

# An Old Remedy for a New Problem? Identification of *Ooencyrtus kuvanae* (Hymenoptera: Encyrtidae), an Egg Parasitoid of *Lycorma delicatula* (Hemiptera: Fulgoridae) in North America

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## Abstract

Spotted Lanternfly, *Lycorma delicatula* (White) is a recently introduced pest of Tree-of-Heaven, *Ailanthus altissima* (Mill.) Swingle in North America. Natural enemy surveys for this pest in Pennsylvania in 2016 recovered an encyrtid egg parasitoid from both field collections and laboratory rearing of field-collected *L. delicatula* egg masses. Both molecular and morphological data confirm that the egg parasitoids are *Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae). *Ooencyrtus kuvanae* (Howard) is primarily an egg parasitoid of gypsy moth, *Lymantria dispar* (L.), and was introduced to North America in 1908 for gypsy moth biological control. Although *O. kuvanae* is known to attack multiple host species, to our knowledge, this is the first report of *O. kuvanae* as a primary parasitoid of a non-lepidopteran host. Potential of *O. kuvanae* in the biological control of *L. delicatula* in North America and research needs are discussed.

**Key words:** spotted Lanternfly, *Lycorma delicatula*, *Ooencyrtus kuvanae*, egg parasitoid, identification, North America

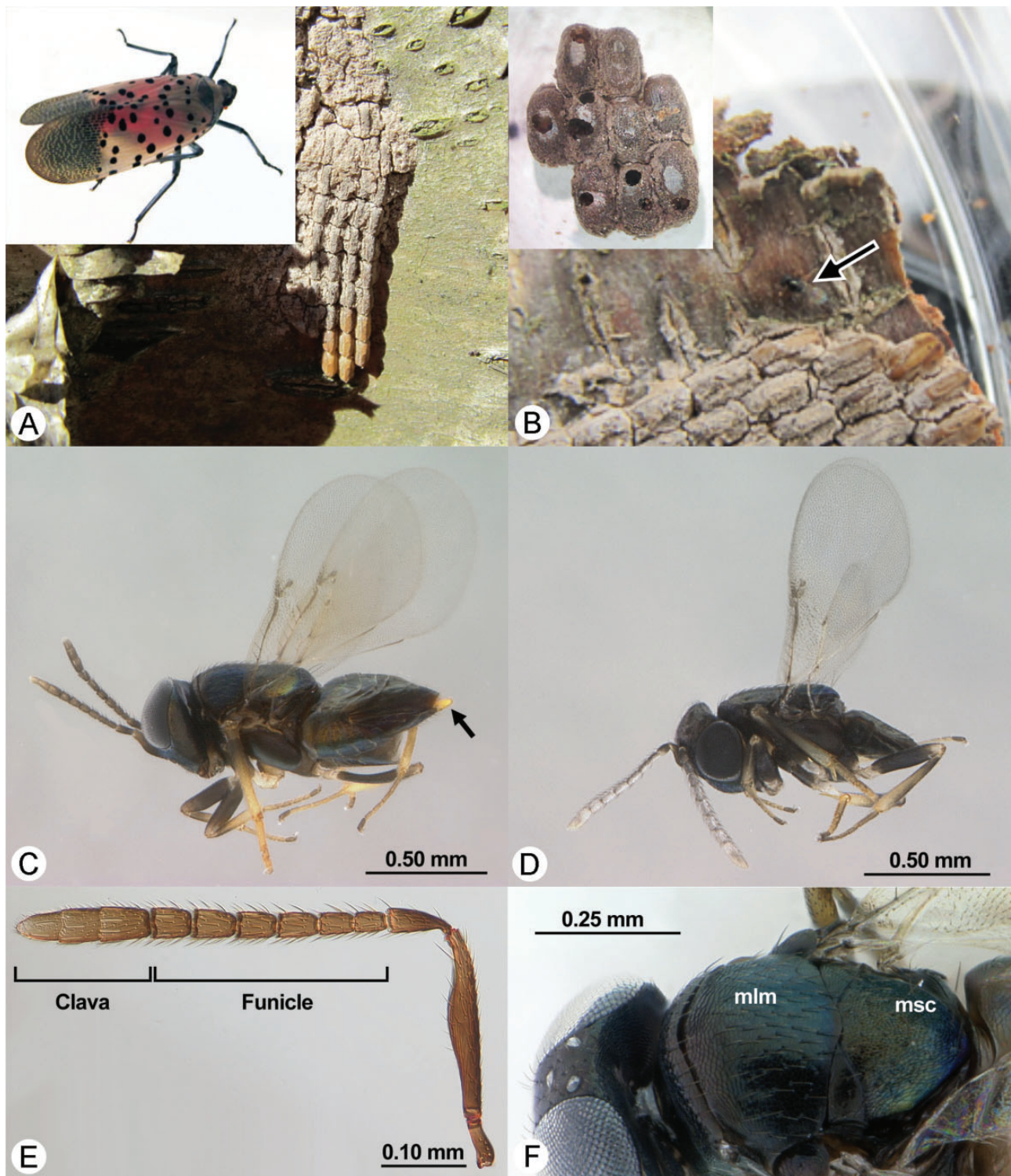
Spotted Lanternfly, *Lycorma delicatula* (White) (Hemiptera: Fulgoridae), is a sporadic pest of Tree-of-Heaven, *Ailanthus altissima* (Mill.) Swingle (Simaroubaceae). The natural distribution of *L. delicatula* includes most of China (especially northern China), Taiwan and Vietnam (White 1845; Liu 1939; Zhou 1992; Han et al. 2008). As an exotic pest, *L. delicatula* invaded Korea in 2004 (Kim and Kim 2005), Japan in 2009 (Tomisawa et al. 2013), and was first detected in Pennsylvania in 2014 (Barringer et al. 2015; Dara et al. 2015). Host species in its native range as well as introduced areas include more than 70 woody plants and vines in 25 families, such as apple, birch, grape, cherry, lilac, maple, poplar, and stone fruits (Zhou 1992; Kim et al. 2011; Dara et al. 2015). Feeding by nymphs and adults on phloem tissue causes oozing wounds on trunks and branches, resulting in wilting or death of the branches. Nymphs and adults also excrete large amounts of honeydew, attracting ants, bees, hornets, and promoting the growth of sooty mold (Han et al. 2008; Dara et al. 2015). Significant damage has been recorded in vineyards in Korea (Han et al. 2008; Lee et al. 2009; Park et al. 2009), and it is a potential threat to the grape and fruit industries in Pennsylvania and beyond in North America (Barringer et al. 2015; Dara et al. 2015).

In China, *L. delicatula* is univoltine, and overwinters in the egg stage on tree trunks or nearby man-made structures. Eggs start to

hatch in mid-April and peak in early May. Nymphs pass through four instars and become adults between mid-June and early July. Adults (Fig. 1A, inset) mate and lay eggs in mid-August and continue feeding until October. Eggs are laid in masses arranged in 5–10 rows with 10–30 eggs/row. Egg masses are covered by a layer of gray wax (Fig. 1A). Both nymphs and adults aggregate on leaves and tree trunks and will jump off when disturbed (Zhou 1992). In Korea, nymphs emerge in May and become adults in late July, with egg-laying starts in August and lasts until early November (Park et al. 2009; Lee et al. 2011). The life cycle of *L. delicatula* in Pennsylvania is similar to that reported in China and Korea, except eggs do not appear in the field until mid-October (Dara et al. 2015; H. Liu, personal observation).

Natural enemies of *L. delicatula* in its native range include a solitary egg parasitoid, *Anastatus orientalis* Yang & Choi (Hymenoptera: Eupelmidae) (Kim et al. 2015; Yang et al. 2015); and a solitary ecto-parasitoid on nymphs, *Dryinus browni* Ashmead (Hymenoptera: Dryinidae) (Yan et al. 2008).

*Ooencyrtus kuvanae* (Howard) (Hymenoptera: Encyrtidae) is an egg parasitoid of gypsy moth (*Lymantria dispar* (L.) (Lepidoptera: Erebidae)) from Japan (Howard 1910). It has also been reared from field-collected eggs of other lepidopterans such as *Dendrolimus spectabilis* Butler (Lasiocampidae) (Hirose 1964) and *Eriogyna*



**Fig. 1.** (A) *Lycorma delicatula* egg masses on bark of sweet birch; inset showing *L. delicatula* adult. (B) *Lycorma delicatula* egg mass with arrow indicating *Ooencyrtus kuvanae* female; inset showing *L. delicatula* eggs with parasitoid emergence holes. (C) *Ooencyrtus kuvanae*, female habitus. Arrow indicating exposed portion of ovipositor sheath. (D) *Ooencyrtus kuvanae*, male habitus. (E) *Ooencyrtus kuvanae*, female antenna. (F) *Ooencyrtus kuvanae*, female showing relative sculpture of the midlobe of the mesoscutum (mlm) and the mesoscutellum (msc).

*pyretorum* Westwood (Saturnidae) (Koidzumi and Shobata 1940). The natural distribution of *O. kuvanae* includes Japan, China, Korea, and Taiwan (Huang and Noyes 1994; Zhang et al. 2005). As a hyper-parasitoid, *O. kuvanae* parasitizes *Anastatus disparis* Ruschka (Hymenoptera: Eupelmidae) and *Apanteles melanosceus*

Ratzburg (Braconidae), egg and larval parasitoids of gypsy moth, respectively (Howard and Fiske 1911; Muesebeck and Dohanian 1927). *Ooencyrtus kuvanae* was introduced into the United States in 1908 for gypsy moth biological control (Crossman 1917). Successful establishment was achieved in Massachusetts by 1911 (Crossman

**Table 1.** Primers used for this study

Primer	Sequence <sup>a</sup>	Reference
28S D2-3551 F	5'-CGG GTT GCT TGA GAG TGC AGC-3'	Mod. 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'	Mod. from Nunn et al. (1996)
28S D3-4413 R	5'-TCG GAG GGA ACC AGC TAC TA-3'	Mod. 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'	Mod. from Simon et al. (1994)
COI MD-2614 R	5'-ATT GCA AAT ACT GCA CCT AT-3'	Dowton and Austin (1997)

<sup>a</sup>Numbers following Ribosomal primers refer to the complimentary 5' start position in *Drosophila melanogaster* (Tautz et al. 1988). Numbers following cytochrome oxidase subunit I primers refer to the complimentary 5' start position in *Drosophila yakuba* (Folmer et al. 1994).

1925). Since then, millions of *O. kuvanae* have been released throughout the areas infested by gypsy moth in North America (Brown 1984). Release and subsequent recovery of *O. kuvanae* in Pennsylvania occurred between 1969 and 1971 (Smilowitz and Rhoads 1973).

No parasitoids have been recorded from *L. delicatula* in North America since its introduction in 2014. In this study, we report the identification process of *O. kuvanae* as an egg parasitoid of this pest based on morphological and molecular characteristics as part of the natural enemy surveys in Pennsylvania. The direction of future studies and potential for *O. kuvanae* as a biological control agent for *L. delicatula* in North America are discussed.

## Materials and Methods

### Collection of Parasitoids

Parasitoids were either collected in the field near *L. delicatula* egg masses (Fig. 1B, indicated by arrow) or reared from *L. delicatula* eggs that were brought into the laboratory (Fig. 1B, inset showing *L. delicatula* eggs with parasitoid emergence holes). Field collections occurred during a natural enemy survey of two sites near Boyertown in Berks County Pennsylvania (40.40361°N, 75.67333°W and 40.41417°N, 75.68575°W). Specimens were collected between 30 March and 19 April 2016. For field collections, *O. kuvanae* adults observed on the surface of *L. delicatula* egg masses were collected into 50-ml S-centrifuge tube (VWR international, Radnor, PA) and killed in 95% ethanol. For laboratory rearing, field-collected *L. delicatula* eggs were incubated at 22 ± 1°C for two months for parasitoid emergence. *Ooencyrtus kuvanae* adults emerging from host eggs were collected and preserved in 95% ethanol and stored at -20°C until further processing.

### DNA Sequencing

DNA was extracted from one male and five female specimens. Three of the specimens (USNMENT01119482, USNMENT01119483, and USNMENT01231105) were collected from *L. delicatula* egg masses in the field, but were not observed emerging from or ovipositing into *L. delicatula* eggs. Three additional specimens (USNMENT01231083, USNMENT01231084, and USNMENT01231085) were reared from field-collected *L. delicatula* egg masses. DNA was extracted non-destructively using the DNeasy Blood and Tissue Kit (Qiagen) following the protocol of the manufacturer. After incubation, 4 µL RNase A (Thermo Fisher Scientific, Waltham, MA) was added to each extract in order to remove RNA. Gene amplification was performed using standard three-step PCR protocols optimized for Chalcidoidea (the superfamily to which Encyrtidae belongs) DNA (Heraty et al. 2004). Because the relationship between genetic sequence divergence and

species boundaries is not always clear, we chose to sequence multiple genes that often used for species-level molecular phylogenetics and taxonomy. Amplified gene regions included 28S rDNA expansion regions D2-3, a 651-bp fragment of the barcoding region of cytochrome oxidase subunit I (*COI*), and an additional 390-bp fragment of *COI*. Primers used for this study are found in Table 1. All extraction, amplification and sequencing were performed at the Smithsonian Institution Laboratories of Analytical Biology. Sequences were verified by comparing forward and reverse reads, and mitochondrial DNA was examined for stop codons using the software package Geneious v8.1.7 (Biomatters, available from <http://www.geneious.com/>). All sequences used for this study have been uploaded to GenBank, and accession numbers are listed in Table 2.

### Parasitoid Identification

Following DNA extraction, specimens were dried using HMDS (Heraty and Hawks 1998), and then either slide-mounted in Canada balsam or point mounted. Specimens in ethanol (pre-extraction) and point-mounted specimens were photographed using the Entovision Imaging Suite, which includes a firewire JVC KY-75 3CCD digital camera. Individual planes of focus were captured using ARCHIMED 5.6.0 (Microvision Instruments, France), and the resulting focal planes were merged into a single, in-focus composite image using Zerenestacker v.1.04 (Zerene Systems, LLC, Richland, WA). Slide-mounted specimens were imaged using a Leica DMRB with Nomarski differential interference contrast optics, coupled with the Entovision Imaging Suite.

We took extra care in establishing and confirming the identity of *O. kuvanae* as it was previously only known to attack lepidopteran eggs. Specimens were initially identified using the key to species in Huang and Noyes (1994). This identification was further confirmed by morphological comparison with *O. kuvanae* specimens in the United States National Museum of Natural History (USNM) collection. Images were also sent to Dr. John Noyes at the Natural History Museum, London, who concurred with our identification. All specimens are deposited in the USNM under the specimen identification numbers listed in Table 2.

## Results and Discussions

### DNA Sequencing

All four gene regions were successfully amplified and sequenced for four of the six specimens. We were not able to amplify 28S D3 for USNMENT01231085 and the *COI* barcode region for USNMENT01119482, so not all specimens have the same gene coverage. However, there was no sequence variation among any of the specimens for any of the gene regions that we were able to amplify.

**Table 2.** Specimen details and Genbank accession numbers

Specimen ID <sup>a</sup>	Sex	28S D2	28S D3	COI (LCO1490/HCO2198)	COI (NJ2197/MD2614)
USNMENT01119482 C	F	KX868554	KX868560	N/A	KX868570
USNMENT01119483 C	F	KX868555	KX868561	KX868565	KX868571
USNMENT01231083 R	M	KX868556	KX868562	KX868566	KX868572
USNMENT01231084 R	F	KX868557	KX868563	KX868567	KX868573
USNMENT01231085 R	F	KX868558	N/A	KX868568	KX868574
USNMENT01231105 C	F	KX868559	KX868564	KX868569	KX868575

<sup>a</sup>Specimens with ID numbers followed by “R” were reared from field-collected *L. delicatula* eggs in the laboratory, whereas those followed by “C” were collected in the field in association with *L. delicatula* eggs.

A nucleotide search in Genbank for “*Ooencyrtus kuvanae*” yielded a single 946bp COI fragment (GenBank: KP676668.1), which was identical with our COI sequences across the 751bp where the sequences shared coverage. Therefore, the molecular evidence strongly suggests that all *Ooencyrtus* specimens collected with *L. delicatula* eggs and reared from *L. delicatula* eggs are the same species, and all are *O. kuvanae*.

### Parasitoid Identification

*Ooencyrtus kuvanae* was originally described as *Schedius kuvanae* by Howard (1910). The most thorough subsequent description is included in Huang and Noyes (1994), and this work should be consulted for a complete list of diagnostic features. Briefly, *O. kuvanae* females may be identified by a combination of their size (body length 0.87–1.35 mm); dark body color with coppery blue and green metallic sheen (Fig. 1C); visible portion of the ovipositor sheath yellow (Fig. 1C, indicated by arrow); antenna with all funicular segments longer than broad, funicle and clava uniform brown (clava sometimes lighter distally), and clava without distinct obliquely truncate sensory region (Fig. 1E); mesoscutellum with relatively deep reticulate sculpture, more deeply sculptured than the midlobe of the mesoscutum (Fig. 1F). The male is generally similar to the female, though the antenna is lighter in color and has longer setae (Fig. 1D).

*Ooencyrtus kuvanae* is primarily an egg parasitoid of gypsy moth although it has been collected from a few lepidopteran species (Koidzumi and Shobata 1940; Hirose 1964). In the laboratory, it has been successfully reared on eggs of four species of Lymantriidae, four species of Saturniidae, and one species of Lasiocampidae (Brown 1984; Hofstetter and Raffa 1997). Our discovery represents a significant increase of the reported host range of *O. kuvanae* to include another insect order, even when laboratory hosts are considered.

*Ooencyrtus kuvanae* is a multivoltine arrhenotokous parasitoid with a development time of 21 days at 25 °C from egg to adult (Crossman 1925). On gypsy moth, it has at least four generations a year (Crossman 1925). It overwinters as fertilized adult females in forest litter layers. Females become active in mid-April. The 1st spring generation takes 4–6 weeks to mature. Adults of the 1st spring generation emerge in late May to early June and females parasitize any suitable gypsy moth eggs that have not hatched. Adults of the 2nd spring generation will not emerge until July when new generation gypsy moth eggs become available. *Ooencyrtus kuvanae* populations peak in August and September as adults of the 1st and the 2nd summer generation start to emerge. Populations then decline rapidly toward November when adults prepare to overwinter (Crossman 1925; Weseloh 1972; Brown et al. 1982).

*Ooencyrtus kuvanae* is an important biological control agent for gypsy as it generally exerts 10–40% parasitism on host eggs (Brown

1984). It deepens population collapse and prolongs outbreak intervals for gypsy moth by influencing population behavior at outbreak and post-outbreak levels (Brown et al. 1982). Factors limiting its efficacy include overwintering mortality, poor dispersal ability, and the inability to reach eggs beneath the upper layer of egg masses (Crossman 1925; Brown 1984; Hofstetter and Raffa 1998).

It is possible that *O. kuvanae* is part of the larger cryptic species complex and that the specimens attacking *L. delicatula* are not the same species as those imported for gypsy moth biological control over a century ago. Cryptic species complexes are not uncommon among hymenopteran parasitoids (Hoy et al. 2000; Desneux et al. 2009; Chesters et al. 2012; Zhou et al. 2012; Wang et al. 2016). However, to thoroughly address this issue would require a large integrative taxonomic study that includes targeting sequencing of *O. kuvanae* populations (and closely related species) in Asia and North America. Based on current taxonomy, all morphological and molecular evidence suggests that these specimens are *O. kuvanae*.

There are similarities in life cycles between *L. delicatula* and gypsy moth, such as voltinism, overwintering patterns, and substrates for egg deposition. However, there are obvious differences between them as well, such as insect order, metamorphosis, host plants, and behavior. It is unclear if *O. kuvanae* will behave the same on *L. delicatula* eggs as on gypsy moth eggs. In addition, there appears to be differences in egg-laying strategies for *L. delicatula* between Asian and North America populations. Females lay their eggs shortly after emergence in China and Korea, whereas no eggs were observed in the field until approximately 2 months after emergence in Pennsylvania. A key question is whether or not *O. kuvanae* will complete multiple generations on *L. delicatula* in a season despite available gypsy moth eggs nearby. If yes, it could play an important role in the population dynamics of *L. delicatula* in North America. Information on the seasonal abundance of *O. kuvanae* on *L. delicatula* eggs will provide helpful answers to these questions.

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