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Highly diverse and rapidly spreading: *Melanagromyza sojae* threatens the soybean belt of South America

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Abstract The soybean stem fly, *Melanagromyza sojae,* an Asian native insect, has successfully established in Brazil, Argentina, Paraguay and Bolivia. These countries are among the lead global soybean producing nations, being collectively known as the soybean belt of South America. Infestation levels of *M. sojae* grow by the year, facilitated by the lack of efficient management strategies. Previous studies have revealed a high number of maternal lineages in *M. sojae* populations from Southern Brazil and Paraguay, but a comprehensive survey on genetic diversity combining samples from all countries within the South American soybean belt remains absent. We used the mitochondrial DNA cytochrome oxidase I partial gene (mtCOI) to characterize specimens of *M. sojae* collected in fourteen Brazilian sites and one Argentine site, and then combined our mtCOI data with previously published data from Australia, Bolivia, Paraguay, and other Brazilian sites, to investigate genetic diversity in this invasive agricultural pest species. Based on the molecular characterisation of the mtCOI gene, haplotypes Msoj-COI-01 and Msoj-COI-02 have the highest frequencies in the continent. The

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high genetic diversity found is evidence of introductions involving multiple female founders into the continent, and the high proportion of unique mtDNA haplotypes identified from Brazil, Paraguay and Bolivia (~ 50%) suggests potential novel introductions have taken place. The findings from our study will contribute to a better understanding of *M. sojae* genetic diversity in South America, supporting the development of management strategies for this highly invasive pest and assisting with biosecurity preparedness of other emerging Agromyzidae flies of economic importance.

Keywords Agromyzidae · Genetic diversity · *Glycine max* · Molecular characterization · Soybean stem fly

Introduction

Soybean production in South America is concentrated on Brazil, Argentina, Paraguay and Bolivia, in that order. Together, these four countries grow 187 million metric tons of soybean on 58 million hectares annually (50% of the world production; FAOSTAT 2020, USDA 2020), being collectively known as the 'soybean belt' of the continent. South American farmers have historically struggled against many pests and pathogens, which thrive successfully in this tropical/subtropical region and severely impair soybean yield. Non-native arthropod species are among the most damaging and hard-to-control pests within soybean agroecosystems, frequently leading to increased insecticide usage following its introduction into the new environment (Pozebon et al. 2020). Among the long list of arthropod invasions in South America is included the soybean stem fly, *Melanagromyza sojae* (Zehnter) (Diptera: Agromyzidae). Native to East Asia (Spencer 1973), this species was confirmed as occurring in Southern Brazil in 2015 (Arnemann et al. 2016a) and Central-West Brazil in 2018 (Czepak et al. 2018), but is probably present in the country since 1983 (Gassen and Schneider 1985). Field reports also indicate that the pest has reached Brazilian states of Minas Gerais (Southeast region) and Bahia (Northeast region) (CESB 2018). Molecular characterization subsequently confirmed its occurrence in Paraguay (Guedes et al. 2017), Bolivia (Vitorio et al. 2019) and Argentina (Trossero et al. 2020) (see Fig. 1 for the species' current known worldwide distribution).

M. sojae larvae injure soybean plants by boring the main stem and feeding on the pith, leading to yield losses estimated to range between *ca.* 2% in Indonesia (van den Berg et al. 1998) to 40% in India (Jadhav et al. 2013), and reaching as high as 50% in untreated plots in Thailand (Pitaksa et al. 1996). High oviposition rate (170 eggs female^{-1} on average; Wang 1979) and short lifecycle (16–26 days; Spencer 1990) allow the occurrence of at least five generations per crop cycle. Infestation outbreaks are correlated with sowing date and maturation rate, being more frequent on late season (Talekar and Chen 1983) and more damaging on early-maturing soybean cultivars (Talekar 1989). As such, *M. sojae* has become the main pest of second-season soybean (i.e. soybean sowed after maize harvest, from December 31th onwards; Follmann et al. 2017), typically grown on *ca.* 250 thousand hectares of Rio Grande do Sul and Santa Catarina states (Southern Brazil). Late soybean cultivation and survival of volunteer plants in the fields, coupled with presence of alternative overwintering plant hosts (e.g. Persian clover *Trifolium resupinatum;* Ferreira et al. 2020), have allowed the species' populations to survive winter conditions and hit the summer cropping season at production-threatening levels. While some studies have proposed chemical (e.g. Curioletti et al. 2018) and biological (e.g. Beche et al. 2018) strategies for *M. sojae* management, control efficiency is often impaired by the concealed feeding habit of the larvae.

Increasing international trade and human travel activities have facilitated the dispersal of invasive arthropod species (Hurley et al. 2016; Tay et al. 2017; Seebens et al. 2017). The spreading of an invasive species over space and time increases control costs and decreases control effectiveness (Harvey and Mazzotti 2014; Pozebon et al. 2020), as observed in the recent spread of *Spodoptera frugiperda* across Africa and Asia (Goergen et al. 2016; Tay and Gordon 2019). Variability analysis within standard DNA barcode region has provided preliminary evolutionary genetic understanding of invasive pests and enabled a starting point to determine dispersal patterns, while also

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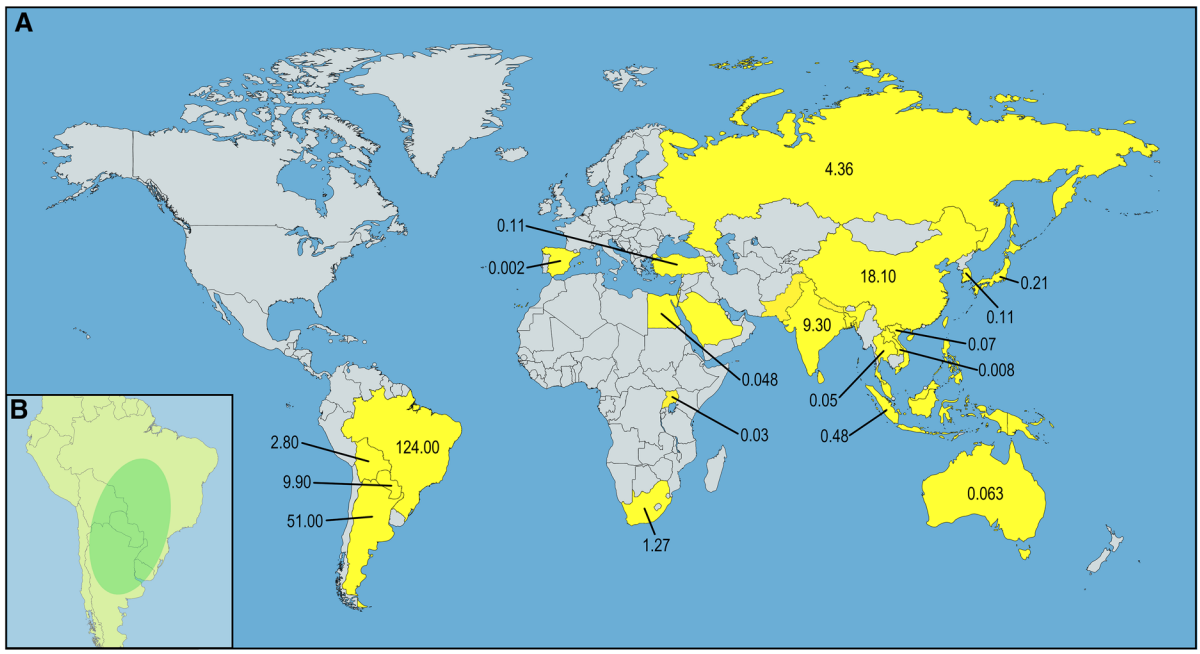

Fig. 1 Worldwide distribution of *Melanagromyza sojae* (yellow colour) and soybean production (million metric tons) of each country in the 2019/20 cropping season (A). In detail (B) the 'soybean belt' of South America, where soybean production in the continent is concentrated. *M. sojae* distribution data obtained from CABI (2020). Soybean production data obtained from FAOSTAT (2020) and USDA (2020)

assisting with molecular species diagnostics (Kang et al. 2018; Madden et al. 2019; Piper et al. 2019). Examples of such approach, as applied to non-native arthropod species in Brazil, include *Helicoverpa armigera* (Tay et al. 2013; Arnemann et al. 2019) and the eucalyptus pest *Thaumastocoris peregrinus* (Machado et al. 2020), while Arnemann et al. characterised the full mitochondrial genome of *M. sojae* (2016b) and developed the cytochrome oxidase I (mtDNA COI) gene as DNA marker (2016a) to facilitate further analyses of the pest genetic diversity in South America. Previous studies have revealed the presence of several mtCOI haplotypes in *M. sojae* populations from Southern Brazil (Arnemann et al. 2016a), Paraguay (Guedes et al. 2017) and Bolivia (Vitorio et al. 2019), hinting at a multiple invasion scenario involving either multiple female founders, and/or involving more than one incursion pathway into the continent. However, a comprehensive analysis combining samples from all countries within the South American soybean belt, and unravelling dispersal routes among them, is still lacking.

In this study, we sequenced part of the mtDNA COI gene of 18 *M. sojae* specimens collected in different localities of Rio Grande do Sul state (Southern Brazil), Goiás state (Central-West Brazil) and Córdoba province in Argentina. Previously published sequences from Brazil, Paraguay and Bolivia were added to the dataset. Haplotype patterns, genetic diversity and the current status of management strategies available for this highly invasive pest, based on data from literature and collected in the present work, are analysed and discussed.

Material and methods

Sampling procedure

Fly larvae were collected from the stems of soybean plants that showed characteristic symptoms of injury by *M. sojae* (van den Berg et al. 1998), in 13 municipalities of Rio Grande do Sul state (Southern Brazil), one municipality of Goiás state (Central-West Brazil) and one municipality of Córdoba province (Argentina), from March 2019 to April 2019 (Table 1, Fig. 2). At each site, the larvae found within soybean stems were manually detached and placed into 1.5 mL

Table 1 Sample list, countries of origin, sample size, host plants and collection dates of *Melanagromyza sojae* individuals used/sequenced by population. Mitochondrial DNA sequences from this study were combined with other sequences deposited in Genbank (Arnemann et al. 2016a; Guedes et al. 2017; Vitorio et al. 2019). Haplotype ID numbers refer to Msoj-COI-01 to 23. Newly identified haplotypes are indicated by '†'. Population acronyms refer to Brazilian states of Rio Grande do Sul (RS), Santa Catarina (SC) and Goiás (GO); Paraguayan departments of Alto Paraná (AP), Canindeyú (CA) and Itapúa (IT); Bolivian department of Santa Cruz (SC); Argentine province of Córdoba (CO) and Australian state of New South Wales (NSW)

Country	Population (sample size)	Municipality	Source	Sampling date	Haplotypes	Reference
Brazil	Rio Grande do Sul (31)	Boa Vista do Buricá	Soybean	Apr.2015	01, 08	Arnemann et al. (2016a, b, c)
		Campo Novo	Soybean	Apr.2015	02	Arnemann et al. (2016a, b, c)
		Catuípe	Soybean	Apr.2019	21†	This study
		Condor	Soybean	Apr.2019	09	This study
		Cruz Alta	Soybean	Apr.2015 (3)	01, 02, 06	Arnemann et al. (2016a, b, c)
				Apr.2019 (1)		This study (1) (3)
		Dois Irmãos das Missões	Soybean	Apr.2015	13	This study
		Giruá	Soybean	Apr.2019	22†	This study
		Itaqui	Soybean	Apr.2019	02	This study
		Novo Machado	Soybean	Apr.2015	04	Arnemann et al. (2016a, b, c)
		Porto Lucena	Soybean	Apr.2019	09	This study
		Santa Maria	Persian clover	Aug.2019	02	Ferreira et al. (2020))
		Santa Rosa	Soybean	Apr.2019	01	This study
		Santo Augusto	Soybean	Apr.2019	13	This study
		Santo Cristo	Soybean	Apr.2019	23†	This study
		São Borja	Soybean	Apr.2019	13	This study
		Seberi	Soybean	Apr.2019	02	This study
		Três de Maio	Soybean	Apr.2015	03, 04	Arnemann et al., (2016a, b, c)
		Três Passos	Soybean	Apr.2019	02, 09	This study
		Tucunduva	Soybean	Apr.2015	01	Arnemann et al. (2016a, b, c)
	Santa Catarina (12)	Descanso	Soybean	Apr.2015	02, 07	Arnemann et al., (2016a, b, c)
		Iporã do Oeste	Soybean	Apr.2015	01, 02, 03	Arnemann et al., (2016a, b, c)
		Mondaí	Soybean	Apr.2015	05, 09	Arnemann et al. (2016a, b, c)
		Riqueza	Soybean	Apr.2015	03, 10	Arnemann et al. (2016a, b, c)
		Tunápolis	Soybean	Apr.2015	01, 04	Arnemann et al. (2016a, b, c)
	Goiás (3)	Silvânia	Soybean	Mar.2019	02, 13	This study

Table 1 continued

Country	Population (sample size)	Municipality	Source	Sampling date	Haplotypes	Reference
Paraguay	Alto Paraná (10)	San Alberto	Soybean	Dec.2015	01, 03, 11, 12, 16	Guedes et al. (2017)
		Santa Rita	Soybean	Dec.2015	01, 09, 14, 15	Guedes et al. (2017)
	Canindeyú (5)	Corpus Christi	Soybean	Dec.2015	01, 07, 13	Guedes et al. (2017)
	Itapúa (8)	Pirapó	Soybean	Dec.2015	01, 02, 08, 09, 13, 17	Guedes et al. (2017)
Bolivia	Santa Cruz (7)	Cuatro Cañadas	Soybean	Jan.2017	13, 19	Vitorio et al. (2019)
		Fernández Alonso	Soybean	Sep.2016	02, 13	Vitorio et al. (2019)
		Santa Rosa del Sara	Soybean	Mar.2017	02	Vitorio et al. (2019)
		Santa Cruz de la Sierra	Soybean	Jan.2017	13	Vitorio et al. (2019)
		Yapacani	Soybean	Nov.2016	20	Vitorio et al. (2019)
Argentina	Córdoba (1)	Cañada de Luque	Chickpea	Nov.2019	11	This study
Australia	New South Wales (2)	Casino	Soybean	Mar.2013	02	Arnemann et al. (2016a, b, c)

vials containing 98% ethanol to preserve their DNA. Each vial was labelled with the municipality, date, and coordinates of the collection site. Vials from all sample collection sites were sent to the Crop Protection Department of Federal University of Santa Maria, Santa Maria, RS, Brazil, where they were stored at –20 °C until genomic DNA extraction.

DNA extraction, PCR amplification, and COI-gene sequencing

The identification of all *M. sojae* specimens was initially confirmed based on morphological traits and feeding behaviour within the plants (Talekar and Chen 1985) prior to being used in DNA extraction. DNA extraction was performed individually for each specimen using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each ethanol-preserved larva was removed from the specimen vial and left to air dry on a paper towel. The entire body was individually macerated in a 1.5 mL tube containing 180 µL of buffer ATL and 20 µL of proteinase K and incubated at 56 °C for 24 h. Subsequently, genomic DNA was purified in a silica-based matrix and eluted in 35 µL of buffer AE. The concentration of DNA was assessed in a spectrophotometer (NanoDrop^{TM} 1000, Thermo Scientific, Wilmington, DE, USA).

A fragment of the mitochondrial COI gene was amplified from 18 individuals through polymerase chain reaction (PCR) using the primers SSF-COI-F and SSF-COI-R and PCR conditions as described in Arnemann et al. (2016a). Briefly, each PCR reaction was performed with a final volume of 25 µL composed by 2.5 µL of JumpStartTM 10X reaction buffer; 1.25 µL of dNTP mix (10 nM of each); 2.0 µL of each primer (10 pM); 0.25 µL of JumpStartTM DNA Polymerase (2.5 U/µL) (Sigma-Aldrich, St. Louis, MO, USA); 2 µL of template DNA (05–100 ng/µL); and 15.0 µL of ultra-pure water. PCR amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by 34 cycles at 95 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplified products were resolved on 1.0% agarose electrophoresis gel, pre-stained with Nancy-520 DNA gel stain (Sigma-Aldrich) and visualized using a gel documentation system. Successfully amplified PCR products were sequenced by ACTGene Molecular Analyses (Alvorada, RS, BR), using the BigDye Terminator method on an ABI 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Data analysis

Quality assessment, trimming, editing, and analysis of each DNA sequence were performed using the softwares Pregap4 and Gap4 within the Staden package (Staden and Bonfield 2000). Geneious R9 (Biomatters

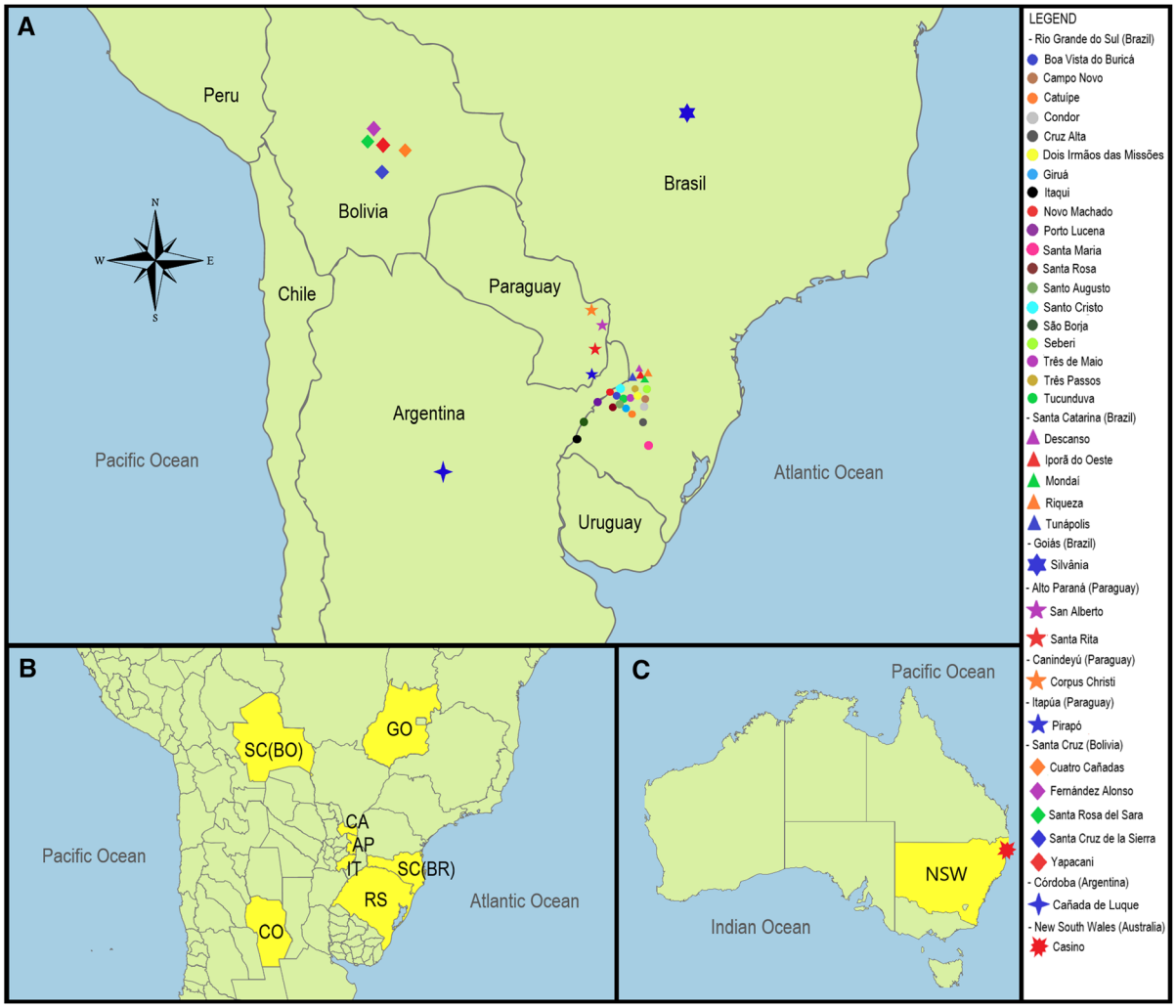

Fig. 2 Municipalities (A) and states, departments or provinces (B) where the specimens of *Melanagromyza sojae* were collected in South America and Australia (C), in the present and previous studies. Acronyms refer to Brazilian states of Rio Grande do Sul (RS), Santa Catarina (SC) and Goiás (GO); Paraguayan departments of Alto Paraná (AP), Canindeyú (CA) and Itapúa (IT); Bolivian department of Santa Cruz (SC); Argentine province of Córdoba (CO) and Australian state of New South Wales (NSW)

Ltd., New Zealand) was used to retrieve and align sequences with a length of 740 base pairs (bp). Presence of premature stop codons was checked through translation of the partial mtCOI contigs into protein sequences, by selecting the invertebrate genetic code 5 for amino acid translation in Geneious R9. The 18 COI sequences generated in this study were combined with 61 published sequences from different municipalities of Rio Grande do Sul state (Brazil; 17 sequences), Santa Catarina state (Brazil; 12 sequences), Alto Paraná department (Paraguay; 10 sequences), Canindeyú department (Paraguay; 5 sequences), Itapúa department (Paraguay; 8 sequences), Santa Cruz department (Bolivia; 7 sequences), and from New South Wales state (Australia; 2 sequences). The final dataset therefore consisted of 79 COI sequences from South America and Australia.

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). Tajima's D (Tajima 1989) was computed for all populations with at least

four samples, to evaluate deviations from the standard neutral model. The inference and visualization of genetic relationships among intraspecific sequences used to generate a haplotype network were conducted using TCS network (Clement et al. 2000) within the program PopART (Leigh and Bryant 2015). Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was conducted on Arlequin 3.5 (Excoffier et al. 2005); the sequences from Argentina and Australia were excluded from AMOVA test due to small sample size. Separate analyses of unlinked loci were also performed, using the locus-by-locus option in Arlequin 3.5. Pairwise uncorrected (p) nucleotide distances (i.e. estimates of evolutionary divergence) between all *M. sojae* haplotypes and populations were calculated in MEGA X (Kumar et al. 2018) with 1000 bootstrap replications. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option).

Results

Fragments of mtDNA from 18 individuals of *M. sojae,* collected in 14 Brazilian sites (Rio Grande do Sul and Goiás states) and one Argentine site (Córdoba province), were amplified and sequenced. Combining our data with sequences previously deposited in GenBank (Arnemann et al. 2016a; Guedes et al. 2017; Vitorio et al. 2019), we obtained a total of 79 *M. sojae* mtDNA COI sequences. The trimmed 740 bp sequences (nucleotide positions 46 to 786 of the mitochondrial *M. sojae* genome, GenBank accession number KT597923) showed no ambiguity and no premature stop codons. Based on a consensus SNP (single nucleotide polymorphism) profile matching haplotype Msoj-COI-02 (Arnemann et al. 2016a), a total of 24 SNPs were identified from the 740 bp partial COI region (Fig. 3). This resulted in 22 mtDNA haplotypes, three of which (named Msoj-COI-21, Msoj-COI-22 and Msoj-COI-23) were novel haplotypes. Haplotype Msoj-COI-18, previously identified in Paraguay (Guedes et al. 2017), was found to be identical to Msoj-COI-01, and thus removed from our haplotype list; all sequences matching it were renamed as Msoj-COI-01. The new mtDNA haplotypes Msoj-COI-21, M-soj-COI-22 and Msoj-COI-23 generated from this study were submitted to GenBank (accession numbers: MW222178, MW222179 and MW222180, respectively). All base substitutions identified involved transition (i.e. purine/purine; pyrimidine/pyrimidine) substitutions (sixteen T/C, eight G/A). Also, of the 24 variable (polymorphic) sites, 14 were singleton variable sites (two variants) and 10 were parsimony informative sites (two variants); there were no variable sites with three or four variants.

The observed nucleotide diversity between countries was low and ranged from 0.0037 to 0.0043 (Table 2). The highest nucleotide and haplotype diversities were found in Santa Catarina state (Brazil) and Alto Paraná department (Paraguay), probably due to larger sample size in these localities. For the Tajima's D estimates, all countries analysed (Brazil, Paraguay and Bolivia) showed negative D-values, indicating high level of low frequency polymorphisms (i.e. excess of rare alleles). This indicates that *M. sojae* populations within these countries are not constant in size or close to mutation-drift equilibrium, which is expected from an invasive species outside its native range. However, all D-values were associated with non-significant *P*-values (> 0.10), making further interpretations unreliable. Unique haplotypes within Brazil, Paraguay and Bolivia constituted *ca.* 50% of all haplotypes identified in each country (Table 2), indicating that separate invasion events may have introduced the species in each country from overseas.

The pairwise uncorrected (p) genetic distances between all *M. sojae* haplotypes were low (ranging from 0.00 to 0.014; see Fig. 4) as expected for the intra-species level comparison (e.g. Scheffer 2000; Arnemann et al. 2016a). Paraguay presented the highest evolutionary divergence from Argentina, Bolivia and Brazil (0.005; see Fig. 5). Analysis of Molecular Variance (AMOVA) detected no genetic structure at various hierarchical levels (Table 3), with almost all variation (98.82%) accounted for at the within population (i.e. within states) level; the variation observed among separate geographical groups (i.e. among countries) was small, and among populations within groups (i.e. among states), negligible. Locus-by-locus AMOVA presented similar results, with the majority of variation detected within populations for all 24 polymorphic loci (Supplementary Table 1). The associated *P*-values were non-significant for all loci except 192 and 603 (at the among groups level), further supporting the lack of genetic structure in the invaded range.

SNP position	54	72	81	99	126	144	237	258	264	273	276	288	291	334	354	360	388	414	549	552	601	648	675	771	n
Consensus	T	C	A	G	C	T	G	T	C	T	C	C	T	G	T	T	C	A	G	G	G	T	T	T	
Haplotype																									
Msoj-COI-1	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	16
Msoj-COI-2	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	21
Msoj-COI-3	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	4
Msoj-COI-4	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	T	G	*	*	*	*	C	*	3
Msoj-COI-5	*	*	*	A	*	C	*	*	*	*	*	*	*	*	*	*	T	G	*	*	*	*	C	*	1
Msoj-COI-6	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	1
Msoj-COI-7	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	2
Msoj-COI-8	*	*	*	*	*	*	A	*	*	*	*	*	*	*	C	*	*	G	*	*	*	C	*	*	2
Msoj-COI-9	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	6
Msoj-COI-10	C	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	1
Msoj-COI-11	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	G	*	*	*	*	*	*	2
Msoj-COI-12	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	C	*	*	1
Msoj-COI-13	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	T	G	*	*	*	*	*	*	10
Msoj-COI-14	*	*	*	*	*	*	A	C	*	*	*	T	*	*	*	*	*	*	*	A	*	C	*	*	1
Msoj-COI-15	*	*	G	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	1
Msoj-COI-16	*	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	1
Msoj-COI-17	*	*	*	*	*	*	A	*	*	*	*	*	C	*	*	*	*	*	*	*	*	C	*	*	1
Msoj-COI-19	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A	A	C	*	*	1
Msoj-COI-20	*	*	*	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	1
Msoj-COI-21	*	T	*	*	*	C	*	*	*	*	*	*	*	*	*	*	T	G	*	*	*	*	*	*	1
Msoj-COI-22	*	*	*	T	*	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	*	*	C	*	1
Msoj-COI-23	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	C	1

Fig. 3 Single nucleotide polymorphisms (SNPs) and haplotypes identified from this study and previous sequences deposited in Genbank (Arnemann et al. 2016a; Guedes et al. 2017; Vitorio et al. 2019). Consensus SNPs were determined by population majority, corresponding to the mtCOI haplotype Msoj-COI-02. SNP nucleotide positions were numbered according to the *Melanagromyza sojae* mitogenome (KT597923), corresponding to the mtCOI haplotype Msoj-COI-01. Nucleotide changes identical to the consensus base substitution patterns are indicated by an asterisk. The final column shows the total individuals identified for each mtCOI haplotype (n). Msoj-COI-02 includes the two individuals sampled from Australia

The proportions of the different haplotypes among countries and among states are presented on a haplotype network (Fig. 6) and a haplotype distribution map (Fig. 7). The most prevalent haplotype was Msoj-COI-02, found in all countries except Argentina. Msoj-COI-01 and Msoj-COI-13 follow as second and third most frequent haplotypes, respectively, attesting to their ecological competence and adaptability to South American conditions. Together, haplotypes Msoj-COI-01 and Msoj-COI-02 represent 47% of all specimens sampled in the continent, and 54% of those sampled in the Brazilian state of Rio Grande do Sul. Accordingly, these two haplotypes (Msoj-COI-01 and Msoj-COI-02) occupied central positions in the network distribution. Ten nucleotide substitutions separated the haplotypes most apart from each other (Msoj-COI-14 and Msoj-COI-05; see Fig. 6), but they were located geographically near (Alto Paraná department in Paraguay and Santa Catarina state in Brazil, respectively; see Fig. 7). Furthermore, these two haplotypes diverged with five mutation steps from the major haplotype Msoj-COI-02, more than any other.

Discussion

Haplotype patterns

Our study updates information on the genetic diversity and mtDNA COI haplotype distribution of *M. sojae* in the soybean belt of South America. Samples from 14 Brazilian sites were collected and analysed, alongside one sample from Argentina. We combined our mtDNA data with published sequences of *M. sojae* from 21 other South American sites (Table 1), revealing the presence of 22 different haplotypes in the continent, three of which were previously unidentified (Msoj-COI-21, Msoj-COI-22 and Msoj-COI-23, from municipalities of Catuípe, Giruá and Santo Cristo, Rio Grande do Sul state, Brazil). The specimen from Argentina was identified as haplotype Msoj-COI-11, also reported in Paraguay (municipality of San Alberto, Alto Paraná) but not in Bolivia and Brazil. The specimens from Goiás state (Brazil) matched haplotypes Msoj-COI-02 and Msoj-COI-13, the two most frequent haplotypes alongside Msoj-COI-01. Msoj-COI-02 was also the haplotype identified in Australia (Arnemann et al. 2016a) and found overwintering on *T. resupinatum* plants from Southern Brazil (Ferreira et al. 2020), thus appearing to thrive particularly well under South American conditions.

Table 2 *Melanagromyza sojae* partial (740 bp) mtDNA COI gene nucleotide diversity (π), haplotype diversity (*h*), Tajima's D, number of unique and shared haplotypes identified in different states, departments and provinces of Brazil, Paraguay, Bolivia, Argentina and Australia. Diversity analyses were conducted on DNA Sequence Polymorphism (DnaSP) version 5.10.01 (Librado and Rozas 2009)

Country	Population	Number of samples	Nucleotide diversity	Haplotype diversity	Tajima's D[a]		Number of haplotypes	Number of unique haplotypes	Number of shared haplotypes
					D-value	*P*-value			
Brazil	Rio Grande do Sul (RS)	31	0.00362 ± 0.00041	0.834 ± 0.050	− 0.0857	ns	11	4	7
	Santa Catarina (SC)	12	0.00446 ± 0.00064	0.924 ± 0.057	− 0.0104	ns	8	3	5
	Goiás (GO)	3	0.00270 ± 0.00127	0.667 ± 0.314	–	–	2	0	2
	Brazil (RS + SC + GO)	46	0.00371 ± 0.00033	0.846 ± 0.037	− 0.4257	ns	14	8	6
Paraguay	Alto Paraná (AP)	10	0.00489 ± 0.00080	0.933 ± 0.077	− 1.2277	ns	8	4	4
	Canindeyú (CA)	5	0.00459 ± 0.00092	0.800 ± 0.164	1.241	ns	3	0	3
	Itapúa (IT)	8	0.00396 ± 0.00070	0.929 ± 0.084	0.4034	ns	6	1	5
	Paraguay (AP + CA + IT)	23	0.00433 ± 0.00047	0.897 ± 0.050	− 1.1037	ns	13	5	8
Bolivia	Santa Cruz (SC)	7	0.00386 ± 0.00087	0.810 ± 0.130	− 0.0634	ns	4	2	2
Argentina	Córdoba (CO)	1	–	–	–	–	1	0	1
Australia	New South Wales (NSW)	2	–	–	–	–	1	0	1
Overall	79	0.00391 ± 0.00026	0.870 ± 0.023	–	–	22	–	–	

[a]Neutrality test conducted for populations with sample size ≥ 4. *ns* non-significant *P*-value (> 0.10) (Tajima 1989)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	19	20	21	22	23
Msoj-COI-01		0.002	0.003	0.004	0.004	0.003	0.003	0.002	0.003	0.002	0.003	0.001	0.003	0.003	0.002	0.003	0.001	0.003	0.003	0.004	0.004	0.000
Msoj-COI-02	0.003		0.002	0.003	0.003	0.001	0.001	0.003	0.002	0.002	0.002	0.003	0.002	0.003	0.003	0.001	0.003	0.003	0.001	0.003	0.003	0.002
Msoj-COI-03	0.006	0.003		0.003	0.003	0.003	0.002	0.004	0.003	0.003	0.003	0.003	0.003	0.004	0.004	0.002	0.003	0.003	0.003	0.003	0.001	0.003
Msoj-COI-04	0.008	0.006	0.006		0.001	0.003	0.003	0.004	0.002	0.004	0.002	0.004	0.001	0.004	0.004	0.003	0.004	0.004	0.003	0.002	0.003	0.004
Msoj-COI-05	0.010	0.007	0.007	0.001		0.003	0.003	0.004	0.002	0.004	0.002	0.004	0.002	0.005	0.004	0.003	0.004	0.004	0.004	0.002	0.003	0.004
Msoj-COI-06	0.004	0.001	0.004	0.007	0.008		0.002	0.003	0.002	0.003	0.002	0.003	0.003	0.004	0.003	0.002	0.003	0.003	0.002	0.003	0.003	0.003
Msoj-COI-07	0.004	0.001	0.004	0.007	0.008	0.003		0.003	0.002	0.002	0.002	0.003	0.003	0.004	0.003	0.002	0.003	0.003	0.002	0.003	0.003	0.003
Msoj-COI-08	0.003	0.006	0.008	0.008	0.010	0.007	0.007		0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.002	0.003	0.003	0.004	0.003	0.002
Msoj-COI-09	0.006	0.003	0.006	0.003	0.004	0.004	0.004	0.008		0.003	0.002	0.003	0.001	0.004	0.004	0.002	0.003	0.003	0.003	0.002	0.003	0.003
Msoj-COI-10	0.003	0.003	0.006	0.008	0.010	0.004	0.004	0.006	0.006		0.003	0.002	0.003	0.003	0.003	0.003	0.002	0.003	0.003	0.003	0.003	0.002
Msoj-COI-11	0.006	0.003	0.006	0.003	0.004	0.004	0.004	0.006	0.003	0.006		0.003	0.001	0.004	0.004	0.002	0.003	0.003	0.002	0.002	0.003	0.003
Msoj-COI-12	0.001	0.004	0.007	0.010	0.011	0.006	0.006	0.004	0.007	0.004	0.007		0.003	0.003	0.003	0.003	0.002	0.003	0.003	0.004	0.004	0.001
Msoj-COI-13	0.007	0.004	0.007	0.001	0.003	0.006	0.006	0.007	0.001	0.007	0.001	0.008		0.004	0.004	0.003	0.004	0.004	0.003	0.001	0.003	0.003
Msoj-COI-14	0.004	0.007	0.010	0.013	0.014	0.008	0.008	0.007	0.010	0.007	0.010	0.006	0.011		0.003	0.004	0.003	0.003	0.004	0.004	0.004	0.003
Msoj-COI-15	0.003	0.006	0.008	0.011	0.013	0.007	0.007	0.006	0.008	0.006	0.008	0.004	0.010	0.007		0.003	0.002	0.004	0.003	0.004	0.004	0.002
Msoj-COI-16	0.004	0.001	0.004	0.007	0.008	0.003	0.003	0.007	0.004	0.004	0.004	0.006	0.006	0.008	0.007		0.003	0.003	0.002	0.003	0.003	0.003
Msoj-COI-17	0.001	0.004	0.007	0.010	0.011	0.006	0.006	0.004	0.007	0.004	0.007	0.003	0.008	0.006	0.004	0.006		0.003	0.003	0.004	0.004	0.001
Msoj-COI-19	0.004	0.004	0.007	0.010	0.011	0.006	0.006	0.007	0.007	0.007	0.007	0.006	0.008	0.006	0.007	0.006	0.006		0.003	0.004	0.004	0.003
Msoj-COI-20	0.004	0.001	0.004	0.007	0.008	0.003	0.003	0.007	0.004	0.004	0.004	0.006	0.006	0.008	0.007	0.003	0.006	0.006		0.003	0.003	0.003
Msoj-COI-21	0.008	0.006	0.008	0.003	0.004	0.007	0.007	0.008	0.003	0.008	0.003	0.010	0.001	0.013	0.011	0.007	0.010	0.010	0.007		0.004	0.004
Msoj-COI-22	0.007	0.004	0.001	0.007	0.008	0.006	0.006	0.007	0.007	0.007	0.007	0.008	0.008	0.011	0.010	0.006	0.008	0.008	0.006	0.010		0.004
Msoj-COI-23	0.000	0.003	0.006	0.008	0.010	0.004	0.004	0.003	0.006	0.003	0.006	0.001	0.007	0.004	0.003	0.004	0.001	0.004	0.004	0.008	0.007	

Fig. 4 Pairwise uncorrected (p) nucleotide distances between all *Melanagromyza sojae* haplotypes. The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). This analysis involved 22 nucleotide sequences, with a total of 740 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018)

	ARG-CO	AUS-NSW	BOL-SC	BRA-GO	BRA-RS	BRA-SC	PAR-AP	PAR-CA	PAR-IT
ARG-CO		0.002	0.001	0.001	0.002	0.002	0.002	0.001	0.002
AUS-NSW	0.003		0.001	0.001	0.001	0.001	0.001	0.001	0.001
BOL-SC	0.003	0.003		0.001	0.001	0.001	0.002	0.001	0.001
BRA-GO	0.002	0.001	0.003		0.001	0.001	0.001	0.001	0.001
BRA-RS	0.004	0.002	0.004	0.003		0.001	0.001	0.001	0.001
BRA-SC	0.004	0.003	0.004	0.003	0.004		0.001	0.001	0.001
PAR-AP	0.005	0.003	0.005	0.004	0.005	0.005		0.001	0.001
PAR-CA	0.004	0.003	0.004	0.003	0.004	0.004	0.005		0.001
PAR-IT	0.004	0.003	0.004	0.003	0.004	0.004	0.004	0.004	

Fig. 5 Pairwise uncorrected (p) nucleotide distances over sequence pairs between *Melanagromyza sojae* populations from: Córdoba province, Argentina (ARG-CO); New South Wales state, Australia (AUS-NSW); Santa Cruz department, Bolivia (BOL-SC); Goiás state, Brazil (BRA-GO); Rio Grande do Sul state, Brazil (BRA-RS); Santa Catarina state, Brazil (BRA-SC); Alto Paraná department, Paraguay (PAR-AP); Canindeyú department, Paraguay (PAR-CA); Itapúa department, Paraguay (PAR-IT). The number of base substitutions per site from averaging over all sequence pairs between groups are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). This analysis involved 79 nucleotide sequences, with a total of 740 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018)

The mtDNA COI haplotype network (i.e. haplotype clustering patterns) revealed a pattern of genetic variation consistent between regions, with each haplotype (when present in two or more individuals) showing a wide geographic distribution (see Fig. 6). Such pattern is common in species capable of long-range movement (Avise et al. 1987) and has also been detected on mitochondrial DNA analysis of *H. armigera* (Behere et al. 2007). Together with the low among-group haplotype variance (6.03%; see Table 3) and low nucleotide diversity (0.0037—0.0043; see Table 2) observed between countries, this could be evidence of high mobility and long distance gene flow in this pest species; however, further *F*-statistic analysis are necessary to support this hypothesis. Similar heterogeneous haplotype patterns have also been detected on *H. armigera* populations across the Cone Sul region of South America (i.e. Argentina, Paraguay, Uruguay, and the Southern Brazilian states of Rio Grande do Sul, Santa Catarina and Paraná;

Table 3 Analysis of molecular variance (AMOVA) reflecting the correlation of *Melanagromyza sojae* mtDNA haplotype diversity at different levels of hierarchical subdivision. Groups correspond to countries and populations correspond to states/departments within each country. Distance method used was by pairwise difference. The significance of the variance components and fixation indices (ϕ–statistics) was tested using 1000 permutations. Analyses were conducted in Arlequin 3.5 (Excoffier et al. 2005)

Source of variation	*df*[a]	Sum of squares	Variance component[b]	% variation	Significance tests (1000 permutations)[c]			Φ-statistics
						P (rand. > obs.)	*P* (rand. = obs.)	*P*-value	
Among groups	2	5.056	σ_a^2	0.09041	6.03	$\sigma_a^2 \times \Phi_{CT}$	0.01564	0.01271	0.02835 ± 0.00561	Φ_{CT} = 0.06027
Among populations	4	3.268	σ_b^2	-0.07278	-4.85	$\sigma_b^2 \times \Phi_{SC}$	0.95699	0.00000	0.95699 ± 0.00592 ns	Φ_{SC} = -0.05164
Within populations	69	102.282	σ_c^2	1.48234	98.82	$\sigma_c^2 \times \Phi_{ST}$	0.53959	0.00000	0.53959 ± 0.01954 ns	Φ_{ST} = 0.01175
Total	75	110.605		1.49997	100.00					

[a]Degrees of freedom

[b]Negative values indicate excess of heterozygotes and should be interpreted as zero (Schneider et al. 2000)

[c]Statistical significance: probability of random value > observed Φ-value and of random value = observed Φ-value (Excoffier et al. 1992). *ns* non-significant *P*-value (> 0.05)

Arnemann et al. 2019) and Asian citrus psyllid *Diaphorina citri* in Brazil (Guidolin et al. 2014). Both studies suggested multiple introductions of these species as the main factor leading to mtDNA genetic signatures resembling a rapid population expansion.

Long distance migration and gene flow could also explain why the *M. sojae* haplotype clusters observed in the haplotype network (Fig. 6) are seemingly unrelated to the geographical separation between the populations (i.e. country and state boundaries). Alternatively, this could be evidence of a potential selection process occurring over a long timescale and within the species' native range, with the resulting haplotype diversity being introduced in South America through a minimum of two separate introduction events. Although haplotype distribution was not location-specific for South America, whether they might be related to geographic distribution in Asia remained to be investigated. Nonetheless, care should be taken to draw strong evolutionary conclusions based on such short sequences as the mtDNA COI, especially when presenting low number of SNPs. Combined evidence from mtDNA COI and rapidly evolving nuclear non-coding sequences (i.e. intron sequences) will be needed to correctly ascertain the distribution of common and rare *M. sojae* haplotypes among different South American regions.

Genetic diversity

The overall nucleotide diversity ($\pi = 0.00391 \pm 0.00026$) and haplotype diversity ($h = 0.870 \pm 0.023$) observed in South American *M. sojae* populations were higher than those observed in genetic diversity studies of other dipterans (e.g. *Liriomyza sativae* in China; Du et al. 2014) and similar to other invasive pests that have established in South America (e.g. *Helicoverpa armigera;* Mastrangelo et al. 2014, Arnemann et al. 2016c). The high number of mtDNA haplotypes identified in the relatively small sample sizes within South America resembles the invasive genetic signature of *H. armigera* detected in Brazil (Tay et al. 2013; Mastrangelo et al. 2014) and cannot be explained simply by mutation or divergence of subpopulations, as invasive species require a long evolutionary timeframe to increase their genetic diversity in a new environment (Dlugosch and Parker 2008; Garnas et al. 2016). Mutation rates in the insect mtDNA COI gene are typically around 2% between

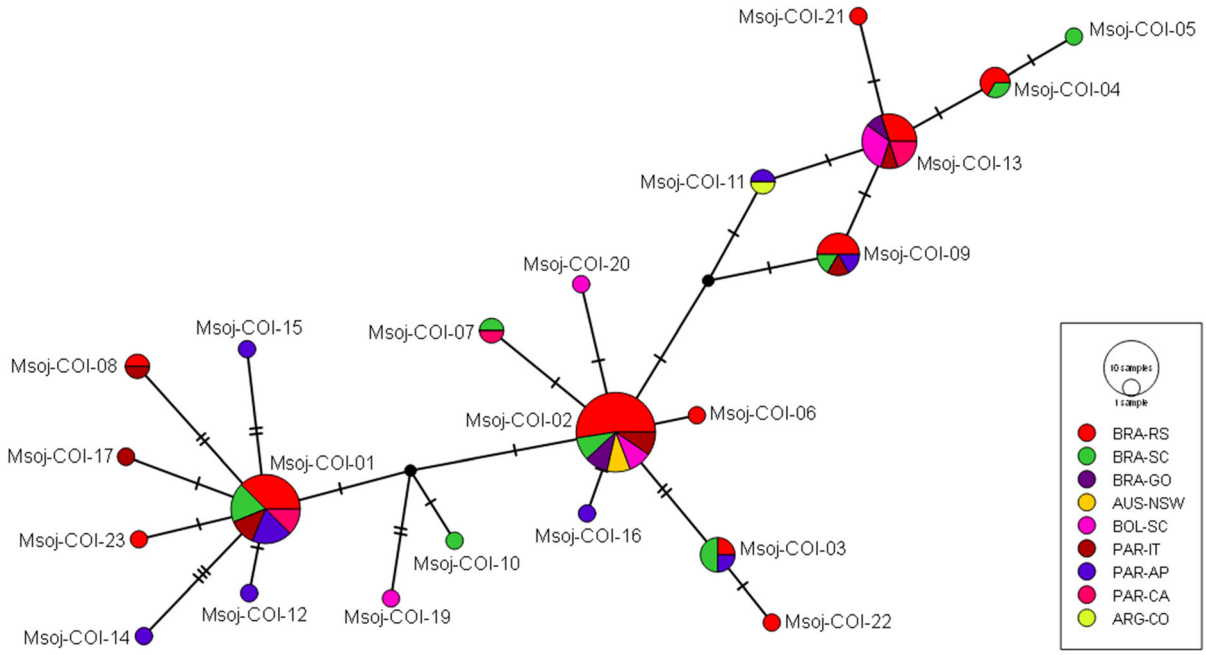

Fig. 6 Haplotype network based on partial mtDNA COI (740 bp) of *Melanagromyza sojae*. Circle sizes indicate approximate proportion of haplotype frequencies. Each mark represents one nucleotide difference. Population acronyms refer to Rio Grande do Sul state, Brazil (BRA-RS); Santa Catarina state, Brazil (BRA-SC); Goiás state, Brazil (BRA-GO); New South Wales state, Australia (AUS-NSW); Santa Cruz department, Bolivia (BOL-SC); Itapúa department, Paraguay (PAR-IT); Alto Paraná department, Paraguay (PAR-AP); Canindeyú department, Paraguay (PAR-CA); Córdoba province, Argentina (ARG-CO). Analyses were conducted in PopART (Leigh and Bryant 2015)

lineages per million years, or approximately 1 mutation per 100,000 years (e.g. Powell 1986; Behere et al. 2007; Papadopoulou et al. 2010).

The rates of nucleotide substitution in *Drosophila* for both mitochondrial DNA and nuclear DNA are similar (Powell 1986) and have been estimated at 2.8×10^{-9} per site per generation (Keightley et al. 2014), including both coding and non-coding regions, and it is reasonable to presume that other dipterans (such as *M. sojae*) would present similar rates. Assuming that *M. sojae* was not present in South America prior to 1983 (Gassen and Schneider 1983), post-introduction mutations and/or population divergence from a single founder could not account for the high number of maternal lineages found in the continent. On the other hand, if multiple introductions of *M. sojae* took place in South America instead of just one, this haplotype diversity can be explained as being the result of high propagule pressure (Simberloff 2009; Simberloff et al. 2013) that ensured successful establishment of the populations, as reported for other invasive pest species in Brazil (e.g. *Bemisia tabaci* MED, Barbosa et al. 2015; *H. armigera*, Arnemann et al. 2019). The results from Tajima's neutrality test point to the same conclusion.

The positive Tajima's D values found for Canindeyú and Itapúa populations (Paraguay) could be indicative of a recent introduction of different populations (Tay et al. 2020), while the negative D values found for most populations (and for countries as a whole) likely reflect a population expansion after a recent selective sweep or bottleneck process. This discrepancy (i.e. positive values for Canindeyú and Itapúa and negative for the others) is probably linked to the small sample sizes from these two Paraguayan departments, as Tajima's D values are expected to become more negative with increasing number of individuals due to introduction of new mutations occurring in low frequencies or even as singletons (Larsson et al. 2013). Tajima's D estimates are highly sensitive to sample size (Nelson et al. 2012; Larsson et al. 2013) and should be considered with caution when sample size is small (< 50, according to Simonsen et al. 1995 and Marroni et al. 2011),

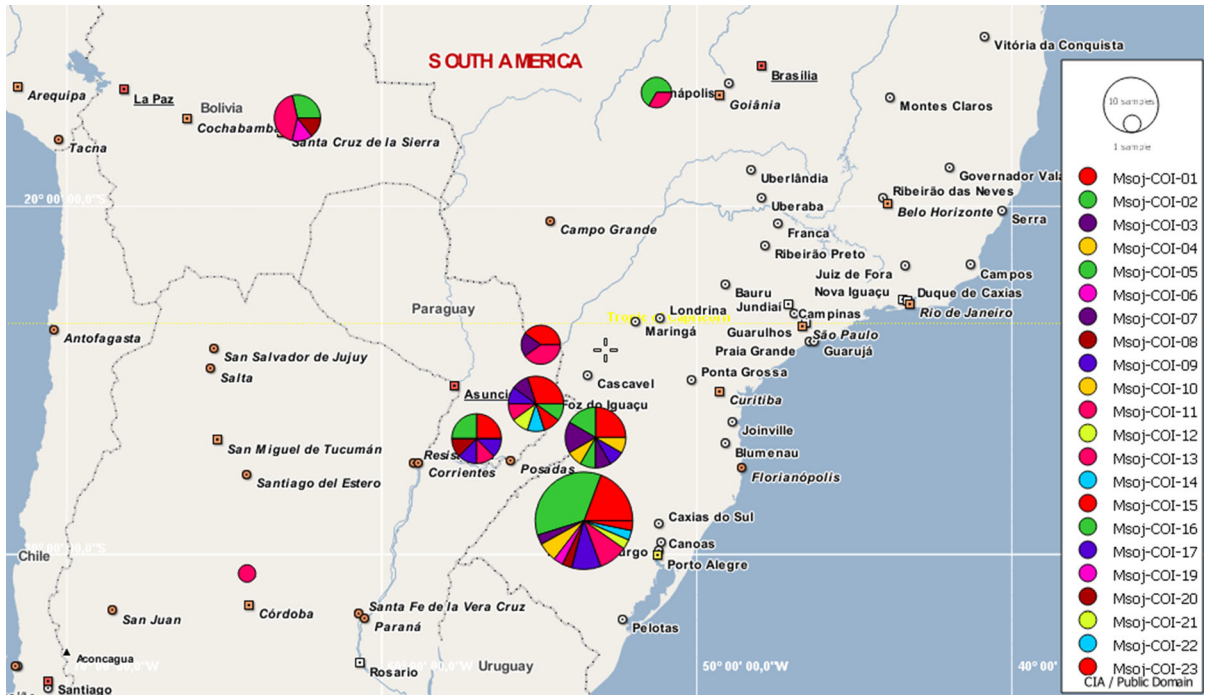

Fig. 7 Haplotype distribution of *Melanagromyza sojae* in South America. Localities: Rio Grande do Sul state, Brazil (BRA-RS); Santa Catarina state, Brazil (BRA-SC); Goiás state, Brazil (BRA-GO); Santa Cruz department, Bolivia (BOL-SC); Itapúa department, Paraguay (PAR-IT); Alto Paraná department, Paraguay (PAR-AP); Canindeyú department, Paraguay (PAR-CA); Córdoba province, Argentina (ARG-CO). The size of pie charts is relative to the number of individuals sequenced at each locality. Analyses were conducted in PopART (Leigh and Bryant 2015)

especially when the associated *P*-values are non-significant (> 0.10; see Table 2). Thus, a larger number of individuals per population as well as a larger number of loci would be required before firm conclusions are drawn regarding *M. sojae* intra-species diversity in South America.

Population structure

The results from the AMOVA test (Table 3) support the hypothesis of multiple recent introductions from different *M. sojae* source populations, associated with limited genetic structure at the invaded range. Almost all haplotype diversity (98.82%) was identified as occurring within each population, rather than among groups or among populations ($P = 0.53959 \pm 0.01954$). Populations among groups were shown to be significantly ($P = 0.02835 \pm 0.00561$) but minimally differentiated, whereas the variation among populations within groups was negligible and not statistically significant ($P = 0.95699 \pm 0.00592$). Locus-by-locus AMOVA test (Supplementary Table 1) detected non-significant *P*-values for all 24 polymorphic loci, except for loci 192 and 603 at the among groups level. Non-significant *P*-values are often linked to small sample sizes per group, as a minimum of six total populations and 20 unique permutations are needed to detect $P < 0.05$ at higher-level population structure (Fitzpatrick 2009). For three groups and seven populations (set in 3–3-1 scheme as in the present study), the minimum number of unique permutations according to the multinomial coefficient (Ross 1998) is 70 and the minimum expected *P*-value is 0.0143 (see Table 2 in Fitzpatrick 2009), assuming that the hypothesized nesting maximizes the value of F_{CT}.

Accordingly, the other possible groupings were tested (schemes 5–1-1, 3–2-2 and 4–2-1) and presented equal or lower F_{CT} than the proposed grouping (0.00874, 0.06604 and -0.00941, respectively). Therefore, the sample size used is within the minimum range needed to detect hierarchical differentiation with $\alpha = 0.05$, supporting the inference that differentiation among groups and populations was not large. Greater

statistical power could be obtained by increasing the number of sites (populations) per group, but a larger sample of individuals per site would probably be uninformative about hierarchical structure. Although a complete F_{ST} analysis would be needed to correctly assess genetic differentiation between *M. sojae* populations in South America, they do not appear to be distributed according to a clear-cut pattern of geographical separation. Thus, the geographical criteria used to define regional groups and populations (i.e. country and state boundaries) do not accurately represent the species' mtDNA COI diversity and distribution within South America, and could be replaced by an alternate partition of populations or a clustering of all populations into a continental supra-population group.

Identifying incursion pathways into a new landscape, and the routes of dissemination within it, is a key step on mitigating the detrimental impacts of a pest invasion and preventing the occurrence of new ones (Essl et al. 2015). Although the fraction of variation found within *M. sojae* populations from South America was very high (see Table 3) and, apparently, not correlated with the distance of geographical separation between them, any solid conclusion on the matter would depend upon the knowledge of when and where the first introduction(s) occurred. Unfortunately, such knowledge is lacking, as the evidence might have been lost over time when movements assisted by humans and/or natural dispersal took place across the landscape. Further studies should increase sample size and include a genotype-by-sequencing approach (e.g., RAD) to assess regional differentiation more accurately and increase statistical significance.

Management strategies

The ongoing spread of *M. sojae* across South America poses a considerable challenge for soybean pest management in the continent. Within its native range (i.e. East Asia), *M. sojae* is usually managed by soybean growers through the use of resistant cultivars (Talekar 1980) and sowing dates outside population peaks (Talekar and Chen 1983). The use of parasitoids (e.g. Talekar 1990, van den Berg et al. 1995) and insecticides (e.g. Adak 2012, Jadhav et al. 2013) has also been reported. Curioletti et al. (2018) recommended spraying Chlorantraniliprole as seed treatment, followed by foliar spraying of Chlorpyrifos until 10 days after plant emergence, and repeated once in an interval shorter than 10 days. On the other hand, control strategies for *M. sojae* in South America remain scarce and poorly tested. Considering the strategies used in other countries, at least two of them are impracticable in Brazil, where soybean cultivars resistant to *M. sojae* are not available and sowing dates can hardly be shifted due tight succession of soybean and maize crops. While Brazilian soybean growers do not employ insecticides targeting specifically at *M. sojae,* populations have probably been indirectly controlled by seed treatments and foliar sprayings aiming at other early-season pests, such as *Sternechus subsignatus, Elasmopalpus lignosellus* and *Phyllophaga capillata.*

Melanagromyza sojae was officially detected in Paraguay in 2017 (Guedes et al. 2017), but is probably present in the country at least since 2015 (Benitez 2015). The highest infestations have been detected on second-season soybean, in the Southern departments of Alto Paraná, Canindeyú and Itapúa, which represent the main agricultural region of the country. Field monitoring carried out from January, 2020 to March, 2020 in several localities of Itapúa and Alto Paraná detected high presence of *M. sojae* adults in second-season soybean, followed by injury symptoms in the plants, such as leaf yellowing and early dropping of upper leaves. Further investigation revealed an incidence level of 100%, with all sampled plants presenting two to three holes in the main stems and three to five *M. sojae* pupae within each stem. Although control measures for this pest are still under development, the high incidence of adults led soybean growers to fight off infesting populations with sequential sprays of pyrethroids.

In Bolivia, *M. sojae* was first identified in 2019 in the department of Santa Cruz–where almost all the country's soybean is grown (Vitorio et al. 2019). The highest infestations have been recorded during low-precipitation seasons (Aguilar 2018) and when two soybean crops are grown per year instead of just one (Vitorio 2020). Field reports also highlight sowing date as an important factor, with higher incidence of attacked plants reported at early sowings–unlike the Brazilian and Paraguayan scenarios, where high infestation levels are apparently correlated with late sowing (Pozebon et al. 2020). In conditions of low precipitation and early sowing, damage in Bolivian

soybean fields can be severe, with up to 100% of plant incidence and soybean stems tunneled in 70% of their length (Vitorio 2020). Several unidentified parasitoids and one fly species (Diptera: Muscidae) have been observed preying on *M. sojae* adults in Bolivia. Insecticides applied as seed treatment presented better results compared to three foliar applications in the seedling stage (Vitorio 2020).

In Argentina, *M. sojae* was first detected in 2019 in the municipality of Jesús María (north of Córdoba province; Vera et al. 2020). Previously, dipteran larvae of an unidentified species with characteristics similar to those of the genus *Melanagromyza* were detected in several localities of Córdoba province, damaging chickpea plants in 2013 and winter weeds in 2014. During the 2019/20 cropping season, larvae and pupae of *M. sojae* were found in stems of soybean plants in the provinces of Santa Fé (Trossero et al. 2020), Entre Ríos (Saluso 2020) and Tucumán (Murúa et al. 2020). During the 2019 winter cropping season, different chickpea varieties were compared in four field trials, planted in different sites in the north of Córdoba, and preliminarily results reveal 30 to 80% of variation in *M. sojae* incidence among the varieties. Chickpea producers have applied systemic insecticides (e.g. imidacloprid and dimethoate) when observing the presence of *M. sojae* larvae within the stems, but no differences in incidence were observed when comparing with untreated areas.

Melanagromyza sojae continues to expand across the Old World as well as the New. It was identified as part of a 3-year (2004–2007) biodiversity study in Spain, being the first and only record of this species from the European continent so far (Gil-Ortiz et al. 2010). The species was also found in Turkey in 2018 in Çukurova region (Özgür et al. 2020), the largest soybean production area in the country (TUIK 2020). Two soybean crops are grown per year in this area, the first sowed in April and the second one in June, after wheat harvesting. Although *M. sojae* occurs in both soybean crops, pest population is higher in the second season, similarly to what occurs in Brazil and Paraguay. Mart (2018) observed that *M. sojae* population established after three weeks of soybean sowing and continued until the tenth week. There are no insecticides registered for *M. sojae* control in Turkey, but infesting populations have probably been indirectly controlled by foliar sprayings of malathion, bifenthrin, novaluron, beta-cyfluthrin, and methomyl targeted at other pests such as *Bemisia tabaci*, *Nezara virudula*, *Spodoptera exigua* and *Helicoverpa armigera*.

Three of the six lead soybean producers in the world (Brazil, Argentina and Paraguay) are located within the so-called South American soybean belt, with Bolivia following close behind and Uruguay making its way into the group. Caught between high propagule pressure on both country borders (Rio Grande do Sul state in the north and Argentina in the west), Uruguay is on the target for *M. sojae* invasion coming from its neighbouring countries, as similarly occurred with *H. armigera* (Castiglioni et al. 2016; Arnemann et al. 2016c). During our sampling of *M. sojae* specimens across Rio Grande do Sul state, we found that this insect was present at high incidence levels (95–100%) in all soybean fields sowed after December 31th (i.e. second-season soybean), with injury often covering the whole length of the soybean stems. If such scenario expands into main-season soybean as well, the main export commodity of these countries would be put at risk, with few alternatives available to manage it. While chemical and biological control alone may not be enough to interrupt the spread of *M. sojae* across the continent, management programs could be strengthened by measures aiming to reduce pest survival in the winter, such as eradication of post-harvest volunteer soybean plants and prohibition of second-season soybean cultivation. The findings from our study contribute to a better understanding of *M. sojae* genetic diversity and population dynamics in this important soybean growing region, and will assist in the development of efficient management measures for this highly damaging invasive pest.

Conclusions

Single nucleotide polymorphisms (SNPs) within the partial (740 bp) COI gene differentiated *M. sojae* populations from South America into 22 mtDNA haplotypes, based on samples from Brazil, Argentina, Paraguay and Bolivia. Msoj-COI-01 and Msoj-COI-02 were the haplotypes most prevalent in the continent, representing 47% of all individuals sampled across South American countries. Msoj-COI-02 was also the haplotype identified in two individuals sampled from Australia. Three novel haplotypes (Msoj-COI-21, Msoj-COI-22 and Msoj-COI-23) were identified in Rio Grande do Sul state (Brazil). There is

evidence of *M. sojae* introductions involving multiple female founders into South America, but no location-specific genetic structure was detected. Pest management measures are still under development for this pest in the South American soybean belt, and there is paucity of data regarding soybean yield losses due to *M. sojae* attack in the New World. No molecular basis was found for treating country-specific populations of *M. sojae* differently, and they could be grouped together as a continental supra-population for future analysis and pest management purposes.

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Data availability The mtDNA sequences generated during and/or analysed during the current study are publicly available in the Genbank repository. Accession numbers to each mtDNA sequence are provided in the text. Other supporting datasets are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest.

Human and animals rights This research did not involve human participants and/or animals.

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