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Identity and origins of introduced and native *Azolla* species in Florida

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ABSTRACT

Azolla pinnata, an introduced aquatic fern, is spreading rapidly causing concern that it may displace native *Azolla*. It is now present in the Arthur R. Marshall Loxahatchee National Wildlife Refuge, the northernmost portion of the Florida Everglades. Because *A. pinnata* subspecies are native to Africa, Southeast Asia, and Australia, determining the actual geographic origin of the Florida exotic is important to the discovery of efficacious biological control agents. Both the exotic and native *Azollas* were examined using morphological and molecular criteria. Both criteria distinguished three *A. pinnata* subspecies with the Florida exotic matching the Australian *A. pinnata* subsp. *pinnata*. Molecular divergence between the *A. pinnata* subspecies indicates the three types should be considered separate species. The Florida native was characterized by both molecular and morphological methods as *Azolla caroliniana*. The discovery of a previously uncharacterized Ecuadorian *Azolla*, which appears to be a paternal ancestor of *A. caroliniana*, indicates that *A. caroliniana* is a hybrid species.

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1. Introduction

The floating aquatic fern *Azolla pinnata* R. Br. (Azollaceae), a listed Federal Noxious Weed (U.S.A.), was discovered in Palm Beach County, Florida during 2007 (Bodle, 2008). The South Florida Water Management District immediately but unsuccessfully sought eradication by treatment with diquat dibromide. The District's concern stemmed from the invasive potential demonstrated by various species of *Azolla* worldwide. Quick regeneration, rapid growth, broad distribution, and dense surface mats of *Azolla* can obstruct weirs, locks, and water intakes impeding irrigation, boating, fishing, and recreational activities (McConnachie et al., 2003; Hashemloian and Azimi, 2009; Baars and Caffrey, 2010). A dense surface cover may also reduce aquatic oxygen levels (Janes et al., 1996) and submersed animal populations (Gratwicke and Marshall, 2001). There is also concern that if *A. pinnata* becomes invasive, it will compete for the same niche and suppress native populations of *Azolla* in Florida. This concern seems warranted inasmuch as introduced *A. pinnata* has largely replaced the native *Azolla rubra* R. Br. in northern New Zealand (Owen, 1996).

Florida's native *Azolla* has historically been considered to be *Azolla caroliniana* Willd. (Godfrey et al., 1961; Wunderlin and Hansen, 2003). However there is a paucity of information as to how this identification was determined. Evrard and Van Hove (2004) have noted that the taxonomy of New World *Azolla* species has been "the subject of much debate and remains unsatisfactory". The status of *A. caroliniana* is perhaps the most debatable within the genus because Willdenow (1810) described the holotype *A. caroliniana* ("habitat in aquis Carolinae") from a sterile specimen. This has led to various interpretations including that the original specimen was actually *Azolla filiculoides* Lamarck (Dunham and Fowler, 1987; Evrard and Van Hove, 2004). Regardless of what the holotype represents, an "*A. caroliniana*" species with a unique megaspore perine structure exists (Perkins et al., 1985; Pereira et al., 2001).

The broad geographic range of *A. pinnata* encompasses Africa, Madagascar, India, China, southeast Asia, Japan, Malaysia and Australia. Saunders and Fowler (1992), in a morphological taxonomic revision of *Azolla* Lam. section *Rhizosperma*, identified three geographically distinct subspecies of *A. pinnata*: (1) *A. pinnata* R.Br. subsp. *pinnata* R.Br. from Australia and New Caledonia; (2) *A. pinnata* R.Br. subsp. *asiatica* Saund. & Fowl. from India, southern China, Southeast Asia, and southern Japan; and (3) *A. pinnata* subsp. *africana* (Desv.) Saund. & Fowl., from tropical Africa and Madagascar. However, Pereira et al. (2011), using morphological

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and molecular (RAPD) criteria, suggested that *A. pinnata* is actually best described as two varieties, *A. pinnata* R.Br. var. *imbricata* (Roxb.) Bonap. from Asia and *A. pinnata* var. *pinnata* R.Br. from Africa and Australia.

Classical biological control is often used to address the complex and multi-faceted problems of exotic alien plant invasions by introducing specialized natural enemies derived from native regions. This process usually begins with determinations of the identity of the plant and its native range followed by surveys for potential agents, an expensive and often difficult process. Because exotic invasive species frequently have large native ranges (Lodge, 1993) the actual geographic origin may not be apparent. Biological control agents that have coevolved with the invasive host plant are most likely to be successful in suppressing it (Hufbauer and Roderick, 2005; Ward, 2006; Mills and Kean, 2010). When regional intra-specific genetic variation is ignored, both within the invasive plant and co-evolved natural enemies, it has the potential to confound the efficacy of the insects chosen (Goolsby et al., 2006; Le Roux and Wieczorek, 2009) resulting in failure to control the target weed (Palmer and Witt, 2006). Weed biological control programs are increasingly using molecular techniques to determine the origins of the targeted plants and to geographically match biotypes of prospective agents with the target weed (Von Senger et al., 2000; Bond et al., 2002; Goolsby et al., 2006; Paterson et al., 2009).

Identification of the subspecies (or variety, *implied hereafter*) of *A. pinnata* recently found in Florida (Pemberton and Bodle, 2009) would enable a search for biocontrol agents in the appropriate geographic area. The primary goal of our study, therefore, was to identify the alien *A. pinnata* subspecies using molecular and morphological methods. The secondary goal was to clarify the taxonomic status of a Florida native *Azolla* species using similar methods.

2. Materials and methods

2.1. Samples and reference sequences

We analyzed 14 samples of the genus *Azolla* collected from Australia, Ecuador, Thailand, United States of America, and Zambia. Field samples were preserved on silica gel. Sample collection information and National Center for Biotechnology Information (NCBI) accession numbers are presented in Table 1. NCBI samples and sequences from Reid et al. (2006) and Metzgar et al. (2007) are also presented. All reference samples except *A. filiculoides* come from the highly examined International Rice Research Institute (IRRI) reference collections (Watanabe et al., 1992).

2.2. Morphological identification of Azolla specimens

Dried voucher *Azolla* specimens were hydrated overnight in warm soapy water (2 drops dish soap in 40 mL DI water) and examined morphologically. Both fresh and dried samples of Florida *A. pinnata* were examined. Evaluation of *A. pinnata* subspecies utilized the vegetative characters of Pereira et al. (2011) and Saunders and Fowler (1992). Observations were made through a MZ1b binocular stereomicroscope and a DM LB light microscope (Leica Microsystems®, Germany). Characters distinguishing *A. pinnata* subspecies include unicellular/bicellular rhizome papillae and dorsal lobe shape (Pereira et al., 2011), also the ventral lobe ratio of length to width and the dorsal lobe ratio of hyaline margin to leaf width (Saunders and Fowler, 1992). Ten measurements were taken for each ratio type/specimen type. A one-way ANOVA (Systat, 2011) was conducted for each ratio along with a pairwise multiple comparison using the Holm–Sidak Method, a method more powerful than but as conservative as the Tukey Test.

Fresh material of the Florida native sample was also examined using the vegetative criteria in Pereira et al. (2011). Additionally a VHX-600 3-dimensional digital microscope (Keyence Corporation, USA) was used to examine several microsporocarps and a single megasporocarp. Massulae were isolated from microspores and their glochidia (barbed hairs) examined. A single megaspore was teased from its megasporocarp wall and examined.

2.3. Molecular methods

DNA was extracted using the DNeasy Plant Mini kit (Qiagen Inc., Valencia, CA, USA). Two plastid genes, *trnL-trnF* (includes the *trnL* intron and the *trnL-F* intergenic spacer) and *trnG-trnR* (includes *trnG* and the *trnG-R* intergenic spacer), as well as the nuclear *ITS1* sequence were amplified and sequenced. Primers used for amplification and sequencing were *TrnL*C, *TrnL*D, *TrnL*E and *TrnL*F for *trnL-trnF* (Taberlet et al., 1991) and *TrnG*1F, *TrnG*43F1, *TrnG*63R, and *TrnR*22R for *trnG-trnR* (Nagalingum et al., 2007). The nuclear *ITS1* primers were the *ITS*-A, *ITS*-B, *ITS*-C and *ITS*-D primers of Blattner (1999). PCR annealing temperatures were 56 °C for *trnL-trnF*, 52 °C for *trnG-trnR* and 58 °C for *ITS1*. Plastid reaction mixtures contained 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 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 contained 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. Amplifications were carried out on a PTC-100 thermocycler (MJ Research, Inc.) PCR products were visualized using 1.5% agarose gels stained with ethidium bromide. PCR products were cleaned using DNA Clean & Concentrator (Zymo Research, Orange, CA, USA). Cycle sequencing was performed at the University of Florida DNA Sequencing Core Lab (Gainesville, FL, USA) using BigDye™ terminator (Life Technologies Corp., Carlsbad, CA, USA), with analysis on an Applied Biosystems 3730 Genetic Analyzer (Life Technologies Corp., Carlsbad, CA, USA).

2.4. Molecular data analysis

Trace files were compiled and viewed using SEQUENCER 4.1.4 (Gene Codes Corporation, Ann Arbor, MI, USA). Reference sequences were downloaded from the NCBI "Taxonomy" window and originated from the two taxonomic studies of *Azolla* by Reid et al. (2006) and Metzgar et al. (2007). CLUSTAL W (Thompson et al., 1994) was chosen for alignment. Various gap opening (GO) and gap extension (GE) costs were examined from GO = 4, GE = 2 to GO = 16, GE = 4, noting alignment length changes and looking for stable alignments over local changes of the parameters. Parameters chosen were: for *trnL-trnF* (GO = 10, GE = 3), for *trnG-trnR* (GO = 10, GE = 4), and for *ITS1* (GO = 9, GE = 3). A partition homogeneity test using the PAUP "homopart" command to compare the *trnL* intron, *trnL-trnF* spacer, *trnG*, and the *trnG-trnR* spacer. It showed no significant difference so the plastid sequences were analyzed together. The *ITS1*, *5.8S*, and partial *ITS2* nuclear data was analyzed separately. Neither analysis was significant. The "best fit" model parameters determined using corrected Akaike weighting (AICc) from jMODELTEST 0.1.1 (Posada, 2008) was TVM + G for the plastid dataset and GTR + G for the nuclear dataset. "Lset" was used in PAUP to generate a maximum likelihood (ML) tree using tree-bisection-reconnection "Hsearch start = NJ nchuck = 2 chuckscore = 5 dstatus = none", otherwise defaults were used. Maximum likelihood pairwise distances were generated with the "dset distance = ml" command. Internal branch reliability was assessed with 1000 bootstraps.

Table 1
Azolla sample, collection, identification and GenBank information.

Sample IDs	Species	NCBI accessions			Sample location	Province/State	Nation[a]	Latitude/longitude
		trnCF	trnGR	ITS1				
38	*A. pinnata pinnata*	HQ909779	JN590211	JN604555	Private pond where first seen	Palm Beach	USA	26.897, −80.141
39	*A. pinnata pinnata*	HQ909780	JN590212		Canal, nr. Loxahatchee Slough	Palm Beach	USA	26.895, −80.144
40	*A. pinnata pinnata*	HQ909781	JN590213		IPRL culture, Davie	Broward	USA	26.085, −80.240
41	*A. pinnata pinnata*	HQ909782	JN590214	JN604556	Pond, Griffin Rd & Turnpike	Broward	USA	26.064, −80.216
42	*A. pinnata pinnata*	HQ909783	JN590215	JN604557	Pond, Donald Ross Rd., WPB	Palm Beach	USA	26.883, −80.130
34	*A. pinnata pinnata*	HQ909775	JN590207	JN604551	Culture, ABCL, Indooropilly	Queensland	Au	−27.512, 152.996
35	*A. pinnata pinnata*	HQ909776	JN590208	JN604552	Culture, ABCL, Indooropilly	Queensland	Au	−27.513, 152.997
36	*A. pinnata pinnata*	HQ909777	JN590209	JN604553	Wappa Dam, Sunshine Coast	Queensland	Au	−26.577, 152.923
37	*A. pinnata pinnata*	HQ909778	JN590210	JN604554	Wappa Dam, Sunshine Coast	Queensland	Au	−26.577, 152.924
24	*A. pinnata asiatica*	HQ909786	JN590198		Queen Siriki Bot. Gdn.	Chiang Mai	Th	18.888, 98.862
25	*A. pinnata asiatica*	HQ909787	JN590199	JN604550	Queen Siriki Bot. Gdn.	Chiang Mai	Th	18.888, 98.862
66	*A. pinnata africana*	JX273515	JX280876	JX297305	Lake Bangweulu	Luapula	Za	−11.084, 29.863
67	*A. species*	JX273521	JX280878	JX297306	L. Mica, Antisana Ecological Res.	Pichincha	Ec	−0.547, −78.210
33	*A. caroliniana*	HQ909770	JN590206	JX297319	Florida Aquatic Nurseries, Davie	Florida	USA	26.111, −80.313
NCBI[b]								
PI0022[c]	*A. pinnata asiatica*	EF520895	EF520883	DQ066489	Bai Kai-Ze, Tancheng District	Shandong	Ch	
NI5001[c]	*A. nilotica*	EF520888	EF520900	DQ066507		Kosti	Su	
CA3001[c]	*A. caroliniana*	EF520882	EF520894	DQ066475		Ohio	USA	
ME2001[c]	*A. mexicana*	EF520886	EF520898	DQ066477	Graylodge State Area, Butte Co.	California	USA	
MI4075[c]	*A. microphylla*	EF520891	EF520903	DQ066486	Logo Canada, Nuevo Italia	Central	Pa	
2112[d]	*A. filiculoides*	EF520889	EF520901		Rolândia	Paraná	Br	
68[e]	*A. filiculoides*			DQ066494			Br	

[a] Nation: Australia (Au), Brazil (Br), China (Ch), Ecuador (Ec), Paraguay (Pa), Sudan (Su), Thailand (Th), United States (USA), Zambia (Za).
[b] Reference sequences: trnCF/trnGR, Metzgar et al. (2007); ITS1, Reid et al. (2006).
[c] International Rice Research Institute (IRRI), Manila.
[d] Fern DNA database number at http://www.Pryerlab.net/DNA_database.shtml.
[e] Reid vouchers at Virginia Commonwealth University.

3. Results

3.1. Morphological examination of Azolla pinnata subspecies

All twelve samples of *A. pinnata* met the following criteria of Pereira et al. (2011) for section *Rhizosperma*: deltoid sporophyte (Fig. 1A), sub-round dorsal lobe apex (Fig. 1A–C) and an acute angle of the dorsal lobe. Criteria pointing to *A. pinnata* included a sub-pinnate alternate deltoid branching pattern (Fig. 1A) and the absence of stomata on the ventral lobe. Samples from Asia (Thailand, sample ID 24–25; *sample ID henceforth implied by number in parenthesis unless otherwise indicated*), Australia (34–37), and Florida (38–42, Fig. 1C) all had reasonably symmetrical dorsal lobe hyaline borders with mostly 2–3 layers of cells and also had pubescent rhizomes. In contrast, the African *A. pinnata* sample (66) had asymmetrical hyaline borders of mostly 3–5 layers of cells (Fig. 1B) and mostly glabrous rhizomes.

Pereira et al. (2011) lists two vegetative characters for distinguishing the two varieties of *A. pinnata* they recognize. *Azolla pinnata* var. *imbricata* (Asian) has an obovate dorsal lobe shape and bicellular rhizome papillae while *A. pinnata* var. *pinnata* (Australian and African) has an elliptical dorsal lobe shape and unicellular rhizome papillae. Our Asian *A. pinnata* sample shows an obovate dorsal lobe shape with at least some bicellular rhizome papillae indicating it is *A. pinnata* var. *imbricata*. We display images showing unicellular rhizome papillae (Fig. 1D and E) for both the African and the Florida *A. pinnata*. African, Australian and Florida *A. pinnata* also show an elliptical dorsal lobe shape with Florida *A. pinnata* dorsal lobes tending toward a rectangular or trapezoidal form. Following Pereira et al. (2011) these criteria point to an identity of *A. pinnata* var. *pinnata* (encompassing both *A. pinnata* subsp. *pinnata* and *A. pinnata* subsp. *africana*) for Florida *A. pinnata*.

ANOVA results ($F = 13.7$; $P < 0.001$) for 10 measurements of the ratio of the ventral lobe length to width for each subspecies produced ratios (mean, standard error) of *A. pinnata* subsp. *asiatica* (1.21, 0.031), *A. pinnata* subsp. *pinnata* (1.53, 0.061), Florida *A. pinnata* (1.59, 0.067) and *A. pinnata* subsp. *africana* (1.71, 0.063). Thailand *A. pinnata* subsp. *asiatica* proved significantly different from Florida *A. pinnata* ($P < 0.001$), Australian *A. pinnata* subsp. *pinnata* ($P = 0.002$) and Zambian *A. pinnata* subsp. *africana* ($P < 0.001$) in the pairwise Holm–Sidak test (overall $P = 0.05$). There was no significant difference between the Florida *A. pinnata*, *A. pinnata* subsp. *pinnata*, and *A. pinnata* subsp. *africana*. Previous studies (Saunders and Fowler, 1992) produced similar ratios (*A. pinnata* subsp. *asiatica*, ratio = 1.2; *A. pinnata* subsp. *pinnata* and *A. pinnata* subsp. *africana*, ratio = 1.7.

ANOVA results ($F = 7.96$; $P = 0.002$) for the ratio of the width of the dorsal leaf to the dorsal leaf hyaline margin resulted in ratios (mean, standard error) for *A. pinnata* subsp. *pinnata* (10.0, 0.25) and *A. pinnata* subsp. *africana* (7.6, 0.41). Ratios for Florida *A. pinnata* (9.2, 0.60) were significantly different in the Holm Sidak test (overall $P = 0.05$) from those for *A. pinnata* subsp. *africana* ($P = 0.002$) but not from *A. pinnata* subsp. *pinnata* ($P = 0.224$). Saunders and Fowler (1992) reported a ratio of 9.1 for *A. pinnata* subsp. *pinnata* and 6.2 for *A. pinnata* subsp. *africana*. The results of both ANOVAs are consistent with those reported previously and indicate the Florida introduction is *A. pinnata* subsp. *pinnata*.

3.2. Morphological characterization of a Florida native Azolla

Both the Florida native sample (33) and the Ecuadorian sample (67) (Table 1) were placed in section *Azolla* using the following vegetative criteria of Pereira et al. (2011). They display polygonal sporophytes, glabrous rhizomes, round dorsal lobe apexes, an obtuse angle of the dorsal lobes, symmetrical hyaline borders, and 2–6 layers of cells on the hyaline border [Florida *Azolla* (33) – mostly 2 cell layers, Ecuadorian *Azolla* (67) – mostly 6 cell layers].

The Florida native *Azolla* sample (33) displays some bicellular papillae (Fig. 1F), as does the Ecuadorian *Azolla* (67), indicating an identity as *Azolla microphylla* Kaulf, *Azolla mexicana* Presl. or *A. caroliniana* according to one vegetative criterion of Pereira et al. (2011). Microscopy of reproductive features on Florida's native *Azolla* using the Keyence dimensional digital microscope show pluriseptate glochidia (Fig. 1G). They also show, using a low gamma setting,

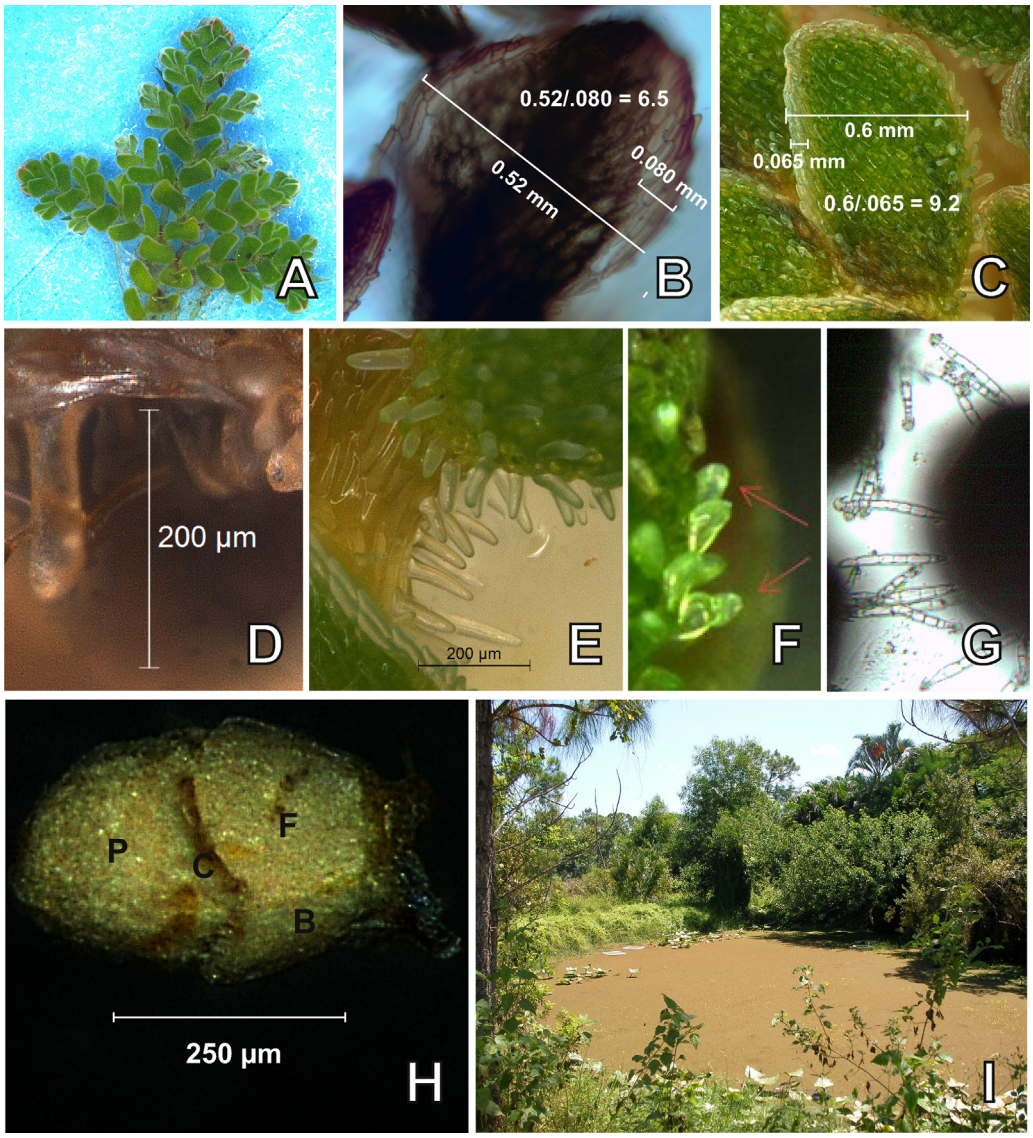

Fig. 1. (A) Sub-pinnate "Christmas tree" branching pattern of Florida exotic *A. pinnata* subsp. *pinnata*. (B and C) Ratio of leaf width to hyaline layer width on dorsal lobe for (B) *A. pinnata* subsp. *africana* and for (C) Florida *A. pinnata*. (D and E) Unicellular rhizome papillae (trichomes) for (D) *A. pinnata* subsp. *africana* and for (E) Florida *A. pinnata*. (F–H) Native Florida *A. caroliniana* displays (F) bicellular dorsal lobe papillae, (G) pluriseptate glochidia and a (G) megaspore showing dense pilosum on the perine {P} extending densely between{B}the floats {F}. (H) A small pond in Palm Beach County in Florida with surface completely covered by exotic *A. pinnata* subsp. *pinnata*.

a perine surface of the megaspore covered by dense filosum with filosum also covering the collar and extending densely between the floats (Fig. 1H).

3.3. Molecular taxonomic results

The sequences of *A. pinnata* African and Australian samples can now be added to a scaffold of the existing molecular taxonomies for *Azolla* section *Rhizosperma* (Reid et al., 2006; Metzgar et al., 2007). Maximum Likelihood (ML) analyses are presented for the chloroplast data in Fig. 2A and for the nuclear data in Fig. 2B using midpoint rooting. Each analysis resulted in a single most likely tree. There is strong bootstrap support for the positions of all taxa in the trees with the exception of the African sample (*A. pinnata* subsp. *africana*), with 81% of chloroplast bootstrap trees placing it outside of the Australian (*A. pinnata* subsp. *pinnata*) and Asian (*A. pinnata* subsp. *asiatica*) samples, but only 48% support for that position in the nuclear sequences.

Chloroplast and nuclear sequences from five exotic Florida samples (38–42) were identical to the four *A. pinnata* subsp. *pinnata* samples from Australia (34–37) (Fig. 2). They differ from the Asian *A. pinnata* subsp. *asiatica* (24–25, PI0022) samples with chloroplast ML distances averaging 0.042 and nuclear distances averaging 0.069. They also differ from the African *A. pinnata* subsp. *africana* sample (66) with chloroplast/nuclear distances of 0.058/0.078. The African to Asian *A. pinnata* distances average 0.051/0.073. Contrast these distances between *A. pinnata* sub-species with several section *Azolla* "between species" distances. The *A. filiculoides*/*A. rubra* chloroplast/nuclear distance is 0.007/0.028 and the average for *A. caroliniana*/*Azolla cristata* Kaulf. is 0.015/0.078 (*A. cristata* lumps *A. microphylla* and *A. mexicana*, see discussion). Representative longer distances within section *Azolla* include *A. rubra*/*A. cristata* (average) = 0.044/0.094 and *A. filiculoides*/*A. cristata* (average) = 0.045/0.084.

The chloroplast sequence for the Florida native *Azolla* (33) is identical to that provided for *A. caroliniana* by Metzgar et al. (2007).

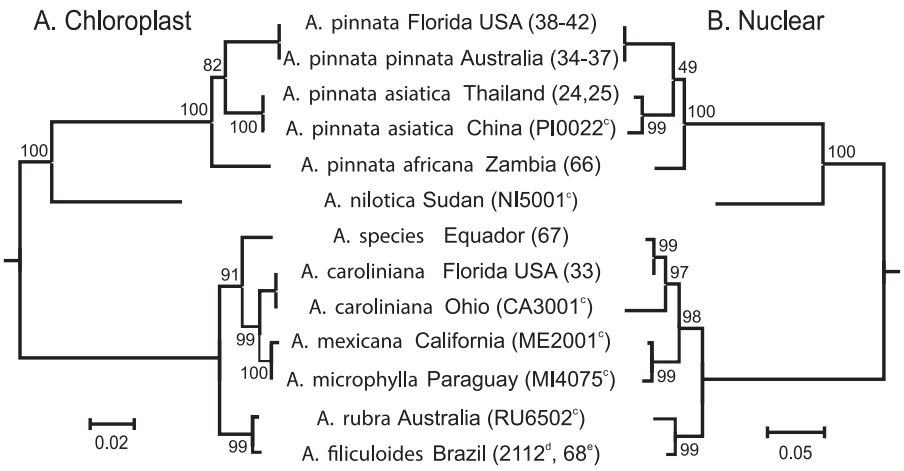

Fig. 2. Maximum likelihood analysis and bootstrap for Chloroplast (A) and Nuclear (B) sequences. Chloroplast sequences include the *trnL-trnF* (*trnL* intron and *trnL-F* intergenic spacer) and *trnG-trnR* (*trnG* and *trnG-R* intergenic spacer) sequences. Nuclear sequences include the *ITS1* (*ITS1*, *5.8S* ribosomal, partial *ITS2*) sequence. Bootstrap values (1000×) occur in the nodes. Study samples and referenced NCBI sequences are assembled to integrate *A. pinnata* subsp. *pinnata* and *A. pinnata* subsp. *africana* into existing *Azolla* phylogenies. Sample ID numbers and reference sample IRRI IDs (except for *A. filiculoides*) may be cross referenced with Table 1.

Our nuclear sequence is closest in identity to the *A. caroliniana* sequence presented by Reid et al. (2006) but differs by a nuclear distance of 0.046. The chloroplast sequence for the Ecuadorian *Azolla* (67) is unique from any reported previously, at a distance of approximately 0.03 from both *A. caroliniana* and *A. cristata* reference sequences. By contrast, the nuclear sequence for the Ecuadorian *Azolla* (67) is closest to the Florida *Azolla* sample (33) at a distance of 0.007 and next closest to *A. caroliniana* (CA3001) at a distance of 0.054.

4. Discussion

4.1. Effectiveness of vegetative morphology for Azolla species identification

The vegetative criteria of Pereira et al. (2011) proved useful in placing samples in either section *Rhizosperma* or section *Azolla*. Within section *Rhizosperma* they also helped identify *A. pinnata* (versus *Azolla nilotica* Decne. ex Mett.). Within *A. pinnata*, the criteria of bicellular vs. unicellular rhizome papillae and obovate vs. elliptical dorsal lobe shape were useful for separating Asian from Australian and African *A. pinnata*. The vegetative ratios (ventral lobe length/width; dorsal lobe width/hyaline layer) presented by Saunders and Fowler (1992) also proved useful, distinguishing between Australian and African *A. pinnata*. Morphological features therefore concurred with the three subspecies described by Saunders and Fowler (1992).

Pereira et al. (2011) proposed two subclusters within section *Azolla*, IIa (*A. filiculoides*, *A. rubra*, *A. microphylla* Kaulf.) and IIb. (*A mexicana* Presl., *A. caroliniana*). They based these clusters on four criteria, each of which has exceptions and/or variable characters. In contrast, *A. microphylla* and *A. mexicana* have been considered by molecular (Reid et al., 2006; Metzgar et al., 2007) and morphological (Evrard and Van Hove, 2004) evidence as a single species. If *A. microphylla* and *A. mexicana* are considered a single species, then the criterion (Pereira et al., 2011) of unicellular dorsal lobe papillae (*A. filiculoides*, *A. rubra*) versus bicellular dorsal lobe papillae (*A. microphylla*, *A. mexicana*, *A. caroliniana*) is useful in the vegetative identification of samples into the two major section *Azolla* sub-clades found in molecular taxonomy (Fig. 2).

4.2. Utility of sexual morphology for the identification of a Florida native Azolla

Light microscopy of the Florida native (33) shows dense filosum hiding the perine surface (Fig. 1H) in a manner which resemble the Scanning Electron Micrograph (SEM) of Perkins et al. (1985) for *A. caroliniana*. Pereira et al. (2001) also displays *A. caroliniana* SEMs and describes them as having "a regular perine surface, unpitted and densely covered with filaments making it almost impossible to distinguish other structures". Curiously, Dunham and Fowler (1987) display an SEM of a specimen they designate as "*Azolla* species", which displays dense filosum, but choose not to identify it as *A. caroliniana* since they believe the type specimen was actually *A. filiculoides*. Having identified our perine specimen (Fig. 1H) as *A. caroliniana* several cautions are in order. While heavy filosum appear on the perine surface and between the floats, final confirmation of the perine structure classically requires an SEM of both the surface and of the stratification of the perine, neither of which is available. Also, the small perine size in contrast to float size and the presence of filosum on the floats indicate the specimen may be immature (with unknown implications for identification).

Microscopy (Fig. 1G) also clearly shows pluriseptate glochidia. Both Godfrey (1961) and Pereira et al. (2001) report that *A. caroliniana* displays both unseptate and septate glochidia, and that this criterion is too variable to be useful. Dunham and Fowler (1987) report, for their "*Azolla* species" with heavy perine filosum, that the number of glochidia range from zero to eight, resembling the higher septation numbers seen in the Florida native *Azolla* (Fig. 1G). Given the bicellular dorsal lobe papillae and pluriseptate glochidia in our specimen we agree with Evrard and Van Hove (2004) that *A. caroliniana* auct. non Willd. should be the preferred nomenclature. However, we do not indicate synonymy with *A. cristata* (Evrard and Van Hove, 2004) given the different and unique molecular sequences of *A. caroliniana*, both here and in previous molecular taxonomies (Reid et al., 2006; Metzgar et al., 2007).

4.3. Reflections on Azolla molecular taxonomy

Saunders' and Fowler's (1992) revision of section *Rhizosperma* grouped African, Asian and Australasian samples into the subspecies utilized here. Pereira et al. (2011), using RAPD markers and morphological characteristics, interpreted their results as showing

two varieties, *A. pinnata* var. *pinnata* and *A. pinnata* var. *imbricata*. Note, however, their branch lengths (Jaccard Similarity, RAPDs) connecting the African and Australian *A. pinnata* var. *pinnata* showed less than 50% similarity. Additionally, no bootstrap or jackknife confidence values were published. In contrast, our sequence data shows maximum likelihood genetic distances between the three *A. pinnata* subspecies greater than those of some "between species" distances in section *Azolla* and only somewhat less than the larger "between species" distances within that section. Clearly the African, Asian and Australian samples represent, at a minimum, three separate subspecies. While no molecular clock was enforced, we would argue that the distance data, along with the geographical isolation of the subspecies, suggest they would better be considered as separate species.

Both Reid et al. (2006) and Metzgar et al. (2007) consider *A. mexicana* and *A. microphylla* to represent a single species, providing combined sequence data from one nuclear and three chloroplast sequences. When their sequencing data is viewed holistically, the evidence that *A. microphylla* and *A. mexicana* are paraphyletic, not separate evolutionary lineages, is strong. Evrard and Van Hove (2004) also concluded that *A. microphylla* and *A. mexicana* are conspecific based on morphology. However, Pereira et al. (2011) interpret their RAPD phenetic data as supporting separate phylogenetic clades. As with their case for *A. pinnata*, many of the samples cluster in the phenetic analysis at less than 50% similarity and again with no confidence values. Additionally, RAPD markers, while useful for many applications, are less than ideal for assessing interspecies phylogenetic relationships (Smith et al., 1994; van de Zande and Bijlsma, 1995; Dowling et al., 1996), especially when between species distances increase, similarity decreases and the homology of bands decline. Generally the use of Southern analysis to confirm the homology of RAPD bands is recommended for interspecific cladistic claims in RAPD analyses (Smith et al., 1994; van de Zande and Bijlsma, 1995).

4.4. Molecular evidence for a new Azolla species and implications for A. caroliniana taxonomy

The Ecuadorian sample from Lake Mica in the Antisana Ecological Reserve is a unique and previously unreported molecular specimen. Its nuclear sequence appears closest to *A. caroliniana* while its chloroplast sequence appears distinct. When viewed in a chromatogram viewer the *ITS1* sequence is clean with no ambiguities. In comparison, the sequence (JX297319) in the Florida native *A. caroliniana* has ambiguities and, on close examination in Sequencher, many calls have high backgrounds. The *A. caroliniana* nuclear sequences (DQ066473- DQ066476) reported by Reid et al. (2006) averaged 16 ambiguities. This is typical of the superimposed sequences found in hybridization events that then have ameliorated over time through concerted evolution. The Ecuadorian sample may represent the paternal ancestor of *A. caroliniana*, displaying a similar but "clean" nuclear paternal sequence but not a similar mitochondrial (maternal) one. Natural interspecies *Azolla* hybridization has been suggested previously (Di Fulvio, 1961) for *A. mexicana*, however, this report provides the first molecular evidence for such a natural event.

The use of molecular taxonomy has proved useful in this study for characterizing the hybrid nature of *A. caroliniana*. It has also shown that three subspecies of *A. pinnata* exist. Previous molecular studies (Reid et al., 2006; Metzgar et al., 2007) have indicated, at least for the samples looked at, that *A. mexicana* and *A. microphylla* are the same species. Unfortunately, molecular biologists have not displayed megaspore SEMs while classical taxonomists have not used molecular barcoding. In the final analysis, only the combination of both molecular characterization and megaspore SEMs from the same samples will clarify *Azolla* taxonomy in a way satisfying to both groups. Since *Azolla* reproductive features are only occasionally available such paired observations would also allow greater confidence in the identification of non-reproductive specimens by the use of their sequence barcodes.

4.5. Implications of A. pinnata pinnata in Florida

Since *A. pinnata*'s 2007 discovery in a minor canal it has subsequently been observed along a major canal (C18), in agricultural areas, and of more significance, bordering the Arthur R. Marshall Loxahatchee National Wildlife Refuge, the remaining northernmost portion of the Florida Everglades. *A. pinnata* has also been found 90 km. south in Broward County (2010) implying long distance transport, probably by human vectors or waterfowl (Brochet et al., 2009).

Fig. 1I displays a heavy mat formed on a small pond. Similar heavy cover has been observed elsewhere, indicating substantial potential for invasive impacts. No information is yet available on the competition between exotic and native *Azolla*. Two native Florida insects are dietary specialists feeding on *Azolla*: the flea beetle *Pseudolampsis guttata* (LeConte) and the weevil *Stenopelmus rufinasus* Gyllenhal (Richerson and Grigarick, 1967; Buckingham and Buckingham, 1981; Hill, 1998; Center et al., 2002). Both can devastate mats of *A. caroliniana* (Center et al., 2002) which could skew inter-specific competition in favor of *A. pinnata*. Pemberton and Bodle (2009) found *S. rufinasus* feeding, mating, ovipositing, and emerging as adults on field collected *A. pinnata* kept in aquaria (without introducing the weevil independently). This agrees with studies by Hill (1998) showing *S. rufinasus* developing on *A. pinnata*, albeit more slowly and producing fewer progeny than on *A. filiculoides*, a host species in the weevil's native range. Therefore, even though feeding and development occurs on both the native and exotic *Azolla*s, herbivore preference for native *Azolla* may skew interspecies competition in favor of *A. pinnata*.

This leads the discussion full circle, to the primary reason we conducted molecular and morphological studies on Florida exotic *A. pinnata* and on the Asian, Australian and African subspecies. In biological control, the search for the most efficacious, coevolved agents is guided by determination of where in the native range the invasive originated from. This study clearly points to Australia as the origin of Florida's exotic *A. pinnata* and therefore the place to begin the search for biocontrol agents. One potential agent, *Bagous clarenciensis* Blackburn, has already been identified in S.E. Queensland and northern New South Wales. [M.F.P.].

5. Conclusions

Azolla pinnata, an exotic aquatic fern, is now in the northern Everglades and surrounding agricultural areas where it displays invasive characteristics. Both molecular and morphological tools indicate there are three *A. pinnata* subspecies and identify the Florida exotic as *A. pinnata* subsp. *pinnata*, the Australian subspecies. Specialist biological control agents are therefore most likely to be found in Australia. Molecular/morphological tools also indicate the identified Florida native is *A. caroliniana* auct. non Willd., a hybrid species.

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