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Research

The First Record of *Ooencyrtus nezarae* (Hymenoptera: Encyrtidae) on Kudzu Bug (Hemiptera: Plataspidae) in North America

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Abstract

The kudzu bug, *Megacopta cribraria* (F.) (Heteroptera: Plataspidae), is an invasive insect pest introduced from Asia in 2009 that poses a threat to soybeans (*Glycine max* [L.] Merr. [Fabales: Fabaceae]) and other legume crops in the United States. Initially discovered in Georgia, *M. cribraria* rapidly expanded across the southeast until 2014 when a significant decline in its population was observed across many locations. This notable decline in *M. cribraria* populations is attributed to the emergence of new parasitoids and pathogens in its new invasive range. So far, only a single egg parasitoid, *Paratelenomus saccharalis* (Dodd) (Hymenoptera: Platygastridae), is known to parasitize the eggs of *M. cribraria* in the United States. Here, we report a new egg parasitoid of *M. cribraria* identified as *Ooencyrtus nezarae* Ishii, 1928 (Hymenoptera: Encyrtidae), recovered from egg masses of *M. cribraria* collected from soybean in Alabama. *O. nezarae* is reported to parasitize eggs from a variety of heteropteran families and has been observed parasitizing *M. cribraria* in China. To the best of our knowledge, this is the first report of *O. nezarae* in North America. The potentials of *O. nezarae* for biological control of *M. cribraria* in the United States and the direction of future studies are discussed.

Key words: Egg parasitoid, biological control, kudzu bug, ecological guild

Megacopta cribraria (F.) (Hemiptera: Plataspidae), also known as kudzu bug, is an invasive insect pest accidentally introduced from Asia to North America in 2009. Although it feeds on kudzu (*Puereria montana* var. *lobata* (Willdenow) Ohwi, (Fabales: Fabaceae) – an economically important invasive weed native to Asia – it also feeds on soybeans (*Glycine max* [L.] Merr. [Fabales: Fabaceae]) and other legume crops, causing significant yield loss in highly infested soybeans (Fig. 1A and B) (Eger et al. 2010, Ruberson et al. 2013, Seiter et al. 2013). *M. cribraria* and other closely related species have been previously reported in many countries across the continents of Asia and Australia (Eger et al. 2010). However, *M. cribraria* is the only member of the family Plataspidae reported in North America (Eger et al. 2010, Suiter et al. 2010). Since its first detection in 2009 in Georgia, the distribution of *M. cribraria* has rapidly expanded across many states in the southern United States, including Alabama, South Carolina, North Carolina, Florida, Tennessee, Mississippi, Virginia, Kentucky, Louisiana, Arizona, Maryland, and Delaware (Gardner 2016). This rapid spread is attributed to its ability as strong flyer and good hitchhiker (Suiter et al. 2010). *M. cribraria* has emerged as the top yield-limiting pest of soybean, which is the second most planted field crop in the United States, having an estimated annual market value of about $39 billion (USDA-ERS 2016). *M. cribraria* can cause up to 60% yield loss in soybean (Seiter et al. 2013). In urban areas, *M. cribraria* is considered a nuisance pest as it invades homes to overwinter (Suiter et al. 2010). Apart from being a nuisance and an agricultural pest, *M. cribraria* also affects international trade and commerce. For instance, the Honduran government temporarily banned all agricultural imports from Alabama, Georgia, South Carolina, and North Carolina due to detection of dead *M. cribraria* adults in shipping containers (Ruberson et al. 2013).

Adult *M. cribraria* are 4–6 mm long, oblong, and greenish-brown in color (Fig. 1C) (Eger et al. 2010). Females lay eggs in groups of two parallel rows mostly on the undersides of leaves and apices of shoots (Fig. 1E and F). Nymphs (Fig. 1D) undergo five instars, and depending on temperature, the entire life cycle from egg to adult takes approximately 6–8 wks (Srinivasaperumal et al. 1992,

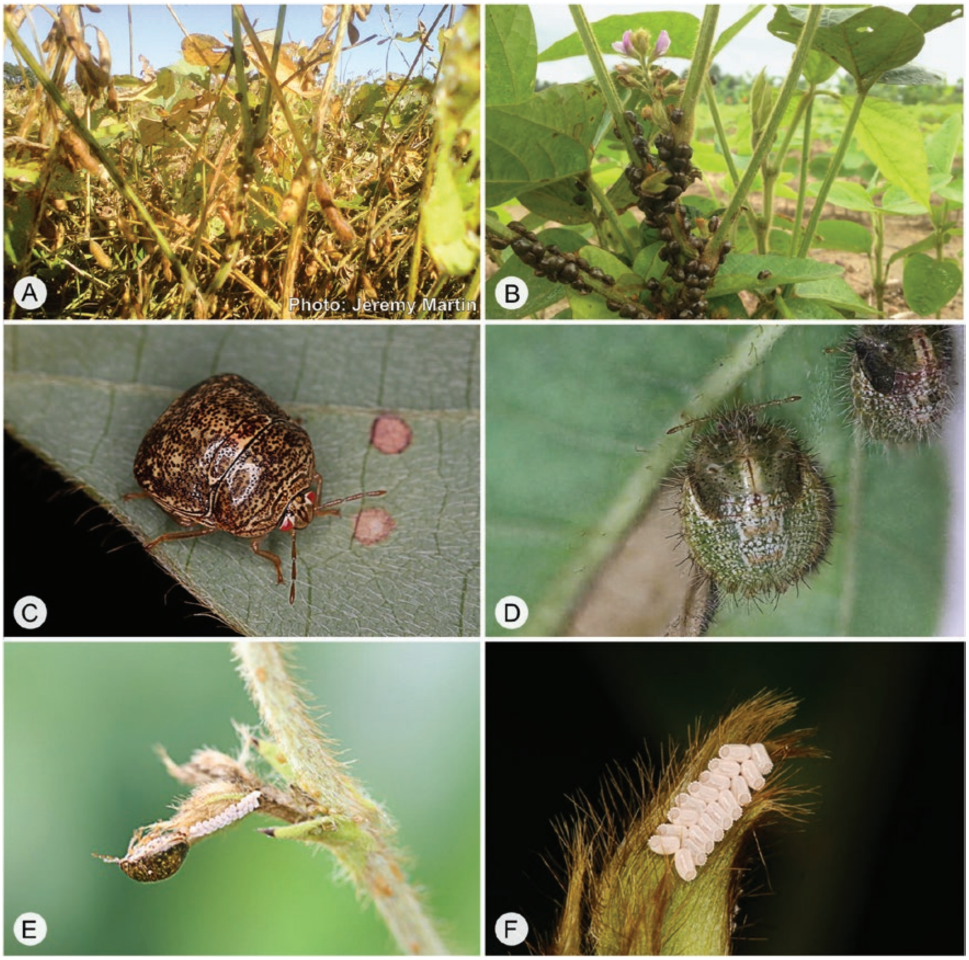

Figure 1. *Megacopta cribraria*. (A) *M. cribraria* damage in soybean. (B) Close-up of *M. cribraria* clustering on a soybean plant. (C) *M. cribraria* adult. (D) *M. cribararia* nymph. (E) adult on egg mass. (F) detail of egg mass.

Thippeswamy and Rajagopal 2005, Zhang et al. 2012, Del Pozo-Valdivia and Reisig 2013). On soybean, both adults and nymphs suck sap from stems, petioles, and leaves (Stubbins et al. 2017), resulting in lower number of pods, reduced pod weight, and poor seed set (Seiter et al. 2013). *M. cribraria* completes two generations per year on both soybean and kudzu in the southeastern United States, although development may occur on secondary hosts such as lima bean (*Phaseolus lunatus* L. [Fabales: Fabaceae]) and pigeon pea (*Cajanus cajan* L. [Fabales: Fabaceae]) (Zhang et al. 2012, Medal et al. 2013, Ruberson et al. 2013, Seiter et al. 2013, Blount et al. 2015). In early spring (March–April), adults emerge from overwintering sites and move on to kudzu and other available legume host plants where they feed and reproduce. The first in-field generation of adults emerge during June, some of which leave kudzu to find new host plants such as soybean where they complete the second in-field generation. However, greenhouse studies have shown that first-generation adults can develop on soybean, mung bean (*Phaseolus radiatus* (L.) R. Wilczek [Fabales: Fabaceae]) and lima bean, an indication that overwintering adults can bypass kudzu to feed and reproduce on early planted soybean (Del Pozo-Valdivia and Reisig 2013, Golec et al. 2015). When temperatures and day length decline in the fall, second-generation adults seek warmer areas such as residential structures and under tree bark to overwinter (Wu et al. 2006, Zhang et al. 2012, Lahiri et al. 2015). In the following spring, the overwintering adults become active again and move into kudzu and other legume host plants.

Although several parasitoids and a pathogen have been reported to attack *M. cribraria* in its native range, none was recovered in the natural enemy surveys conducted in the United States prior to 2013 (Zhang et al. 2012, Ruberson et al. 2013). Egg masses of *M. cribraria* collected in 2010 and 2011, either monitored for parasitoid emergence or exposed to native parasitoids, indicated the inability of native parasitoids in the United States to control *M. cribraria* (Zhang et al. 2012, Ruberson et al. 2013). Natural enemies of *M. cribraria* in its native range include the entomopathogenic fungus *Beauveria bassiana* (Balsamo) (Hypocreales: Clavicipitaceae) (Borah and Dutta 2002), *Ooencyrtus nezarae* Ishi (Hymenoptera: Encyrtidae) (Takasu and Hirose 1985, Tayutivutikul and Yano 1990, Hirose et al. 1996, Wu et al. 2006), *Encarsiella* (=*Dirphys*) *boswelli* (Girault) (Polaszek and Hayat 1990), *P. saccharalis* [= *Asolcus minor*, *Archiphanurus minor* and *Paratelenomus minor* (Johnson 1996)] (Wall 1928, Hirose et al. 1996, Wu et al. 2006). Of these natural enemies, the specialist egg parasitoid *P. saccharalis* was considered for classical biological control and under investigations at the USDA-ARS National Biological Control Laboratory (Ruberson et al. 2013) until its sudden appearance under field conditions in several locations across Georgia and Alabama in 2013 (Gardner et al. 2013). There are speculations that *P. saccharalis* was probably introduced into the United States with parasitized eggs of *M. cribraria* from Asia (Gardner et al. 2013). Subsequent surveys have recovered additional natural enemies, including the entomopathogenic fungi, *Beauveria bassiana* (Balsamo) (Seiter et al. 2013, 2014; Ruberson et al. 2013), three adult parasitoids, *Strongygaster triangulifera* (Loew), a dipteran (Golec et al. 2013), another dipteran, *Phasia robertsonii* (Townsend) (Ruberson et al. 2013), and a mermithid nematode (Stubbins et al. 2015).

In this study, we report the detection of *O. nezarae* parasitizing eggs of *M. cribraria* in soybean crops in Alabama. *O. nezarae* is reported to parasitize eggs of species from several heteropteran families, including Pentatomidae, Coreidae, Alydidae, and Plataspidae (see Zhang et al. 2005 for a detailed host list). Notable hosts include *Homoeocerus unipunctatus* (Thunberg) [Heteroptera: Coreidae], *M. punctatissimum* (Montandon) (Takasu and Hirose 1985 1986), *Riptortus pedestris* (F.) [Heteroptera: Alydidae], *Piezodorus hybneri* (Gmelin) [Heteroptera: Pentatomidae], *Eysarcoris guttiger* (Thunberg) [Heteroptera: Pentatomidae], (Takasu and Hirose 1985, Hirose et al. 1996), *M. cribraria* (Wu et al. 2006), *Leptocorisa chinensis* Dallas [Heteroptera: Alydidae] (Yokosuka et al. 1998), *Nezara viridula* (Linnaeus) [Heteroptera: Pentatomidae] (Jones 1988), *Nezara* sp., *Euschistus* sp., *Acrosternum* sp., *Edessa* sp., and *Thyanta* sp. (Kobayashi and Cosenaza 1987). Until now, the distribution of *O. nezarae* has been limited to China, Japan, Thailand, South Korea, and Brazil (Kobayashi and Cosenza 1987, Zhang et al. 2005, Noyes 2016). To our knowledge, this is the first report of *O. nezarae* in North America and also the first report of this species parasitizing *M. cribraria* in its invasive range.

Materials and Methods

Collection of Parasitoid and Parasitism Rate

Parasitoids were reared from parasitized egg masses of *M. cribraria* collected from soybean fields within the Auburn University campus, Lee County, AL. (32.5934°N, 85.4952°W). Egg masses were collected in the summer (July and August) of 2016. Field-collected egg masses were carefully detached from soybean leaves with a pair of forceps. Each egg mass was placed in a 2 ml Eppendorf microcentrifuge tube (Eppendrof, Hauppauge, NY) and kept under laboratory conditions [25 ± 1°C, 75 ± 5% RH and 14:10 (L: D) h] until emergence. Three to four holes were made on the cap of each Eppendorf tube with an insect pin to allow aeration. Microscopic examination of emerged parasitoids showed a hymenopteran with morphological features strikingly different from *P. saccharalis*. Newly emerged parasitoids were preserved in 95% ethanol and sent to the Systematic Entomology Laboratory at the National Museum of Natural History (Smithsonian Institute, Washington, DC) for identification. Voucher specimens have been deposited at the Auburn University Natural History Museum, Auburn, AL and the Smithsonian Institute, Washington, DC.

To determine field abundance and sex ratio, we sampled two soybean fields located in the Auburn University campus, Lee County, AL (32.5934°N, 85.4952°W) on five dates in July and August of 2016. In each sampling event, egg masses were collected at random by detaching a whole leaf containing egg mass. Field-collected egg masses were incubated individually in petri dishes (35 mm diameter) under laboratory conditions [25 ± 1°C, 75 ± 5% RH and 14:10 (L: D) h] and observed for parasitoid emergence. Parasitoids that emerged were placed in 95% ethanol for later identification. Eggs where nothing emerged or hatched were dissected for signs of underdeveloped or developed parasitoids. Number of eggs per mass, total number of parasitoids that emerged per mass, ratio of male to female (separated based on antennal morphology) and percent parasitism were recorded.

Parasitoid Identification

The two specimens used for gene sequencing were dried using hexamethyldisilazane (HMDS) (Heraty and Hawks 1998), and then slide-mounted in Canada balsam. An additional five females and two males were dried using HMDS and then card or point mounted (USNMENT numbers 01231470–1231471, and 01231597–01231601). Select slide-mounted and pinned specimens were photographed using the equipment and techniques outlined in Liu and Mottern (2017).

There is no recent, comprehensive key to the world *Ooencyrtus* fauna. Therefore, we used major regional keys, including those covering the Indo-Pacific region (Huang and Noyes 1994), China (Zhang et al. 2005), sub-Saharan Africa (Prinsloo 1987), and the Neotropics (Noyes 2007, 2010). We further examined new species descriptions for those species described since the regional keys were published, and for species not included in any of the diagnostic keys. Specimens were also compared against the extensive *Ooencyrtus* collection at the U.S. National Museum of Natural History (USNM). Finally, images of both slide and point-mounted specimens were sent to Dr. John Noyes at the Natural History Museum, London to further confirm our initial identification. All specimens used for species determination are deposited in the USNM.

DNA Sequencing

DNA was nondestructively extracted from one female and one male specimen (USNMENT01231257 and USNMENT01231258, respectively) using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Extraction, PCR amplification, purification, sequencing, and sequence verification/assembly followed the protocols outlined in Liu and Mottern (2017). Amplified gene regions included an 815-bp fragment of the small subunit 18S rDNA (GenBank: KY952632 and KY952633), large subunit 28S rDNA expansion regions D2-5 (GenBank: KY965821 and KY965822; performed in two reactions; see Table 1 for primer sequences) and a 1,041-bp fragment of cytochrome oxidase subunit I (*COI*) (GenBank: KY964494 and KY964495; performed in two reactions; see Table 1 for primer sequences). All molecular work was performed at the Smithsonian Institution Laboratories of Analytical Biology.

Results and Discussion

In the present study, all collected hymenopteran parasitoids from *M. cribraria* eggs were identified as *O. nezarae* – an exotic egg parasitoid reported for the first time in North America. Additionally, an average of 95.6% parasitism rate was recorded for *O. nezarae*, suggesting that this new encyrtid may serve as potential biological control agent of *M. cribraria* in the United States.

O. nezarae is a facultative gregarious egg parasitoid that attacks a variety of important hemipteran pests, including *Homoeocerus unipunctatus* (Thunberg) (Hemiptera: Coreidae), *Megacopta punctatissima* (Montandon) (F.) (Heteroptera: Plataspidae), and *Riptortus pedestris* (F.) (Hemiptera: Alydidae), in Japan (Hirose et al. 1996, Takasu et al. 2002). *O. nezarae* belongs to a large species complex, generally characterized by a completely dark brown head and body (Fig. 2A and B), dark coxae, at least partially dark femora and tibia, and mesoscutellar sculpture that is distinctly deeper and more pronounced than the sculpture of the mesoscutum (Fig. 2C). The species within this group are difficult to distinguish because the diagnostic morphological characteristics often include subtle variations in size ratios among body parts. The only diagnostic keys where our specimens reach a noncontradictory conclusion are those of Huang and Noyes (1994) where they run to *Ooencyrtus hercle* Huang and Noyes (Hymenoptera: Encyrtidae),

Table 1. Primers used for this study

Primer	Sequence	Reference
18S F	5′-AAA TTA CCC ACT CCC GGC A-3′	Munro et al. (2011)
18S R	5′-TGG TGA GGT TTC CCG TGT T-3′	Munro et al. (2011)
28S D2-3551 F	5′-CGG GTT GCT TGA GAG TGC AGC-3′	Modified from Campbell et al. (2000)
28S D2-4039 R	5′-CTC CTT GGT CCG TGT TTC-3′	Mottern and Heraty (2014)
28S D3-4046 F	5′-TTG AAA CAC GGA CCA AGG AG-3′	Modified from Nunn et al. (1996)
28S D5-4625 R	5′-CGC CAG TTC TGC TTA CCA-3′	Modified from Nunn et al. (1996)
COI LCO-1490 F	5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′	Folmer et al. (1994)
COI HCO-2198 R	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′	Folmer et al. (1994)
COI NJ-2197 F	5′-TAT ATT TTA ATT YTW CCW GGA TTT GG-3′	Modified from Simon et al. (1994)
COI MD-2614 R	5′-ATT GCA AAT ACT GCA CCT AT-3′	Dowton and Austin (1997)

Numbers following Ribosomal primers refer to the complimentary 5′ start position in *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) (Tautz et al. 1988). Numbers following cytochrome oxidase subunit I primers refer to the complimentary 5′ start position in *Drosophila yakuba* Burla (Diptera: Drosophilidae) (Folmer et al. 1994). The complete *28S D2-5* was amplified in two reactions: one for D2 and a second for D3-5.

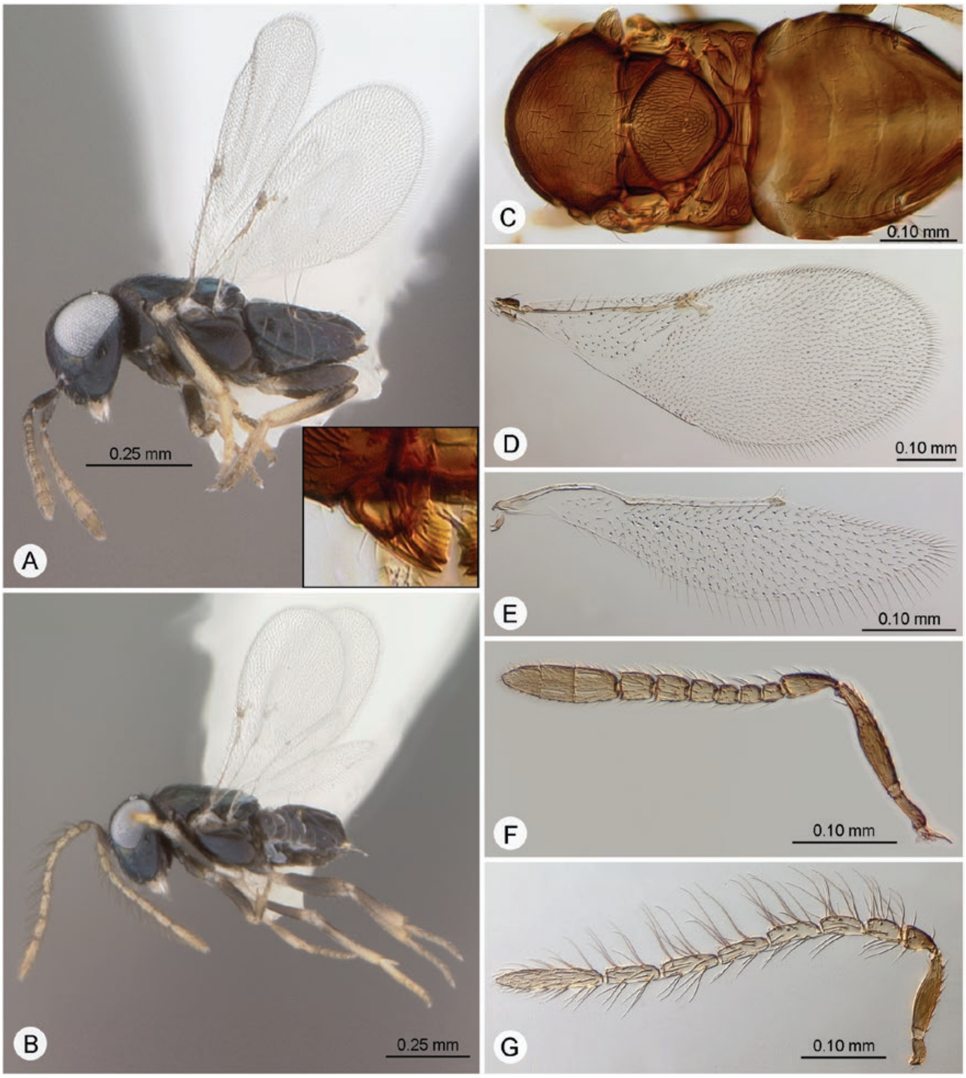

Figure 2. *Ooencyrtus nezarae*. (A) Female habitus with inset showing detail of mandible. Note the long, blade-like truncation with crenulations on the mandible, resulting in a serrated appearance. (B) Male habitus. (C) Female dorsal mesosoma. (D) Female forewing. (E) Female hindwing. (F) Female antenna. (G) Male antenna.

and the key in Zhang et al. (2005), where they run to *O. nezarae*. However, our specimens are a poor match with the description and figures of *O. hercle*, particularly with respect to the form of the male antenna (Fig. 2G). Furthermore, our specimens do not exactly match the original description of *O. nezarae*, which states, in reference to the female antenna, that the first two funicular segments should be slightly longer than wide, whereas they are quadrate, or even slightly wider than long in our specimens (Fig. 2F) are. However, females of our specimens are smaller in overall body length (0.70–0.77mm) than the *O. nezarae* holotype (0.9 mm), upon which the original description is based. To address the possibility that our specimens are unusually small *O. nezarae* that are exhibiting allometric scaling of antennal segments, we sent a series of photographs to Dr. John Noyes, an encyrtid specialist at the Natural History Museum in London (NHML). He examined a series of authoritatively identified *O. nezarae* in their collection (six females and 10 males), and found that smaller specimens do have relatively shorter funicular segments. Apart from overall body size (females ranged from 0.88 to 1.07 mm), the specimens in NHML were a good morphological match with our specimens. These data, combined with the known records of *O. nezarae* attacking eggs of *M. cribraria* in China, led us to conclude that the specimens were best identified as a small form of *O. nezarae*. It is worthy of note that although this parasitoid is gregarious when developing in a large host species, it behaves as solitary in eggs of smaller host species, such as *M. cribraria* and *M. punctatissimum* (Takasu and Hirose 1991).

To the best of our knowledge, ours are the first *O. nezarae* nucleotide sequences to be uploaded to a public database. A BLAST search on the COI barcode sequence indicates that the closest match currently in GenBank is *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae) (GenBank: KX868569.1), with 91% sequence identity over 99% query coverage. BLAST searches on our ribosomal genes confirm that our specimens are *Ooencyrtus*, but do not closely match with any sequences currently in the database, suggesting that GenBank is currently not yet populated with enough *Ooencyrtus* sequence data to help with species identification in this case.

Table 2. Percent parasitism and sex ratio of *O. nezarae* that emerged from field-collected egg masses of *M. cribraria* from a single soybean field in summer 2016

# Eggs/Mass	% Parasitism	# Emerged	#Male	#Female
19	100	17	08	11
08	100	08	02	06
30	100	30	06	24
12	83.3	10	03	07
16	100	16	04	12
15	100	15	05	10
18	100	18	03	15
20	90	18	05	13
26	100	26	09	17
29	82.8	24	08	16

It is not clear whether the *Ooencyrtus* sp. reported attacking *M. cribraria* eggs in Virginia (Dhammi et al. 2016) is also *O. nezarae*. The male specimen figured in Dhammi et al. (2016) appears to have relatively broad antennal flagellomeres compared with our specimens, and the coloration of the midlegs appears much lighter. However, direct examination of the specimens, preferably combined with molecular comparisons, would be required to determine whether or not the wasps reported from Virginia are also *O. nezarae*.

O. nezarae used in this study were collected from soybean between July and August of 2016. Although *O. nezarae* is reported to occur in soybean from May to October in China (Wu et al. 2006) and June to October in Japan (Takasu and Hirose 1985, 1986), the precise period when *O. nazarae* appears in soybean fields in United States is not yet known. Both *O. nezarae* and *P. saccharalis* occupy the same ecological guild and have been observed together in the field parasitizing eggs of *Megacopta* species in Japan and China with *O. nezarae* being the dominant of the two (Takasu and Hirose 1986, Wu et al. 2006). Parasitism rate ranging from 43 to 100% in Japan (Takasu and Hirose 1986) and 22.4 to 76.9% in China (Wu et al. 2006). In the current study, we recorded parasitism rates ranged from 82.8 to 100% with an average of 95.6% (table 2). Here, the parasitism rate is defined as the percentage of the total number of parasitized eggs, including eggs from which parasitoids failed to emerge. It should be noted that samples of parasitized eggs were collected from a single study site and that the high parasitization rate observed may not be the same across different locations. In this study, we observed a sex ratio of 1 male to 2.5 females. A female-biased progeny of one male to three females has been reported for *O. nezarae* on *R. pedestris* in laboratory-reared specimens (Aung et al. 2011a, 2011b). Similarly, Hirose et al. (1996) reported a female-biased sex ratio of 1:2.4 for *O. nezarae* adults in a field study. Parasitism by *O. nezarae* can be distinguished from parasitism by *P. saccharalis* based on the position of the emergence holes on the *M. cribraria* eggs: *O. nezarae* adults emerge from lateral, irregularly shaped openings (Fig. 3A), whereas *P. saccharalis* adults emerge from circular openings at the apices of the eggs (Fig. 3B).

O. nezarae has a broad geographic range. It has been reported in Asia (China, South Korea, Japan, Thailand) and Brazil, where it was introduced to control stink bugs in soybean (Zhang et al. 2005). This range, combined with the morphological variation that we are reporting, suggests the need for additional taxonomic work (integrating molecules and morphology) in investigating the possibility that *O. nezarae* is a cryptic species complex.

The discovery of *O. nezarae* in Alabama under field conditions is not the first egg parasitoid found attacking *M. cribraria* in the United States; *P. saccharalis* was found in Georgia and Alabama

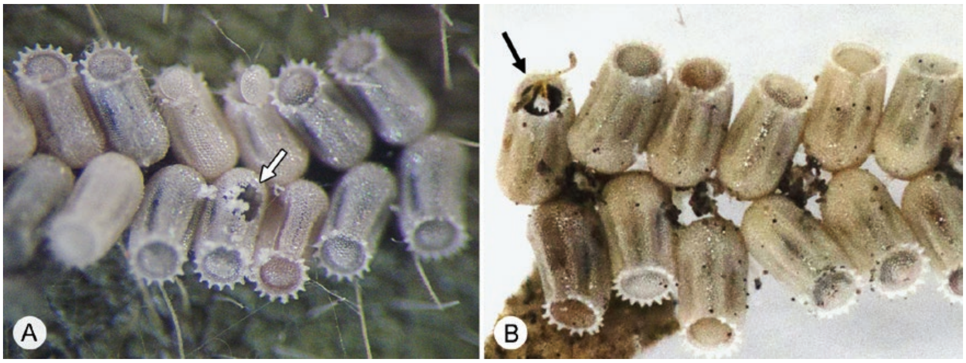

Figure 3. *M. cribraria* egg mass showing emergence holes from *O. nezarae* (A). Wasps emerge from ragged, irregularly shaped holes in the sides of the eggs. *M. cribraria* egg mass with *P. saccharalis* emerging from a circular hole at the apex of the egg (B).

in 2013 (Gardner et al. 2013). As with *P. saccharilis*, the route of introduction of *O. nezarae* to North America is unknown, and we provide two possible explanations. First, we speculate that it came into the United States with parasitized eggs of *M. cribraria* from Asia. Another possibility is that because *O. nezarae* is a generalist parasitoid, it came in with parasitized eggs of other host insects and switched to *M. cribraria* due to availability, proximity, or preference.

In conclusion, two egg parasitoids, *O. nezarae* and *P. saccharalis*, have been identified for the control of *M. cribraria* in soybean in the United States with parasitism rates ranging from 82.8 to 100% and 42 to 95%, respectively, at their reported study sites. Other parasitoids that have been recovered include *Strongygaster triangulifera* (Loew) (Diptera: Tachinidae) (Golec et al. 2013), *Phasia robertsonii* (Townsend) (Diptera: Tachinidae) (Ruberson et al. 2013), and a mermithid nematode (Stubbins et al. 2015). The mean parasitism rate was 5.14% for *S. triangulifera* and 4.7% for the nematode although *P. robertsonii* was observed in a single adult. *O. nezarae* and *P. saccharalis* are successful parasitoids of *M. cribraria* both in their native and new invasive range. Although the parasitism rate is high for both parasitoids, they have a short period of activity in the United States. With the high rate of parasitism and dominance reported for *O. nezarae*, augmentative or inundative releases could be considered to enhance its effectiveness as a biological control agent of *M. cribraria* in soybean production in the United States. Monitoring is on-going in Alabama as well as other localities within *M. cribraria* invasive range for the presence of this wasp.

Acknowledgments

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