Genetic and chemical profiling of *Solenopsis* spp. (Hymenoptera: Formicidae) intercepted in Hawaii

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Hawaii is the most isolated island archipelago in the world, separated by 4,000 km from the nearest continent (Howarth et al. 1988). Only half the insect orders comprising about 15% of insect families worldwide are represented in Hawaii's native terrestrial fauna (Howarth et al. 1988). All 60 species of ants (Hymenoptera: Formicidae) currently recorded in Hawaii have been introduced, including 5 of the most damaging invasive species: *Anoplolepis gracilipes* (Fr. Smith), the yellow crazy ant; *Linepithema humile* (Mayr), the Argentine ant; *Pheidole megacephala* (F.), the big-headed ant; *Solenopsis geminata* (F.), the tropical fire ant; and *Wasmannia auropunctata* (Roger), the little fire ant (Holway et al. 2002; Krushelnycky et al. 2005). The most destructive invasive ant, *Solenopsis invicta* Buren (red imported fire ant) (USDA-ARS 2010; Ascunce et al. 2011; Global Invasive Species Database 2021), has not yet established in Hawaii. Classical taxonomic identification of *Solenopsis* species is very difficult, thus when Hawaii Department of Agriculture quarantine officials intercepted ants identified as a *Solenopsis* spp. and possibly *S. invicta* in 2018 and 2019, the collected ant(s) were submitted to the United States Department of Agriculture-Agricultural Research Service-Center for Medical, Agricultural, and Veterinary Entomology, Imported Fire Ant and Household Insects Research Unit, Gainesville, Florida, USA, for further assessment using genetic and chemical methods.

The 2018 sample consisted of 1 worker ant labelled "Oahu," found on a shipment of flowers from San Diego, California, USA. The 2019 sample consisted of 3 worker ants, designated Lt19 1, 2, and 3. They were found in a peat moss shipment originating in Germany with an undetermined pathway to California and subsequent ship transport to Hawaii. For both interceptions, there is uncertainty as to the actual source of the ants collected.

In all cases, thoraces were used for genetic analyses and gasters were used for venom analyses. For the genetic analysis, thorax DNA was extracted using standard procedures (Ascunce et al. 2011). These DNAs were then subjected to polymerase chain reaction amplification of the mitochondrial COX1 gene using forward primer C1-J-2195 5'-TTGATTTTTTGGTCATCCAGAAGT-3' (Simon et al. 1994) and a new reversed primer Finn 5'-TTCATATCTTCAATATCATTGATGTCC-3' using polymerase chain reaction protocol as described in Ascunce et al. (2011). Polymerase chain reaction products were submitted for Sanger sequencing to service facility. The new sequences (Oahu: GenBank OK071254, 1,109 bp; and Lt19 GenBank OK071253, 1,109 bp) were aligned with previously published sequences of *Solenopsis* species, using Muscle (Edgar 2004). A total of 55 mitochondrial sequences from 6 *Solenopsis* species (Hymenoptera: Formicidae) were analyzed, including *Solenopsis geminata*, *Solenopsis xyloni*, *Solenopsis amblychila*, *Solenopsis aurea*, *Solenopsis invicta*, and *Solenopsis saevissima*. All positions containing gaps and missing data were eliminated. There were 616 positions in the final dataset. A matrix of absolute numbers of pairwise differences among mitochondrial haplotypes was calculated and used to construct a neighbor-joining tree with MEGA version 7 (Kumar et al. 2016). The Oahu sample was closest to a haplotype of *S. xyloni*, with only a 5 bp difference; however, the *S. amblychila* sample also was close (a 6 bp difference). In the neighbor-joining tree analysis, the Oahu sample was positioned with *S. xyloni*, which are native to the shipment's origin, San Diego, California, USA (Fig. 1). The 3 samples from 2019 have the same haplotype, which is only 1 bp different from other *S. geminata* found around the world. In the neighbor-joining tree analyses, the 2019 samples fit within the variation found for *S. geminata* (Fig. 1). *Solenopsis geminata* is a tramp ant species found in many tropical/sub-tropical areas of the world. Commonly known as the tropical fire ant, the native distribution of *S. geminata* extends from northern South America to southern North America (Trager 1991). The tropical fire ant's worldwide dispersal likely began in the 16th century with the commencement of trade routes between the old and new worlds and probably via ballast, e.g., soil in European trading ships, where ants were transported as stowaways (Gotzek et al. 2015). Nowadays this species is found worldwide mainly in tropical zones, with records in Africa, Australia, Borneo, India, Indonesia, Malaysia, Madagascar, Hawaii, Polynesia, China, Japan, and many other areas (Holway et al. 2002; AntWeb 2021). Extensive work is needed to understand the taxonomic and genetic diversity and complexity of this group (Fig. 1).

Fire ant venom alkaloids have been investigated extensively since the early 1970s and their isolation, chemical analysis, mass spectral identification, and their chemotaxonomic value is well documented (Brand et al. 1972, 1973; Vander Meer et al. 1985). The alkaloid profiles from 24 h passive hexane extracts of gasters (see Shi et al. 2015, poison sacs are in the gaster) from both the 2018 and 2019 interceptions were analyzed by Gas Chromatography-Mass Spectrometry, Agilent Intuvo 9000 GC system coupled to a 5977 B mass spectral detector with a Masshunter Data Acquisition Workstation version 10.0.368 (Santa Clara, California, USA). The alkaloid component profiles eliminated *S. invicta* and *S. richteri* as possibilities. The profile of the 2018 intercepted ant had 2 major components, cis-2-methyl-6-undecyl-piperidine

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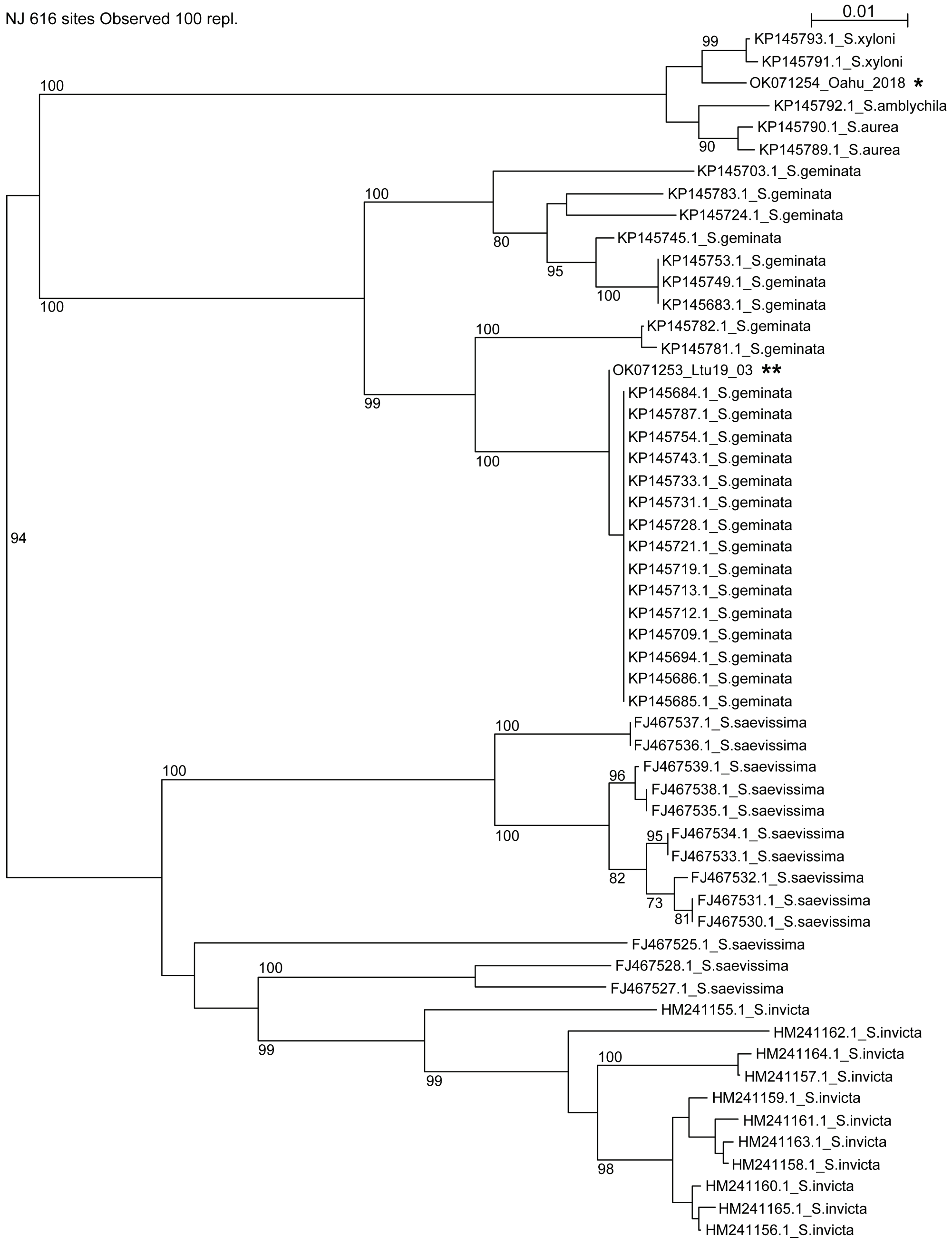

Fig. 1. Unrooted neighbor-joining tree constructed using the matrix of absolute numbers of pairwise differences among 55 mitochondrial COX1 haplotypes from 6 *Solenopsis* species: *S. geminata*, *S. xyloni*, *S. amblychila*, *S. aurea*, *S. invicta*, and *S. saevissima*. All positions containing gaps and missing data were eliminated. Bootstrap values greater than 70% are located below or above nodes. The scale bar distance is shown at the top right corner of the tree indicating number of nucleotide differences. Each sequence was identified by their GenBank number and species name, except for the Oahu sample (GenBank OK071254) marked as Oahu 2018* and the haplotype from the 2019 samples (GenBank OK071253) labelled Ltu19_03**.

and trans-2-methyl-6-undecyl-piperidine (identified by mass spectral fragmentation patterns, peaks 1 and 3; Fig. 2). These alkaloids are common to members of the *S. geminata* species group, which includes *S. xyloni* and *S. geminata* (Brand et al. 1972; Shi et al. 2015). The ratio of the 2 alkaloids (cis/trans) differentiate the 2 species (Brand et al. 1973). For *S. geminata* the ratio of cis to trans is 1.5:1 and for *S. xyloni* the ratio is 3.97:1. The ratio of the Oahu intercepted ant is 4.3:1, most like *S. xyloni* (92% match), and far from the ratio reported for *S. geminata* (35% match). The piperidine alkaloids are very stable, and whereas the amount of alkaloid recovered may vary, the ratio of the major components is expected to remain constant; therefore, the venom alkaloids strongly suggest that this ant is *S. xyloni*.

Venom alkaloids from gaster extracts from the 2019 interception initially appeared to be a typical *S. geminata* group pattern, 2 major components. The second major component (peak 3; Fig. 2) was identified as the expected trans-2-methyl-6-undecyl-piperidine, based on the mass spectral fragmentation pattern; however, the earlier eluting major component (peak 2; Fig. 2) was identified as 2-methyl-6-undecyl-pryidine based on mass spectral fragmentation pattern and library match. This alkaloid has been reported only as a minor component in *S. invicta* and *S. richteri* venom (Chen et al. 2019). In addition, the normally significant peak 1, cis-2-methyl-6-undecyl-piperidine, was reduced to a minor component (Fig. 2). While this is intriguing, the lack of additional samples and origin information limits what we can deduce to suggesting that the three 2019 intercepted ants represent a unique variant within *S. geminata*.

Results from genetic and chemical analyses of the 2018 sample concurred that the ant was *S. xyloni*. The three 2019 samples were identical in their respective genetic and chemical profiles. Genetic methods identified them as *S. geminata*; however, chemical analyses identified a novel major alkaloid component, suggesting that these ants are from a unique *S. geminata* variant. These are excellent examples of the utility of molecular and chemical tools combined to identify intercepted invasive ants, even when the number of samples is limited, and morphological identification is difficult. This study also highlights the need to expand combined genetic and chemical analyses to better understand the diversity of the tropical fire ant and related species. Furthermore, with the expansion of *S. invicta* in China and other countries in the Pacific Rim, it is essential to continue to improve methods for intercepting and identifying potential new ant introductions, especially in Hawaii.

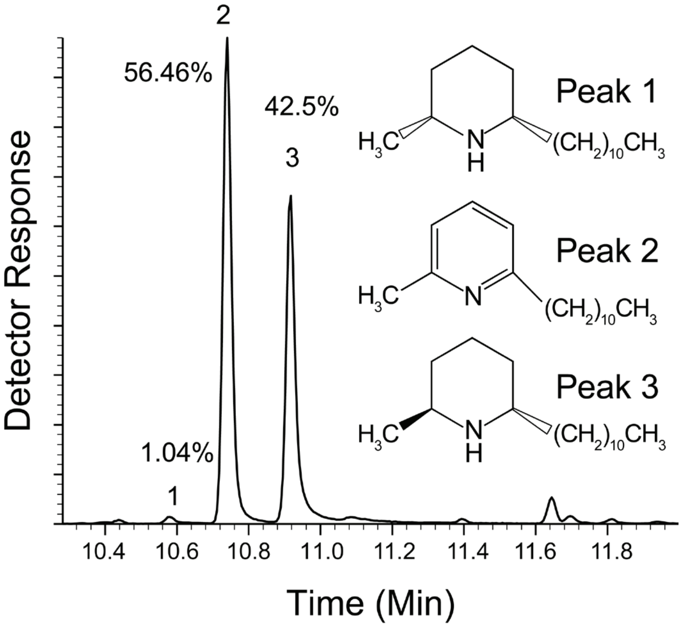

Fig. 2. The Gas Chromatography-Mass Spectrometry (GC-MS), total ion chromatogram (TIC) of a methanol extract of a gaster from a single ant intercepted in 2019 at a port in Hawaii. The reported major venom alkaloids from *Solenopsis geminata* and *Solenopsis xyloni* are cis-2-methyl-6-undecyl-piperidine (peak 1) and trans-2-methyl-6-undecyl-piperidine (peak 3). This intercepted *Solenopsis geminata* worker has 2-methyl-6-undecyl-pyridine (peak 2) as its major component. The areas under peaks 1, 2, and 3 are proportional to their quantity. This is expressed on the graph as the percent composition of the 3 identified peaks. This is the first time that the pyridine (peak 2) has been reported as a major venom component of *Solenopsis geminata*. Components 1 and 3 have been identified in previous literature.

We wish to thank Eileen Carroll, Andrew Nisip, and Erin O'Reilly (USDA-ARS-CMAVE), Michele Nitti (Contractor), and Christopher M. Kishimoto (Hawaii Department of Agriculture, Honolulu, Hawaii, USA) for technical assistance.

Summary

Ants intercepted by the Hawaii Department of Agriculture in 2018 and 2019 identified as *Solenopsis* spp., and possibly *S. invicta*, were submitted to the United States Department of Agriculture-Agricultural Research Service-Center for Medical, Agricultural and Veterinary Entomology, Imported Fire Ant and Household Insects Research Unit for further taxonomic assessment using genetic and chemical methods. The 2018 sample consisted of 1 worker ant, and the 2019 sample consisted of 3 worker ants. Thoraces were used for genetic assessment and gasters were used for venom analyses. It was determined readily from genetic and chemical analyses that the intercepted ants were not *S. invicta* or *S. richteri*. Mitochondrial COX1 gene sequences were compared with previously published data from *Solenopsis* species. The closest match for the 2018 sample was *S. xyloni*, which is native to the shipment's origin (San Diego, California, USA). The 2 major alkaloids found were characteristic of the *S. geminata* species group. In addition, the alkaloid ratio closely matched that of *S. xyloni*. Therefore, the alkaloid analysis of the 2018 intercepted ant concurs with the genetic data that the ant was an *S. xyloni* worker. The COX1 gene sequences of the 2019 interceptions most closely matched *S. geminata*. The corresponding venom alkaloid profile also eliminated *S. invicta* and *S. richteri* as possibilities. However, 1 major alkaloid was unusual (Fig. 2) and normally is found only in trace amounts. We suggest the 2019 specimens are an unusual variant of *S. geminata*.

Key Words: fire ants; mitochondrial DNA; venom alkaloids; *Solenopsis xyloni*; *Solenopsis geminata*

Sumario

Hormigas interceptadas por el Departamento de Agricultura de Hawaii en el 2018 y el 2019 fueron identificadas como *Solenopsis* spp., y posiblemente *S. invicta*. Esas hormigas fueron enviadas al Departamento de Agricultura de los Estados Unidos, Servicio de Investigación Agrícola, Centro de Entomología Medica, Agrícola y Veterinaria, Unidad de Investigación de la Hormiga Importada de Fuego e Insectos Domésticos para corroborar su taxonomía usando métodos genéticos y químicos. La muestra del 2018 consistió en una hormiga trabajadora. Las muestras del 2019 consistieron en tres hormigas trabajadoras. Los toraces fueron usados para asesoramiento genético y los abdómenes fueron usados para análisis químicos de venenos. Fue claramente determinado tanto con los análisis genéticos como químicos que las hormigas interceptadas no eran *S. invicta* ni *S. richteri*.

Secuencias del gen mitocondrial COX1 fueron comparadas con secuencias publicadas anteriormente de especies del genero *Solenopsis*. La secuencia mas similar a la muestra del 2018 fue una secuencia perteneciente a la especie *S. xyloni*, la cual es nativa a la región de origen de la carga en la cual fue interceptada (San Diego, California, USA). Los dos alcaloides principales encontrados fueron característicos de las especies del grupo *S. geminata*. Además, la proporción del alcaloide reflejó una similitud cercana al que se encuentra en las hormigas *S. xyloni*. Por lo tanto, el análisis de alcaloides de la hormiga interceptada en el 2018 concuerda con los datos genéticos determinando que esa hormiga era una hormiga trabajadora de la especie *S. xyloni*. Las secuencias del gen COX1 de la intercepción del 2019 era mas similar a *S. geminata*. El perfil del veneno alcaloide de estas hormigas también elimino a *S. invicta* y *S. richteri* como posibilidades. Sin embargo, un componente alcaloide mayoritario fue inusual y usualmente se halla solo en cantidades mínimas. Nosotros sugerimos que son una variante del grupo *S. geminata*.

Palabras Claves: hormiga de fuego; ADN mitocondrial; venenos alcaloides; *Solenopsis xyloni*; *Solenopsis geminata*

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