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Molecular comparisons of native range collections of *Gadirtha fusca*, a potential biological control agent of Chinese tallowtree

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ABSTRACT
Chinese tallowtree, *Triadica sebifera* (L.) Small (Euphorbiaceae), is one of the worst invasive weeds of the southeastern USA impacting coastal wetlands, forests, and natural areas. A proposed biological control candidate, the defoliating moth *Gadirtha fusca* Pogue (Lepidoptera: Nolidae) shows high specificity for the target weed Chinese tallowtree. A total of 13 field sites were sampled in the native range of this herbivore species. To determine if all individuals were the same taxon, molecular DNA analyses were conducted of these collections. These included collections from 2012, 2015 and 2016, from three regions and two adult color morphs. Molecular COI analysis was performed on thirty colonized individuals from these collections. The results of this analysis arranged all taxa into a single clade with average genetic divergence values of 0.3%. Comparison of these *G. fusca* sequences and those from other *Gadirtha* spp. all had divergence values that were equal to or exceeded 5%. These results indicate that all the *G. fusca* collections were a single species and they were distinct from other known members of this genus.

KEYWORDS
Triadica sebifera; Nolidae; invasive weeds; COI barcode; neighbor joining analysis

1. Introduction

Chinese tallowtree, *Triadica sebifera* (L.) Small (Euphorbiaceae) (hereafter 'tallow'), is one of the most damaging invasive weeds in the southeastern US, impacting wetlands, forests, and natural areas. In California and the states that border the Gulf of Mexico, tallow infests 185,000 ha of southern forests, stranded swamps, flatwoods, and ruderal communities (Rawlins, Griffin, Moorhead, Bargeron, & Evans, 2018). The native range of tallow includes southern China and northern Vietnam (Figure 1; Bingtao & Esser, 2008). In the US, tallow was introduced numerous times into locations including South Carolina and Georgia in the 18th century and throughout the southeastern USA in the early 20th century (Scheld & Cowles, 1981). One of the most common impacts tallow has is the conversion of coastal prairies, where graminoid/herbaceous prairies have been rapidly colonized and transformed to tallow forests (Bruce, Cameron, & Harcombe, 1995). In these eastern Texas prairie habitats, closed canopy forests formed within 10 years

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(Bruce et al., 1995). These infestations have rapidly expanded into upland habitats creating naturalized tallow populations from southern Texas along the Gulf Coast to Florida and north to North Carolina (Rawlins et al., 2018). In many areas, monospecific stands of tallow have developed where few native species survive (Bruce et al., 1995; Jubinsky & Anderson, 1996).

Research on the biological control of tallow began in 2006 with foreign surveys initiated by the USDA/ARS/Invasive Plant Research Laboratory (IPRL) in collaboration with Wuhan Botanical Garden, Chinese Academy of Science (Wheeler & Ding, 2014). These surveys discovered several species of insects and preliminary testing was conducted on three, *Heterapoderopsis* (=*Apoderus*) *bicallosicollis* Voss (Coleoptera: Attelabidae), *Bikasha collaris* (Baly) (Coleoptera: Chrysomelidae), and *Gadirtha fusca* Pogue (Lepidoptera: Nolidae). These preliminary studies showed all three species had narrow specificity to the target weed and close relatives (Huang, Wheeler, Purcell, & Ding, 2011; Wang et al., 2012; Wang, Ding, Wheeler, Purcell, & Zhang, 2009). Following these Chinese studies, all three species were imported sequentially and tested in quarantine at the USDA/ARS/IPRL quarantine facility. Upon testing with North American species, the leaf rolling weevil *H. bicallosicollis* was rejected due to specificity deemed too broad (Steininger, Wright, Ding, & Wheeler, 2013). The flea beetle, *B. collaris,* feed as adults on foliage and the larvae feed on roots. Quarantine testing of *B. collaris* was conducted from 2010 to 2016 and indicated that this species could only complete its life cycle on tallow (Wheeler, Steininger, & Wright, 2017). The third species, *G. fusca* was consigned to quarantine in 2012 and tests indicated it will also be a suitable biological control candidate of tallow (Wheeler unpublished data, Wang et al., 2012).

The original Chinese field collections of this species were thought to be *Gadirtha inexacta* Walker but subsequent analysis indicated that they constituted a new species which was described as *G. fusca* (Pogue, 2014). These original collections were conducted during June 2012 and subsequent collections from many Chinese locations recovered larvae that resembled the original *G. fusca*. Collections of potential agents from disparate field sites in the native range can result in the unintentional recovery of cryptic species (Mound, Wheeler, & Williams, 2010; Paterson et al., 2016; Smith et al., 2018; Tosevski et al., 2011; Tracy & Robbins, 2009). Accurate taxonomic determination of biological control agents is critical to avoid the introduction of mixed or unknown agent species. DNA analysis is becoming a frequent method to confirm species identity of agents being considered for biological control (Gaskin et al., 2011). These molecular determinations have become a useful tool in biological control (Madeira, Facey, Pratt, Maul, & Wheeler, 2016; McCulloch et al., 2018; Paterson et al., 2016; Smith et al., 2018). To minimize risk of introducing unintended taxa, we conducted DNA analysis to determine if these collections included the same species and matched our quarantine colony of *G. fusca*.

2. Methods

2.1. Field collection

Tallow surveys were conducted at 13 sites in south central and eastern China. Collections occurred in several regions from Jiangxi, Hunan, Guangxi, Anhui and Guangdong provinces (Figure 1; Table 1). The first collection of *G. fusca* larvae was made in 2012 and

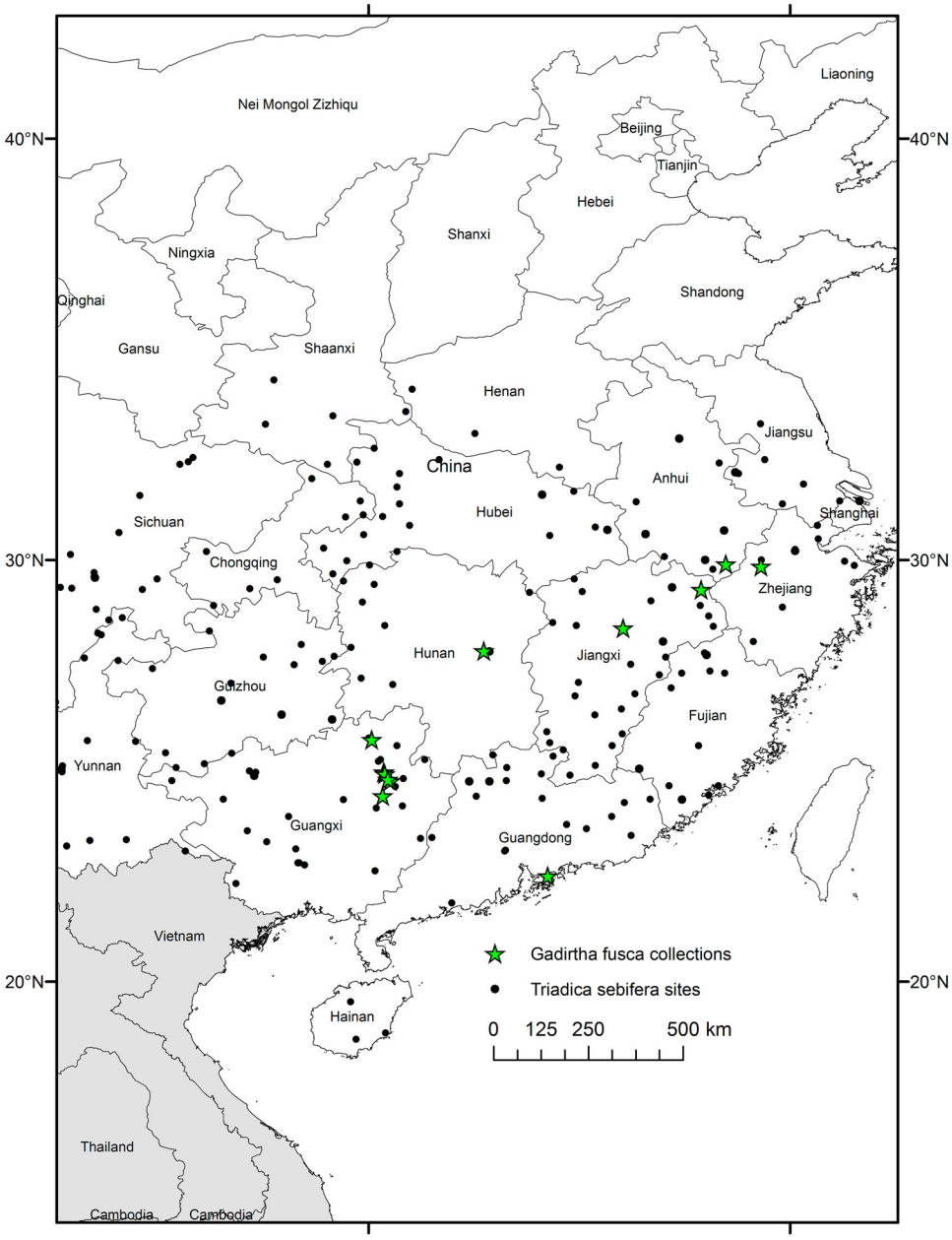

Figure 1. Map of southern China where *Gadirtha fusca* larvae were collected (red stars) and native range location records of the host, *Triadica sebifera* (black dots). DNA analyses were performed on collections made near Guilin, Guangxi, Hong Kong, Guangdong, and Huangshan, Anhui province.

additional collections were made in 2015 and 2016 (Figure 1; Table 1). Collections were made by visual inspection of tallow plants searching for all herbivores. These surveys resulted in the collection of larvae that resembled *G. fusca* found during the initial 2012 collections (Pogue, 2014). The larvae were fed tallow leaves during the 10 to 15-day

Table 1. Records of *Gadirtha fusca* larval collections on Chinese tallow during 2012–2016 surveys of southern China.

Date	Province	Site	Number	Latitude N	Longitude E
June 2012	Jiangxi	Nanchang	1	28.38668	116.03488
June 2012	Hunan	Zhuzhou	1	27.84548	112.73156
June 2012	Guangxi	Guilin	4	24.79834	110.45067
June 2012	Guangxi	Guilin	2	24.97577	110.34844
August 2015	Guangxi	Guilin	7	24.97445	110.34931
August 2015	Guangxi	Guilin	11	24.96914	110.35636
August 2015	Guangxi	Guilin	3	24.76522	110.50437
August 2015	Guangxi	Guilin	3	24.93032	110.37597
August 2015	Anhui	Huangshan	3	29.30302	117.88602
August 2015	Anhui	Huangshan	1	29.90937	118.46834
August 2015	Anhui	Huangshan	17	29.85102	119.30313
June 2016	Guangdong	Hong Kong	1	22.50967	114.24406
June 2016	Guangdong	Hong Kong	2	22.40615	114.32578
September 2016	Guangxi	Guilin	26	24.79834	110.45067

survey and later they were introduced under quarantine at the USDA/ARS/IPRL, Ft Lauderdale, FL, USA. A single colony was established by combining all Guilin 2012 collections. All adults from this colony had gray wing patterns (Figure 2(A)). However, after adults emerged from the 2015 Chinese field collections, two distinct adult color morphs were recognized, gray and copper (Figure 2). Furthermore, the color morph was not

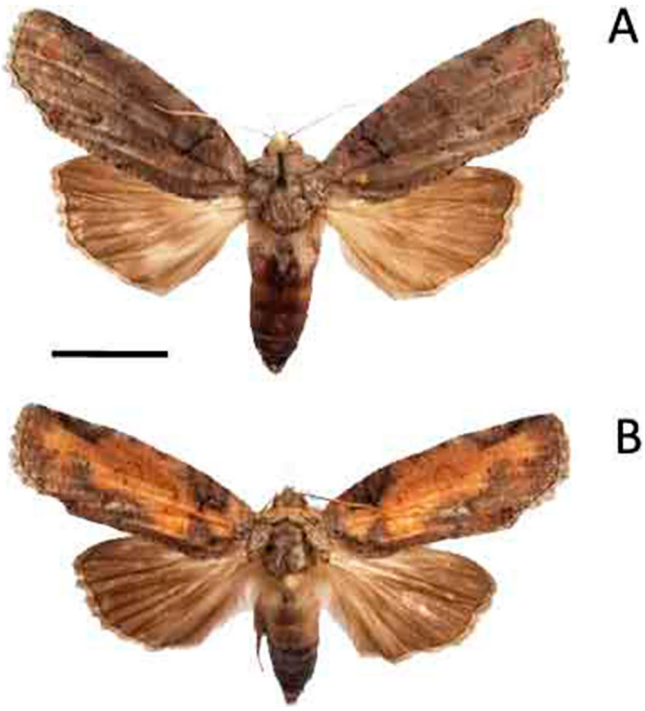

Figure 2. Male *Gadirtha fusca* moths reared from larvae field collected near Guilin, Guangxi province, China. Upon emergence of the adults, two color morphs were noticed, A) the gray and B) the copper varieties. Horizonal bar = 1 cm.

limited to different locations as both the gray and copper colored adults were collected from the Guilin, Guangxi and the Huangshan, Anhui areas. Four separate quarantine colonies were established and maintained from the 2015 collections, one for each source and color morph combination. The following year (2016), additional collections were made near Guilin and Hong Kong and these individuals were introduced and colonized separately under quarantine (Table 1). The 2016 collections resembled the gray color morph from the original 2012 and part of the 2015 collections. DNA analyses were performed on leg samples of 30 individuals that originated from all sources, color morphs and years of collection.

2.2. DNA processing and analysis.

Gadirtha samples were preserved in 95% ethanol prior to analysis. A single leg per insect was removed, blotted dry, ground with a chilled (−20°C) mortar and pestle, and DNA extracted using the OMEGA Bio-Tec E.Z.N.A.® Insect DNA Kit (Norcross, GA, USA). DNA was quantitated using a Qubit fluorometer (Life Technologies, Carlsbad, CA, USA) and adjusted to 10 ng/µL. A portion of the mitochondrial cytochrome c oxidase I (COI) gene was amplified using LepF1 and LepR1 primers (Hebert, Penton, Burns, Janzen, & Hallwachs, 2004). Polymerase chain reactions (PCR) (50 µL) contained 2 µl DNA, 2.5 units BioReady Taq DNA Polymerase (Bulldog Bio, Portsmouth, NH, USA), 1X reaction buffer, 1.5 mM $MgCl_2$, 0.4 µM dNTPs and 0.2 µM of each primer. PCRs utilized a MJ Research PTC-200 thermal cycler (Watertown, MA, USA) and the cycling conditions of Hebert et al. (2004). PCR products were visualized in 1.6% agarose gels stained with ethidium bromide. Unincorporated nucleotides and excess primers were removed from PCR products using DNA Clean & Concentrator (Zymo Research, Orange, CA, USA) and quantitated as before. Cycle sequencing was performed by Eurofins MWG Operon (Huntsville, AL, USA) using BigDye™ terminator technology (Life Technologies Corp., Carlsbad, CA, USA) in both directions using the PCR primers. Sequences were edited using Sequencher v5.0 (Gene Codes, Ann Arbor, MI, USA). Three outgroup sequences of *Gadirtha* sp., six for *Gadirtha impingens* Walker, and five for *G. pulchra* Butler were obtained from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and the Barcode of Life Data System (BOLD; www.boldsystems.org; Ratnasingham & Hebert, 2007). All sequences were aligned and trimmed with MEGA6 (Tamura, Stecher, Filipski, & Kumar, 2013) using Muscle (Edgar, 2004) and default settings. MEGA6 was then utilized for phylogenetic analysis using Neighbor Joining (Saitou & Nei, 1987) and the simple number of differences model [number of base differences per sequence] (Nei & Kumar, 2000).

3. Results

The final sequence alignment was 636 bp and did not contain indels or stop codons which can indicate the presence of pseudo genes. The consensus neighbor-joining tree grouped the different collection years (2012, 2015, 2016), color morphs (gray and copper), and locations (Guilin, Hong Kong, Huangshan) into a single clade suggesting they represented a single taxon (Figure 3). The two samples from Hong Kong were pooled to obtain sufficient DNA material but only provided a partial sequence (447/636 bp). DNA

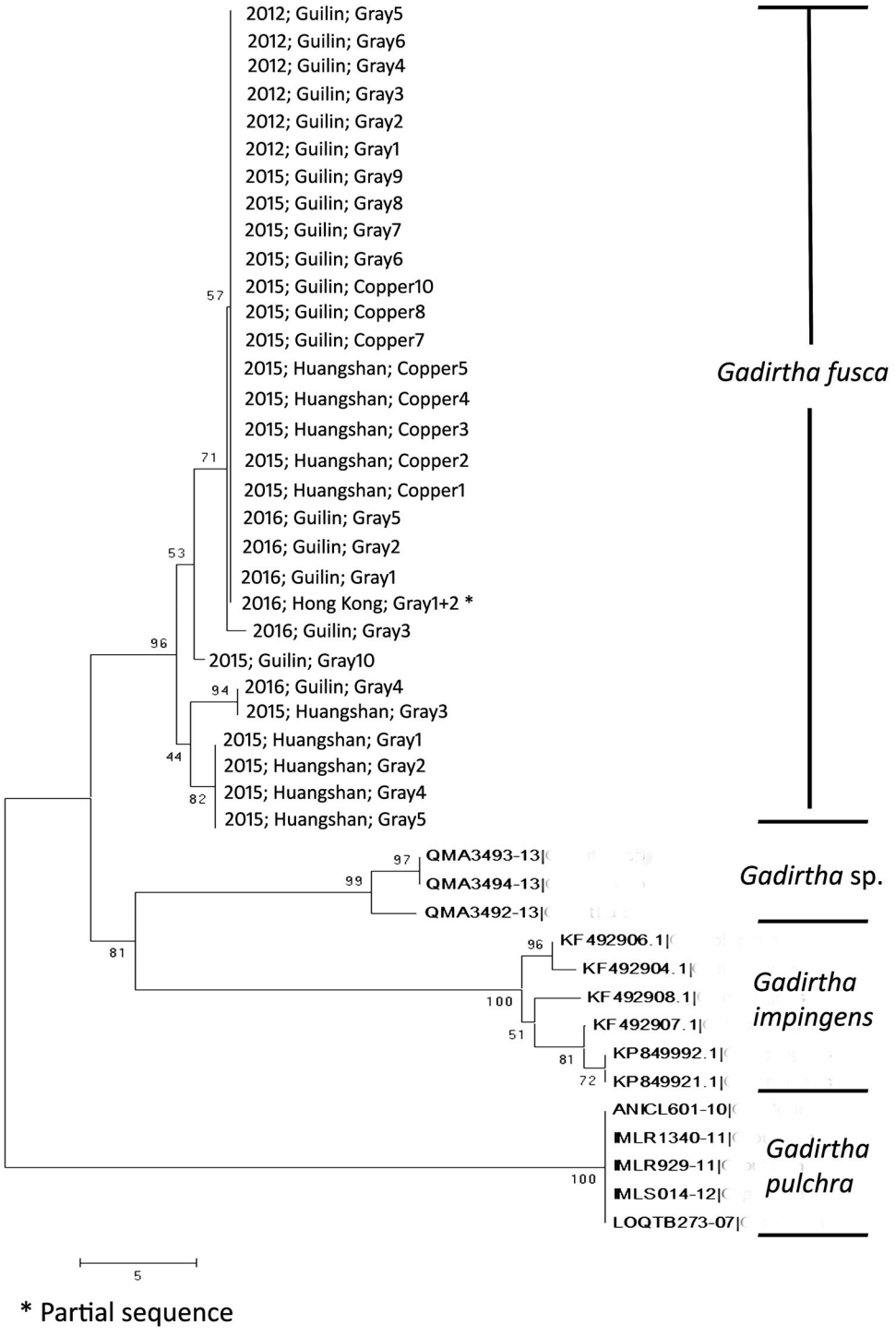

Figure 3. Neighbor joining analysis of COI DNA results using number of differences among sequences. Numbers adjacent to branches are bootstrap values. Number at the end of each label is the specimen replicate. Sequences from *Gadirtha* sp, *G. impingens*, and *G. pulchra* downloaded from the NCBI and BOLD (Ratnasingham & Hebert, 2007).

sequences of the remaining 29 *G. fusca* accessions (MG725697 – MG725725) were deposited on the National Center for Biotechnology Information database (www.NCBI.nlm.nih.gov). The average genetic divergence among these *G. fusca* collections from 2012, 2015 (including two color morphs and two sources) and 2016 was 0.3% (range 0–1.1%). The divergence found in samples of *G. impingens* was 0.1% (range 0–0.6%). Comparison of the *G. fusca* sequences and those from other *Gadirtha* spp. indicated that each was a distinct clade (Figure 3). The average divergence between *G. fusca* and *Gadirtha* sp. was 4.5% (range 4.4–4.7%), *G. fusca* and *G. pulchra* was 5.1% (range: 4.7–5.4%), and *G. fusca* and *G. impingens* was 8.1% (range: 7.9–8.3%). The interspecific distances were, at minimum four times greater than the intraspecific distances.

4. Discussion

These Chinese field collections of *G. fusca* that included different color morphs from different years and locations were assigned to a single clade. Moreover, this collection of *G. fusca* appears to be distinct when compared with the reference outgroups *Gadirtha* sp., *G. impingens* and *G. pulchra*. These results indicate that the colonies of *G. fusca* collected in China during 2012, 2015, and 2016 are a single taxon. Separate colonies based on the factors year, location, wing color, can be combined for further research developing this biological control agent of tallow. The color morphs seen in the 2015 collections apparently represent phenotypic variation within the taxon.

Gadirtha is a small genus whose known species range from India, China, Japan, Australia and parts of the south Pacific (Holloway, 2016; Pogue, 2014). The genus includes four recognized species worldwide and all but *P. fusca* were described prior to 1890. Molecular COI sequences were available for comparison on the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and the Barcode of Life Database System (BOLD; Ratnasingham & Hebert, 2007) only for *Gadirtha* sp., *G. impingens*, and *G. pulchra*. Only the *G. inexacta* sequence was not available; a species name previous used for *G. fusca* (Wang et al., 2012). The three unidentified *Gadirtha* sp. specimens used for outgroup comparisons here were all collected in Xishuangbanna, Yunnan province of China (Ratnasingham & Hebert, 2007) which co-occurs with the host, *T. sebifera* (Figure 1). Possibly *G. inexacta* is represented by these *Gadirtha* sp. sequences but this will need to be confirmed by analysis of identified specimens.

An important precaution of classical biological control of weeds is to confirm the identity of an agent being developed for release (Balciunas & Villegas, 2007). Incorrect identification of species creates considerable confusion that may cloud future research in biological control (Mound et al., 2010; Tosevski et al., 2011; Tracy & Robbins, 2009). Traditionally, this was accomplished by consultation with specialists familiar with the taxonomic group of agents under consideration for release. More recently molecular methods have been developed to compliment these approaches and clarify taxonomy, evolutionary relationships, and to reveal cryptic species that are difficult to separate with morphology alone (Gaskin et al., 2011). The results presented here indicate that despite collections made over 5 years, from various regions of southern China that included two color morphs, a single taxon was colonized for testing in quarantine. The molecular approach allowed colonies and host testing results to be combined for consideration of this potential biological control agent of tallow.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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