South African Journal of Botany 105 (2016) 299–305

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

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

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ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form 18 February 2016
Accepted 11 March 2016
Available online 14 May 2016

Edited by J van Staden

Keywords:
Azolla
Biological control
Exotic
Invasive
Molecular taxonomy

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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http://dx.doi.org/10.1016/j.sajb.2016.03.007
0254-6299/

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).

3. Results

Sample collection information, sample identification numbers, and NCBI accession numbers are presented in Table 1. Fig. 1 displays the sample distribution in southern Africa while the inset displays samples from the broader continent. Sample identities resulting from the Maximum Likelihood analyses are presented in Table 1 with the Maximum Likelihood phylogenies presented in Fig. 2A for *ITS1*, Fig. 2B for

Table 1
The *Azolla* samples used in this study, including the collection, identification and GenBank accession information.

Species symbol	ID #	Species	NCBI accessions			Sample location	Province/State	Country[a]	Latitude	Longitude	Collector	Date collected
			trnCF	trnGR	ITS1							
▲	1	*A. cristata*[b]	HQ909788	JN590175		Save R.	Gaza	Mz	−21.544477	32.954966	S. Langa	Oct-09
▲	2	*A. cristata*[b]	HQ909789	JN590176		Incomati R.	Maputo	Mz	−25.405333	32.809149	S. Langa	Oct-09
▲	3	*A. cristata*[b]	HQ909790	JN590177		Umbeluzi R.	Maputo	Mz	−26.054702	32.327687	S. Langa	Oct-09
▲	4	*A. cristata*[b]	HQ909791	JN590178	JX297309	Limpopo R.	Gaza	Mz	−24.410276	32.877856	S. Langa	Oct-09
▲	5	*A. cristata*[b]	HQ909792	JN590179		White R.	Mpumalanga	SA	−25.317583	31.061916	D. Strydom	Oct-09
▲	6	*A. cristata*[b]	HQ909793	JN590180	JX297310	Primkop Dam	Mpumalanga	SA	−25.384733	31.072500	D. Strydom	Oct-09
▲	7	*A. cristata*[b]	HQ909794	JN590181		Crocodile R.	Mpumalanga	SA	−25.452650	31.057083	D. Strydom	Oct-09
▲	8	*A. cristata*[b]	HQ909795	JN590182		Tekwane	Mpumalanga	SA	−25.465566	31.156350	D. Strydom	Oct-09
▲	9	*A. cristata*[b]	HQ909796	JN590183		Tekwane	Mpumalanga	SA	−25.465567	31.156350	J. Coetzee	Mar-08
▲	10	*A. cristata*[b]	HQ909797	JN590184	JX297311	Karino R.	Mpumalanga	SA	−25.472600	31.096800	D. Strydom	Oct-09
▲	11	*A. cristata*[b]	HQ909798	JN590185	JX297312	Crocodile R.	Mpumalanga	SA	−25.524050	31.330266	D. Strydom	Oct-09
▲	12	*A. cristata*[b]	HQ909799	JN590186	JX297313	Komati R.	Mpumalanga	SA	−25.610200	31.861817	D. Strydom	Oct-09
▲	13	*A. cristata*[b]		JN590187		Nsikazi R.	Mpumalanga	SA	−25.310200	31.258470	D. Strydom	Oct-09
▲	14	*A. cristata*[b]	HQ909800	JN590188		Nsikazi R.	Mpumalanga	SA	−25.308770	31.258050	D. Strydom	Oct-09
▲	15	*A. cristata*[b]		JN590189		Skukuza	Mpumalanga	SA	−24.993300	31.588833	D. Strydom	Nov-09
▲	16	*A. cristata*[b]		JN590190		Great Letaba R.	Limpopo	SA	−23.661120	30.681470	M. Hill	Jan-10
▲	17	*A. cristata*[b]	HQ909801	JN590191		Crocodile R.	Mpumalanga	SA	−25.384340	31.881230	J. Coetzee	Jan-10
▲	18	*A. cristata*[b]	HQ909802	JN590192		Hluhluwe	KwaZulu Natal	SA	−27.736690	32.455300	J. Coetzee	Jan-10
▲	19	*A. cristata*[b]		JN590193		Nahoon R.	Eastern Cape	SA	−32.973920	27.925700	M. Hill	Jan-10
▲	20	*A. cristata*		JN590194		KwaJobe Dam	KwaZulu Natal	SA	−27.915000	32.493620	J. Coetzee	Jan-10
▲	21	*A. cristata*[b]	HQ909803	JN590195		Zambezi R.	Mashonaland	Zw	−16.566950	28.956390	P. Weyl	Jan-10
■	22	*A. pinnata asiatica*	HQ909784	JN590196		Tinley Manor,	KwaZulu Natal	SA	−29.445357	31.240755	J. Coetzee	May-08
■	23	*A. pinnata asiatica*	HQ909785	JN590197		Ashburton	KwaZulu Natal	SA	−29.796690	30.514720	M. Hill	Jan-10
■	43	*A. filiculoides*	JX273522	JX280884		Vals R.	Free State	SA	−27.406666	26.388888	C. Fordham	Oct-10
●	44	*A. filiculoides*	JX273523	JX280885		Westminster	Free State	SA	−29.215480	27.215890	M. Hill	Jan-11
●	45	*A. filiculoides*	JX273524			Century City	Western Cape	SA	−33.888360	18.513530	J. Coetzee	Jan-11
▲	46	*A. cristata*[b]		JX280881		Mtunzini	KwaZulu Natal	SA	−28.969810	31.754951	J. Coetzee	Feb-11
●	47	*A. filiculoides*		JX280886		Bethlehem	Free State	SA	−27.914855	28.526610	J. Coetzee	Apr-10
●	48	*A. filiculoides*	JX273525		JX297314	Misverstand	Western Cape	SA	−33.025000	18.789430	J. Kirsten	Nov-10
▲	49	*A. cristata*[b]	JX273519	JX280882		Mposa R.	KwaZulu Natal	SA	−28.685856	32.019203	J. Coetzee	Feb-11
▲	50	*A. cristata*[b]	JX273520	JX280883		Mposa R.	KwaZulu Natal	SA	−28.690660	32.014527	J. Coetzee	Feb-11
●	51	*A. filiculoides*	JX273526		JX297315	Swartkops R.	Eastern Cape	SA	−33.790000	25.430000	M. Hill	Aug-10
●	52	*A. filiculoides*	JX273527	JX280887		Harrismith	Free State	SA	−28.282030	29.114530	M. Hill	May-11
●	53	*A. filiculoides*	JX273528			Stockdale	Eastern Cape	SA	−32.401990	25.305390	J. Coetzee	Jan-11
●	54	*A. filiculoides*	JX273529	JX280888	JX297316	Heilbron Dam	Free State	SA	−27.277750	27.961460	M. Hill	May-11
●	55	*A. filiculoides*	JX273530	JX280889		Mocke R.	Western Cape	SA	−34.066140	18.474640	J. Coetzee	Feb-11
■	56	*A. pinnata asiatica*	JX273516	JX280877		Brettenwood	KwaZulu Natal	SA	−29.486550	31.245433	J. Coetzee	Feb-11
●	57	*A. filiculoides*	JX273531	JX280890		Jagersfontein	Free State	SA	−29.806360	25.495360	J. Coetzee	Feb-11
●	58	*A. filiculoides*	JX273532	JX280891		Zeekoevlei	Western Cape	SA	−34.034200	18.524720	J. Coetzee	Feb-11
●	59	*A. filiculoides*		JX280892		Petrus Steyn	Free State	SA	−27.575910	28.123920	M. Hill	Apr-10
●	60	*A. filiculoides*	JX273533	JX280893		Sandvlei	Western Cape	SA	−34.087150	18.461130	J. Coetzee	Feb-11
●	61	*A. filiculoides*	JX273534	JX280894		Stockdale	Eastern Cape	SA	−32.398420	25.301950	J. Coetzee	Jan-11
●	62	*A. filiculoides*	JX273535	JX280895	JX297317	Zuurfoutein	Western Cape	SA	−31.704080	24.690760	J. Coetzee	Jan-11
●	63	*A. filiculoides*	JX273536	JX280896	JX297318	Swartviei	Western Cape	SA	−33.993639	22.699895	J. Coetzee	Sep-10
▲	64	*A. cristata*[b]	JX273517	JX280879	JX297307	Tano Lagoon	Western	Gh	5.088687	−2.898490	F. Akpabey	Mar-11
▲	65	*A. cristata*[b]	JX273518	JX280880	JX297308	Accra	Accra	Gh	5.595996	−0.187586	F. Akpabey	Mar-11
□	66	*A. pinnata africana*	JX273515	JX280876	JX297305	L. Bengwelu	Luapula	Za	−11.083740	29.862767	C.Huchzermeyer	Apr-11
□	67	*A. pinnata africana*	KP308215		KP318121	Fiko Village	Cameroon	Cm	4.293180	9.715420	P. Weyl	Jun-14
□	68	*A. pinnata africana*	KP308216		KP318122	Cattle Village	Cameroon	Cm	4.101990	9.615810	P. Weyl	Jun-14
□	69	*A. pinnata africana*	KP308214		KP318120	Kouilou R.	Congo	Cg	−4.411390	11.786670	M. Hill, I.Paterson	Sep-12
▲	70	*A. cristata*[b]	KP308217		KP318123	*L. Victoria*	Uganda	Ug	0.055280	32.480830	I. Paterson	Jan-14
□	71	*A. pinnata africana*	KP308213		KP318119	Bengwelu Swamps	Luapula	Za	−11.968060	30.253610	C.Huchzermeyer	Apr-11

[a] Country: Cameroon (Cm), Congo (Cg), Ghana (Gh), Mozambique (Mz), South Africa (SA), Uganda (Ug), Zambia (Za), Zimbabwe (Zw).
[b] *A. cristata* is synonymous with *A. mexicana* and *A. microphylla*.

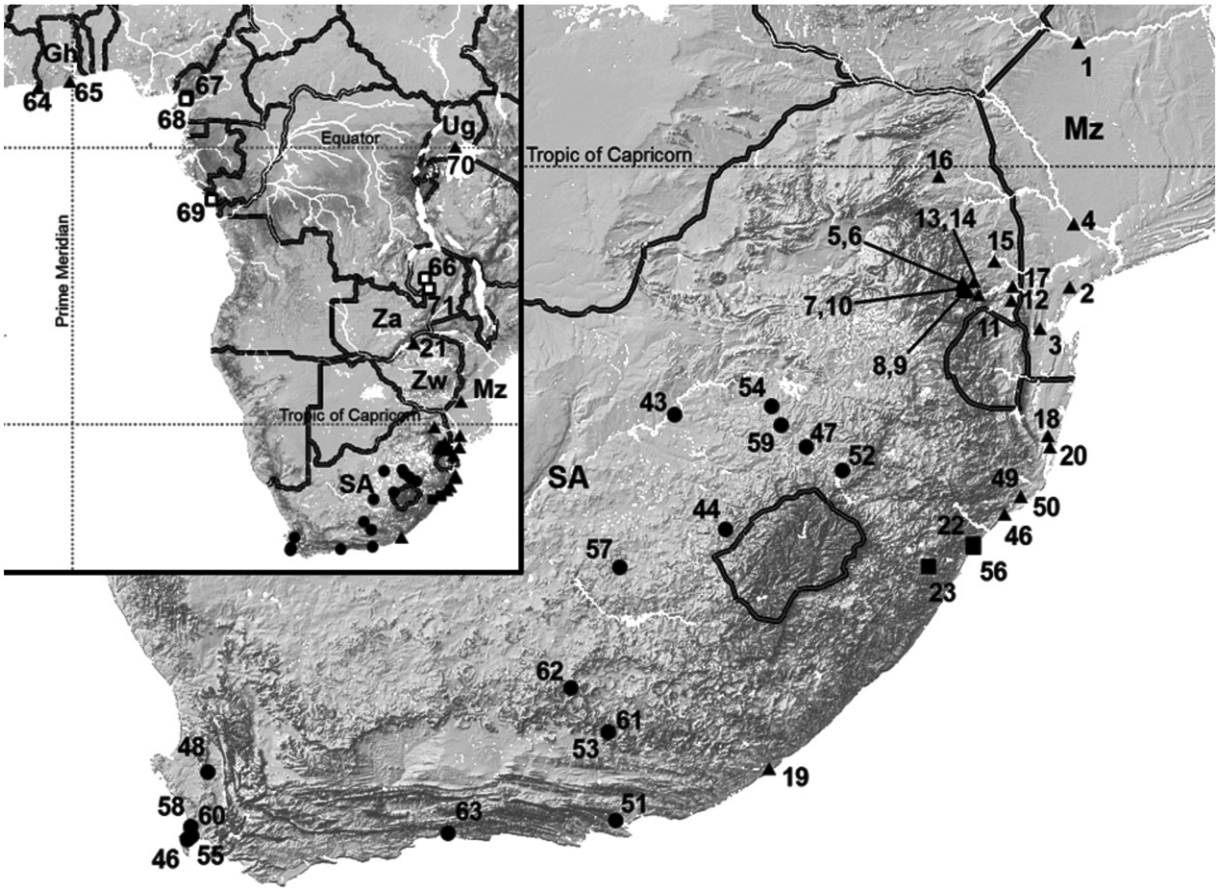

Fig. 1. Distribution of *Azolla* samples collected in South Africa and Mozambique (Inset displays sample locations in rest of Africa). Sample numbers and species symbols may be cross referenced with Table 1 and with analysis in Fig. 2A–C. Note the widespread distribution of *Azolla filiculoides* in South Africa and the presence in NE South Africa, Mozambique, Zimbabwe, Uganda and Ghana of *Azolla cristata*. Additionally, the native *Azolla pinnata africana* was located only in Zambia, Cameroon and Congo, suggesting it may be displaced by invasive *Azollas*. ▲ *Azolla cristata* (*A. mexicana* or *A. microphylla*). ■ *Azolla pinnata* subsp. *Pinnata*. ● *Azolla filiculoides*. □ *Azolla pinnata* subsp. *Africana*.

trnL-trnF, and Fig. 2C for *trnG-trnR*. Samples are identified by sample numbers from 1 to 65. Sample ID numbers not included in Fig. 1 (24–25, 33–42) were part of a previous study (Madeira et al., 2013). OTUs used for taxonomic identification are indicated in the phylogenies by their species name and NCBI accession number(s). Samples of the same species are represented by identical symbols in Table 1 and the maps (Fig. 1). Samples represented in the phylogenies (Fig. 2A–C) may be cross-referenced by their sample numbers to both Table 1 and the maps (Fig. 1). Note that bootstrap values were greater than 80% for all species groupings except for *Azolla microphylla* Auct. non Kaulf. and *Azolla mexicana* Presl., which previous molecular taxonomic studies (Reid et al., 2006, Metzgar et al., 2007) have indicated are actually conspecific. Evrard and Van Hove (2004) also present detailed evidence from microscopy of numerous cultures and specimens that *A. microphylla* and *A. mexicana* are the same species, which by precedent they name *Azolla cristata* Kaulf. In deference to this, and for the sake of brevity, we will refer to this clade (*A. microphylla* and *A. mexicana*) as *A. cristata* in figures and tables. Bootstrap values for the *A. cristata* clade were at 99% or higher in all three analyses (Fig. 2A–C).

In 2008, two samples were collected while surveying in South Africa for *A. pinnata* subsp. *africana*, Sample ID #22, from Tinley Manor Estate in KwaZulu Natal, which morphologically appeared to be *A. pinnata*. When sequenced, this sample was identified by NCBI sequences as the alien subspecies *A. pinnata* subsp. *asiatica*. Additional samples were again collected in 2009/10 and again produced no *A. pinnata* subsp. *africana* specimens. Sample ID #23 from Peach's Farm, Ashburton, not far from the Tinley Estate, was also identified as *A. pinnata* subsp. *asiatica*. The survey also located one additional site (ID #56) with *A. pinnata* subsp. *asiatica*. Twenty-one samples (ID #1–8, 10–21) from north eastern South Africa, Mozambique, and Zimbabwe were identified as *A. cristata*, a western hemisphere species that has been introduced to the region. The long described presence of *A. filiculoides* in the interior of South Africa in the Orange River Catchment, Free State Province, Western Cape Province and Eastern Cape Province was confirmed by the presence of 16 sites (ID #43–45, 47–48, 51–55, 57–63) in the interior where samples were identified as *A. filiculoides*. Samples from two sites in Ghana were also *A. cristata* (ID #64–65) though different in their *trnL-F* and *ITS1* sequences from the southern Africa samples. A reference sample of *A. pinnata* subsp. *africana* was also finally located in Zambia during the 2011 samplings. This sample (ID #66) appears in the molecular taxonomy of Madeira et al. (2013) and is represented here in both Table 1 and in the phylogenies of Fig. 2 as NCBI accessions (JX273515, JX280876, JX297305). Further *A. pinnata* subsp. *Africana* specimens were located in the Republic of Congo and two sites in Cameroon.

4. Discussion

In a pre-introductory survey, prior to the classical biological control program on *A. filiculoides* in South Africa, Hill (1998) reported the presence of *A. pinnata* at three localities in KwaZulu-Natal Province, South Africa. Teixeira et al. (2000) reported that samples from Hammersdale Dam, KwaZulu-Natal were *A. pinnata* subsp. *asiatica*. McConnachie and Hill (2005) also report that samples sent to Generosa Teixeira (University of Lisbon) were identified as *A. pinnata* subsp. *asiatica*. Therefore it is not surprising to find three of the specimens (ID #22–23, 56) from KwaZulu-Natal were confirmed by molecular analysis as the alien *A. pinnata* subsp. *Asiatica*.

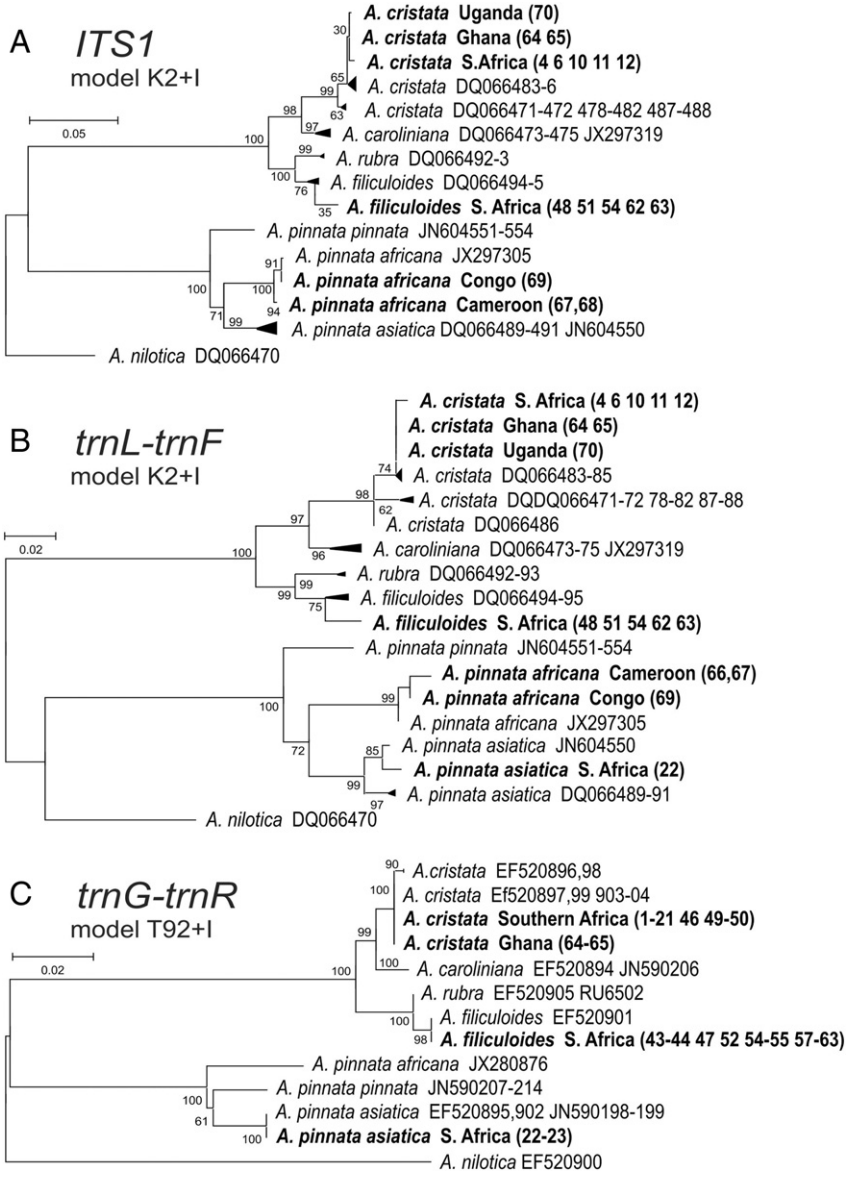

Fig. 2. Maximum Likelihood Tree using nuclear *ITS1* (A), chloroplast *trnL-F* (B) and chloroplast *trnG-trnR* (C) to identify study samples using the NCBI database. Sample numbers and species symbols may be cross referenced with Table 1 and with mapped locations in Fig. 1. Reference sequences are identified by accession number. Maximum Likelihood analysis used partial deletion, "extensive" (SPR level 5) Subtree-Pruning–Regrafting and a "Very Weak" Branch Swap Filter. The nuclear *ITS1* (A) analysis utilized the Kimura 2-parameter plus Invariant (K2 + I) model, the chloroplast *trnL-F* (B) analysis the Tamura 3-parameter plus Invariant (T92 + I), and the chloroplast *trnG-trnR* (C) also the T92 + I model. Tree branches were collapsed to reduce figure sizes (wherever possible without the loss of critical information) by using the subtree collapse command in MEGA Tree Explorer. Branch reliability was tested using bootstrap analysis (1000 replicates) and is shown as a confidence percentage at the nodes.

A large number of samples (ID #1–21, 46, 49–50) from north eastern South Africa, Mozambique and Zimbabwe are identified here as another alien species, *A. cristata*. In addition to the early confusion of these plants as *A. pinnata africana*, in 2007, the Southern African Plant Invaders Atlas mapping project (SAPIA) (Henderson, 2007) reported that it was *A. filiculoides* that was widely dispersed in the KwaZulu Natal, Limpopo, and Mpumalanga provinces of South Africa, as well as in Mozambique and Zimbabwe.

A. cristata is a closer relative to *A. filiculoides* (both Section *Azolla*) than it is to *A. pinnata* (section *Rhizosperma*). Host specificity testing suggested that feeding by *S. rufinasus* on close relatives of *A. filiculoides*, such as *A. cristata*, was to be expected but that damage was likely to be limited (Hill, 1998). The effectiveness of *S. rufinasus* on *A. cristata* should however be further examined in the laboratory and field as *A. cristata* is taxonomically more closely related to *Azolla caroliniana* auct. non Willd., the host plant from which *S. rufinasus* was introduced, than *A. filiculoides*. However, the picture is complicated because *S. rufinasus* is indigenous to both southern and western United States of America (LeConte, 1876). It occurs on *A. caroliniana* in the southern U.S.A. and on *A. filiculoides* in the western U.S.A. (Richerson and Grigarick, 1967). *A. filiculoides* status as an alternate host may confer feeding advantages not present with *A. cristata* despite its close taxonomic relationship to *A. caroliniana*.

No native section *Azolla* species exist in southern Africa (Lumpkin and Plucknett, 1980) so it is unlikely that natural enemies from African *Azolla* species will provide any level of control. It may be necessary to search for *A. cristata* biological control agents within its native range to find an agent which can achieve similar control to that demonstrated by *S. rufinasus* on *A. filiculoides*. In contrast to *A. cristata*, *A. pinnata* subsp. *asiatica* may share some natural enemies with the

native *A. pinnata* subsp. *africana* but will probably be a less suitable host because it is part of a clade of more distantly related congeners (Hill, 1998; Madeira et al., 2013).

The most likely explanation for the current distribution of alien *Azolla* species in southern Africa is that the initial invasion (*A. filiculoides*), reported in the Northern Cape region, slowly spread within the Orange River watershed, which empties westward towards the Atlantic Ocean, while secondary introduction(s), comprising *A. pinnata* subsp. *asiatica* and *A cristata* later occupied rivers emptying eastwards into the Indian Ocean. *A. cristata* populations constituted a separate introduction but the samples were originally mistakenly classified as *A. pinnata* subsp. *africana* (Hill, 1998) then later as *A. cristata* (Madeira et al., 2013) under the assumption that there had been only one introduction into southern Africa. The distribution of *A. filiculoides* is in the higher lying and cooler areas of the country, whereas *A. cristata* and *A. pinnata* subsp. *asiatica* are found in the lower lying, coastal warmer regions extending northward towards more tropical climatic regions. This distribution is likely influenced by the thermal tolerances of the species. Uheda et al. (1999) studied the differential tolerance of six *Azolla* species to transient exposure from high-temperature stress (>40 °C) and concluded the order was: *A. pinnata* > *A. microphylla*, *A. mexicana* > *A. caroliniana*, *A. filiculoides* > *A. rubra*. Talley et al. (1977) report that *A. filiculoides* can tolerate temperatures as low as −5 °C without apparent harm but is less tolerant than *A. mexicana* (*A. cristata*) to high temperatures. Watanabe and Berja (1983) report that *A. filiculoides* requires lower temperatures than other species for its optimum growth.

A. cristata was also sampled in Ghana and Uganda. The two samples from Uganda, identical in haplotype, were found at sites over 300 km apart, inferring a widespread distribution. Asuming-Brempong and Watanabe (1989) report the performance testing of *A. microphylla* as a bio-fertilizer at a University of Ghana Agricultural Research Station in Kpong, Ghana, potentially the source of the introduction. Additionally, Fiogbe et al. (2004) report the introduction of *A. microphylla* as a source of protein in low-cost feeds for tilapia at a research project at Porto-Novo in nearby Benin. We hypothesize that in Africa, *A. cristata*, in the absence of natural enemies has been an excellent competitor in the most tropical regions and has most likely resulted in the exclusion of the native *A. pinnata* subsp. *Africana* with the exception of localities such as the Banguelu Swamps, Zambia and Republic of Congo and Cameroon.

Incorrect identifications and taxonomic confusions can complicate biological control programs and are often only resolved when molecular techniques are utilized (Gaskin et al., 2011). The biological control agent for *A. filiculoides* was reported to be feeding on *A. pinnata* subsp. *Africana* (Hill et al., 2008), a species which should not be a suitable host according to the results of host specificity testing (Hill, 1998). The plants used in the host specificity trials were collected in Zambia on the Kafue River and we are thus confident that *A. pinnata* subsp. *africana* was tested. This study has confirmed that this unpredicted non-target effect reported from the field in the eastern parts of South Africa was in fact an incorrect identification, wherein *A. cristata* was mistaken for *A. pinnata* subsp. *africana*. Additionally, the biological control agent *S. rufinasus* is an excellent taxonomist. It performs best on its native host *A. filiculoides* (and presumably *A. caroliniana*), will accept only the closest relatives (section *Azolla*, *A. cristata*), and will not develop on section *Rhizosperma* (the *A. pinnata* subspecies).

Azolla taxonomists generally consider an SEM of the megaspore surface (preferably with a cross-section) important for accurate determination of species, therefore clarity in *Azolla* taxonomy and identification will only be completely resolved by combining the two tools (SEM and DNA sequencing) in an analysis of the same material, whether cultures or herbarium specimens. Since *Azolla* species only infrequently display reproductive material for morphological analysis, such a correlation of barcodes and morphology would also strengthen the identities of samples identified by molecular barcoding, as in this study.

In conclusion, this study has shown how useful genetic barcoding can be for the identification of *Azolla* species and the importance of correct identifications for the control of alien species, especially when biological control is being used. *A. cristata* was recorded as fairly widespread in southern Africa and the presence of another alien species, *A. pinnata* subsp. *asiatica* has been confirmed. A study to examine the extent of *A. cristata* and *A. pinnata* subsp. *astiatica* infestations in southern Africa, as well as the negative impacts of these species should be conducted and a management plan should be developed. The impact of *S. rufinasus* on *A. cristata* should also be examined. The study also confirms the integrity of the host specificity testing of *S. rufinasus* and the specificity of the biological control agent to section *Azolla* of the genus *Azolla* (Hill, 1998; Madeira et al., 2013).

Acknowledgements

We thank everyone who collected samples (see names in Table 1). Thanks to David Serrano, director of Biological Science Interns at Broward College for his support in providing excellent interns who provided laboratory help. Thanks to the technicians and interns who helped with preparing samples and vouchers, DNA extraction, PCRs and other laboratory work: Sam Camirand, David Fox, Christian Henry, Ligia Jurado, and Elizabeth Mattison. The Department of Environmental Affairs (OR-006146), Natural Resources Management Programs, Working for Water Program of South Africa is acknowledged for financial support to this project.

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