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First record of the soybean stem fly *Melanagromyza sojae* (Diptera: Agromyzidae) in Bolivia

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ABSTRACT. This is the first scientific record of *Melanagromyza sojae* in Bolivia, confirmed through molecular characterization of the mtDNA COI gene. Commonly known as the soybean stem fly, *M. sojae* belongs to the family Agromyzidae and is a highly polyphagous pest, attacking several plant species of the Fabaceae family, such as soybeans and other beans. Previously reported in Brazil and Paraguay, the soybean stem fly presence was confirmed in soybean fields of the municipalities of Fernández Alonso, Cuatro Cañadas, Santa Cruz de la Sierra, Yapacani and Santa Rosa del Sara, which are located in Santa Cruz Department, Bolivia. This soybean stem fly detection in Bolivia will affect soybean crop practices in South America.

Key words: Biosecurity; *Glycine max;* mtDNA COI; Stem borer flies

INTRODUCTION

The soybean (*Glycine max*) is a host plant for several stem borer flies that are highly destructive (Talekar and Chen, 1985; Spencer, 1990), with *Melanagromyza sojae* (soybean stem fly: SSF) being one of the most important (Talekar and Chen, 1985; Talekar, 1989). This insect pest has a wide distribution, mainly throughout much of the Asian continent, Africa and Australia (Spencer, 1973; Dempewolf, 2004). In recent years, its presence has also been recorded in Europe (Gil-Ortiz et al., 2010) and more recently in the Americas (Arnemann et al., 2016a; Guedes et al., 2017). Among the causes that have resulted in these accelerated changes in the geographic distribution of SSF we can include the intensification of trade that favors the colonization of new habitats and provides new and propitious environments for this pest (Régnière, 2009).

In South America, the first occurrences of the *Melanagromyza* genus in soybean crops were reported in 1983 in Brazil, in regions of the southern state of Rio Grande do Sul, including the Passo Fundo region (Gassen and Schneider, 1985), and later in Paraguay during the 2014/15 growing season (Benítez-Díaz, 2015). The presence of the species *M. sojae* was confirmed using molecular tools and morphological characters in Brazil (Arnemann et al., 2016a,b) and Paraguay (Guedes et al., 2017). In Bolivia, the genus *Melanagromyza* was mentioned causing damage to soybean crops in 2006 (Matef, 2006); however, its occurrence has been recorded on numerous occasions in entomological diagnosis reports from the laboratory of the Institute of Agricultural Research "IIA El Vallecito - UAGRM" from 2005 to date.

Several plants of the Fabaceae family are hosts of SSF, but the main one is soybean (Spencer, 1990; Dempewolf, 2004), which suffers great damage due to the drilling of the stem by SSF larvae, resulting in considerable yield losses. Data concerning damage varies among regions, from no direct yield loss observed in Java (Van Der Goot, 1930), to 2% yield loss in early attacks in Indonesia (Van den Berg et al., 1998), 21% in Taiwan (Talekar, 1989), 20-30% in China (Du and Hong, 1982), 33-40% in India (Jadhav et al., 2013) and 34-51% in Thailand (Suwanpornsakul et al., 1996). Given the importance of the soybean crop in the current agricultural scenario, this pest presents a major risk to New World agriculture.

The biological cycle of *M. sojae* comprises 3-6 days of egg phase, 7-12 days of larval phase, 7-12 days of pupal phase, 7-16 days for adult females and 5-14 for adult males. Females have a potential to lay 75-95 eggs (Jadhav et al., 2013). Specimens of *M. sojae* from the same or different host plants may present many variants, according to characteristics of the genitalia of the males (Thapa, 2012). Dempewolf (2004) mentions that there is contradictory information or taxonomic errors in the literature that indicate cryptic species. Arnemann et al. (2016a,b) was able to develop molecular markers for SSF based on the mitochondrial gene of DNA cytochrome oxidase I (mtDNA COI) of this species, in order to facilitate its identification. We identified larvae and adults of suspected *M. sojae* collected from soybean plants in different localities and municipalities of the Santa Cruz Department, Bolivia, identifying the species as *M. sojae* through molecular comparison of the COI gene region of mitochondrial DNA and confirming the presence of this pest in Bolivian soybean fields.

MATERIAL AND METHODS

Samples

Fly larvae and adults were collected from individual soybean plant stems that showed characteristic SSF feeding damage (Figures 1 and 2), as described by Van den Berg

et al. (1998). The samples were collected in various geographic regions of Bolivia in the 2016/17 cropping season (Table 1 and Figure 3) and conserved in 1000 µL of 99.9% ethanol, in the Entomology laboratory of the Agricultural Research Institute "El Vallecito-UAGRM". Parts of the samples were sent to the Integrated Pest Management Laboratory / Molecular Insect Lab of the Federal University of Santa Maria, Santa Maria, RS, Brazil, for molecular characterization and species confirmation.

Figure 1. Damage due to *Melanagromyza sojae*: A) Necrosed unifoliate leaves and apical bud; B) Plant with mummified apex and lateral ramifications; C) Stem drilled with pupa and exit hole; D) Stem drilled with reddish coloration; E) Necrosed leaflet; F) Drilled petiole exhibiting pupa and exit hole; G) Unifoliate leaf showing a mined vein and H) Feeding holes made by female adults (photos by L. Vitorio).

Figure 2. Morphological features of *Melanagromyza sojae*: A) Female adult; B) Egg laying; C) Larva; D) Anterior spiracles of larva; E) Pupa; F - G) Posterior spiracles of pupa; H) Wing and detail of insertion of subcostal vein (photos by L. Vitorio).

Table 1. Collection sites, dates and GeneBank haplotype locus ID of *Melanagromyza sojae* specimens from Bolivia. New haplotypes are indicated by '†'.

Sample ID	Local (municipality)	Coordinates		Collection date	Haplotype locus
		S	W		
1	Cuatro Cañadas	17°27'07,08"	62°36'43,29"	02/01/2017	Msoj-COI-13
2	Cuatro Cañadas	17°27'07,08"	62°36'43,29"	02/01/2017	Msoj-COI-19†
3	Fernández Alonso	16°54'59,28"	63°14'08,99"	12/09/2016	Msoj-COI-02
4	Fernández Alonso	16°48'33,75"	63°19'11,46"	31/09/2016	Msoj-COI-13
5	Santa Rosa del Sara	17°02'48,46"	63°40'04,97"	04/03/2017	Msoj-COI-02
6	Santa Cruz de la Sierra	17°42'10,48"	63°08'52,04"	02/01/2017	Msoj-COI-13
7	Yapacani	17°21'44,24"	63°54'34,9"	15/11/2016	Msoj-COI-20†

Upon reception, the quality of the samples was inspected, since the molecular techniques used for identification require the use of well-preserved tissues and specimens (in general it is used alcohol > 98ºGL), aiming at non-destructive extraction of genomic DNA with quality. Of the total of samples received, seven with adequate DNA content were used for the molecular identification process, as shown in Table 1.

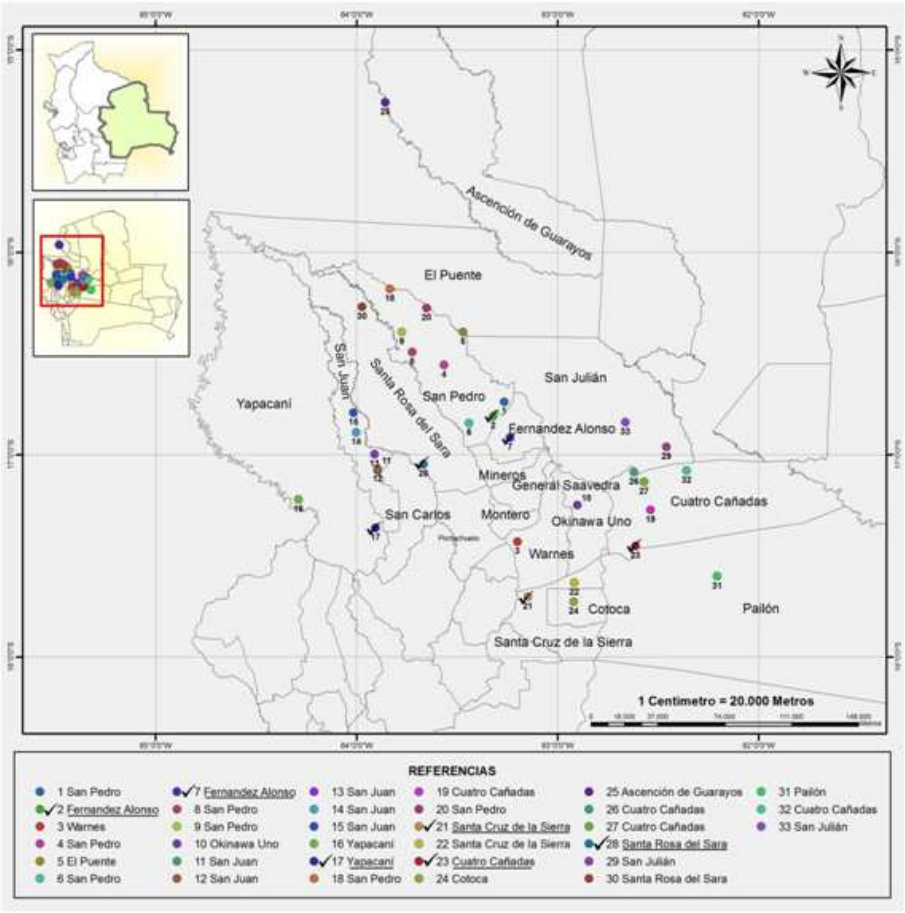

Figure 3. Sampling sites (municipalities) in Bolivia where specimens of *Melanogromyza sojae* were collected. The places where its presence was confirmed are marked with a "✓" symbol.

Total genomic DNA (gDNA) extraction

Individual specimens were washed three times in 1000 µL of fresh laboratory-grade ethanol (99.9%) prior to gDNA extraction. Total gDNA from all specimens was extracted from the whole larval or adult body using a Qiagen DNeasy Blood and Tissue DNA Extraction Kit (Qiagen, Hilden Germany), according to manufacturer's instructions. Final elution volume for the individual gDNA samples was 25 µL Qiagen buffer AE, with gDNA quality ascertained by visualization of the samples electrophoresed on a 1.5% agarose gel.

PCR amplification and sequencing of the partial mtDNA COI gene

Amplification of gDNA samples for molecular characterization of the suspected SSF individuals was done via PCR, using SSF-specific partial mtDNA COI gene PCR primers (SSF-COI-F01: 5'-GACAATGATTATTTTCGACAAAT-3'; SSF-COI-R01: 5'-GTAAAATAAGCTCGTGTATCTACATC-3') and PCR conditions for the mtDNA COI primer pairs, as previously reported (Arnemann et al., 2016b).

The seven selected samples presented satisfactory results with amplification of the N-terminal fragment of the Cytochrome Oxidase subunit I gene (COI I - used in the DNA BarCoding), as follows: sample 1 (Cuatro Canãdas), 2 (Cuatro Canãdas), 3 (Fernández Alonso), 4 (Fernández Alonso), 5 (Santa Rosa del Sara), 6 (Santa Cruz de la Sierra), 7 (Yapacani). All these municipalities are located in different provinces of the Santa Cruz Department of Bolivia. Afterwards, the PCR amplicons were sent for sequencing to ACTGene®, Porto Alegre, RS, Brazil.

Sequence analysis and molecular characterization of the mtDNA COI gene

The programs Pregap and Gap4 within the Staden package (Staden et al., 2000) were used for editing the sequences. Sequence alignments of all mtCOI haplotypes were carried out using Geneious R9 (Biomatters Ltd., New Zealand). Potential presence of premature stop codons was checked through translation of the partial mtCOI sequences into protein sequences, by selecting the invertebrate genetic code 5 for amino acid translation. Nucleotide distances (uncorrected P-distance) between specimens from Bolivia and all previously reported mtDNA COI haplotypes from Brazil (Arnemann et al., 2016b) and Paraguay (Guedes et al., 2017) were calculated in MEGA (Tamura et al., 2013), and maximum likelihood phylogeny was inferred using the web-based program PhyML (Guindon et al., 2010) using the automatic model selection option, followed by 1,000 bootstrap replications to estimate node confidence. Selection of out-group species *Ophiomyia quinta* (EF104665) and *Ophiomyia nasuta* (EF104661) for the phylogenetic analysis was based on the study by Scheffer et al. (2007).

RESULTS

The specimens in the samples from the municipalities of Cuatro Canãdas, Fernández Alonso, Santa Cruz de la Sierra, Santa Rosa del Sara and Yapacani were confirmed as *M. sojae*. Our results, based on molecular characterization of the mtDNA COI partial gene as well as morphological identification, provided the first record of *M. sojae* in Bolivia, and represent the first confirmation of the occurrence of this pest in the Santa Cruz Department, an important soybean growing region of the country.

A 906-bp fragment of the mtDNA COI gene was amplified using the SSF-specific mtDNA COI primers, from seven suspected individuals from Bolivia (Table 1). Post-sequencing trimming of the sequenced amplicons resulted in 740-bp partial mtDNA COI contigs in all samples. The estimates of evolutionary divergence between *M. sojae* sequences were as expected at the intra-species level (e.g. Scheffer, 2000; Arnemann et al., 2016b) and ranged from 0.00 to 0.015% (± 0.001 - 0.004 SE). The evolutionary divergence between SSF haplotypes from Bolivia and Brazil was 0.0047%, and between Bolivia and

Paraguay it was 0.0058%. Both new mtDNA COI sequences of haplotypes 'M-soj-COI-19' and'M-soj-COI-20'generated from this study have been submitted to GeneBank (accession numbers: MK490675 and MK490676, respectively). The inferred partial mtCOI phylogeny (Figure 4) suggested a basal position for *M. sojae* as compared with other Agromyzidae species.

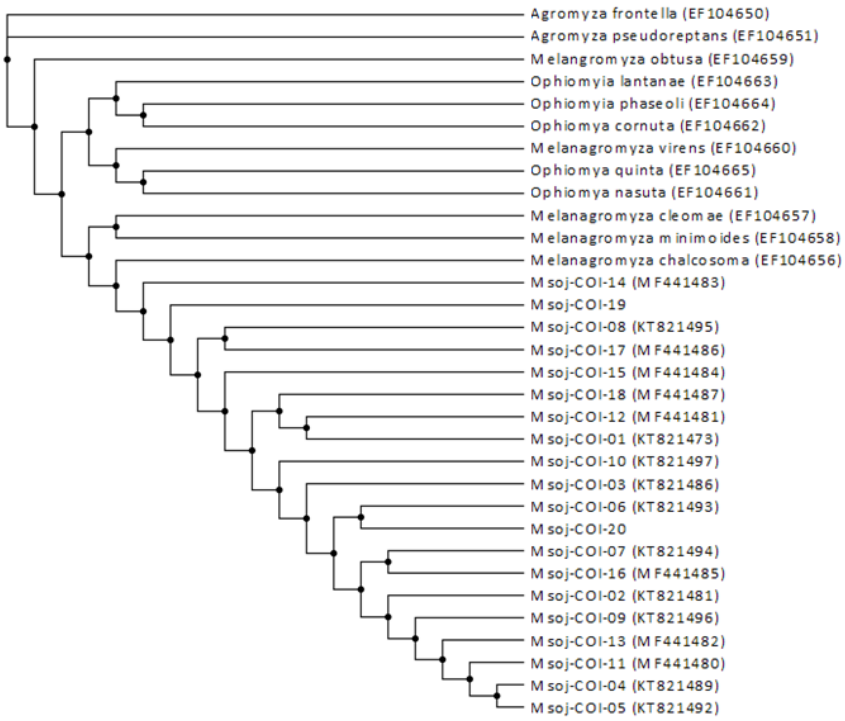

Figure 4. Maximum likelihood (ML) phylogeny analysis using PhyML (Guindon et al., 2010) of Agromyzidae species and *Melanagromyza sojae* haplotypes (substitution model: GTR+G; 7297.43470 (AIC); Gamma shape parameter: 1.075, proportion of invariable sites: 0.608), based on 684bp partial mtCOI gene. The outgroup is *Ophiomyia quinta* (EF104665) and *Ophiomyia nasuta* (EF104661) (Scheffer *et al* 2007). Nodes with bootstrap values of >50% are shown. The GeneBank accession numbers for all samples used are provided.

DISCUSSION

From the seven *M. sojae* specimens collected in Bolivia and selected for DNA characterization, four SSF haplotypes were identified, two of which were novel haplotypes (specimens from Cuatro Cañadas and Yapacani, 'M-soj-COI-19' and 'M-soj-COI-20', respectively; see Table 1) and two identical to previously reported haplotypes from Brazil (haplotype 'Msoj-COI-02' in specimens from Fernández Alonso and Santa Cruz del Sara; see Table 1) and Paraguay (haplotype 'Msoj-COI-13' in specimens from Cuatro Cañadas, Fernández Alonso and Santa Cruz de la Sierra; see Table 1) (haplotypes names from Arnemann et al., 2016b).

Our study enabled a preliminary genetic diversity survey of SSF in Bolivia, providing some insights into potential pathways of biological incursion of this pest in this region of South America. The presence of specimens from the same haplotypes previously identified in Brazil and Paraguay suggests that *M. sojae* may have invaded Bolivia coming from both countries, while the detection of unique haplotypes that are not shared by the neighboring countries points to a potential hypothesis of multiple independent incursions of SSF in South American countries. Nonetheless, studies to date have surveyed only limited populations of SSF in Brazil and Paraguay (Arnemann et al., 2016ab; Guedes et al., 2017), thereby limiting detailed understanding of the genetic diversity of *M. sojae* populations in South America. While the diversity of maternal lineages suggests an establishment of the pest as a result of multiple introductions, the current populations might also represent the descendants of a large initial 'multi-founder' incursion (Arnemann et al., 2016b). Sampling sizes should be increased and molecular characterization of multiple DNA gene regions or NGS carried out in order to better test these hypotheses.

While the potential of establishment of *M. sojae* in the North American continent remains unknown, the species has been detected across very diverse eco-climatic zones (Dempewolf, 2004), suggesting a potential to also establish successful populations throughout the entire New World. By understanding the potential distributional range of *M. sojae* and its patterns of biological incursion, control measures might be developed in order to prevent further dissemination and better manage the populations already introduced. Efforts must be directed to the re-evaluation of biosecurity protocols and phytosanitary practices concerning agricultural commodities that are entering Bolivia, Paraguay and Brazil.

The presence of *M. sojae* in Bolivia confirmed through scientific studies was notified to the sanitary entity SENASAG (Servicio Nacional de Sanidad Agropecuaria e Inocuidad Alimentaria), by the Agriculture Research Institute "El Vallecito" – UAGRM, at June 12th, 2018. Currently, *M. sojae* is placed in the category of quarantine pests for Bolivia, which makes it difficult to address phytosanitary control measures. Nonetheless, it is causing severe damage and is widely dispersed in all soybean growing areas. Bolivia is the ninth largest soybean producer in the world, with an annual production of 3.1 million metric tons grown on 1.3 million hectares. Of the total area cultivated with soybean in the country in the 2017/18 growing season, 330,000 hectares were cultivated in the late sowing season (or Fall season, with sowing starting in March), and these areas typically present the highest rates of infestation by *M. sojae,* with an average of 70% of infestation (against 20-25% of infestation in the early sowing season or Summer season, from October to February), according to reports from Bolivian soybean growers and agronomists.

Soybean is also the main agricultural crop in Bolivia and one of its main sources of income from exports. The municipalities where *M. sojae* was reported are located in the Santa Cruz department, the largest soybean national producer (ANAPO, 2016); therefore, the presence of this pest represents a high risk for national soybean production, due to its high potential to cause yield losses as reported in other regions of the world (Suwanpornsakul et al., 1996; Van den Berg et al., 1998; Jadhav et al., 2013). Furthermore, Santa Cruz department borders the Brazilian Savannah region, where the presence of *M. sojae* has been recently confirmed on soybean fields (Czepak et al., 2018) and may have served as a geographical path of incursion for the pest.

The confirmation of *M. sojae* occurrence in Bolivia is the first step towards the development of regional and country-specific integrated pest management and resistance pest management strategies for this pest. The geographic span of the identified infestation sites, allied to the many reports by agronomists and soybean growers of high incidence of stem-boring flies in the last crop seasons (especially on late sowings), suggests that this insect is substantially established in the Santa Cruz Department of Bolivia; nonetheless, the real dispersion of the pest in the country might be much larger, though further positive confirmation of the species from samples collected in other regions would be needed in order to check this possibility.

Additionally, overall yield losses due to SSF damage and ecological aspects of the insect in the New World remain poorly understood, including the potential existence of native or invasive beneficial insects that could target this pest, as reported by Beche et al. (2018). Currently, containment and eradication measures would be expensive and unachievable, due to the *M. sojae* high reproduction rate and short generation time (four to five generations per year; Wang, 1979); moreover, early infestations are difficult to detect due to the small size of the flies, inconspicuous oviposition scars, and larval damage similar to that caused by other soybean stem-boring pests.

Across the Old World regions where SSF is endemic and is an economically important pest in soybean crops, usual control measures have included the use of resistant cultivars and sowing dates outside the population peaks. The use of endemic beneficial insects, such as parasitoids, has been reported (e.g.Talekar, 1990; Van den Berg, 1998) as well as insecticides applied via seed or foliar sprays (e.g. Adak, 2012; Jadhav et al., 2013; Curioletti et al., 2018).

Future research directions on alternative control methods for SSF might include the breeding of resistant soybean varieties (see Wang and Gai, 2001) and biological control (e.g. by parasitoid *Syntomopus parisii;* see Beche et al., 2018), as well as detailed modeling of its potential economic impact in South American soybean fields. The findings from this study contribute to a better understanding of potential incursion pathways of *M. sojae*, and assist in the development of more efficient management strategies for this important agricultural pest.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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