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IDENTIFICATION OF FIRE ANTS (HYMENOPTERA: FORMICIDAE) FROM NORTHEASTERN MEXICO WITH MORPHOLOGY AND MOLECULAR MARKERS

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ABSTRACT

The invasive red imported fire ant, *Solenopsis invicta* Buren, has successfully dispersed across many countries from its South American homeland and now has reached the US-Mexico border (e.g., Matamoros, state of Tamaulipas, México), where it now coexists with native fire ants, *Solenopsis geminata*, *Solenopsis xyloni,* and others. The morphological identification of *Solenopsis* spp. workers is difficult, particularly small ones. We examined the sequence of the cytochrome oxidase I (COI) mitochondrial gene (mtDNA) as a marker for fire ants collected at several Mexican localities. PCR products from this locus yielded unique sequences and restriction patterns that allowed distinguishing between *S. invicta*, *S. geminata*, and specimens harboring *S. xyloni* sequences. The *S. invicta* sequences obtained were 99% identical to sequences reported from Florida and New Mexico specimens. The *S. xyloni* sequences obtained were 96% identical to New Mexico sequences. The *S. geminata* sequences were similar (93% identity) to those from Florida, and shared a *Hinf I* restriction site with some but not all Florida sequences. The *S. xyloni* sequences were detected in *S. geminata*/*S. xyloni* hybrids identified by morphology; along with other characters, the marker allows their characterization.

RESUMEN

La hormiga de fuego roja importada, *Solenopsis invicta* Buren, especie invasora, se ha dispersado exitosamente a muchos países desde su origen en Sudamérica, y ha alcanzado la frontera Estados Unidos-México (p. ej. Matamoros, estado de Tamaulipas, México) donde ahora coexiste con hormigas de fuego nativas, *Solenopsis geminata* y especies del subcomplejo *Solenopsis xyloni*. La identificación morfológica de las *Solenopsis* spp. es difícil, particularmente de las obreras más pequeñas. Examinamos la secuencia del gene mitocondrial citocromo oxidasa I (COI) como marcador para poblaciones de hormigas de fuego en varios sitios de México. Los productos de PCR de este locus rindieron secuencias únicas y patrones de restricción que permitieron distinguir entre *S. invicta*, *S. geminata* y caracterizar miembros del subcomplejo *S. xyloni*. Las secuencias de *S. invicta* fueron iguales en 99% a secuencias reportadas para especímenes de Florida y Nuevo México. Las secuencias de *S. xyloni* fueron iguales en 96% a las secuencias reportadas de Nuevo México. Las secuencias de *S. geminata* fueron similares (93% identidad) a las de Florida, y comparten un sitio de restricción *Hinf I* con algunas pero no todas las secuencias reportadas para Florida. Las secuencias de *S. xyloni* también se detectaron en hibridos de *S. geminata*/*S. xyloni* identificados por morfología; junto con otros caracteres el marcador permite su caracterización.

The invasive red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) is considered the most destructive ant species in the world (Landcare Research 2007). *Solenopsis invicta* has multiple impacts, including changing food webs (Vinson 1997), threatening endangered species (Wojcik et al. 2001), and negatively impacting human and animal health (Jetter et al. 2002). Red imported fire ants were reported to have the ability to feed on any insect species, and in general will attack and consume any invertebrate species that is not capable of defending itself or escaping attack (Ricks & Vinson 1970). After arriving accidentally to the Gulf coast region of the United States, this species has been enormously successful ecologically; by 1999, it infested more than 121 million hectares in the United States and Puerto Rico (Wojcik et al. 2001). Sánchez-Peña et al. (2005) published the first reports of *S. invicta* in Mexico, at several locations along the USA-Mexico border, all within 1 km from the Rio Grande in the Mexican states of Tamaulipas and Coahuila. In 2007, infestations were observed 3 km south of the Rio Grande, east of Matamoros, Tamaulipas (Sánchez-Peña unpublished observations). All these identifications

of *S. invicta* in Mexico were based on morphology. The municipality of Matamoros includes the most extensive *S. invicta* populations detected in Mexico so far (Sanchez-Peña, unpublished observations) and the area contains the most favorable habitats for unimpeded expansion of *S. invicta* further south into Mexico (Phillips & Thorvilson 1992).

In addition to the numerous urban, medical, veterinarian, and agricultural implications of the *S. invicta* invasion (Jetter et al. 2002), there are several sites of prime ecological importance in Northeastern Mexico that could be severely impacted by this species. One such site is the sea-turtle egg-laying region (e.g., Rancho Nuevo beach) in central and southern Tamaulipas state. Predation by *S. invicta* at Rancho Nuevo could impact the Kemp's ridley sea turtle, (*Lepidochelys kempii* (Garman)), considered one of the most endangered vertebrates in the world. This location is the only site worldwide where large nesting aggregations of this sea turtle are known to occur (Johnson et al. 1999). Parris et al. (2002) documented *S. invicta* predation of sea turtle hatchlings (*Caretta caretta*) in Florida, and considered *S. invicta* to be a serious threat. The unique ecosystems at Cuatrociénegas (state of Coahuila) and El Cielo Biosphere Reserve (state of Tamaulipas) also may be impacted by *S. invicta*.

Fire ants in the *Solenopsis saevissima* species-group generally appear to be a morphologically homogeneous clade, making species identification quite difficult. Native fire ants (4 described species: *Solenopsis amblychila* Wheeler, *Solenopsis aurea* Wheeler, *Solenopsis geminata* (F.), and *Solenopsis xyloni* McCook) and several hybrids between these species are (or were) sympatric over a large portion of the current range of *S. invicta* in North America; *S. invicta* has caused large local extinctions of *S. geminata* and *S. xyloni* (Trager 1991; Wojcik et al. 2001). All 4 native species have been collected in northern Mexico (Trager 1991; Helms Cahan & Vinson 2003). The identification of these polymorphic North American fire ant species by morphology is most easily accomplished by examination of the largest (major) workers; species identification becomes considerably more difficult as worker size diminishes (Trager 1991).

Populations of the tropical fire ant, *S. geminata* exist in Northeastern Mexico. It is a keystone predator in some Latin American agroecosystems, and it has an important effect on the composition of arthropod fauna (Risch & Carroll 1982; Perfecto 1991). The key morphological characteristic for identification of *S. geminata* is soldiers (major workers) with disproportionately enlarged heads (Trager 1991). However, these soldiers comprise a very minor proportion of the workers in colonies, they rarely forage outside the nest, and they tend to retreat deeper into soil when the colony is disturbed (Trager 1991). Thus, samples of *S. geminata* colonies commonly lack soldiers making morphological identification more difficult.

Jacobson et al. (2006) remarked on the difficulty of identifying all size castes of *S. invicta* from those of *S. xyloni*, the southern fire ant, which is an extremely similar native species. They developed a PCR assay for the identification of *S. xyloni* and *S. invicta*, involving specific amplification in the maternally-inherited cytochrome oxidase I (COI) mitochondrial locus, followed by digestion of PCR products with the restriction enzyme *Hinf* I. This method identified unambiguously all *S. invicta* samples tested.

Identification of native fire ant species is not straightforward either, as mentioned. Trager (1991) groups the very similar North American species *S. xyloni*, *S. aurea,* and *S. amblychila* (i.e., non-*S. geminata* native fire ants) into the "*S. xyloni* subcomplex". Although among major workers, eye size and ommatidia number are reported to be adequate characters for separating *S. xyloni* from *S. aurea* and *S. amblychila* (Trager 1991; E. LeBrun personal communication), the morphological discrimination of some samples of *S. xyloni*, *S. aurea,* and *S. amblychila* is very difficult or almost impossible (Trager 1991). Our preliminary observations indicate that in Northeastern Mexico these native, non-*S. geminata* fire ants species do not include bicolored forms (with brownish-black gaster) but mainly rather uniform yellow-reddish forms (Trager 1991; Cook & O´Keefe 2002). Considering the similarity of these 3 species, the lack of comprehensive fire ant taxonomical studies in this region, and the lack of information on COI sequences for *S. aurea* (although Shoemaker et al. (2006) reported having sequenced the COI gene of *S. amblychila*), in this report we take a conservative approach and for purely practical reasons we use the term "*S. xyloni* subcomplex" sensu Trager (1991) regarding our collections of non-*S. geminata* native fire ants. Thus, it is possible that they might correspond to either *S. xyloni*, *S. aurea*, or *S. amblychila*. In this respect it must be mentioned that the cladistic analysis and phylogenetic study of Pitts et al. (2005) failed to support the splitting of the *Solenopsis* fire ants into subordinate divisions; we acknowledge that the "*S. xyloni* subcomplex" of Trager (1991) might not be a phylogenetically supported clade despite the apparent relatedness of its members.

In this study, we adapted the PCR assay of Jacobson et al. (2006) to identify *Solenopsis* spp. samples in northeastern Mexico, initially to verify the presence of *S. invicta* in the municipality of Matamoros. A PCR detection assay could be a valuable tool for identification of *Solenopsis* spp. in northern Mexico.

Materials and Methods

Collection and Morphological Identification of Fire Ants

Table 1 lists the samples analyzed and their origin; localities are shown in Fig. 1. At all sites, samples were obtained by either (1) directed searches of nests or (2) baiting with 5-cm hotdog pieces placed on the ground and collection when *Solenopsis* fire ants were present.

Solenopsis invicta were collected at the cities of Nuevo Laredo and Matamoros, state of Tamaulipas, within 1 km of the Rio Grande river (USA-Mexico border) (Sánchez-Peña et al. 2005). Native fire ants were collected at the following states and municipalities (Table 1): CHIHUAHUA: Chihuahua; COAHUILA: Torreón; NUEVO LEON: Monterrey, Cadereyta, Pesquería (town of Santa María la Floreña); TAMAULIPAS: Matamoros. Ants were transferred to 96% ethanol, stored at room temperature, and processed for molecular analysis within 1 month of collection. *Solenopsis* spp. were identified according to Trager (1991).

Molecular Methods

Total genomic DNA was extracted from 1 to 5 worker ants stored in 96% ethanol with the lithium chloride method for insects (Huang et al. 2000). PCR amplification of cytochrome oxidase I (COI) regions were carried out as described by Jacobson et al.(2006). Reactions were carried out in a volume of 25 µL consisting of 2.5 µL of 10X buffer, 0.75 µL of 50mM $MgCl_2$, 1.25 µL of 2.5mM dNTPs, 1.0 µL of genomic ant DNA, 1.3 µL of CI-J2195 primer (13.6 µM), 1 µL Jerry Garcia-CI primer (15.2 µM) (Jacobson et al. 2006), 0.3 µL of *Taq*DNA polymerase (5U/µL) (all reagents from Invitrogen, Carlsbad CA), and 16.9 µL of MilliQ water (Millipore Corp., Billerica, MA). PCR conditions were 95°C (1 min), 40 cycles of 94°C (1 min each), 43°C (1 min), 68°C (2 min), and 72°C (2 min). Amplification products were visualized on 1.2% agarose gel stained with ethidium bromide. They were digested with *Hinf* I enzyme; standard restriction enzyme digestions were carried out in a 10 µL volume consisting of the following: PCR product (2 µL), 0.3 µL *Hinf* I enzyme (10U/µL), 1 µL of 10X buffer, and 6.7 µL MilliQ water. Each reaction mixture was incubated at 37°C overnight and PCR digestion was visualized on 1.2% agarose gel stained with ethidium bromide to compare the size of the bands obtained with *Msp*I-digested pBlueScript as molecular marker.

Nucleotide sequencing of PCR fragments were performed by cloning into the PGEM-T vector system (Promega, Madison, WI). Recombinant plasmids were characterized with *Pvu* II enzyme (New England Biolabs, Ipswich, MA) and purified by the Wizard SV Gel and PCR Clean-up System (Promega, Fitchburg, WI). PCR fragments were sequenced with pUC/M13 reverse and forward sequencing primers in a Perkin Elmer/Applied Biosystems Model 3730 sequencer at the Universidad Nacional Autonoma de Mexico (UNAM) Sequencing Facility, Mexico City.

The standard genetic analysis of raw sequences data were performed at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/) with the BLAST site (http://www.ncbi.nlm.nih.gov/BLAST/). *Hinf* I sites were verified for all PCR products with New England Biolabs NEBcutter version 2.0.

Results and Discussion

Morphological Identification

Identification according to Trager (1991) indicated that specimens collected in our study included the invasive *S. invicta*, and the following native taxa: *S. geminata*; members of the *S. xyloni* subcomplex of species, and *S. geminata*/*S. xyloni* hybrids (Fig. 2). It was possible to identify specimens as native (non-*invicta*) fire ants based on morphology and color. Identification of native fire ants to species was sometimes not possible because some samples lacked major workers; fire ant morphological taxonomy is mainly based on these workers (Trager 1991, and references therein).

Only sample 2 from the city of Chihuahua, state of Chihuahua (North-Central Mexico) included *S. xyloni* workers (Trager 1991) very similar to *S. invicta* in pigmentation and color pattern (Jacobson et al. 2006). This *S. xyloni* sample from Chihuahua is removed from *S. geminata* distribution sites (Trager 1991; Mackay & Mackay 2002; Helms Cahan & Vinson 2003), reducing the chances that this sample includes *S. xyloni*/*S. geminata* hybrids (Helms Cahan & Vinson 2003). Morphological examination of the remaining native fire ant samples indicated that some were typical *S. geminata*, and others *S. geminata*/*S. xyloni* hybrids. We also collected samples similar to the hybrids but lacking major workers, thus restricting morphological identification.

Fire Ant Molecular Markers Detected

Analysis of PCR products of all our samples yielded 3 main patterns (Fig. 3). These PCR products, digestions and sequences are described below. Regarding *S. invicta* markers, sequencing of the PCR product from sample 3 from Nuevo Laredo, Mexico and homology search (BLAST) in GenBank sequences indicated highest identity (99%) with 18 *S. invicta* COI accessions (i.e., EF620559 from the Wu-Chuan, China population). Comparison of our PCR product with one of these 99% identical sequences (Genbank acces-

TABLE 1. FIRE ANT SAMPLES, LOCALITIES, AND MORPHOLOGICAL AND MOLECULAR IDENTIFICATION.

Sample # and Locality	Coordinates	Sample # and Morphological Identification	Marker detected and (# of assays run on sample)
1. Torreón, Coahuila	N 25°40' 07" W 103°32'26"b	1. G	G (2)
2. Chihuahua, Chihuahua	N 28°30' 44" W 105°54' 07"	2. X*	X (2)
3. Nuevo Laredo, Tam.	N 27°29' 46" W 99°29' 37"	3. I	I (2)
4. Matamoros, Tam.	All Matamoros samples (4-15) collected within a 2-km radius from N 25°52' 09" W 097°24' 24"	4. G	G (4)
5. Matamoros, Tam.		5. I	I (2)
6. Matamoros, Tam.		6. I	I (2)
7. Matamoros, Tam.		7. I	I (2)
8. Matamoros, Tam.		8. H	G (2)
9. Matamoros, Tam.		9. I	I (2)
10. Matamoros, Tam.		10. I	I (2)
11. Matamoros, Tam.		11. I	I (2)
12. Matamoros, Tam.		12. I	I (2)
13. Matamoros, Tam		13. I	I (4)
14. Matamoros, Tam.		14. I	I (5)
15. Matamoros, Tam		15. I	I (2)
16. Monterrey, N.L.	Samples 16-20 from urban area N 25°42' 44", W 100°21' 28"	16. H	X (2)
17. Monterrey, N.L.		17. H	X (2)
18a+. (workers) Monterrey, N.L.		18a+ (workers). H	X (2)
18b+. (alate queens)		18b+. X (alate queens)	X
19. Monterrey, N.L.		19. H	G (4)
20. Monterrey, N.L.		20. H	X (2)
21. Monterrey (west)	N 25°42' 35" W 100°21' 50"	21. H	X
22. Monterrey (west)		22. H	X
23. Monterrey (south)	N 25°36' 11" W 100°15' 32"	23. H	X
24. Monterrey (south)		24. H	X
25. Santa María, Pesquería, N. L.	N 25°44' 05", W99°49' 44"	25. G	G
26. Santa María, Pesquería, N.L.		26. X	X
27. Santa María, Pesquería, N.L. (river)	N 25°44'21" W 99°49' 55"	27. X	X
28a+. (soldiers) Cadereyta	N 25°32'42" W 99°53'15"	28a+. G (soldiers)	G
28b+. (alate queens) Cadereyta	N 25°32'42" W 99°53'15"	28b+. G (alate queens)	G

G = *S. geminata*; I = *S. invicta*; X = *S. xyloni* subcomplex; H = *geminate/xyloni* hybrid; *, typical *S. xyloni* sensu Trager (1991); + = individuals (winged queens and workers) with the same sample number (e.g., 18a and 18b) were collected from the same colony. Santa Maria and Cadereyta are 40-50 km east of Monterrey.

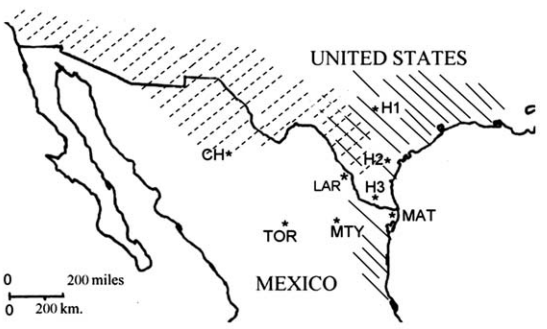

Fig. 1. General distribution and collection localities for fire ants in Northeastern Mexico. CH, Chihuahua, Chihuahua; LAR, Nuevo Laredo, Tamaulipas; MTY, Monterrey, Nuevo León; MAT, Matamoros, Tamaulipas; TOR, Torreón, Coahuila. H1-3, *S. geminata*/*S. xyloni* hybrid collection sites in Texas (from Helms Cahan &Vinson 2003): H1, Austin; H2, San Patricio County; H3, Weslaco. Dashed lines: approximate general distribution for *Solenopsis xyloni*: California to south-central Texas; solid lines, general historical distribution for *Solenopsis geminata*: Gulf of Mexico coast, in the USA and Mexico, and Southeastern USA. Notice the general area where *S. geminata* and *S. xyloni* overlap, north and east from Laredo, Texas. General distribution of *S. geminata* and *S. xyloni* from Trager (1991) and Helms Cahan & Vinson (2003).

sion DQ153014), submitted by Jacobson et al. (2006) indicated that both possess the diagnostic *Hinf* I restriction site, splitting the PCR product into 2 fragments (691 and 249 bp in this work; 687 and 252 bp in Jacobson et al. (2006)) (Fig. 3). The PCR-*Hinf* I digestion of COI in our samples (Table 1) confirms the presence of *S. invicta* populations across hundreds of kilometers in Mexico (Nuevo Laredo and Matamoros), and supports the use of this molecular marker as an identification tool for Mexican populations of *S. invicta* when fire ant morphological identification is limited.

Native Fire Ants with the *S. xyloni* COI Marker

Most of our samples identified as native fire ants harbored the *S. xyloni* marker (i.e., COI digestion pattern) described by Jacobson et al. (2006). These samples were the following.

(1) the Chihuahua *S. xyloni* specimens (sample 2). These were morphologically typical *S. xyloni*, with bicolored major workers having a lighter, brown-reddish head and mesosoma, and uniformly dark gaster lacking light reddish or brown areas, in a pattern very similar to that of U.S. populations of *S. invicta* (Trager 1991; MacKay & MacKay 2002). We did not observe these bi-colored majors of *S. xyloni* in the samples from Nuevo Léon and Tamaulipas described herein, nor in the survey of Sánchez-Peña et al (2005). Trager (1991) reports that *S. xyloni* is very rare or absent along the Gulf of Mexico coast.

(2) Samples identified as *S. geminata*/*S. xyloni* hybrids by morphology. Some native ant samples had large workers that match the description of Trager (1991) for the *S. geminata*/*S. xyloni* hybrids: larger workers with bilobed head having posteriorly divergent sides; head width ≥1.50 mm (larger than that of the parental queens, unlike *S. xyloni* major workers, but smaller than that of *S. geminata* soldiers), and poorly developed propodeal carinae; they are yellowish-red, in a rather uniform color pattern, with a darkened gaster tip. Alate queens from these colonies belonged to the *S. xyloni* subcomplex sensu Trager (1991) (Fig. 3). The color of these putative hybrid workers is similar to, but usually clearly paler than, the orange color of sympatric *S. geminata*. The following comparisons can be made across the hybrid and the parental species *S. geminata* and *S. xyloni*; they might be useful for hybrid identification if the following castes are available. As described in Trager (1991), the head of the majors of these putative hybrids workers' is clearly wider that that of *S. geminata* queens; these were identified morphologically and they have the *S. geminata* COI marker as well (see below). *S. xyloni* major's heads are not wider than those of *S. geminata* queens (Trager 1991).

(3) We also found the *S. xyloni* marker in native fire ant samples lacking major workers and sexuals; these samples resembled the hybrid samples in coloration; and they were collected within meters of hybrids. As mentioned, fire ant taxonomy is based on the major workers (Trager 1991; Jacobson et al. 2006); to our knowledge, there are no descriptions of non-major workers of the *S. geminata* / *S. xyloni* hybrid and morphological identification is limited at this point.

The sequence of sample 18 from Monterrey (identified as a *S. geminata*/*S. xyloni* hybrid by morphology) was 96% identical to the *S. xyloni* COI sequence (Genbank accesion DQ153015) from New Mexico (Jacobson et al. 2006). Both sequences had an identical restriction pattern as indicated by virtual and actual *Hinf* I digestion of our samples. Bands generated are 18, 108, 138, 327 and 347 kb, resulting in a 2-band pattern on gel (347/327 kb) and (138/108 kb) (Jacobson et al. 2006; Fig. 3). Our *S. xyloni* sequence (sample 18) had a maximum of 88% identity with our *S. geminata* marker (see next section), and with Genbank COI accessions of U.S. (Florida) *S. geminata* (Ross et al. 2003) and South American accessions of the *Solenopsis saevissima* species group.

Solenopsis geminata

A third band pattern (Fig. 3) was obtained from samples morphologically identified as typical reddish *S. geminata* (Trager 1991). These were sample 1 from Torreón, sample 4 from Matamoros and sample 27 from Cadereyta (Table 1). This pat-

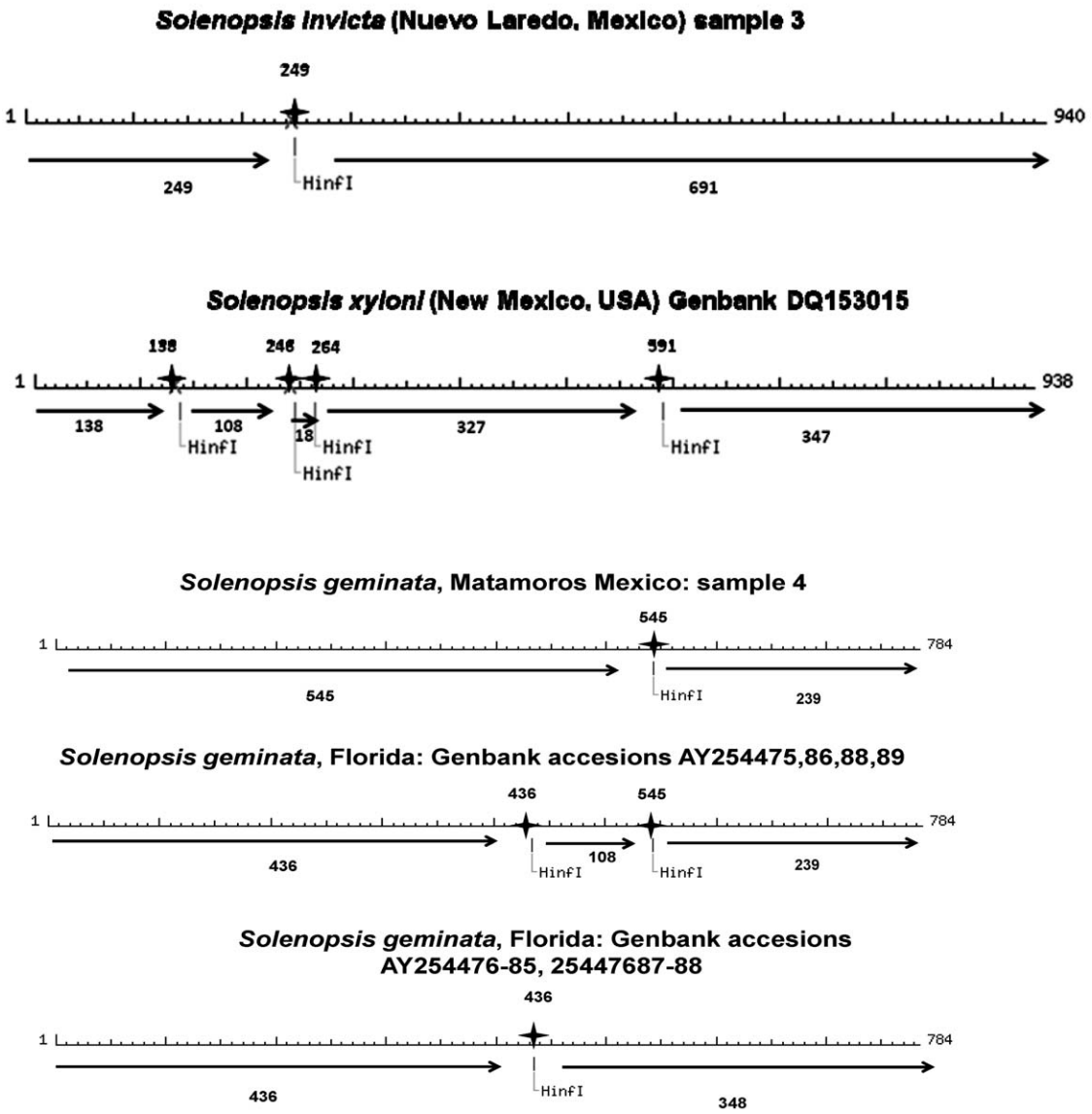

Fig. 2. Comparison of *Hinf I* digestion of COI PCR products of fire ants from the USA (Genbank accessions) and Northern Mexico (this report). Scaled lines represent PCR products. Top line: *S. invicta* digestion pattern (Nuevo Laredo, Mexico). Fragments produced are very similar to those reported by Jacobson et al. (2006) for New Mexico specimens of *S. invicta.* See text for details. Second line from top: *S. xyloni* digestion pattern. Product size and *Hinf I* sites were identical for the Chihuahua, Mexico (this work) and the New Mexico (Jacobson et al. 2006) specimens. Bottom three lines: *Solenopsis geminata* digestion patterns. The Northern Mexico material shared a *Hinf I* site with four Genbank accessions of *S. geminata* from Florida. The remaining Florida accessions did not share a restriction site with the Mexican material reported herein (bottom line). Numbers under the arrows = fragment size after *Hinf I* digestion; numbers above the scaled lines (stars) = position of the *Hinf I* restriction site. Number to the right of the scaled line = PCR product size in kb. See text for sequence comparisons and

tern was obtained also from samples lacking the typical *S. geminata* soldiers (likely due to limited sampling) from Monterrey (sample 19) and Matamoros (sample 8). The PCR product sequence from *S. geminata* (sample 4) from Matamoros was 938 bp long. The 15 *S. geminata* CO1 accessions in Genbank originate from 2 Northern Florida populations (Alachua and Leon Counties), about 1000 km NE from Matamoros (Ross et al. 2003); these accessions are 795 bp because they were

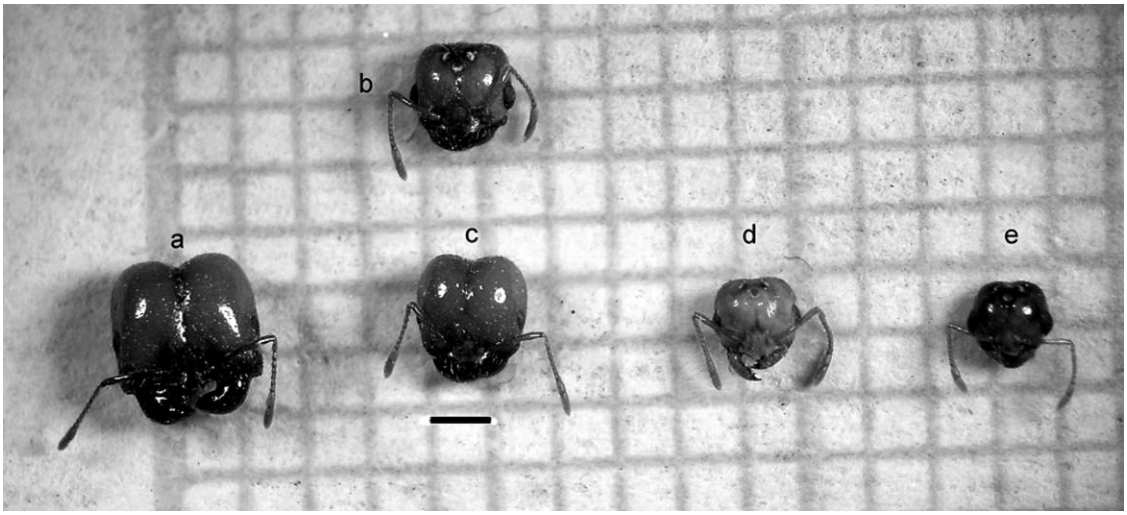

Fig. 3. Native *Solenopsis* heads and genotypes from Northeastern Mexico. a, *S. geminata* soldier (individual has *S. geminata* COI marker; sample 28a in Table 1, from Cadereyta, Nuevo León); b, *S. geminata* alate queen (individual has *S. geminata* COI marker; sample 28b in Table 1, from Cadereyta, Nuevo León) (the soldier and queen labeled a and b respectively are from the same colony, thus presumably sisters); c, *S. geminata* x *S. xyloni* hybrid (individual has *S. xyloni* COI marker; sample 18a in Table 1, from Monterrey, Nuevo León). Notice that head of hybrid "soldier" (c) is distinctly wider (above the eyes) than that of the *S. geminata* queen (b), a parental species; d, *S. xyloni* subcomplex winged queen from Monterrey (not genotyped, thus not in Table 1); e, *S. xyloni* subcomplex winged queen (individual has *S. xyloni* COI marker; sample 18b in Table 1, from Monterrey, Nuevo León) (the worker and alate queen labeled c and e respectively are from the same colony, thus presumably sisters). Reference bar = 1 mm.

generated with primers different from the ones we used, and they are nested within our longer sequence. In the overlapping regions, our *S. geminata* sample had 93% sequence homology with GenBank accession AY254488, the most similar. Our *S. geminata* sequence shares a *Hinf* I restriction site on position 545 with 4 out of the aforementioned 15 *S. geminata* Genbank accessions (haplotypes 1, 12, 14, and 15). All 15 Genbank accessions have a *Hinf* I site on position 436; our *S. geminata* samples (1, 4, 6, 19 and 27) lack this site as shown by analogous digestion (Fig. 2). Eleven Genbank accessions have only 1 site at position 436. Haplotype 1 is restricted to a polygyne form and haplotypes 12, 14 and 15 are variants from monogyne colonies (Ross et al. 2003). We do not know if our samples come from polygyne or monogyne *S. geminata.* Our Sample 4 of *S. geminata* and the Genbank accessions from Ross et al. (2003) of this species had 88% identity with our *S. xyloni* (sample 18). The sequences obtained from all these Mexican populations of fire ant species will be accessed in GenBank.

The molecular characterizations described in this work have implications for regional fire ant identification. Considering the wide geographical distribution of this *S. invicta* marker (i.e., New Mexico, Jacobson et al. 2007); Southeastern USA (Shoemaker et al. 2006); and this report) it is probably useful for general *S. invicta* identification. Jacobson et al. (2006) emphasized the difficulties in accurately discriminating between *S. invicta* and *S. xyloni* from New Mexico. They suggested that several morphological characteristics should be used to distinguish *S. xyloni* from *S. invicta*, a task to be performed by "taxonomists familiar with *Solenopsis*". Although somewhat time-consuming and expensive, the molecular method described herein makes use of methodologies from standard assays in *Drosophila* genetics that might be available to a considerable number of researchers. Molecular methods constitute a significant tool for species identification when certainty is critical (Jacobson et al. 2006).

Regarding *S. geminata*, our samples spanned more than 600 km; therefore, this marker can probably be used for identification of local samples. However, considering our data and the Genbank sequences from Florida, *S. geminata* appears to be a variable species regarding this marker.

In conclusion, the modification described to the assay of Jacobson et al. (2006) can be a useful tool for identification of *S. invicta*, *S. geminate,* and the *S. xyloni* subcomplex from northeastern Mexico. The mtDNA polymorphism in *S. geminata* and particularly the widespread presence of the *S. geminata*/*S. xyloni* hybrid in this area (Helms Cahan & Vinson 2003) adds a facet to fire ant identification. This assay might be particularly

useful for accurate hybrid identification, because yellow-orange major hybrid workers with conspicuously enlarged heads and black mandibles (Fig. 3) could rather easily be misidentified as *S. geminata*. It must be considered, however, that widely distributed fire ant morphospecies might comprise cryptic species or species complexes with diagnostic genetic regional differences reflected in their mtDNA haplotype. Ross et al. (2007) show evidence of this in *S. invicta*; *S. geminata* could be a similar case considering the mtDNA polymorphism detected. Thus, although the reported bioassay (and that of Jacobson et al. 2006) are probably useful for the geographical areas they analyzed, we cannot be sure that they are universally useful in all areas where the nominal species might co-occur without further documentation of the mtDNA diversity in these species.

Our morphological and molecular identifications of native fire ants are consistent with the report of Helms Cahan & Vinson (2003) regarding native fire ant populations from central and extreme south Texas. Using allozyme and microsatellite markers, they reported that native fire ant workers collected in these regions were *S. geminata* and *S. geminata*/*S. xyloni* hybrids only. *Solenopsis xyloni* is very rare or absent along the Gulf of Mexico coast in the USA (Trager 1991); the Matamoros site herein is about 10 km from the Gulf coast, and very close to the Texas, USA border.

According to Helms Cahan & Vinson (2003), the *S. geminata*/*S. xyloni* hybrid workers from these populations are all F1 and heterozygotic; however they possess the *S. xyloni* mitochondrial genome almost exclusively, because this hybrid is unidirectional and the result of a *S. geminata* male/*S. xyloni* female cross. Helms Cahan & Vinson (2003) found that in Texas the frequency of hybrid colonies relative to that of *S. geminata* increased in a north-south direction, from 51% in Central Texas (Austin, Travis county) up to 82% at the Mexican border (Weslaco, Hidalgo county); this last location is about 50 km west (inland) from the Matamoros site reported herein. Likewise, most (89%, $n = 9$) of our hybrid-looking samples (most lacking major workers) had the *xyloni* marker. See Helms Cahan & Vinson (2003) for the description of social hybridogenesis in these *Solenopsis* populations.

Accurate identification of *S. invicta* will support management strategies to mitigate the environmental, urban and economic impact of this invasive ant species in Mexico. Also, regarding native fire ant populations, a more complete knowledge of their genetic markers will contribute to our understanding of local biodiversity. Our analysis of the COI marker appears to confirm the very restricted gene flow previously reported between *S. geminata* and *S. xyloni* sexuals, thus supporting their validity as "good" biological species. These *Solenopsis* hybrids are still abundant and geographically widespread (Hung & Vinson 1977; Trager 1991; Helms Cahan & Vinson 2003). However, native *Solenopsis* species and their hybrids have been greatly reduced or eliminated from large areas (such as the southeastern USA) after the *S. invicta* invasion in the 60s and 70s (Trager 1991; Wojcik et al. 2001; Helms Cahan & Vinson 2003). The clarification of the identity of invasive and native fire ant species and of their hybrids will contribute towards our understanding of their ecology and their interactions, like displacement of native ants by the *S. invicta*.

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