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The noncoding trnH-psbA spacer, as an effective DNA barcode for aquatic freshwater plants, reveals prohibited invasive species in aquarium trade in South Africa

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ABSTRACT

The negative effects of invasive alien aquatic plants raise concern globally. These effects are predicted to increase in the future due to a rapid development in transport modes (air, terrestrial, and water) that facilitate connection between continents, difficulties inherent to identification of potentially problematic species, and for aquatic systems, insufficiently regulated aquarium and ornamental pond industries. In this study, we aimed to assist traditional species identification methods with genetic methods and provide ways of verifying the status of alien plants (invasive or non-invasive) sold in aquarium market in South Africa. To this end, the best DNA barcode for South Africa's freshwater plants was identified; a DNA barcode library was assembled and aquarium plants sold in Johannesburg (South Africa) were screened using this DNA barcode. We found that trnH-psbA was a reliable single DNA barcode for freshwater plant species in South Africa. We therefore assembled a trnH-psbA library on BOLD (Barcode Of Life Database) to assist in future identification of unknown or taxonomically doubtful freshwater alien plants in South Africa. Using this region to screen aquarium species, we found surprisingly that some prohibited aquatic invaders are already in circulation in the local aquarium trade, including *Hydrilla verticillata* (L.f.) Royle, *Egeria densa* Planch., *Myriophyllum spicatum* L., and *Echinodorus cordifolius* (L.) Griseb. This raises concern and calls for a strict regulation of species traded in aquarium industries to be implemented, as well as a need for public environmental education on the threat posed by invasive and potentially invasive plants for South Africa's natural aquatic ecosystems.

1. Introduction

The vulnerability of South Africa's unique flora to invasion of alien species is well established (Richardson and Van Wilgen, 2004; Coetzee et al., 2009; Government Gazette, 2014). The most recent list of invasive species in South Africa comprises 559 species or groups of species, including 379 terrestrial and freshwater plant species (Government Gazette, 2014). Because aquatic and semi-aquatic alien plants are more likely to become invasive in comparison to terrestrial alien plants (see Daehler, 1998; Andreu and Vilà, 2010), they deserve particular attention when defining control measures. In South Africa's freshwater systems, the most damaging invasive alien plants—generally known as the "bad five" (Henderson and Cilliers, 2002)—are of South American origin and includes water hyacinth (*Eichhornia crassipes* (Mart.) Solms), water lettuce (*Pistia stratiotes* L.), parrot's feather (*Myriophyllum aquaticum* (Vell.) Verdc.), Kariba weed (*Salvinia molesta* D.S. Mitch.), and red water fern (*Azolla filiculoides* Lam.) (Van Wilgen et al., 2001; Richardson and Van Wilgen, 2004). One impact of invasive alien plants is the loss of local species richness. In addition, the rapid spread of invasive aquatic species disrupts a number of important activities such as the navigation of boats, fishing, and recreational activities. Also, their rapid spread reduces water flow and causes damage to hydroelectric infrastructures (Henderson and Cilliers, 2002).

Current measures to control alien invasion are costly and generally unaffordable for most countries. For example, the South African government spends ~US$ 620 million annually to fight invasive species (De Lange and Van Wilgen, 2010). Of this expenditure, the management of water systems invaded by only *Azolla filiculoides* and *Eichhornia crassipes* using a physical removal approach costs the South African government ~US$ 58 million and US$ 78 000, respectively (Van Wilgen et al., 2001; Van Wyk and Van Wilgen, 2002). Unfortunately, the economic commitment of governments to preventive measures against invasive species is far below what is needed, and this is a general trend globally (Leung et al., 2002).

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Controlling alien species in South Africa is a difficult task for a number of reasons. Firstly, there is no efficient border control that prevents new introduction of problematic alien species into the country. Secondly, border officers generally do not have botanical training and cannot provide accurate and rapid species identification. Thirdly, the identification of alien plants can be problematic even for experts particularly if only sterile material is available (Henderson and Cilliers, 2002; Pyšek et al., 2013). Furthermore, unregulated aquarium and ornamental pond industries pose additional risks by facilitating the introduction of numerous alien aquatic species into a country (Strecker et al., 2011). Even in the developed world (e.g. southern Ontario in Canada), species in the aquarium trade are not well regulated (Funnell et al., 2009), raising the risk of aquarium industries becoming an uncontrolled pathway of introducing problematic aquatic plants into the country (Funnell et al., 2009; Strecker et al., 2011; Azan et al., 2015).

In a recent study, Pyšek et al. (2013) showed that accurate and rapid identification of alien taxa is critical for biosecurity strategies, legislation on invasive species, quarantine, weed surveillance and monitoring, as well as ecological studies of alien invasion. Consequently, they called for an integrative approach that combines classical alpha-taxonomy and modern genetic approaches to improve the identification speed and accuracy of alien species (Pyšek et al., 2013). From this perspective, several attempts have already confirmed the efficacy of genetic approaches, particularly the DNA barcoding approach, in improving species identification (e.g. Packer et al., 2009) or in accelerating the identification of invasive aquatic plants (e.g. Ghahramanzadeh et al., 2013). However, in South Africa, the applicability of DNA barcoding in assisting identification of alien aquatic plants is yet to be tested.

In this study, our objectives are to identify a reliable DNA barcode for alien plants of South Africa's freshwater systems, to establish a DNA barcode library for these plants, and to facilitate species identification and test the performance of the identified DNA barcode with species on sale in local aquaria.

2. Materials and methods

2.1. Taxon sampling

Twenty-one (21) species are currently recorded as invasive alien plants of freshwater systems in South Africa (Henderson and Cilliers, 2002). In this study, 19 species (~90%) representing 11 families were sampled. In addition, seven species (seven plant families) identified as native opportunistic aquatic plants that may become invasive in disturbed aquatic ecosystems (Henderson and Cilliers, 2002) were also included in our dataset. Furthermore, we included seven aquatic plants purchased from aquaria in Johannesburg, South Africa. Of these aquarium species, three are found among the 19 invasive species that we sampled from the field. In total, 30 aquatic plant species were analyzed in this study, and this comprises 19 alien plant species sampled from the field, seven native but potentially invasive (following Henderson and Cilliers, 2002), and seven aquarium species (three of which are already in the 19 species sampled from the field). These species were collected from several watercourses in five of the nine provinces of South Africa (Gauteng, Limpopo, Mpumalanga, KwaZulu Natal, and Eastern Cape). Voucher specimens for the taxa used in this study and GenBank/EBI accession numbers are listed in Supplementary Table S1. Images and other associated meta-data for all taxa included in the analyses are available on the BOLD systems v. 3 (http://www.boldsystems.org/). Species were identified by experts and using relevant literature on aquatic weeds in South Africa (Henderson and Cilliers, 2002; Gerber et al., 2004; Milton, 2004; Madeira et al., 2007; Coetzee et al., 2009; Henderson, 2009) including the Southern African Plant Invaders Atlas (SAPIA: www.arc.agric.za; http://www.invasives.org.za/).

2.2. DNA extraction

Total genomic DNA was isolated from 0.5 g of silica-dried leaves using the 2× CTAB (Cetyltrimethylammonium bromide) extraction methods of Doyle and Doyle (1987). Polyvinylpyrrolidone (2% PVP) was added to reduce the effects of high polysaccharide concentration in the samples. Isolated DNA was precipitated with 100% ethanol and stored at −20 °C for a minimum of 2 weeks (Fay et al., 1998). Purification of samples (DNA cleaning) was done using QIAquick silica columns (Oiagen Inc., Hilden, Germany) following the manufacture instructions.

2.3. DNA amplification

The amplification reactions (PCR) were performed using 1 μl of clean DNA template in 24 μl of reaction mixture, which included Ready Mix Master (Advanced Biotechnologies, Epson, Surrey, UK), bovine serum albumin (3.2% BSA), and 4.5% dimethyl sulfoxide (DMSO). DMSO was added only for the amplification of *matK* to improve PCR efficiency. To amplify *rbcLa* and trnH-psbA, the primer sets *rbcLa-F*: *rbcLa-R* and *trnH-F*: *psbA-R* (Sang et al., 1997) were used, respectively. Four different primer combinations were, however, used for *matK*, including 1R Kim-f - 3 F-Kim; 390 F -1326R (Cuénoud et al., 2002); matK_MALPR 1 - matK X F (Dunning and Savolainen, 2010); and 472 F -1248R (Mort et al., 2009). The following DNA amplification protocol was used for the *rbcLa* region: pre-melting at 94 °C for 60 s, denaturation at 94 °C for 30 s, annealing at 50 °C for 40 s, and extension at 72 °C for 40 s. For *matK*, the protocol is described as follows: pre-melting at 94 °C for 3 min, denaturation at 94 °C for 60 s, annealing at 52 °C for 60 s, and extension at 72 °C for 2.5 min. Finally, the amplification protocol of the trnH-psbA spacer comprises pre-melting at 94 °C for 60 s, denaturation at 94 °C for 60 s, annealing at 48 °C for 60 s, and extension at 72 °C for 60 s. The resulting PCR products were purified using QIAquick columns following the manufacturer's instructions.

2.4. DNA sequencing and alignment

Cycle sequencing was done on purified PCR products using Big Dye TM v.3.2 Terminator mix (Applied Biosystems, Inc., Warrington, Cheshire, UK) and the same primers used in PCR reactions. Cycle-sequenced products were purified with EtOH-NaCl and sequenced on an ABI 3130X1 Genetic Analyser. Complementary DNA strands were edited and assembled using Sequencer 3.1 (Gene Code, Ann Arbor, Michigan, USA). The *rbcLa* and *matK* sequences were aligned manually in PAUP* v.4.0b.10 (Swofford, 2002). The trnH-psbA sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE v. 3.8.31) (Edgar, 2004), followed by manual adjustments. Finally, all sequences generated were combined to form single-locus and combined-locus DNA matrices. These matrices represent a DNA database or library for invasive plant species of South Africa's freshwaters.

2.5. Statistical data analysis, species monophyly, and BLAST analysis

All statistical analyses were conducted using the R library Spider 1.1-1 (Brown et al., 2012). First, we tested for the best DNA barcode for all aquatic plants of South Africa's freshwater systems analyzed. The search of the best DNA barcode focused on core barcodes (i.e. *rbcLa* + *matK*), core + trnH-psbA, and trnH-psbA. Four criteria were used for this search: i) presence of a barcode gap i.e. presence of a statistically significant difference between intra- and interspecific genetic distances; ii) discriminatory power i.e. the proportion of successful species identification for each individual marker and combined markers; iii) PCR success rate; and iv) species monophyly examined based on the topology of species along a phylogenetic tree.