Biology of Parasitoids (Hymenoptera) Attacking *Dasineura oxycoccana* and *Prodiplosis vaccinii* (Diptera: Cecidomyiidae) in Cultivated Blueberries

BLAIR J. SAMPSON,[1] TIMOTHY A. RINEHART,[1] OSCAR E. LIBURD,[2] STEPHEN J. STRINGER,[1] AND JAMES M. SPIERS[1]

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ABSTRACT The blueberry gall midge, *Dasineura oxycoccana* (Johnson), and blueberry tip midge, *Prodiplosis vaccinii* (Felt) (Diptera: Cecidomyiidae), are recurring cecidomyiid pests of cultivated blueberries in the southern United States and Mediterranean Europe. Insecticides can give short-term control, but overlap in parasitoid phenologies indicates the potential for natural control of midge populations. Using a combination of laboratory rearing and mitochondrial DNA analysis of field samples, we identified five species of solitary endoparasitoids that killed 30–40% of midges. These species include at least three undescribed platygastrids in the genera *Synopeas*, *Platygaster*, and *Inostemma*. An undescribed prepupal idiobiont, *Aprostocetus* sp. (Eulophidae: Tetrastichinae) was the only midge parasitoid that was consistently active when rabbiteye blueberries, *Vaccinium ashei* Reade, were in flower. Six percent of midge prepupae, half of which already contained platygastrid larvae, were parasitized by *Aprostocetus*.

KEY WORDS biological control, Platygastridae, Eulophidae, high-fidelity polymerase chain reaction, parasitism

BLUEBERRY GALL MIDGE, *Dasineura oxycoccana* (Johnson), and blueberry tip midge, *Prodiplosis vaccinii* (Felt), are two species of univoltine gall midges (Diptera: Cecidomyiidae) that feed exclusively on *Vaccinium* buds. Midges are not easily detected because their feeding damage only becomes visible 7–14 d after larvae have left the blueberry bush. Furthermore, bud injury is often mistaken for freeze damage, plant disease, or nutrient deficiency (Driggers 1926, Lyrene and Payne 1992, Bosio et al. 1998, Sampson et al. 2002, Wei 2004). *D. oxycoccana* is the first of these species to break winter dormancy in January or February in the southeastern United States. Females principally oviposit between the developing scales of flower buds. Newly hatched larvae crawl deeper into blueberry buds where they feed on the innermost meristematic tissue and can reduce up to 80% of flower production and potential fruit yield (Lyrene and Payne 1992, Sampson et al. 2002). As damaged floral buds disintegrate, *D. oxycoccana* move to leaf buds, but they are soon superseded by *P. vaccinii* (Driggers 1926; Gagné 1986, 1989; B.J.S., unpublished data). Crop losses to *P. vaccinii* have yet to be assessed. However, injury to blueberry leaf buds and meristems by *P. vaccinii* often induces excessive suckering, leaf distortions, and leaf drop, which could result in lighter bud sets (Gagné 1986, Williamson and Miller 2000).

Little is known about the interactions between midges and their parasitoids on fruit crops. Proctotrupoid wasps such as those belonging to the family Ceraphronidae parasitize *D. oxycoccana* on Wisconsin cranberries, *Vaccinium macrocarpon* L. (Barnes 1948). In the southern United States (Florida, Georgia, Alabama, Mississippi, and Louisiana), endoparasitic Platygastridae seem to replace ceraphronids as the key natural enemies of midges (Vlug 1976, Yoshida and Hirashima 1979, Jeon et al. 1985, Soné 1986, Sampson et al. 2002, Sarzynski and Liburd 2003, Legner 2004). Chalcids, like tetrastichine eulophids, are also midge parasitoids found in both midwestern and southern habitats, where they attack midges on cranberries and blueberries, respectively (Sampson et al. 2002).

Here, we studied the ecology of parasitoids found in rabbiteye, *Vaccinium ashei* Reade, and southern highbush blueberry (*Vaccinium corymbosum* × *Vaccinium darrowii* Camp) ecosystems. Larval development, adult reproductive behavior and parasitoid–host phenology was investigated during 2003–2004. Mitochondrial DNA analysis was used to confirm the identity of adult parasitoids as well as to assist in interpreting interactions between parasitoids and their midge hosts.

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[1] USDA–ARS Small Fruit Research Station, Poplarville, MS 39470.
[2] Department of Entomology and Nematology, University of Florida, Gainesville, FL 32611–0620.

Materials and Methods

Host and Parasitoid Sampling. *Host Eggs and Larvae.* Juvenile midges were sampled from two blueberry nurseries at the USDA–ARS Small Fruit Research Station, Poplarville MS (N 30° 50.21′, W 89° 32.65′). Nursery 1 contained 950 2- to 6-yr-old potted bushes representing ≈50 clones of rabbiteye blueberry, southern highbush blueberry, and wild blueberries, *Vaccinium elliottii* Chapman. Nursery 2 housed ≈11,000 second- and third-year seedlings representing 300 clones of *V. ashei* and southern highbush blueberry. Additional midge hosts were collected from 10 commercial rabbiteye blueberry farms in southern Louisiana, Mississippi, and Alabama.

A single sample consisted of ≈10 terminals bearing 30 flower or leaf buds enclosed in a resealable plastic bag. Five or 10 samples were taken from random bushes in each nursery every 3 to 4 d over a 2-yr period. Smaller samples were collected when bud availability waned in the winter. Bags were held for 2 to 3 d to allow larvae to abandon buds. The bags were then partly filled with water and shaken to flush out remaining host eggs and larvae as well as larvae that were diseased, heavily parasitized, or dead. Eggs and larvae were pipetted into clean petri dishes, counted, and preserved in 100% ethanol. The number of host larval stages (instars) was established from a frequency histogram generated by PROC UNIVARIATE (SAS Institute 1990) by using the body width measurements of 474 midge larvae. Mature midge larvae (third instars or prepupae) were identifiable by a dark Y-shaped ventral sclerite called a spatula (Gagné 1989).

Juvenile Parasitoids. Preparing a parasitoid brood for slide mounting sometimes required varying a host larva's exposure to the clearing, staining, and fixing agents. Normally, host fat tissues were adequately solubilized with exposure to solutions of 5 and 10% KOH for 24 h and 30 min, respectively. Two or three drops of diluted double stain (BioQuip Products, Inc., Rancho Dominguez, CA) or acid fuschin in lactophenol was sufficient to clear, stain, and temporarily fix specimens. Smearing host larvae by applying mild pressure to the coverslip entirely expulsed many parasitoids from the host or more clearly exposed them. Live parasitized hosts, compressed by a coverslip, made it easier to identify which host organs nourished the parasitoid brood (e.g., midgut, brain, or ganglia). Using an ocular micrometer, we measured the cephalothorax and caudal segments of juvenile parasitoids that were fixed on glass slides.

Adult Parasitoids. Adult wasps were collected from the plastic bags and from parasitized midge prepupae and pupae that were raised on moist peat in dark insect growth chambers at 30°C (I-30 BLL, Percival Scientific Inc., Perry, IA). Randomly placed sticky yellow traps (16 by 23 cm, Trecé, Salinas, CA) were hung in the canopies of 10 blueberry bushes to collect foraging parasitoids. The tip of an insect pin was used to remove parasitoid adults from the traps. Residual glue that adhered to the insect was dissolved by agitating specimens for 24–48 h in Histoclear (National Diagnostics, Atlanta, GA). Wings were removed from adults, labeled, and separately slide mounted in water to protect fine hairs during the clearing process. Adults were cleared using 10% KOH, neutralized, and stained with acid fushin in lactophenol, and then slide mounted in euparal or silicon oil. The identity of parasitoids on sticky traps that belonged to species capable of parasitizing immature midges was confirmed by comparing them with specimens captured among host eggs and neonates inside blueberry buds or reared from larval or prepupal hosts inside the insect growth chambers.

Host and Parasitoid Identification. The following taxonomic keys and records were used for midge and parasitoid identification: Cecidomyiidae (Diptera): Barnes (1948) and Gagné (1986, 1989). Platygastridae (Hymenoptera): Ashmead (1887, 1893), Marchal (1906), Fouts (1924), Kieffer (1926), Masner and Muesbeck (1968), Krombien et al. (1979), Velikan et al. (1984), Vlug (1984, 1995), Masner and Huggert (1989), and Buhl (1998, 2001). Eulophidae (Hymenoptera): Crawford (1907), Girault (1917), Myers (1930), Walter (1941), Burks (1943), Piore and Viggiani (1965), Bouček (1986), Krombien et al. (1979), Velikan et al. (1984), LaSalle (1994), and Shauff et al. (1997). Taxonomic experts affiliated with the USDA–ARS Systematic Entomology Laboratory Communications and Taxonomic Services Unit (Beltsville, MD) confirmed our identifications. Vouchers were deposited in the U.S. National Collection by Raymond J. Gagné (Cecidomyiidae), Michael E. Shauff (Eulophidae), Michael W. Gates (Eulophidae and Platygastridae), and Terry Nuhn (Platygastridae).

Ecological Data: Analysis of Host Parasitism and Parasitoid Reproductive Behavior. We calculated the average parasitism rate expected for each month of a single year (Fig. 1). Parasitism rates were expressed as the percentage of host eggs and larvae with parasitoid eggs, larvae, or pupae (Figs. 2 and 3). The chi-square goodness-of-fit analysis (PROC FREQ) was used to test whether parasitoid eggs, neonates, and older first instars were randomly or uniformly distributed among host larvae (SAS Institute 1990).

Foraging activity of adult parasitoids throughout the day was evaluated using hourly sticky trap (16 by 23-cm) catches. The resulting data were fitted to a Gompertz function [$y = 163\,e^{-49e^{(-0.26x)}}$], where x is the cumulative hours that elapsed starting at 0800 hours, and y is hourly cumulative catch expressed as a percentage of the total daily catch (PROC NLIN, SAS Institute 1990). The behavior of female parasitoids in or around buds and host larvae also was noted.

Polymerase Chain Reaction (PCR) Procedure and Sequencing Mitochondrial DNA from Adult Parasitoids. Randomly chosen adult wasps of both sexes (Fig. 4a–i) were sorted by taxon and placed into 1.5-ml microcentrifuge tubes filled with 100% ethanol before analysis. Sequencing mitochondrial DNA revealed diagnostic mutations useful for calculating genetic similarity in midge endoparasitoids. A bead-beating technique and Ultraclean soil DNA isolation kit (MoBio,

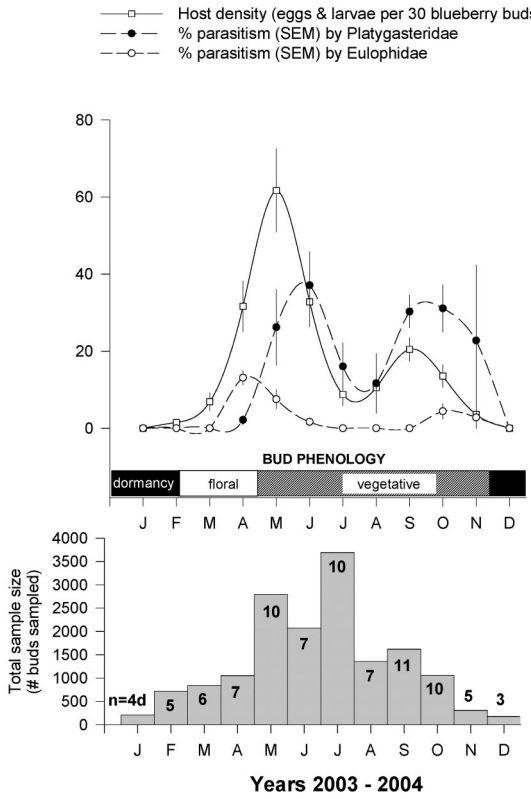

Fig. 1. Seasonal changes in host density (□) and the percentage of parasitism of midge larvae by Platygastridae, largely *Synopeas* and *Platygaster* (●) and Eulophidae, exclusively *Aprostocetus* (○) sampled from cultivated southern blueberry. Phenology of blueberry bud development is depicted as horizontal boxes. SEM is standard error of the means represented by vertical bars. The lower figure summarizes monthly sampling effort. Numbers inside the bars are the sample sizes for monthly percentage of parasitism.

Solana Beach, CA) extracted total DNA from adult parasitoids from each taxa (see Fig. 5 for taxon designations). The degenerate primer pairs C1-J-1763 (TATAGCATTCCCACGAATAAATAA) with C1-N-2096D (GANGTATTWARRTTTCGRTCWGTTA) and C1-J-2090 (AGTTTTAGCAGGAGCAATTACTAT) with C1-N-2395D (TTAATWCCWGTWGGNACNGCAATRATTAT) amplified part of the mitochondrial cytochrome oxidase I (COI) gene (Simon et al. 1994). Reactions consisted of 4 μl of MasterMix *Taq*DNA polymerase (Brinkmann-Eppendorf, Westbury, NY) and 5 ng of total insect DNA in 6.4 μl of water. Cycling parameters were 95°C for 2 min followed by 43 cycles of 95°C for 1 min, 56°C for 2 min, 72°C for 3 min, and then 72°C for 10 min with storage at 4°C. Amplified PCR products were purified using a QIAquick PCR kit (QIAGEN, Valencia, CA), and the resulting DNA strands were sequenced using BigDye version 3.1 (Applied Biosystems, Foster City, CA).

Partial sequences of the COI gene consisting of 364 bp were edited using Sequencher (Gene Codes, Ann Arbor, MI) and aligned with MegAlign (DNAstar, Madison, WI). Phylogenies based on parsimony were generated using PAUP*4.0 (Swofford 2000). Because our analysis was limited to adult identifications, we selected the most parsimonious tree (Fig. 5) to depict the phylogeny among midge parasitoids based on 114 informative characters. Boot strap values from 100 replicates and percentage of sequence similarity are shown above and below each branch, respectively (Fig. 5). COI sequences and alignment for the 19 wasp specimens used to produce a preliminary phylogeny of our small parasitoid assemblage can be found in National Center for Biotechnology Information GenBank under the accession nos. AY843313–AY843331.

Results

Host–Parasitoid Ecology and Identification. *Synopeas* were the most common midge parasitoids. Adults of this genus were distinguished from other midge parasitoids by a posterior scutellar spine; short and pointed in *S. Sactogaster* (Fig. 4a, akin to Ashmead's original description of *S. anomaliventre*), longer and blunter in *S. Synopeas* [Fig. 4c similar to *S. pennsylvanicum* (Fouts)]. Ninety percent of adult *Synopeas* actively flew between 1100 and 1700 hours. Females were observed to crawl deep inside blueberry buds. Female *Synopeas* dispersed their eggs (Fig. 2a) and young first instar brood (Fig. 2i) uniformly among hosts (goodness-of-fit test: $\chi^2 = 258.8$, df = 6, $P < 0.005$, $n = 355$ parasitized hosts) and positioned eggs near the host's midgut (Fig. 2j and k; Ganin 1869, Marchal 1906). Nine of 10 broods contained only one or two offspring (mean, 1.5 ± 1.0 per host, $n = 354$ parasitized hosts). When broods were larger, offspring were typically staggered in the posterior half of the host (Fig. 2k).

First instars of *Synopeas* were cyclopiform (Fig. 2i–l), each with a light yellow-brown cephalothorax. Head capsules bore broad sickle-shaped mandibles as well as maxillae and ligulae spiked with short rasping teeth. Newly hatched larvae measured 210 ± 50 μm ($n = 38$) wide at the head and 390 ± 120 μm between apices of the head and furca ($n = 17$; Fig. 2i). Instars II (Fig. 2n) and III (Fig. 2o) were hymenopteriform with reduced mandibles, and before pupating (Fig. 2p and q) they grew five-fold. Instar II: (L) 550 ± 110 μm, (W): 280 ± 50 μm, $n = 13$). Instar III: (L): 1000 ± 120 μm, (W): 440 ± 80 μm, $n = 10$).

Female *Inostemma* sp. (Fig. 4e) likely oviposited in *P. vaccinii* eggs. Embryonic development occurred in the brain and first five ganglia of third instar hosts (Fig. 2t). Early first instars of *Inostemma* (Fig. 2g and h) were recognized by fine serrations along the inner margins of furcae (Marchal 1906, Jeon et al. 1985). Adults (Fig. 4e) possessed a prominent marginal vein in the forewing and a long arching horn that sheathed the ovipositor. Parasitism by *Platygaster* (Fig. 4g) was indicated by the presence of oval eggs (Fig. 2r), mandibulate-type first instars with 12 abdominal and thoracic segments (Fig. 2b–d) and a smaller cyclopiform larva (Fig. 2e and f). Clutches of one or two progeny typify *Platygaster* broods, but as many as seven were

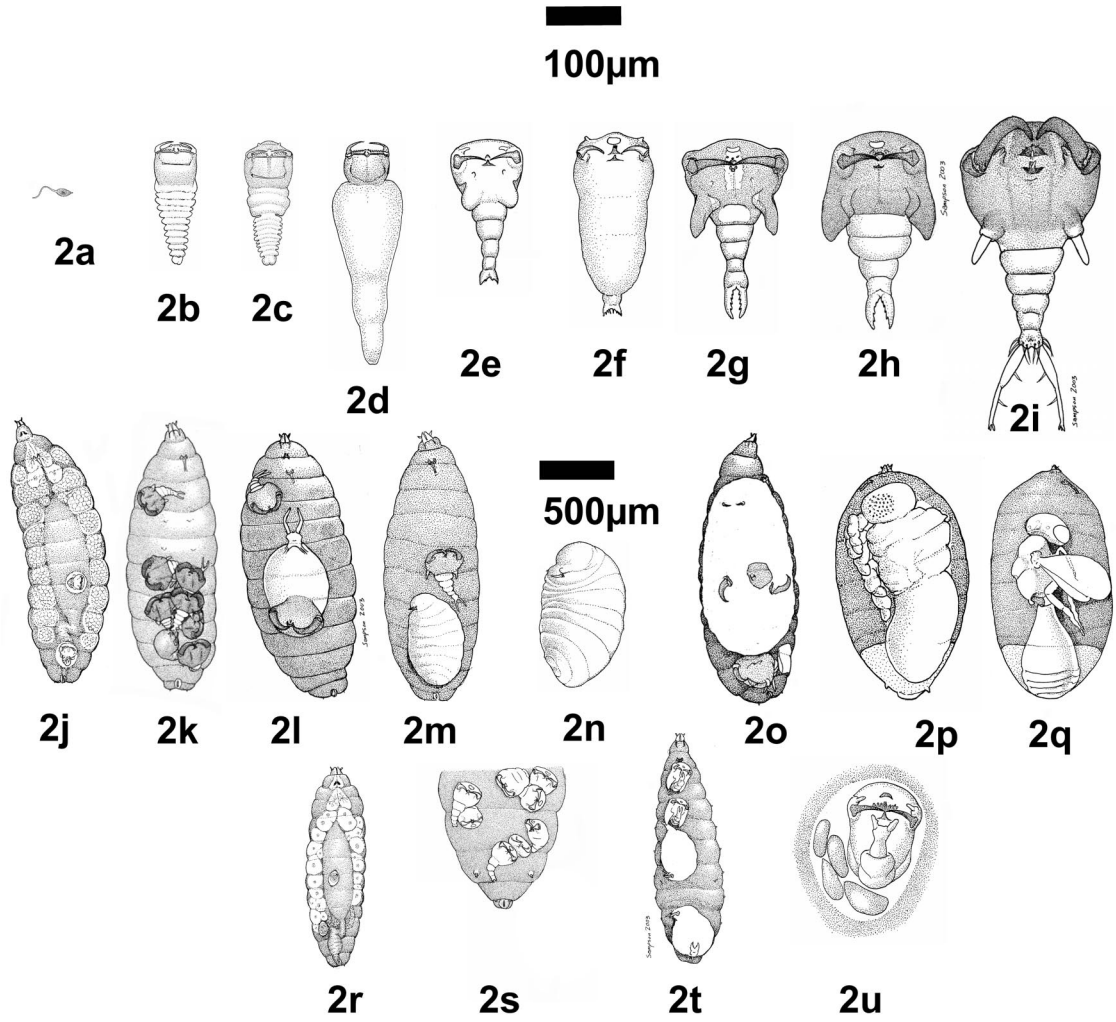

Fig. 2. Eggs, larvae, and pupae of Platygastrinae found in *D. oxycoccana* and *P. vaccinii* hosts. (a–i) Egg and first instars. (a) Newly inserted *Synopeas* egg. (b) Early first instar of *Platygaster* sp.1. (c) Same partially engorged. (d) Same fully engorged. (e) First instar of *Platygaster* sp. 2. (f) Same fully engorged. (g) *Inostemma* first instar. (h) Same partially engorged. (i) Early first instar of *Synopeas* sp. Top scale bar represents larva size in micrometers. (j–q) Development of *Synopeas* brood inside host larvae. (j) Fully developed embryos. (k) Superparasitism produces nine first instar progeny. (l) Early and late (engorged) first instars. (m) Early first instar and second instar. (n) Second instar of *Synopeas*. (o) Third instar. (p) Early pupa. (q) Late pupa. Scale is provided by brood shown inside their hosts as well as a lower bar for j–m and o–q. (r–u) Immature stages of other platygastrids, scale not shown, but can be inferred from figures. (r) *Platygaster* eggs. (s) superparasitism in *Platygater*. (t) Early and late (engorged) first instars of *Inostemma*. (u) Early *Synopeas* first instar still surrounded by its trophamnion.

possible per host (Fig. 2s). First instars had a membranous cephalothorax that was 120 ± 30 µm ($n = 15$) in width and a body 220 ± 30 µm in length ($n = 9$; Fig. 2e and f). Adult *Platygaster* lacked a horn or scutellar spine, but basal tergites were embossed with prominent longitudinal grooves. A male *Synopeas* or *Platygaster* had an abdomen shorter than the female, but his antennae were longer and more filiform (Fig. 4b, d, f, and g). In *Inostemma*, males lacked horns and were consistently larger than females.

Mismatching descriptions of our specimens with those of males and females of previously described species of *Aprostocetus* indicated that our eulophid wasp was newly discovered. Oviposition by female *Aprostocetus* principally occurred in prepupal (third instar) midges (Fig. 3g) and occasionally in younger host larvae. Ninety-four percent of *Aprostocetus* clutches were small with one or two eggs [(Fig. 3a, g, and h); mean $1.3 \pm 0.6, n = 129$)]. Eggs were uniformly dispersed among hosts and typically inserted in the lumen of the host midgut (goodness-of-fit test: $\chi^2 = 90.6$, df = 6, $P < 0.005$; Fig. 3g). Larval *Aprostocetus* were hymenopteriform, hyaline, and sometimes had a dark dorsal spot (Fig. 3b). Earlier instars bore 13 cau-

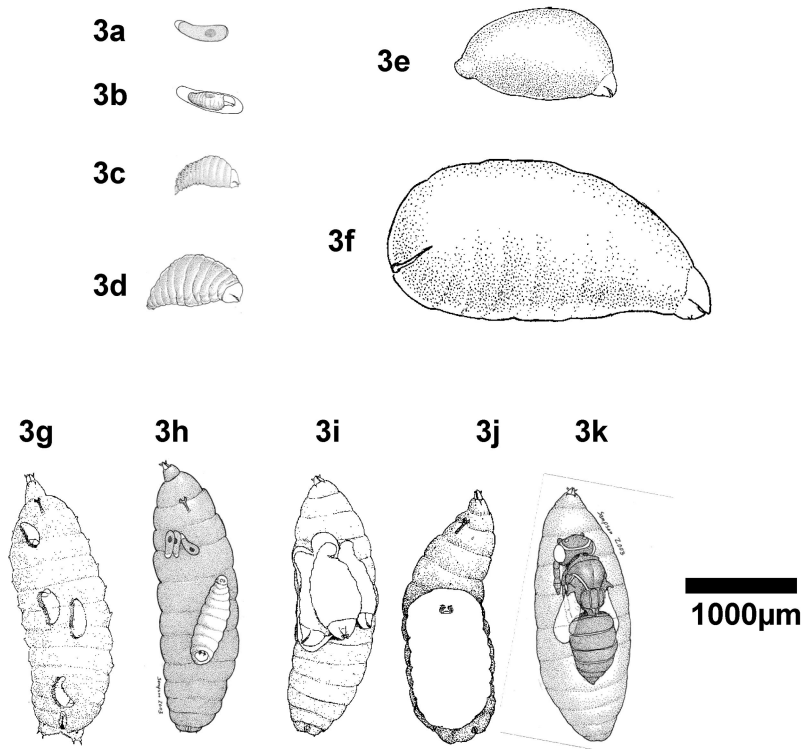

Fig. 3. Eggs, larvae, and pupae of *Aprostocetus* sp. found in *D. oxycoccana* and *P. vaccinii* hosts. (a) Detail of egg. (b) Neonate first instar. (c) First instar. (d) Partially engorged first instar. (e) Second instar. (f) Third instar. (g) One egg and three first instars in a host larva. (h) Three eggs and one second instar. (i) Three partially engorged second instars. (j) Third instar. (k) Pupa. Bottom bar represent size scale in micrometers for g–k.

dal segments and a bulbous head laterally divided by an incomplete line or suture (Fig. 3b–d). Older and engorged instars lacked any obvious surface segmentation (Fig. 3e and f). Only one first instar (Fig. 3b–d) per host survived to undergo two more molts (Fig. 3e, f, i, and j) before pupating (Fig. 3k). Adult *Aprostocetus* (Fig. 4h) were ≈1.0 mm in length with a shiny black head with hints of green iridescence. A finely reticulated scutum was subdivided by a median line and arrayed laterally with three pairs of long bristles. The scutellum was fully subdivided by two parallel submedian grooves and flanked by two rows of stout bristles. The basal area of the antennal scape in the male (Fig. 4i) was marked by a prominent circular carina. The annual parasitism rate of *Aprostocetus* was 6% and stretched from late March to November. Half of the hosts that received *Aprostocetus* eggs already contained platygastrid larvae.

Mitochondrial DNA Analysis of Adult Parasitoids. Sequence variation in the COI gene depicted as a parsimonious tree (Fig. 5) supported the generic and subgeneric categories to which we identified our adult midge parasitoids (Fig. 4a–i). No insertions or deletions were present in the sequence alignment except for a six base deletion in *Aprostocetus* samples, consistent with the coding status of this COI region (GenBank accession nos. AY843313–AY843314). Sequence similarity within taxonomic groups was high and included an abundance of informative sites. Taxa-specific characters, or diagnostic mutations, were identified for each group, including 39 substitutions specific to *Aprostocetus*, 20 specific to *Inostemma*, 27 specific to *Platygaster*, four specific to *Synopeas* with an additional nine defining *S. Synopeas*, and three more specific to *S. Sactogaster*.

Discussion

Midge parasitoids, especially the Platygastrinae and Inostemmatinae, are endemic to North America and show a strong specificity for cecidomyiid eggs and larvae (Ashmead 1893, MacGown 1979). Their occurrence in European blueberry plantings has yet to be confirmed, but two midge hosts seem to have survived the passage from North America to Europe most likely as larvae on nondormant blueberry plants or as pupae in soil (Bosio et al. 1998, Molina 2004). To date, few accounts detail the ecology and natural histories of proctotrupoid and chalcidoid wasps associated with cultivated fruits in North America (Vlug 1976, Yoshida and Hirashima 1979, Jeon et al. 1985, Soné 1986). Our research expands the knowledge of ecological and developmental interactions between parasitoids and their cecidomyiid hosts on cultivated blueberries.

Five previously undescribed parasitoid wasps were found to parasitize the eggs and neonates (Platygas-

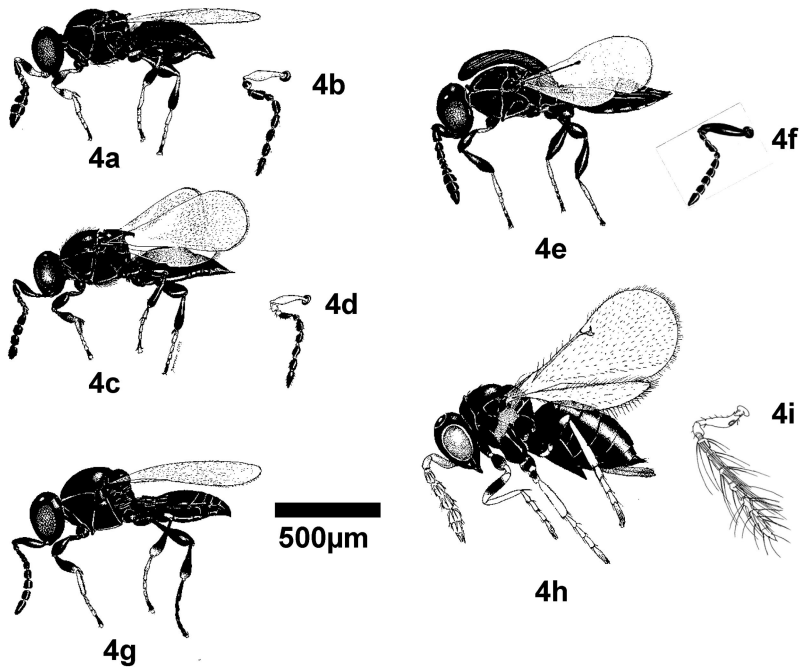

Fig. 4. Adult Platygastrinae and Tetrastichinae (Eulophidae) that parasitized *D. oxycoccana* and *P. vaccinii* in the southeastern United States. (a) *Synopeas* (*Sactogaster*) female and (b) antenna of male. (c) *Synopeas* (*Synopeas*) female and (d) antenna of male. (e) *Inostemma* female and (f) antenna of male. (g) *Platygaster* male. (h) Adult *Aprostocetus* female. (i) Detail of antenna of male *Aprostocetus*. Bar represents size scale in micrometers.

trinae) or third instars (Tetrastichinae) of *D. oxycoccana* and *P. vaccinii*. *Aprostocetus* (Tetrastichinae) is the first parasitoid to attack *D. oxycoccana* infesting blueberry flower buds and seems to be more widely distributed than any of the other blueberry parasitoids in North America (Crawford 1907, Dean 1911, Girault 1917, Myers 1930, Breland 1939, Burks 1943, Barnes 1948, LaSalle 1994, Sampson et al. 2002). At the end of bloom and as leaf buds break, Platygastrinae, especially *Synopeas*, begin to actively parasitize midge eggs and larvae. Unfortunately, the biology and host affiliations of the few described species of related Platygastrinae and Synopeadines in North America remain obscure or unknown (Ashmead 1893, Kieffer 1926, Vlug 1995). However, the clustering of both sexes into each of two subgroups or clades based on 12 different molecular characters and two morphological traits (scutellar spine and abdominal pouch) show two distinct lineages of *Synopeas* attacking southern populations of midges. Both *Synopeas* species were active during the day and from May to November females sought newly hatched larvae deep inside unfurling leaf buds. Eggs were inserted in the host larva's midgut, and brood developed as solitary koinobionts (Marchal 1906, Vlug 1976, MacGown 1979, Yoshida and Hirashima 1979). Other solitary koinobionts, such as *Platygaster*, initiated larval development in the host stomach and hemocoel, whereas embryogenesis and early larval development for *Inostemma* occurred inside cyst-like outgrowths of the host's central nervous system (Marchal 1906, Jeon et al. 1985). Morphological differences among first instars and some divergence in the mitochondrial sequences of adult *Platygaster* indicate that two species probably parasitize midges, one common (near *P. striaticollis*) and the other rare (B.J.S., unpublished data).

Thirty to 40% of larval midges were paralyzed or killed in the blueberry nurseries; rates considered very high in cecidomyiid populations (Gagné 1989). *Aprostocetus* was the only midge parasitoid active for most of the bloom period, but without parasitism from other wasp species, this wasp may have produced too few broods to adequately control *D. oxycoccana.* If properly applied, prebloom applications of registered insecticides, including spinosad, malathion, and diazinon, can reduce 50–95% of *D. oxycoccana* larvae (Sampson et al. 2003; B.J.S and O.E.L., unpublished data), and any midge survivors and their descendants can be later eliminated by *Aprostocetus* and other parasitoids (Grover 1986, Sampson et al. 2002).

Knowing exactly when to apply an insecticide for midge control without harming parasitoids can be difficult for scientists and impossible for most berry producers. Visual crop scouting is limited by the small size of midges and their parasitoids. The presence of parasitoids and parasitism rate can be obtained from field sampling, rearing and lengthy host preparation, as was done in this study. However, we could more quickly identify parasitoids and derive parasitism rates from the percentage of eggs and larval hosts that test positive for the unique DNA signatures of their parasitoids. We have partially developed a high-fidelity

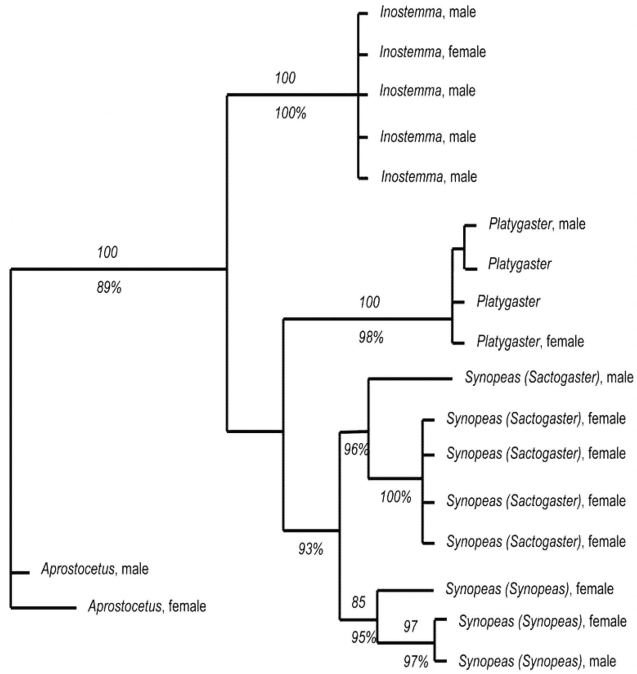

Fig. 5. First step of our high-fidelity PCR assay was to establish consistency in our parasitoid clades based on the most parsimonious tree generated from the alignment of 364 bases of the COI gene. Generic and subgeneric names correspond to 19 adult wasps shown in Fig. 4a and i, and *Aprostocetus* served as an outgroup. Bootstrap values >50% are shown above branches, and percentage of genetic similarity is shown below branches.

PCR assay for rapid parasitoid detection by successfully extracting and sequencing picogram quantities of adult mitochondrial DNA in the COI region. The next step is to develop taxa-specific or species-specific PCR primers from these COI sequences, which will give us the means to amplify and separate brood DNA from midge DNA, similar to work by Persad et al. (2004) and Weatherbee et al. (2004). Our DNA sequences also will aid the systematic study of these midge parasitoids.

Acknowledgments

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