana* plants: top left: leaf and petiole; top right: seed after shedding the appendage; centre: outermost and innermost stamens; bottom: clonal plants joined by a stolon (Cook 2004). E: Bright yellow flowers with lanceolate petals. F: Large tubers resemble bananas, with fleshy rhizomes (Photo: Prinavin Naidu). G: Fruit.

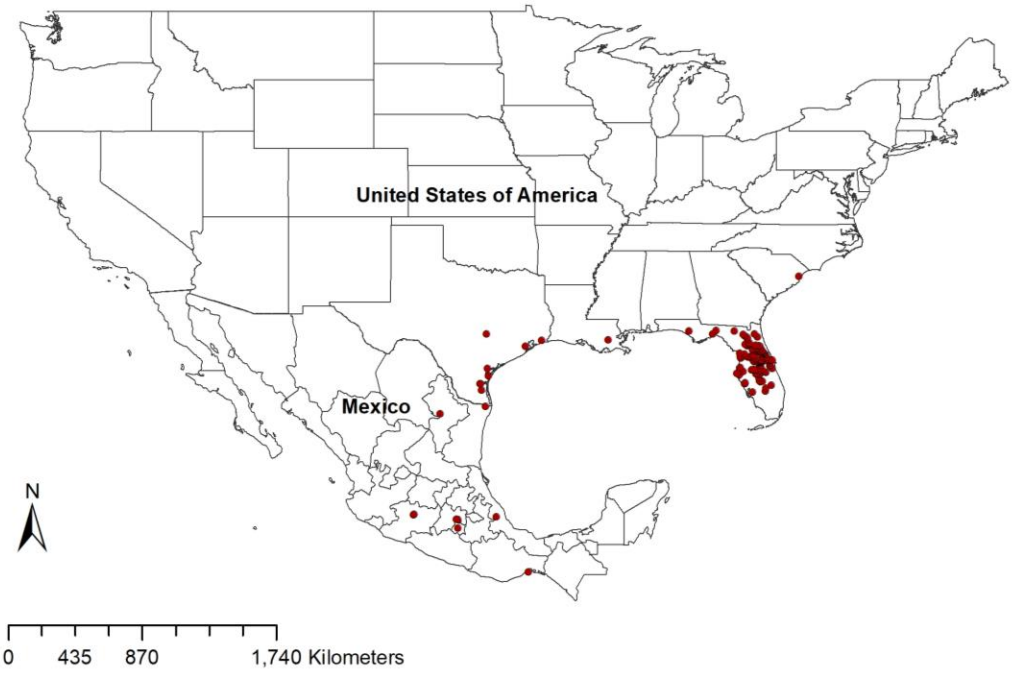



Figure 1.3: Native range of *Nymphaea mexicana*. Mapped in ArcMap (Environmental Systems Research Institute 2014) using distribution data from GBIF (GBIF.org 2019).

*Nymphaea mexicana* has become invasive in numerous regions, including Australia, New Zealand, India, Europe, Spain, and South Africa (Johnstone 1982; Garcia-Murillo 1993; Henderson 2010; Newfield and Champion 2010; Hussner 2012; Shah and Reshi 2012) (Figure 1.4). Due to the aesthetic appeal of this plant, many introductions have occurred through ornamental trade, and its rapid growth has allowed *N. mexicana* to spread rapidly and overwhelm aquatic ecosystems, thereby reducing ecosystem function and recreational value (Capperino and Schneider 1985; Marcos 1985), although its effects in South Africa are not yet well quantified. With the successful control of other invasive floating macrophytes such as salvinia, water lettuce, parrot's feather, and red water fern (Coetzee et al. 2011a), *N. mexicana* as a rooted floating aquatic plant has been able to take advantage of the freed aquatic environments, and has the potential to become increasingly invasive. Furthermore, the biology of this plant makes mechanical removal difficult because unlike floating weeds, the roots must be removed from submerged soils or *N. mexicana* will re-sprout from its rhizomes and stolons (Johnstone 1982). As such, this waterlily is classified as a Category 1b invasive weed in South Africa according to the National Environmental Management: Biodiversity Act (No. 10 of 2004) (NEM:BA). Under

this category, trade or planting is prohibited and this species must be controlled, and where possible, removed and destroyed.


Figure 1.4: Global distribution of *Nymphaea mexicana*. From The Catalogue of Life Partnership (2018).

Distribution in South Africa

*Nymphaea mexicana* was first recorded in the Vaal River near Vanderbijlpark in Johannnesburg in 1968 and has since spread to other regions along the Vaal River and other parts of the country. In 2008 it was recorded in Cape Town in the Westlake and Keyser's Rivers. In 2010, extensive growth was noted in the Keyser's River, and the plants expanded along the length of the Westlake River by 2016 (Fowkes 2016). Other sites have been found in the Western Cape, Gauteng, near Port Alfred, and the North West (Prinavin Naidu pers. comm.) (Figure 1.5).

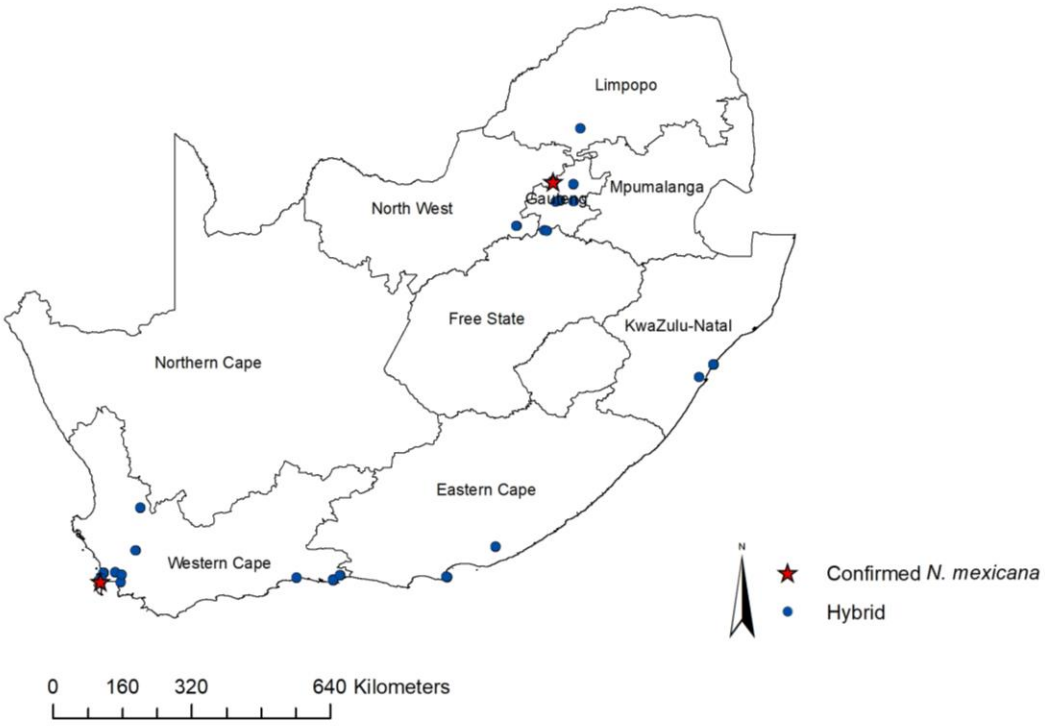



Figure 1.5: Distribution of *Nymphaea mexicana* in South Africa according to morphological and genetic work by Naidu (2018). Mapped using ArcMap (Environmental Systems Research Institute 2014).

Hybrids

It is documented that horticultural hybrids of numerous *Nymphaea* species have been created especially by Joseph Bory Latour-Marliac in the 19$^{th}$ century (Holmes 2015; Sheldon 2017). Hence, various hybrids of *Nymphaea* species are known and have been bred and sold in the ornamental trade. The flowers of the waterlilies found at the sites infested with *N. mexicana* in South Africa possess morphological characteristics that suggest that they are hybrids of *N. mexicana* and other *Nymphaea* species (Prinavin Naidu pers. comm.). The population at Westlake in Cape Town appears to consist of *N. mexicana* in its original form, while other populations in the Western Cape and Gauteng seem to consist of *Nymphaea* X *marliacea* Latour-Marl. ex Gardn. or *Nymphaea marliacea* var. *chromatella* (*N. chromatella* Hort.) (Naidu 2018). These are believed to be hybrids of *Nymphaea alba* Linneaus x *N. mexicana*, or *Nymphaea tuberosa* Paine x *N. mexicana* (Verdcourt 1989). *Nymphaea* X moorei Hort., a hybrid of *N. mexicana* x *N. alba* (different flower morphologies are produced depending on which species

provides the pollen when the species hybridise), as well as *N. alba mexicana* x *Nymphaea pubescens* Willd. (also known as *N. rubra)* hybrids are also found in Gauteng, Western Cape, Eastern Cape, and North West in South Africa (Naidu 2018). No hybridisation appears to have occurred between *N. mexicana* and native waterlilies found in South Africa, namely *Nymphaea lotus* Linneaus and *Nymphaea nouchali* Burm.f. (Prinavin Naidu pers. comm.).

Impact

*Nymphaea mexicana* is capable of colonising nutrient rich waters up to 2 m deep, and can reduce gas exchange, restrict water movement, increase siltation, and decrease recreational value (Capperino and Schneider 1985). In addition, water quality may be reduced in the presence of *N. mexicana* populations, leading to foul odour and decreased habitat value for flora and fauna (Finlay 2008 cited in Hofstra et al. 2013). Another problem associated with *N. mexicana* infestations is the depletion of oxygen levels in occupied waterbodies, especially after rhizome death due to herbicidal treatment (Hofstra et al. 2013). This in turn has negative impacts on fauna living in the water, and may be linked to fish deaths downstream of infestations in Goulburn River in Victoria, Australia (G-MW 2009; Hofstra et al. 2013), which in turn affects birds that feed on fish. Like water hyacinth (*P. crassipes*), *N. mexicana* forms dense mats on water surfaces and blocks out light for submerged plants, though it cannot be removed as easily because it is rooted in the soil. As such, *N. mexicana* may pose a higher threat than water hyacinth (Fowkes 2016). Without sufficient control, *N. mexicana* will likely spread and move into other freshwater bodies, as rhizomes, tubers and seeds are dispersed by water, while boats and machinery can spread fragments (Fowkes 2016). While the economic impacts caused by *N. mexicana* have not yet been quantified in South Africa, it is important to develop control programmes for this weed before its range is expanded and prospects of control are further encumbered.

Current control

In Australia, the herbicide Roundup Biactive, which contains glyphosate as the active ingredient, is used to provide control of *N. mexicana* infestations (G-MW 2009). Mechanical control is also effective, but regrowth occurs within 8-12 months with both methods of control (G-MW 2009; Hofstra et al. 2013). Low cover of *N. mexicana* is maintained for a longer period of time after herbicide application during midsummer to autumn, as translocation of solutes into

the rhizomes occurs more effectively at this time, while growth is triggered only in spring conditions (Hofstra et al. 2013). Maintaining low cover of *N. mexicana* in summer months and for long durations requires two applications per year (in spring and autumn), with mechanical removal of rhizomes once the plants begin to die off (Hofstra et al. 2013).

There are currently no registered herbicides for use against *N. mexicana* in South Africa (Fowkes 2016). Infestations of *N. mexicana* in Westlake, Cape Town (34°04'54.9"S 18°27'23.4"E) were originally sprayed with glyphosate as in Australia (Gaertner et al. 2016), but authorities have since resorted to manual removal as a result of public outcry against the use of glyphosate (Naidu 2018). Manual removal is also used in Emmarentia Dam through hand-pulling of the leaves by a contracted company (Naidu 2018). The lower costs and lower environmental impacts of biological control compared to chemical and mechanical control makes it a desirable option for managing *N. mexicana,* but no biological control programmes have been developed to manage this species yet. The implementation of biological control requires that researchers proceed through numerous stages to collect information and prevent impacts on non-target species (Figure 1.6). Completion of these steps in numerical order maximises the likelihood that the biological control programme will be successful.

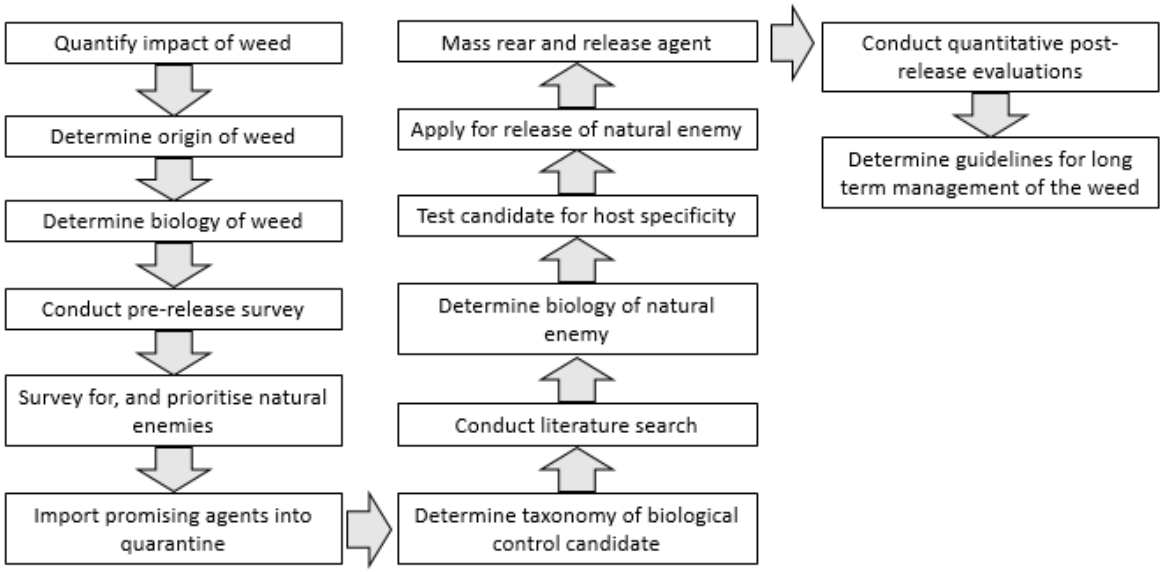



Figure 1.6: 14 steps to 'biological control happiness' as per Martin Hill (pers. comm.).

Potential impact of biological control

Many invasive weeds have far-reaching effects in invaded regions. These effects include reduction of natural water supplies, inhibition of agricultural and recreational activities, reduction in native biodiversity, blocking of waterways used for transport and fishing, and spread of disease through the creation of desirable habitat for insects such as mosquitoes. Economically, these effects can be devastating, requiring millions of rands to mitigate effects and remove populations. Biological control provides a long-term, environmentally friendly, and relatively inexpensive means of controlling invasive alien plants, and is thus beneficial through reducing the negative effects. Biological control in general has created job opportunities for many people, through Working for Water schemes and the employment of disabled individuals from poorer communities to maintain plant and insect cultures at Waainek Research Facility at Rhodes University in Grahamstown. Continuing research and implementation of biological control research will allow these schemes to continue and grow, thereby providing further socio-economic benefits.

## 1.5 THESIS OUTLINES AND AIMS

This thesis aims to cover the first steps of the initiation of a biological control programme for *N. mexicana* in South Africa. Firstly, it is important to understand the genetic structuring of invasive plant populations and to compare this to native populations. Naidu (2018) began to conduct genetic analyses of *N. mexicana* populations in the invaded range using AFLPs, but only included samples from two sites in the native range with which to compare the invaded range samples. Native range samples were collected in south-eastern USA and these were utilised with invaded range samples collected by Naidu to conduct ISSR analyses in Chapter 2. Obtaining an improved understanding of the genetic structuring of invasive populations of *N. mexicana* will enable more informed decisions about source populations of biological control agents (Goolsby et al. 2006a; Paterson et al. 2009; Sutton et al. 2017).

No insects native to South Africa are known to feed on *N. mexicana* (Prinavin Naidu, Julie Coetzee pers. comm.). Hence, it was necessary to conduct surveys for natural enemies across the native range of *N. mexicana,* with focus placed on the regions that match climatically to invaded regions in the introduced range (Briese and Walker 2002; Goolsby et al. 2003). These surveys are detailed in Chapter 3. Chapter 4 presents a discussion of the importance of the

genetic studies in Chapter 2, and of the field surveys conducted in Chapter 3. A discussion of the predicted efficacy of the agents prioritised in Chapter 3, biological control of hybrids, and the limitations of the studies in this thesis are also included. Finally, recommendations for the next stages of programme development are made.

# Chapter 2: Matching invasive *Nymphaea mexicana* to native range populations

## 2.1 INTRODUCTION

The success of biological control programmes may be improved by matching populations of an invasive species in the introduced range to populations in the native range, and by ensuring that the taxonomy of invasive species is well studied. Resolving taxonomic uncertainties will facilitate more efficient management of invasive populations by: identifying source populations from which potential biological control agents should be collected and tested for host specificity; predicting and understanding the performance of biological control agents at different sites; determining genetic diversity and gene flow in invasive populations; and understanding if there has been any hybridisation of the target plant with other invasive or native species (Vilà et al. 2000; Le Roux and Wieczorek 2009; Gaskin et al. 2011; Hulme et al. 2013). Such analyses are usually conducted in the early phases of the initiation of biological control programmes.

*Nymphaea mexicana* is targeted for biological control in South Africa, but it and many of the Nymphaeles hybridise readily with other *Nymphaea* species including *N. odorata*, which is found in the native range of *N. mexicana*. Some hybrids created through horticulture have naturalised in the wild and may displace indigenous 'pure' bred forms (Dkhar et al. 2013; Nierbauer et al. 2014). These hybrids can be difficult to distinguish based solely on morphological characteristics, and this difficulty becomes evident and problematic where invasions of *Nymphaea* species have occurred (Padgett 2001; Nierbauer et al. 2014; Dana et al. 2017).

Hybridisation of invasive weeds can have important implications for their control (see Chapter 4). For example, hybridisation can result in novel genotypes that are better adapted to the environment (Snow et al. 1999; Whitney et al. 2006; Rieseberg et al. 2007), while other studies have recorded larger size or higher fecundity in hybrid species compared to their parent species (Campbell et al. 2006; Whitney et al. 2006; Ridley and Ellstrand 2010). Differences in life history traits such as these increase the invasive potential of some species through hybridisation. (Ellstrand and Schierenbeck 2000). Additionally, hybrids may be more, or less, resistant to insect attack compared to their parent species (Fritz et al. 1999; Moody and Les 2002; Krebs et al. 2011). For example, greater tolerance to defoliation, higher investment in roots, and lower resistance to insect attack were recorded as a result of higher

levels of introgression within *Tamarix* spp. L. (Tamaricaceae) (Williams et al. 2014), while novel chemical combinations in hybrids may deem the plants unrecognizable or unpalatable to specialized herbivores (Schoonhoven et al. 2005). Reduced insect resistance in hybrids has also been reported (Fritz et al. 1999). Nevertheless, these differences in life history and herbivore resistance in hybrids impact ecological interactions with specialized natural enemies, and thus affect the success of biological control efforts (Williams et al. 2014).

Genetic variation in invasive and native plant populations may also influence biological control. Studies show that natural enemies are often locally adapted to their hosts, and demonstrate better performances on genotypes from their local populations than other populations (Jarosz and Burdon 1991; Lively and Jokela 1996; Mopper and Strauss 1998; Cory and Myers 2004; Kawecki and Ebert 2004). For example, Goolsby et al. (2004) found strong evidence that *Floracarus perrepae* Knihinicki and Boczek (Eriophyidae), a mite considered for the biological control of *Lygodium microphyllum* (Cav.) R.Br. (Lygodiaceae), is locally adapted to certain genotypes of the plant. Other studies have similarly shown that herbivores can distinguish between hybrid genotypes in native systems (Fritz et al. 1998; McGuire and Johnson 2006). However, the importance of genetic variation for biological control differs case by case, as some biological control agents may vary in terms of their preference and performance among genotypes, but others do not (Kaltz et al. 1999; Hufbauer 2002; Goolsby et al. 2006b).

Understanding the genotypes of invasive plant populations, and how they compare to native populations is important to determine the most suitable biological control agents for invasive populations and increase the likelihood of the success of biological control programmes. For example, *Chromolaena odorata* (L.) R.M. King & Rob. (Asteraceae: Eupatorieae) exists as two genotypes in its invaded range: the Asian/West African genotype found in West and Central Africa, India, Southeast Asia, and Oceania, and the southern African genotype found in southern Africa (Paterson and Zachariades 2013). It is believed that the limited success of biological control of *C. odorata* in South Africa can be explained, in part, by the incompatibility of the agents with the southern African genotype of the plant (Zachariades et al. 2011). In contrast, substantial control of the same plant in Southeast Asia and Oceania has been achieved using biological control agents collected from the Asian/West African genotype (Zachariades et al. 2009). To improve the biological control of *C. odorata* in South Africa, agents should be collected from the origin of the South African genotype, which was determined to be Cuba or Jamaica using molecular methods (Paterson and

Zachariades 2013). Molecular techniques were necessary to identify the origin of this genotype and thus to improve the compatibility of the biological control agents used.

In the *C. odorata* example, there were some morphological differences between the Asian/West African genotype and the southern African genotype, but this was not enough to distinguish between genotypes alone as some morphologically distinct samples grouped together genetically (Paterson and Zachariades 2013). Hence, morphological characteristics are often not sufficient to distinguish between species, and underlying differences between populations of plants and insects can have major implications for biological control (Verloove 2010; Hulme et al. 2013; Madeira et al. 2016). Resolving taxonomic differences using molecular techniques can therefore be critical for the successful selection and establishment of biological control agents. The use of molecular techniques enables the parent plants of each hybrid to be determined and closely related genetic groupings to be matched to improve the selection and establishment of biological control agents (Urban et al. 2011).

Molecular markers 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 (Esselman et al. 1999; Li et al. 2006; Paterson and Zachariades 2013). Various genetic markers may be used to study population genetics, including restriction length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) markers, amplified fragment length polymorphisms (AFLP), and inter-simple sequence repeats (ISSRs) (Le Roux and Wieczorek 2009). Of the various markers available, simple sequence repeats (SSRs) or microsatellites are some of the most informative markers currently available, owing to high levels of polymorphism and codominant Mendelian inheritance, and are becoming increasingly popular in studies investigating genetic diversity within populations, genome mapping, parentage, and kinships (Le Roux and Wieczorek 2009; Gaskin et al. 2011).

SSRs are sets of a few nucleotides repeated in tandem throughout an organism's genome (Hamada et al. 1982; Tautz 1989; Weber and May 1989). Inter-simple sequence repeats (ISSR) make use of microsatellite anchored primers annealed to SSR regions to amplify regions between adjacent SSRs. ISSR markers are desirable for their robustness and cost efficiency, and have the advantage that prior knowledge of the genome sequence is not required for them to assess genetic variability in plant species (Gui et al. 2008). ISSRs are rapid, have good reproducibility, and have been used successfully to differentiate between closely related individuals (Zietkiewicz et al. 1994; Bornet and Branchard 2001; Paterson et

al. 2009; Barker et al. 2015). ISSR markers overcome numerous technical limitations presented by RFLP (Rafalski et al. 1991) and RAPD (Devos and Gale 1992) analyses both in animal and plant DNA (Tsumura et al. 1996).

ISSRs have been used to determine genetic structuring in other *Nymphaea* species. For example, Woods et al. (2005) used ISSR analysis to confirm that *N. odorata* consists of two subspecies, namely *Nymphaea odorata* subsp. *odorata* and *Nymphaea odorata* subsp. *tuberosa*, and that these should not be distinguished at the species level. Similarly, they determined that *N. odorata* and *N. mexicana* could be clearly distinguished based on ISSR data, despite suspected introgression between the two species. These results were further supported by sequence data obtained using nuclear internal transcribed spacer (ITS) and plastid trnL-trnF region molecular analyses (Woods et al. 2005a).

Genetic structuring of the introduced populations of *N. mexicana* in South Africa has been investigated previously using AFLPs (Naidu 2018). Naidu (2018) compared samples from invasive populations in South Africa to *N. mexicana* samples collected at two sites in the native range of the plant: Lake Kissimmee, Florida, and Lewisville, Texas in the south-eastern USA. Results from these analyses revealed the presence of multiple hybrid groups of *N. mexicana* in South Africa, as well as pure *N. mexicana* that grouped with the two samples from the US. However, the presence of natural hybrids in the wild and in the invaded range, and possible genetic differences between populations in the native range, may reveal more useful information about the genetic structuring of this species and its hybrids. Moreover, only two sites from the native range were used in Naidu's (2018) analysis. Hence, it is necessary to build on to Naidu's work using samples from more populations within the native range of *N. mexicana*. This forms the aim of this chapter, using samples from *N. mexicana* populations that were sampled during the first surveys for biological control agents of *N. mexicana* in the US. Conducting these analyses and adding them to Naidu's work will improve our understanding of the native and invasive populations of *N. mexicana* and allow us to refine efforts to initiate a biological control programme to manage invasions of this plant in South Africa, and other sites around the world.

## 2.2 MATERIALS AND METHODS

### Sampling

Samples from the invaded range in South Africa were collected from natural and artificial water bodies by Prinavin Naidu (Naidu 2018). Whole leaves were collected 5 m

apart from each other, rinsed with water and dried before being wrapped in paper towel and stored in sealed bags with silica gel. DNA was extracted from four dry leaf samples from each site using QIAGEN Mini Plant Extraction kits (QIAGEN Inc.) as detailed below (Naidu 2018), except the samples from Westlake in which DNA was extracted from 12 leaves due to the larger sampling area (Table 2.1).

In the native range, 17 populations of *N. mexicana* were sampled across south-eastern USA including Florida, Louisiana, and Texas (Table 2.1). At each site, approximately 10 leaf segments were collected from plants at least 10 m apart to ensure sufficient sampling of the population and to reduce the collection of clonal plants. The leaf segments were wrapped in paper towelling and stored individually in clear plastic Ziploc bags containing about 30 g of silica gel or equivalent desiccant, which was changed as needed to desiccate the leaf material and ensure dry storage. A further eight samples collected from Lewisville, Texas, and 20 samples from Kissimmee, Florida, were couriered to Rhodes University from colleagues residing in the native range (Naidu 2018).

DNA extraction

Each leaf sample was ground in a mortar and pestle using liquid nitrogen to produce 30-40 mg of dry leaf powder. Total genomic DNA was then extracted from each sample using QIAGEN Mini Plant Extraction kits (QIAGEN Inc.). A NanoDrop™ 2000 spectrophotometer (Thermo scientific™) was used to measure DNA quality and concentration, and the extracted DNA was stored at -20 $^{o}$C for later use.

ISSR PCR protocol and analysis

Two primers were used in the analyses: the universal primer HB15 manufactured by Applied Biosystems Inc., U.K. (Paterson et al. 2009; Barker et al. 2015) and UBC-852 manufactured by Integrated DNA Technologies, WhiteSci Whitehead Scientific (Pty) Ltd., RSA (Poczai et al. 2011) (Table 2.2). 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). Other primers that produced fewer or inconsistent bands during pilot tests were 17899A (Wolfe et al. 1998), ISSR-19 (Poczai et al. 2011), and 901 (Woods et al. 2005b).

The ISSR PCR reactions utilised 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, $MgCl_2$, enhancers, stabilisers, and dyes), 3 µL of plant DNA, and 6.2 µL denucleated water. The ISSR PCR protocol was as follows: an initial denaturing step of 2 minutes at 94 °C, and then 35 cycles of 94 °C for 30 s, 44 °C for 45 s and 72 °C for 90 s (Paterson et al. 2009). A final extension of 20 min at 72 °C ended the cycle.

Similar concentrations were used to make up the reaction volumes for the UBC-852 primer, except that half the volumes were utilised to make up 10 µL per reaction. Hence, 0.4 µM of 852 primer, 5 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1.5 µL plant DNA, and 3.1 µL denucleated water were used. These lower volumes were used to reduce costs as preliminary tests suggested that 10 µL reactions were sufficient to obtain acceptable results. The ISSR PCR protocol had the following parameters as per Poczai et al. (2011): initial denaturation step of 2 minutes at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 minute at 53 °C, and 2 minutes at 72 °C. Final extension occurred at 72 °C for 5 minutes.

Table 2.1: Details of invasive and native samples of *Nymphaea mexicana* used for genetic matching using ISSR analysis. The number of samples varied due to unequal sampling and removal of low-quality samples during analysis by Naidu (2018). Where large numbers of samples were used (more than 4 samples for the invaded range), they were collected from multiple sites within the same area.

| **Province/State** | **Locality** | **Latitude** | **Longitude** | **Number of samples** | **Sample code** |
|---|---|---|---|---|---|
| **Invaded range: South Africa*** | | | | | |
| Western Cape | Muizenberg, Westlake | -34.0841 | 18.4437 | 17 | WL |
| | Neil Ellis, Stellenbosch | -33.9249 | 18.8912 | 2 | NE |
| | Century City | -33.8878 | 18.5128 | 4 | CC |
| | Kluitjieskraal | -33.0622 | 20.3562 | 2 | KK |
| | Maynardville Wynberg | -34.0053 | 18.4643 | 4 | MAYN |
| | George | -33.9945 | 22.5262 | 3 | GEO |
| | Dam 1, Plettenberg, Knysna | -34.0448 | 23.2919 | 2 | KNY |
| | Yellowwood dam, Somerset | | | 2 | SOM |
| | West | -34.0941 | 18.8651 | | |
| | Bellevue wine estate, | | | 4 | BELL |
| | Stellenbosch | -33.8791 | 18.7630 | | |
| | Cottage dam farm, Kromrivier | -32.5417 | 19.2811 | 4 | KR |
| Eastern Cape | Boardwalk, Port Elizabeth | -33.9830 | 25.6574 | 3 | PE |
| Gauteng | Benoni | -26.1705 | 28.2890 | 1 | BE |
| | Morelata Park, Pretoria | -25.8139 | 28.2848 | 2 | PRET |

| | | | | | |
|---|---|---|---|---|---|
| | Emmarentia, Randburg | -26.1501 | 28.0058 | 8 | EMM |
| | Florida lake | -26.1783 | 27.9065 | 3 | FLLAKE |
| North West | Potchefstroom NWU Botanical gardens | -26.6823 | 27.0950 | 3 | POT |
| KwaZulu-Natal | Paradise Valley | -29.8321 | 30.8922 | 2 | D |
| **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 |
| | Emeralda 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 |

* Samples collected in Naidu (2018).

Table 2.2: Details of primers successfully used in ISSR analysis.

| Primer name | Primer sequence (5’ to 3’) | Average number of replicable bands |
|---|---|---|
| HB15 | GTGGTGGTGGC | 36 |
| 852 | TCTCTCTCTCTCTCTCAGA | 15 |

Electropherograms

The primers were fluorescently labelled with 6-FAM dye on the 5' end, and the PCR products were sent to Central Analytical Facilities (CAF) at Stellenbosch University, Stellenbosch, South Africa to visualise banding patterns. This was carried out by capillary electrophoresis using an ABI 3130 genetic analyser. There were two replicates for each sample to ensure replicability of the PCR reactions and a binary matrix was generated based on the absence or presence of bands. The electropherograms were analysed using GeneMarker® ver. 2.7.4 (SoftGenetics LLC.). 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, after they had been analysed and sized in GeneMarker.

ISSR scoring parameters

Band scoring differs depending on the setting used in the analytical software, so preliminary tests were conducted to determine the settings that produced the lowest error rates as suggested by Holland et al. (2008). A subset of 30 samples run using HB15 was analysed using minimum peak heights of 20, 50, and 100 in GeneMarker. The stutter peak filter and AFLP normalisation was unchecked, smoothing was selected, and the 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 created by Clarke van Steenderen (site can be accessed through https://clarkevansteenderen.shinyapps.io/BINMAT/). This site was also used to generate Euclidean and Jaccard's average error rates as well as data summaries and non-metric Multidimensional Scaling (nMDS) plots to test different settings.

Standard (Euclidean) and Jaccard's replicate error rates were calculated for the resulting dataset after analyses with each of the settings (Bonin et al. 2004; Pompanon et al. 2005). In the equations below, $N_{(1,1)}$ and $N_{(0,0)}$ represent the number of calls in which both of the two replicates either do or do not have a peak respectively; and $N_{(1,0)}$ and $N_{(0,1)}$ represent the incorrect calls

where one of the two replicates has a peak, and the other does not. These values are calculated by obtaining the sum of all the replicate pair values in the data from the consolidated matrix. The equations are as follows, as defined by Bonin et al. (2004) and Holland et al. (2008):

Standard replicate error (average Euclidean distance between replicate pairs):

$$\frac{N_{(0,1)} + N_{(1,0)}}{N_{(0,0)} + N_{(1,0)} + N_{(0,1)} + N_{(1,1)}}$$

Jaccard's error (average Jaccard's distance between replicate pairs):

$$\frac{N_{(0,1)} + N_{(1,0)}}{N_{(1,0)} + N_{(0,1)} + N_{(1,1)}}$$

Analysis of full dataset

The settings that produced the lowest error rates and greatest number of peaks for the subset of samples were more likely to elicit higher quality results. For the preliminary tests, the error rates were similar for the matrices generated using a minimum peak height of 20 and 50, and those generated at a peak height of 100 produced greater error rates and a lower number of peaks. For the final dataset, a minimum peak height of 20 was used to analyse the data in GeneMarker, and other parameters were set as in the preliminary tests. The first 160 base pairs were excluded from the analysis for HB15, and the first 80 base pairs were excluded for UBC-852, as most of the samples shared the same peaks between these base pairs. 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 later analyses and plots and were considered to have failed to amplify. After data from each primer had been analysed separately, the binary matrices were combined and analysed as a whole.

Non-metric Multidimensional Scaling (nMDS) plots were produced using the final consolidated binary matrix in the programme PAST: Paleontological Statistics package ver. 3 (Hammer et al. 1999). This analysis was appropriate for the binary data as it is categorical. PAST was also utilised to convert the binary matrix to a pairwise similarity matrix using the Jaccard's similarity index. The Jaccard's index is the most suited coefficient to this data, as it excludes shared absence as a character (Sokal and Sneath 1963). A phylogenetic network was constructed 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). Network analyses such as these consider intraspecific and population level phenomena such as recombination, unlike more traditional phylogenetic analyses such as NJ, MP, and Bayesian analyses (Posada and Crandall 2001).

The mean pairwise Jaccard's genetic distances were calculated for all the invasive samples, native samples, and the invasive samples that grouped with the native samples in the nMDS and SplitsTree analyses and were used as a measure of genetic diversity. Significant differences between the populations were tested using a Wilcoxon signed-rank test (native vs invasive), and a Kruskal-Wallis ANOVA (invasive samples that didn't group with native vs native vs invasive samples that did group with native) in R ver. 3.5.3 (R Development Core Team 2013) as the data were not normally distributed. Non-parametric post-hoc analyses were used to examine genetic differences between the groups.

AMOVAs (Analysis of Molecular variance) were conducted using the Gen-AlEx ver, 6 software package in Microsoft® Excel (Peakall and Smouse 2006) to determine genetic variation between and among the three groups identified in the MDS and SplitsTree analyses: a) native samples, b) invasive samples, and c) invasive samples that grouped with the native population (mixed group). This analysis was chosen to allow comparison with AMOVA results obtained by Naidu (2008) and Woods et al. (2005). Permutations were set at 999, and the population estimator PhiPT ($\phi$PT) 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).

STRUCTURE analysis

STRUCTURE 2.3.4 (Pritchard et al. 2000), by use of a Bayesian clustering algorithm, was used to assign individuals to genetic clusters (K) based on similarities between genotypes. Estimates of the probability of assignment (Q) for each individual for values of K from 1 to 5 were obtained by running five iterations of 100 000 Monte Carlo Markov Chain (MCMC) followed by 50 000 burnins. The admixture model was used, based on evidence that demonstrates this as the most efficient model for intra-specific variation studies (Falush et al. 2003), and other settings were left at default. StructureSelector (Li and Liu 2018) was used to determine the optimal number of K-clusters, using the ad hoc statistic $\Delta$K (Evanno et al. 2005) as

well as the Puechmaille Method of K selection (Puechmaille 2016). Graphical outputs of the results from STRUCTURE for the selected K values were prepared using CLUMPAK software (Kopelman et al. 2015).

Samples that were assigned a Q-value greater than or equal to 0.80 to a genetic cluster were considered as "pure" individuals belonging to that cluster. Samples with Q-values below 0.80 to any genetic cluster were considered to be of hybrid origin (Andersen and Mills 2016; Sutton et al. 2017). The simulations were run using the full dataset, as well as restricting the analysis to the native samples as well as those that clustered with the native samples in the MDS plot.

## 2.3 RESULTS

Preliminary tests

The preliminary tests showed that a minimum peak setting of 100 in GeneMarker elicited a slightly lower average number of peaks, and a slightly higher error rate compared to the peaks and errors generated using a minimum peak height of 20 and 50 (Table 2.3). The average number of peaks and average error rates were identical for the 30 sample subset used for the preliminary analysis at minimum peak heights of 20 and 50 so a minimum peak height of 20 was used for the full dataset as it would likely increase the number of peaks in the full dataset.

Table 2.3: Results from preliminary tests using three different minimum peak height settings in GeneMarker.

| Min peak height | Min/max bin width in RawGeno | Average number of peaks | Min/max number of peaks | Number of loci | Average Euclidean error | Average Jaccard's error |
|---|---|---|---|---|---|---|
| 20 | 1/1.5 | 24.4516 | 2/47 | 168 | 0.2732 | 0.6472 |
| 50 | 1/1.5 | 24.4516 | 2/47 | 168 | 0.2732 | 0.6472 |
| 100 | 1/1.5 | 23.6774 | 2/46 | 160 | 0.2802 | 0.6484 |

ISSR data

The final dataset (after low quality samples were removed and data from both primers were combined) had a mean Euclidean error rate of 7.24 %, and a mean Jaccard's error rate of 50.58% (Bonin et al. 2004, 2007; Holland et al. 2008; Whitlock et al. 2008). The mean number of replicable peaks was 51.77, with 732 loci ranging in size from ~85 to 1190 bp. There was a minimum of 21 and maximum of 112 peaks. Overall 628 sites were polymorphic (85.80 %).

Two clusters were observed on the nMDS plot (Figure 2.1). In the first main group, all of the native range samples grouped with most of the invasive range samples, including those from Westlake. The second grouping was not as tightly clustered as the first, and consisted of samples from Knysna, Emmarentia, Potchefstroom, Bellevue Wine Estate, George, Krom Rivier, and Neil Ellis in Stellenbosch. Some samples from Emmarentia also grouped with the native samples.

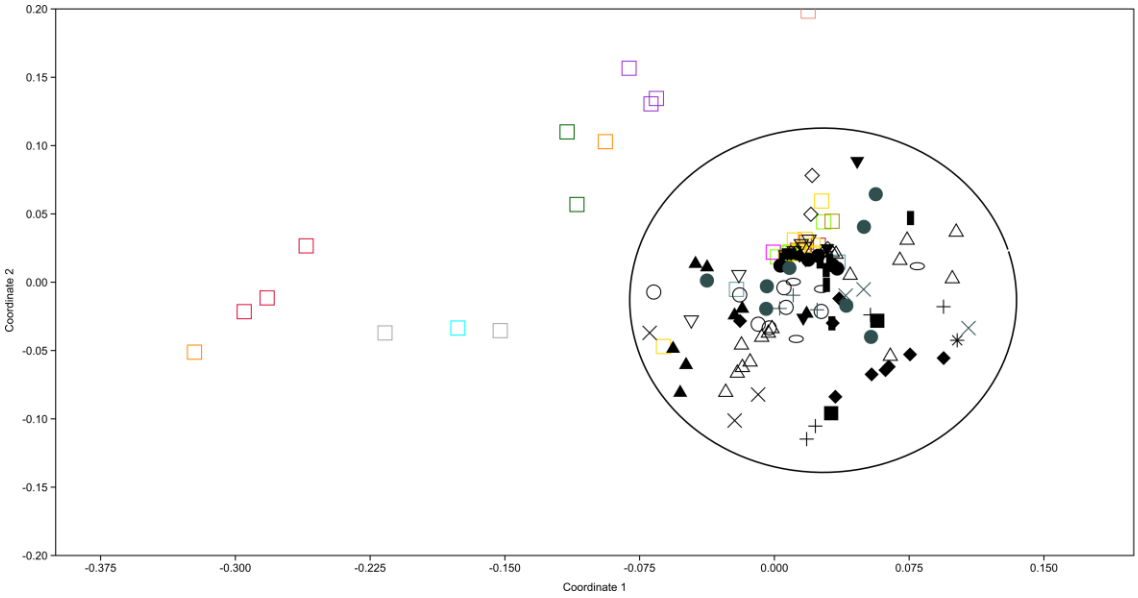
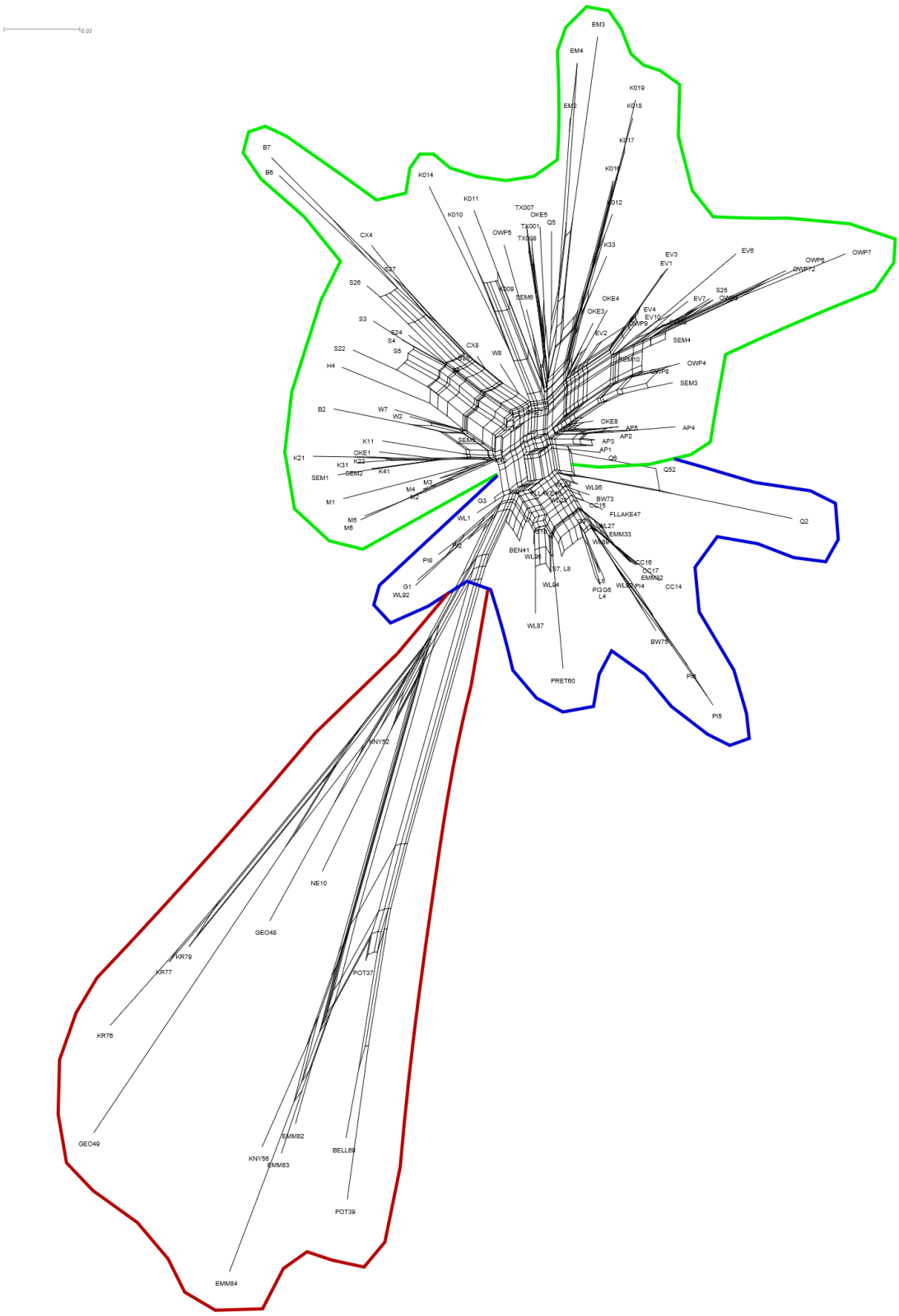



Figure 2.1: Non-metric MDS for the combined data. Black and dark grey symbols represent the native samples, with different symbols representing different sites. Coloured square outlines represent invasive samples, with colours representing different sites. Stress = 0.293 (a value greater than 0.2 is close to random). $R^2$ axis 1 = 0.5836. Axis 2 = 0.1868.

The SplitsTree analysis indicated the presence of two major groups, with one of these groups further separated into two subsets (Figure 2.2). The first major cluster consisted of the same samples that were grouped separately in the nMDS plot, namely samples from Knysna,

Emmarentia, Potchefstroom, Bellevue Wine Estate, George, Krom Rivier, and Neil Ellis in Stellenbosch. The second major grouping consisted of the same native and invasive samples that grouped together in the nMDS. Within this group, a subset was formed by most of the native range samples, while a second subset consisted of the remaining invasive samples mixed with native samples from Lake George, Pine Island, Lake Lawne, and some overlap with samples from Quinta, and Lake Apopka.


0.01
EM3
EM4
EM2
K019
K018
K017
K016
K012
K014
K011
K010
TX007
OKE5
Q5
TX001
TX008
OWP5
K33
EV3
EV1
EV6
OWP6
OWP7
OWP12
S26
S27
K09
SEM6
OKE4
OKE3
EV4
EV10
EV7
OWP5
EV2
SEM4
SEM10
OWP4
OWP9
SEM3
CX4
S3
S24
S4
S5
S22
H4
CX8
W8
B2
W7
W2
OKE8
AP5
AP4
AP2
AP3
AP1
Q6
SEM5
K11
OKE1
K21
K22
K31
K41
SEM1
SEM2
M3
M4
M2
M1
M5
M6
WL1
G3
Q52
WL95
BW73
CC15
FLLAKE47
Q2
WL27
EMM33
WL88
BEN41
WL96
PI8
G2
G1
WL92
S7, L8
WL94
CC16
CC17
EMM32
PI3G5
L4
WL88F4
CC14
WL87
BW75
PRET60
PI6
PI5
KNY52
NE10
GEO48
KR79
KR77
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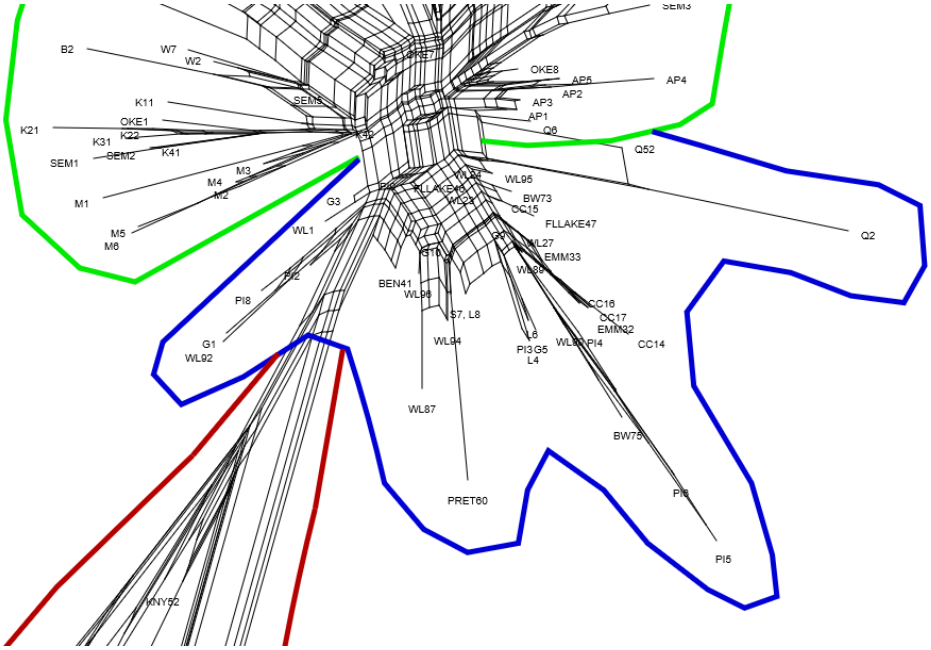
POT37
GEO49
EMM82
KNY56
EMM83
BELL69
POT39
EMM84

A




B

Figure 2.2: NeighbourNet tree using Jaccard's distance constructed in SplitsTree. Green outline represents native range data, blue outline represents mixed invasive and native sites that group together, and red outline represents invasive data. A: full view. B: zoomed view of mixed group. Site codes are given in Table 2.1.

Within the invasive distribution, although 57 % ($P$ = 0.001) of the genetic variability could be attributed to within population variation, a significant amount of genetic variability was attributed to amongst population variation (43 %, $P$ = 0.001). Similarly, for the native range, 20% ($P$ = 0.001) of the variation was significantly attributed to amongst population variation, and 80% ($P$ = 0.001) to within population variation. There was moderate support for population differentiation between the invasive and native samples ($\phi$PT = 0.097, $P$ = 0.001), and strong support for differentiation between the native samples, the invasive samples that grouped with the native samples (mixed group), and the other invasive samples ($\phi$PT = 0.189, $P$ = 0.001). When comparing the invasive samples that grouped with natives (mixed group), with the remaining invasive samples, levels of variation were 30% among populations and 70% within populations, with significant differentiation between these populations ($\phi$PT = 0.299, $P$ = 0.001).

The mean (± S.D.) Jaccard's genetic differences of the invasive population (0.41 ± 0.19, n = 101) was slightly lower than that of the native populations (0.48 ± 0.06 n = 37), but this

difference was not statistically significant (W = 1930, *P* = 0.7694). However, significant differences were observed between all groups when comparing the native samples (0.48 ± 0.06, n = 101), invasive samples (0.18 ± 0.09, n = 14), and the mixed invasive samples that grouped with the natives in the SplitsTree analysis (0.55 ± 0.04, n = 23) (H = 58.11, d.f. = 2, *P* < 0.05), as revealed by non-parametric post-hoc analysis (Figure 2.3). The invasive samples had the lowest mean genetic diversity, with the mixed group having the greatest genetic diversity.

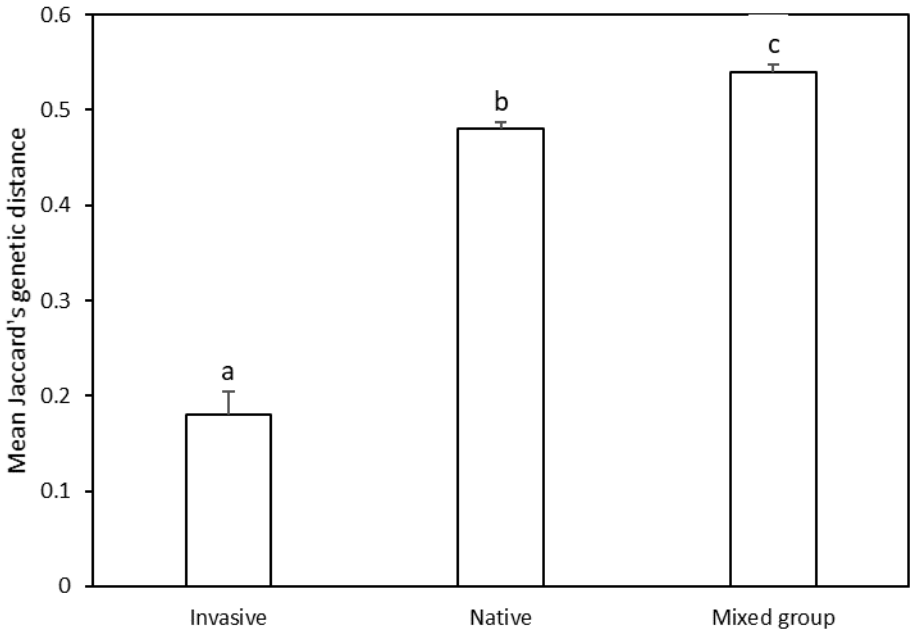



Figure 2.3: Mean (±S.E.) Jaccard's genetic distances of *Nymphaea mexicana* samples from the native and invasive range. Invasive samples are those that clustered separately from the native cluster on the nMDS and SplitsTree plots, the native bar represents the native samples excluding those that grouped with the other invasive samples, and the mixed group bar represents the native and invasive samples that grouped together on the SplitsTree plot. The letters above the bars represent significant differences between the groups.

STRUCTURE analysis

The Puechmaille Method of K selection (Puechmaille 2016), as determined by StructureSelector, provided support for a K value of 5, and the Evanno Method (Evanno et al. 2005) provided statistical support for K = 2 when simulations were run using the full dataset. Although these methods of K selection contradict each other, it is important to consider the biology of the study organism when selecting optimal K values (Gilbert et al. 2012), and to practise caution when interpreting the STRUCTURE output (Pritchard 2007). Hence, K = 2 and K = 3 are presented here, as plots with K values higher than 3 become complicated.

The same invasive range samples clustered with the native samples as in the SplitsTree and nMDS analyses for simulations run with two genetic clusters, with some of the invasive populations grouping with the native range (blue) and others grouping separately with some genetic mixing (orange) (Figure 2.4). For K = 3, the native range samples formed two clusters. One cluster grouped the invasive samples from the mixed group shown in the nMDS and SplitsTree analyses with samples from Lake George (G), Lake Lawne (L), most samples from Pine Island (PI), Lake Apopka (AP), Lake Maggiore (M), and Quinta (Q), and random samples from the other populations (blue). The second cluster grouped half the samples of Lake Kissimmee (K), and most samples from Orlando Wetlands Park (OWP), Salvador (S), Lake Boeuf (B), and Cote Crossing (CX), with some genetic mixing (dark purple). The third cluster (orange) grouped the invasive samples that were separated from the other samples in the previous analyses and in the K = 2 clustering.

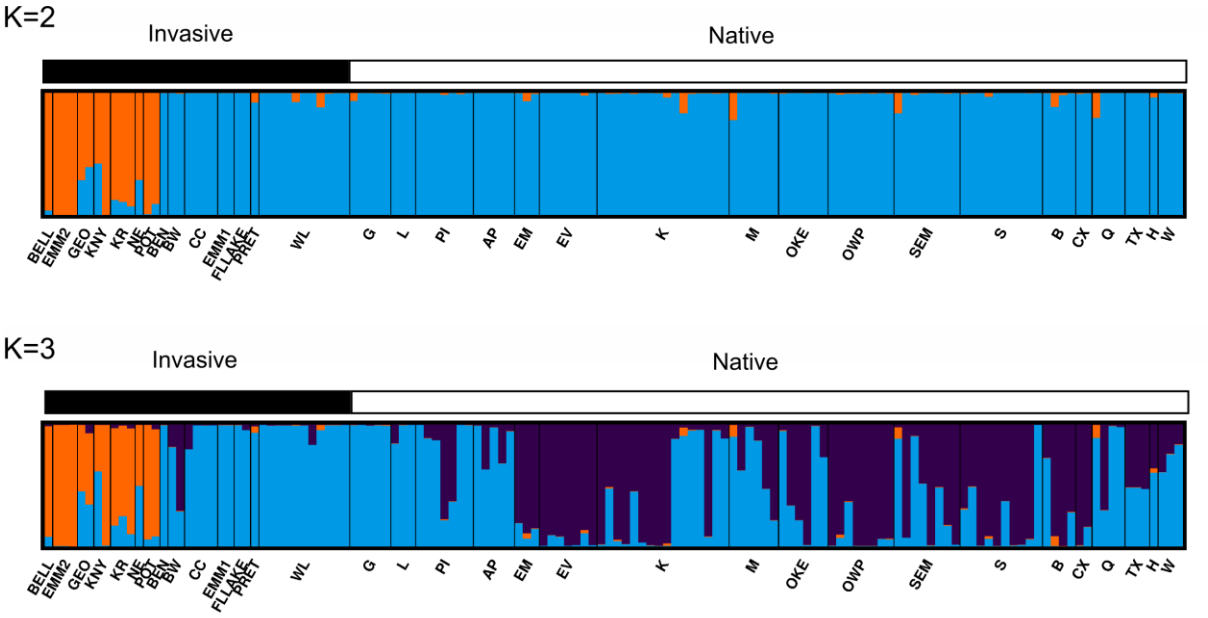



Figure 2.4: Results from STRUCTURE analysis of invasive and native populations of *Nymphaea mexicana*. Letters represent each site as shown in Table 2.1. The coloured bars represent the genetic similarity of samples from each site, and the width of the bars represent sample sizes.

## 2.4 DISCUSSION

The results from the analyses suggest that many of the *N. mexicana* plant populations from the invaded range in South Africa are genetically similar to the native populations from south-eastern USA. In contrast, some of the samples from the invaded range, including samples from Knysna, Emmarentia, Potchefstroom, Bellevue Wine Estate, George, Krom Rivier, and Neil Ellis in Stellenbosch, are more genetically distant from samples collected in the native range. These sites therefore likely contain hybrids of *N. mexicana*, and it is predicted that these sites may be more difficult to control using biological control, if the selected agent is specifically adapted to particular genotypes of the plant. Understanding genetic data such as these may also be utilised to establish links with morphological characteristics of plants collected from different sites in the invasive and native range. For example, it is interesting that a few samples from Emmarentia were genetically distant from the native range and other invaded range samples, but other samples from Emmarentia grouped with the native range samples. Morphological observations suggest that there are multiple genotypes/hybrids occurring at the Emmarentia site, as one patch of plants produced yellow flowers, while the other patch produced pink flowers

(Prinavin Naidu pers. comm.). In this case, it is likely that the patch that produced yellow flowers are the samples from Emmarentia that were more closely related the native range samples, as indicated by the analyses in this study.

The results in this study differed to the AFLP results obtained by Naidu (2018). While the Westlake samples grouped with the native range samples in this study as they did in the analyses by Naidu (2018), other samples such as those from Century City, Boardwalk in Port Elizabeth, and others also grouped with the native range. This could be explained by the fact that only two native range samples were used in Naidu's study. Hence, the greater number of native samples used in this study provided a better representation of the genetic diversity in the native range and allowed more insight into the genetic makeup of the invasive plants.

The genetic diversity (as measured by mean Jaccard's genetic distances within each population) of plant samples from the invasive range was lower than that of native range populations, but this was only statistically significant when these samples were treated as a separate group to the invasive samples that grouped with the native range. This is in contrast to the results obtained by Naidu (2018), but concurs with findings from other studies that have recorded higher levels of genetic diversity in the native compared to the introduced ranges of invasive alien plants (Hofstra et al. 2000; Li et al. 2006; Paterson et al. 2009). This lower genetic diversity in the introduced range 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). However, the mixed group – that is the group containing the invasive samples that grouped with the native samples – had the highest genetic diversity. This corroborates the findings by Naidu (2018) and may be attributed to multiple introductions of the plant bringing additional genotypes in from the invasive range (for example, as was shown to occur with *L. camara* in India (Ray and Quader 2014)), or to the presence of multiple forms of the plant (Ward et al. 2008). 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 both of these explanations for higher genetic diversity would be true, especially considering that many of the sampled sites are located near major ports and highly populated cities (Figure 2.5).

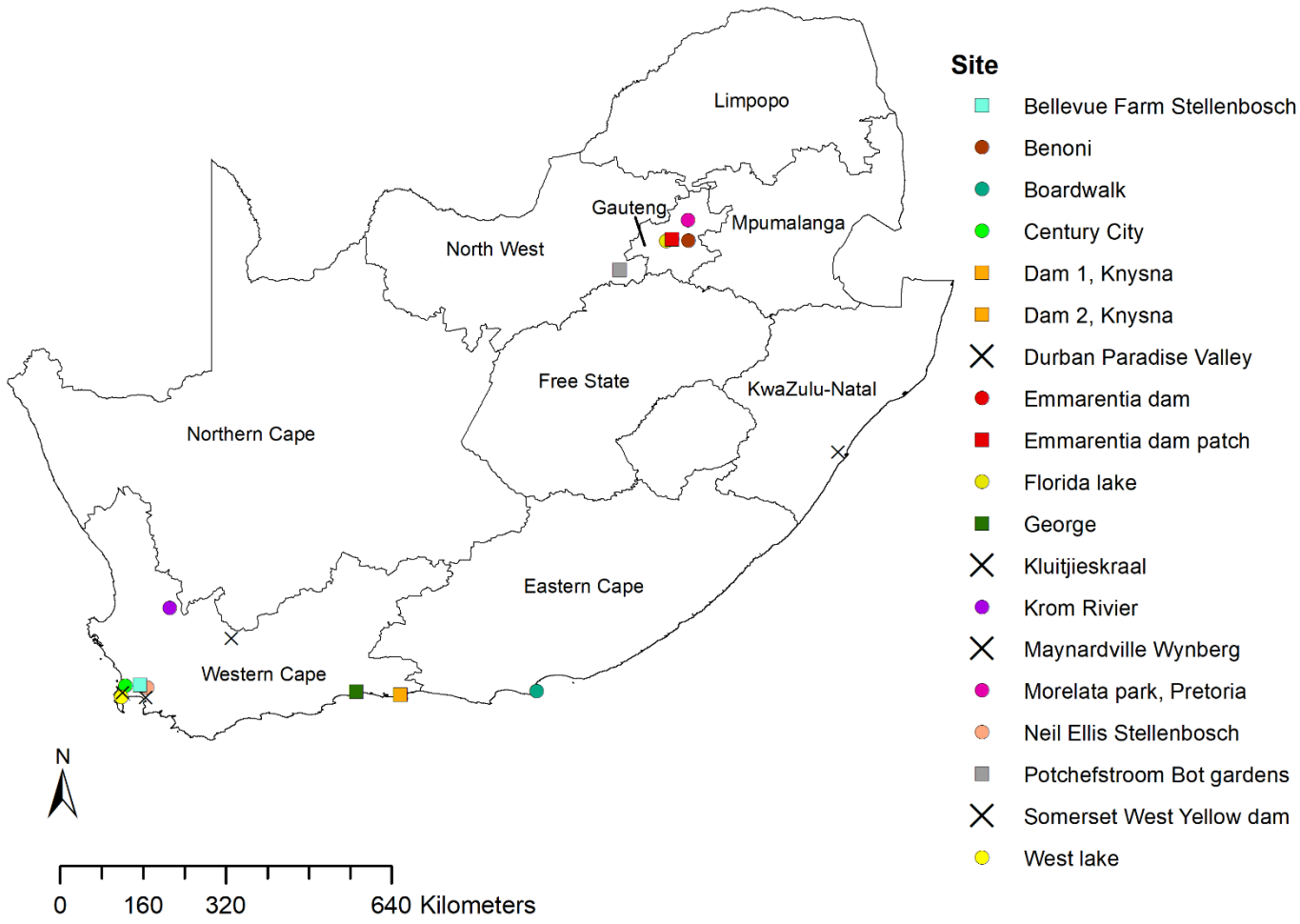



Figure 2.5: *Nymphaea mexicana* sites in the invaded range (South Africa) from which genetic samples were collected for ISSR analyses. Squares indicate sites that were separated from the other invasive and native samples in the nMDS and SplitsTree plots. X symbols represent samples that were excluded in the final combined dataset due to failed amplification.

Lower overall genetic diversity (and higher levels of within-population genetic variability) may be expected for clonal plants, but has also been reported for plants with sexual reproduction as well as asexual reproduction, such as *Solidago canadensis* L. (Asteraceae) and *Linaria vulgaris* Miller (Plantaginaceae) (Ward et al. 2008). *Nymphaea mexicana* reproduces sexually and asexually both in the invaded and native range. Some evidence suggests that the hybrids of *N. mexicana* do not reproduce sexually in the invaded range, though further study is needed to confirm this (Naidu 2018). Hence, an alternative explanation for the high genetic

diversity of the mixed *N. mexicana* group of invasive populations that grouped with the natives could be that these are more likely to be "purer" *N. mexicana* forms that are able to reproduce sexually as well as asexually, thus increasing genetic diversity, while the invasive group that was separated from the remaining samples in the nMDS and SplitsTree analyses could be horticultural hybrid forms. Indeed, in the breeding system studies carried out by Naidu (2018), plants from Westlake were used as a representative of the "pure" *N. mexicana* form, while plants from Potchefstroom and Knysna were used as representatives of the hybrid form. The differences in morphological characteristics for the plants from Knysna, Potchefstroom, and the pink flowered plants from Emmarentia, corroborate the genetic results found in this study.

Elevated genetic diversity of introduced populations may increase the likelihood of invasion success. Indeed, multiple introductions of a plant may be responsible for triggering plant invasion, but there are many exceptions where invasions have resulted from only a few, or a single introduction event (Frankham 2005). Increased genetic diversity improves the chances that beneficial genotypes will be selected for to improve survival and reproductive success, and that improves invasion success (Courchamp et al. 1999; Hughes et al. 2008; Crawford and Whitney 2010). If invasive populations have undergone significant selection, such that they evolve different genotypes compared to the native range, finding appropriately adapted biological control agents may be more challenging.

Pine Island, Lake Lawne, and Lake George grouped with the invasive samples in the SplitsTree analysis, with some overlap with samples from Lake Apopka, and Quinta in Texas. The first four sites are relatively close to each other in the more northern parts of Florida, USA, compared to other sites sampled within the native range (Figure 2.6), so it is possible that some of the invasive populations originated from these sites. Gene flow could be maintained at these sites through pollination facilitated by insects travelling between sites, transportation of plant material by boat, or via waterfowl through the ingestion of seeds (Woodyard and Bolen 1984). The overlap with Quinta is interesting, as this site is in Texas. However, Quinta is an urban park, and the *N. mexicana* population was planted there in an artificial pond (Ken King, pers. comm.). Nevertheless, all of these native sites were grouped with the rest of the native sites in the Structure analysis (when K = 2), so it may not be accurate to assume that the invasive plants in South Africa necessarily originated from Pine Island, Lake Lawne, and Lake George in particular, though perhaps they are more likely to have originated in Florida. Similarly, more

samples were collected from Florida compared to Louisiana and Texas, and this may cause bias. Further surveying in the native range, and the use of other molecular techniques such as sequencing of plastid *trnL-trnF* regions and nuclear internal transcribed spacers (ITS) (Woods et al. 2005a; Sutton et al. 2017) may provide higher resolution in this regard. Furthermore, including closely related *Nymphaea* species in genetic analyses, especially those that hybridise readily with *N. mexicana* in the wild, may provide further clarity as to the parentage and levels of gene flow in the invasive *Nymphaea* populations (also see Chapter 4).

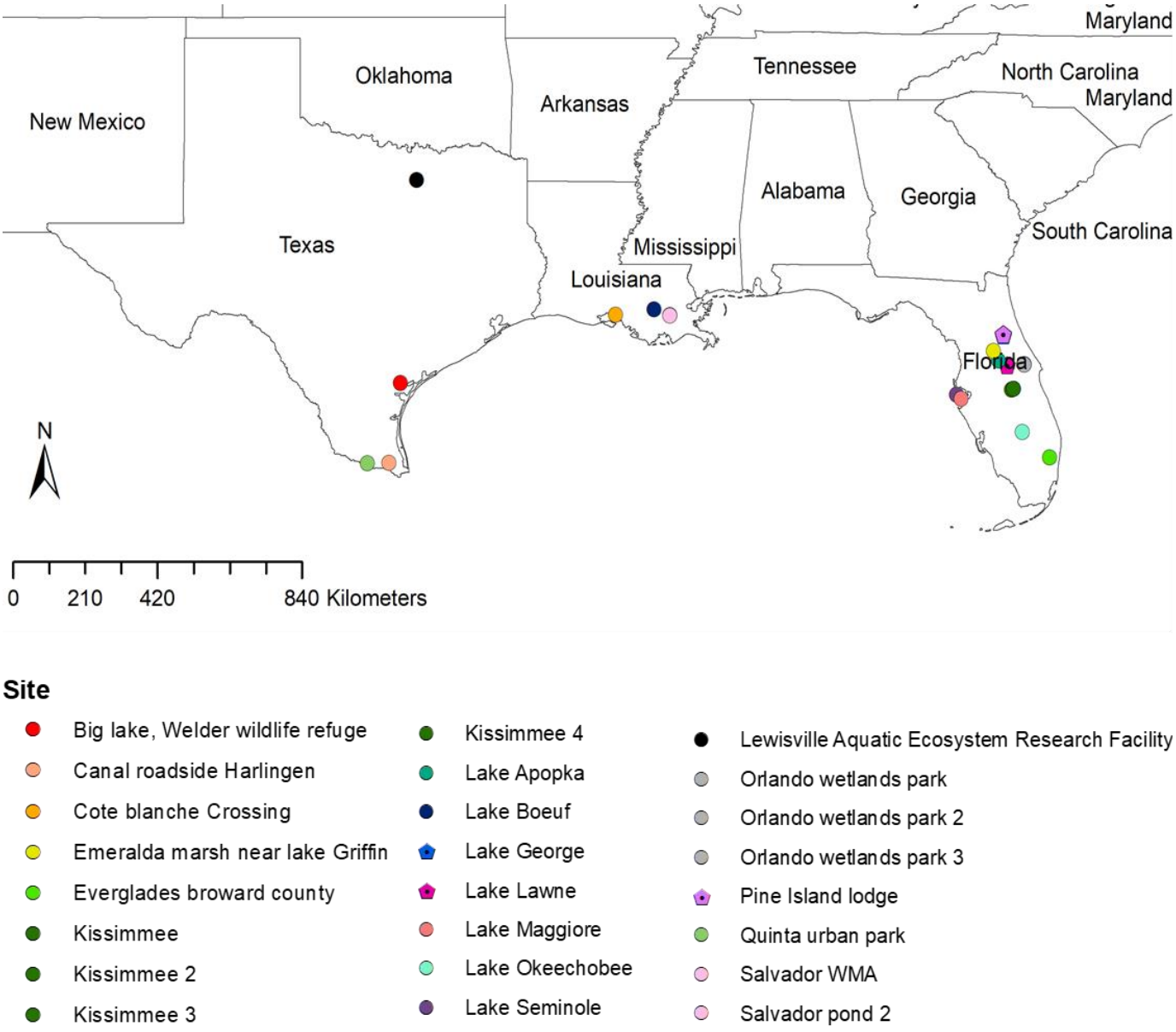



Figure 2.6: *Nymphaea mexicana* sites in the native range in the southeastern USA from which genetic samples were collected for ISSR analyses. Pentagon symbols with dots in the middle represent those samples that grouped with the invasive samples in the SplitsTree analysis (Lake George symbol hidden beneath Lake Lawne symbol).

It is important to note that the unequal sampling of *N. mexicana* populations in this study, especially in the introduced range, could confound the results obtained. Indeed, many DNA

samples collected from the invaded range were discarded due to their failure to amplify. Should future genetic work be carried out on the invasive and native populations of *N. mexicana*, it may be necessary to resample the invaded range to reduced bias due to sampling errors (Muirhead et al. 2008).

In summary, the results from this study suggest that the plant populations at the Boardwalk in Port Elizabeth; Century City and Westlake in the Western Cape; Florida Lake in Pretoria; and parts of the population at Emmarentia Dam in Gauteng are genetically similar to plant populations in the native range, and are possibly most similar to populations in Florida. Biological control is thus more likely to succeed at managing these populations, and efforts to collect biological control agents should be focused on sites in Florida, USA. The remaining populations of invasive *N. mexicana* are likely to be hybrid forms and are thus more likely to be challenging to manage using biological control. However, determining the parentage of these plants may improve attempts to identify suitable biological control agents. Alternatively, other management strategies, such as chemical control, could be considered to control hybrid populations and prevent them from replacing *N. mexicana* populations if biological control is successful. Studies such as this are important to better understand the genetic makeup of alien invasive plant invasions in order to better plan biological control efforts. *Nymphaea mexicana* and *Nymphaea* hybrids are becoming increasingly problematic around the world (Garcia-Murillo 1993; Nierbauer et al. 2014; Dana et al. 2017), especially owing to the ease with which hybridisation occurs within the genus (Jacobs and Hellquist 2010; Dkhar et al. 2013; Borsch et al. 2014). With a firm genetic and morphological understanding of invasive alien plant populations, biological control efforts may become more efficient and effective (see Chapter 4).

# Chapter 3: Surveys for potential biological control agents for *Nymphaea mexicana*, with notes on field host specificity*

## 3.1 INTRODUCTION

Biological control is largely built on the Enemy Release Hypothesis (ERH) which is based on the assumptions that: (1) natural enemies regulate plant populations, (2) greater regulation occurs on native species compared to exotics, and (3) that plants can divert more resources to growth in the absence of regulation by natural enemies. The hypothesis suggests that alien plants are able to increase in abundance and expand their distribution in an exotic area as a result of decreased or no regulation by natural enemies (McFadyen 1998; Keane and Crawley 2002). One proposed mechanism for this phenomenon is the EICA (Evolution of Increased Competitive Ability) hypothesis (Blossey and Notzold 1995), which states that invasive alien plants (IAPs) allocate more resources to growth and reproduction, as fewer resources are needed for allocation to defence against natural enemies. Considerable evidence exists to support this hypothesis at a biogeographical scale, although other factors such as climatic variables, selection for invasive genotypes, and human disturbance also likely explain increases in vigour of invasive alien species (reviewed in Colautti et al. 2004). Indeed, while plants may shift resource allocation to enable increased growth as a result of lower allocation to defences against specialist predators, more resources may instead be allocated to defences against generalist predators found in the introduced range (Joshi and Vrieling 2005). Nevertheless, irrespective of the mechanism, biological control has proved overall to be a successful means of managing invasive weed populations if carried out with the correct procedure (Wapshere 1985; Zimmermann et al. 2004; Hill and Coetzee 2017; Zachariades et al. 2017).

It is estimated that one in three to one in five biological control agents are successful after release on IAPs, with higher proportions of successful control occurring with multiple introductions (McFadyen 1998; Sheppard et al. 2003a; van Klinken and Raghu 2006). The initiation of a biological control programme requires that a series of steps are taken (Figure 3.1), such that if natural enemies are prioritised, the probability of effective control by a selected agent



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is increased (Harley and Forno 1992; Sheppard 2003). Exploration and prioritisation of natural enemies is thus imperative to reduce the risks associated with biological control.

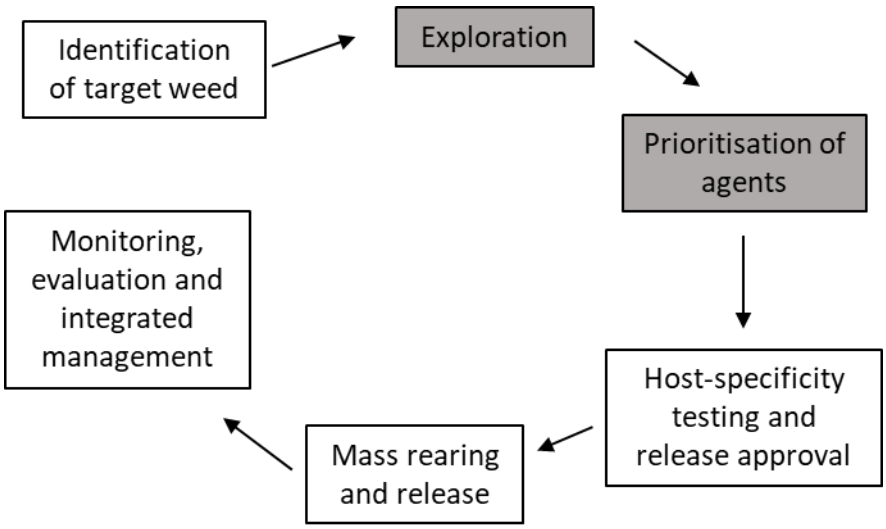


Figure 3.1: Steps taken during the initiation of biological control programmes. Steps shaded in grey are those focussed on in this chapter. From Van Klinken and Raghu (2006).

Prioritising natural enemies may be based on a plethora of factors, which in the past have relied on 'rules of thumb', scoring systems, and modelling (van Klinken and Raghu 2006). For example, some authors suggest that new associations between natural enemies and IAPs are more effective because the target plant has not evolved defences as strong and specific as those in plants with co-evolved associations with the enemies (Hokkanen and Pimentel 1984). Other hypotheses state that effective control agents can be found in regions that are eco-climatically similar to the introduced range of the plant (Wapshere 1983, 1985). While such hypotheses may hold promise, they should be approached with caution, as most of these 'rules of thumb' are insufficiently tested and present ample opportunity for error (van Klinken and Raghu 2006). Scoring systems developed by Harris (1973), Goeden (1983), and Forno and Julien (2000) may have some value for prioritisation and attempt to make agent selection more meticulous and methodical. However, scoring systems such as these have not been adopted, and are often an oversimplification of the numerous factors that should be considered (van Klinken and Raghu 2006). Sheppard et al. (2003a) discussed a few factors that could potentially improve the efficiency of agent selection. Firstly, by studying the target weed's population dynamics (in terms of which stage of the life cycle is most vulnerable) and identifying the type of damage needed to effectively suppress the population (i.e., which part

of the plant should be targeted), agents can be prioritised based on their ability to exert the type of control needed. Agent establishment and rates of spread are also important factors and should be considered during prioritisation (Groves et al. 2001).

Another factor that is likely to contribute to the success of biological control agent selection is agent diversity. Once selected, Sheppard et al. (2003a) suggest that it is important to capture maximum diversity by sampling as widely as possible, to ensure that the populations tested in quarantine are representative of the genetic makeup of the native populations. However, the presence of cryptic species in geographically distinct populations of natural enemies is also a consideration, as this can cause considerable confusion. For example, host specificity test results of potential agents could be complicated if there are two cryptic species masked within one population, whereby one of the species is specific to one plant, and another is more general or is specific to another plant (Toševski et al. 2011; Paterson et al. 2016). In this way, false host specificity data could be generated. Therefore, when importing potential biological control agents into quarantine, it may be best to initially import agents from only one site, or to keep sites separated until cryptic species are ruled out. Additionally, if there are distinct genetic variations of the target weed, agents should be sampled from populations that are adapted to weeds of that particular genetic makeup, as these are often more likely to exert better control (Sheppard et al. 2003a). In the case of *N. mexicana*, some of the invasive populations grouped with samples from the native range in Florida, Louisiana, and Texas, so it was appropriate to sample insects from sites in these states (Chapter 2). The genetic analyses in Chapter 2 suggested that the genetic structure of the invasive populations in South Africa may match more to native populations in Florida, so surveys should be focused in this region.

Furthermore, sampling focus should be placed on regions that are climatically similar to the invaded range, as the natural enemies found there are adapted to those conditions (Robertson et al. 2008; but see Van Klinken et al. 2003). For example, Cameron et al. (1993) suggest that lack of climate-matching is likely to be the main reason explaining why parasitoids of *Forficula auricularia* L. (Dermaptera: Forficulidae) failed to establish in New Zealand even though they were successful in Canada, and why *Leucopis tapiae* Blanchard (Diptera: Chamaemyiidae) failed to establish on *Pineus laevis* Maskell (Hemiptera: Adelgidae) in Australia, but did establish in New Zealand. Finally, it may be considered that agents that are highly parasitized and have adapted defences to predation should be selected as potential biocontrol agents for the target weed (Harris 1991; Sheppard et al. 2003a). However, control of an invasive plant species may be hindered when the insect herbivore has

high levels of parasitism and/or predation, and while release from these natural enemies may improve levels of control in the invaded range, parasitoids native to the invaded range may still affect the biological control agents and thus hinder their effectivity. For example, Siebert (1975) suggested that parasitoids of species within the subfamily Cassidinae (Chrysomelidae) in the invaded range of *Solanum elaeagnifolium* Cav. (Solanaceae) could reduce the effectivity of *Gratiana* species being considered for use as biological control agents. This led Hill and Hulley (1995) to predict the same of *Gratiana spadicea* Klug for use in control of *Solanum sisymbriifolium* Lamarck. Indeed, one parasitoid species was found to affect over 80% of *G. spadicea* summer pupae in Mpumalanga (Byrne et al. 2002). Hence, although parasitism of introduced biological control agents in the native range is usually low (Cornell and Hawkinst 1993), and it is rare that biological control programmes fail as a result of parasitism (Goeden and Louda 1976), it is nevertheless something to be wary of if choosing highly parasitized biological control agents. For example, *Megamelus scutellaris* Berg (Hemiptera: Delphacidae) released into Florida to control water hyacinth, *P. crassipes*, is being utilized by a native parasitoid, *Kalopolynema ema* (Schauff and Grissell) (Hymenoptera: Mymaridae) as a new host, and an indigenous parasitoid wasp has also been recorded on *M. scutellaris* in South Africa (Minteer et al. 2016; Kraus et al. 2019).

Initiation of a biological control programme for the management of *N. mexicana* began in 2016, so it is necessary to conduct field surveys and select an agent that is most likely to succeed at managing this plant in South Africa. *Nymphaea mexicana* predominantly exhibits vegetative reproduction or vegetative propagules in the invaded range, so it is likely that a control agent that targets the leaves and stems of this plant will be successful. Furthermore, Harris (1973) suggests that natural enemies that damage vascular and mechanical support tissue are more likely to be effective biological control agents. Hence, leaf- or stem-feeding natural enemies found in the native range of *N. mexicana* hold promise as biological control agents. However, the situation is complicated by the existence of hybrids of *N. mexicana* (Naidu 2018; Chapter 2). While some of the invasive populations match genetically to populations from the native range, others form a separate genetic grouping and it may be more challenging to find specific biological control agents for these populations. Ideally, field surveys would reveal an insect that feeds on and causes significant damage to *N. mexicana* as well as the hybrid forms that occur in South Africa,but does not feed on the two native *Nymphaea* species and other species within Nymphaeaceae in South Africa. Further genetic work may be necessary to achieve this result (see Chapters 2 and 4),

but initially surveys should be conducted to find biological control agents for those invasive populations that do group with native *N. mexicana* populations.

A few insect species have been observed visiting *N. mexicana* in its native range. *Chauliognathus marginatus* Fabr. (Coleoptera: Cantharidae) was frequently found in the flowers of *N. mexicana* by Capperino and Schneider (1985), with foraging damage to the stigmas and stamens. These beetles spent long periods visiting individual flowers and were thus not associated with cross pollination. However, while adults may feed on pollen in flowers, the larvae are carnivorous (Riley 1892), so this species is unsuitable for biological control. Another species, *Ceratomegilla fuscilabris* Mulsant (Coleoptera: Coccinellidae), was observed on non-reproductive flower parts, and is known to feed on leafhopper nymphs (Hefley 1937). Aside from an unidentified dipteran found on flower parts, and common pollinating Hymenoptera such as *Apis mellifera* L. (Apidae), no other insects were observed visiting *N. mexicana* flowers in the study by Capperino and Schneider (1985).

Another more recent study by Nachtrieb et al. (2007) recorded the presence of two snail genera on *N. mexicana* which feed on epiphytic growth or detritus, thereby impacting little damage to aquatic plants. *Rhopalosiphum* spp. (Hemiptera: Aphididae) were also recorded, but these are generalist aphids and are thus unsuitable for biological control. Aside from odonate egg deposition, the larvae of *Synclita* spp. Walker (Lepidoptera: Crambidae; reclassified and hereafter referred to as *Elophila* Walker) also inflicted damage both on *N. mexicana* and other plants in the study. The only insect that may have potential as a biological control mentioned in Nachtrieb et al. (2007) is *Donacia cincticornis* Newman (Coleoptera: Chrysomelidae), which caused considerable damage only on *N. mexicana* (and not the other plants in the study, namely American pondweed *Potamogeton nodosus* Poir. (Potamogetonaceae) and Illinois pondweed (*P. illinoensis* Morong)). However, *D. cincticornis* feeds on other *Nymphaea* spp., *Nuphar variegata* Engelm. ex Durand, and *Brasenia* Schreb. (Cronin et al. 1998), so while they may be specific to Nymphaeaceae, they are likely to be unsuitable as biological control agents due to the damage they will likely exert on *Brasenia* and *Nymphaea* species native to South Africa.

Harms and Grodowitz (2009) listed several insects that feed on *Nymphaea odorata* Aiton. Although a list of insect herbivores has not been published for *N. mexicana* as a host, the list for *N. odorata* may be useful to identify insect genera that may occur on *N. mexicana*. Taxa mentioned in the list compiled for *N. odorata* include several *Donacia* Fabricius and *Parapoynx* Hübner (Lepidoptera: Crambidae) species, three *Bagous* spp. including *Bagous americanus* LeConte and *Bagous magister* LeConte (Coleoptera: Curculionidae), a few

species of *Elophila* including *Elophila obliteralis* Walker, and numerous chironimid and lepidopteran species. Having a knowledge of the natural enemies of other species of *Nymphaea* is useful to attempt to understand the host specificity of the insects found feeding on *N. mexicana.*

This study aimed to identify the phytophagous insect species that feed on *N. mexicana* in its native range, and prioritise species based on feeding damage and host range as potential biological control agents of *N. mexicana*. Consideration of the factors that play a role in the effectiveness of biological control agents will enable careful selection of agents to be imported into quarantine and tested for their suitability to manage *N. mexicana* in South Africa.

## 3.2 MATERIALS AND METHODS

MaxEnt modelling and surveys

Surveys for natural enemies took place in southeastern USA and were focused mainly in Florida due to the abundance of sites and closer genetic match to invasive populations, but sites in Louisiana and Texas were also surveyed. Sites within the native range of *N. mexicana* were selected using MaxEnt modelling (Phillips et al. 2004, 2006) to match regions with a similar climate to areas in South Africa infested with *N. mexicana* (Goolsby et al. 2006b). Locality data were obtained from the Global Biodiversity Information Facility (GBIF.org 2019), Southern African Plant Invaders Atlas (SAPIA) on the Botanical Database of Southern Africa (BODATSA) online website (Henderson 2007; South African National Biodiversity Institute 2016), and records from field surveys (Naidu 2018). Locality coordinates were filtered by removing invalid points (e.g. points found on land in dry areas are inappropriate for aquatic plants), and the model was generated based on coordinates from the native range and invaded sites in South Africa. Bioclimatic predictor variables were downloaded from the WORLDCLIM database (Hijmans et al. 2005) (http://www.worldclim.org/) and the following predictor values were selected from 'bioclimatic variables' (BIO x): mean annual temperature (BIO 1); mean diurnal range (mean of monthly (maximum temperature - minimum temperature)) (BIO 2); isothermality (BIO 3); temperature seasonality (BIO 4); maximum temperature of warmest month (BIO 5); minimum temperature of coldest month (BIO 6); temperature annual range (BIO 7); mean temperature of wettest quarter (BIO 8); mean temperature of driest quarter (BIO 9); mean temperature of warmest quarter (BIO 10); mean temperature of coldest quarter (BIO 11); and annual precipitation (BIO 12) (Martin 2013).

Surveys were conducted from August to October 2018. Sites were identified by contacting personnel at state universities and state wildlife and fisheries departments via email, and by telephone. This was necessary as herbarium and survey records did not provide GPS coordinates of the sites, and populations of *N. mexicana* fluctuate with changes in water levels due to rainfall, flooding, and hurricanes. Sites were accessed by motorboat or airboat, and the leaves, stems, roots, and flowers of *N. mexicana* were inspected for damage. All life stages of insects were collected using an aspirator, Berlese funnel extractions, and manually by inspection under microscope in the laboratory. Specimens were frozen and/or preserved in 70 – 95% isopropyl or ethyl alcohol for later identification. An attempt was made to rear live collected insects under laboratory conditions. Whole potted plants were placed in 68 L black mesocosm tubs in a greenhouse at University of Florida Center for Aquatic and Invasive Plants and United States Department of Agriculture facility in Davie, Florida, and insects collected in the field were placed on the plants and left to feed while observations were made. Temperatures in the greenhouse are estimated to fluctuate around 27 $^{o}$C. Leaf samples were taken from each site and dried in ZipLoc bags with silica gel or Drierite for DNA analysis. Eighteen sites were surveyed at 17 different water bodies (Figure 3.2). In addition, leaf length was measured from five leaves from each site (excluding two sites). These measurements were used to compare mean leaf length between sites to account for geographical differences in habitat quality. Kruskal–Wallis tests and post-hoc analyses determined statistical differences between the mean leaf lengths at different sites using R version 3.5.3 (R Development Core Team 2013).

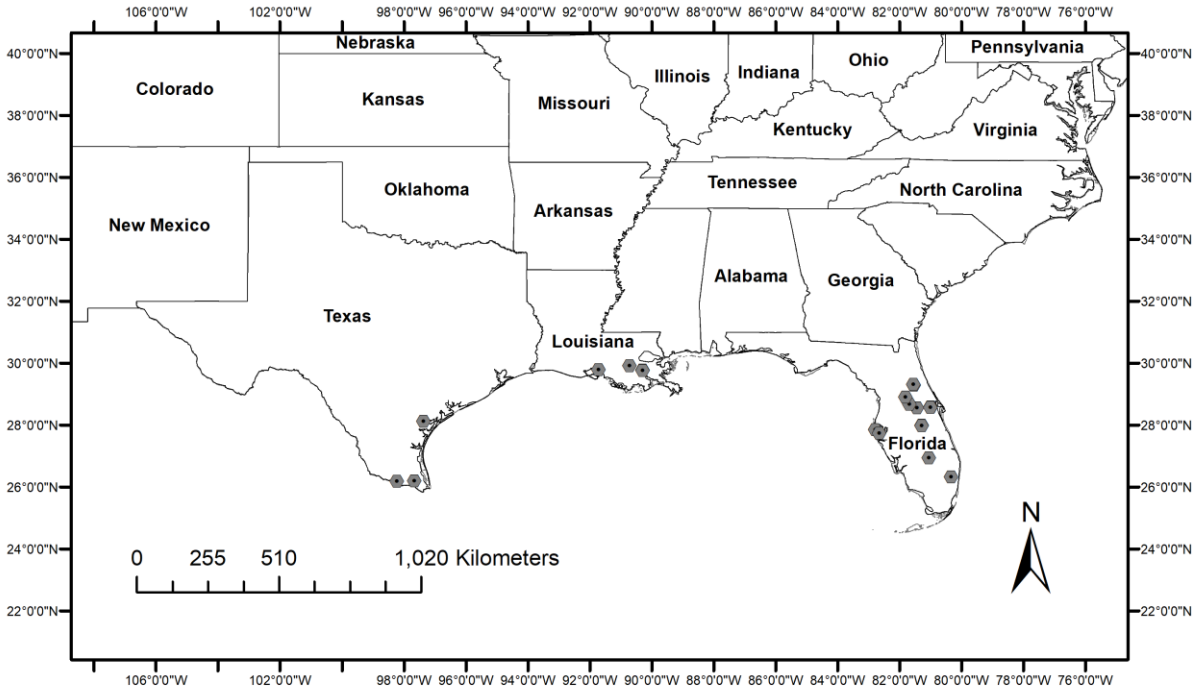


Figure 3.2: Sites surveyed for natural enemies of *N. mexicana* in southeast USA in August-October 2018. Map created using ArcMap (Environmental Systems Research Institute 2014).

Where possible, all parts of *N. mexicana* plants were searched for all life stages of insect natural enemies, including root tubers, stems, leaves, and flowers. However, this was not always possible as the roots and tubers could not be successfully pulled from the sediment in deeper areas. Individual based rarefaction species accumulation curves were generated using the Chao 2, ICE (Incidence Coverage Estimator) and MMRuns estimators in EstimateS version 8.0 (Colwell 2006) as these estimators are effective at estimating true species diversity when the abundances of species are unknown. Incidental insect visitors to *N. mexicana* (that is those that did not feed on the plant, as determined by observation and literature searches) were excluded from the analyses. Species were prioritised based on extent of feeding damage and mode of damage, incidence, field host range, and climate matching (Spafford et al. 2003; Paterson 2010). Information from literature searches for each species, or similar species in the same genus if information at the species level was unavailable, was used to score the potential agents when not enough information was known from field observations. The scoring system developed by Forno and Julien (2000) (Table 3.1) was utilized as an additional means of prioritisation. Scores were allocated only to the species that

were likely to have narrow host ranges based on observations in the field and literature searches. Where scores did not fit exactly into a category, intermediate scores were used. For example, if adults caused minimal damage, they received a score of 1 (between 0 for no damage, and 2 for damage). Prioritisation based on genotype matching was limited as genetic work has only been conducted on material from two sites (Naidu 2018).

Table 3.1: Scoring system developed by Forno and Julien (2000) to prioritise potential phytophagous biological control agents, based on initial assessments from observations in the native range.

| Criterion | | Score |
|---|---|---|
| 1. Damage by adults | | |
| A | No damage (adults do not feed) | 0 |
| B | Damage leaf or stem tissue | 2 |
| C | Destroy vascular or structural tissue | 4 |
| 2. Damage by immatures | | |
| A | Damage leaf or stem tissue | 2 |
| B | Destroy vascular or structural tissue | 4 |
| 3. Duration of attack | | |
| A | Limited period of attack not increasing plant susceptibility to attack by other agents, waterlogging or sinking | 1 |
| B | Prolonged attack increasing plant susceptibility to waterlogging or sinking | 2 |
| C | Prolonged attack increasing plant susceptibility to waterlogging or sinking | 4 |
| 4. Number of generations | | |
| A | Obligate univoltine species | 1 |
| B | Two or three generations a year | 2 |
| C | Four or more generations a year | 4 |

Host specificity pilot study

As a preliminary assessment of the host specificity of the most promising agent, *B. americanus*, five outside concrete crypt tanks (2.4 m length x 0.9 m height x 0.9 m width) with lids were prepared by adding one plant each of *N. mexicana*, *N. odorata*, *Nuphar lutea* (L.) Sm. (Nymphaeaceae), *Sagittaria latifolia* Wiild. (Alismataceae), *Nymphoides indica* (L.) Kuntze (Menyanthaceae) and *Cabomba caroliniana* A.Gray (Cabombaceae), to each tank. The tanks were situated at the University of Florida Center for Aquatic and Invasive Plants and United States Department of Agriculture facility in Davie, Florida, USA. Eight *B. americanus* adults (unsexed) were added to the tanks one day after the plants, to allow the plants to acclimatize. All plants were inspected and photographed, and observations and measurements of the mines were taken every two to three days over a period of 20 days. An additional 68 L black mesocosm tub with two *N. mexicana* plants was kept in a greenhouse and covered with netting. Sixteen *B. americanus* weevils were added to this mesocosm and observations and measurements of mines were made over the same time period. The mean (± SE) length of the mines was measured from photographs using Image J (Schneider et al. 2012). At the end of the experiments, all plants were thoroughly inspected, and stems dissected to detect the presence of *B. americanus* larvae and pupae.

## 3.3 RESULTS

MaxEnt modelling

MaxEnt modelling indicated that the regions most climatically similar to the invaded range of *N. mexicana* in South Africa were south-eastern USA, from the southern half of Georgia, into Florida and along the lower southern parts of Alabama, Mississippi, Louisiana, and south-eastern Texas. Climatically suitable areas also occur in the eastern and western regions of central Mexico (Figure 3.3).

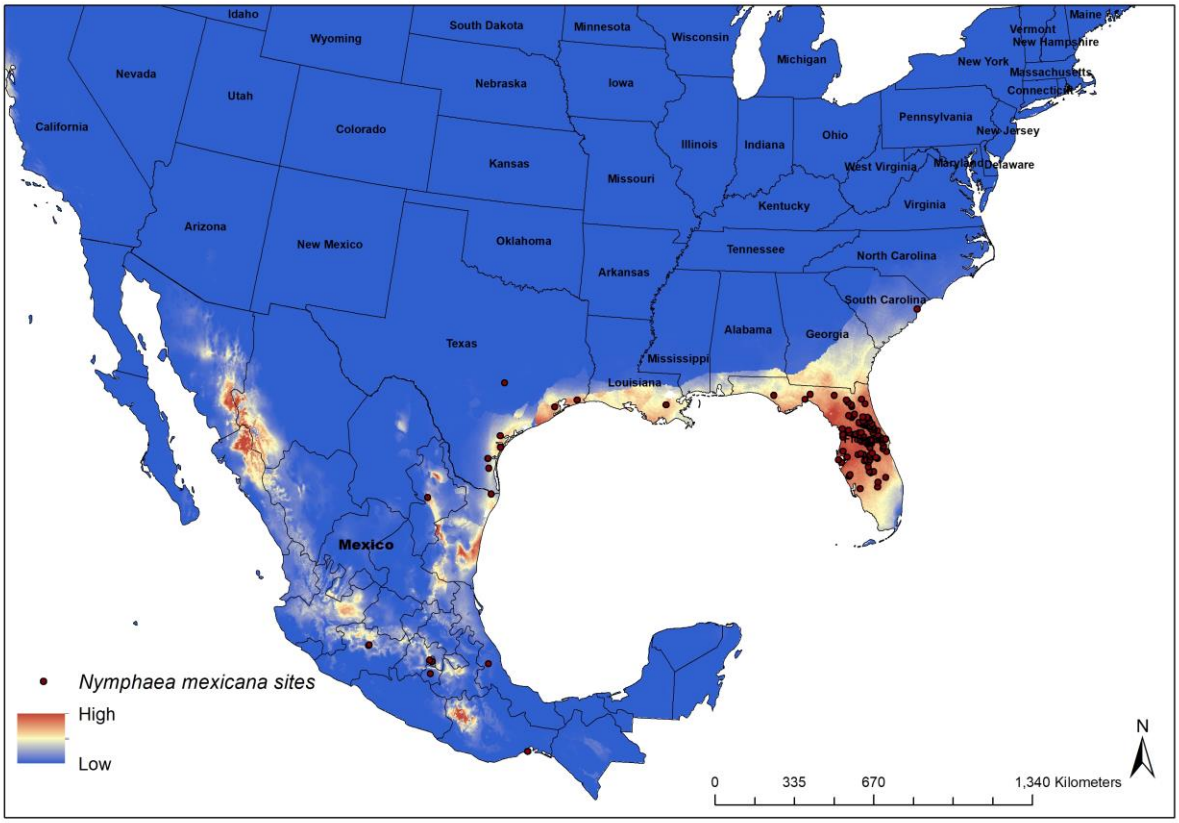


Figure 3.3: Climatic similarity of the native distribution and introduced distribution of *Nymphaea mexicana* in South Africa modelled using MaxEnt (Phillips et al. 2004, 2006). Red colours indicate regions that are climatically similar to South Africa, and lighter yellow colouring indicates regions of lesser similarity. Red circle icons indicate the native range sites considered in the analysis.

## Surveys

### *Sampling Effort*

Most of the species present on *N. mexicana* were sampled during these surveys, as the species accumulation curve almost approached the asymptote (Figure 3.4). The ICE mean was slightly higher than the S(est) curve and estimated that one or two species were missed during the surveys, while the MMRuns mean had a slightly steeper curve, and also estimated that further surveys may reveal the presence of one or two more species. Similarly, the Chao 2 mean was higher than the S(est) curve but reached the same point as the curve ends, whereas the Chao 2 upper 95% confidence interval suggests that up to five species may be unrecorded on *N. mexicana*. Hence, overall two to five species could have been missed during the surveys.

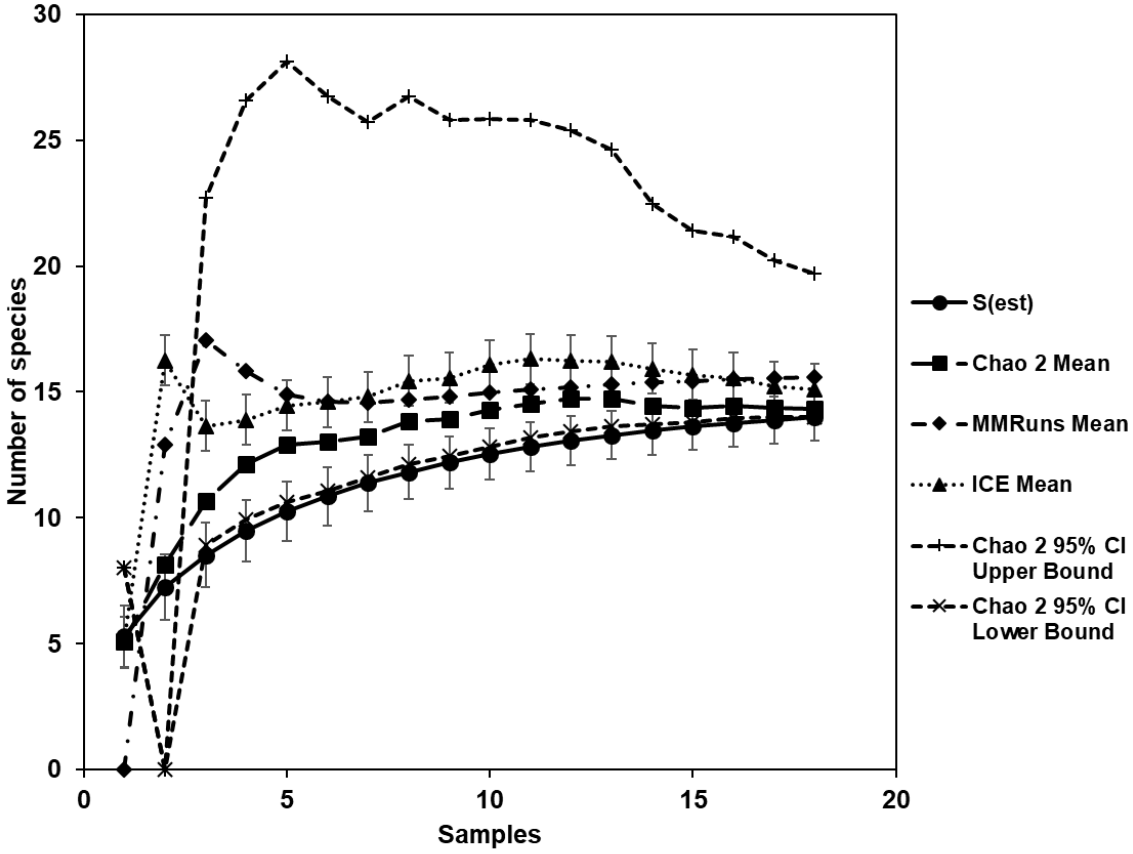


Figure 3.4: Species accumulation curve showing ICE mean, Chao 2 mean and the Chao 2 95% upper and lower confidence intervals, and MMRuns mean indicators for species richness (error bars indicate standard deviations). Chao 2 mean estimates the true species diversity based on incidence data; ICE mean is the incidence coverage estimator, and the MMRuns (Michaelis-Menton) mean calculates the mean score after 100 randomisations (Colwell 2006; Gotelli and Colwell 2011).

*Survey outcomes*

The surveys took place in the mid to late summer months in the USA. Many of the sites surveyed had healthy populations of *N. mexicana* isolated from other *Nymphaea* species, although a few sites, namely Big Lake in Welder Wildlife Management Area (WMA), Texas, Lake Okeechobee, Lake Kissimmee, and the Everglades in Florida, were near to, or mixed with, populations of *Nymphaea elegans* Hook (Big Lake) or *N. odorata* Aiton (Nymphaeaceae) (Table 3.2). The sites surveyed consisted of large natural lakes and wetlands accessible only by airboat, smaller lakes accessible by motorboat or from the bank, and a few canals and artificial ponds. Almost all *N. mexicana* populations were exposed to full sunlight, and the water depth at the sites ranged from knee deep to about 1.8 m.

Table 3.2: Sites surveyed for natural enemies of *N. mexicana* in south-eastern United States, and other plant species present at the sites in 2018. In the table, the common plant names are listed.[a]

| Site | State | Latitude | Longitude | Plants present |
|---|---|---|---|---|
| Cote Blanche Crossing | Louisiana | 29.7774 | -91.7155 | Hornwort, water hyacinth, sawgrass, other grasses. |
| Lake Boeuf | Louisiana | 29.9111 | -90.7117 | Water hyacinth, hydrilla, frog's bit, duckweed, hornwort, salvinia, smartweed, american water lotus, alligatorweed. |
| Salvador site 1 | Louisiana | 29.7657 | -90.2930 | Hornwort, hydrilla, water hyacinth, grasses, salvinia, american water lotus. |
| Salvador site 2 | Louisiana | 29.7623 | -90.2917 | Hornwort, hydrilla, american water lotus, water hyacinth, fanwort, pennywort, common water nymph. |
| Harlingen roadside canal | Texas | 26.1903 | -97.6636 | Water hyacinth, some grasses. |
| Big lake, Welder Wildlife Management Area | Texas | 28.1216 | -97.3650 | Tropical royalblue waterlily, american water lotus, cattail, *Naja sp*., bladderwort, sesbania, dollarweed, cattail, smartweed. |
| Quinta urban park | Texas | 26.1767 | -98.2298 | Cattail, various bank plants. |
| Lake Lawne | Florida | 28.5579 | -81.4381 | Spatterdock, water hyacinth, hydrilla, pickerelweed, spikerush. |
| Lake Apopka | Florida | 28.6722 | -81.6748 | Sagittaria, pickerelweed, hydrilla. |
| Lake George | Florida | 29.2828 | -81.5408 | Alligatorweed eelgrass, hornwort, hydrilla, water lettuce, salvinia, cattail. |
| Lake Kissimmee | Florida | 27.9792 | -81.2743 | Frog's bit, hydrilla, salvinia, ludwigia, hornwort, spatterdock, water lettuce. |

| | | | | |
|---|---|---|---|---|
| Lake Okeechobee | Florida | 26.9329 | -81.0503 | White waterlily, pickerelweed, bladderwort, hornwort, smartweed, cattail. |
| Lake Seminole | Florida | 27.8414 | -82.7740 | Pickerelweed, grasses, eelgrass, sagittaria, spikerush, hydrilla. |
| Lake Maggiore | Florida | 27.7373 | -82.6475 | Hydrilla, cattail. |
| Pine Island lodge | Florida | 29.3119 | -81.5458 | Hornwort, alligatorweed, cattail. |
| Emeralda marsh near Lake Griffin | Florida | 28.9039 | -81.8087 | Hydrilla, sagittaria, alligatorweed. |
| Orlando wetlands park | Florida | 28.5824 | -81.0022 | Hornwort, pickerelweed, pennywort, common water nymph, hydrilla, duckweed, ludwigia. |
| Everglades | Florida | 26.3205 | -80.3300 | White waterlily, dollarweed, common water nymph, spikerush, pickerelweed. |

[a] The species names and families can be found in the following descriptions, which are listed alphabetically by common name: **Alligatorweed**, *Alternanthera philoxeroides* (Mart.) Griseb (Amaranthaceae); **american water lotus**, *Nelumbo lutea* Wiild. (Nelumbonaceae); **bladderwort**, *Utricularia* L. (Lentibulariaceae); **cattail**, *Typha spp.* L. (Typhaceae); **common water nymph**, *Naja guatalupensis* (Spreng.) Magnus (Hydrocharitaceae); **hornwort**, *Ceratophyllum demersum* L. (Ceratophyllaceae)*;* **dollarweed**, *Hydrocotyle umbellata* L. (Araliaceae); **duckweed**, *Lemna spp.* L. (Araceae); **eelgrass**, *Vallisneria* L. (Hydrocharitaceae); **fanwort**, *Cabomba caroliniana* A. Gray (Cabombaceae); **floating primrose willow**, *Ludwigia peploides* (Kunth) P.H.Raven; **frog's bit**, *Limnobium spongia* (Bosc) L.C. Rich. ex Steud. (Hydrocharitaceae); **hydrilla**, *Hydrilla verticillata* (L.f.) Royle (Hydrocharitaceae); **ludwigia**, *Ludwigia spp.* L. (Onagraceae); **pennywort**, *Hydrocotyle spp.* L. (Araliaceae); **pickerelweed**, *Pontederia cordata* L. (Pontederiaceae); **sagittaria**, *Sagittaria spp.* L. (Alismataceae); **salvinia**, *Salvinia molesta* D.Mitch. or *Salvinia minima* Baker (Salviniaceae); **sawgrass**, *Cladium* P.Browne (Cyperaceae); **sesbania**, *Sesbania spp.* Scopoli (Leguminosae); **smartweed**, *Polygonum* L. (Polygonaceae); **spatterdock**, *Nuphar advena* (Aiton) W.T.Aiton (Nymphaeaceae); **spikerush**, *Eleocharis spp*. (L.) Roem. & Schult. (Cyperaceae); **tropical royalblue waterlily**, *Nymphaea elegans* Hook (Nymphaeceae); **water hyacinth**, *Pontederia crassipes* Mart. [≡ *Eichhornia crassipes (*Mart.) Solms] (Pontederiaceae)*;* **waterlettuce**, *Pistia stratiotes* L. (Araceae); **white waterlily** *Nymphaea odorata* Aiton (Nymphaeaceae).

Populations of *N. mexicana* ranged from patches of one or two plants to stands of hundreds of large, healthy flowering plants covering areas of over 50 $m^2$. Statistical differences were observed among the mean leaf lengths between sites ($H_{17} = 59.06$; $P < 0.001$) (Figure 3.5). The longest leaves (20.8 ± 1.7 cm [SE]; n = 5) were recorded at Lake Okeechobee in Florida whereas those from Lake George in Florida were the shortest (10.6 ± 0.5 cm [SE]; n = 5).

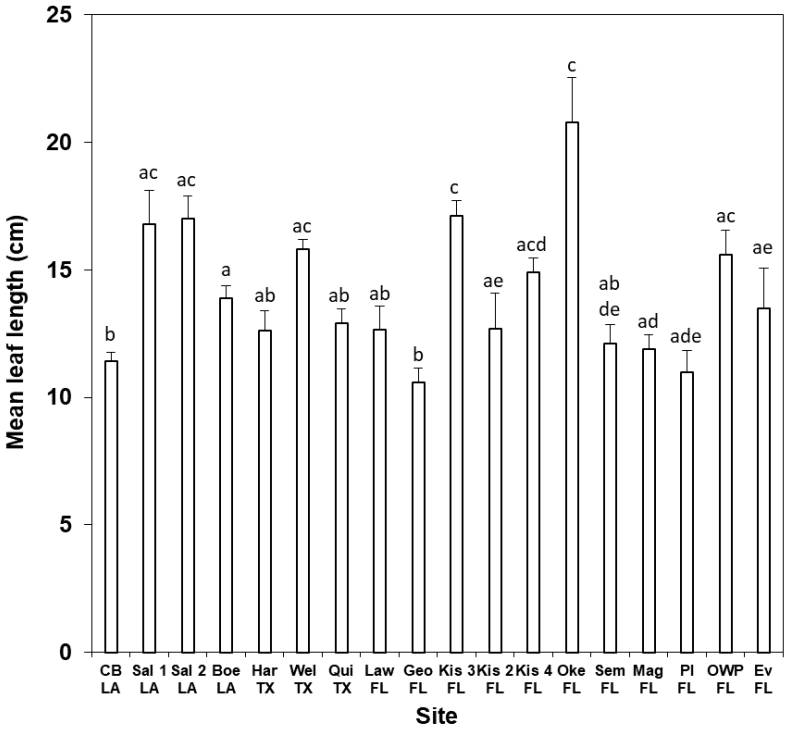


Figure 3.5: Mean (±SE) leaf lengths of *Nymphaea mexicana* leaves measured from sites in Louisiana (LA), Texas (TX), and Florida (FL) in the United States during field surveys for potential biological control agents (n = 5 leaves per site). Leaves from 2 sites were not measured due to time constraints. Letters indicate significant differences between sites ($P \leq 0.05$), medians compared by Kruskal Wallis ANOVA. CB = Cote Blanche crossing; Sal 1 = Lake Salvador site 1; Sal 2 = Lake Salvador site 2; Boe = Lake Boeuf; Har = Harlingen roadside; Wel = Welder Wildlife Management Area; Qui = Quinta urban park; Kis 2, 3, and 4 = Lake Kissimmee sites 2, 3, and 4; Oke = Lake Okeechobee; Sem = Lake Seminole; Mag = Lake Maggiore; PI = Pine Island; OWP = Orlando Wetlands Park; Ev = Everglades.

Numerous aquatic insects were encountered during the surveys, and several caused feeding damage on *N. mexicana* (Table 3.3). At most sites, there was considerable damage

associated with the presence of generalist lepidopteran species such as *Elophila* spp. and *Parapoynx* spp. (Figure 3.6 and 3.7), as determined by literature searches. Generalist herbivore insects were found during the surveys, including *Notiphila latigena* Mathis (Diptera: Ephydridae) (Figure 3.8) (identified by Dr. Richard Zack, Washington State University); *Draeculacephala* sp. (Hemiptera: Cicadellidae)*;* a black planthopper, suspected to be *Megamelus davisi* Van Duzee (Hemiptera: Delphacidae); and *Donacia cincticornis* Newman and *D. hypoleuca* Lacordaire (Coleoptera: Chrysomelidae) (identified by Dr. Chris Carlton and Victoria Bayless, Louisiana State University) as well as other unidentified *Donacia* spp. (Figure 3.9). Chironomid larvae were collected at several sites, created 'trenches' in the leaf surface and caused considerable damage (Figure 3.6), but were also recorded from Berlese funnel extractions on *Nymphaea pubescens* Wiild (synonym *Nymphaea rubra* Roxb. ex Andrews), and *N. odorata*. However, these larvae were not identified to species level as larvae are difficult to distinguish. Coccinellids believed to be feeding on the aphids present at a site in Texas, a small number of lampyrids, luminous green dipterans, Sciaridae, Gerridae, and a few parasitoids (Ichneumonidae), were all recorded at one or more sites.

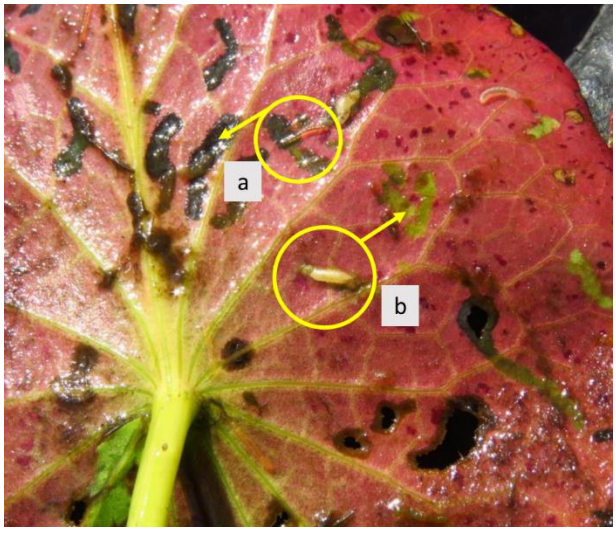


Figure 3.6: Feeding damage on *Nymphaea mexicana*: a: Feeding 'trenches' associated with chironomid larvae; b: feeding damage associated with Lepidoptera larvae (*Paraponyx sp.?*).

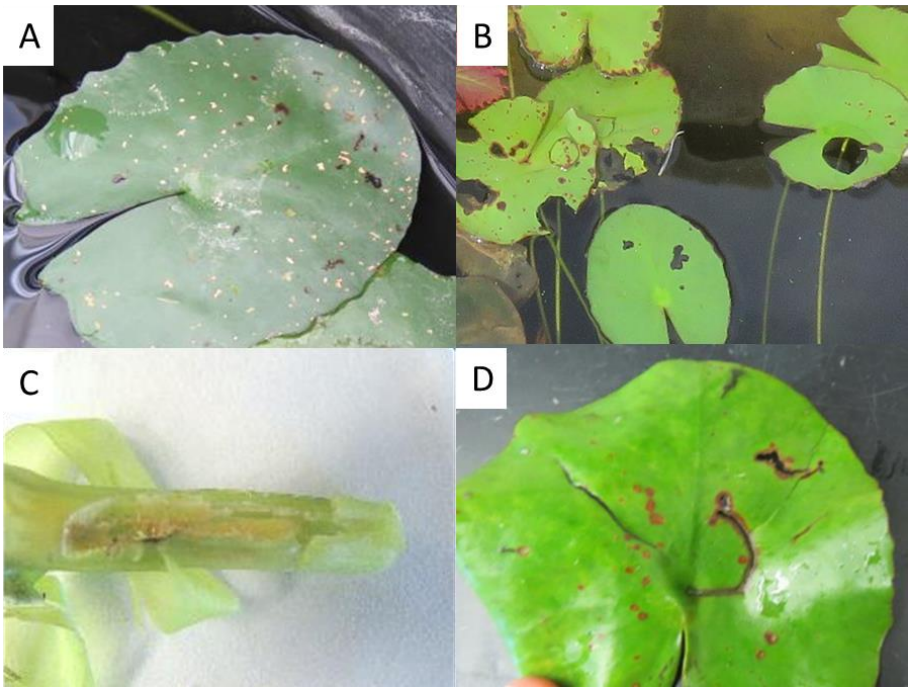


Figure 3.7: Feeding damage on *Nymphaea mexicana*. A: Feeding damage of *Lysathia ludoviciana* Fall (Coleoptera: Chrysomelidae) larvae in laboratory; B: feeding damage associated with *Elophila obliteralis* Walker (Lepidoptera: Crambidae). C: lepidopteran larva in petiole (*Paraponyx sp.* Hübner (Lepidoptera: Crambidae*?*). D: Mining damage from *Bagous americanus* Le Conte (Coleoptera: Curculionidae) larvae.

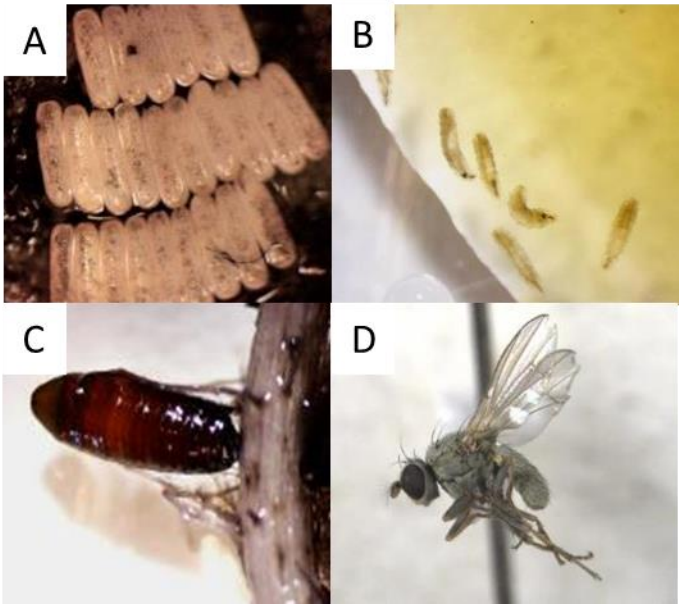


Figure 3.8: Life stages of *Notiphila latigena* Mathis. (Diptera: Ephydridae) found on *Nymphaea mexicana.* A: Eggs laid on emergent vegetation; B: larvae hatched from eggs in

laboratory, which immediately moved to the edge of the leaf towards the roots; C: pupa found on roots; D: adult (often found flying in abundance around plants and collecting in flowers).

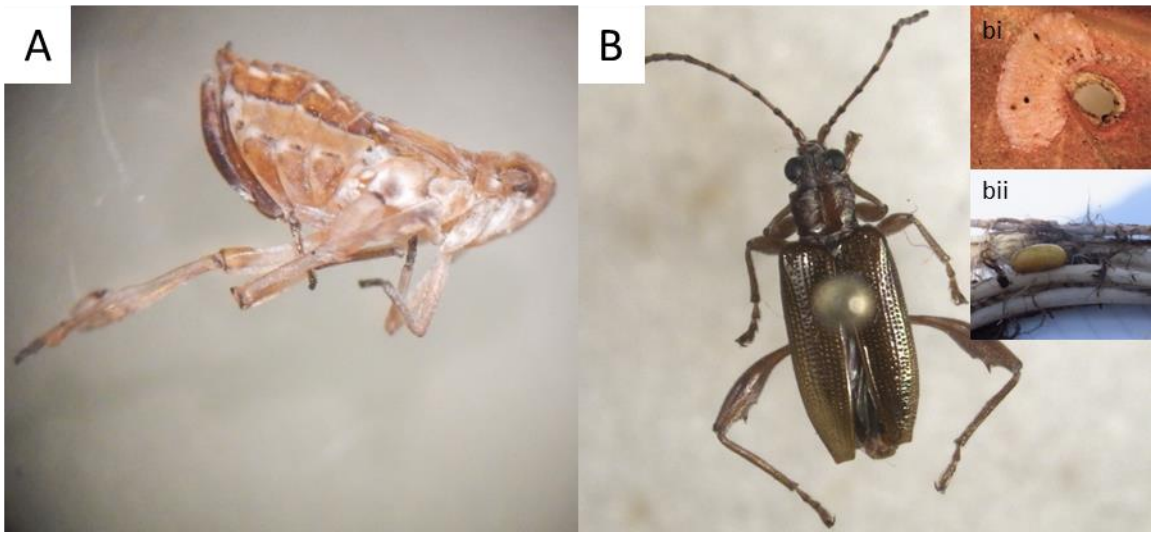


Figure 3.9: A: *Megamelus toddi* Beamer (Hemiptera: Delphacidae). B: Adult *Donacia spp.* Fabricius (Coleoptera: Chrysomelidae) found on *Nymphaea mexicana*; bi: eggs found on the underside of the leaf near oviposition hole; bii: pupa found on root (larvae collected in burlese funnel extractions).

*Megamelus toddi* Beamer (Hemiptera: Delphacidae) (identified by Dr. Andy Boring and Dr. Susan Halbert, Florida Department of Agriculture and Consumer Services) (Figure 3.9) was found at 39% of the sites (Table 3.3). In Louisiana, *M. toddi* populations of several thousand were found at the Cote Blanch Crossing site, and this species was also relatively abundant at Lake Boeuf.

Table 3.3: Summary of species found on *Nymphaea mexicana* during surveys in south-eastern United States during 2018. Species identifications marked by an asterisk were not confirmed by a taxonomist. Incidence values indicate the number of sites at which each species was present, expressed as a percentage. Levels of feeding damage were estimated subjectively and comparatively, and potential for use in biological control is based on feeding damage and incidence, as well as likely host specificity as determined from information in the literature and observations of host range during the surveys.

| Family | Species (Author) | Life stage | Feeding | Level of feeding damage | Incidence (%) | Potential for use in biological control |
|---|---|---|---|---|---|---|
| Curculionidae | *Bagous americanus* (LeConte) | Adult | Leaf chewer | Low | 17 | - |
| | | Larvae | Mines leaves and petiole | High | 17 | High |
| Delphacidae | *Megamelus toddi* (Beamer) | Adults and nymphs | Sap sucker | Low to medium | 39 | Medium to high |
| | *Megamelus* sp. *(davisi?)* (Van Duzee) | Adults and nymphs | Sap sucker | Low | 11 | Low |
| Chrysomelidae | *Donacia* spp. (F.) | Larvae | Root feeder | Uncertain | - | - |
| | | Adult | Leaf feeder | Medium | 67 | Medium |
| | *Donacia hypoleuca* (Lacordaire) | | As above for *Donacia spp.* | Uncertain | Uncertain | Medium |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *Donacia cincticornis* (Newman) | | As above for *Donacia spp.* | Uncertain | Uncertain | Low |
| | *Lysathia ludoviciana* | Larvae | Not observed in field but leaf chewing in laboratory | - | - | - |
| | | Adult | Leaf chewing | Uncertain | 11 | Low |
| Crambidae | *Parapoynx* spp.* (Hübner) | Larvae | Leaf chewing, bore into petiole | High | 83 | Low |
| | *Elophila/Synclita* spp.* (Walker) | Larvae | Leaf chewing | High | 89 | Low |
| Ephydridae | *Notiphila latigena* (Mathis) | Pupae | On roots | Uncertain | 100 | Low |
| Diptera | Unknown. | | Uncertain. Possibly *Hydrellia* fly associated with *Hydrilla verticillata.* | Uncertain | 17 | Probably low |
| Arctiidae | *Spilosoma virginica* * (F.) | Larvae | Leaf chewing | High | 6 | Low |

| | | | | | | |
|---|---|---|---|---|---|---|
| Cicadellidae | Unknown (suspected *Draeculacephala* sp. (Ball)) | Adults and nymphs | Sap sucker | Uncertain | 39 | Uncertain |
| Aphididae | Unknown | Adults and nymphs | Sap sucker | Uncertain | 6 | Low |
| Chironomidae | Unknown | Larvae | Leaf grazing (produce trenches) | High | 28 | Unclear |

*Lysathia ludoviciana* Fall (Coleoptera: Chrysomelidae) (identified by Dr. Paul Skelley, Florida Department of Agriculture and Consumer Services, and Dr. Brian Bahder, University of Florida) was also found feeding on *N. mexicana,* though only at two of the sites. Only adults were found, and these were taken back to the laboratory where they laid eggs, and the larvae fed on *N. mexicana* leaves (Figure 3.7). However, after only one or two days, the larvae were no longer seen on the leaves. It is possible that the larvae moved away or that they died, but no adults emerged in the remaining two weeks before the experiments were terminated. A single adult survived on *N. mexicana* for several weeks in the laboratory. However, *L. ludoviciana* is known to complete its life cycle on *Myriophyllum aquaticum* (Velloso) Verde (Haloragaceae) even though this is an introduced plant in the beetle's native range (Habeck and Wilkerson 1980), as well as *Ludwigia* species (McGregor et al. 1996), so it is unlikely to be suitable as a biological control agent for *N. mexicana.*

*Bagous americanus* (Dr. Robert Anderson, Canadian Museum of Nature) was found completing its life cycle on *N. mexicana* at all four sites surveyed in Louisiana, causing considerable damage to the plants (Figure 3.10). The adults were found in curled up, browning leaf edges, but the larvae and pupae were much more commonly found by dissecting the petioles of leaves showing characteristic mining patterns towards the centre of the leaf (Figure 3.7). This mining behaviour could be potentially highly damaging to the mesohyll and stomata, thus resulting in reduced photosynthesis and increased probability of leaf and petiole decomposition. Adults were collected and taken back to the laboratory, where they successfully completed a life cycle.

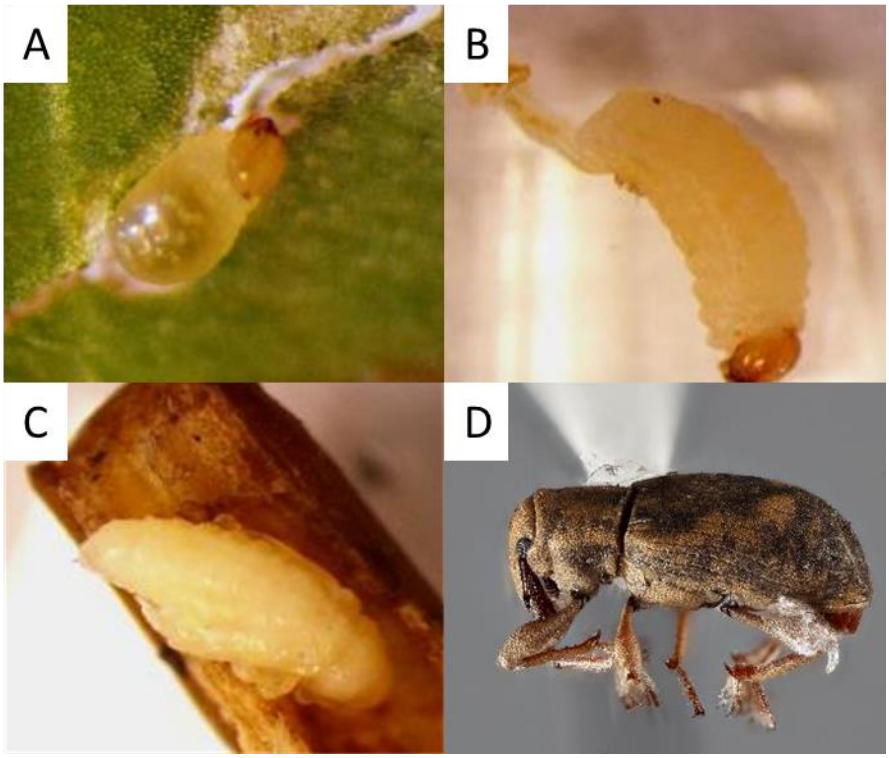


Figure 3.10: Life stages of *Bagous americanus* Le Conte (Coleoptera: Curculionidae) found in *Nymphaea mexicana* during surveys. A: early instar larva dissected from leaf mine; B: extracted later instar larva from petiole; C: pupa found in stem; D: adult.

Although *N. odorata* is a known host for *B. americanus* (McGaha 1954; Cronin et al. 1998), there are no records of this weevil species occurring on *N. mexicana* or any other species. Additionally, there were no *N. odorata* plants at any of the sites where *B. americanus* was recorded on *N. mexicana* during the surveys. *Bagous americanus* was not found during the initial surveys in Florida, but one site (Lake Seminole) was resurveyed opportunistically, and plant material from two other sites (Lake Lawne and Lake Okeechobee) were mailed from staff at Florida Fish and Wildlife Conservation Commission to be searched for the weevils towards the end of the surveying trip. Plant material from one of these sites (Lake Okeechobee) contained weevil larvae suspected to be *B. americanus*, but confirmation is needed. This site had a large population of *N. odorata* growing with *N. mexicana*, so it is possible that these plants could be hybrids.

Host specificity pilot studies

The first signs of mining by *B. americanus* during preliminary host specificity trials conducted in outside tanks, were noticed on 10 Oct 2018, four days after the weevils had been added. By 15 Oct 2018, many of the mines reached the petiole. Oviposition and larval

mining only occurred on *N. mexicana* and *N. odorata*. Mines were recorded on 15 of approximately 86 *N. mexicana* leaves (17.44%), and three of approximately 11 *N. odorata* leaves (27.27%) across the five tanks.

In the greenhouse mesocosm, mining by *B. americanus* larvae was noticed on 10 Oct 2018, four days after the weevils had been added. By 15 Oct 2018, most mines had reached the petioles. Eleven leaves had mines, and some had three or four mines on one leaf. The mean mine length, measured from the outside tanks as well as the greenhouse mesocosm once mines had reached the petiole, was 2.7 ± 0.4 cm (S.E., n = 12). The mean boring distance in the petiole was 0.8 ± 0.1 cm (S.E., n = 5). At the end of the experiment, the petioles were dissected from the outside tanks and the mesocosm, and pupae were found in two *N. mexicana* petioles, one *N. mexicana* petiole had a newly formed adult, and a larva was found in a *N. odorata* petiole. Other dissected petioles showed signs of mining/boring but no larvae or pupae, and some showed evidence of emergence holes.

Prioritisation

The highest prioritisation scores (as per Forno and Julien (2000)) were obtained for *B. americanus* and *M. toddi* (Table 3.4), with *M. toddi* obtaining a slightly higher score of 12 compared to a score of 10 for *B. americanus.* Hence, these two species were prioritised as potential biological control agents for *N. mexicana.* The preliminary host specificity studies conducted on *B. americanus* suggested that it may be specific at least to *Nymphaea*, and its abundance and levels of damage supported the decision to import this species into quarantine at Rhodes University in Grahamstown, South Africa (Permit no.: P0098640) for rearing and further study. The weevils were collected from Salvador site 1 in Louisiana, as this site had a large healthy population of *N. mexicana* and was relatively easy to access, with mostly other invasive aquatic plant species in the area aside from some *Nelumbo lutea, Ceratophyllum demersum* and a few native grasses. *Megamelus toddi* should also be considered as a biological control agent for *N. mexicana,* although it was not imported into quarantine with *B. americanus.*

Table 3.4: Prioritisation scores for *Bagous americanus* and *Megamelus toddi* according to the scoring system developed by Forno and Julien (2000). Only scores for the two highest priority species are shown. Where scores did not fit exactly into a category, intermediate scores were used. For example, if adults cause minimal damage, they received a score of 1 (between 0 for no damage, and 2 for damage). Sections marked with an asterisk are estimated from observations and literature on similar species but need confirmation through life history studies.

| Criterion | | Score |
|---|---|---|
| ***Bagous americanus*** | | |
| Damage by adults | No damage (adults feed, but cause minimal damage) | 1 |
| Damage by immatures | Damage leaf and stem tissue, enough to cause leaves to break off | 3 |
| Duration of attack | Prolonged attack but not increasing plant susceptibility to waterlogging or sinking | 2 |
| Number of generations | Four or more generations a year * | 4 |
| *Total* | | *10* |
| ***Megamelus toddi*** | | |
| Damage by adults | Damage vascular or structural tissue through sap sucking | 3 |
| Damage by immatures | Damage vascular or structural tissue | 3 |
| Duration of attack | Prolonged attack but not increasing plant susceptibility to waterlogging or sinking (but may increase susceptibility to disease) | 2 |
| Number of generations | Four or more generations a year * | 4 |
| *Total* | | *12* |

## 3.4 DISCUSSION

The species richness of natural enemies found on *N. mexicana* is lower than that of *N. odorata*, which hosts various Trichoptera, Lepidoptera and Coleoptera (Harms et al. 2011). However, the insect taxa found on *N. odorata* have been recorded more extensively, probably because this species seems to be more common than *N. mexicana* (personal observation). It is possible that Mexico is the centre of origin for *N. mexicana*, and that more species would

have been found if surveys were conducted here. Additionally, sampling was biased in Florida compared to Texas and Louisiana, owing to the greater number of sites in Florida. Hence, future surveys for natural enemies in Mexico may reveal more potential biological control agents for *N. mexicana*. Furthermore, many of the species found during these surveys were not identified to species and thus cannot be taken as an accurate representation of the insects associated with *N. mexicana.* The species accumulation curve suggests that only about two to five species were likely to have been missed during the surveys. These indicators are useful to estimate species richness, and the Chao 2 and ICE estimators may present higher accuracy than asymptotic estimators such as the Michaelis-Menton, and Bootstrap estimators (Hortal et al. 2006). However, only 17 sites were sampled during the surveys, and it is recommended that rarefaction should be based on 20 samples or more (Gotelli and Colwell 2011). Leaf length varied per site suggesting phenotypic response to local conditions such as water quality and nutrition, sediment type, and community interactions (Crossley et al. 2002; Bornette & Puijalon 2011). Alternatively, morphometric differences may be caused by genetic differences, varying length of growing season between states, or phenotypic plasticity (Via & Lande 1985; Schmid 1992).

More species were likely to be found towards the end of the surveys as the surveyor became more skilled at identifying the insects present, so some species may have been present at earlier sites but were not recorded as they were not yet noticed. Sampling effort, sampling periods, and seasonal variability may have affected the species abundance and diversity, and many of the natural enemies observed hosted by *N. mexicana* were identified only to family, while some individual species identifications, especially with regards to the Chironomids, *Parapoynx*, and *Elophila* species, were not confirmed. Hence, it is possible that there could be several additional species hosted by *N. mexicana* that were not identified during these surveys, and that it may be useful to conduct additional surveys at a greater number of sites. Additionally, there may be plant pathogens that were present but not investigated that may be specific to *N. mexicana*.

Identification of the chironomids found on *N. mexicana* may reveal species that hold promise to act as biological control agents. For example, the chironomid *Polypedilum tuburcinatum* Andersen *et* Bello González (Diptera: Chironomidae) showed promise as a biological control agent for *Lagarosiphon major* (Ridl.) Moss ex Wager (Hydrocharitaceae) after preliminary host range and biology studies (Earle et al. 2013; Earle 2015), while *Cricotopus lebetis* Sublette (Diptera: Chironomidae) may also be considered as a biological control agent for *Hydrilla verticillata*, though laboratory host range studies show damage on

other plant so its suitability is unclear (Stratman et al. 2013). On the other hand, *Parapoynx* and *Elophila* species are less likely to have potential as biological control agents on account of their wide host range on aquatic plants including *Potamogeton spp., Sagittaria spp., Brasenia schreberi, Lemna spp., Nuphar spp.,* and several other species (Habeck 1974; Buckingham and Bennettl 1989; Center et al. 1999; Harms and Grodowitz 2009; Nachtrieb et al. 2011).

Most of the species found on *N. mexicana* during the surveys are either known to be generalists based on literature searches or are not associated with plant damage. For example, the *N. latigena* fly adults, eggs, and/or pupae were found at all the sites surveyed but it was unclear if or how they fed on the plant. Information from literature searches indicates that larvae of various species within the genus move to the roots of various aquatic plants immediately after hatching and live and pupate in the submerged substrate, where they obtain oxygen by attaching to the roots of the plants (Mathis 1976, 1979). Gut dissections of adult specimens of three *Notiphila* species, namely *Notiphila macrochaeta* Cresson, *Notiphila olivacea* Cresson, and *Notiphila solita* Walker showed that these species feed mostly on algae with some traces of diatoms (Deonier 1972), while third-instar larval gut contents of *Notiphila aenigma* Cresson and *N. solita* showed bacteria and large amounts of detritus had been consumed (Busacca and Foote 1978). Adults have been suggested to feed on microflora on plant surfaces, and have been observed feeding on slightly decomposing animal material as well as a mixture of honey and brewer's yeast (Mathis 1979). Hence, it is highly unlikely that *N. latigena* is suitable as a biological control agent for *N. mexicana*.

According to old records, *Donacia spp.* are known to feed on a variety of different plants, though some species may show preference for one host plant (Marx 1957). The adults feed on the leaves, while the larvae feed externally on the roots and stems. Species from *Sparganium*, *Nymphaea*, and *Nuphar* are most commonly found associated with species of *Donacia* (Marx 1957). Hoffman (1940) stated that the adults of seven *Donacia* species preferred one plant, and only two confined to more than one plant. Similarly, the larvae of six *Donacia* species were only found on the host plant associated with the adult, while in three other species, the larvae could be found on up to five additional different plants (Hoffman 1940; Marx 1957).

There are few records in the literature of *Donacia* species hosted by *N. mexicana.* As previously mentioned, Nachtrieb et al. (2007) recorded *D. cincticornis* on *N. mexicana,* and this species, as well as numerous others including *Donacia liebecki* Schaeffer, and *Donacia megacornis* Blatchley, are found on *N. odorata* (this is well summarised in Harms and

Grodowitz (2009)). *Donacia hypoleuca* however, is not mentioned as a herbivore of *N. odorata*, though it is mentioned that it regulates aquatic weed populations without specific details on host specificity (Martin 1953 cited by Shah and Tyagi 1985). In terms of feeding damage, there did not seem to be high levels of damage associated with *Donacia* in the field, although it was difficult to quantify this and differentiate between feeding damage from other species. Hence, it may be necessary to further investigate and identify the *Donacia spp.* found feeding on *N. mexicana* to confirm their potential use as biological control agents.

*Megamelus toddi* was found in abundance at all the sites surveyed in Louisiana, as well as a few sites accompanied by what is thought to be *M. davisi* in Florida. *Nuphar advena* and *Nymphaea odorata* are known hosts of *M. davisi* (McGaha 1952; Harms and Grodowitz 2009) so it is unlikely to be host specific to *N. mexicana*, and its abundance was not sufficient on the plants to warrant collection. Furthermore, *N. advena* and/or *N. odorata* was often present at the sites where *M. davisi* was found. However, *M. toddi* is not well studied. There is minimal information available about *M. toddi* in the literature, and very little is known about this species and its plant hosts (Dr. Susan Halbert and Dr. Charles Bartlett, Florida Department of Agriculture and Consumer Services pers. comm.). The record acquired from these surveys was the first record of *M. toddi* being hosted by waterlilies and was also a new county record (S. Halbert Department of Agriculture and Consumer Services pers. comm). *Megamelus toddi* was particularly abundant at a few of the sites surveyed, though the level of damage was not very high at the time of sampling. However, it is possible that effects on the plant would only be seen later, and studies on woody plants suggest that sap sucking insects may have greater impacts on plant performance than defoliators (Zvereva et al. 2010). Furthermore, *Megamelus scutellaris* has been released in South Africa to control water hyacinth *P. crassipes* and is well established and damaging in cooler regions of the country where other biological control agents have struggled to establish (Hill & Coetzee 2017). Therefore, due to the lack of information on its biology and host range, and the known success of *M. scutellaris, M. toddi* has a lot of potential as a biological control agent of *N. mexicana*.

Although *B. americanus* is known to be hosted by *N. odorata*, a feeding selectivity study conducted by Cronin et al. (1998) demonstrated that *B. americanus* preferred to feed on *N. odorata* when offered a choice of *Nuphar variegata* Engelman and *Nuphar pumila* (Pers.) Fernald (Nymphaeaceae), *Pontederia cordata, Potamogeton amplifolius* Tuckerman (Potamogetonaceae), *Typha latifolia* Linneaus (Typhaceae), and *Calla palustris* Linneaus (Araceae). Results from the host specificity pilot tests conducted in this study similarly

suggest that the species is specific at least to the genus *Nymphaea*. In the literature, there are no published records of *B. americanus* being hosted by other *Nymphaea* species aside from *Nymphaea tuberosa* (McGaha 1954; Harms and Grodowitz 2009). However, it has since been determined that *N. tuberosa* does not possess enough genetic difference to *N. odorata* to be classified as a separate species, and should therefore be demoted to subspecies level (*Nymphaea odorata subsp. tuberosa* (Paine) Wiersma & Hellq) (Woods et al. 2005b).

There is a possibility that cryptic species could be present and misidentified based on morphological characteristics (Toševski et al. 2011; Paterson et al. 2016). Hence, the *B. americanus* collected from *N. mexicana* in this study could be genetically different to those hosted by *N. odorata* and thus perhaps more adapted to feeding on *N. mexicana* as opposed to other plant species. It may be useful to conduct molecular studies of the *B. americanus* collected in this study to confirm its identity, as taxonomic error has occurred in the past and can have considerable effects on biological control programmes. For example, *Cyrtobagous singularis* Calder and Sands (Coleoptera: Curculionidae) was introduced from Trinidad into Zimbabwe and Botswana to control *Salvinia molesta* D. Mitch Salviniaceae, and what was thought to be the same species was later imported to Australia from Brazil. The weevil was very effective at controlling *S. molesta* in Australia but was unsuccessful in Botswana and Zimbabwe. Later, taxonomic studies revealed that the weevils introduced into Australia had been misidentified and were actually a new species, *Cyrtobagous salviniae* Calder and Sands (reviewed in Center et al. 1999).

In conclusion, field densities, damage, climatic adaptations and field host associations in the native range suggest that *B. americanus* and *M. toddi* should be prioritised for importation, and their host specificity studied under quarantine conditions at Rhodes University, South Africa. While it may be necessary to conduct additional surveys to better understand the diversity of natural enemies (including mites and fungi) that control populations of *N. mexicana* in its native range, these surveys are a starting point for identifying potential biological control agents for *N. mexicana* in South Africa, and in other invaded regions in the rest of the world. Indeed, these surveys are the most extensive to date known for *N. mexicana*. Not only is this information useful to initiate a control programme for this plant, but also to better understand the aquatic diversity present in the south-eastern US.

Although surveys in the native range of invasive species can be logistically challenging and expensive, they form the basis for biological control programmes and are thus critical for biological control to be successful (Goolsby et al. 2006b). Refining survey

techniques and the use of modern technology and taxonomy, as well as international collaboration can reduce the difficulties involved in host range surveys and maximise the success of biological control programmes (Goolsby et al. 2006b), which remain a very effective, cost-efficient, long term means of managing alien invasive species and protecting natural ecosystems (Wapshere 1985; van Driesche et al. 2010; Hill and Coetzee 2017).

# Chapter 4: General discussion

## 4.1 INTRODUCTION

The aims of this thesis were to develop a biological control programme for *N. mexicana* by developing a better understanding of the origin of invasive *N. mexicana* populations in South Africa and conducting field surveys to identify and prioritise potential agents in the native range. DNA samples were collected in the native range and utilised to match invasive populations to native populations. Results indicated that while some of the populations in South Africa grouped with a few of the native *N. mexicana* populations, there are high levels of genetic diversity and some of the invasive populations are likely hybrids. At least 15 species of potential natural enemy were identified during the field surveys, and two of these, *B. americanus* and *M. toddi*, were prioritised based on abundance, damage, and host range data. These results provide insight into the way forward for managing invasive *N. mexicana* populations in South Africa.

In the past many biological control researchers have followed either the lottery model, in which a number of agents are introduced to increase the probability that an effective agent is released (Myers 1985), or the cumulative stress model, in which biological control success is believed to be achieved as a result of different types of attack from different species released one after the other (Harris 1985). A study by Denoth et al. (2002) suggests that a single agent was responsible for biological control success in over 50% of the successful multiple-agent projects included in the analysis. Observations such as these, and concerns about non-target impacts and indirect effects of agent releases has led to more focus being placed on prioritising agents based on factors such as climate, impact, and local adaptation (Sheppard 2003; van Klinken and Raghu 2006). Making predictions about the efficacy of biological agents before they are released will enable researchers to release only the most effective agents, thus reducing non-target risks. Predictive modelling will also allow researchers to plan ahead to deal with challenges in different areas of the invaded range. Hence, the importance of field surveys and genetic studies for implementing biological control programmes is discussed, as well as the factors that affect predictions about biological control efficacy. Finally, the factors affecting the biological control of hybrids, importance of anthropogenic influences, and study limitations are considered before recommendations are made for future research.

## 4.2 MATCHING INVASIVE *NYMPHAEA MEXICANA* TO ITS NATIVE RANGE

Understanding the genetic composition of invasive alien species within the native and introduced range can be important to maximise the success of biological control agents, to reduce the risks of non-target effects by reducing the number of agents released (Balciunas 2004), and by selecting agents with high host specificity and effectivity (Strong and Pemberton 2001). Molecular methods help resolve taxonomic difficulties of identifying the target weed as well as the insect herbivores by providing information that is not reflected in the morphology of various species, including hybridisation (Vilà et al. 2000), population structure (Culley and Wolfe 2001), and cryptic speciation (Toševski et al. 2011; Paterson et al. 2016). In addition, molecular techniques allow scientists to pinpoint the source populations from which plants in the invasive range were likely introduced, as well as invasion routes (Amsellem et al. 2000; Sutton et al. 2017). Studies such as these reveal information about the evolution of host-insect relationships, and enable scientists to prioritise sites that should be surveyed for biological control agents (Briese 1996a). Furthermore, molecular techniques enable scientists to understand the genetic diversity of invasive populations compared to native populations, by determining the presence or absence of founder effects, genetic bottlenecks, or indeed greater genetic diversity of invasive populations as a result of multiple introductions and adaptive evolution in the invaded range (Amsellem et al. 2000; Lawson Handley et al. 2011).

Determining the native range of an IAP can be achieved using extensive literature and herbaria searches, but finer scale resolutions of distributions require the use of molecular methods. More detailed understanding of the genetic structure of invasive populations is often imperative to locate biological control agents that are specific enough for use in the invaded range. For example, determining the native origin of the invasive haplotype of *Phragmites australis* (Cav.) Trin. ex Steud. (Poaceae) in Europe allowed researchers to discover specialised herbivore complexes that made a significant contribution to the advancement of biological control of the species (Häfliger et al. 2006). Furthermore, basic processes in invasion ecology may be better understood by determining geographic sources of invaders (Le Roux and Wieczorek 2009).

While previous work confirmed the identity of the invasive populations of *N. mexicana* in South Africa and determined the presence of high genetic diversity and hybridisation (Naidu 2018), the greater number of samples from the native range used in this thesis (Chapter 2) enabled us to gain more detailed insight into population level genetic matching of invasive and native populations of *N. mexicana.* Ideally, such detail should have

been explored without the need to visit the native range, but DNA samples of the target plant from the native range may be difficult to acquire unless there are well formed collaborative relationships with research centres in foreign countries. Nevertheless, matching the invasive populations to the native populations surveyed in the native range allows us to determine the most ideal site for collecting biological control agents for further testing. This will also enable us to understand which sites are most likely to be well controlled using biological control. In addition, population matching studies allow us to make decisions regarding the control of the invasive populations that did not group with samples from the native range. Much time and money might be wasted on attempting to control various invasive populations without understanding their genetic structure.

## 4.3 SURVEYS FOR POTENTIAL BIOLOGICAL CONTROL AGENTS

Valuable data can be collected in the native range of invasive alien plants (IAPs) and this information can be utilised to reduce the costs and risks associated with biological control, and effectively prioritise potential biological control agents (Goolsby et al. 2006b). It is desired that field surveys will accomplish at least two goals: one, to develop an extensive checklist of natural enemies on the target weed; and two, to obtain information that will assist in prioritising potential agents (Goolsby et al. 2006b). Obtaining a comprehensive checklist of natural enemies for a target weed increases the probability of identifying an effective agent and assists with making decisions about the sequence in which to release agents (Denoth et al. 2002). In addition, this information can aid decisions about when options for biological control have been exhausted for a particular invasive species (Müller-Schärer et al. 1991) and also adds scientific value to other disciplines. Species accumulation/rarefaction curves are useful in this regard to understand how extensive surveys are, and whether sufficient sampling has occurred (Müller-Schärer et al. 1991). At least 15 species were identified during the surveys for biological control agents for *N. mexicana* (Chapter 3), and many of these were only identified to family level. While two of these species were prioritised as biological control agents, the rarefaction curve generated in Chapter 3 suggested that an additional 2-5 species were unsampled, so there is potential that more species would be appropriate for consideration. The use of rarefaction curves in this thesis enable us to develop an idea of the extent of the surveying efforts, and the possible need for more surveys in different areas and seasons to identify more potential agents should they be needed.

Many hypotheses exist about the importance of various factors for selecting biological control agents during surveys in the native range. A recurring theme is the climatic

compatibility of the potential agent in the native compared to the invaded range, as climate can play a large role in affecting the establishment and success of biological control agents (see next section on predicting efficacy of biological control agents). For example, humidity stress likely resulted in the failure of *Gratiana spadicea* (Klug) (Coleoptera: Chrysomelidae), a biological control agent released in South Africa, to control *Solanum sisymbriifolium* Lamarck (Solanaceae) (Byrne et al. 2002). Field surveys in the native range can be costly and logistically challenging, so restricting surveys to regions in the native range that match climatically to the invaded range enables one to conserve time and money, and target agents that are likely to be well adapted to the conditions in the invaded range. Indeed, many of the sites in South Africa that have proven difficult to manage using biological control are a result of climatic conditions that are not conducive to the survival of introduced agents (Coetzee et al. 2011a).

MaxEnt modelling was used to identify regions that were climatically similar to the invaded range, and this allowed targeted surveying in regions with high probabilities of producing compatible agents (Chapter 3). However, it should be noted that selecting agents based on climate matching may not always produce optimal results as each species is likely to have specific climatic requirements (van Klinken et al. 2003; van Klinken 2004). Furthermore, predicting agent performance based only on climate matching is difficult as the climate in the release environment is likely to vary. In South Africa, *N. mexicana* is found in the Western Cape (34°S), Eastern Cape, on the coast of KwaZulu-Natal, in the high altitude (highveld) regions of Gauteng (>1 500 m), and in Limpopo. These regions vary climatically, with the cold winters of the highveld regions being particularly problematic for biological control agents that are not adapted to deal with such cold temperatures (Byrne et al. 2002; Coetzee et al. 2011a). In addition, other factors are likely to affect establishment in the introduced range, and data quality will affect model predictions (Samways et al. 1999; Ulrichs and Hopper 2008) (see Section 4.4). While some scientists may advise surveying across the entire native range of a plant for this reason, practical, political, and financial limitations often prevent such widespread surveys (Goolsby et al. 2006b).

Collecting environmental data in the native range assists with understanding possible factors that regulate the abundance of an IAP, and to identify environmental preferences or constraints that affect agent abundance (Paynter et al. 2003). For example, differences in plant ecology, insect activity, and rates of egg parasitism were recorded across different climatic zones and habitats for the seed-feeding beetle *Penthobruchus germaini* Pic. (Coleoptera: Bruchidae) on *Parkinsonia aculeata* (Mimosaceae). Data from the native range

can also help to assess sampling efforts across environmental gradients and determine the need for additional surveying (Goolsby et al. 2006b). These data can be entered into relational databases for a more comprehensive checklist of species, and to allow access by other scientists and for future reference (Palmer and Pullen 1995; Colwell 2004). Site data were collected in Chapter 3, including information on plant parameters to attempt to understand environmental variability between sites. These data will be beneficial especially in later stages of the biological programme for *N. mexicana*.

Host specificity is an important requirement of a biological control agent to reduce non-target risks (McFadyen 1998; Louda et al. 2003). Host specific natural enemies have the greatest likelihood to be found in the native range of the target weed, particularly in centres of diversity for the genus, and Pleistocene refugia (Wapshere et al. 1989; Müller-Schärer et al. 1991). Field surveys are useful to develop an idea of host specificity using host range data. In Chapter 3, neighbouring and closely related plants were surveyed opportunistically to determine the likely host range of the natural enemies encountered on *N. mexicana*. These observations are useful, as they reflect real field conditions that cannot be reproduced in laboratories, and assist in the prioritisation of potential agents, thus conserving time and resources. For example, *Notiphila latigens* Mathis (Diptera: Ephydridae) and *Megamelus* sp. Duzee (Hemiptera: Delphacidae) were found hosted by other plant species in the field and were thus deemed low priority as biological control agents for *N. mexicana* (Chapter 3). The pilot studies conducted in Chapter 3 to determine the preliminary host specificity of *B. americanus* similarly demonstrate the usefulness of performing studies in the native range. Such experiments could not have been carried out in the invaded range and would have been logistically challenging to mimic in quarantine. While improvements in quarantine facilities are reducing challenges of conducting studies under these conditions, it is generally accepted that biosecurity is enhanced when potential agents are thoroughly studied in their native range before importation into quarantine (Ferrar et al. 2004).

It is clear that field surveys in the native range, as well as molecular techniques, provide much useful data that is necessary to maximise the success of biological control. Data from both field surveys and genetic studies can be used to develop a broader understanding of the factors that drive the successes and failures of biological control programmes. For example, unique genotypes can occur as a result of environmental differences in the native range. Populations of *Floracarus perrepae* separated by biogeographical barriers had unique genotypes, and this knowledge was critical for selecting the best adapted genotype for the control of Old World climbing fern *Lygodium*

*microphyllum* (Goolsby et al. 2004). Although both field surveys and molecular methods can be expensive and time consuming, and logistically challenging, these costs are worth the benefits of improving the likelihood of finding potential agents as well as adding knowledge of the fauna and flora in the native range. The improved efficacy and success of biological control projects that include extensive native range and genetic research will outweigh the initial costs and time in the long run (Goolsby et al. 2006b).

## 4.4 PREDICTING EFFICACY OF BIOLOGICAL CONTROL AGENTS

The efficacy of biological control agents on targeted IAPs is dependent on numerous factors. However, the success of a biological control agent depends on the way in which one defines and evaluates what "success" is. Such evaluations require pre- and post-release assessments to allow comparisons, and can include measures of ecological, economic, and social impacts of the IAP with focus placed on the insect agent itself, the target weed, or the ecosystem responses to invasions by the weed (Morin et al. 2009). Similarly, the development of standardised categorisations of biological control success will be useful to ensure that these categories are clarified and internationally understood to allow comparisons between systems and on different scales (Hoffmann et al. 2019). Nevertheless, the ideal scenario is that a biological agent demonstrates the following main characteristics (Harris 1973, 1991):

- Host specificity;
- Abundance, such that it establishes and proliferates in the invaded range;
- High levels of damage inflicted on the target weed such that the weed decreases;
- Relative ease of rearing in quarantine and mass rearing facilities;
- Ability to survive in climatic conditions of the invaded range.

Many of these factors are determined during the pre-release stages of biological control. However, conditions in the introduced range once agents are approved for released may limit the establishment and success of biological control agents. Of these factors, climate is the primary driver as it directly affects each component individually, as well as the interactions

between the components (Zalucki and van Klinken 2006) (Figure 4.1).

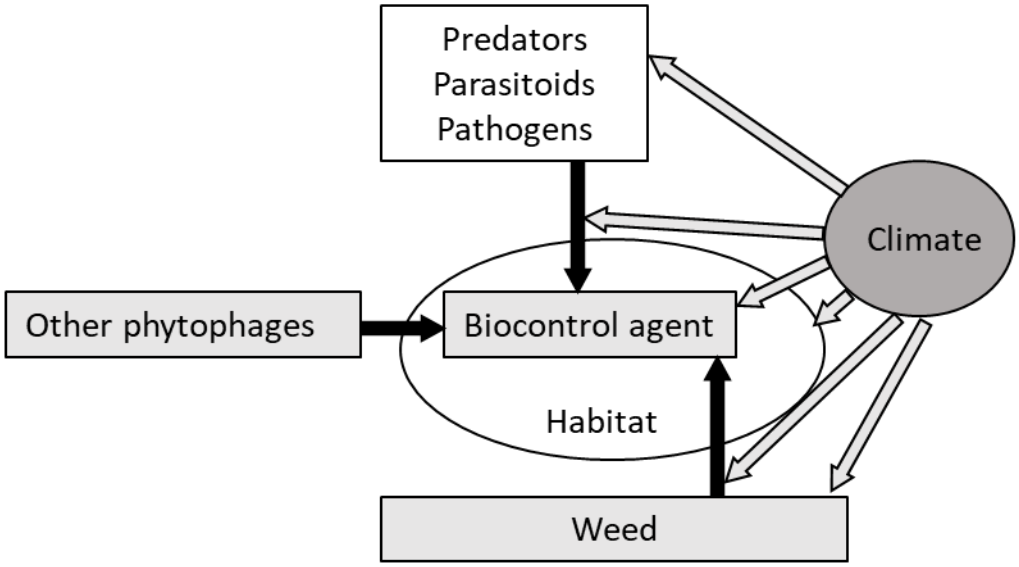


Figure 4.1: Basic interacting components that affect populations dynamics of phytophagous biological control agents. Each component and the interactions between components are affected by climate. Redrawn from Zalucki & van Klinken (2006).

Climatic variables such as temperature and moisture have various impacts on physiological processes such as thermoregulation, osmoregulation, and growth. It follows that extremes of these variables significantly impact organism development, survival, and reproduction. Hence, the distribution and success of biological control agents can be predicted to some extent using climate data and population dynamics of the potential agent. Indeed, a review of case histories for the failures of 199 biological control agents released 148 times against 84 pests suggests that 34.5 % of the investigated failures were believed to be related to incompatibilities with climate (Stiling 1993). Zalucki and van Klinken (2006) detail the use of CLIMEX to make predictions by integrating physiological processes and climate data that may influence an organism's distribution and abundance. It should be noted that CLIMEX assesses the suitability of a region for a species using climate data to predict distributions, climate similarity, and seasonal phenology (Kriticos et al. 2015; Jung et al. 2016). On the other hand, MaxEnt, which was used in this study, uses species presence information to predict distributions based on maximum entropy distributions, and can apply and assess the contribution of environmental variables such as geographical factors, unlike CLIMEX (Phillips et al. 2006; Byeon et al. 2018). The use of modelling programmes such as this can contribute significantly to understanding the limiting factors that affect the success of biological control agents. Similarly, these models can be used to plan ahead when they

predict the failure of insects to establish in certain regions of the country. For example, climate matching and pre-release evaluations of *Eretmocerus* spp. Haldeman (Hymenoptera: Aphelinidae) effectively predicted parasitoid establishment on *Bemisia tabaci* biotype "B" Gennadius (Hemiptera: Aleyrodidae) in a retrospective analysis of the biological control programme in the USA (Goolsby et al. 2005).

*Nymphaea mexicana* has invaded seven of the nine provinces in South Africa. While these regions showed climatic similarities to the native distribution of *N. mexicana* according to MaxEnt modelling, it may be important to investigate differences and similarities between the native and invaded range on a finer level and perhaps per site in order to predict the success of the potential agents identified in this thesis. For example, the biological control of water hyacinth (*P. crassipes*) has had varying levels of success in different parts of South Africa as a result of variable climate and eutrophication, interference from herbicide control, and varying hydrological features (Hill and Olckers 2001). In particular, high lying areas of South Africa (the Highveld) and the Western Cape have had less effective control by biological control agents introduced there. The populations of *N. mexicana* at Westlake in the Western Cape have been particularly problematic sites, and populations exist in the Highveld province (Gauteng), so it is possible that these regions might pose challenges for biological control of *N. mexicana* as they did for water hyacinth.

Host plant attributes are likely to influence the success of biological control agents in the invaded range (Cortesero et al. 2000). While host abundance is unlikely to limit agent survival in the growing season, during periods of little or no growth the agent will need to survive without a stable food source, depending on the phenology and synchronicity of both the plant and the agent. This can be challenging at sites with more extreme environmental conditions where plant growth and reproduction are synchronised with specific environmental cues, especially for seed or flower feeding insects. Persistence of biological control agents in these scenarios require the development of mechanisms to survive when the food source is absent (van Klinken 2005). Similarly, host quality affects the abundance and impact of herbivorous insects (Egan and Ott 2007; Center and Dray 2010), and this in turn is influenced by soil attributes, climate, plant density, and the possible presence of generalist herbivores. Understanding the plant population dynamics of an invasive plant and how these dynamics vary in contrasting habitats is useful to predict where IAPs are likely to thrive, and thus infer at which sites control is most needed, and where plant quality is likely to be high. Zalucki and van Klinken (2006) suggest that building weed population models and overlapping these with models of biological control agents could be useful to infer where the

impact of biological control is likely to be the greatest. Understanding the methods by which herbivory limits plants dynamics would enable such predictions to be made (Raghu et al. 2006). The presence of multiple or unique genotypes of the target plant may similarly affect biological control efficacy, especially with the presence of hybrids, and where biological control agents are locally adapted to particular genotypes (Day & Neser 2000; Zalucki et al. 2007; Urban et al. 2011).

Natural enemies can impact the effects insect herbivores have on their host plants through regulation of population numbers (Berryman et al. 1987; Rosenheim et al. 1993; Shea and Chesson 2002; Knight et al. 2005). As a result, natural enemies are suspected to be responsible for the failure of agents in some biological control programmes (Goeden and Louda 1976). It follows that agents that are likely to experience enemy release are more likely to be successful in biological control programmes (Paynter et al. 2010). However, agent effectiveness does not always require enemy release in the introduced range, and agents can still demonstrate high abundance with high levels of parasitism and predation from diverse faunas (Bess and Haramoto 1972; van Klinken and Burwell 2005). Nevertheless, knowledge of the natural enemies that affect similar taxa in the introduced range can be useful to predict enemy release for biological control agents in the introduced range. For example, researchers were able to predict that *Evippe* sp. #1 Chambers (Gelechiidae), a leaf-tying moth introduced into Australia from Argentina to control *Prosopis* L. (Fabaceae), would be parasitized by diverse fauna in the introduced range because Australia has a diversity of Lepidoptera similar to *Evippe* sp. #1 (van Klinken and Burwell 2005). Susceptibility to natural enemies should still be considered (Zalucki and van Klinken 2006) as predicting the likelihood that an agent will experience effects from natural enemies can assist with agent prioritisation and make biological control safer and more effective (Paynter et al. 2018).

Biological control agents may be more likely to experience enemy release if the imported agent belongs to a taxon that is not well represented in the introduced range or if the agent is biologically unique (McFadyen and Spafford-Jacob 2004). In other words, predation and parasitism of introduced agents are more likely to occur when ecological analogues are present (Paynter et al. 2010). Knowledge of the fauna in the introduced range can thus be useful to predict the success of biological control agents, but this may not always be possible if the fauna are not well studied. For example, there are records of *Megamelus scutellaris* (the biological control agent released against water hyacinth *P. crassipes*), being parasitised by the indigenous *Echthrodelphax migratorius* Benoit (Hymenoptera: Dryinidae) in South

Africa (Kraus et al. 2019). Before these records, alternative hosts of *E. migratorius* were not known (Kraus et al. 2019), so potential ecological analogues of *M. scutellaris* were not recorded and thus could not be utilised to make predictions about parasitism before *M. scutellaris* was released in South Africa. However, the case of *E. migratorius* being hosted by *M. scutellaris* means that it is highly probable that *M. toddi*, one of the agents prioritised in Chapter 3, will be affected by South African parasitoids should it be appropriate for release. Parasitism of biological control agents may negatively affect control efficacy as outlined above, but agent populations may also be unaffected (Hill and Hulley 1995b; Norman et al. 2009) or indeed stabilised by natural enemies through the prevention of population crashes (Kraus et al. 2019). Possible effects of natural enemies on *B. americanus* may be more difficult to predict, but the points made above highlight the importance of having a comprehensive knowledge of the fauna in the native and introduced ranges of invasive species.

In summary, the efficacy of biological control agents can be difficult to predict, as it is influenced by a plethora of factors which are likely to vary considerably between sites in the invaded range. Nevertheless, understanding the roles each factor plays and utilising modelling techniques such as CLIMEX may enable scientists to have some ability to predict the success of biological control agents in the field. Climate matching was utilised in the field surveys conducted in this thesis and genotype matching has provided insight regarding the most appropriate sites from which to import biological control agents to target specific sites in the invaded range. However, more information regarding the phenology of the insects prioritised in this study and their ability to impact and survive on *N. mexicana* under varying circumstances is needed to make more informed predictive statements about the likely efficacy of these agents in the invaded range.

## 4.5 BIOLOGICAL CONTROL OF HYBRIDS

Hybridisation has been linked to the evolution of invasiveness in multiple plant taxa, and human interference worsens the scenario (Schierenbeck and Ellstrand 2009). For example, investigations carried out by Moody & Les (2002) between invasive and native watermilfoil (*Myriophyllum* spp.) showed that populations that exhibited very high levels of invasiveness were hybrids, while parental populations did not grow aggressively. Hybridisation leads to the introduction of genetic variation in populations and may result in the inheritance of traits that lead to higher fitness, and are selected for in the invaded environment (Latta et al. 2007; Arnold et al. 2008). Elevated levels of invasiveness contribute

to the rapid spread of invasive weeds, and thus make controlling them even more difficult. The economic and environmental costs of chemical and mechanical control emphasise the importance of establishing effective biological control agents to manage hybrid populations, but the variable genetic makeup of hybrid plants may make it difficult to find agents with appropriate host specificity. If herbivores are not well-adapted to specific genotypes, they may be ineffective as biological control agents (Gaskin and Schaal 2003; Urban et al. 2011; Williams et al. 2014).

It follows that understanding the genetic structure of invasive species is critical for their biological control, as it determines where surveys for biological control agents should be targeted to find species adapted to hybrids (Chapters 2 and 3). For example, the nomenclature and taxonomic status of *Lantana camara* Linneaus (Verbenaceae) (commonly referred to just as 'lantana'), which is generally regarded as a complex of species/hybrids/varieties, has been a challenging topic of biological control research as a result of genetic modification through hybridisation and horticultural selection (Zalucki et al. 2007; Urban et al. 2011). As a result, biological control of this plant is an ongoing challenge, in addition to constraints presented by climatic incompatibility, natural enemies, and the number of agents introduced (Zalucki et al. 2007; Urban et al. 2011). Nevertheless, retrospective analyses of the biological control agents released against invasive lantana in Australia showed that a greater proportion of the agents that were collected from *L. camara* and *Lantana urticifolia* Mill established compared to agents collected from other species of lantana (Zalucki et al. 2007). Similarly, agents that were collected from Mexico and the Caribbean were more likely to establish than agents collected elsewhere, as Mexico and the Caribbean are thought to be the origin and native range of the weedy form of *L. urticifolia* (Winder and Harley 1983; Palmer and Pullen 1995). Cases of local adaptations have been recorded in other plants (Boecklen and Mopper 1998; Hufbauer and Roderick 2005; Egan and Ott 2007), and understanding such processes is important to aid the success of biological control of hybrids.

Finding biological control agents that are adapted to wild type hybrids, or finding agents adapted to either or both hybrid parents, improves the chances of hybrid biological control success. Similarly, collecting each natural enemy from several sites and genotypes might be useful by providing a genetic stock that is capable of utilising a few different varieties of the target plant is and that is adapted to variable climate zones (Urban et al. 2011). In the case of *N. mexicana*, the genetic matching from Chapter 2 provides insight as to where to collect potential agents for importation into quarantine. Host specificity testing using both native plants at risk of non-target effects and non-native hybrids of *N. mexicana*

will enable us to determine the suitability of the agents identified in Chapter 3 and establish whether more surveys need to take place. Should more surveys be necessary, the parentage of the hybrids present in South Africa should be confirmed using molecular studies so that these parents can be targeted in their native range. Targeting natural hybrids in the wild for natural enemy surveys may also be useful. An alternative option is to grow hybrids from the invaded range in the native range of the parent plants, and record what insects colonise them *in situ* (Cilliers and Neser 1991). However, this method would need to be carried out with caution to prevent these hybrids from becoming invasive in the native range.

Hybrid plants may show varying levels of resistance to herbivory with hybrids either displaying increased, decreased, or unchanged resistance to herbivory compared to parent plants (Fritz et al. 1994, 1999; Whitham et al. 1994). Varying resistance to herbivory occurs as a result of changes in chemical defences (Fritz et al. 1999; Cheng et al. 2011), while variation in characters such as leaf pubescence, flowering times, plant size, and temperature affiliation will also impact relationships with natural enemies (Briese 1996b; O'Hanlon et al. 1999; Cortesero et al. 2000). Increased levels of resistance are likely to negatively affect biological control efforts, while reduced resistance will favour herbivory by biological control agents (Williams et al. 2014). Overcoming resistance to herbivory might require the integrated use of chemical and mechanical as well as biological control. Similarly, utilising multiple biological control agents that target different parts of the plants (leaves, flowers, stems, and roots, for example) could be a means of overwhelming defences and suppressing growth on multiple fronts (Denoth et al. 2002; Mewis et al. 2006; Huot et al. 2014). However, a better way of doing this might be to determine which feeding guilds exert better control, and to introduce only those agents that are likely to be met with less resistance (Denoth et al. 2002; Zalucki et al. 2007; Dhileepan et al. 2009). Herbivory simulation studies might be useful to determine which type of herbivory creates more impact on hybrids, although there is controversy about the applicability of such studies (Raghu and Dhileepan 2005; Hjältén 2008). Finally, possible consideration of the use of new association biological control may be another way to overcome resistance to herbivory by insects that share an evolutionary history with the plant (Hokkanen and Pimentel 1984, 1989; Waage and Greathead 1988).

A concern of biological control is the possibility of host shifting or host range expansion once biological control agents have been released, and introgressive hybridisation may provide a means for this to occur (Fritz et al. 1994; Marohasy 1996; Holt and Hochberg 1997). This could be particularly problematic in cases where exotic species hybridise with

native species, as the risk of introduced agents feeding on the native hybrid parent could increase with such introgression. Evidence suggests that introduced and native waterlilies do not hybridise in South Africa, so introgression is unlikely to negatively control efforts of *N. mexicana* hybrids in this regard. Nevertheless, of the numerous insect species introduced as biological control agents, relatively few cases document changes in host plant range, and these cases can be explained in terms of established and pre-adapted behaviours, effects of experience (learning), and threshold changes as a result of host deprivation (Marohasy 1996). Furthermore, cited examples of host-shifts in biological control were not found to result from genetic changes due to evolutionary adaptation of the released insects, and agent fundamental host range has not been found to change in the introduced range (van Klinken and Edwards 2002). Risk assessments and nontarget effects in biological control have been well studied (Pemberton 2000; Louda et al. 2003; Sheppard et al. 2003b; Suckling and Sforza 2014), so it is unlikely that nontarget effects will become problematic with hybrid plants, so long as strict protocols are followed.

In the case of *N. mexicana*, it may be necessary to conduct further genetic analyses to determine the parentage of the hybrids in South Africa, and more field surveys to identify and test agents with varying local adaptations and thus varying host specificity (see Chapter 2 and recommendations below). The extent of specificity required in biological control programmes is case dependent, as greater agent specificity would be required in scenarios where there are many non-target species closely related to the IAP in the invaded range, while less specificity may be acceptable in the opposing case. For example, there are no native Cactaceae in South Africa, so host specificity testing of biological control agents for this group may be considered less challenging compared to agents tested for invasive plants with many South African congeners (Paterson et al. 2011). In this case, it may be advantageous that there are only two native waterlilies in South Africa (*N. nouchali* and *N. lotus*), and few species closely related to *Nymphaea.*

Potentially elevated levels of invasiveness, increased resistance to herbivory, and challenges associated with finding biological control agents with appropriate specificity to safely manage invasive hybrids account for the difficulties faced by researchers in controlling hybrids. Alternative management strategies such as chemical control using herbicides may also face challenges. For example, increased herbicide resistance has been recorded in some hybrids (Jasieniuk et al. 1996; Snow et al. 1999; LaRue et al. 2013). Effective control of hybrids may be achieved by carefully considering the factors that affect their biological control, and also by utilising integrated and compatible management methods simultaneously.

Advances in modelling will enable researchers to predict probabilities of hybrid formation, rates of hybrid genotype spread, and impacts of hybridisation on population genetic structure in order to focus control efforts and reduce future problems (Hall et al. 2006; Hall and Ayres 2009).

## 4.6 IMPORTANCE OF ANTHROPOGENIC INFLUENCE ON INVASIONS

Human activity plays a major role in the spread of invasive species across the globe. While routes of invasion caused by human activity are highlighted as key factors in the spread of invasive species (Hulme 2009), it is becoming clear that anthropogenic influences also affect invasions in other ways. Humans induce changes that create highly favourable conditions for species to become invasive (van der Wal et al. 2008), and are also largely responsible for the dispersal of invasive species (Hulme 2009; Tabak et al. 2017) as well as increased invasibility of natural sites (Ervin et al. 2006). In addition, human introduction of hybrid plants into natural environments is hugely problematic, as the existence of horticultural varieties of plants makes biological control efforts very difficult (see previous sections of this Chapter). While hybridisation and subsequent evolution of invasiveness may occur under natural conditions, anthropogenic influences accelerate the process and encourage biotic homogenization by mediating gene flow between congeners (Olden et al. 2004; Schierenbeck and Ellstrand 2009).

Another way in which human activity contributes to the spread of invasive species is through climate change. Human mediated climate change may make previously unsuitable habitats appropriate for colonisation by invasive species, which may in turn accelerate hybridisation between invasive and native species (Ervin et al. 2006; Thuiller et al. 2008; Chunco 2014), while extreme weather and disturbance may favour the spread and establishment of propagules (Alston and Richardson 2006; Murphy and Metcalfe 2016). Disturbance may include habitat destruction and eutrophication, which can cause considerable problems with controlling aquatic species as the plants often grow so vigorously under high nutrient conditions that biological control may be less effective (Coetzee and Hill 2012). Similarly, elevated carbon dioxide levels could affect invasive plants and their biological control agents in various ways, including increased competitive success of invasive species (Manea and Leishman 2011), improved fitness through elevated plant growth and altered phenology, but also possible increases in agent abundance which may temper these effects (Reeves et al. 2015). Elevated carbon dioxide levels may also reduce plant defences against insects (Zavala et al. 2008), but these predicted effects will depend on interactions

between various factors of climate change including changes in temperatures, carbon dioxide, and precipitation (Mooney and Hobbs 2000).

The anthropogenic forces that influence invasive species have only briefly been discussed here, but what is important to note is that while biological control is the most environmentally friendly, economical way of managing invasive species to date, human activity remains a crucial driving factor for species invasions. Biological control research should continue, but it is also important to focus on eliminating the anthropogenic aspect through raising awareness of invasive species, monitoring trade routes, and implementing safety measures to reduce the spread of invasive species. This is especially important with the occurrence of new invasive species occupying niches that are freed with the effective control of other invasive species (Chapter 1, Hill & Coetzee 2017).

## 4.7 LIMITATIONS AND RECOMMENDATIONS

The use of ISSRs have multiple advantages (Sarwat 2012, Chapter 2) but may produce ambiguous fingerprints as a result of lower specificity to the genome, and may have poor reproducibility if low quality DNA is used (Sarwat 2012). Nevertheless, ISSRs were appropriate for the level of detail required in this study and have previously been successfully used to match invasive populations with samples from the native range (Paterson et al. 2009; Paterson and Zachariades 2013; Sutton et al. 2017). However, higher resolution may be obtained when using other molecular techniques, such as through the use of of plastid *trnL-trnF* regions and nuclear internal transcribed spacers (ITS), while repeating this study using other markers could provide more reliable confirmation (Woods et al. 2005a; Le Roux and Wieczorek 2009; Paterson et al. 2009).

The samples included in these analyses were limited, as they did not include samples from all sites of the invaded range, nor did they include samples from some parts of the native range of *N. mexicana.* There were also varying numbers of samples analysed from each site, so these may not be representative of the genetic diversity of each population. While samples were replicated and were collected from three different states in the US, genetic analysis of samples collected from Mexico might result in different genetic groupings. For example, some populations of *N. mexicana* might match more closely genetically to Mexican populations than to US populations, and this could have important implications for biological control efforts.

Because of the high genetic diversity in *N. mexicana* and the presence of hybrids in the invaded range, it would be worthwhile to conduct further studies to investigate the

parentage of these hybrids. This has been done to some extent using morphology (Naidu 2018) but confirmation may be necessary using plants from the native range of *N. mexicana*, especially in cases where hybridisation occurs in the wild (Borsch et al. 2014; Nierbauer et al. 2014). Pinpointing the parentage of the hybrids that are problematic in South Africa will allow us to target other *Nymphaea* species for potential biological control agents. Further studies should therefore include samples of possible parents of each of the hybrids found in South Africa, including but not limited to *Nymphaea tetragona* Georgi, *Nymphaea alba* L., and *N. odorata* as these species were deliberately used to create horticultural hybrids.

Field surveys in the native range can be expensive and logistically challenging, and this may affect the research quality that can be obtained, unless collaborative long-term studies are run by scientists who live in the native range of an invasive plant. The field surveys that took place in this study occurred over three months, and while useful information was obtained, there are a few ways in which the study could have been improved. Seventeen sites were sampled during late summer/early autumn. Repeated surveys of these sites during different seasons and during different times of day may have revealed the presence of other insect species and would have provided a better indication of damage levels exerted by each species on *N. mexicana*. The species accumulation curves generated in Chapter 3 suggested that between two and five species were unsampled, but a minimum of 20 sites are recommended for species accumulation curves (Gotelli and Colwell 2011). Indeed, there were many sites that were not sampled in the native range, including sites in Mexico which were not surveyed at all. While climate matching assists one to prioritise sites that are most likely to host suitable biological control agents, sampling over a range of habitat types under varying climatic conditions may still be worthwhile, especially with limitations of climate matching techniques (Goolsby et al. 2006b).

With regards to the prioritisation of the species listed in Chapter 3, while some host range data is known about *B. americanus,* very little is known about *M. toddi,* and some of the insects found on *N. mexicana* were not identified to species level. It is possible that some of these taxa (for example, the chironomids) may prove to be appropriate for consideration as biological control agents once they are properly identified and further studied. Furthermore, while measurements of plants were taken at each site, other parameters such as water quality and nutrient composition, soil type, and temperature, would have provided additional useful information (Goolsby et al. 2006b). It should also be noted that it is difficult to ascertain the probability of success for the biological control of *N. mexicana* as there are no other biological control programmes for this species in the world. The presence of the two

congeners, *N. nouchali* and *N. lotus*, presents risks in terms of the possible impacts that introduced biological control agents could have on these species. However, it is possible that these agents will display sufficient host specificity so as to not pose significant threats to native congeners. Furthermore, allowing *N. mexicana* to spread in the absence of biological control, and outcompete native vegetation may pose a greater risk to congeners and other native species than the release of biological control agents.

With this in mind, it is recommended that: 1) *B. americanus* and *M. toddi* are imported into quarantine for host specificity and biology studies; 2) the unidentified insects sampled during the surveys are identified and researched; and 3) impact studies of *B. americanus* and *M. toddi* are conducted should they prove to be sufficiently host-specific, prior to application for release. Following these recommendations will determine the suitability of the agents prioritised in this study, clarify whether they cause sufficient damage to control *N. mexicana* populations, and provide insight into the potential of the other taxa if the agents are inappropriate. Understanding the host specificity of *B. americanus* and *M. toddi* will enable researchers to make recommendations for the use of these species to control *N. mexicana* and its hybrids. The information obtained from Chapter 3 will be useful to compare conditions between sites, and to provide a baseline for possible future surveys. Continued collaboration with international colleagues will be important to improve the quality and ease of research needed to implement biological control of *N. mexicana.*

## 4.8 CONCLUSION

The results from this study contribute significantly to the development of a biological control programme for *N. mexicana* in South Africa. As *N. mexicana* is also invasive in other countries, this study provides a baseline for future management strategies across the world. The high genetic diversity and presence of *N. mexicana* hybrids highlight the importance of using genetic studies in combination with valuable information collected from the native range during field surveys. Consideration of the numerous factors that affect the efficiency of biological control during the early stages of programme development reduces costs and allows researchers to develop a holistic understanding of the processes that govern plant-insect interactions. In conclusion, while South African water systems and water bodies in other countries will benefit from biological control of *N. mexicana* once the programme is in place, there is still much work to be done, and this should be carried out carefully. Furthermore, it is important to tackle invasive alien plant invasions on multiple fronts, to

prevent future invasions by raising awareness, and to solve problems caused by anthropogenic influences.

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