

Parasitoids, Hyperparasitoids, and Inquilines Associated With the Sexual and Asexual Generations of the Gall Former, *Belonocnema treatae* (Hymenoptera: Cynipidae)

Andrew A. Forbes,^{1,2} M. Carmen Hall,^{3,4} JoAnne Lund,^{3,5} Glen R. Hood,^{3,6} Rebecca Izen,⁷ Scott P. Egan,⁷ and James R. Ott³

¹Department of Biology, University of Iowa, Iowa City, IA 52242 (andrew-forbes@uiowa.edu), ²Corresponding author, e-mail: andrew-forbes@uiowa.edu, ³Population and Conservation Biology Program, Department of Biology, Texas State University, San Marcos, TX 78666 (Myra.Hall@gpc.edu; lundbug@newnorth.net; ghoo@nd.edu; jimott@txstate.edu), ⁴Current address: Science Department, Georgia Perimeter College, Decatur, GA 30034, ⁵Current address: 4223 Bear Track Lane, Harshaw, WI 54529, ⁶Current address: Department of Biological Sciences, University of Notre Dame, Galvin Life Sciences, Notre Dame, IN 46556, and ⁷Department of BioSciences, Anderson Biological Laboratories, Rice University, Houston, TX 77005 (rebecca.m.izen@rice.edu, scott.p.egan@rice.edu)

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Abstract

Insect-induced plant galls are thought to provide gall-forming insects protection from predation and parasitism, yet many gall formers experience high levels of mortality inflicted by a species-rich community of insect natural enemies. Many gall-forming cynipid wasp species also display heterogony, wherein sexual (gamic) and asexual (agamic) generations may form galls on different plant tissues or plant species. Despite broad interest in using these systems to study parasitism, enemy escape, and community assembly, few studies have provided complete characterizations of the natural enemy diversity associated with gall-forming wasp species, and fewer still have done so for both alternating generations. Here, we characterize the parasitoids, inquilines, and hyperparasitoids associated with the alternating sexual and asexual generations of the cynipid gall former, *Belonocnema treatae* Mayr in Texas, USA. We find 24 species associated with the asexual generation in leaf galls and four species associated with the sexual generation in root galls. We provide photographs and mitochondrial sequences for most species, discuss implications of these findings for hypotheses that consider heterogony as an adaptation for escape from natural enemies, and contemplate the relative incidence of specialized versus generalist feeding habits among gall former natural enemies.

Key words: oak gall, *Quercus virginiana*, *Quercus fusiformis*, DNA barcode, heterogony

Gall-forming insects are found in seven orders distributed across 24 families (Dreger-Jauffret and Shorthouse 1992) and comprise an estimated number of species ranging from 13,000 (Buhr 1965) to as many as 211,000, which are collectively known to gall a majority of the flowering plant species worldwide (Espírito-Santo and Fernandes 2007). Gall formers are important, as they sit at the center of complex food webs (Veldtman et al. 2011, Visser et al. 2013) linking trophic levels below and above them (Schoenrogge et al. 1995, Abrahamson 1997, Malinga et al. 2014) and can have either positive or negative effects on plant fitness (Weis et al. 1988, Fernandes and Negreiros 2001). Gall formers are also models for the study of both extended phenotypes (Crespi and Worobey 1998, Stone and Cook 1998, Bailey et al. 2009) and natural enemy community assembly (Sanver and Hawkins 2000, Stone and Schonrogge 2003, Hayward and Stone 2005).

Among the parasitic Hymenoptera, some taxa have evolved secondary phytophagy (Quicke 1997) and induce plant galls. The family Cynipidea (the gall wasps) is the most diverse group of hymenopteran gall formers, with at least 1,300 species (Ronquist 1999, Abe et al. 2007, Liljeblad et al. 2011). Gall wasps have unusual life cycles; upon oviposition, plant growth is manipulated to form morphologically distinct outgrowths of nutrient-rich plant tissue within which the immature insect feeds and develops (Rohfritsch 1992). While the adaptive nature and ecological significance of galls is debated (Miller et al. 2009), one popular hypothesis is that galls help the developing gall former avoid natural enemies (Price et al. 1987, Stone and Schonrogge 2003). Many Cynipid species also display heterogony (i.e., cyclical parthenogenesis) whereby sexual (gamic) and asexual (agamic) generations alternate to complete a single bivoltine life cycle (Folliot 1964, Crozier 1975,

Pujade-Villar et al. 2001). Individual gall-forming species induce galls on specific host plant tissue (e.g., leaf, stem, root, flower, bud) on a single hostplant species or group of closely related host plants. However, the alternating generations of heterogonic species often use different host plant tissue within the same plant. The galls produced by the alternating generations typically differ in morphology as well (Felt 1965, Stone and Cook 1998).

Despite the defensive role galls play in protecting gall formers from predation (Price et al. 1987, Hawkins 1988, Price and Pschorn-Walcher 1988, Stone and Schonrogge 2003), high levels of mortality among gall formers inflicted by a diverse and species-rich community of insect natural enemies is evident (Eliason and Potter 2001, Hood and Ott 2010, Joseph et al. 2011). Comparatively few studies, however, have provided detailed descriptions of the entire suite of natural enemies associated with specific gall-former species; or examined the community composition across the geographic range (e.g., Schonrogge et al. 1995, Menendez et al. 2008, Prior and Hellmann 2013). Less frequent still are studies that compare the natural enemy communities between generations of heterogonic gall formers (e.g., Evans 1967, Askew 1984, Schonrogge et al. 1995, Bird et al. 2013). The paucity of information for the natural enemy community associated with gall formers is particularly evident for North American Cynipids.

Here we characterize the community of insects associated with the alternating sexual and asexual generations of the gall former, *Belonocnema treatae* Mayr, living on live oaks in central and southeast Texas, USA. This community of associates is based on identifications of insects directly reared from galls produced by both gall-former generations and includes parasitoids of *B. treatae*, hyperparasitoids, associated inquilines, and possibly parasitoids of inquilines. Thus, this community is inclusive of *B. treatae* natural enemies (*sensu stricto*). Since adult *B. treatae* do not survive to emerge from galls from which any community associate has emerged (Lund 1998, Hall 2001) and because niche relations within galls for most of these species are not resolved, we refer to the entire community of associates as natural enemies (*sensu lato*).

To aid future research in the system, we provide photographs and mitochondrial cytochrome oxidase (mtCOI) sequence data for most species. We review known associations of the parasitoids in this community, and identify those that have previously been found in association with other gall formers versus those for which *B. treatae* is the only known host. The extent to which specific natural enemies specialize on particular gall-former species, or galls on specific plant parts, or act as generalists across many gall former species has yet to be comprehensively addressed across a sufficiently large number of taxa to test for generalities. The results of such a long-term goal have important implications for understanding coevolution between gall formers and their natural enemies (e.g., Abrahamson 1997).

Study System

Belonocnema is a North American genus of gall formers (Hymenoptera: Cynipidae: Cynipini), consisting of two described species: *B. treatae* and *B. quercusvirens* (Osten-Sacken 1861, Melika and Abrahamson 2002, Liljebblad et al. 2008). Lund et al. (1998) examined the sexual and asexual generations of *B. treatae* in Texas and established that a third species, *B. kinseyi*, (Weld 1921) was actually the asexual generation of *B. treatae*. Both generations of *B. treatae* are known from Texas to Florida (Lund et al. 1998, Egan et al. 2012a); however, *B. quercusvirens* is only known as an asexual

generation in Florida and Georgia (Melika and Abrahamson 2002). Whether *B. treatae* and *B. quercusvirens* are simply geographic populations or different generations of the same species as postulated by Weld (1921) is currently unresolved. *Belonocnema* overlap geographically and ecologically with many other gall-forming cynipid genera that use live oaks (genus *Quercus*, series Virentes), (Muller 1961) as hosts, including *Disbolcaspis*, *Andricus*, *Bassetia*, *Neuroterus*, and *Callirhytis*, and galls of these genera can be found on the same individual host trees and host organs (Felt 1965, Egan et al. 2013).

Based on our sampling in Texas, Louisiana, Mississippi, Alabama, and Florida, *Belonocnema treatae* appears to form locally adapted populations to individual host plant species along the Gulf Coast (Egan et al. 2012a,b, 2013; S.P.E. and J.R.O., unpublished data). Host plants include the plateau live oak, *Q. fusiformis*, in central Texas and Oklahoma, the southern live oak, *Q. virginiana*, in southeastern Texas and across the U.S. Gulf coast in mesic soils, and the sand live oak, *Q. geminata*, in a more restricted range across the Gulf coast in xeric soils (Cavender-Bares and Pahlisch 2009). Host plant associations of *B. treatae* with other live oaks (e.g., *Q. minima* in Florida, *Q. oleoides*, distributed from northern Mexico to Costa Rica, and *Q. sagraena* in western Cuba) are unknown. The present study focuses on the community of insects associated with the asexual and sexual generations of *B. treatae* for populations developing on two host plants: *Q. fusiformis* in central Texas and *Q. virginiana* in southeastern Texas.

The asexual generation of *B. treatae* develops within single-chambered leaf galls while the sexual generation develops within multichambered root galls (Lund et al. 1998; Fig. 1). Coincident with spring leaf flush, sexual generation females oviposit into young leaves from March through May (Hood and Ott 2010). Asexuals develop within the leaf galls through the summer, emerge from November through February, and oviposit into oak rootlets (Cryer 2003). Both asexual and sexual generations are attacked by parasitoids and inquilines. In addition, some *B. treatae* natural enemies are attacked by hyperparasitoids. Adult gall formers do not emerge from single-chambered leaf galls from which known inquiline species have emerged or from individual chambers within root galls from which any other insects emerge (Lund 1998, Hall 2001); thus, both parasitoids and inquilines result in mortality of *B. treatae* and function as direct and indirect natural enemies, respectively.

Published information about the natural enemies (*sensu stricto*) of gall formers in the genus *Belonocnema* is incomplete for several reasons: 1) sampling across the broad geographic range and diverse host associations is incomplete, 2) a general lack of available taxonomic information for many groups of hymenopteran parasitoids makes identification to the species level difficult, 3) uncertainty regarding the ecological role of organisms that emerge from galls (e.g., some inquiline species might move in and out of galls through emergence holes created by other species that previously developed within galls, creating false-positive counts of gall-former natural enemies, and some parasitoids may be developing in associated insects, not *B. treatae*). Despite these problems, some natural enemies have been described. Peck (1963) lists eight chalcid associates, all reared from *B. treatae* galls on *Quercus virginiana* in Florida. More recent work by Lund et al. (1998) lists 12 additional associates of *B. treatae* in central Texas. Herein, we use the collections of Lund et al. (1998), Hall (2001), and Cryer (2003), alongside additional collection efforts described below, to characterize the natural enemy community associated with both *B. treatae* leaf and root galls across these two host plant species in Texas.

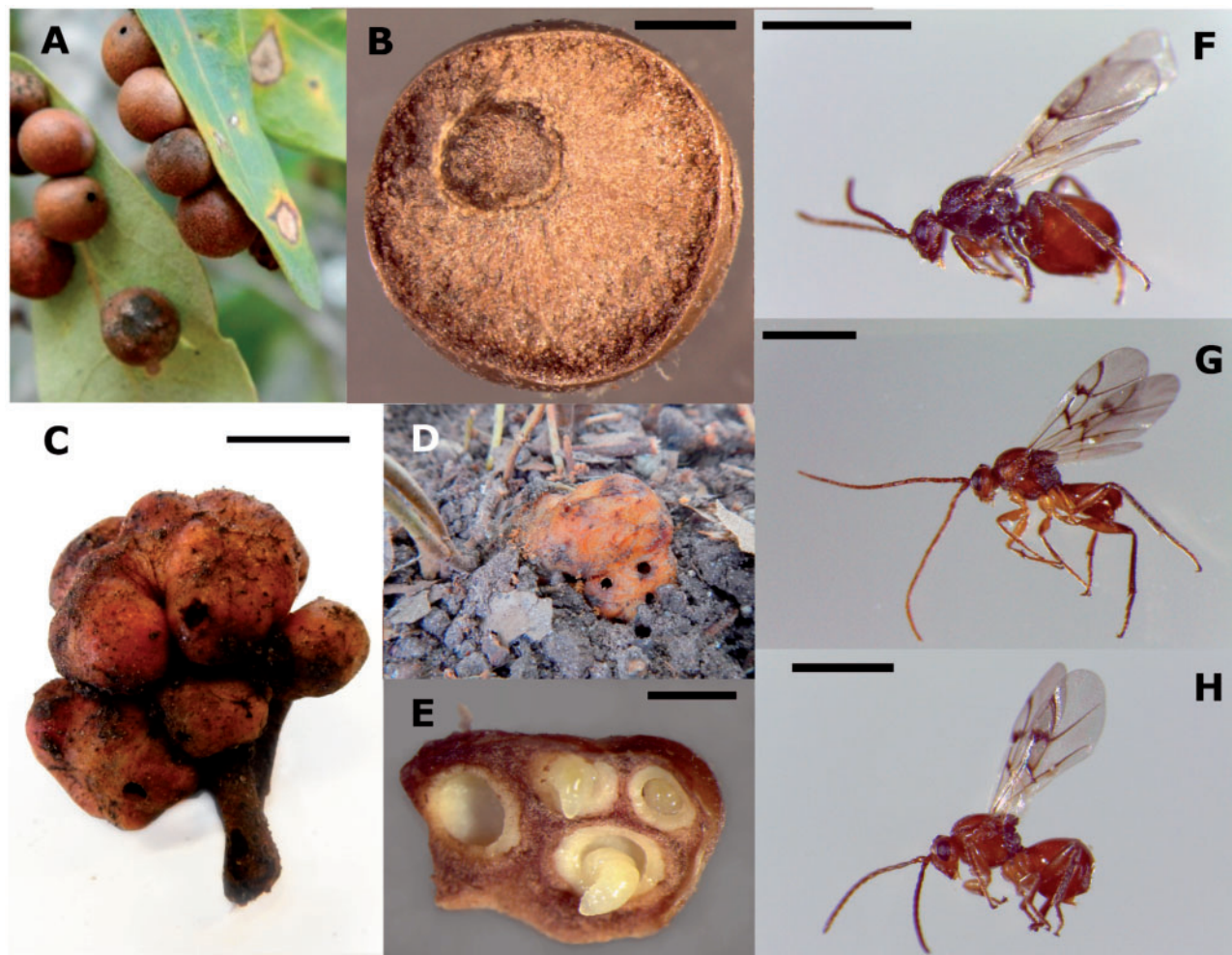


Fig. 1. Galls and adults of *B. treatae*. (A and B) Leaf galls, (C and D) Root galls, (E) Root gall section with larvae, (F–H) Asexual female, sexual male, sexual female; scale = 1 mm for all images in this and all subsequent figures.

Materials and Methods

Collections and Characterization of the Natural Enemy Community

We surveyed natural enemy communities associated with the galls produced by the sexual and asexual generations of *B. treatae* on two closely related live oak species: *Q. fusiformis* (central Texas) and *Q. virginiana* (southeastern Texas). Information presented here is based on studies conducted in multiple years at multiple sites. Galls were collected from natural host plant populations, plant stands along highways, and trees on the campus of Rice University (Table 1). The collection sites for galls on *Q. fusiformis* (Table 1) were typical of the Texas Hill Country, which is characterized as a live oak – juniper savannah, where live oaks occur as mottes interspersed with *Juniperus ashei*. The primary collection site for *B. treatae*-induced galls on *Q. virginiana* was the campus of Rice University, Houston, TX. Additional locales were distributed east of the Texas Hill Country.

Asexual Generation

In 1995–1996 and 1996–1997, we collected asexual generation leaf galls on *Q. fusiformis* bimonthly from late July to early March of the following year at Texas State University's Freeman Center, Hays County, Texas, USA (29° 55' N, 98° 00' W). In 2000 from

April–November we again collected leaf galls from this site and monitored the fate of individually stored galls (Hall 2001). In October 2014, we collected leaf galls from a natural population at the Devil's Backbone, Comal County, TX (29° 55'30.5" N, 98° 09'04.1" W), 12.5 miles from the Freeman Center. From August 2014 until January 2015, we collected leaf galls on *Q. virginiana* monthly on the campus of Rice University, Houston, TX (29° 43'03.4" N, 95° 24'06.5" W), supplemented with smaller collections southeast of Houston, TX, along highway 288 near Angleton, TX (29° 09'00.5" N, 95° 27'01.3" W), and west of Houston in Luling, TX (29° 40'31.7" N, 97° 38'08.6" W). For these latter three studies, leaf galls were bulk stored as described above. Collection of leaf galls across broad time intervals allowed us to capture species that use the galls during different stages of development (Hall 2001). Collection sites, dates, and gall numbers are summarized in Table 1.

We reared the occupants of leaf galls that were either stored individually in gelatin capsules or from bulk storage of leaf galls in ~0.95-liter jars fitted with collection tips or swept every 2 d with an aspirator. The gelatin capsule method definitively captures insect associates of *B. treatae*, and provides detailed information on the fates of galls as a function of associated insects, patterns of co-occurrence of insects, and species-specific phenology (to be reported elsewhere).

Table 1. Primary collections of *Belonocnema treatae* leaf (asexual generation) and root (sexual generation) galls examined in this study, with counts of natural enemies reared

Year	Location	Generation	No. of galls monitored	No. of natural enemies examined	Reference
1995	FR, Hays Co., TX	Asexual	2,658	783	Lund 1998
1996	FR, Hays Co., TX	Asexual	1,965	729	Lund 1998
2000	FR, Hays Co., TX	Asexual	21,690	957	Hall 2001
2014	DB, Comal Co., TX	Asexual	8,341	1171	This study
2014	Rice University, TX	Asexual	2,524	102	This study
2014	Luling (Hwy 288), TX	Asexual	1,460	355	This study
2014	Angleton, TX	Asexual	1,502	31	This study
2002	FR, Hays Co., TX	Sexual	^a	365	J.R.O., unpub.
2003	FR, Hays Co., TX	Sexual	913	668	Cryer 2003
2012	DB, Comal Co, TX	Sexual	85	331	J.R.O., unpub.
2015	Rice University, TX	Sexual	543	229	This study

^aParasitoids captured in collection traps placed over root galls attached to oak roots in the field.

Sexual Generation

Insect associates of *B. treatae* root galls developing on *Q. fusiformis* were monitored in six studies. In the springs of 1997, 1999, 2002, and 2003, root galls were collected at the Freeman Center, and in 2012, from the Devils Backbone site. Galls were collected each year just prior to emergence of adult gall formers and associated insects (galls containing penultimate stage *B. treatae* are recognizable by chamber size). Root galls were stored individually in *Drosophila* vials and monitored daily in each study with the exception that in 2002 emergent insects were collected in traps placed over rootlets of naturally occurring live oak heavily infested with root galls. In spring 2015, we collected root galls weekly at Rice University between March 1 and April 30. These galls were placed in bulk emergence traps. All emerged insects from galls of both generations were either frozen at -80°C or fixed in 95% ethanol for future morphological and genetic work. Collection sites, dates, and gall numbers are summarized in Table 1.

We sent representatives of unique specimens in 1995, 1997, and 2001 to the United States Department of Agriculture's (USDA) Systematic Entomology Laboratory (SEL) in Beltsville, MD, for identification and voucher. We also keyed out all Hymenoptera collected in 2014 and 2015 to superfamily using Mason (1993), then to genus using Gibson et al. (1997) [for Chalcidoidea] or Townes (1969) [for Ichneumonoidea]. Where keys existed (see individual species listings in results section for keys used), we also determined species names. Cynipid inquilines (*Synergus*) were not identified below the genus level, as no appropriate keys were available. Representatives of most species were mounted on card points and photographed using a Canon EOS 60D camera with a Canon MP-E 65 mm macro lens and a Canon Macro Ring Lite MR-14EX (Canon USA, Melville, NY), mounted on a StackShot Automated Focus Stacking Macro Rail (Cognysis Inc., Traverse City, MI). Stacked images were processed using Zerene Stacker (Zerene Systems LLC., Richland, WA) and Adobe Photoshop (Adobe Systems Inc., San Jose, CA). Wings of the same species were removed, placed on temporary slides, and photographed using a Leica M125 stereomicroscope (Leica Inc., Switzerland).

When samples were of sufficient quality for genetic work, we complemented morphological taxonomy with molecular barcodes. Barcodes can reveal cryptic species and circumvent inadequacies in taxonomic resources that may exist for certain genera (e.g., Smith et al. 2008, 2012), and are therefore useful for the study of diverse natural enemy communities (Fernandez-Triana et al. 2011, Forbes

and Funk 2013, Hrcek et al. 2013). Genomic DNA of a subset of individuals from each species in the 2014 collection was extracted using DNeasy Blood and Tissue kits (Qiagen Inc., Valencia, CA). We used the "universal" primers LepF1 (ATTCAACCAATCATAAA GATATTGG) and LepR1 (TAAACTTCTGGATGTCCAAAAA TCA) or RonII (GGAACIGGATGAACAGTTTACCCICC) and MLepR1 (CCTGTTCAGCTCCATTT) to amplify a segment of the mitochondrial cytochrome oxidase (mtCOI) gene using standard PCR protocols (Smith et al. 2008). We treated amplified fragments with Exonuclease I (New England Biolabs, Ipswich, MA) and Shrimp Alkaline Phosphatase (Fermentas Life Sciences, Glen Burnie, MD) and sequenced in both forward and reverse directions on an ABI 3730 DNA analyzer using BigDye 3.1 sequencing chemistry (ThermoFisher Inc., Waltham, MA). We edited raw sequences and assembled forward and reverse reads using Geneious v.6.1.8 (Kearse et al. 2012). Final sequences varied in length from 295–685 bp, a function of amplification and sequencing success, which were highly variable across the diverse set of study taxa. We ran each sequence through the "identification request" module on the Barcode of Life Database (BOLD; Ratnasingham and Hebert 2007) to identify the highest percentage matches from previously identified taxa. All sequences were deposited in GenBank (accession numbers provided below).

Results

Community Associates of *B. treatae*

A total of 25 insect species were reared from the leaf and root galls produced by *B. treatae* on *Q. fusiformis* in central Texas and *Q. virginiana* in southeastern Texas: Hymenoptera (21), Coleoptera (2), Diptera (1), Lepidoptera (1); (Table 2). Included among these community associates are parasitoids of *B. treatae*, hyperparasitoids, inquilines, and likely the parasitoids of inquilines. Collections are summarized below beginning with the most diverse order, the Hymenoptera. Species are reported alphabetically by superfamily, family, subfamily, and genus within each order.

Hymenoptera

Galeopsomyia nigrocyanea (Ashmead) (Hymenoptera: Chalcidoidea: Eulophidae: Tetrastichinae; Fig. 2A) was reared from four of the five leaf gall collections (Table 2). This species was identified by the SEL, and we corroborated the identification in 2014

Table 2. Natural enemies of *Belonocnema treatae* in leaf and root galls of *Quercus fusiformis* (Qf) and *Quercus virginiana* (Qv) in central and southeastern Texas

Family within order	Species or subfamily	Leaf galls (Asexual generation)					Root galls (Sexual generation)					
		'95 Qf	'96 Qf	'00 Qf	'14 Qf	'14 Qv	'97 Qf	'99 Qf	'02 Qf	'03 Qf	'12 Qf	'15 Qv
Coleoptera												
Anobiidae	<i>Tricorynus</i> sp.			81	54	3						
Curculionidae	<i>Conotrachelus juglandis</i>					1			2	3		6
Hymenoptera												
Eulophidae	<i>Galeopsomyia nigrocyanea</i>		98	10	644	22						
Eupelmidae	<i>Brasema</i> sp.1	54	62	197	121	6						
Eupelmidae	<i>Brasema</i> sp.2	25	33	14	7							
Eupelmidae	<i>Eupelmus cushmani</i>				1							
Eurytomidae	<i>Eurytoma</i> (3 species)	52	36	23	302	10						
Eurytomidae	<i>Sycophila texana</i>	67	53	35	6	43						
Eurytomidae	<i>Sycophila varians</i>			83	11	8						
Eurytomidae	<i>Sycophila dorsalis</i>			1		2						
Ormyridae	<i>Ormyrus labotus</i>	26	20	73	2	19						
Pteromalidae	<i>Acaenacis lausus</i>	54	10	10		20						
Pteromalidae	<i>Anisopteromalus</i> sp.			2								
Pteromalidae	<i>Cecidostiba</i> sp.			3				200	70			16
Torymidae	<i>Torymus tubicola</i>	46	61	69	52	38						
Torymidae	<i>Torymus fullawayi</i>						28	234	287	659	331	14
Bethylidae	<i>Goniozus</i> sp.				1							
Cynipidae	<i>Synergus</i> (3 species)	440	329	228	244	29						
Braconidae	<i>Allorhogas</i> sp.	19	27	124	13	2						
Diptera												
Cecidomyiidae	Cecidomyiinae			1	17	17						
Lepidoptera												
Gelechiidae				3				1	6	3		

Totals in some columns may be less than the totals in Table 1, reflecting individuals that were collected but were not available to ID.

using a key by Schauff et al. (1997) and the original description by Ashmead (1886). Five COI sequences were 99–100% identical to one another and 90.5% identical to an existing but unnamed eulophid sequence on BOLD (Supp. Table 1 [online only]). *G. nigrocyanea* is a known associate of *B. treatae* in Florida (Peck 1963). Other *Galeopsomyia* wasps have been reared as hyperparasitoids of wasp genera found in the *B. treatae* community, including species of *Eurytoma* (Herting 1977) and *Torymus* (Stiling and Rossi 1994), suggesting that *G. nigrocyanea* may be a hyperparasitoid in the *B. treatae* system.

Brasema sp.1 (Hymenoptera: Chalcidoidea: Eupelmidae: Eupelminae; Fig. 2B) were reared from all five leaf gall collections (Table 2).

Brasema sp.2 (Hymenoptera: Chalcidoidea: Eupelmidae: Eupelminae; Fig. 2C) were reared from the four *Q. fusiformis* leaf gall collections but not from the *Q. virginiana* leaf gall collection (Table 2).

Brasema wasps were identified to genus using Gibson (1995), but no species name was resolved for either. Identification by the SEL split *Brasema* sp. into three species, one exclusively female with yellow bodies, another exclusively male with brown bodies, and a third brown species with both sexes. MtCOI sequences from 2014 samples of the two unisexual “species” were identical. As wasps in subfamily Eupelminae typically show extreme sexual dimorphism (Gibson 2011), we have collapsed these into a single species. Eight mtCOI sequences, including six from a supplemental collection made in Louisiana, were obtained from 2014 collections of *Brasema* sp. 1 (Supp. Table 1 [online only]). These sequences were 99.7–100% identical to one another and were a 91.1% match to an

existing sequence on BOLD from an unnamed eupelmid. Two mtCOI sequences from *Brasema* sp. 2 were obtained and were 91.2% identical to an existing *Brasema* species on BOLD. Sequences from *Brasema* sp.1 and *Brasema* sp.2 were 88–89% identical. *Brasema* and its putative sister genus *Eupelmus* are typically primary or secondary parasitoids of larval or pupal holometabolous insects (Gibson 1997), but the role of the *Brasema* species reared from *B. treatae* galls is currently unknown. Wasps in the genus *Brasema* have been reared from galls of 13 cynipid wasp species, as well as from Diptera, Hemiptera, Eurytomidae (as a secondary parasitoid), Lepidoptera, and Orthoptera (Noyes 2015).

A single male *Eupelmus cushmani* (Crawford) (Hymenoptera: Chalcidoidea: Eupelmidae: Eupelminae; no figure) was reared from a *Q. fusiformis* leaf gall collected in 2014 (Table 2). A mtCOI sequence was 99.2% identical to an existing sequence on BOLD. *E. cushmani* is considered a broad generalist, attacking a wide breadth of hosts from across seven insect orders (Gibson 2011).

Three species of *Eurytoma* (Hymenoptera: Chalcidoidea: Eurytomidae: Eurytominae; Fig. 3A) were reared from all five leaf gall collections (Table 2). *Eurytoma* species were identified by the SEL and we corroborated this identification using a key by Bugbee (1967). The three species, *Eurytoma bugbeeii* Grissell, *Eurytoma furva* Bugbee, and *Eurytoma* sp. can be distinguished from one another primarily by antennal morphology, but because antennae were often damaged, the above counts of the three species are lumped together. Three mtCOI sequences were collected from *E. furva* and one from *E. bugbeeii*. No samples of *E. sp.* were of DNA quality. *Eurytoma furva* and *E. bugbeeii* sequences were 88.7% and 91.8% similar to *Eurytoma* sequences on BOLD. *Eurytoma bugbeeii* has

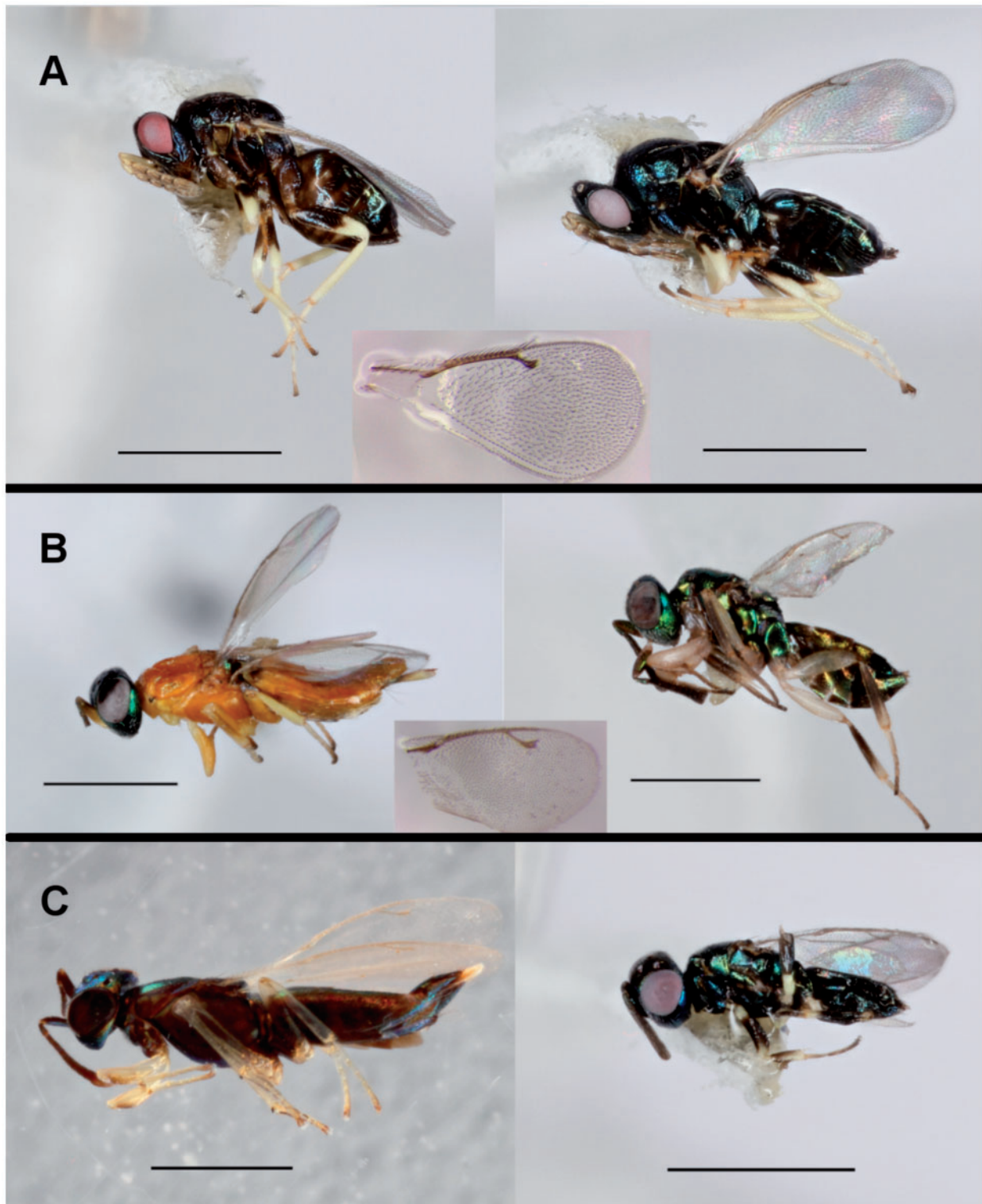


Fig. 2. (A) Female (left) and male (right) *Galeopsomyia nigrocyanea* reared from *B. treatae* leaf galls. Inset: detail of female forewing. (B) *Brasema* sp. 1 female (left) and male (right) reared from *B. treatae* leaf galls. Inset: detail of female forewing. (C) *Brasema* sp. 2 female (left) and male (right) reared from *B. treatae* leaf galls.

been previously reared from galls induced by three species of the cynipid genus *Dishlocaspis* and from galls of another cynipid, *Andricus kingi*, all found on oaks (Noyes 2015). The only recorded host of *E. furva* is the cynipid gallformer *Dishlocaspis washingtonensis* on *Quercus douglassii* (Bugbee 1967).

Three species of *Sycophila* (Hymenoptera: Chalcidoidea: Eurytomidae: Eurytominae; Fig. 3B) were reared from leaf gall collections (Table 2). *Sycophila* were identified to genus by the SEL, and we keyed them to species using a key by Balduf (1932). The three species, *Sycophila texana* (Balduf), *Sycophila varians* (Walsh),

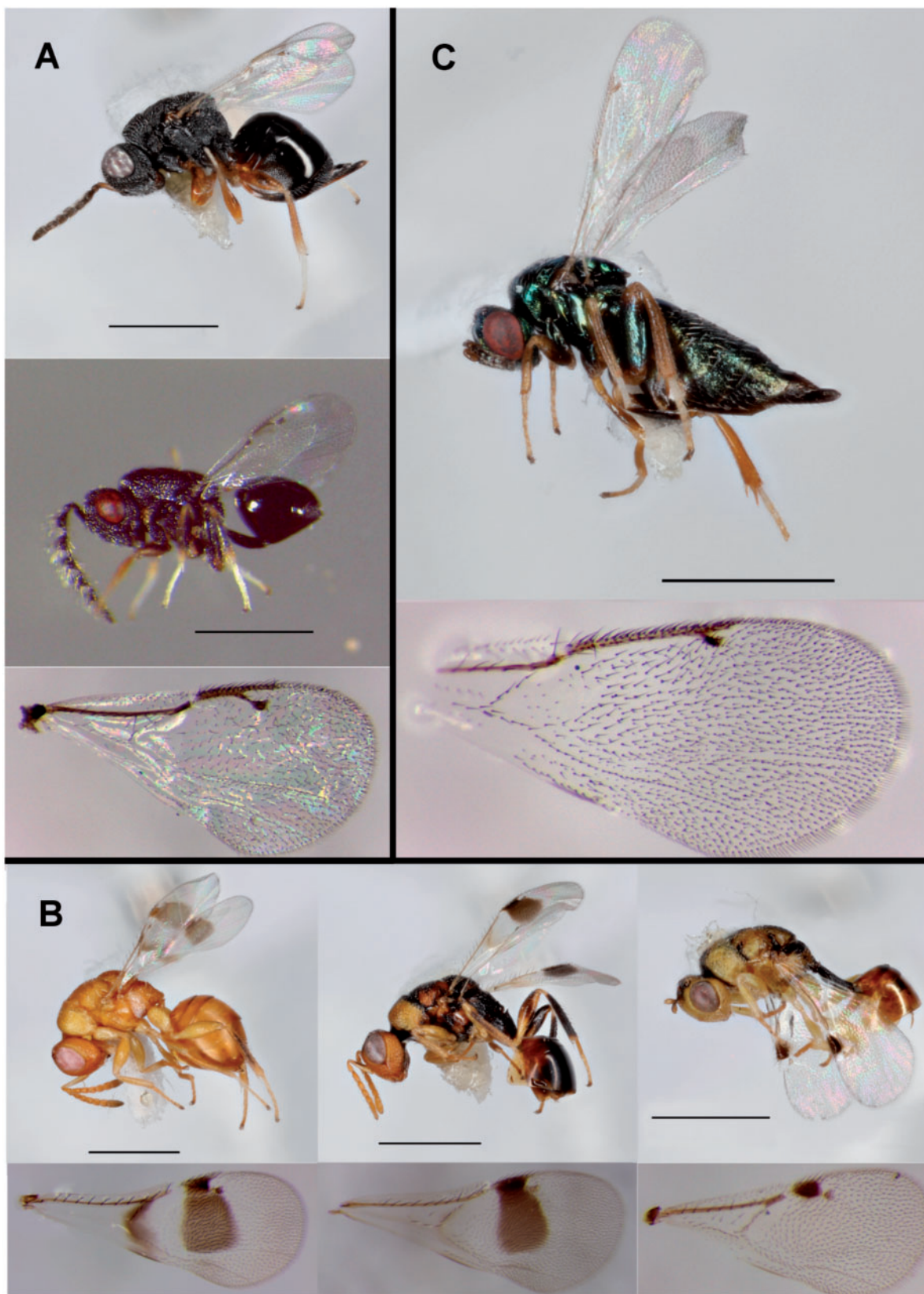


Fig. 3. (A) *Eurytoma bugbeeii*, female (top) and male (middle) reared from *B. treatae* leaf galls. Bottom: detail of female wing. (B) *Sycophila texana* female (left), *Sycophila varians* male (middle), and *Sycophila dorsalis* male (right) reared from *B. treatae* leaf galls. Details of forewings shown below each species. (C) Female *Ormyrus labotus* reared from *B. treatae* leaf galls. Includes detail of forewing.

and *Sycophila dorsalis* (Fitch) can be readily distinguished from one another by their coloration and wing markings. We obtained three mtCOI sequences, two from *S. texana* and one from *S. varians* (Supp. Table 1 [online only]). Sequences from *S. texana* and *S. varians* differed by ~8%. The *S. texana* COI sequences were 91.5% identical to a *Sycophila* sp. sequence on BOLD. *S. texana* was reared from all five leaf gall collections. No known hosts have been reported for *S. texana* (Balduf 1932, Noyes 2015), but a morphologically similar species, *Sycophila flava*, has been reared from galls of 14 cynipid species across seven genera (Noyes 2015). *S. varians* was reared from three leaf gall collections. *S. varians* has previously been reared from galls of 17 species of cynipid gall wasps across eight genera (Noyes 2015). Tree associates are all oaks: *Q. alba*, *Q. chrysolepis*, *Q. phellos*, and *Q. virginiana* (Noyes 2015). This appears to be the first record from *B. treatae* (Noyes 2015). *S. dorsalis* was reared from two leaf gall collections. *S. dorsalis* has been reared from cynipid galls across six genera, but there are no previous records from *B. treatae*.

Ormyrus labotus Walker (Hymenoptera: Chalcidoidea: Ormyridae: Ormyrinae; Fig. 3C) was reared from all five leaf gall collections (Table 2). *O. labotus* was identified to species by the SEL, and we corroborated this identification using a key by Hanson (1992). Four COI sequences were obtained from the 2014 sample (Supp. Table 1 [online only]). These sequences were 98.5–99.3% similar to one another, and one was a 93.3% match with an existing *O. labotus* sequence on BOLD. *O. labotus* has been associated previously with 16 species of Cynipidae on four species of *Quercus*, while another record associates *O. labotus* with galls on plants of genus *Rubus* (Burks 1979). A record of *Ormyrus labotus* attacking the gall wasp *Acraspis hirta* on *Quercus prinus* lists a *Eurytoma* wasp as a hyperparasitoid (Washburn and Cornell 1979).

Acaenacis lausius (Walker) (Hymenoptera: Chalcidoidea: Pteromalidae: Pteromalinae; Fig. 4A) was reared from four of the five leaf gall collections (Table 2). This species was identified by the SEL, and we corroborated the identification in 2014 using a key by Boucek and Heydon (1997) and a description by Ashmead (1885). Five COI sequences were 99.6–100% identical to one another and 90.5% identical to an existing but unnamed pteromalid sequence on BOLD (Supp. Table 1 [online only]). *Acaenacis* are known only as parasitoids of cynipid gall wasps in oaks. *Acaenacis lausius*, in particular, has previously been reared from *Dishlocaspis quercusvirens* on *Q. virginiana* in Florida (Bird et al. 2013) and from *Cynips ficigera* (association unknown; Ashmead 1885), while another species, *A. agrili*, is known as a parasitoid of *Agrilus angelicus* on *Q. agrifolia* in California (Rohwer 1919). The only other species currently in the genus, *A. tacti*, has no associated host record (Noyes 2015).

Anisopteromalus sp. (Hymenoptera: Chalcidoidea: Pteromalidae: Pteromalinae; no figure) was reared from one leaf gall collection (Table 2). This species was identified by the SEL, but not independently verified in this study. *Anisopteromalus* is not previously known as an associate of galls or gall-forming insects; most hosts are bruchid beetles (Noyes 2015). This wasp may be a parasitoid of a beetle inquiline with which it co-occurred (see below). Alternatively, a recent revision of this genus removed some species from *Anisopteromalus* and placed them in sister genera (Baur et al. 2014). Species in one of these genera, *Cyrtoptyx*, are quite often associated with Cynipid wasps, so it is possible that our *Anisopteromalus* species belongs to *Cyrtoptyx* or a different genus more commonly associated with gall wasps.

Cecidostiba sp. (Hymenoptera: Chalcidoidea: Pteromalidae: Pteromalinae; Fig. 4B) was reared from one leaf gall collection and from three root galls collections (Table 2). The SEL identified

samples from leaf galls as members of genus *Cecidostiba*, and we identified root gall samples using a key by Boucek and Heydon (1997). *Cecidostiba* is primarily a Palearctic genus (Nieves-Aldrey and Askew 1988) and currently has no named species in North America (Noyes 2015), though sequences on BOLD representing this genus have a Canadian origin. A mtCOI sequence from an individual reared from a root gall was 88.2–87.4% identical to existing Pteromalidae sequences on BOLD. European *Cecidostiba* have been frequently associated with cynipid galls (Boucek and Heydon 1997).

Torymus tubicola (Osten-Sacken) (Hymenoptera: Chalcidoidea: Torymidae: Toryminae; Fig. 4C) was reared from all five leaf gall collections (Table 2). This species was identified by the SEL and corroborated by us using a key by Grissell (1976). Seven mtCOI sequences were obtained from the 2014 sample (Supp. Table 1 [online only]). These sequences were 98.3–99.7% similar, and were 89.8–90.9% matches to existing sequences on BOLD from unnamed wasps in family Torymidae. *T. tubicola* has been reared previously from galls of 61 cynipid species, across 16 genera (Noyes 2015). One previous record mentions *B. treatae* as a host (Grissell 1979, using the synonym *Belonocnema kinseyi*). Oaks are the only known plant associates (Noyes 2015).

Torymus fullawayi (Huber) (Hymenoptera: Chalcidoidea: Torymidae: Toryminae; Fig. 4D) was reared from all six root gall collections (Table 2). The species was keyed to species using Grissell (1976). A mtCOI sequence was 94.0% similar to an existing *Torymus* sequence on BOLD. MtDNA sequences from *T. fullawayi* and *T. tubicola* were >10% different, indicating that each species is restricted to parasitizing a single generation of *B. treatae*. *T. fullawayi* has been previously associated with woody stem galls of cynipid wasps on oaks and willows in California.

A single *Goniozus* sp. (Hymenoptera Chrysoidea: Bethylinae: Bethylinae; Fig. 5A) was reared from a *Q. fusiformis* leaf gall in 2014. We keyed this individual to genus using Polaszek and Krombein (1994). A mtCOI sequence was 90.6% similar to an existing *Goniozus* sequence on BOLD. This is likely a parasite of the microlepidopteran inquiline described below. Most members of subfamily Bethylinae parasitize lepidopteran larvae (Polaszek and Krombein 1994), and a previous record associates *Goniozus gallicola* with *Cydia latiferreans* (Lepidoptera: Tortricidae) found in galls of *Cynips maculipennis* (Hymenoptera: Cynipidae) on oaks in Oregon (Fouts 1942).

Three species of *Synergus* (Hymenoptera: Cynipoidea: Cynipidae: Synerginae; Fig. 5B) were reared from all five leaf gall collections (Table 2). These species were identified to genus by K. Schick, Essig Museum of Entomology, University of California Berkeley. Seven COI sequences representing all three of the species were obtained from the 2014 *Synergus* samples (Supp. Table 1 [online only]). Sequences from the three *Synergus* species were 7–12% different from each other. Sequences from *Synergus* sp. 1 were 96.9% similar to a sequence on BOLD identified as a species of *Dryocosmus* (Hymenoptera: Cynipidae), a gall-forming wasp. Sequences from *Synergus* sp. 2 were 95.4% similar to a sequence on BOLD from an unidentified cynipid, and 88.4% similar to an existing *Synergus* sequence. Sequences from *Synergus* sp. 3 were 98.8% similar to an unidentified hymenopteran sequence on BOLD, and 95.3% similar to an existing *Synergus* species. *Synergus* are almost always inquilines, and known host associations are primarily the galls of oak gallwasps in tribe Cynipini, including *Andricus quercusradicis*, *Neuroterus numismalis*, and *Biorbiza pallida* (Ronquist 1994).

Allorhogas sp. (Hymenoptera: Ichneumonoidea: Braconidae: Doryctinae; Fig. 5C) was reared from all five leaf gall collections

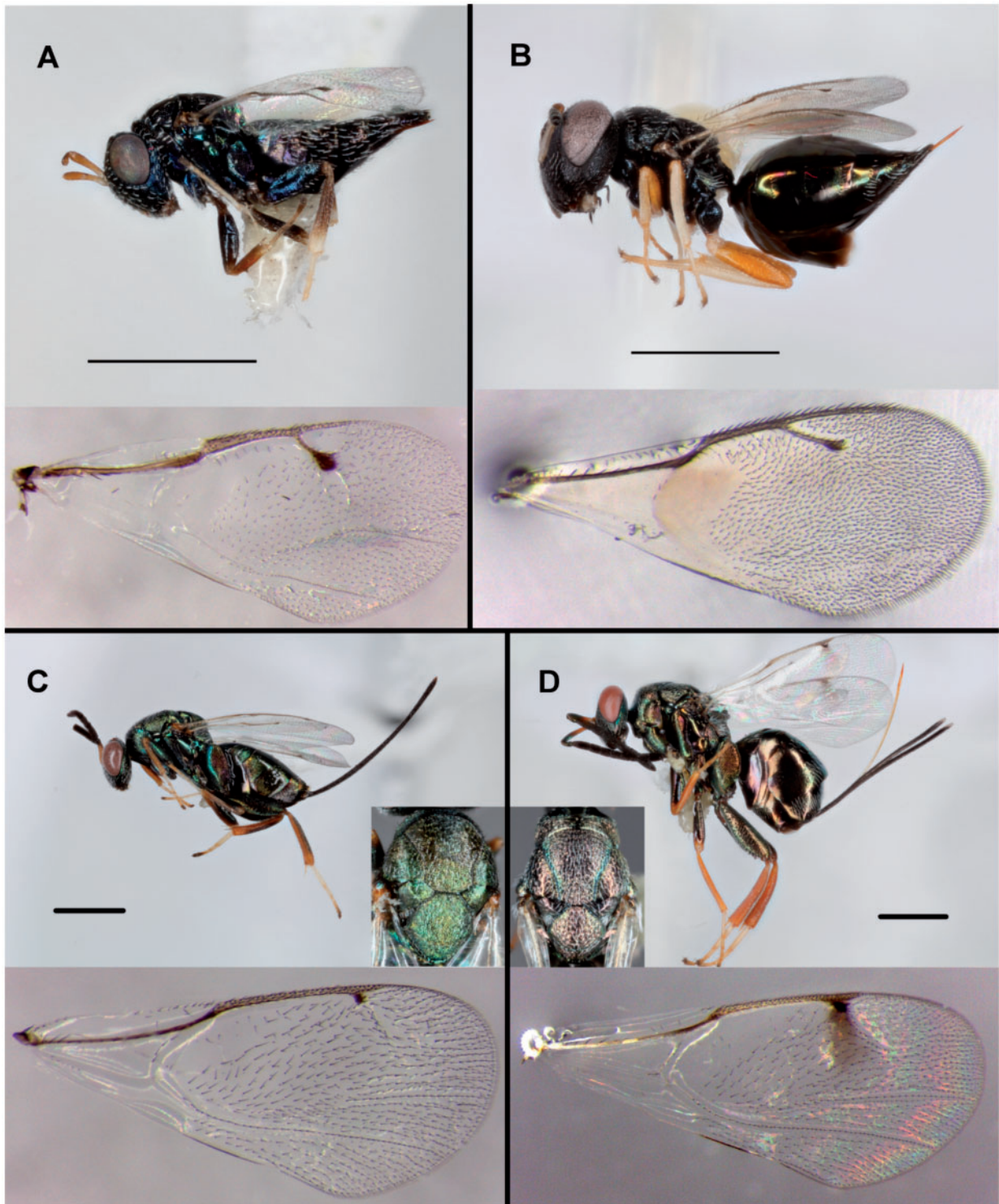


Fig. 4. (A) Female *Acaenacis lausus* reared from *B. treatae* leaf galls. Includes detail of forewing. (B) Female *Cecidostiba* sp. reared from *B. treatae* root galls. Includes detail of forewing. (C) Female *Torymus tubicola* reared from leaf galls with detail of scutum (inset) and forewing. (D) *Torymus fullawayi* reared from root galls (right), with detail of scutum (inset) and forewing.

(Table 2). *Allorhogas* sp. was identified by the SEL, and we corroborated this identification using a key by Marsh (1993). A mtCOI sequence was procured from a single *Allorhogas* (Supp. Table 1 [online only]). This sequence was a 89.4% match to an existing *Allorhogas* sp. sequence on BOLD. Another *Allorhogas* species, *A. gallicola*, has been reared from twig galls infested with the gall inquiline *Synanthedon*

(Lepidoptera: Sesiidae) on *Quercus pinifolia* in Maryland, and from twig-galls in another collection from Arizona (Gahan 1912).

Coleoptera

Tricorynus sp. (Coleoptera: Bostrichoidea: Anobiidae: Mesocoelopodinae; Fig. 6A) was reared from three leaf gall

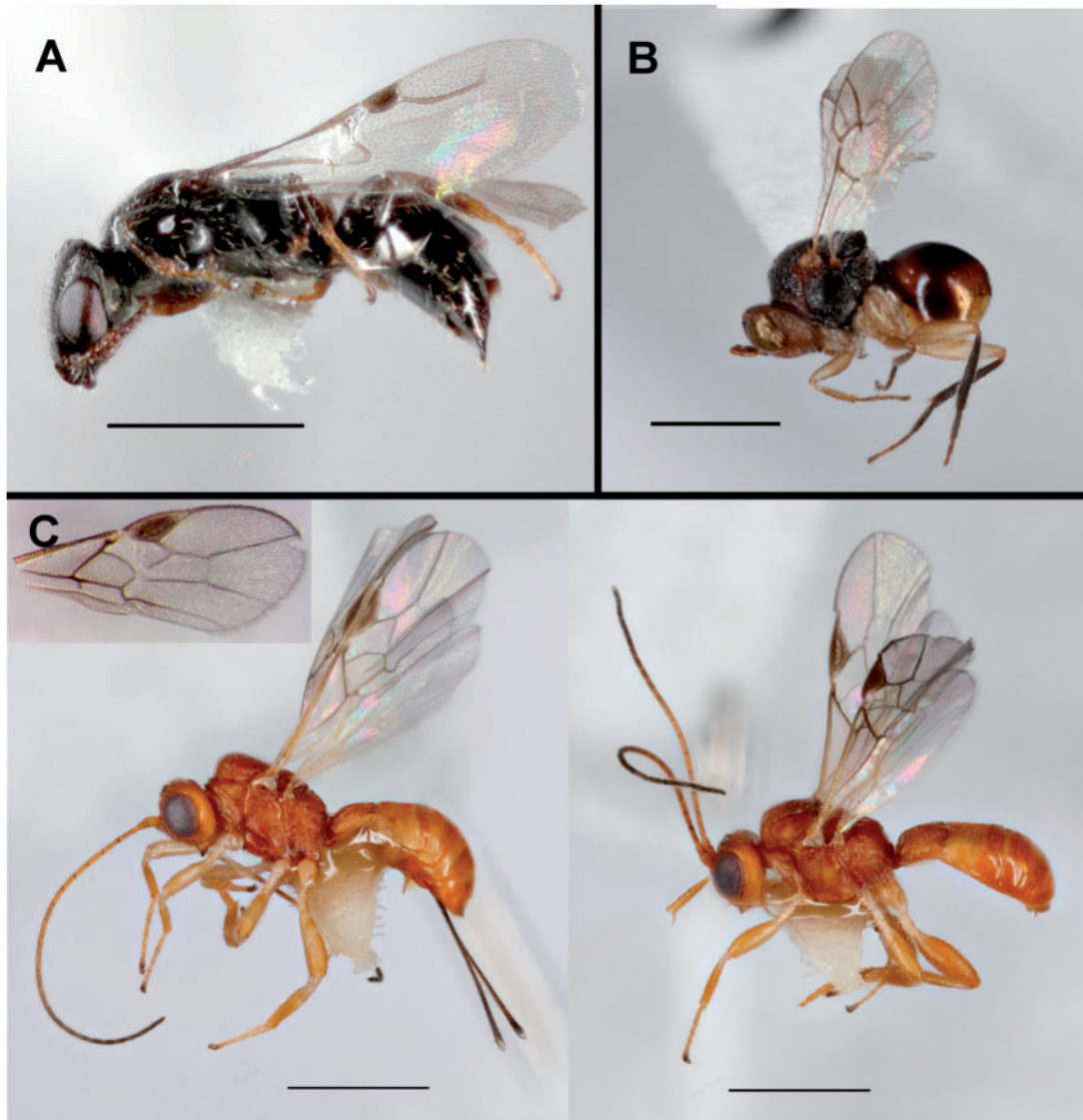


Fig. 5. (A) Female *Goniozus* sp. reared from a *B. treatae* leaf gall. (B) Female *Synergus* reared from a *B. treatae* leaf gall. (C) Female (left) and male (right) *Allorhogas* sp. reared from *B. treatae* leaf galls. Includes detail of forewing (inset).

collections (Table 2). Tentative identification of this beetle as *Tricorynus moderatus* White was provided by the SEL, but our own morphological identification using a key by White (1963) was inconclusive. We are hesitant to definitively refer to our sample as *T. moderatus*, because while other *Tricorynus* species have been reared from oak galls, *T. moderatus* has been previously reared only from fungi (White 1963). A mtCOI sequence was 98.4% identical to an existing Ptinidae (\approx Anobiidae) sequence on BOLD.

The butternut curculio, *Conotrachelus juglandis* LeConte (Coleoptera: Curculionidae: Curculionidae: Molytinae; no figure) was reared from one leaf gall collection and three root gall collections (Table 2). A mtCOI sequence from a root gall-associated individual was 100% identical to a *C. juglandis* sequence on BOLD. A mtCOI sequence from the leaf gall-associated larval individual was 93.7% identical to another *C. juglandis* sequence on BOLD. MtCOI sequences from the leaf and root gall-associated *C. juglandis* were 7.2% different, including one nonsynonymous substitution. It is possible, based on the sequence differences between the leaf- and root-gall associated individuals, that these represent two different

species. *C. juglandis* is a common associate of walnut trees and their relatives, but other species are associated with *Quercus*, and the genus is in need of revision (Arnett et al. 2002).

Diptera

An unidentified gall midge in subfamily Cecidomyiinae (Diptera: Sciaroidea: Cecidomyiidae; Fig. 6B) was reared from three leaf gall collections. A mtCOI sequence was 91.5% identical to an existing cecidomyiid sequence on BOLD. We were not able to key these specimens out to genus, but the closest named genus on BOLD was *Ozirhincus* (91.0%). Gall midges are best known as gall formers, but some are known to be inquilines in galls of other insects, including cynipids on oaks (Mamaev and Krivosheina 1992).

Lepidoptera

An unidentified member of the family Gelechiidae (Lepidoptera: Gelechioidea: Gelechiidae Fig. 6C) was reared from one leaf gall collection and three root gall collections (Table 2). A mtCOI sequence

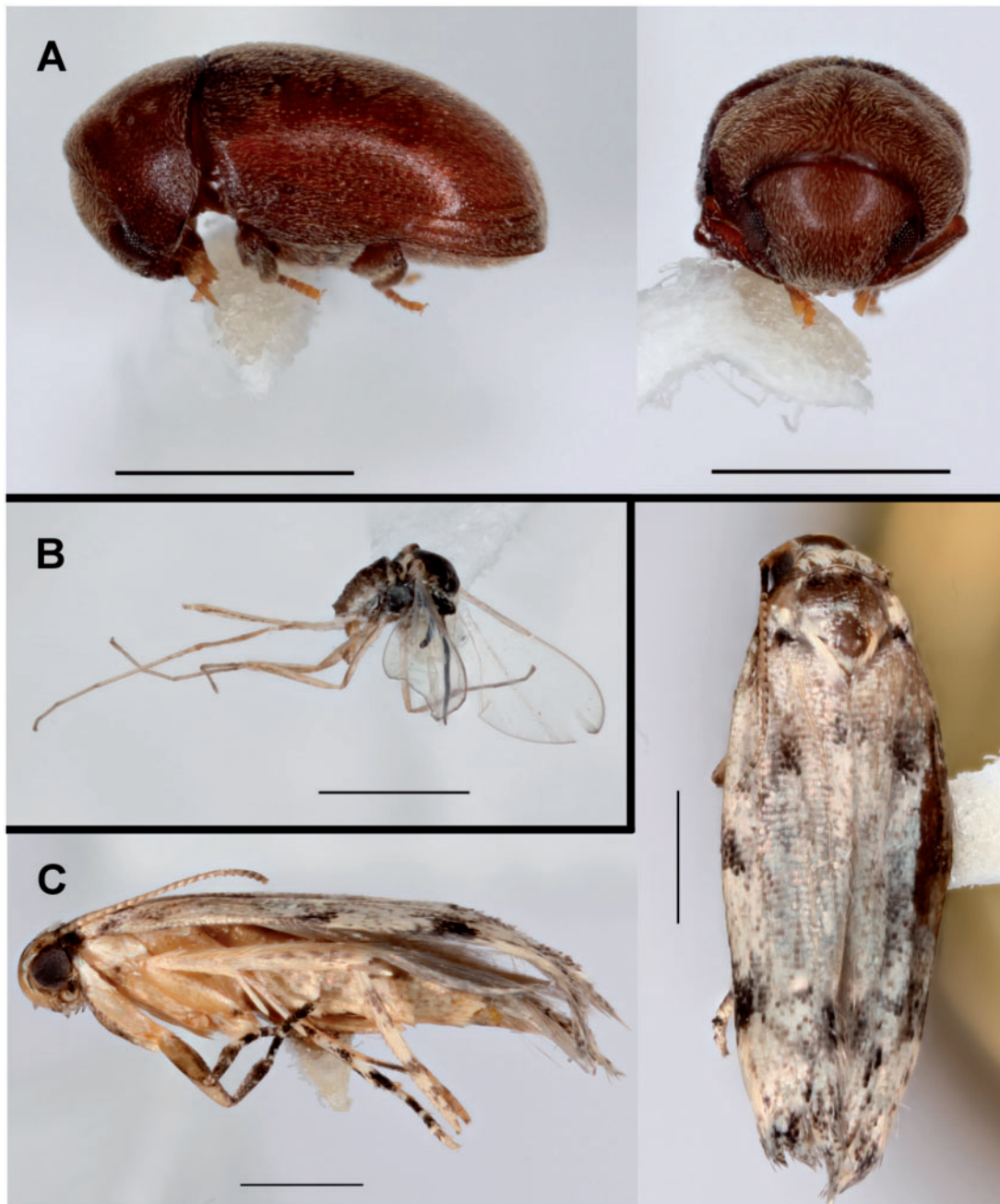


Fig. 6. (A) *Tricorynus* sp. reared from *B. treatae* root galls. (B) Cecidomyiinae fly reared from a *B. treatae* leaf gall. (C) Gelichid moth reared from a *B. treatae* leaf gall.

was 100% identical to an unnamed moth in family Gelechiidae. The two genera closest in sequence to our sample were *Simoe* and *Coleotechnites*. One species of *Coleotechnites* (*C. quercivorella*) has been previously found in association with oaks (Lee and Brown 2008).

Discussion

The community of insects associated with the leaf and root galls within which the asexual and sexual generations of *B. treatae* each develop is both species-rich, with at least 25 species, and phylogenetically diverse, with representatives from 10 hymenopteran families and three additional insect orders. Such diversity may be typical

of oak gall systems (Askew 1980, 1984), although to date too few oak galling cynipid species have been comprehensively studied to be definitive. The diversity we have documented is likely a minimum estimate of the number of insect associates of *B. treatae* given its geographic range and host range: we sampled only two host plant species in only a portion of the combined geographic range of the oak species on which *B. treatae* can develop.

The diversity of natural enemies associated with leaf galls (24 species overall including 20 species of Hymenoptera) greatly exceeded the diversity (4 species) associated with the root galls. Moreover, of the species reared from root galls, Hymenoptera (2), Coleoptera (1), Lepidoptera (1), only the two hymenoptera, *T. fullawayi* and *Cecidostiba* sp., were common. *T. fullawayi* is also the

primary source of mortality (from insect natural enemies) within root galls of the sexual generation (Cryer 2003). The curculionid beetle, *C. juglandis*, a pteromalid wasp, *Cecidostiba* sp., and the unnamed gelechiid moth were found in galls of both generations. These species were reared out of individually isolated leaf and root galls so despite their rarity in one or both generations, their occurrence is definitive. The degree to which these species are genuinely rare or whether their rarity could reflect the timing of sample collections relative to timing of their attack is unknown.

A long-standing hypothesis for the evolution of heterogony is that alternate generations have evolved as a means to escape parasitism (Askew 1984, Stone et al. 2002). Under this scenario, the derived generation experiences lower rates of attack from natural enemies (both in total mortality and the number of species that pose threats). Evidence of asymmetries in natural enemy-induced mortality between generations is available for *B. treatae* (Hood and Ott 2010) and other heterogonic cynids (Eliason and Potter 2001). Hayward and Stone (2005) further suggested that imbalances between natural enemy communities should even out with time, making it difficult to assess the validity of this hypothesis without an estimate of the age of the derived generation. In this context, our results demonstrating the striking asymmetry in diversity of natural enemies between generations is especially noteworthy since *B. treatae* represents a basal group within the cynipid clade (Liljeblad et al. 2008). Thus, relative to other members of the group, a comparatively long period of time has been available for equilibration of natural enemy communities to have been achieved between the generations of this heterogonic species.

If the relative age of the derived generation cannot explain differences in the natural enemy diversity of the natural enemies associated with the galls of the asexual and sexual generations of *B. treatae*, perhaps we can look to other differences for answers. The alternate generations develop in galls that differ dramatically in: 1) timing of oviposition (asexuals oviposit in the fall to initiate the sexual generation phase of the life cycle while sexuals oviposit in the spring to begin the asexual phase); 2) onset of gall growth (sexual generation galls delay visible growth until late winter, asexuals galls initiate visible growth in June); 3) the development period (i.e., the time from oviposition to adult emergence; sexual generation: 5 mo total, 2 mo visibly growing; asexual generation: 8 mo total, 5 mo visibly growing); 4) accessibility: asexual galls are apparent on leaves, sexual galls are buried underground on roots; and 5) structure: asexual galls are single-chambered; sexual galls are multichambered. Any or all of these factors could influence susceptibility to parasitism and the accumulation of natural enemies as shown in other gall formers (Askew 1984, Weis et al. 1988)—in this case limiting expansion of the natural enemy community associated with sexual generation root galls.

While the natural enemy communities of root and leaf galls differed considerably, the communities associated with *B. treatae* on *Q. fusiformis* and *Q. virginiana* among the sites sampled in Texas did not appear to be substantially different. The 2014 collections from *Q. virginiana* and *Q. fusiformis* shared 15 species, including all of the common community members. One caveat is that we only sequenced mtCOI in both tree habitats for three species, so some morphological species may actually be different between the two trees, but cryptic. For the three species where COI allowed us to assess this possibility (*T. tubicola*, *Brasema* sp. and *Synergus* sp.3), we found no sequence divergence indicative of cryptic diversity. However, another study that used mtDNA to barcode individuals across a cynipid gall wasp community did find evidence for cryptic

diversity (including among wasps of genus *Torymus*; Lima 2012), so further investigation may be warranted.

It is also interesting that all but one of the previously known natural enemies of *B. treatae* reported by Peck (1963) were not found in this study. Eight natural enemies had been previously described: *Galeopsomyia nigrocyanea* (Hymenoptera: Eulophidae: Tetrastichinae), *Eupelmus dryorhizoxeni* (Eupelmidae: Eupelminae), *Eurytoma profunda* (Eurytomidae: Eurytominae), *Sycophila bicolor* (Eurytomidae: Eurytominae), *Ormyrus dryorhizoxeni* (Ormyridae), *Lariophagus dryorhizoxeni* (Pteromalidae: Pteromalinae), *Torymus flavicoxa* (Torymidae: Toryminae), and *T. lissus*. Only *G. nigrocyanea* was listed by Peck (1963) and also found in this study. Most reports listed in Peck (1963) were rearings from *B. treatae* galls in Florida (Ashmead 1881, 1885, 1886), so the most straightforward explanation for these differences is that *B. treatae* natural enemy communities in Florida differ from the communities from central and southeast Texas studied here. Alternatively, differences in these reports may represent misidentifications of species, or the use of synonyms. The latter does not generally appear to be the case—all of our collections were cross-checked against known synonyms reported in Noyes (2015) and some species are clearly not present in our collections (e.g., *E. dryorhizoxeni* is a wingless eupelmid, and all parasitic wasps in this collection had fully developed wings). However, two previous reports do seem to fit the misidentification scenario: *Eurytoma profunda* was listed by Peck (1963), but all other reported hosts of *E. profunda* are weevil larvae found in plant stems (Bugbee 1967). Similarly, *Sycophila bicolor* seems likely to have been previously misidentified. This species name was synonymized with *Anidarnes biocolor*, an associate of figs (Noyes 2015), and it seems more likely that the true identity of this wasp is one of the *Sycophila* species we describe here. Comparative studies of *B. treatae* natural enemy communities from across its range will be helpful in determining its complete list of associates, a necessary step in the analysis of comparative community assembly across geography and host plants, which is one future goal for research in this system.

A second goal for future research is to identify how many of the species of natural enemies of *B. treatae* are specialists on this gall-former. This is not a trivial task. Several of the natural enemies reported here have also been reported from other cynipid species (Noyes 2015). While this may imply that many species in this community are generalists, we are more cautious. On the one hand, previous reports of shared hosts might be considered a conservative estimate of overlap, considering that most guilds of natural enemies have not been exhaustively described and the hosts described here may represent a small fraction of the total hosts attacked by any given species. On the other hand, it is also possible that previous reports grossly overestimate the amount of overlap between communities—apparent generalist parasitoids may instead represent highly speciose guilds of morphologically similar species (Smith et al. 2008, 2011).

Resolution of the above problem will be aided by morphological and molecular characterization of the natural enemy communities associated with each of the many cynipid species that form galls on *Q. virginiana* and *Q. fusiformis* in Texas and with the other oak species that are member of the series *Virentes* distributed across the range occupied by live oaks. Individual oak species are known for their diversity of gall-forming species, all developing within the same tree and even on the same individual plant (Hayward and Stone 2005). Currently, we have collected nine gallforming species from *Q. virginiana* or its close relatives *Q. fusiformis* (central Texas) and *Q. geminata* (southeastern United States), including

B. treatae, *Disholcaspis cinerosa*, *D. quercusvirens*, *Neuroterus minutissimus*, *N. christi*, *Callirhytis batatoides*, *Andricus quercuslanigera*, *Andricus quercusfoliatus*, and *Bassetia pallida* (Egan et al. 2013, Egan et al., unpublished data). There are other gall formers that have yet to be collected that could add to this number, including *Loxaulus pattersoni*, *Odontocynips nebulosa*, *Disholcaspis fungiformis*, and *Neuroterus saltatorius texanus* (Weld 1959). In addition, one or more common galls formed by members of the Cecidomyidae (Diptera) have been observed on live oaks in Texas, including *Arnoldia atra* (Gagné and Riley 1999). Unification of morphological and molecular identification of gall associates will provide valuable evidence about specialization and how multitrophic communities of parasites coevolve.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

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