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Molecular identification of *Azolla* invasions in Africa: The *Azolla* specialist, *Stenopelmus rufinasus* proves to be an excellent taxonomist

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ABSTRACT

Biological control of *Azolla filiculoides* in South Africa with the *Azolla* specialist *Stenopelmus rufinasus* has been highly successful. However, field surveys showed that the agent utilized another *Azolla* species, thought to be the native *Azolla pinnata* subsp. *africana*, which contradicted host specificity trials. It is notoriously difficult to determine *Azolla* species based on morphology so genetic analyses were required to confirm the identity of the *Azolla* used by the agent. Extensive sampling was conducted and samples were sequenced at the *trnL-trnF* and *trnG-trnR* chloroplastic regions and the nuclear *ITS1* region. Current literature reported *A. filiculoides* as the only Section *Azolla* species in southern Africa but 24 samples were identified as *Azolla cristata*, an introduced species within Section *Azolla* that was not used during host specificity trials. *A. pinnata* subsp. *africana* was only located at one site in southern Africa, while the alien *A. pinnata* subsp. *asiatica* was located at three. What was thought to be *A. pinnata* subsp. *africana* was in fact *A. cristata*, a closer relative of *A. filiculoides* and a suitable host according to specificity trials. This study confirms that *S. rufinasus* is a proficient *Azolla* taxonomist but also supports the use of molecular techniques for resolving taxonomic conundrums.

1. Introduction

Azolla species, small aquatic ferns (family Azollaceae), live in symbiotic association with nitrogen fixing cyanobacteria (Papaefthimiou et al., 2008). The nitrogen-fixing capabilities of these symbionts have led to the broad introduction of *Azolla*, mainly *Azolla filiculoides* Lam. as a "green manure" for rice cultivation (Lumpkin and Plucknett, 1980; Peters and Meeks, 1989; Wagner, 1997), and as a source of protein in low-cost feeds for tilapia fish (Fiogbe et al., 2004). In the first half of the 1900s, *Azolla* spp. were introduced into parts of Europe and the United States under the theory that they would create a heavy water surface cover thereby suppressing mosquito larvae (Benedict, 1923; Massol, 1950; Cohn and Renlund, 1953). Subsequently, this group has become problematic, following escape from botanical gardens (Chevalier, 1926), as well as ornamental and aquarium plant dealers (Oosthuizen and Walters, 1961; Bodle, 2008). The ballast tanks of ships may have served as a source in Europe (Szczesniak et al., 2009; Hussner, 2010), as well as epizoochory on domesticated animals, for example, on cattle in New Guinea (Pagad, 2010). Following introduction, *Azolla* is readily transported locally by human and animal activities, with waterfowl frequently considered facilitators (Brochet et al., 2009).

A dense surface cover of *Azolla* spp. can reduce aquatic oxygen levels by inhibiting air/water diffusion and also reduce sub-surface light levels, which in turn may cause submerged macrophytes and algae to die (Janes et al., 1996). Additionally, *Azolla* mats can reduce submersed animal populations (Gratwicke and Marshall, 2001). Exotic *Azolla* populations, lacking natural enemies, have also out-competed native *Azolla* species. For example, *Azolla pinnata*, invasive in New Zealand, has mostly replaced the native *Azolla rubra* R. Br. over most of northern New Zealand (Owen, 1996). The most notorious member of the group, *A. filiculoides* is a damaging invasive alien in many parts of the world. It was introduced into northern Iran and parts of Africa, and South East Asia for use as a natural fertilizer for rice agriculture, and as an aquatic ornamental plant in many countries throughout the world (Lumpkin and Plucknett, 1980). Quick regeneration and rapid growth generated a broad distribution of dense surface mats impeding boating, fishing, and recreational activities (Hashemloian and Azimi, 2009). In South Africa, McConnachie et al. (2003) report substantial economic losses to farming and recreational uses caused by thick mats. In Ireland, thick mats also obstruct weirs, locks, and water intakes (Baars, 2008; Baars and Caffrey, 2010).

In South Africa, *A. filiculoides* has been successfully controlled by the biological control agent *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae) (McConnachie et al., 2004). The females of this host-specific weevil lay eggs in the tips of the fronds, the first instar larvae feed here and then migrate to the rhizomes where the majority of the damage to the plant is inflicted. Pupal chambers are constructed on

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the surface of the plant, in amongst the fronds (Hill, 1998). Following its release in South Africa in 1997, the weevil spread unaided throughout the country, and within five years, *A. filiculoides* was no longer considered a problem plant (McConnachie et al., 2004). The biological control program against *A. filiculoides* is regarded as one of the most successful biological control programs in South Africa and the species is now considered under complete control where it no longer poses a threat to aquatic ecosystems (Coetzee et al., 2011). However, it was observed that *S. rufinasus* persisted on an *Azolla* species occurring in north eastern South Africa, which looked different and was first considered to be *A. pinnata* subsp. *africana* (Hill et al., 2008). This non-target effect was unexpected because the original host specificity trials showed no utilization of *A. pinnata* subsp. *africana* (Hill, 1998), raising concerns about the level of host specificity of the agent, as well as the validity of the host specificity testing results. Clearly, proper identification of the host *Azolla* species is critical to biological control studies.

However, the identification of *Azolla* species is notoriously difficult and replete with historical, nomenclatural, and taxonomic issues and complications (Evrard and Van Hove, 2004). Reid et al. (2006) state that, "The morphological similarity of *Azolla* species, together with their diminutive stature, have led to a long history of mistaken identifications, some of which have added to the taxonomic confusion." The best identifications require the identification of reproductive features such as the glochidia from the microspore and the perine structure of the megaspore (Perkins et al., 1985). Unfortunately, reproductive structures are seldom available at the time when identifications are needed. Some literature attempts to address identification using vegetative features (*Azolla* species in Pereira et al. (2011) and Madeira et al. (2013); *A. pinnata* subspecies in Saunders and Fowler (1992) and Madeira et al. (2013)), however these criteria alone often seem insufficient for confidence in identification (Madeira et al., 2013). Fortunately, in recent years, a number of authors have published molecular taxonomies for *Azolla* species which have helped to clarify the taxonomy, as well as providing molecular barcodes for the identification of field samples (Reid et al., 2006; Metzgar et al., 2007; Madeira et al., 2013).

The aim of this paper was to complete a thorough molecular analysis of *Azolla* in southern Africa in order to understand which native and alien species are present, their distributions in the region, and to understand the patterns of utilization of *S. rufinasus* in the field. This knowledge is essential in order to develop control or conservation strategies for either alien or native species.

2. Materials and methods

2.1. Plant material, DNA extraction, amplification and sequencing of PCR products

This study analyzed 52 samples of the genus *Azolla* collected from Ghana (2 samples), Mozambique (4 samples), South Africa (39 samples), Zambia (2 samples), Republic of Congo (1 sample), Cameroon (2 samples), Uganda (1 sample) and Zimbabwe (1 sample). Samples collected in the field were placed directly on silica gel. Up to 20 mg of dried sample was extracted for DNA using the DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA, USA).

Two plastid amplifications, *trnL-trnF* and *trnG-trnR*, were attempted for all samples. *TrnL-trnF*, including the *trnL* intron and the *trnL-F* intergenic spacer, used the universal primers "*TrnLC*" (CGA AAT CGG TAG ACG CTA CG) and "*TrnLF*" (ATT TGA ACT GGT GAC ACG AG) of Taberlet et al. (1991). For some samples that did not successfully amplify using the *trnLC and trnLF* primers, the internal primers "*trnLD*" (GGG GAT AGA GGG ACT TGA A) and "*trnLE*" (GGT TCA AGT CCC TCT ATA CC) were used for amplification of the regions separately (Taberlet et al., 1991). The Nagalingum et al. (2007) primers "*TrnG*1F" (GCG GGT ATA GTT TAG TGG TAA) and "*TrnR*22R" (CTA TCC ATT AGA CGA TGG ACG) were used to amplify the *trnG-trnR region*. The nuclear *ITS1* sequence (Blattner, 1999) was obtained for a subset of the samples using primers "*ITS*-A" (GGA AGG AGA AGT CGT AAC AAG G) and "*ITS*-B" (CTT TTC CTC CGC TTA TTG ATA TG). We used annealing temperatures of 56 °C for *trnL-trnF*, 52 °C for *trnG-trnR* and 58 °C for *ITS1*. The plastid reaction mixtures contained 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.5 mM Betaine, 0.001% BSA, 0.2 mM dNTPs, 0.5 μM each primer, and 0.06 U/μl EconoTaq polymerase (Lucigen Corp., Middleton, WI, USA). The *ITS1* reaction utilized 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10% DMSO, 0.2 mM dNTPs, 0.5 μM each primer, and 0.04 U/μl EconoTaq polymerase.

PCR products were visualized in 1.5% agarose gels stained with ethidium bromide. PCR products were excised and cleaned using DNA Clean & Concentrator (Zymo Research, Orange, CA, USA). Sequencing external primers were the same as for the PCR. Internal primers included for *trnL-trnF* were (Taberlet et al., 1991) — "*TrnLD*" and "*TrnLE*" (primer sequences shown above), for *trnG-trnR* (Korall et al., 2007; Nagalingum et al., 2007) — "*TrnG*43F1" (GCC GGA ATC GAA CCC GCA TCA) and "*TrnG*63R" (TTG CTT MTA YGA CTC GGT G). Cycle sequencing was performed at either the University of Florida DNA Sequencing Core Lab (Gainesville, FL, USA), by Eurofins MWG Operon (Huntsville, AL, USA) or Stellenbosch University (Stellenbosch, South Africa) using BigDye™ terminator technology (Life Technologies Corp., Carlsbad, CA, USA).

2.2. NCBI search, alignment parameters, gap coding, and phylogenetic analysis

The identities of the samples were determined using molecular taxonomy. Reference sequences were obtained from the NCBI "Taxonomy" window and originated from three taxonomic studies of *Azolla* by Reid et al. (2006), Metzgar et al. (2007) and Madeira et al. (2013). SEQUENCER 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA) was used to view and compile trace files. The gap opening (GO) and gap extension (GE) costs were varied in CLUSTAL W (Thompson et al., 1994) from GO = 4, GE = 2 to GO = 16, GE = 4. Final parameters chosen by looking for stable alignments/alignment lengths were: for *trnL-trnF* (GO = 10, GE = 3), for *trnG-trnR* (GO = 10, GE = 4), and for *ITS1* (GO = 9, GE = 3).

The species identity of unknown samples was investigated using the Maximum Likelihood routine in MEGA5.2 (Tamura et al., 2011). The *trnL-trnF* and *trnG-trnR* and *ITS1* sequences were analyzed independently using partial deletion, "extensive" (SPR level 5) Subtree-Pruning–Regrafting and a "very weak" Branch Swap Filter. Partial deletion was chosen to better show small differences between accessions hidden by complete deletion and produced alignments of 732 bp for *trnL-trnF*, 849 bp for *trnG-trnR* and 653 bp for *ITS1*. Identical sequences were represented as a single sequence unless their inclusion as separate sequences was informative, for example, because they represented a sample with the same sequence as a reference sequence, or, in the case of given *A. microphylla* and *A. mexicana* identities, the sequences were identical. The optimum Maximum Likelihood model for each analysis was chosen from 24 different nucleotide substitution models using BIC criteria. Models chosen were Tamura 3-parameter plus Gamma (T92 + I) for *trnL-trnF*, Tamura 3-parameter plus Invariant (T92 + I) for *trnG-trnR* and Kimura 2-parameter plus Invariant (K2 + I) for *ITS1*. Branch reliability was tested using bootstrap analysis (1000 replicates). Branches within the phylogenies produced were collapsed where possible using the subtree collapse command in MEGA Tree Explorer.

Once the identities of the samples were determined, their distribution was mapped by importing geographic coordinates acquired at each *Azolla* collection site into ArcMap™ 9.3 (ESRI 2008, Redlands, CA). Layers were constructed containing sample sites for each *Azolla* species, and these layers were overlain on layers comprising geographical feature data (country borders, rivers, lakes, etc.), symbols and topographical relief maps contained in the ArcGIS® 9 media kit for Africa (Fig. 2).