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DNA Barcodes and Targeted Sampling Methods Identify a New Species and Cryptic Patterns of Host Specialization Among North American Coptera (Hymenoptera: Diapriidae)

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ABSTRACT Fewer than half of the 80–100 North American species in parasitoid genus Coptera Say (Hymenoptera: Diapriidae) have been described. Hosts are known for just nine of these. The taxonomy of Coptera has been complicated by its cryptic morphology and a life history that includes parasitism of pupae beneath the surface of soils. Here, we describe collections targeting the host genus with which Coptera have most frequently been associated: flies in genus Rhagoletis (Diptera: Tephritidae). DNA barcodes, morphology, and ecology (host associations) were used to understand species limits for Coptera collected from Rhagoletis. Four species of Coptera were recovered from five species of Rhagoletis, including a new species: Coptera n. sp. 1. Two of the associations with particular species of Rhagoletis were previously unknown, and no two species of Coptera were found to be attacking the same host, suggesting these four Coptera are specialist parasites. As several of the 25 North American species of Rhagoletis are agricultural pests, a better understanding of their natural associations with Coptera may prove valuable to biological control efforts.

KEY WORDS Rhagoletis, parasitoid, genetic barcode, biological control, cryptic species

The parasitic wasp genus Coptera Say (Hymenoptera: Diapriidae) is species-rich and common in North America. However, most species remain undescribed, while host-associations and other relevant biology are unknown for all but a handful of species (Muesebeck 1980, Masner and Garcia 2002). Coptera has great potential as an economically important genus. A native Mexican species, Coptera haywardi (Ogloblin), is cultured for control of the Caribbean fruit fly, Anastrepha suspensa (Loew) (Diptera: Tephritidae) (Sivinski et al. 1998), and African Coptera have been bred and released in Hawaii for control of the Mediterranean fruit fly Ceratitis capitata (Wiedemann) (Diptera: Tephritidae) (Silvestri 1914, Yoder and Wharton 2002). Nevertheless, just 29 of the estimated 80–100 native North American species of Coptera have been described, and host associations are known for only nine (Muesebeck 1980).

Our poor understanding of the genus Coptera may be a symptom of a “perfect storm” of taxonomic impediments. First, many of the most commonly applied character suites are uninformative for Coptera. Coptera always have uniformly black bodies, and their sclerites rarely bear surface sculpturing (Yoder and Wharton 2002). Characters used in distinguishing species are often quantitative and continuous, such as the relative lengths and shapes of body parts, which can become cumbersome if many species are under consideration (Muesebeck 1980). Second, Coptera are sexually dimorphic, making the association of males with females in sympathy problematic and necessitating the creation of separate keys for each sex (Muesebeck 1980, Masner and Garcia 2002).

A third complication is that Coptera have a life history that results in their host associations being mostly unknown or overlooked. Adult Coptera walk along fruit fly larval trails as they search for pupal hosts buried in the soil (Buckingham 1975, Granchietti et al. 2012). Females dig into the soil, unearth concealed hosts, and oviposit a single egg (Buckingham 1975). Because hosts are attacked in soil, definitive host associations for Coptera are only known from cases when insect pupae were floated from soil samples and both host and parasitoid were allowed to eclose (Cameron and Morrison 1974, Maier 1981). As soil-based collecting is far less common (and much more difficult) than other trapping methods, Coptera are almost exclusively collected as adults in low-to-the-ground traps, resulting in their host-associations being mostly anonymous.

It is unclear whether most Coptera are specialist or generalist parasites. Muesebeck (1980) reports multiple pupal hosts for some species, including some...
from different Dipteran families. Few, if any, of these have been confirmed in the time since that revision of the genus. Several were reared solely from lab cultures, indicating that these may not be “natural” hosts. Other spurious associations are because of errors in host fly identification, such as the reference to C. occidentalis Muesebeck having been reared from Ragoletis cingulata (Loew) (Diptera: Tephritidae) in Oregon (Hagen et al. 1980, Muesebeck 1980). R. cingulata is an Eastern species that does not occur in Oregon (Foote et al. 1993). Studies of host acceptance in lab settings have indicated that Coptera may have the ability to develop on several different host species, but nevertheless have a more limited host range than other pupal parasitoids (Sivinski et al. 1998). Additionally, behavioral experiments indicate a preference both for ancestral hosts and for volatile odors associated with fruit habitats (Sivinski et al. 1998, Granchi et al. 2012), suggesting specialized host-searching behavior.

One method that can be used to determine the diversity and understand host specificity for North American Coptera is to conduct directed sampling efforts from their most frequently reported hosts, which are flies in genus Ragoletis Loew (Diptera: Tephritidae). Of the nine host records for North American Coptera, five are with Ragoletis. Ragoletis are specialist frugivores with nonoverlapping host ranges whose third instar larvae emerge from fruit hosts and pupate in soil. The genus has 25 named North American species, each with well characterized host associations and geographic ranges (Bush 1966, Foote et al. 1993). Further, egg and larval stage parasitoids of each Ragoletis species are well known for many taxa, but pupal parasitoids are not (Forbes et al. 2010). The standing store of knowledge about this genus of flies provides an opportunity for targeted sampling of their pupal parasitoids, which will in turn reveal more about Coptera.

Here, we report the collection of Coptera from six species of Ragoletis associated with eight different host plant species using two different sampling techniques. We used DNA barcodes (mtDNA COI) as a manner by which to identify preliminary species limits (Hebert et al. 2003). Specifically, we asked 1) whether new Coptera-Ragoletis associations would be revealed by targeting Ragoletis for which no such association has been previously published, and 2) whether Coptera associated with Ragoletis appear to be specialist or generalist.

Materials and Methods

Six different species of Ragoletis were identified as targets for our collections, three of which had been previously associated with Coptera taxa, and three that had not. R. pomonella Walsh infests haws (Crataegus spp.) and apples (Malus domestica Borkhausen), and it is a documented host of C. pomonellae Muesebeck (Maier 1981). R. cingulata infests black cherries (Prunus serotina Ehrhart) and is host to C. cingulatae Muesebeck (Muesebeck 1980). R. suavis (Loew) infests husks of walnuts (Juglans spp.) and is another host of C. pomonellae (Muesebeck 1980). R. juniperina Marcovitch, R. mendax Curran and R. zephyria Snow infest Eastern redbacder (Juniperus virginiana L.), blueberries (Vaccinium corymbosum L.), and snowberries (Symphoricarpos spp.), respectively. No Coptera associations have been reported from any of these potential hosts. All six Ragoletis species included here are found sympatrically in the Midwestern United States, which is where this study was conducted.

Coptera collections were performed in the summer of 2011. The primary trapping method used was the deployment of 4 cm deep, 18 cm diameter yellow plastic pans (Solo Cup Co., Lake Forest, IL) half-filled with a dilute water/detergent mixture, for 24 h under Ragoletis host fruit plants. Only fruiting plants known by the authors to be infested with Ragoletis flies in previous years were used for this study. Pans were checked daily for Coptera, then emptied and refilled with new liquid. Different numbers of pans were deployed, and for varying numbers of total days, as dictated by time and space constraints. Pans under Eastern redbacder and hawthorns in East Lansing, MI, and under black cherries in Iowa City, IA, were maintained for the longest duration (see Table 1 for deployment details).

At three of the same sites where yellow pan traps were used (hawthorn and juniper in East Lansing, MI, and walnut in Riverside, IA), Coptera were also collected by excavating soil samples, which were then air dried for 2 d and sifted through a #10 soil sieve (Hubbard Scientific Co., Chippewa Falls, WI) to isolate Ragoletis puparia. Ragoletis pupae were held at room temperature (≈23°C) until all flies and parasitoids emerged. The number of Ragoletis flies, Coptera wasps, and other parasitoid taxa emerging from each pupal collection was recorded, and the identities of Ragoletis hosts were confirmed using Foote et al. (1993).

Live adult Coptera were also collected from beneath walnuts in Riverside, IA. R. suavis-infested walnut fruits were moved into small piles to attract host-searching Coptera. After 1 h, Coptera found searching on and around fruits were collected using an aspirator. These, and all Coptera collected in this study, were frozen at −80°C.

For a subset of the yellow pan-trapped Coptera, and for all Coptera collected from soil under juniper and hawthorn in East Lansing, MI, whole genomic DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen Sciences, Germantown, MD). A 648 bp segment of the mitochondrial COI gene was polymerase chain reaction (PCR)-amplified using the primers LepF1 and LepR1 (Smith et al. 2007) and using the following cycling parameters: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 46°C for 1 min, and 72°C for 2 min, with a final extension of 72°C for 4 min. Reactions were cleaned using Shrimp Alkaline Phosphatase (USB, Swampscoall, MA) and Exonuclease I (New England Biolabs, Ipswich, MA), and cycle sequencing was performed in both forward and reverse directions.
on an ABI 3730 DNA Analyzer using BigDye 3.1 (Applied Biosystems, Foster City, CA) sequencing chemistry. Forward and reverse sequences were used to create consensi for each individual, which were then aligned by hand in BioEdit (Hall 1999). Bayesian phylogenetic analysis was performed using MrBayes 3.1.2 (Ronquist et al. 2012) (5,000,000 generations, 1,250 burnin, GTR/G model). The best-fit substitution model was determined to be GTR/G (general time reversible with gamma rates) using the Phylogenetic Inference with Automatic Likelihood model Selectors (PALM) web server, which integrates PhyML and MODELTEST to select a model via maximum likelihood methods (Chen et al. 2009). An individual wasp from genus Aneurhynchus (Hymenoptera: Diapriidae) was also sequenced at COI and used as an outgroup. Sequences were deposited into GenBank (Accession Nos.: JQ889990-JQ890048). All individuals from which DNA was extracted were pinned and labeled for future taxonomic work.

## Results

In total, 831 individual Coptera were collected in this study. Yellow pan collections yielded 803 Coptera across all sites (Table 1). Coptera were collected in yellow pans associated with all hosts except for snowberry, the host plant of R. zephyria. Rhagoletis pupae sifted from soil collections yielded 25 individual Coptera, and three host-searching Coptera were aspirated from the surface of R. suavis-infested walnuts. Bayesian analysis of COI sequences resolved four different clades of Coptera among our collections (Fig. 1). Each clade fits the definition of a ‘barcode species’ based on interclade sequence divergence; pairwise divergence between clades ranged from 9.7–13.7%, far exceeding the conservative 2% divergence limit typically used in barcoding studies (Smith et al. 2007, 2011). Intraclade sequence divergence was also low, ranging between 0 and 0.59%. Pairwise comparisons also therefore satisfy the “10x rule” threshold for barcode species, wherein inter-
specific variation must exceed intraspecific variation by a factor of ten (Hebert et al. 2004), though sample sizes of genotyped individuals within each clade were often small ($n = 2–41$).

The first species cluster, which keyed out as *C. pomonellae* in the key by Muesebeck (1980), was pan-trapped under fruit hosts of *R. pomonella*, *R. suavis*, and *R. juniperina*. A second cluster, keyed to *C. cingulatae*, was pan trapped only under black cherries (*P. serotina*), the fruit host of *C. cingulata*. A third cluster, keyed to *C. pilosa* (Ashmead), was pan-trapped under highbush blueberries, the fruit host of *R. mendax*. Finally, the fourth cluster also keyed morphologically to *C. pomonellae* based on Muesebeck’s (1980) key. However, based on the extent of divergence of the COI sequence from all other clusters ($\geq9.7\%$), and the fidelity of the host association with *J. virginiana*, we putatively identify individuals in this fourth cluster as a new species. This species (from here on ‘*Coptera* n. sp. 1’) was trapped in yellow pans under hawthorns and junipers in Michigan, and under apple trees in both Michigan and Iowa. Soil-collections, however, showed that only *Coptera* n. sp. 1 was reared from *R. juniperina*, while only *C. pomonellae* was reared from *R. pomonella* (Table 2). We were not able to determine diagnostic characters to distinguish *Coptera* n. sp. 1 from *C. pomonellae* morphologically. Some variation in setal distribution across the dorsal mesosoma was observed, but these traits were not uniformly different.

### Discussion

Our sampling effort targeting *Rhagoletis* fly host pupal environments was effective in uncovering new diversity and host records for *Coptera*. We collected one new species of *Coptera* (*Coptera* n. sp. 1), morphologically. This new species was cryptic with *C. pomonellae*, but differed both in haplotype and host use. Further, *C. pilosa*, a species described first by Ashmead (1893), revised by Muesebeck (1980), and unmentioned in any subsequent peer reviewed literature was collected under blueberries, the host plant of the blueberry maggot, *R. mendax*. This is the first suggestion of a host association for *C. pilosa*, though we hesitate to firmly associate it with *R. mendax* until it has been reared from pupae of this fly. We also confirmed published host associations for *C. cingulatae* (*R. cingulata*) and *C. pomonellae* (*R. pomonella*) (Muesebeck 1980, Maier 1981). *C. pomonellae* was also aspirated from above walnuts, but *Coptera* reared from *R. suavis* pupae were not of sufficient quality for genetic work, and so we consider the association of *C. pomonellae* with *R. suavis* unconfirmed.

This study also revealed new information about host specialization. *Coptera* n. sp. 1 was collected in pan traps beneath plant hosts of both *R. pomonella* and *R. juniperina*, but reared exclusively from pupae of the latter (Table 2). Conversely, soil-collected *R. pomonella* pupae yielded only *C. pomonellae*. These results indicate that *Coptera* may search for hosts over a wide area (the two sites in question were both in East Lansing, MI, $\approx0.8$ km apart), but only attack certain hosts. Alternatively, *Coptera* may attack many hosts, but fail to develop in all but a subset. In either case, pan trapping beneath black cherry trees and blueberries in the same city each produced just a single species of *Coptera*, strongly suggesting that different species of *Coptera* specialize on one or a few hosts. Many larval parasitoids of *Rhagoletis* flies show evidence of specialization on just one or a few fly species (Forbes et al. 2009, 2010), but all of these also interact directly with the fruit host. Here, we demonstrate the first evidence that direct interaction with the plant environment may not be necessary for specialization among *Rhagoletis* parasitoids.

We note that without mtDNA sequences, *Coptera* n. sp. 1 would not have been distinguished from *C. pomonellae* because of their extreme morphological similarity. Parasitoids as a whole tend to be morphologically cryptic, especially to nonexperts, and genetic barcodes have shown great value in revealing hidden diversity, even in temperate regions (Smith et al. 2011). Critics of the use of barcode sequences to define species boundaries argue that incomplete lineage sorting or introgression of mitochondrial genomes across species boundaries may result in a misrepresentation of true species limits (Funk and Omland 2003). Here, however, the extremely divergent haplotypes ($>10\%$ in all pairwise comparisons), as well as the 100% host fidelity between mtDNA haplotypes and the *Rhagoletis* pupae from which these *Coptera* were reared via soil collections (Table 2), strongly support *Coptera* n. sp. 1 as a new species, parasitizing only *R. juniperina*, and being different from *C. pomonellae*. We do not formally describe *Coptera* n. sp. 1 here, as we anticipate its inclusion in a future formal circumscription of North American *Coptera*.

This and future efforts to identify *Coptera–Rhagoletis* associations may prove informative to biological control of *Rhagoletis* flies. For instance, *C. pilosa*, which we can now tentatively associate with pupae of the blueberry maggot, *R. mendax*, might be a completely overlooked, yet potentially beneficial control organism for that commercially important pest species. Additionally, several other economically relevant *Rhagoletis* flies have not yet been assessed for potential *Coptera* associations, including *R. fausta* and *R. indifferens*, which both infest cultivated cherries, *R.ribicola* (currants) and *R. striatella* (tomatillos). Attempts have been made to use *Coptera* as biological control agents, but the results to date have been mixed. An African *Coptera* species, *C. silvestri*, was released in Hawaii in the early 1900s to control the Mediterranean fruit fly (*Silvestri 1914*), but its success has not been
measured, and it is known to (counterproductively) hyperparasitize other introduced larval and egg parasitoids. More promisingly, C. haywardii is being cultured in Mexico for control of its apparently ancestral hosts in the genus Anastrepha (Tephritidae) in citrus fruits (Sivinski et al. 1998, Guillen et al. 2002). Another potential success has been the use of C. occidentalis to control its natural host, R. completa, which has invaded walnuts in Europe (Granchietti et al. 2012). These latter two examples highlight the promise for control of pest species using ancestrally associated Coptera.

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