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Soybean Stem Fly, *Melanagromyza sojae* (Diptera: Agromyzidae), in the New World: detection of high genetic diversity from soybean fields in Brazil

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ABSTRACT. Soybean Stem Fly (SSF), *Melanagromyza sojae* (Zehntner), belongs to the family Agromyzidae and is highly polyphagous, attacking many plant species of the family Fabaceae, including soybean and other beans. SSF is regarded as one of the most important pests in soybean fields of Asia (e.g., China, India), North East Africa (e.g., Egypt), parts of Russia, and South East Asia. Despite reports of Agromyzidae flies infesting soybean fields in Rio Grande do Sul State (Brazil) in 1983 and 2009 and periodic interceptions of SSF since the 1940s by the USA quarantine authorities, SSF has not been officially reported to have successfully established in the North and South Americas. In South America, *M. sojae* was recently confirmed using morphology and its complete mitochondrial DNA (mtDNA) was

characterized. In the present study, we surveyed the genetic diversity of *M. sojae*, collected directly from soybean host plants, using partial mtDNA cytochrome oxidase I (COI) gene, and provide evidence of multiple (>10) maternal lineages in SSF populations in South America, potentially representing multiple incursion events. However, a single incursion involving multiple-female founders could not be ruled out. We identified a haplotype that was common in the fields of two Brazilian states and the individuals collected from Australia in 2013. The implications of SSF incursions in southern Brazil are discussed in relation to the current soybean agricultural practices, highlighting an urgent need for better understanding of SSF population movements in the New World, which is necessary for developing effective management options for this significant soybean pest.

Key words: Soybean Stem Fly; Soybean Stalk Fly; Biosecurity; mtDNA COI; Haplotype diversity; Invasive pest

INTRODUCTION

The arrival of invasive species is increasingly associated with human activities such as trade, tourism, and migration. Globally, the rapid spread of invasive pests such as the red imported fire ants, *Solenopsis invicta*, and the *Bemisia tabaci* whitefly 'MED' and 'MEAM1' cryptic species complexes represent excellent examples of the introduction and establishment of alien species as a direct consequence of increased international trade activities (Ascunce et al., 2011; De Barro et al., 2011). The spread of invasive pests has significant negative socio-economic and agricultural impacts. Similarly, the spread of the Yellow Crazy Ant, *Anoplolepis gracilipes*, caused significant destruction of keystone species and the modification of ecosystems, and was likely associated with human migration (Lowe et al., 2001).

Brazilian agriculture has had a long history of exotic pest incursions that significantly affected agricultural production, with increasing frequencies of invasive agricultural pest incursions observed since the start of 1900s (Oliveira et al., 2013). Recent invaders, with significant impact on the agricultural sector in Brazil and neighboring countries, included the Asian Soybean Rust *Phakosphora pachyrhizi*, the spider mite, *Schizotetranychus hindustanicus*, the invasive whitefly, *Bemisia tabaci*, MEAM1 (previously known as 'B biotype'), and the Old World cotton bollworm, *Helicoverpa armigera*.

Another group of invasive insect pests is that of agriculturally important Agromyzidae flies, which are often intercepted at the borders during plant quarantine inspections (e.g., USDA, 1940; Kamiji and Iwaizumi, 2013). The soybean stem fly (SSF; also known as the soybean stem miner), *Melanagromyza sojae* (Zehntner), belongs to the family Agromyzidae and is highly polyphagous, attacking plants such as *Glycine max*, *Phaseolus vulgaris*, *Pisum sativum*,*Vigna angularis*, and other members from the family Fabaceae (Dempewolf, 2004). *M. sojae* has been reported across a wide geographic range but not from North and South Americas (Dempewolf, 2004). It is regarded as one of the most important pests in soybean fields in parts of Russia (Strakhova et al., 2013), in Asia including China (Wang and Gai, 2001), India and Nepal (Thapa, 2012), North East Africa (e.g., Egypt), in parts of South East

Asia (e.g., Indonesia; Van Den Berg et al., 1998), and Australia (Brier and Charleston, 2013). The precise time of the arrival of SSF in Australia is not known, but the fly was first detected in the Lockyer Valley (southeastern region of Queensland) of Australia and was flagged as a potential invasive pest by Shepard et al. (1983) during the survey period between October, 1978 and April, 1981. Since then, a significant SSF outbreak has occurred in soybeans in the Mackay region of tropical North Queensland in 2009, followed by a larger outbreak, much further south in the Casino region of northern New South Wales, in 2013 (Brier and Charleston, 2013). In Brazil, SSF is regarded as a potential invasive pest of soybean (Hirose and Moscardi, 2012).

Soybean (*G. max*) is one of the most important vegetable crops worldwide, which is heavily traded in international market, and has the largest harvested area for any global commodity (Macdonald et al., 2015). In Brazil, 31.5 million hectares of soybean is grown (CONAB, 2015) and over 55 million hectares are cultivated across the South American continent (FAOSTAT, 2015). The first record of Agromyzidae flies infesting soybean fields in Brazil was in the State of Rio Grande do Sul in 1983, in the Municipality of Passo Fundo (Gassen and Schneider, 1985). Link et al. (2009) subsequently published a report that described the occurrence of Agromyzidae flies in five soybean fields at the Municipality of São Francisco de Assis (Figure 1A). Whether these two incidents represented different species and/or were separate incursion events, or if populations in the Municipality of São Francisco de Assis were the progenies of the Rio Grande do Sul populations, is not known.

Morphological identification of Agromyzidae species can be accomplished by assessing the shape of the male genitalia or morphological characters of the larva, and can be aided by the larval feeding habits (Dempewolf, 2004; Thapa, 2012). Using the larval morphological characters and combining the larval feeding behavior, Arnemann et al. (2016a) reported the presence of the soybean stem fly, *M. sojae*, in Brazil, and provided the annotation of a complete mitochondrial DNA (mtDNA) genome of this agriculturally invasive pest. Over 2000 species of Agromyzidae flies have been reported (Thapa, 2012). The development of molecular markers could be a useful tool for identification of these species. Knowledge of mtDNA genes, such as the widely used cytochrome oxidase subunit I (COI), will greatly assist in the confirmation of species, besides providing an opportunity to ascertain the population diversity and to infer possible patterns associated with this incursion.

In the present study, suspected *M. sojae* (SSF) specimens were collected directly from *G. max* host plants to identify the species and to survey for the population diversity via sequencing of the partial mtDNA COI gene. We present preliminary results of Brazilian *M. sojae* population genetic diversity and discuss the potential implications of incursions by *M. sojae* in Brazil.

MATERIAL AND METHODS

Samples

Fly larvae were collected from individual soybean plants that showed characteristic SSF feeding damage, as described by Van Den Berg et al. (1998). The samples were collected from Rio Grande do Sul (RS) and Santa Catarina (SC) states, in the cropping season of 2014/15 (Table 1; Figure 1A-C). The flies were reared in the lab to obtain adults, prior to

preserving them in individual 1.5-mL Eppendorf tubes in 1000 µL 99.9% ethanol, for molecular characterization. Two *M. sojae* samples previously collected from *G. max* at Casino, New South Wales, Australia (Brier and Charleston, 2013) were also included to enable direct comparison of the mtDNA COI gene.

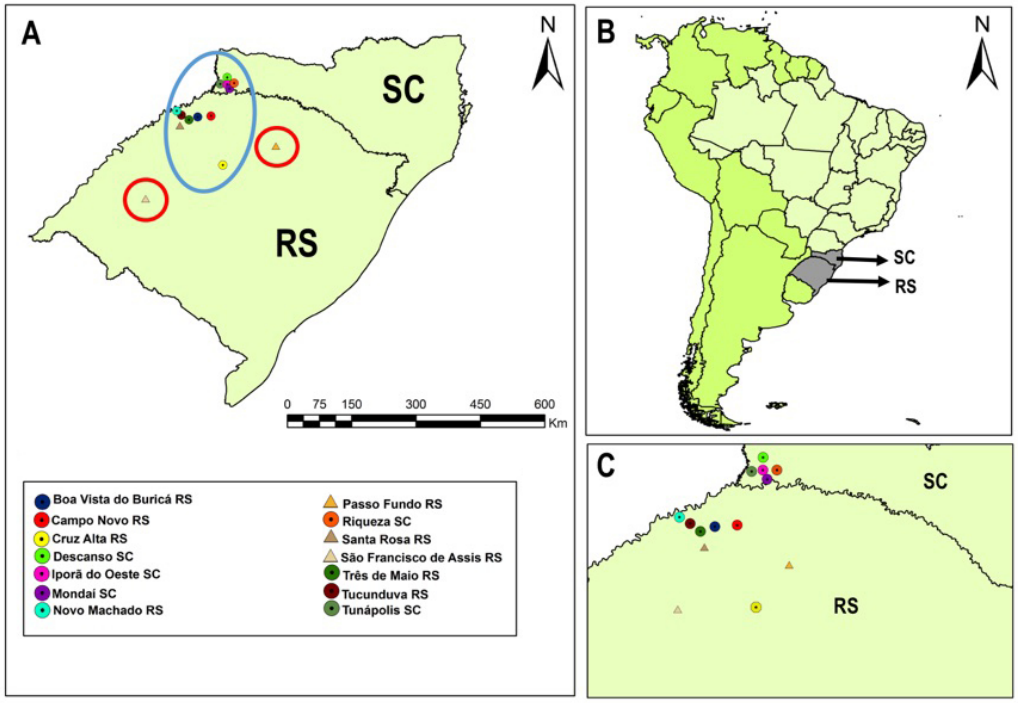

Figure 1. Map of sampling sites in Brazil from which suspected *Melanagromyza sojae* were collected (blue circle; **A**). The red circles in 'A' indicate the locations where individuals of *Melanagromyza* sp were found in 1983 and 2009 but were not identified at the species level. Brazilian states are Santa Catarina (SC) and Rio Grande do Sul (RS; **B**). Enlarged map detailing *M. sojae* collection sites in SC and RS in southern Brazil (**C**).

Total genomic DNA (gDNA) extraction

Individual specimens were washed three times in 1000 µL fresh 99.9% ethanol prior to gDNA extraction. With the exception of three suspected adult *M. sojae* flies where non-destructive gDNA extraction method was used (Tay et al., 2014), total gDNA from all the remaining specimens was extracted either from the legs or partial thorax (for adult Brazilian samples) or from the whole larval body (in the case of Australian samples), using Qiagen DNasy Blood and Tissue DNA Extraction Kit (Qiagen, Hilden, Germany). Final elution of the individual gDNA samples was done in 35 µL Qiagen buffer EB and the gDNA quality was ascertained by visualization of the samples electrophoresed on a 1.5% agarose gel. The DNA was quantified using Qubit 2.0 Fluorometer (Invitrogen Life Technologies, Carlsbad, CA, USA).

PCR amplification and sequencing of partial mtDNA COI gene

Based on the SSF mtDNA COI gene sequence (GenBank accession No. KT597923) characterized from a Brazilian *M. sojae* individual (Arnemann et al., 2016a), we designed an SSF-specific partial mtDNA COI gene PCR primer pairs using the Oligo Primer Analysis Software version 7.60 (Molecular Biology Insights, Inc., Cascade, CO, USA). The primer design was based on the considerations of minimal propensity for primer-dimer and heteroduplex structure formation, minimal false-priming sites to avoid non-specific PCR amplification, and a theoretical melting temperature (Tm) >60°C, calculated using the formula Tm = 2(A+T) + 4(G+C).

PCR conditions for amplification using the designed primer pairs (SSF-COI-F01: 5'-GACAATGATTATTTTCGACAAAT-3'; SSF-COI-R01: 5'-GTAAAATAAGCTCGTGTAT CTACATC-3') were optimized by gradient PCR (at temperatures from 48° to 62°C increased in 8 steps) on a Bio-Rad PCR machine (model C1000 Thermal Cycler, Hercules, CA, USA). The amplified products were visualized on a 1.5% 1X TAE agarose gel after staining with GelRed™ (Biotium, Cat. # 41003, Hayward, CA, USA). The final optimized and standardized PCR profile for the SSF-COI-F/R primers comprised of the following steps: 95°C for 5 min (one cycle), 30 s each at 95°, 61°, and 72°C (34 cycles), followed by a final extension at 72°C for 5 min. The amplicons were held at 4°C after the PCR and stored at -20°C until needed. PCR amplification of individual DNA samples was carried out in a 25-µL total reaction volume that contained 25 ng genomic DNA, 0.5 µM each forward and reverse primer, 0.2 mM dNTPs, 1X Phusion HF Buffer (NEB, Ipswich, MA, USA), and 1.25 U Phusion DNA polymerase (NEB).

Amplicons were purified using a QIAquick® PCR purification Kit (Qiagen) prior to being used as a DNA template for Sanger sequencing reaction using the ABI BigDye® Termination v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction and post-sequencing reaction clean-up was done as specified by the sequencing facility at the Australian National University Biomolecular Resource Facility.

Sequence analysis and molecular characterization of mtDNA COI gene

The programs Pregap and Gap4 within the Staden package (Staden et al., 2000) were used for editing and analyzing the DNA sequences and to generate sequence contigs. Assembled partial mtDNA COI contigs were checked for premature stop codons that could indicate a pseudogene (e.g., nuclear mtDNA) using Geneious R8 (Biomatters Ltd., New Zealand) and BLASTp. Sequences that differed by one or more nucleotides were considered as different haplotypes, whereas sequences exhibiting identical single nucleotide polymorphisms (SNPs) at same nucleotide positions were considered as the same haplotype.

Network mtDNA haplotypes and COI phylogenetic analysis

An mtDNA COI haplotype network for *M. sojae* was constructed manually and verified using the statistical parsimony phylogenetic network estimation program TCS v1.21 (Templeton et al., 1992). The distribution map of the SSF haplotypes was built using PopArt (http://popart.otago.ac.nz). Estimates of evolutionary divergence between all *M. sojae* individuals (N = 25) were from a 740-bp region in the 5'-(N-terminal)-end of the mtDNA

COI gene (corresponding to nucleotide positions 5-911 of KT597923). Evolutionary genetic analyses between *M. sojae* haplotypes were conducted using the MEGA6 software (Tamura et al., 2013). Estimates of haplotype diversity ($h \pm$ SE) and nucleotide diversity ($\pi \pm$ SE) were carried out using the molecular evolution software package DNA Sequence Polymorphism (DnaSP) version 5.10.01 (Librado and Rozas, 2009).

Intra-species nucleotide diversity in Agromyzidae

Estimates of intra-species nucleotide diversity between all *M. sojae* haplotypes involved trimming of all sequences to 740 bp in the final dataset. The evolutionary divergence and the average intra-species evolutionary diversity were estimated using the Maximum Composite Likelihood model (Tamura et al., 2004), and included all codon positions (i.e., 1st + 2nd + 3rd). The partial mtDNA COI dataset (740 bp) did not have gaps and/or missing nucleotide bases for all SSF samples. Evolutionary genetic analyses were conducted using MEGA6 (Tamura et al., 2013).

RESULTS

Species confirmation

Previously, Arnemann et al. (2016a) identified four *M. sojae* individuals from Cruz Alta, Rio Grande do Sul (RS), Brazil, based on the larval morphological characters, notably the "distinctive posterior spiracles, which have a blunt, somewhat atrophied central horn" (Dempewolf, 2004). This was followed by complete sequencing of the mitochondrial genome of an additional *M. sojae* individual (Sample 11 - Table 1). Australian *M. sojae* individuals were also similarly identified based on the morphological characters of their larvae (Brier and Charleston, 2013).

In the present study, the partial mtDNA COI gene region of specimens originating from Brazil were analyzed by comparing against the mtDNA sequences of two confirmed *M. sojae* specimens from Casino (New South Wales), Australia. This comparison identified a shared mtDNA COI haplotype (i.e., Msoj-COI-02; Table 2) between three Brazilian flies (N = 1 from RS; N = 2 from SC) and the two Australian *M. sojae* samples, adding support to the morphological analysis of *M. sojae* invasion in Brazil. The three *M. sojae* adults from which gDNA was non-destructively extracted were deposited as voucher samples in the Australian National Insect Collection (ANIC) at CSIRO, Canberra (ANIC Database numbers 29035952, 29035953, and 29035954).

PCR amplification and sequence analysis

The mtDNA COI gene was chosen because of existing sequence database (e.g., from iBoL: http://www.boldsystems.org) for other Agromyzidae flies, and because surveying the mtDNA COI gene region of SSF would enable future comparison with the existing Agromyzidae sequence database. This is expected to help build the mtDNA COI database for this agriculturally important fly family. A 906-bp fragment of the mtDNA COI gene was PCR amplified using the SSF-specific mtDNA COI primers from 23 suspected individuals as well as from the two Australian *M. sojae* specimens (Table 1). Post-sequencing trimming of the sequenced amplicons resulted in 740-bp partial mtDNA COI contigs in all samples, with no SNPs being present at nucleotide (nt) positions 631 to 740 in all analyzed SSF individuals.

The low estimates of evolutionary divergence between *M. sojae* sequences were as expected at the intra-species level (for example, see Scheffer, 2000) and ranged from 0 to 0.01% (± 0.001-0.004 SE).

Table 1. Collection sites and dates, mtDNA COI GenBank accession numbers and GenBank haplotype locus ID of *Melanogromyza sojae* specimens from Brazil and Australia.

Sample ID	City	State	Sampling date	Accession No.	Haplotype locus
1	Boa Vista do Buricá	RS	April 18, 2015	KT821473	Msoj-COI-01-1
2	Boa Vista do Buricá	RS	April 18, 2015	KT821495	Msoj-COI-08
3	Campo Novo	RS	April 18, 2015	KT821481	Msoj-COI-02-1
4	Cruz Alta	RS	April 18, 2015	KT821474	Msoj-COI-01-2
5	Cruz Alta	RS	April 18, 2015	KT821475	Msoj-COI-01-3
6	Cruz Alta	RS	April 18, 2015	KT821493	Msoj-COI-06
7	Novo Machado	RS	April 17, 2015	KT821489	Msoj-COI-04-1
8	Três de Maio	RS	April 17, 2015	KT821486	Msoj-COI-03-1
9	Três de Maio	RS	April 17, 2015	KT821490	Msoj-COI-04-2
10	Tucunduva	RS	April 17, 2015	KT821476	Msoj-COI-01-4
11	Tucunduva	RS	April 17, 2015	KT821477	Msoj-COI-01-5
12	Descanso	SC	April 15, 2015	KT821483	Msoj-COI-02-3
13	Descanso	SC	April 15, 2015	KT821494	Msoj-COI-07
14	Iporã do Oeste	SC	April 16, 2015	KT821478	Msoj-COI-01-6
15	Iporã do Oeste	SC	April 16, 2015	KT821482	Msoj-COI-02-2
16	Iporã do Oeste	SC	April 16, 2015	KT821487	Msoj-COI-03-2
17	Mondaí	SC	April 15, 2015	KT821496	Msoj-COI-09
18	Mondaí	SC	April 15, 2015	KT821492	Msoj-COI-05
19	Riqueza	SC	April 15, 2015	KT821488	Msoj-COI-03-3
20	Riqueza	SC	April 15, 2015	KT821497	Msoj-COI-10
21	Tunapólis	SC	April 15, 2015	KT821479	Msoj-COI-01-7
22	Tunapólis	SC	April 15, 2015	KT821480	Msoj-COI-01-8
23	Tunapólis	SC	April 15, 2015	KT821491	Msoj-COI-04-3
24	Casino	NSW	March 26, 2013	KT821484	Msoj-COI-02-4
25	Casino	NSW	March 26, 2013	KT821485	Msoj-COI-02-5

Brazilian states are Rio Grande do Sul (RS) and Santa Catarina (SC). The Australian samples were from Casino New South Wales (NSW). All the M. sojae specimens were collected from soybean (Glycine max).

Haplotype patterns

A total of 10 haplotypes (see Table 1 for GenBank accession Nos.) were identified from 23 individuals collected from SC and RS States of Brazil, and two individuals from NSW, Australia (Table 2). From the trimmed 740-bp partial mtDNA COI gene, 12 base substitutions were identified and all involved transition (i.e., purine/purine; pyrimidine/pyrimidine) substitutions (nine T/C; three C/A). Based on the SSF population-wide base substitution patterns, a 'consensus' SNP profile was determined (Table 2) that also matched the Msoj-COI-02 haplotype.

The base substitutions within this haplotype were representative of the majority of SNPs across all the detected haplotypes and were shared between Brazil and Australian populations (albeit at a lower frequency, possibly due to low sampling size). This suggests that the Msoj-COI-02 haplotype is potentially an ancestral haplotype, although accurate inference of phylogenetic relationship between the haplotypes will require multigene analysis (Winkler et al., 2009). Unique and shared haplotypes at the national level (i.e., between SC and RS States, see Figures 2 and 3) and between countries (Brazil and Australia) were also ascertained, with unique haplotypes within Brazil constituting 50% of all the haplotypes identified in the individuals sampled to-date (Table 3).

Table 2. Single nucleotide polymorphisms (SNPs) and haplotypes identified in the field-collected *Melanagromyza sojae* samples from soybean crops in Brazil's Santa Catarina (SC) and Rio Grande do Sul (RS) States, and from Casino, New South Wales (NSW), Australia.

SNP nucleotide position		9	64	81	99	192	231	309	315	343	369	603	630	
Consensus		T	G	C	T	G	C	T	T	C	A	T	T	
Sample ID	State													Haplotype
1	RS	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
4	RS	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
5	RS	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
10	RS	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
14	SC	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
21	SC	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
22	SC	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
11	RS	.	.	.	.	A	.	.	.	.	.	C	.	Msoj-COI-01
3	RS	.	.	.	.	.	.	.	.	.	.	.	.	Msoj-COI-02
15	SC	.	.	.	.	.	.	.	.	.	.	.	.	Msoj-COI-02
12	SC	.	.	.	.	.	.	.	.	.	.	.	.	Msoj-COI-02
24	NSW	.	.	.	.	.	.	.	.	.	.	.	.	Msoj-COI-02
25	NSW	.	.	.	.	.	.	.	.	.	.	.	.	Msoj-COI-02
8	RS	.	.	T	.	.	.	.	.	.	.	.	C	Msoj-COI-03
16	SC	.	.	T	.	.	.	.	.	.	.	.	C	Msoj-COI-03
19	SC	.	.	T	.	.	.	.	.	.	.	.	C	Msoj-COI-03
7	RS	.	.	.	C	.	.	.	.	T	G	.	C	Msoj-COI-04
9	RS	.	.	.	C	.	.	.	.	T	G	.	C	Msoj-COI-04
23	SC	.	.	.	C	.	.	.	.	T	G	.	C	Msoj-COI-04
18	SC	.	A	.	C	.	.	.	.	T	G	.	C	Msoj-COI-05
6	RS	.	.	.	.	.	T	.	.	.	.	.	.	Msoj-COI-06
13	SC	.	.	.		.	.	.	C	.	.	.	.	Msoj-COI-07
2	RS	.	.	.	.	A	.	C	.	.	G	C	.	Msoj-COI-08
17	SC	.	.	.	C	.	.	.	.	T	.	.	.	Msoj-COI-09
20	SC	C	.	.	.	A	.	.	.	.	.	.	.	Msoj-COI-10

Consensus SNP (= Msoj-COI-02 haploytpe) were determined by population majority, nucleotide changes identical to the consensus base substitution patterns and are indicated by a period.

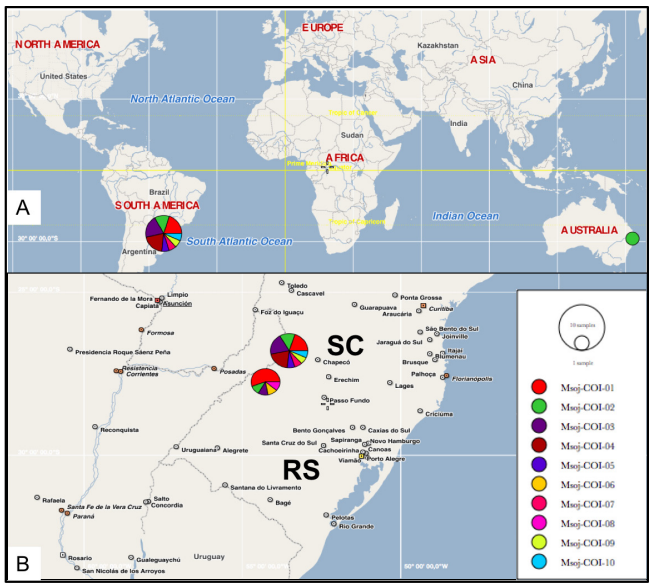

Figure 2. Haplotype distribution patterns and diversity of *Melanagromyza sojae* in Australia and Brazil (**A**) and enlarged map detailing *M. sojae* collection sites in the States of Santa Catarina (SC) and Rio Grande do Sul (RS) in Southern Brazil (**B**).

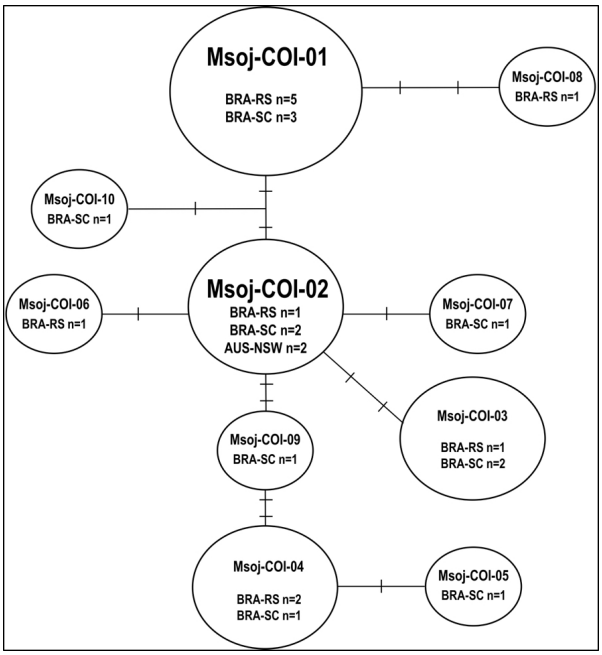

Figure 3. Haplotype network of *Melanagromyza sojae* based on partial (740 bp) mtDNA COI gene, and the included samples from Australia (AUS-NSW) and Brazil (BRA-RS, BRA-SC). Each haplotype is represented by a circle and is identified by 'Msoj-COI-01' to 'Msoj-COI-10'. Haplotype 'Msoj-COI-01' included eight individuals; haplotype 'Msoj-COI-02', 'Msoj-COI-03', and 'Msoj-COI-04' had 5, 3, and 3 individuals, respectively. All the remaining haplotypes had one individual each. Number of base changes that differentiated between the haplotypes are represented by a black bar.

Table 3. Number of unique and shared haplotypes identified in different states of Brazil and Australia.

Country	State (individuals sampled)	Number of haplotypes	Number of unique haplotypes	Number of shared haplotypes
	SC (N = 12)	8	4	4
Brazil	RS (N = 11)	6	2	4
Brazil	SC + RS (N = 23)	10	5	4
Australia	NSW (N = 2)	1	0	1

Number of individuals (N) examined are listed. States within Brazil are Santa Catarina (SC) and Rio Grande do Sul (RS), and that in Australia is New South Wales (NSW).

The highest SSF nucleotide and haplotype diversity was found in SC (Table 4). The pairwise uncorrected (p) genetic distances between all *M. sojae* haplotypes were low (ranging from 0.00 to 0.01) as expected for the intra-species level comparison (Table 5).

Table 4. Comparison of *Melanagromyza sojae* mtDNA partial COI gene nucleotide diversity (π) and haplotype diversity (h) between different Brazilian states.

Local	Nucleotide diversity	Haplotype diversity
Santa Catarina State (SC)	0.00446 ± 0.00064	0.924 ± 0.057
Rio Grande do Sul State (RS)	0.00428 ± 0.00081	0.800 ± 0.114
Brazil (SC + RS)	0.00408 ± 0.00047	0.853 ± 0.048

Table 5. Estimates of evolutionary divergence (nucleotide distances) between all *Melanagromyza sojae* haplotypes based on 740 bp partial mtCOI gene.

	Msoj-COI-01	Msoj-COI-02	Msoj-COI-03	Msoj-COI-04	Msoj-COI-05	Msoj-COI-06	Msoj-COI-07	Msoj-COI-08	Msoj-COI-09	Msoj-COI-10
Msoj-COI-01	-	0.002	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.002
Msoj-COI-02	0.003	-	0.002	0.003	0.003	0.001	0.001	0.003	0.002	0.002
Msoj-COI-03	0.005	0.003	-	0.003	0.003	0.002	0.002	0.003	0.003	0.002
Msoj-COI-04	0.008	0.005	0.005	-	0.001	0.003	0.003	0.003	0.002	0.003
Msoj-COI-05	0.010	0.007	0.007	0.001	-	0.003	0.003	0.003	0.002	0.004
Msoj-COI-06	0.004	0.001	0.004	0.007	0.008	-	0.002	0.003	0.002	0.002
Msoj-COI-07	0.004	0.001	0.004	0.007	0.008	0.003	-	0.003	0.002	0.002
Msoj-COI-08	0.003	0.005	0.008	0.008	0.010	0.007	0.007	-	0.003	0.003
Msoj-COI-09	0.005	0.003	0.005	0.003	0.004	0.004	0.004	0.008	-	0.003
Msoj-COI-10	0.003	0.003	0.005	0.008	0.010	0.004	0.004	0.005	0.005	-

Standard error (SE) estimates from 500 bootstrap replications are shown in the above diagonal.

DISCUSSION

In the present study, we report the development of an SSF-specific mtDNA COI gene PCR marker. We identified 10 mtDNA COI haplotypes in the SSF populations from Brazil and demonstrated that the haplotype Msoj-COI-02 was shared by individuals from both Brazilian states as well as by individuals collected from Australia in 2013.

The nucleotide diversity (π) and haplotype diversity (h) observed in Brazilian *M. sojae* populations (π = 0.0045 and 0.0043, and h = 0.924 and 0.800 for SC and RS, respectively) were higher than those observed in the genetic diversity studies in various dipterans (e.g., *Drosophila lacertosa* lineages in China, He et al., 2007; *Liriomyza sativae* in China, Du et al., 2014). The SSF high genetic diversity was similar to those of other invasive pests that have established in the South America, e.g., Asian citrus psyllids, *Diaphorina citri* (Hemiptera: Liviidae; Guidolin et al., 2014) and the invasive and highly polyphagous lepidopteran pest *H. armigera* (Mastrangelo et al., 2014; Arnemann et al., 2016b). The number of haplotypes identified in the relatively small sample sizes within Brazil suggests high number of maternal lineages in the populations of *M. sojae*, and is reminiscent of the invasive genetic signature of *H. armigera* detected in Brazil to date (e.g., Tay et al., 2013; Mastrangelo et al., 2014). The high number of mtDNA haplotypes detected cannot be explained simply by mutation or divergence of subpopulations. If we assume that *M. sojae* was present in 1983 in Brazil but was not correctly identified (Gassen and Schneider, 1985), approximately 150,000 years would be needed to result in the observed nucleotide diversity from a single founder, if applying the estimated divergence rate of 2.69% per million year in the insect mtDNA COI gene (Papadopoulou et al., 2010). The diversity of maternal lineages can be explained as being the result of multiple introductions that contributed to high propagule pressure and ensured successful establishment of the populations. Alternatively, the current populations might represent the descendants of a large initial 'multi-founder' incursion.

Findings from Arnemann et al. (2016a) and this study confirmed the presence of *M. sojae* in soybean fields in both SC and RS States, possibly with substantial established populations. This study reports the high genotype diversity in the populations of *M. sojae* from soybean fields in two southern Brazilian states. Previously, Gassen and Schneider (1985) reported the occurrence of *Melanogromyza* sp in Brazil, but the species was not determined. Interestingly, the occurrence of *Melanagromyza* sp as reported by Gassen and Schneider (1985) was in the Municipality of Passo Fundo (RS State), only 100 km from some of the current sampling sites (see Figure 1A). Where circumstances permit (e.g., voucher samples were located and deemed to be of sufficiently good quality for molecular genetic analysis),

investigators of future studies should revisit historical Agromyzidae samples [i.e., specimens collected in 1983 and 2009 by Gassen and Schneider (1985) and Link et al. (2009), respectively] to enable a definitive identification of species [e.g., similar to the study of Tay et al. (2013) to confirm the real *B. tabaci* from museum specimens], and therefore better understanding of the history of SSF incursion into South America.

SSF is a polyphagous species feeding on soybean plants as its main host, along with other cultivated legume crops (Verma et al., 1989). In southern regions of South America (i.e., the 'Cone Sul' region), soybean represents one of the 55 economically important crops that are available to *M. sojae*. Together with the substantial soybean area available in the USA (30.7 million hectares in 2013; FAOSTAT, 2015), Brazil (31.5 million hectares in 2015; CONAB, 2015) and neighboring countries, such as Argentina (20.3 million hectares in 2015; USDA, 2015), Paraguay (3.6 million hectares in 2014; USDA, 2015), and Uruguay (1.3 million hectares in 2014; USDA, 2015), there is a significant likelihood that *M. sojae* can establish and sustain population growth. The high reproduction rate (with each female laying an average of 171 eggs) and short generation time (four to five generations per year; Wang, 1979) probably render containment or eradication expensive and unachievable. In addition, early infestations are difficult to detect because of small size of flies, inconspicuous oviposition scars, and larval stem-boring damage, similar to that caused by other soybean stem boring pests. Currently, knowledge on this pest in the New World is lacking, with regard to its potential to establish and spread in the Cone Sul region, its adaptability to native non-agricultural host plants of the region, and its resistance profile to insecticidal chemicals. Nevertheless, the fact that *M. sojae* has been detected across diverse eco-climatic zones at a global scale (Dempewolf, 2004) suggests that it has the potential to establish across the North and South America continents. To assist with biosecurity preparedness, its potential distribution range may benefit from pre-emptive population modeling, as has been carried out for *H. armigera* (Kriticos et al., 2015).

At the global scale, there is a paucity of data relating to life history, evolutionary genetics, ecology, and parameters associated with integrated pest management strategies and insecticide responses in SSF. This information is necessary for better understanding and management of SSF in the affected countries, including Brazil and regions across South America. Future investigations in southern Brazil and neighboring countries will need to focus on the management of this pest. Seed treatments, breeding resistant soybean varieties (see, Wang and Gai, 2001), *Bt* soybeans, biological control [e.g., by parasitoids *Cynipoidea* sp and *Eurytoma melanagromyza*; Jayappa et al. (2002)], and detailed modeling of its potential economic impact in Brazilian soybean fields are all potentially important research directions. Findings from the present study should serve as the basis for establishing a more comprehensive mtDNA database for future comparisons with both native populations endemic to specific regions, as well as with populations representing invasive ranges. This is expected to contribute to better understanding of potential historical and future global incursion pathways of *M. sojae*.

Conflicts of interest

The authors declare no conflict of interest.

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