final report

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Genetic Variation and the Origin of Lippia Populations in Australia

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Abstract

Lippia (*Phyla canescens*: Verbenaceae) is a serious weed of wetlands, riparian zones and floodplains, particularly in eastern Australia where many Ramsar wetlands are threatened by hydrological changes precipitated by soil accreting lippia mats. Understanding the genetic structure of lippia population is crucial in finding and developing bio-control agents. Microsatellite markers were developed for *Phyla canescens*. Genetic structures of Australia populations of lippia were compared to that of native range. ITS sequences analyses revealed that Australian populations of lippia have originated from at least two regions in Argentina. Considerable advances have been made in our genetic understanding of this species, which will have an important influence in its management and control. Low levels of diversity have been detected in 15 populations of lippia in Australia. In addition genetic analysis show that there is minimal genetic exchange occurring among these populations.

Executive Summary

Lippia (*Phyla canescens*: Verbenaceae) is a serious weed of wetlands, riparian zones and floodplains, particularly in eastern Australia where many Ramsar wetlands are threatened by hydrological changes precipitated by soil accreting lippia mats

Three distinct *P. canescens* genotypes have been identified, two from the native range, and one that has so far only been found in France (presumably from an as yet unidentified American population).

The two genotypes in South America are geographically isolated (SE coastal and NW inland Argentina).

French and Australian populations are represented by numerous genotypes, suggesting multiple introductions (or considerable crossing/domestication prior to introduction)

Glasshouse experiments revealed that *P. canescens* does not set seed without pollinators (is not autonomous). Experiments are underway to study more aspects of reproductive biology of the species. On the other hand *P. nodiflora* is autonomous and produces seeds without the need for pollinator.

Contents

1 Background

The introduction of exotic species into new environments can have serious consequences on ecosystem function. Lippia (*Phyla canescens*) was introduced as a garden plant to Australia in the first half of 20th century but is now a serious environmental weed. It is currently estimated that lippia is distributed across 5 million hectares in the Murray Darling Basin in Australia (Earl 2003), where it clogs waterways and degrades grazing lands. Lippia is a severe threat to riparian areas where it displaces native vegetation by forming dense mats. It also consumes vast amounts of water and caused soil erosion. It is estimated that lippia is costing $38 million in lost annual production to the grazing industry and over $3 billion in lost ecosystem services.

Lippia is a prostrate plant that grows rapidly and produces dense mats covering large areas in wetland and river banks (Figure 1). It reproduces both asexually and sexually by producing massive amounts of seeds (Macdonald 2008).

Use of chemical or low density grazing management has not been proved to be effective in the long term. Bio-control seems to be only viable option to control lippia (Earl 2003).

Figure 1. Lippia infestation along the creek banks in Macquarie Marshes (M. Fatemi).

2 Project Objectives

The aim of this study was to identify the level of genetic diversity within and among the lippia infestations across Australian and compare them to the native range in South America. Another objective of this study was to reveal the source population of Australian lippia, which would help with the selection of bio-control agents in the native range.

3 Methodology

3.1 Development of Microsatellite Primers

To date, no population genetic studies on lippia are known thus we developed microsatellite primers to study the population genetic structure of infested populations in detail. DNA was isolated from leaf tissue samples using the DNeasy Plant Kits (QIAGEN). Microsatellite loci were isolated following the standard protocol of Glenn and Schable (2005). Restriction enzymes *Rsa*I and *BstU* I (New England Biolabs) were used to digest genomic DNA. Double-stranded SNX linkers were ligated to DNA fragments and used to amplify the genomic DNA. Amplified DNA was hybridized with a mixture of biotinylated oligos with AG, TG, AAC, AAG, AAT, ACT and ATC repeats. Hybridized DNA was mixed with magnetic beads (Bynabeads) and incubated at room temperature for 30 min. Recovered DNA fragments were amplified using SNX-forward linker as a primer. DNA fragments were ligated into pCR®2.1-TOPO® (Invitrogen) according to the manufacturer's instructions. Following transformation into One Shot® TOP10 Chemically competent *E. coli*, 200 positive clones were selected and screened by PCR using M13 primers. In total 75 PCR products with the right size of inserts (300-1000 bp) were sequenced. Sequences were examined by an SSR identification tool (Temnykh *et al.* 2001) to locate microsatellite motifs. Twenty three pairs of flanking primers were designed using Primer 3 (Rozen and Skaletsky 2000) to amplify the repeated regions. A M13 universal sequence was appended to each forward primer for fluorescent labeling of PCR products according to Schuelke (2000). Microsatellite PCR was carried out on a set of 40 individual plants from two populations, Hurlingham in Argentina and Goondiwindi in Australia. A fluorescent M13 primer (FAM, PET, NED or VIC dyes) was used to label the PCR products. The cycling profile was: 10 min initial denaturation at 95°C; 30 cycles of denaturation at 95°C for 30s, annealing at 59°C for 45s, and extension at 72°C for 1min; followed by 8 cycles of denaturation at 95°C for 30s, annealing at 53°C for 45s, and extension at 72°C for 1 min; and final 10 min of extension at 72°C. PCR products were visualized on 1% agarose stained with SYBR® safe DNA gel stain (Invitrogen).

PCR products were multiplexed and profiled with ABI 3730 DNA Analyzer. Diversity levels for each locus, number of alleles (N), number of effective alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using GenAlex 6 (Peakall and Smouse 2006). Nine loci gave clearly scorable and polymorphic products. Between four and 20 alleles were detected and values for expected and observed heterozygosities ranged from 0.32 to 0.906 and from 0.0 to 1.0, respectively.

Linkage disequilibrium across the loci was tested using GENEPOP on the web version 3.4 (http://genepop.curtin.edu.au) employing Markov chain parameters. No significant linkage disequilibrium was detected in pairwise comparisons across loci. Significant deviation from Hardy-

Weinberg equilibrium was observed which given that *Phyla canescens* is reproducing both sexually and asexually is not surprising.

Despite the low level of genetic diversity, which results from the limited number of founder events and also clonal reproduction, these markers display adequate levels of polymorphism and provide valuable tools to investigate the genetic structure of populations.

3.2 Population Genetics Using ISSR Markers

Collections of lippia were made from 12 populations in infested regions in Australia and from the native range of the species in South America (five populations). Samples were also obtained from non-native populations in France (five populations).

Silica gel dried samples were used for total genomic DNA isolation. For each sample 20-50 mg of leaf tissue was crushed in a 2 ml micro-tube using a mixer mill. Genomic DNA was extracted using DNeasy Plant Minikit (QIAGENE, Inc.) following the manufacturer's instructions.

Inter-simple sequence repeat (ISSR) markers were used to estimate the level of genetic diversity within and among the populations of lippia. In total 12 primers were used to fingerprint individuals from all populations.

PCR was performed in a 25 µL reaction mixture per sample (1 x *Taq* Polymerase PCR buffer, 400 µM dNTPs, 6 mM magnesium chloride, 0.2 µM of primer, 1 U of Taq DNA polymerase and 100 ng of genomic DNA). The amplification was performed in a PC-960C Thermal Cycler (Corbet Research). Initial denaturation was for 5 min at 94°C, followed by 30 cycles of 45s at 94°C, 45s at 46 or 41°C, 90s at 72°C, and a final extension of 10 min at 72°C. The PCR products were separated electrophoretically on 1.5% agarose gels in 1x TBE buffer containing SYBR safe DNA gel stain (Invitrogen). The gels were illuminated under UV light and digitally photographed. The presence or absence of bands was scored from the gel photographs. Duplicates and negative controls were included in each gel for quality control. Bands were scored based on their reproducibility and consistency to determine the ISSR phenotype for each individual sampled.

3.3 Biogeographic analyses using DNA sequencing of ITS regions

DNA sequences from internal transcribed spacer (ITS1 and ITS2) regions of ribosomal DNA were amplified in 25 µL volumes using standard polymerase chain reaction (PCR) protocols. ITS4i and ITS5 primers were used to amplify ITS regions. This resulted in ~700 base pairs (bp) of sequences. Resulting PCR products were purified by adding Exonuclease and Antarctic Phosphatase and incubating at 37°C for 15 min and 80°C for 15 min. Purified PCR products were sequenced using the ITS4i primer.

Obtained sequences were aligned using the online ClustalW tool from European Bioinformatics Institute (EBI). In total 592 bps were included in the analysis.

3.4 Population analyses

As a dominant marker, ISSR bands were scored as present (1) or absent (0) and a data matrix was created. Data were analysed using GenAlex version 6 (Peakall and Smouse 2006) and genetic diversity indices were calculated for 18 populations (Table 1). Analyses were undertaken for the countries separately and collectively. Analysis of molecular variance (AMOVA) was used to overcome the assumption of Hardy-Weinberg equilibrium. AMOVA makes no assumption about Mendelian gene frequencies (Chapman *et al.* 2004). AMOVA is now regularly used to analyse ISSR and RAPD data (Huff *et al.* 1998; Chapman *et al.* 2004). Principal component analyses (PCA) was also used to further investigate the structure of lippia populations and their similarities.

3.5 Biogeographic analyses

The origin of Australian populations was investigated by conducting a phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacers (ITS) sequences using PAUP* version 4.10b (Swofford 2002) with a sample from each region to determine relatedness. Five equally parsimonious trees were found by executing a heuristic search with gaps treated as missing data and characters unordered and weighted equally.

3.6 Polyploidy and Chromosome Number

To determine the number of chromosomes and the level of ploidy in lippia, fresh root tips were harvested from glasshouse grown specimens and fixed in glacial acetic acid and ethanol (1:3) for at least 2 hours. Roots were stained in aceto-orcein for 4 hours. Root tips (1mm) were placed on a microscope slide, macerated, covered with cover slip and squashed between filter paper. Slides were examined under microscope and chromosomes were counted in metaphase of mitosis.

4 Results and Discussion

4.1 Microsatellite Primer Development

In total nine informative microsatellite primers were developed for *Phyla canescens* (Fatemi and Gross In press). These markers are currently being used for the study of population structure of lippia. A paper on the population structure of lippia is in preparation by Fatemi and Gross.

4.2 Population genetics

A total of 155 bands ranging from approximately 200 to 2000 bp were detected from 12 selected ISSR primers. The percentage of polymorphic loci (P) was as low as 16% in the Goondiwindi region and as high as 56% in the French populations. The percentage of polymorphic loci in Argentinean populations ranged from 18% to 52%. Analysis of molecular variance (AMOVA) (Figure 2) revealed that most of the genetic variation is distributed within populations. In the Principal Coordinate

Analysis (PCA) two groups of the Argentinean populations were formed, with the Australian populations being represented in both (Figure 2). Therefore, it is reasonable to suggest that Australian populations have originated from at least two regions in Argentina.

The results indicate that some of the Australian populations of lippia show low levels of genetic diversity, whereas other populations have levels of diversity comparable to that found in Argentina and France. In the PCA analysis the Australian populations were nested among Argentinean populations, suggesting that the Australian populations may have originated from these Argentinean populations or regions.

Analysis of molecular variance (AMOVA) showed that most of the variation is distributed within populations. This is supported by a low G_{ST} of 0.0261 which indicates that populations are genetically separated from one another. This also supports our view that Australian populations have originated from multiple introduction events.

Higher genetic variation within populations in Australia and France suggests that there is significant gene flow among the populations. This gene flow could be due to the fact that these populations are introduced and may have originated from the same introduction event. In other words this pattern of partitioning of genetic variation may not be as a direct result of current gene flow but rather genetic exchange at founder events. This suggests that lippia may have been introduced via seed sourced from different locations in native range.

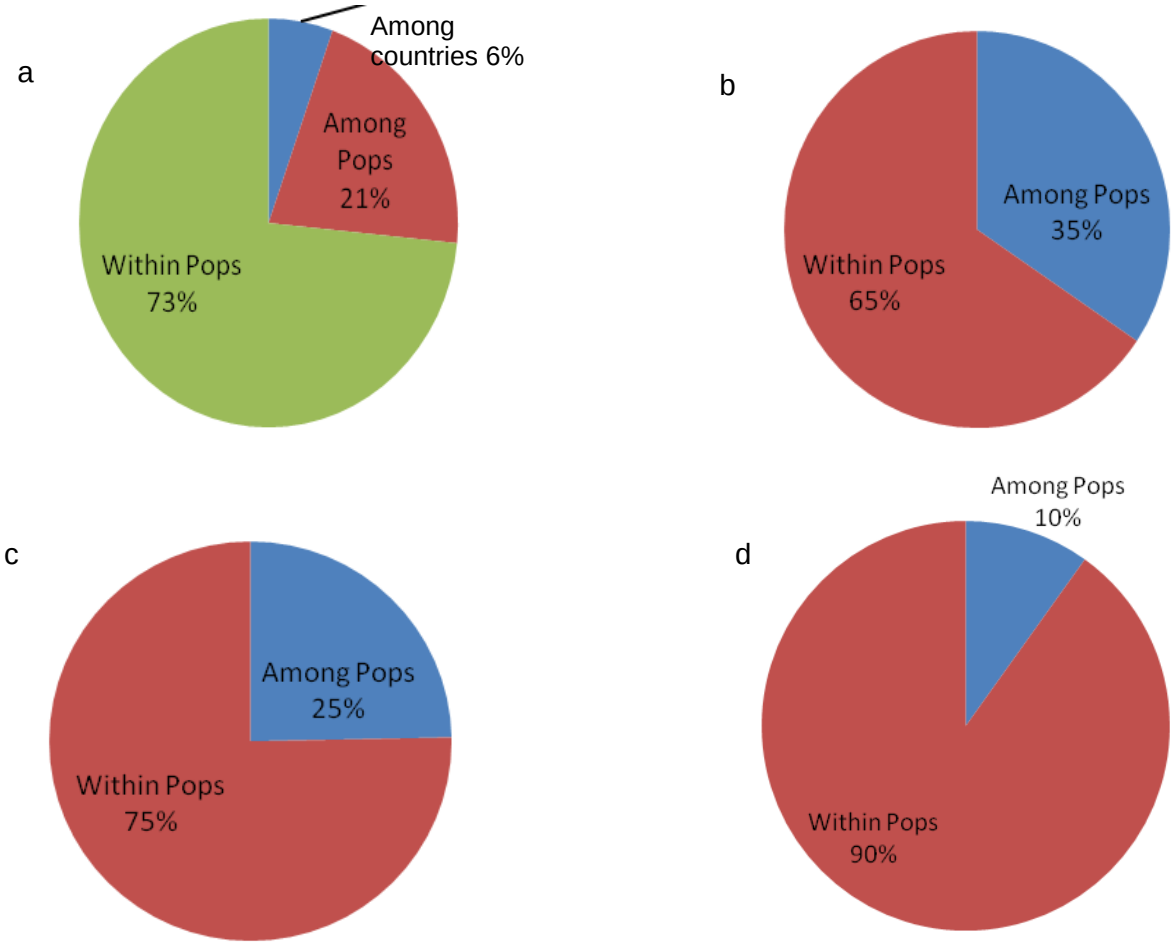
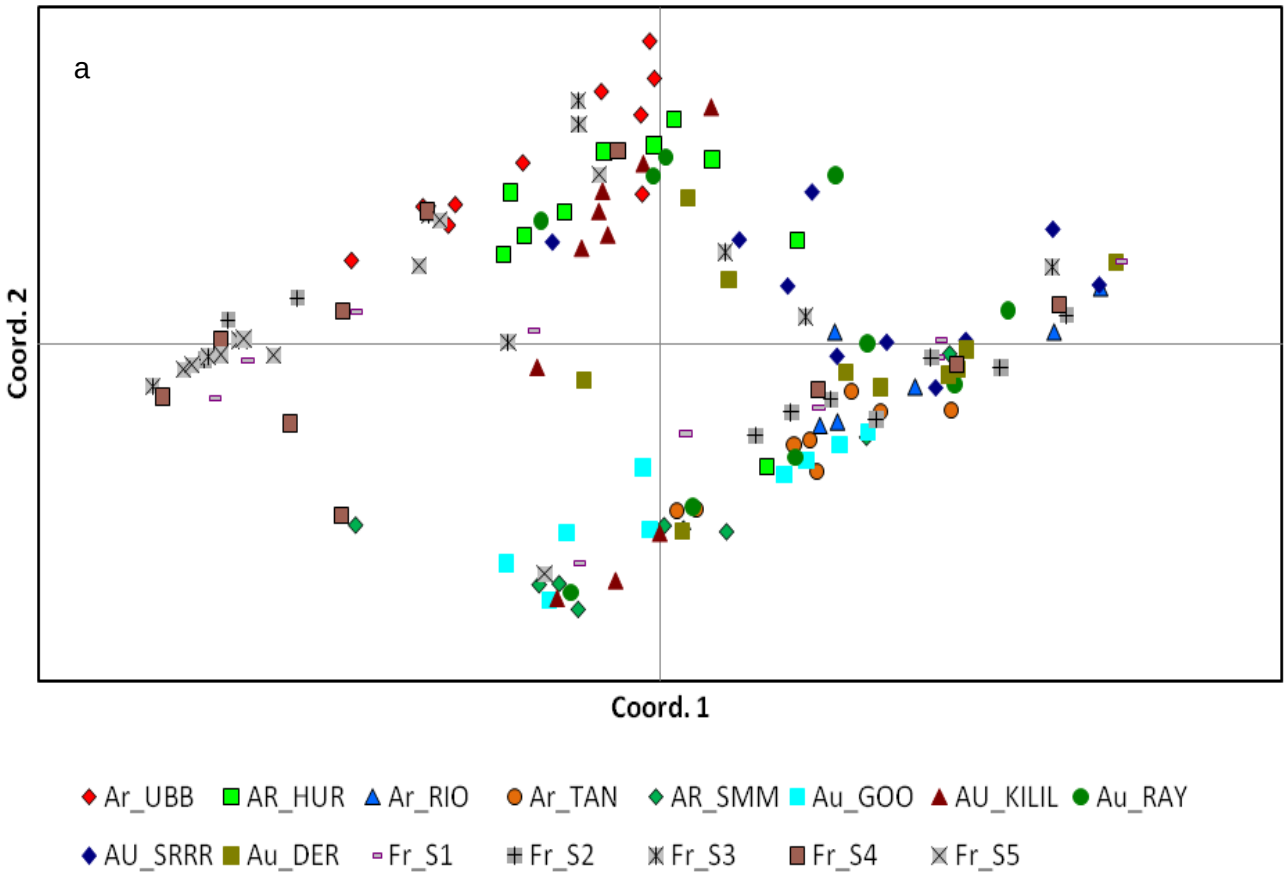


Figure 2. Partitioning of molecular variance in different levels of populations of *Phyla canescens*; a = in three countries; b = Argentina; c = Australia; and d = France.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Coord. 1
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AR_HUR
Ar_RIO
Ar_TAN
AR_SMM
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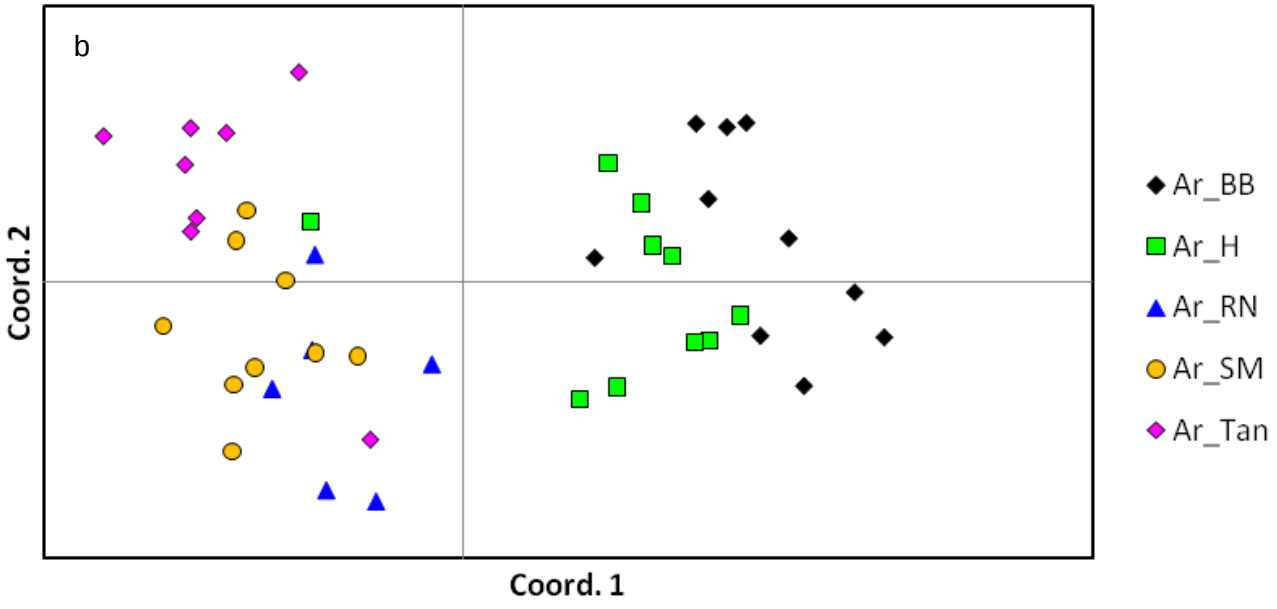
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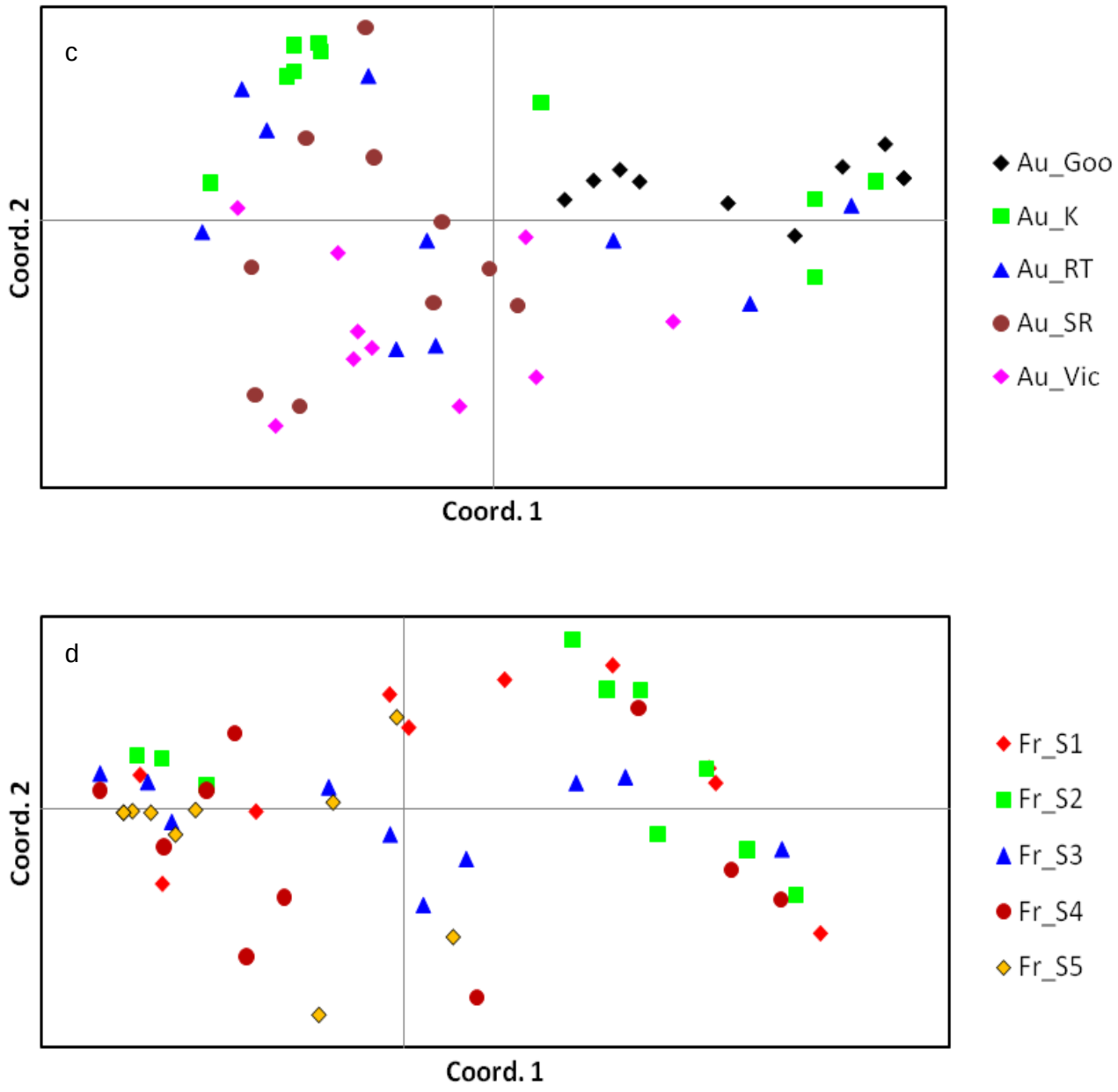

Figure 3 Principal Coordinates Analysis of *Phyla canescens* populations in a = three countries; b = Argentina; c = Australia; d = France.

Table 1 Summary of Genetic Variation Statistics for All Loci and Nei's Analysis of Gene Diversity in Subdivided Populations in Argentina.

N = Number of individuals; Na = Observed number of alleles; Ne = Effective number of alleles ;I = Shannon's Information index; He = Nei's gene diversity; UHe = Nei's unbiased gene diversity; P = Polymorphic loci

Pop		N	Na	Ne	I	He	UHe	%P
Arg_Bahia Blanca	**Mean**	10	1.541	1.412	0.362	0.243	0.255	67.57
	SE		0.120	0.060	0.046	0.033	0.034	
Arg_Hurlingham	**Mean**	10	1.568	1.516	0.430	0.293	0.309	75.68
	SE		0.132	0.062	0.045	0.032	0.034	
Arg_Rio Negro	**Mean**	6	1.027	1.277	0.249	0.164	0.179	48.65
	SE		0.162	0.059	0.046	0.032	0.035	
Arg_San Miguel del Monte	**Mean**	9	1.243	1.386	0.320	0.218	0.231	56.76
	SE	0	0.152	0.067	0.050	0.035	0.037	
Arg_Tandil	**Mean**	8	1.541	1.487	0.391	0.268	0.286	70.27
	SE		0.126	0.069	0.049	0.036	0.038	
Total	**Mean**	8	1.384	1.416	0.350	0.237	0.252	63.78
	SE	0.11	0.064	0.029	0.021	0.015	0.016	4.88
						Ht = 0.3448 (0.0178)		
						Hs = 0.2373 (0.0179)		
						Gst = 0.3118		
						Nm = 1.1036		

Table 2 Summary of Genetic Variation Statistics for All Loci and Nei's Analysis of Gene Diversity in Subdivided Populations in Australia.

N = Number of individuals; Na = Observed number of alleles; Ne = Effective number of alleles ;I = Shannon's Information index; He = Nei's gene diversity; UHe = Nei's unbiased gene diversity; P = Polymorphic loci

Pop		N	Na	Ne	I	He	UHe	%P
Au_Goondiwindi	**Mean**	10	1.000	1.288	0.223	0.156	0.164	35.14
	SE		0.140	0.067	0.051	0.036	0.038	
Au_Kilcoy	**Mean**	10	1.622	1.567	0.455	0.313	0.330	78.38
	SE		0.125	0.065	0.045	0.033	0.034	
Au_Raymond Terrace	**Mean**	10	1.405	1.362	0.335	0.221	0.233	64.86
	SE		0.142	0.056	0.045	0.031	0.032	
Au_St Ruth Reserve	**Mean**	10	1.459	1.374	0.337	0.220	0.232	72.97
	SE		0.148	0.063	0.044	0.032	0.034	
Au_Reedy Lake	**Mean**	10	1.459	1.423	0.368	0.245	0.258	72.97
	SE		0.148	0.064	0.045	0.033	0.034	
Total	**Mean**	10	1.389	1.403	0.344	0.231	0.243	64.86
	SE		0.064	0.029	0.021	0.015	0.016	7.74
						Ht = 0.3061 (0.0219)		
						Hs = 0.2311 (0.0117)		
						Gst = 0.2450		
						Nm = 1.5411		

Table 3. Summary of Genetic Variation Statistics for All Loci and Nei's Analysis of Gene Diversity in Subdivided Populations in France.

N = Number of individuals; Na = Observed number of alleles; Ne = Effective number of alleles ;I = Shannon's Information index; He = Nei's gene diversity; UHe = Nei's unbiased gene diversity; P = Polymorphic loci

Pop		N	Na	Ne	I	He	UHe	%P
Fr_S1	**Mean**	10	1.838	1.599	0.515	0.348	0.366	91.89
	SE		0.091	0.050	0.033	0.024	0.026	
Fr_S2	**Mean**	10	1.730	1.482	0.433	0.288	0.303	81.08
	SE		0.100	0.056	0.040	0.028	0.030	
Fr_S3	**Mean**	10	1.811	1.636	0.507	0.350	0.368	86.49
	SE		0.085	0.061	0.040	0.030	0.031	
Fr_S4	**Mean**	10	1.811	1.696	0.544	0.379	0.399	89.19
	SE		0.094	0.055	0.037	0.027	0.029	
Fr_S5	**Mean**	10	1.622	1.554	0.425	0.297	0.313	70.27
	SE		0.105	0.070	0.051	0.036	0.038	
Total	**Mean**	10	1.762	1.593	0.485	0.332	0.350	83.78
	SE		0.043	0.027	0.018	0.013	0.014	3.82
						Ht = 0.3898 (0.0159)		
						Hs = 0.3324 (0.0119)		
						Gst = 0.1471		
						Nm = 2.8996		

Table 4. Summary of Genetic Variation Statistics for All Loci and Nei's Analysis of Gene Diversity in Subdivided Populations in 15 populations in three countries.

N = Number of individuals; Na = Observed number of alleles; Ne = Effective number of alleles ;I = Shannon's Information index; He = Nei's gene diversity; UHe = Nei's unbiased gene diversity; P = Polymorphic loci

Pop		N	Na	Ne	I	He	UHe	%P
Ar_UBB	**Mean**	10	1.541	1.412	0.362	0.243	0.255	67.57
	SE		0.120	0.060	0.046	0.033	0.034	
AR_HUR	**Mean**	10	1.568	1.516	0.430	0.293	0.309	75.68
	SE		0.132	0.062	0.045	0.032	0.034	
Ar_RIO	**Mean**	6	1.027	1.277	0.249	0.164	0.179	48.65
	SE		0.162	0.059	0.046	0.032	0.035	
Ar_TAN	**Mean**	8	1.162	1.375	0.303	0.209	0.222	51.35
	SE		0.153	0.069	0.051	0.036	0.039	
AR_SMM	**Mean**	9	1.568	1.508	0.406	0.279	0.295	72.97
	SE		0.126	0.069	0.049	0.035	0.038	
Au_GOO	**Mean**	10	1.000	1.288	0.223	0.156	0.164	35.14
	SE		0.140	0.067	0.051	0.036	0.038	
AU_KIL	**Mean**	10	1.622	1.567	0.455	0.313	0.330	78.38
	SE		0.125	0.065	0.045	0.033	0.034	
Au_RAY	**Mean**	10	1.405	1.362	0.335	0.221	0.233	64.86
	SE		0.142	0.056	0.045	0.031	0.032	
AU_SRR	**Mean**	10	1.459	1.374	0.337	0.220	0.232	72.97
	SE		0.148	0.063	0.044	0.032	0.034	
Au_DER	**Mean**	10	1.459	1.423	0.368	0.245	0.258	72.97
	SE		0.148	0.064	0.045	0.033	0.034	
Fr_S1	**Mean**	10	1.838	1.599	0.515	0.348	0.366	91.89
	SE		0.091	0.050	0.033	0.024	0.026	
Fr_S2	**Mean**	10	1.730	1.482	0.433	0.288	0.303	81.08
	SE		0.100	0.056	0.040	0.028	0.030	
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	SE		0.085	0.061	0.040	0.030	0.031	
Fr_S4	**Mean**	10	1.811	1.696	0.544	0.379	0.399	89.19
	SE		0.094	0.055	0.037	0.027	0.029	
Fr_S5	**Mean**	10	1.622	1.554	0.425	0.297	0.313	70.27
	SE		0.105	0.070	0.051	0.036	0.038	

Total	**Mean**	9.533	1.508	1.471	0.393	0.267	0.282	70.63
	SE	0.046	0.034	0.017	0.012	0.009	0.009	4.03
						Ht = 0.3639 (0.0129)		
						Hs = 0.267 (0.0081)		
						Gst = 0.2662		
						Nm = 1.3786		

4.3 Phylogenetic analyses

The results of the phylogenetic analyses using ITS sequences revealed that Australian populations of lippia group with two groups of populations from different geographic regions in South America. (Figure 4).

Other species of *Phyla* from South America form a non-resolved basal clade and are the subject of further studies.

Finally, results of phylogenetic analysis of ITS data again support the hypothesis that *Phyla canescens* has been introduced to Australia from multiple source populations.

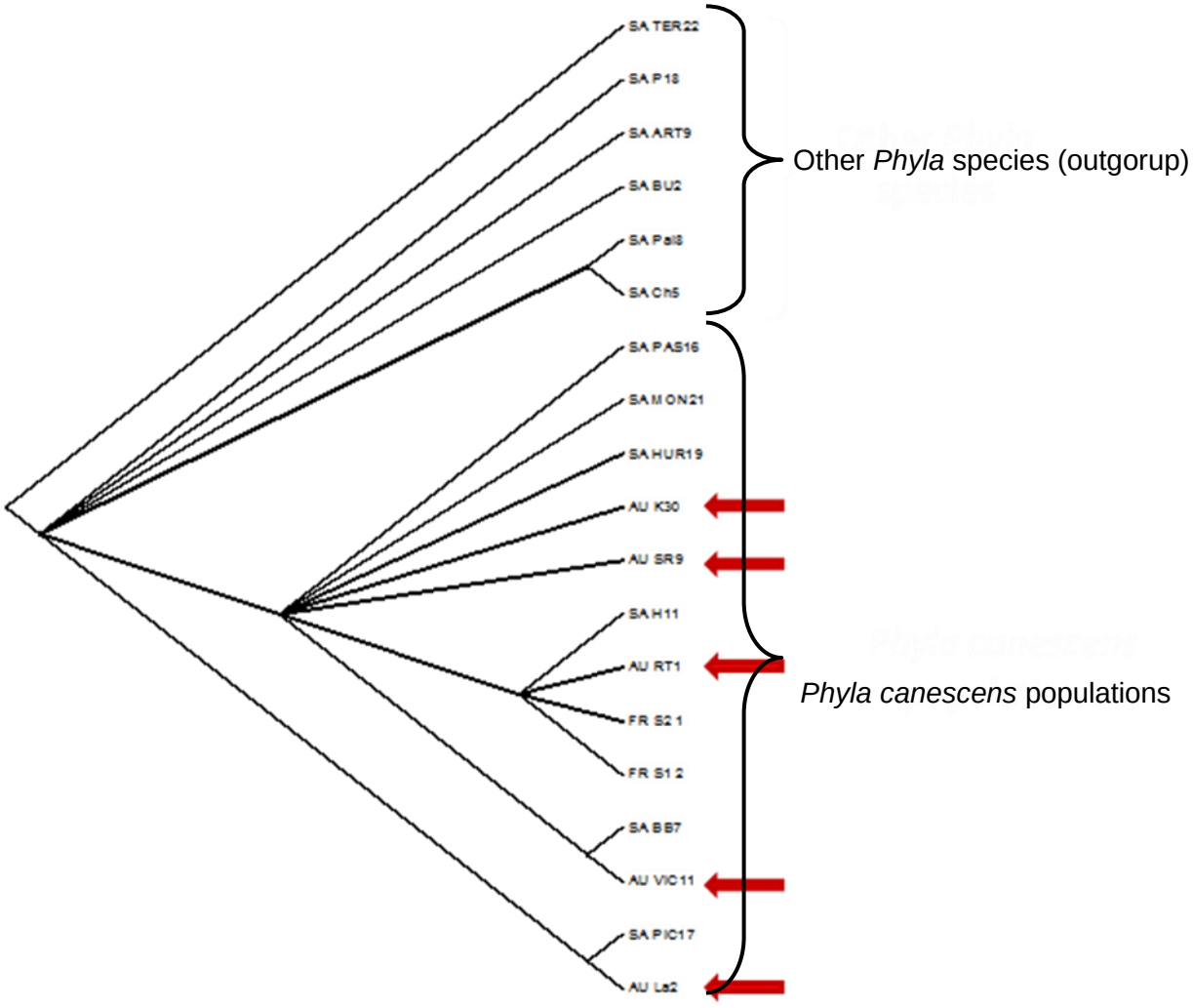

Figure 4. One of 5 equally parsimonious cladograms; arrows indicate Australians entities.

These results are consistent with multiple introductions and/or a large founder populations and high gene flow among populations. Especially in France with lowest G_{ST} value and lowest variation partitioned among populations. These results are supported by PCA analyses, for example in the French populations PCA (Figure 3d), symbols from different populations are overlapping and populations cannot be separated. Argentinean populations are relatively discrete and 35% of variations is partitioned among the populations, which is supported by the PCA.

4.4 Chromosome number and ploidy level

A change in ploidy level could increase invasiveness of introduced plant species in new habitats. To examine this question for *Phyla canescens*, we compared its ploidy level in its South American native range and in the Australian populations. Overall 36 chromosomes were counted in Australian samples (Figure 5), which is consistent with the number reported from the native range (Xu et al. unpublished). Therefore there is no change in ploidy level in the introduced *Phyla canescens* populations. Some irregularities have been observed in the meiosis of *Phyla canescens* in Goondiwindi and St Ruth Reserve populations (Xu et al. unpublished) but further investigations are needed to reveal the nature of these irregularities and their effect on the reproduction of the populations.

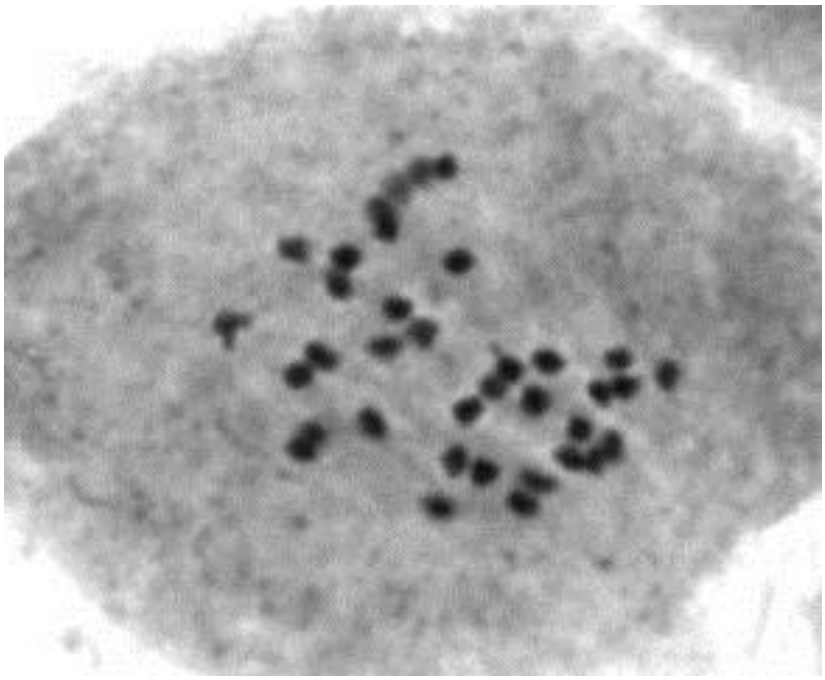

Figure 5 Micrograph of chromosomes in *Phyla canescens*.

5 Success in Achieving Objectives

5.1 Development of molecular markers for *Phyla canescens*

Species specific microsatellite markers were developed for *Phyla canescens* and are being used to study the genetic structure of the populations (Fatemi and Gross In press).

5.2 Determine the level of genetic variation in the populations of *Phyla canescens*

We used ISSR markers to study the level of genetic variations within and among the populations of *Phyla canescens* in Australia and in its native range in South America. Genetic variation in Australia was slightly lower than that of Argentinean populations, but it was revealed that Australian populations are less differentiated which indicates not only multiple introductions but also minimal gene movement among the populations.

5.3 Determine the origin of *Phyla canescens*

Using ISSR markers and phylogeographic analysis of ITS sequences we determined that lippia has been introduced to Australia from at least two regions in Argentina. This has implications on the process of selecting and introducing bio-control agents for the control of lippia.

Table 5 Population genetic structure in weeds. **Still working on this table**

Author	Species	Family	Marker	No. Pops	No. Specimen	P %	H_T	H_S	He	F_{ST}	Gst	I
Xu *et al.* (2003)	*Alternanthera philoxeroides*	Amaranthaceae	RAPD	8	67	0			-		-	-
Amsellem *et al.* (2000)	*Rubus alceifolius*	Rosaceae	AFLP									
Dodet *et al.* (2007)	*Cyperus esculentus*	Cyperaceae	AFLP	49	147	50	0.140	0.006		0.95		
Green *et al.* (2001)	*Anisantha sterilis*	Poaceae	Microsatellite	14	211							
Hollingsworth and Bailey (2000)	*Fallopia japonica*		RAPD	3+	150	0	0	0	0			
Wang *et al.* (2005)	*Alternanthera philoxeroides*	Amaranthaceae	ISSR, RAPD	7	112	0	0	0	0			
Chapman *et al.* (2000)	*Pilosella officinarum*	Asteraceae	ISSR	5	50	50						
Kreher *et al.* (2000)	*Vaccinium stamineum*		RAPD	1	99							
Ash *et al.* (2003)	*Carthamus lanatus*	Asteraceae	ISSR	11	60							0.22-0.33

6 Impact on Meat and Livestock Industry – now & in five years time

7 Conclusions and Recommendations

The current study provides the first molecular-based population genetic analysis of lippia. This study demonstrated that genetic diversity in lippia is comparable to the genetic diversity of species reproducing both sexually and asexually (Table 5).

The results indicate that some of the Australian populations of lippia show low levels of genetic diversity, whereas some other populations are genetically diverse. This may be related to the mode of propagation in the populations as well as founder events. Populations in which the main mode of reproduction is by cloning, the level of genetic diversity is relatively low, but population in which vegetative growth is interrupted by environmental factors such as drought, the level of genetic diversity is high. In these populations the spread of lippia is mainly by seed production, which in turns results in higher genetic diversity. This also suggests that more diverse populations may have been introduced by seeds thus having more genetic diversity.

8 Bibliography

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9 Appendices

9.1 Appendix 1

The following paper was presented and published in the 16th Australian Weed Conference Proceedings. 18-22 May 2008. Cairns, Australia.

Fatemi M, Gross CL, Duggin JA, Julien M (2008) *Phyla canescens*: Multiple introductions into Australia as revealed by ISSR Markers and Nuclear Ribosomal DNA Internal Transcribed Spacers (ITS) In '16th Australian Weed Conference'. Cairns Convention Centre, North Queensland. (Eds RD van Kilnken, VA Osten, FD Panetta and JC Scanlan) pp. 247-249. (Queensland Weed Society, Brisbane).

Phyla canescens: Multiple introductions into Australia as revealed by ISSR Markers and Nuclear Ribosomal DNA Internal Transcribed Spacers (ITS)

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Summary Lippia (*Phyla canescens* (Kunth) Greene: Verbenaceae) is a very invasive weed in wetlands and riparian zones in Australia and especially in the Murray Darling Basin. The use of chemicals as control method is inappropriate in such environmentally sensitive areas particularly the Macquarie Marshes and Gwydir Wetlands. Biological control will be the only option in many areas. We investigated genetic diversity in 12 populations of lippia from four different catchments in Australia, eight populations from the native range in South America and five populations from France where the species is non-native and invasive. Low levels of genetic diversity were detected within some Australian regions in contrast to the Argentinean and French populations. In the analyses the Australian material segregated with two disjunct regions in Argentina suggesting that Australia has experienced multiple introductions of lippia.

Keywords Weeds, biogeography, biological control.

INTRODUCTION

Exotic species have caused considerable economic and environmental damage worldwide (Pimentel *et al.* 2001). The invasiveness of alien species and the ability of ecosystems to be invaded and colonised are among the main considerations for any weed control program (Dong *et al.* 2006). Understanding the genetic structure of populations of invasive weed species is important, especially for biological control programs involving host-specific pathogens (Burdon and Marshall 1981; Chapman *et al.* 2004).

Lippia (*Phyla canescens* Verbenaceae) is a serious weed of wetlands, riparian zones and floodplains, particularly in eastern Australia where many Ramsar wetlands are threatened by hydrological changes precipitated by soil-accreting lippia mats (Lucy *et al.* 1995, Earl 2003). A key area to investigate is the level of genetic diversity existing within and among infestations and whether novel alleles are appearing in introduced populations. The aim of this study is to evaluate the level genetic diversity within and among the lippia infestations in Australia and study the origin of Australian lippia in its native range in South America. The levels of genetic diversity in lippia play a major role in determining the specificity of

biological control agents. Medium to low levels of genetic diversity within and among lippia populations would increase the chances of biological control agents being effective in a range of environments.

Here we describe results on inter-population variability in lippia from Australia and two regions overseas.

MATERIALS AND METHODS

Collections of lippia were made from 12 populations in infested regions in Australia and from the native range of the species in South America. Samples were also obtained from non-native populations in France.

Silica gel dried samples were used for total genomic DNA isolation. In total, 20-50 mg of leaf tissue was crushed using a mixer mill. Genomic DNA was extracted using DNeasy Plant Minikit (QIAGENE, Inc.) following the manufacturer's instructions.

Inter-simple sequence repeat (ISSR) markers were used to estimate the level of genetic diversity within and among the populations of lippia. In total 12 primers were used to fingerprint individuals from all populations.

DNA sequences from internal transcribed spacer (ITS1 and ITS2) regions of ribosomal DNA were amplified in 25 µL volumes using standard polymerase chain reaction (PCR) protocols. ITS4i and ITS5 primers were used to amplify ITS regions. This resulted in ~700 base pairs (bp) of sequences. Resulting PCR products were purified by adding Exonuclease and Antarctic Phosphatase and incubating at 37°C for 15 min and 80°C for 15 min. Purified PCR products were sequenced using the ITS4i primer.

Obtained sequences were aligned using the online ClustalW tool from European Bioinformatics Institute (EBI). In total 592 bps were included in the analysis.

Population analyses As a dominant marker, ISSR bands were scored as present (1) or absent (0) and a data matrix was created. Data were analysed using GenAlex version 6 (Peakall and Smouse 2006) and genetic diversity indices were calculated for 18 populations (Table 1) .

Biogeographic analyses The origin of Australian populations was investigated by conducting a phylogenetic analysis of nuclear ribosomal DNA internal transcribed spacers (ITS) sequences using PAUP* version 4.10b (Swofford 2002) with a sample from each region to determine relatedness. Five equally parsimonious trees were found by executing a heuristic search with gaps treated as missing data and characters unordered and weighted equally.

RESULTS

A total of 155 bands ranging from approximately 200 to 2000 bp were detected from 12 selected ISSR primers. The percentage of polymorphic loci (P) was as low as 16% in the Goondiwindi region and as high as 56% in the French populations. The percentage of polymorphic loci in Argentinean populations ranged from 18% to 52%. Analysis of molecular variance (AMOVA) (Figure 1) revealed that most of the genetic variation is distributed within populations. In the Principal Coordinate Analysis (PCA) two groups of the Argentinean populations were formed, with the Australian populations being represented in both (Figure 2). Therefore, it is reasonable to suggest that Australian populations have originated from at least two regions in Argentina.

The results of the phylogenetic analyses using ITS sequences also revealed that Australian populations of lippia group with two groups of populations from different geographic regions in South America. (Figure 3).

Other species of *Phyla* from South America form a non-resolved basal clade and are the subject of further studies.

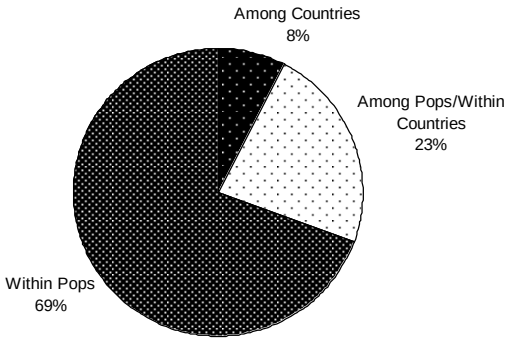

Figure 1. Partitioning of molecular variance in populations from Argentina, Australia and France

Table 1. The number of observed (N_a) and effective alleles (N_e), Nei's genetic diversity (H), Shannon's information index (I) and percentage of polymorphic (P) loci. Argentina (Ar), Australia (Au) and France (Fr).

Population	Na	Ne	H	I	P
Ar-BB	1.4286	1.2598	0.1507	0.2251	40.54%
Ar-BU	1.3056	1.1814	0.1064	0.1594	29.73%
Ar-H	1.3611	1.2354	0.1349	0.1993	35.14%
Ar-MR	1.5278	1.2987	0.1756	0.2657	51.35%
Ar-P	1.2414	1.2043	0.1101	0.1565	18.92%
Ar-RN	1.2857	1.2189	0.121	0.1747	21.62%
Ar-SM	1.3235	1.2386	0.1298	0.1877	29.73%
Ar-Tan	1.4706	1.3341	0.1876	0.2739	43.24%
Au-Goo	1.2	1.1652	0.0896	0.1278	16.22%
Au-K	1.3333	1.2136	0.1205	0.1781	32.43%
Au-RT	1.3333	1.2575	0.1383	0.1988	32.43%
Au-SR	1.3714	1.1834	0.1107	0.1709	35.14%
Au-Vic	1.2778	1.2076	0.1152	0.1669	27.03%
Fr-S1	1.5676	1.4515	0.2434	0.3489	56.76%
Fr-S2	1.3056	1.0236	0.1183	0.1746	29.73%
Fr-S3	1.4324	1.2935	0.1634	0.2393	43.24%
Fr-S4	1.3056	1.2088	0.1165	0.1706	29.73%
Fr-S5	1.2432	1.1747	0.0942	0.1363	24.32%
Total	1.508	1.471	0.267	0.393	70.63%
			H_T = 0.1305		
			H_S = 0.1271		
			D_{ST} = 0.0034		
			G_{ST} = 0.0261		

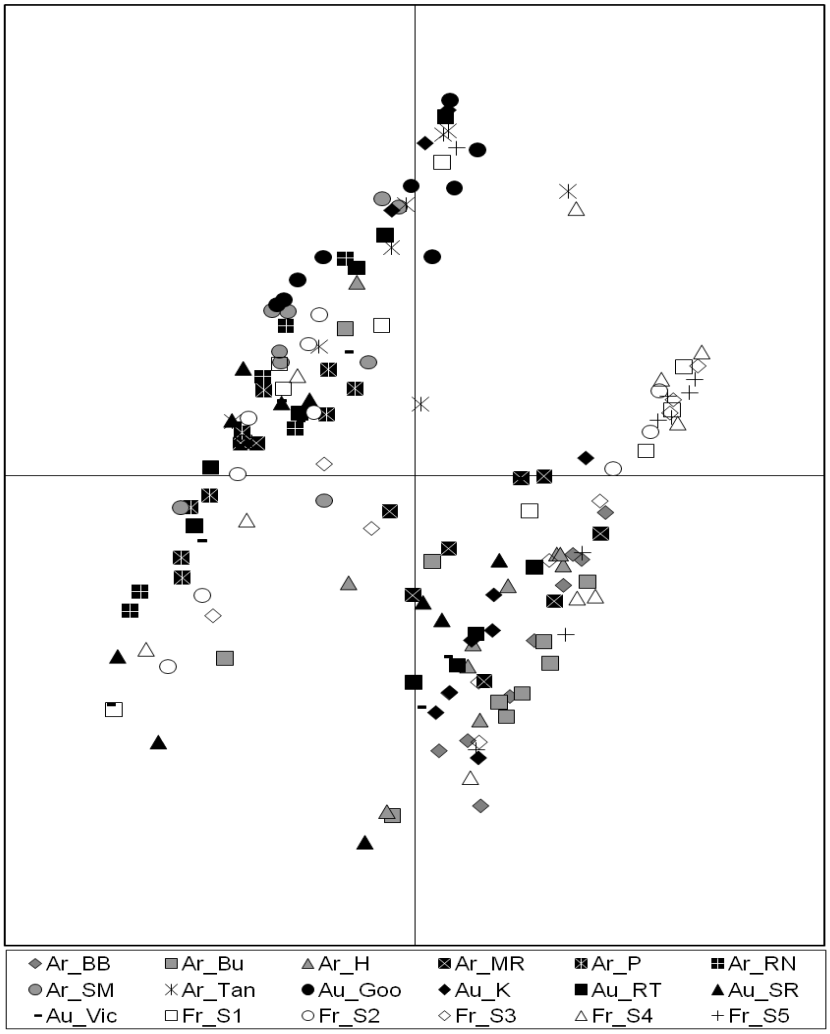

Figure 2. Principal coordinate analysis of lippia populations in Argentina (Ar), Australia (Au) and France (Fr).

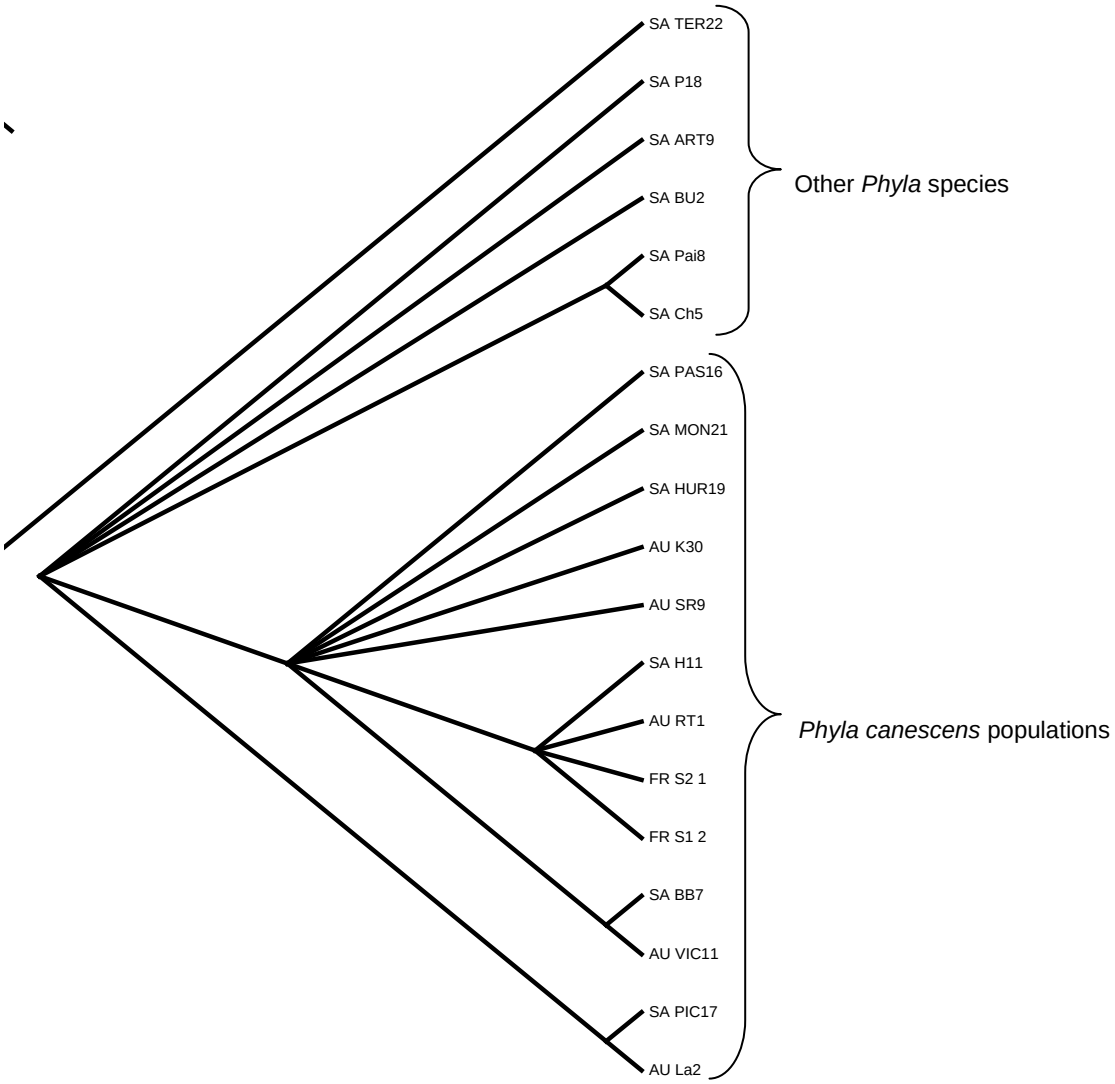

Figure 3. One of the 5 equally parsimonious cladograms. AU = Australia; FR = France; SA = South America.

DISCUSSION

The results indicate that some of the Australian populations of lippia show low levels of genetic diversity, whereas other populations have levels of diversity comparable to that found in Argentina and France. In the PCA analysis the Australian populations were nested among Argentinean populations, suggesting that the Australian populations may have originated from these Argentinean populations or regions.

AMOVA showed that most of the variation is distributed within populations. This is supported by a low G_{ST} of 0.0261 which indicates that populations are genetically separated from one another. This also supports our view that Australian populations have originated from multiple introduction events.

Finally, results of phylogenetic analysis of ITS data again support the hypothesis that *Phyla canescens* has been introduced to Australia from multiple source populations.

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9.2 Appendix 2

The following paper has been submitted and accepted for publication in *Molecular Ecology Resources*.

Fatemi M, Gross CL (**In Press**) Development and characterization of microsatellite markers for lippia (*Phyla canescens*: Verbenaceae). *Molecular Ecology Resources*.

Development and characterization of microsatellite markers for lippia (*Phyla canescens*: Verbenaceae)

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Abstract

Lippia (*Phyla canescens*: Verbenaceae) is a serious weed of wetlands, riparian zones and floodplains, particularly in eastern Australia where many Ramsar wetlands are threatened by hydrological changes precipitated by soil-accreting lippia mats. Enriched genomic DNA libraries were used to develop nine informative microsatellite markers. These markers will be valuable tools to understand the genetic structure of the lippia populations in different regions throughout the world.

Keywords: Lippia, *Phyla canescens*, microsatellites, wetlands, weed invasions.

The introduction of exotic species into new environments can have serious consequences on ecosystem function. Lippia (*Phyla canescens*) is a perennial herbaceous, ground creeper, native to South American countries from southern Ecuador, through Peru, Chile, Argentina, Uruguay, Paraguay, and Bolivia (Kennedy 1992). Lippia was introduced as a garden plant to Australia in the first half of 20th century, from South America, but is now a serious environmental weed. It is

currently estimated that lippia is distributed across 5 million hectares in the Murray Darling Basin in Australia (Earl 2003), where it clogs waterways and degrades grazing lands. To date, no population genetic studies on lippia are known so we developed microsatellite primers to study the population genetic structure of infested populations in detail.

DNA was isolated from leaf tissue samples using the DNeasy Plant Kits (QIAGEN). Microsatellite loci were isolated following the standard protocol of Glenn and Schable (2005). Restriction enzymes *Rsa*I and *BstU* I (New England Biolabs) were used to digest genomic DNA. Double-stranded SNX linkers were ligated to DNA fragments and used to amplify the genomic DNA. Amplified DNA was hybridized with a mixture of biotinylated oligos with AG, TG, AAC, AAG, AAT, ACT and ATC repeats. Hybridized DNA was mixed with magnetic beads (Bynabeads) and incubated at room temperature for 30 min. Recovered DNA fragments were amplified using SNX-forward linker as a primer. DNA fragments were ligated into pCR®2.1-TOPO® (Invitrogen) according to the manufacturer's instructions. Following transformation into One Shot® TOP10 Chemically competent *E. coli*, 200 positive clones were selected and screened by PCR using M13 primers. In total 75 PCR products with the right size of inserts (300-1000 bp) were sequenced. Sequences were examined by an SSR identification tool (Temnykh *et al.* 2001) to locate microsatellite motifs. Twenty three pairs of flanking primers were designed using Primer 3 (Rozen and Skaletsky 2000) to amplify the repeated regions. An M13 universal sequence was appended to each forward primer for fluorescent labeling of PCR products according to Schuelke (2000). Microsatellite PCR was carried out on a set of 40 individual plants from two populations, Hurlingham in Argentina and Goondiwindi in Australia. A fluorescent M13 primer (FAM, PET, NED or VIC dyes) was used to label the PCR products. The cycling profile was: 10 min initial denaturation at 95°C; 30 cycles of denaturation at 95°C for 30s, annealing at 59°C for 45s, and extension at 72°C for 1min; followed by 8 cycles of

denaturation at 95°C for 30s, annealing at 53°C for 45s, and extension at 72°C for 1 min; and final 10 min of extension at 72°C. PCR products were visualized on 1% agarose stained with SYBR® safe DNA gel stain (Invitrogen).

PCR products were multiplexed and profiled with ABI 3730 DNA Analyzer. Diversity levels for each locus, number of alleles (N), number of effective alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using GenAlex 6 (Peakall and Smouse 2006). Nine loci gave clearly scorable and polymorphic products. Between four and 20 alleles were detected and values for expected and observed heterozygosities ranged from 0.32 to 0.906 and from 0.0 to 1.0, respectively (Table 1).

Linkage disequilibrium across the loci was tested using GENEPOP on the web version 3.4 (http://genepop.curtin.edu.au) employing Markov chain parameters. No significant linkage disequilibrium was detected in pairwise comparisons across loci. Significant deviation from Hardy-Weinberg equilibrium was observed which given that *Phyla canescens* is reproducing both sexually and asexually is not surprising.

Despite the low level of genetic diversity, which results from the limited number of founder events and also clonal reproduction, these markers display adequate levels of polymorphism and provide valuable tools to investigate the genetic structure of populations.

Acknowledgement

This work was funded by the Australian Government through the Natural Heritage Trust through the Department of Environment and Conservation – NSW; Meat and Livestock Australia and the CRC for Australian Weed Management. This project was an initiative of the NSW Ramsar Managers Network, a group of private and Government wetland managers.

Table 1. Characterization of the nine microsatellite loci developed for *Phyla canescens*. N/N_A, number of alleles/number of effective alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; Ar, Argentina; Au, Australia; * significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$).

Locus	Repeat Motif	Primer sequence 5'-3'	Clone size (bp)	N/N_A Ar - Au	H_E Ar - Au	H_O Ar - Au	*P* values (HWE) Ar - Au	GenBank Accession No.
Phc07	$(GT)_8$	F: TCCGAGGATGCAAGAGTTGA	162	15/4 - 20/2	0.522 - 0.32	0.2 - 0.0	0.018 - 0.000*	EU286572
		R: CCAAAAATGCAATTCCCAATC						
Phc12	$(TG)_7$	F: CGGGGTTATCCTTGTTGATG	208	19/12 - 20/7	0.819 - 0.713	1.0 - 0.75	0.281 - 0.003*	EU286567
		R: GCTTCCCCTCAGAATCCAAT						
Phc13	$(AT)_8$	F: AAGAACTTTACTCTGGCGTACCA	244	4/2 - 14/4	0.5 - 0.61	1.0 - 1.0	0.046 - 0.03	EU286566
		R: CCACCTACAGGTGAAGAATTGG						
Phc15	$(TC)_{12}$	F: TTTAATGCGGCTTCCTTCTG	241	20/9 - 17/4	0.721 - 0.673	0.75 - 0.529	0.034 - 0.151	EU286564
		R: TTGCACGGCCAAATTACAG						
Phc17	$(TC)_{14}$	F: GGAGGCCGTTTCTTTGTTTT	245	18/10 - 16/7	0.833 - 0.783	0.833 - 0.938	0.002 - 0.001*	EU286562
		R: CCAAAGAATATGCTGATCAAAGAG						
Phc18	$(ATG)_6$	F: TGGTCCTTGATGGCATTTTT	227	19/6 - 14/3	0.755 - 0.666	1.0 - 0.643	0.137 - 0.000*	EU286561
		R: CCCAGAGCGAGCTTAATCAG						
Phc20	$(AC)_6$	F: TTGTCCTTTTCCTGCTCCTG	217	19/4 - 17/3	0.544 - 0.32	0.316 - 0.0	0.001 - 0.337*	EU286559
		R: GGCGACTTTTTCCATCGTTA						
Phc21	$(GT)_6$	F: CAATTTGTAAGGCAGAAGATGC	177	19/10 - 6/4	0.823 - 0.583	0.895 - 0.667	0.000 - 0.041*	EU286558
		R: GCAGAATCACCAAACTCTCTCC						
Phc22	$(GA)_{22}$	F: AAAAATGGCTCCTTCCCTTC	296	19/14 - 18/8	0.906 - 0.762	0.684 - 0.389	0.008 - 0.000*	EU286557
		R: GGGTCCCCAAAATACCAAAT						

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