ABSTRACT

Title of Dissertation: FOREST MICRO-HYMENOPTERA, INCLUDING THOSE ATTACKING TREES (CYNIPIDAE OAK GALL WASPS) AND THOSE POTENTIALLY DEFENDING THEM (PARASITIC PTEROMALIDAE)

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Dissertation directed by: Professor, Charles Mitter, Department of Entomology

The first part of this dissertation details studies involving an important group of insects attacking oaks, the oak gall wasps (Cynipidae: Cynipini). The second part deals with pteromalid parasitoids involved in thousand cankers disease affecting walnuts. Oak gall wasps are parasites that mainly attack oaks and induce highly differentiated plant growths, called galls, in which they develop. Most gall wasps alternate between dimorphic asexual and sexual generations. However, most species are only known from one generation with the other remaining undescribed. The taxonomy across much of Cynipini is in need of revision. Ultraconserved elements are used to collect phylogenomic data (average 956loci per specimen) for the oak gall wasps. Numerous genera were found to be polyphyletic with separate Nearctic and Palearctic lineages. The ancestor to Cynipini is thought to have been Palearctic based on the taxa sampled. One of the genera found to be polyphyletic, *Disholcaspis* Dalla Torre and Kieffer, is being thoroughly evaluated in preparation for revision. These

efforts include the discovery and identification of new sexual generations, discovery of a new species, locating type specimens, and imaging of species in the genus. The molecular tools used for the identification of sexual generations exposed a great need for more loci, lending support for why phylogenomics is a valid option for this group.

Thousand cankers disease is threatening cultivated and natural walnut tree populations. The disease is caused by a phytopathenogenic fungus, *Geosmithia morbida* Kolařík , Freeland, Utley, and Tisserat, that is vectored by the walnut twig beetle, *Pityophthorus juglandis* Blackman. Studies that have reared insects from trees infected with thousand cankers disease have resulted in the discovery of two new species of parasitoid wasps in the subfamily Cerocephalinae (Pteromalidae). The first was *Theocolax americanus* McEwen which was found in Colorado, USA though the natural origin and host are unknown. The second was *Cerocephala flavus* Cooke-McEwen which was found in the Piemonte region of Italy though its natural origin is also unknown. Cerocephaline wasps are known to attack wood boring beetles so these parasitoid wasps are thought to be associated with *P. juglandis*.

FOREST MICRO-HYMENOPTERA, INCLUDING THOSE ATTACKING TREES (CYNIPIDAE OAK GALL WASPS) AND THOSE POTENTIALLY DEFENDING THEM (PARASITIC PTEROMALIDAE)

by

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Chapter 1: Forest micro-Hymenoptera, including those attacking trees (Cynipidae oak gall wasps) and those potentially defending them (parasitic Pteromalidae)

Introduction

This dissertation has two parts. One deals with a group of Hymenoptera that attack oaks, *Quercus* Linnaeus. The other deals with a group of Hymenoptera that are thought to attack a wood boring beetle that vectors a disease that is deadly to black walnuts, *Juglans nigra* Linnaeus. Together they relate to forest entomology and pest management.

Part I. Wasps attacking oaks

My projects all stem from my Master's Degree research that was performed at Colorado State University (CSU). One of my projects was describing an unknown portion of the life cycle of the gall wasp, *Disholcaspis quercusmamma* (Walsh and Riley) (McEwen et al. 2015). Working with this system exposed me to not only gall wasps, but also their parasitoids. My second and third chapters stem from this research.

In the second chapter I discuss a phylogenomic study on classification of the oak gall wasps (Cynipidae: Cynipini) that emphasizes the genus *Disholcaspis* Dalla

Torre and Kieffer in its sampling. This chapter is a broad look at the overall tribe and the evolutionary patterns I found in the phylogeny produced from genome-scale data. There is emphasis on the genus *Disholcaspis* since one of my overarching goals is to prepare this genus for revision.

The third chapter is a closer look at *Disholcaspis* and current taxonomic and morphological knowledge about that genus. I once again describe the missing portion of the life cycle for two more *Disholcaspis* species and I also describe a new species from Texas. This chapter also summarizes the efforts to locate all the type specimens and image a majority of the species in the genus.

Part II. Wasps potentially defending walnuts

Before I left from my Master's degree I was given a parasitoid from another system for identification. This parasitoid was reared from a black walnut tree, *J. nigra*, infected with thousand cankers disease. These parasitoid wasps were thought to attack the disease vector, *Pityophthorus juglandis* Blackman. These parasitoids were identified as a new species of *Theocolax* Westwood (Pteromalidae: Cerocephalinae). My fourth chapter describes this new species discovered in Colorado. New morphological traits were discovered using scanning electron microscopy. Some of these morphological traits were found to correspond to subgroupings previously found in the genus.

Since that chapter was published (McEwen 2015, Chapter IV), I was contacted to identify other cerocephaline wasps reared from thousand cankers disease infested wood in Italy. A second paper involving the results of those and other insect

identifications has been submitted to the Bulletin of the Entomological Society of Italy. The fifth chapter is an extension of this second paper in that it describes a new species of *Cerocephala* Westwood (Pteromalidae: Cerocephalinae) found the Piemonte region.

Literature cited

McEwen C., Digweed S., Nicholls, J.A., Cranshaw W. 2015. Description and biology of the sexual generation of *Disholcaspis quercusmamma* (Walsh and Riley) (Hymenoptera:Cynipidae), with notes on associated parasitoids. Proceedings of the Entomological Society of Washington 116(3): 294–310.

McEwen C. 2015. A new species of *Theocolax* Westwood (Hymenoptera: Pteromalidae: Cerocephalinae) reared from *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae). Proceedings of the Entomological Society of Washington 117(2):162-178.

Part I. Wasps attacking oaks

Chapter 2: Phylogeny and classification of the oak gall wasps (Hymenoptera: Cynipidae: Cynipini): evidence from phylogenomics

Abstract

Cynipini (Hymenoptera: Cynipidae) are a diverse group of wasps known for their ability to induce galls mainly on oaks (Fagaceae: *Quercus*). Cynipini includes a few large and polyphyletic genera in need of revisions and reliable molecular markers are of interest for this endeavor. Most phylogenies involving Cynipini have been biased towards the Palearctic taxa, though the Nearctic is more diverse for Cynipini, and these phylogenies were limited in the number of molecular markers used due to older sequencing technologies. Ultraconserved elements (UCEs) and their flanking regions have been shown to be useful phylogenetic markers for both deep and shallow evolutionary relationships. This method was previously developed within Hymenoptera and the probes were shown to work within Cynipini, providing several hundred loci. The current study sequenced UCEs for 67 individuals across 19 Cynipini genera and one outgroup. An average of 956 UCE loci was captured per specimen and analysis of data resulted in a strongly supported tree. The data has weak to moderate support for a Nearctic origin of Cynipini. Previously published relationships were corroborated by UCE data and a few previous polytomies were resolved between taxa with high support. Seven of the 19 genera sampled were found to be polyphyletic and most of those are currently Holarctic in distribution. The pairwise distance analyses suggest that this method will be suitable for species level work in this group.

Introduction

Gall wasps (Hymenoptera: Cynipidae) are a charismatic group of parasitic wasps that are obligate parasites on plants. They are characterized by their induction of tumor-like growths, called galls, which they inhabit and feed on during larval development. This intimate relationship with their host plants and their ability to induce gall growth while avoiding, often co-opting, the host's defenses are the basis for the hypothesis of coevolution between gall wasps and their hosts (Abrahamson et al. 1998, Stone et al. 2002, Stone et al. 2009). Due to the diversity of oaks in North and Central America (Manos 1999, Nixon 1993, Stone et al. 2002), this region currently has the highest gall wasp diversity in the world, with an estimated 700 Nearctic species (Liljeblad et al. 2008, Stone et al. 2002). However, most phylogenetic studies have concentrated mainly on the Palearctic species (Liljeblad et al. 2008, Rokas et al. 2003, Stone and Cook 1998, Stone et al. 2009).

Most of the larger genera within Cynipini, are known to be either polyphyletic or paraphyletic (Drown and Brown 1998, Liljeblad et al. 2008, Nicholls et al. 2017, Rokas et al. 2003, Stone and Cook 1998, Stone et al. 2002). The first molecular phylogenies involving Cynipini came from Drown and Brown (1998, Fig. 1) using eastern Nearctic taxa and cytochrome oxidase I (COI) and from Stone and Cooke (1998, Fig. 2) using European taxa and cytochrome b (cyt b). The former called into question the monophyly of *Andricus*, *Dryocosmus*, and *Callirhytis* (Drown and Brown 1998). The latter study also concluded that *Andricus* was polyphyletic (Stone and Cook 1998) even though the species *A. gallaeurnaeformis* Fonscolombe, and

perhaps *A. hystrix* Trotter, were found to be misidentified inquiline gall wasps from Synergini rather than Cynipini (Liljeblad et al. 2008).

Rokas et al. (2003, Fig. 3) again examined Palearctic taxa, emphasizing *Andricus*, using cyt b. This study included mostly European taxa with a few Asian taxa and one North American taxon. *Andricus* was again demonstrated to be polyphyletic, as was *Neuroterus*. The North American taxon, *Disholcaspis spectabilis* (Kinsey) (refered to as *Andricus spectabilis*) was found to be grouped with *Biorhiza* and *Trigonaspis*. This was the first time *D. spectabilis* is included in a phylogeny.

Liljeblad et al. (2008, Fig. 4) performed a morphology based phylogeny that showed *Andricus*, *Plagiotrochus*, *Biorhiza*, *Neuroterus*, and *Disholcaspis* as polyphyletic or paraphyletic. This study proposed higher level groupings that included a *Neuroterus*-group, *Cynips*-group, and a Protobalanus/Lobatae (PL) group based on the host plants for those species. In this study *D. spectabilis* was placed in this PL–group. This study also presented a phylogeny that used cyt b for a subset of the taxa (tree not shown) that showed a polyphyletic *Andricus*. The strict consensus tree in this study had multiple unresolved relationships.

Stone et al. (2009, Fig. 5) performed an analysis of host plant conservation across Palearctic taxa and looked at patterns of diversification across the region. This study used molecular data from cyt b, opsin, and 28S. In addition to finding that lineages diversified from Asia into Europe, they also showed a polyphyletic *Andricus* and the previously mentioned *Cynips*-group. This study showed a large white oak group in which at least one generation induces galls oaks in the section *Quercus*. A

similar group can be seen in the phylogeny from Liljeblad et al. (2009) with mapped host plant data (mapped phylogeny not shown) but was not discussed since it showed up as a paraphyletic group with the PL-group nested within. The maximum clade credibility tree presented in this study included numerous unresolved relationships.

Most recently, Nicholls et al. (2017, Fig. 6) produced a phylogeny that involved Holarctic taxa and concentrated on the evolution of nectar secretion from galls. This study used molecular data from cytb, opsin, and 28S for the Cynipini tree but also included the second internal transcribed spacer of the ribosomal gene complex (ITS2) for a *Disholcaspis* only phylogeny. Their study showed a polyphyletic *Andricus*, *Callirhytis*, *Disholcaspis,* and *Neuroterus*. . There were multiple large polytomies that appeared in this study. This study would have also shown a polyphyletic *Cynips* if they had correctly attributed species from Antron and *Besbicus* to *Cynips* since they had been previously synonymized (Melika and Abrahamson 2002). In this study the species *Disholcaspis spectabilis* (referred to as *Andricus spectabilis*) was found in yet another new position, this time as one of the earlier branches in the tree.

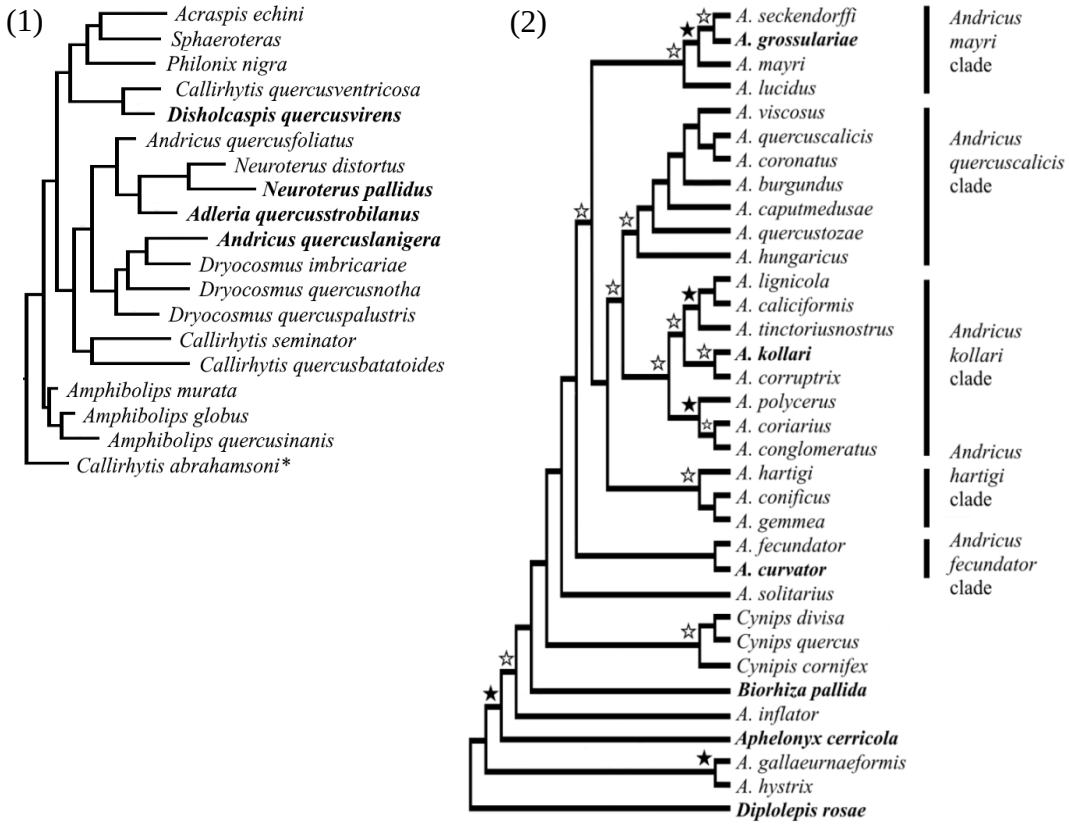
These studies showed that many Cynipini genera need revision but they also demonstrated a need for better molecular tools to aid in this process since polytomies were found in multiple studies. Previously used loci leave much to be desired as far as their ability to determine relationships, particularly relationships below the genus level (Chapter III). Rokas et al. (2002) suggested that the mitochondrial loci cytb and cytochrome oxidase subunit 1 would be informative at the species level. However, Nicholls et al. (2012) found that mitochondrial loci grouped individuals

based on shared historical refugia rather than by morphospecies leading researchers to use those loci cautiously. They instead suggested using multiple loci approach, including nuclear loci. Finding sufficient molecular markers is fundamental for phylogenetic reconstruction and next-generation sequencing (NGS) technologies offer numerous possible solutions.

Ultraconserved Elements (UCEs) are sections of DNA of various lengths that are highly conserved (nearly 100%) across divergent groups of organisms and numerous throughout the genome (Bejerano et al. 2004, Faircloth et al. 2012). Using these regions for phylogenetic purposes is now becoming more common, particularly within Hymenoptera. The UCEs are used as targets that are universal across divergent taxa and that have variable flanking regions. These flanking regions have been shown to be informative at multiple taxonomic levels from deep to shallow evolutionary time scales (Blaimer et al. 2015, Blaimer et al. 2016, Branstetter et al. 2017a, Branstetter et al. 2017b, Branstetter et al. 2017c, Crawford et al. 2012, Faircloth et al. 2012, Faircloth et al. 2013, Jesovnik et al. 2017, McCormack et al. 2012, McCormack et al. 2013, and Smith et al. 2014). This method was developed within Hymenoptera (Faircloth et al. 2015) and further expanded to include representation from every superfamily (Branstetter et al. 2017a) including two gall wasps that served as a test run for the current study. The use of UCEs has aided in multiple phylogenomic studies looking at relationships involving ants and bees (Blaimer et al. 2015, Blaimer et al. 2016, Branstetter et al. 2017a, Branstetter et al. 2017b, Branstetter et al. 2017c, and Jesovnik et al. 2017). There are also ongoing projects or current plans using UCEs across much of Aculeata (pers. comm. E.

Sadler) and the parasitic lineages (pers. comm. M. Gates). Within Hymenoptera, the current study is the first known to use UCEs below the family level outside of Aculeata.

The purpose of this study is to establish a foundation of UCE data for the tribe Cynipini in hope that it will elucidate relationships that have been troubling in the past and give gall wasp researchers more robust molecular tools for future systematic works at any taxonomic level. Special emphasis is placed on the genus *Disholcaapis* Dalla Torre and Kieffer, since it is of interest for revision and a need has been demonstrated for more loci that are informative at the species level.



Figures 1–2. Previous phylogenetic hypotheses. (1) Tree modified from one of two maximum parsimony trees presented in Drown and Brown (1998) that used cytochrome oxidase I to infer relationships. Non-Cynipini were trimmed and taxa in bold are also included in the current study. (2) Tree modified from the maximum parsimony tree presented in Stone and Cook (1998) that used gall morphology and cytochrome b to infer relationships. Black stars at nodes indicate ≥95% bootstrap values, white stars indicate 70–94% bootstrap values, and no star means the bootstrap value was under 70%. Taxa in bold are taxa also included in the current study.

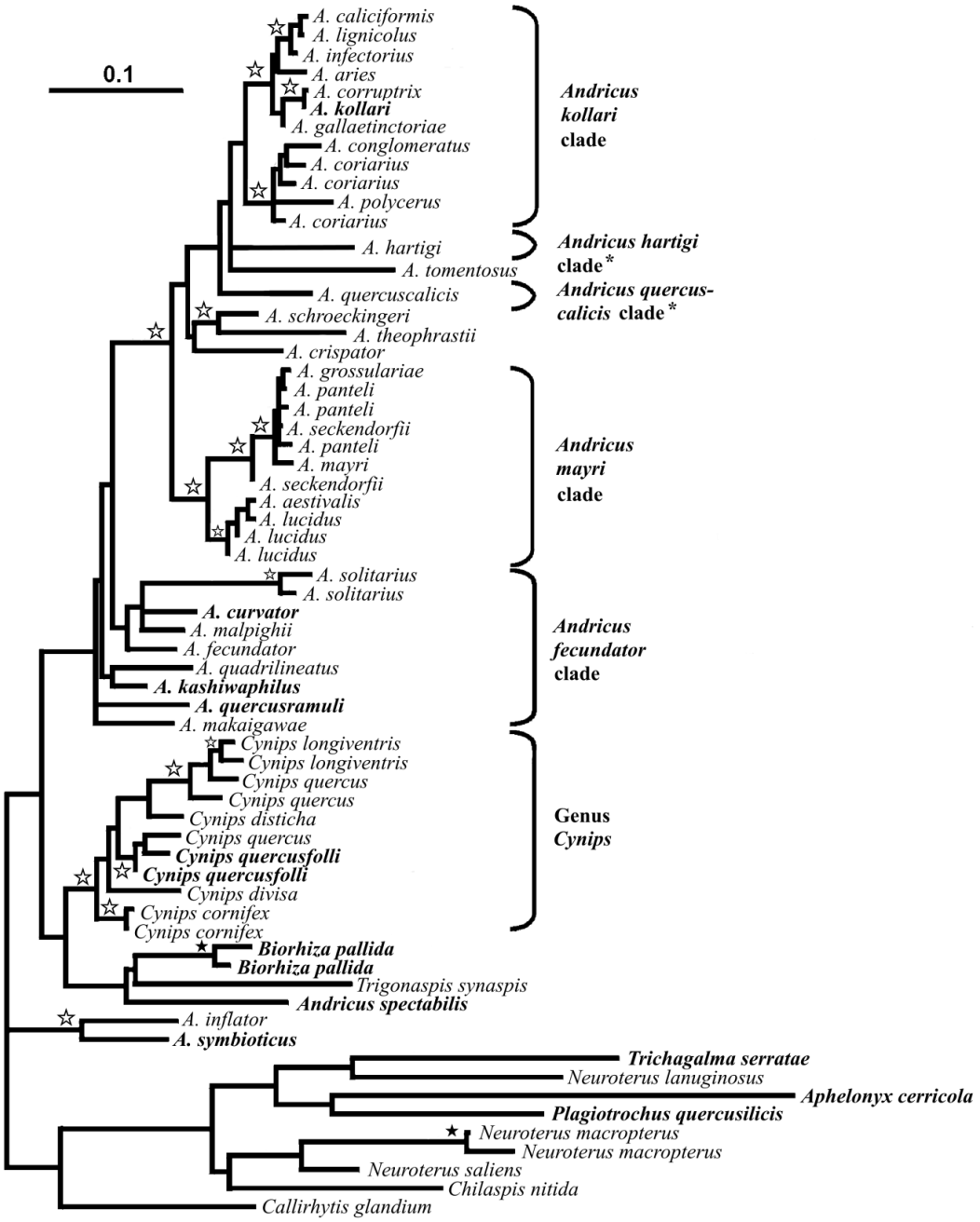


Figure 3. Previous phylogenetic hypothesis from Rokas et al. (2003). Tree modified from the maximum likelihood tree that used cytochrome b data to infer relationships. Black stars at nodes indicate ≥95% bootstrap values, white stars indicate 70-94% bootstrap values, and no star means the bootstrap value was under 70%. Taxa in bold are also included in the current study.*Taxa in the *Andricus hartigi* and *Andricus quercuscalicis* clades were reduced to simplify tree.

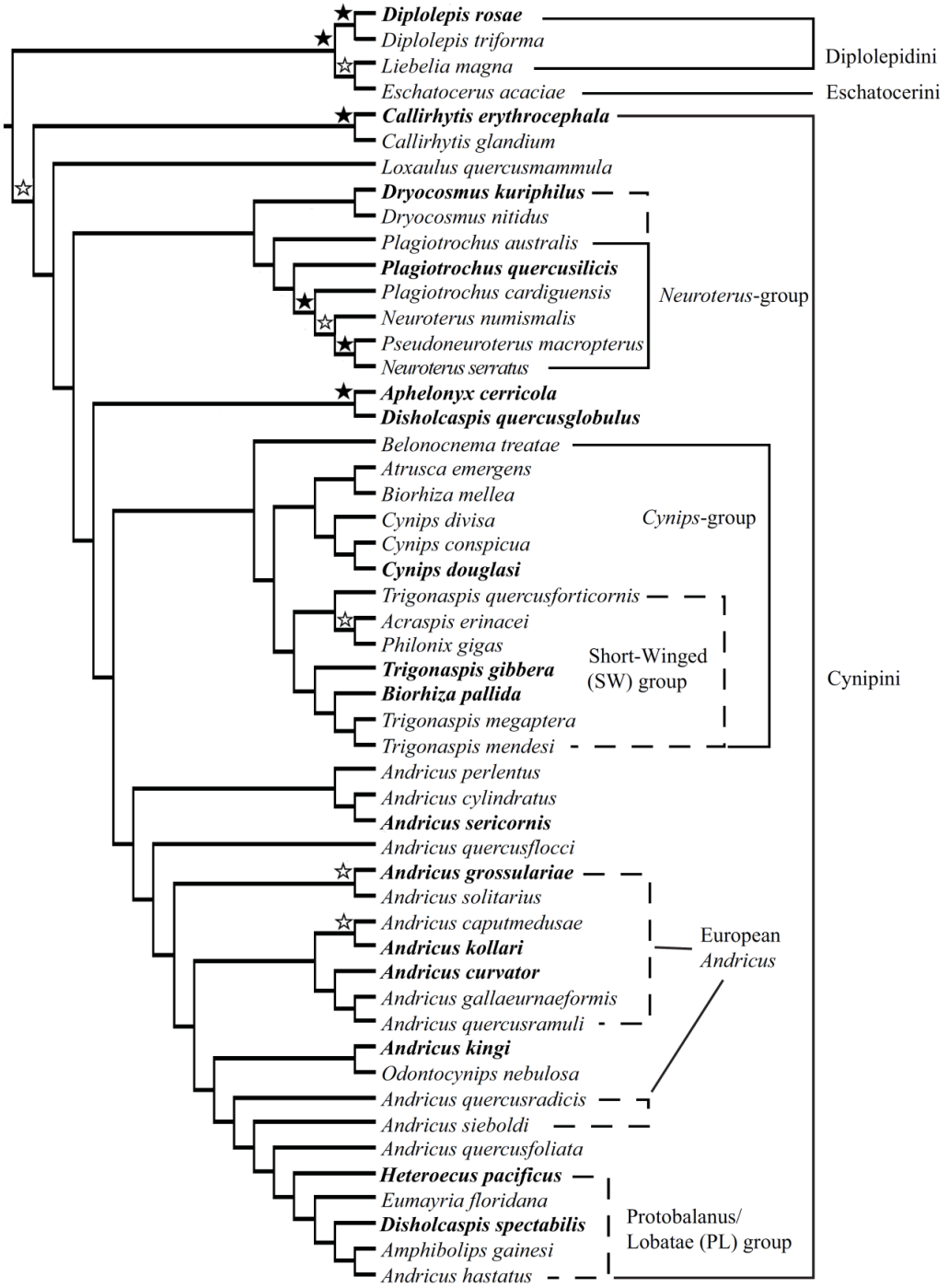


Figure 4. Previous phylogenetic hypothesis from Liljeblad et al. (2008). Tree modified from the shortest parsimony tree based on morphology. Black stars at nodes indicate ≥95% bootstrap values (5000 replicates), white stars indicate 70–94% bootstrap values, and no star means the bootstrap value was under 70%. Taxa in bold are also included in the current study.

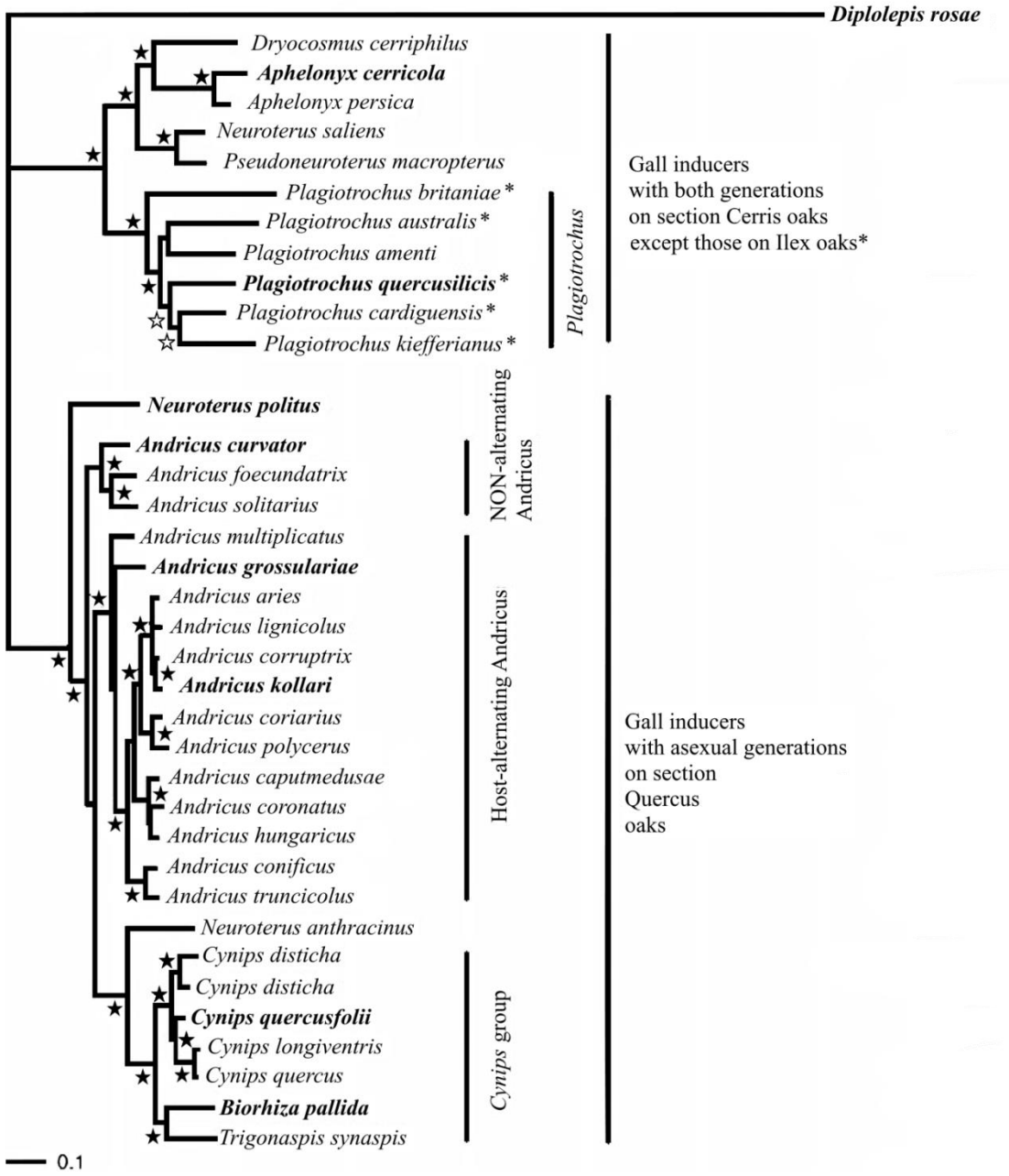


Figure 5. Previous phylogenetic hypothesis from Stone et al. (2009). Tree modified from the Bayesian majority rule consensus tree based on cytochrome b, opsin, and 28s rDNA. Black stars at nodes indicate ≥0.95 posterior probabilities, white stars indicate 0.70–0.94 posterior probabilities, and no star means the posterior probability was under 0.70. Taxa in bold are also included in the current study.

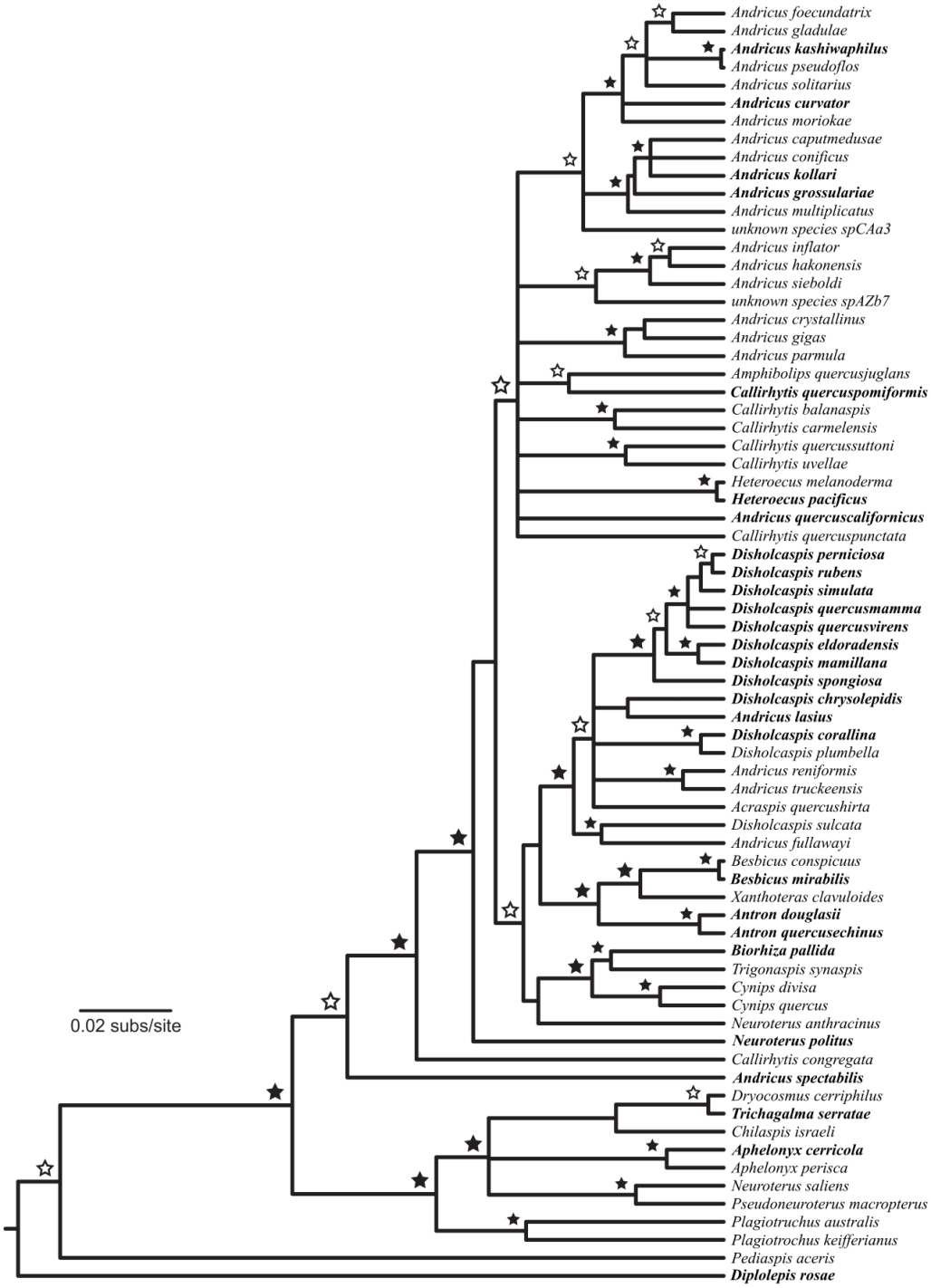


Figure 6. Previous phylogenetic hypothesis from Nicholls et al. (2017). Tree modified from the majority rule consensus species tree based on cytochrome b, opsin, and 28S rDNA sequence data. Black stars at nodes indicate ≥95% bootstrap values, white stars indicate 70–94% bootstrap values, and no star means the bootstrap value was under 70%. Taxa in bold are taxa also included in the current study.

Methods

Taxon sampling. —

Collections were made between 2007 and 2014 by harvesting galls and rearing out the contained wasp. Specimens were then collected and stored in either ≥95% ethanol or RNAlater. In the case of *Callirhytis erythrocephala* (Giraud) adults were too difficult to rear so larvae were removed from the galls. When possible, sexual generation males were used but most specimens were asexual females since that is the primary generation documented for most species.

Taxa (Table 1) from most major Cynipini genera were sampled with special attention to genera that have New and Old World distributions. Nineteen of the 41 genera were sampled (Table 2). Of the approximately 957 species in the tribe 127 are in genera that are not represented in this analysis.

Sampling is slightly biased towards New World species since this is where Cynipini are most diverse. *Disholcaspis* is highly sampled due to its interest for revision and to test UCE loci for ability to differentiate close relationships. The outgroup chosen is *Diplolepis rosae* (Linnaeus) following Stone et al. (2009). Two taxa were used from Branstetter et al. (2017), *Disholcaspis quercusmamma* (Walsh and Riley) and *Disholcaspis lasia* (Ashmead).

Table 1. Cynipidae sampled for UCE sequencing.

Specimen	Origin	Host Plant Species	Host *Quercus* Section
Acraspis macrocarpae	Alberta, Canada	*Quercus macrocarpa*	*Quercus*
Acraspis pezomachoides	Maryland, USA	*Q. alba*	*Quercus*
Acraspis sp. E60	Colorado, USA	*Q. gambelii*	*Quercus*
Amphibolips quercusracemaria	Florida, USA	*Q. laurifolia*	*Lobatae*
Andricus brunneus	California, USA	*Q. douglasii*	*Quercus*
Andricus curvator	Hungary	*Q. robur*	*Quercus*
Andricus grossulariae	United Kingdom	*Q. cerris*	*Cerris*
Andricus kashiwaphilus	Japan	*Q. dentata*	*Quercus*
Andricus kingi	California, USA	*Q. lobata*	*Quercus*
Andricus kollari	Hungary	*Q. petraea*	*Quercus*
Andricus pattersonae	California, USA	*Q. douglasii*	*Quercus*
Andricus quercuscalifornica	California, USA	*Q. lobata*	*Quercus*
Andricus quercuslanigera	Texas, USA	*Q. virginiana*	*Quercus*
Andricus quercusstrobilana	Maryland, USA	*Q. bicolor*	*Quercus*
Andricus sp.	California, USA	Unknown	Unknown
Andricus symbioticus	Japan	*Q. dentata*	*Quercus*
Aphelonyx cerricola	Hungary	*Q. cerris*	*Cerris*
Atrusca aggregata	Arizona, USA	*Q. oblongifolia*	*Quercus*
Atrusca bella	Arizona, USA	*Q. arizonica*	*Quercus*
Atrusca brevipenenata	Colorado, USA	*Q. gambelii*	*Quercus*
Belizinella gibbera	Russia	*Q. dentata*	*Quercus*
Belonocnema quercusvirens	Florida, USA	*Q. virginiana*	*Quercus*
Biorhiza pallida	United Kingdom	*Q. robur*	*Quercus*
Biorhiza trimaculosum	California, USA	*Q. lobata*	*Quercus*
Callirhytis erythrocephala	Hungary	*Q. cerris*	*Cerris*
Callirhytis perfoveata	California, USA	*Q. wislizeni*	*Lobatae*
Callirhytis quecuspomiformis	California, USA	*Q. wislizeni*	*Lobatae*
Callirhytis serricornis	California, USA	*Q. wislizeni*	*Lobatae*
Callirhytis tumifica	Germany (Nearctic species)	*Q. rubra*	*Lobatae*
Cerroneuroterus vonkuenburgi	Japan	*Q. acutissima*	*Cerris*
Cynips douglasii	California, USA	*Q. lobata*	*Quercus*
Cynips mirabilis	British Columbia, Canada	*Q. garryana*	*Quercus*
Cynips quercusechinus	California, USA	*Q. duglasii*	*Quercus*
Cynips quercusfolii	Hungary	*Q. robur*	*Quercus*
Diplolepis rosae	Hungary	*Rosa* sp.	NA
Disholcaspis chrysolepidis	California, USA	*Q. chrysolepis*	*Protobalanus*

Table 1 Continued. Cynipidae sampled for UCE sequencing.

Specimen	Origin	Host Plant	Host Plant Section
Disholcaspis cinerosa E61	Texas, USA	*Q. fusimformis*	*Quercus*
Disholcaspis cinerosa E63	Texas, USA	*Q. virginiana*	*Quercus*
Disholcaspis corallina	California, USA	*Q. douglasii*	*Quercus*
Disholcaspis edura	Arizona, USA	*Q. arizonica*	*Quercus*
Disholcaspis eldoradensis	California, USA	*Q. lobata*	*Quercus*
Disholcaspis erugomamma	Texas, USA	*Q. havardii*	*Quercus*
Disholcaspis lasia	California	*Q. chrysolepidis*	*Protobalanus*
Disholcaspis pedunculoides	Arizona, USA	*Q. turbinella*	*Quercus*
Disholcaspis perniciosa	Colorado, USA	*Q. gambelii*	*Quercus*
Disholcaspis quercusglobulus	Maryland, USA	*Q. alba*	*Quercus*
Disholcaspis quercusmamma	Colorado, USA	*Q. macrocarpa*	*Quercus*
Disholcaspis quercusvirens	Florida, USA	*Q. virginiana*	*Quercus*
Disholcaspis rubens	Colorado, USA	*Q. gambelii*	*Quercus*
Disholcaspis rubens E36	Arizona, USA	*Q. gambelii*	*Quercus*
Disholcaspis rubens E37	Arizona, USA	*Q. gambelii*	*Quercus*
Disholcaspis nr. *rubens*	Utah, USA	*Q. gambelii*	*Quercus*
Disholcaspis simulata	British Columbia, Canada	*Q. garryana*	*Quercus*
Disholcaspis sp. E64	Mississippi, USA	*Q. stellata*	*Quercus*
Disholcaspis sp. E65	Texas, USA	*Q. stellata*	*Quercus*
Disholcaspis spectabilis	California, USA	*Q. chrysolepis*	*Protobalanus*
Disholcaspis spissa	Arizona, USA	*Q. turbinella*	*Quercus*
Dryocosmus kuriphilus	Italy (Asian species)	*Castanea sativa*	NA
Dryocosmus rileypokei	California, USA	*Chrysolepis chrysophylla*	NA
Heteroecus pacificus	California, USA	*Q. chrysolepis*	*Protobalanus*
Heteroecus pacificus E68	California, USA	*Q. chrysolepis*	*Protobalanus*
Neuroterus fragilis	California, USA	*Q. lobata*	*Quercus*
Neuroterus politus	Spain	*Q. pyrenaica*	*Quercus*
Neuroterus saltatorius	California, USA	*Q. lobata*	*Quercus*
Phylloteras cupella	Arizona, USA	*Q. gambelii*	*Quercus*
Plagiotrochus quercusilicis	Spain	*Q. ilex*	*Ilex*
Trichagalma serratae	Japan	*Q. acutissima*	*Cerris*
Trigonaspis eburneum	Arizona, USA	*Q. gambelii*	*Quercus*
Trigonaspis polita	Texas, USA	*Q. sinuata*	*Quercus*

Table 2. Distribution of taxonomic sampling across Cynipini genera. Approximate number of species based on counts from Liljeblad et al. (2008), Melika and Abrahamson (2002), Medianero and Nieves-Aldrey (2013), Pujade-Villar et al. (2012a), Melika et al. (2013), Tang et al. (2011), Ide et al. (2012), Tang et al. (2016), Pénzes et al. (2018), Pujade-Villar and Melika (2014), Pujade-Villar et al. (2010, 2012a, b, 2014, 2017), Wang et al. (2016), Pujade-Villar et al. (2013), Pujade-Villar et al. (2012b), Buffington et al. (2016), and Melika et al. (2010), pers. com. G. Melika.

Genus	Approximate # spp	# Exemplars
Acraspis	20	3
Amphibolips	51	1
Andricus	around 260	12
Aphelonyx	3	1
Atrusca	56	3
Barucynips	1	0
Bassetia	9	0
Belizinella	2	1
Belonocnema	2	1
Biorhiza	15	3
Callirhytis	114	4
Cerroneuroterus	9	1
Chilaspis	2	0
Coffeikokkos	2	0
Cyclocynips	2	0
Cycloneuroterus	17	0
Cynips	60	4
Disholcaspis	54	22
Dros	3	0
Dryocosmus	46	2
Eumayria	5	0
Eumayriella	2	0
Femuros	7	0
Heteroecus	12	2
Holocynips	4	0
Kinseyella	2	0
Kokkocynips	1	0
Latuspina	9	0
Loxaulus	17	0
Melikaiella	13	0
Neuroterus	73	3
Odontocynips	3	0
Philonix	7	0
Phylloteras	9	1
Plagiotrochus	21	1
Pseudoneuroterus	4	0

Table 2 Continued. Distribution of taxonomic sampling across Cynipini genera. Approximate number of species based on counts from Liljeblad et al. (2008), Melika and Abrahamson (2002), Medianero and Nieves-Aldrey (2013), Melika et al. (2013), Tang et al. (2011), Ide et al. (2012), Tang et al. (2016), Pénzes et al. (2018), Pujade-Villar and Melika (2014), Pujade-Villar et al. (2010, 2012a, b, 2013, 2014, 2017), Wang et al. (2016), Buffington et al. (2016), and Melika et al. (2010), pers. com. G. Melika.

Trichagalma	3	1
Trigonaspis	20	1
Ussuraspis	1	0
Zapatella	10	0
Zopheroteras	6	0

Library preparation, target enrichment, and sequencing of UCEs. —

Total DNA was extracted from whole specimens soaked overnight in extraction buffer and Proteinase K. The standard DNeasy® spin column protocol (QIAGEN 2006) was followed with the last step modified to result in 200uL volume. Samples were quantified using a Qubit fluorometer (Life Technologies, Inc.) and ran on a gel to see the size range of DNA strands.

DNA Libraries were prepared following the protocol of Faircloth et al. (2015). DNA template was sheared using a Qsonica DNA Shearer at 25% amplitude for cycles of ten seconds on and ten seconds off for a total of 60 seconds. This gave a size range that had been shown to work for UCE sequencing in ants (Person. comm. M. Lloyd). Sheared DNA was combined with Sera-Mag Carboxylate-Modified speedbeads (Thermo Scientific, Rohland and Reich 2012) in a PEG solution, incubated to allow the DNA to attach to the bead coating, and then placed on a rare earth magnet to pull the beads/DNA to the side wall of the tube. The samples were then washed with 80% ethanol two times before using an elution buffer to pull the DNA from the beads. End repair and A-tailing enzyme mixtures (Kapa Hyper Prep kit, Kapa Biosystems) were added to the washed DNA sample and underwent a thermal cycle of 20°C for 30 minutes followed by 65°C for 30minutes and hold at 10°C to allow the end repair and A-tailing reactions. Stub adapter sequences used for Illumina sequencing were then ligated to the DNA using universal stub mixture (from Glenn Lab, University of Georgia, http://baddna.uga.edu/services_unlinked.html), ligation buffer, and DNA ligase (Kapa Hyper Prep kit). The mixture was then resuspended in speedbeads and PEG solution.

Samples underwent another set of two ethanol washes and then were separated from the beads using elution buffer. The resulting samples were saved as Pre-PCR libraries.

A PCR reaction was set up using 2X Kapa HiFi HotStart Ready Mix, water, two coordinated dual index iTru adapters (from Glenn Lab, University of Georgia), and 15uL of the Pre-PCR library. The i5 and i7 adapters are unique 8bp sequences and the combination of i5 and i7 primers is unique to each specimen for identification purposes. The thermocycler program for the PCR reaction was 98°C for 45 seconds; 13 cycles of 98°C for 15seconds, 60°C for 30seconds, 72°C for 60 seconds; 72°C for 5 minutes; and then hold at 10°C. For a few samples that proved more difficult to get usable concentrations of DNA from using this protocol, 15 cycles instead of 13 were run, with good success.

The PCR product was then combined with more speedbeads in PEG solution and the samples were washed with ethanol twice. The resulting stub and adapter ligated DNA was eluted from the beads and saved as the post-PCR library. The post-PCR libraries were checked for sequence size range to confirm it is over 300bp, by running the samples on an agarose gel. The samples were also quantified using a QuBit Fluorometer. Goal QuBit values were above 5 ng/uL and these values were used to pool samples together at equimolar ratios. Six pools of 10 samples and one pool of 8 samples were pooled together and these mixtures were then used for target enrichment.

Pools were enriched using a custom probe set (MYbaits from MYcroarray) developed in Faircloth et al. (2015) consisting of 2749 probes targeting 1510 UCE loci. Protocols for the library enrichment using the MYbaits kit (MYcroarray, Inc.; Blumenstiel et al. 2010) were followed except the concentration of the MYbaits was

reduced to 0.1X and custom blocking oligos were added. Library pools were combined with a custom Block Mix to prevent the DNA strands from hybridizing. Probes were combined with an RNase Block Mix to create a Capture Library. A combination of library pools and Block mix was incubated in a thermocycler at 95°C for five minutes. The hybridization buffer was then added to a different row in the thermocycler and both mixtures incubated at 65°C for 5minutes. The Capture Library was then added to a different row in the thermocycler and ran at 65°C for two minutes. Then the hybridization buffer and Pool/Block mixture are transferred to the Capture libraries and incubated at 65°C for 24 hours to allow the probes to hybridize with the DNA. The enriched pools then underwent washing on streptavidin beads (MyOne C1; Life Technologies) and were then added to a PCR reaction using HiFi Ready Mix (Kapa Biosystems), P5 and P7 primers for Illumina, and water. The thermal cycle was 98°C for 45seconds; 18cycles of 98°C for 15 seconds, 60°C for 30seconds, 72°C for 60seconds; 72°C for 5 minutes; and then hold at 4°C. The PCR product was then washed on speedbeads with ethanol 3 times. Once the enriched, post-PCR library pools were eluted from the speedbeads, this product is saved and used for qPCR verification of enrichment.

Three standard dilutions were made of the enriched library pools for use in the qPCR reaction. The reaction was set up on a qPCR ViiA 7 (Applied Biosystems) using Sybr Fast qPCR Master Mix (Kappa Biosystems), Primer Premix, and each of the standard dilutions. The qPCR values were used to calculate concentrations for pooling the enriched library pools. The pools were dried and resuspended in an amount that allowed for equimolar concentrations across all seven pools as they are combined to

make 100uL total volume. This final pool then underwent a final size selection that was set to 300–600bp. This gave the final product that was sent to the High Throughput Genomics lab at the Huntsman Cancer Institute of the University of Utah for sequencing using Illumina HiSeq 125 Cycle Paired-End Read Sequencing.

Processing of UCE data and phylogenetic inference. —

The FASTQ files were trimmed of low quality bases and adapter contamination using Illumiprocessor (Faircloth 2013) version 2.0.62017–03–30. The PHYLUCE package (Faircloth 2015) v. 1.5.02017 was used to perform the *de novo* assembly using Trinity (Grabherr et al. 2011) v. r2013_02_25, identify contigs representing enriched UCE loci, create FASTA files for each locus, and align those loci using MAFFT (Katoh et al. 2009) v. 7.130b. PHYLUCE was then used to trim the alignments using Gblocks (Castresana J. 2000) v. 0.91b with the following settings: b1=0.5, b2=0.5, b3=12, b4=7. The trimmed alignments were then filtered for loci that had at least 70% representation across the taxa. The concatenated matrix was then created for use in phylogenetic analysis.

The CIPRES Science Gateway (Miller et al. 2010) was used for the phylogenetic analysis. The concatenated matrix was run through PartitionFinder2 (Lanfear et al. 2014, Lanfear et al. 2016, Stamatakis 2006) with the following settings: use RAxML; branch lengths set to linked; models set to GTR, GTR+G, GTR+I+G; model selection set to aicc; and search set to rcluster. The best scheme from PartitionFinder2 involved 330 partitions. The concatenated matrix was used in RAxML v.8 (Stamatakis 2014) to find the Maximum Likelihood (ML) best tree with the following settings: partitioned based on the best scheme found in PartitionFinder2; GTRGAMMA; rapid bootstrapping;

and the outgroup was set to *Diplolepis rosae* Linnaeus. Proportional pairwise distances, excluding ambiguous sites, were determined for select taxa using PAUP* v. 4.0b10 (Swofford 2002).

Host plant data and biogeographical realm were mapped to the tips of tree to show patterns in those characters. The host plant data follows the classification set by Nixon (1993) with the exception of the section *Quercus* which is now divided into the sections *Quercus*, *Ilex*, and *Cerris* (Denk and Grimm 2009). Stochastic character mapping was done using Phytools package in R v. 3.4.1.

Results and Discussion

The total number of loci captured per specimen ranged from 514–1025 with an average of 956. The reduced concatenated alignment was 426,840 bp long and included 946 loci. The loci were an average of 450 bp long. Overall the topology found in this study is well supported with the bootstrap proportions all being 75% or larger with most values being 100% (Fig. 7).

Character mapping.—

Biogeographical realm is mapped onto the UCE tree in Fig. 7. The stochastic character mapping weakly to moderately suggests a Nearctic origin for Cynipini based on the taxa sampled. This is not consistent with Liljeblad et al. (2008). The posterior probabilities for the ancestral state reconstruction at the base of Cynipini favor a Nearctic origin with five lineages switching over to the Palearctic realm.

Host plant data was also mapped to the UCE tree but there is so much host variation at the base of the tree that the model could not predict a particular host so it is not shown. Perhaps with more taxon sampling at the base of the Cynipini tree, ancestral

states will become more apparent. This study sampled some of the species with particularly odd hosts and they all came out near the base of the tree. There are multiple lineages that evolved to attack hosts in the *Quercus, Lobatae*, and *Protobalanus* sections as well as multiple lineages that alternate hosts.

Higher level relationships.—

Within Cynipini as sampled here, the basal divergence separates *Dischol caspis spectabilis* from all others (Fig. 8, node 1, bootstrap percentage [BP] = 100). The problem with this species is that we know that it morphologically does not belong within *Disholcaspis* (Melika and Abrahamson 2002 and Chapter III) but where it does belong has been somewhat of a mystery since our understanding of its phylogenetic placement keeps changing. Rokas et al. (2003, Fig. 3) was the first study to include this species in a phylogeny and they found this species to be closely allied with *Biorhiza pallida* and *Trigonaspis*, near *Cynips*. Liljeblad et al. (2008, Fig. 4) had this species deeply nested in their phylogeny and in their Protobalanus/Lobatae-group with *Heteroecus pacificus*, *Eumaryia*, *Andricus hastatus*, and *Amphibolips*. Nicholls et al. (2017, Fig. 6) showed this species to be an early branch within Cynipini but not sister to the rest of Cynipini. This new hypothesis based on the UCE analysis shows that this species is quite distant from all other species in *Disholcaspis* and from the other species in this analysis known from hosts in the section *Protobalanus*.

The UCE phylogeny showed a monophyletic Nearctic Lobatae-group (Fig. 8, Node 15, BP = 100) that comprises of *Amphibolips* and the Nearctic *Callirhytis*. Sister to that grouping (Fig. 8, node 14, BP = 100) is a large monophyletic white oak-group (Fig. 8, node 18, BP = 100) that is largely known from hosts in the section *Quercus*.

This clade was also documented in Stone et al. (2009, Fig. 5) and was visible on the host data mapped tree in Liljeblad et al. (2008, tree not shown). The main *Andricus* clade is at the base of the white oak-group being sister to the rest of the clade (Fig. 8, node 18, BP = 100).

Previous studies found a monophyletic group of genera referred to as the *Cynips*-group, however the current study found this grouping to be polyphyletic. The UCE phylogeny showed the *Cynips*-group to be divided into two lineages, here referred to as the Palearctic *Cynips*-group and the Nearctic *Cynips*-group (Figs. 7, 9). Interestingly, both groups include species from *Cynips*, *Trigonaspis*, and *Biorhiza*. The Nearctic *Cynips*-group (Fig. 8, node 34, BP = 100) also includes *Atrusca* and *Belonocnema* similar to Liljeblad et al. (2008). However, they also included *Acraspis* in their *Cynips*-group, which is excluded from this group in the UCE phylogeny. The *Cynips*-group occurred in multiple previous phylogenies (Rokas et al. 2003, Ronquist et al. 2015, Liljeblad et al. 2008). Rokas et al. (2003, Fig. 3) found that *Cynips quercusfolii* is closely allied with *Biorhiza pallida* (Oliver) and *Trigonaspis* in agreement with the current study however they also included the species *Disholcaspis* (*Andricus*) *spectabilis* in this group. Ronquist et al. (2015, tree not shown) also found *Cynips* to be closely allied with *Trigonaspis* and *B. pallida* but also *Neuroterus numismalis* (de Fourcroy).

Previous studies found a *Neuroterus*-group comprised of *Neuroterus*, *Trichagalma, Aphelonyx, Plagiotrochus, Dryocosmus, Chilaspis,* and *Pseudoneuroterus* (Liljeblad et al. 2008, Rokas et al. 2003, Stone et al. 2009). Most taxa from these genera are grouped together in the UCE analysis (Fig. 8, node 2, BP = 100). However, this group as a whole is found to be polyphyletic due to both *Neuroterus* and *Dryocosmus*

being polyphyletic and having species outside of this core clade. This is in agreement with Nicholls et al (2017). The Nearctic *Neuroterus* are found to be sister to the Palearctic *Callirhytis* (Fig. 8, node 7, BP = 100).

Phylogenetic review of existing genera. —

Acraspis Mayr

The monophyly of *Acraspis* has not previously been tested. The UCEs showed the three *Acraspis* species sampled to be monophyletic and sister to the species *D. corallina* (Bassett) which is known to not belong within *Disholcaspis* morphologically (Melika and Abrahamson 2002) (Figs. 7, 9).

Amphibolips Reinhard

Only the morphological phylogenetic study from Liljeblad et al. (2008) has previously sampled from *Amphibolips*. They placed it in the Protobalanus/Lobatae group. The current study showed *Amphibolips* to be closely allied with the Nearctic *Callirhytis*. It should be noted that *Callirytis quercuspomiformis* is sister to *Amphibolips quercusracemaria* (Ashmead) instead of grouping with the other Nearctic *Callirhytis* supporting the proposed transfer of *quercuspomiformis* to *Amphibolips* by Melika and Abrahamson (2002). However, while transfer of this species to *Amphibolips* was discussed in that paper, the new combination never occurred in text so this species remains in *Callirhytis*. General collection specimens were examined for *quercuspomiformis* and it is in agreement with *Amphibolips*. Based on the morphological evidence provided by Melika and Abrahamson (2002) and the stong phylogenetic placement of this species in the UCE analysis, that species is hereby

transferred to *Amphibolips* as *A. quercuspomiformis* (Bassett) **comb. nov.** This makes the Nearctic *Callirhytis* monophyletic and sister to *Amphibolips* (Figs. 7, 9).

Andricus Hartig

Andricus as a whole is polyphyletic. It is shown to include at least two Palearctic lineages and four Nearctic lineages (Fig. 9). The Palearctic *Andricus* are polyphyletic with at least two lineages.

Multiple studies (Nicholls et al. 2017, Rokas et al. 2003, Stone and Cook 1998, and Stone et al. 2009, Figs. 2–3,5–6) concluded that within *Andricus* the *kollari* group (represented here by *A. kollari* (Hartig)) is more closely related to the *mayri* clade (represented here by *A. grossulariae* Giraud) than to the *fecundator* clade (represented here by *A. curvator* Hartig, *A. kashiwaphilus* Abe, and *A. quercusramuli* (Linnaeus)) and the current study is in agreement based on the few taxa from these groups that were included in this analysis. However, the species in the UCE analysis that represent the *fecundator* clade showed a monophyletic relationship but these same three species were previously thought to have a paraphyletic relationship based on Rokas et al. (2003, Fig. 3).

Aphelonyx Mayr

Only one species of *Aphelonyx* was included in this study. It was found to be sister to *Cerroneuroterus vonkuenburgi* (Dettmer) and *Trichagalma serratae* (Ashmead) (Figs. 7, 9).

Atrusca Kinsey

All three *Atrusca* species were strongly grouped together. They were sister to the rest of the Nearctic *Cynips*-group (Fig. 7).

Belizinella Kovalev

Only one species represented *Belizinella* in this study but it was the type species for the genus. It was nested in the *Cynips*-group (Fig. 2).

Belonocnema Mayr

Belonocnema quercusvirens (Osten-Sacken) was the only species sampled in this genus. It was found to be nested within the Nearctic *Cynips*-group (Fig. 7).

Biorhiza Westwood.

Biorhiza was shown to be polyphyletic with two Nearctic lineages and one Palearctic lineage. The Nearctic species *B. trimaculosum* (McCracken and Egbert) was sister to the Nearctic Cynips and the species *B. eburneum* (Bassett) was sister to its previous conager Trigonaspis (Xanthoteras) polita (Bassett). The Palearctic *B. pallida* (Oliver) was placed in the Palearctic *Cynips*-group sister to *Belizinella gibbera* Kovalev (Fig.7). It therefore appears that by encompassing species across the Holarctic realm this genus is being made polyphyletic.

Callirhytis Foerster

Callirhytis was shown to be polyphyletic (Figs. 7, 9). Interestingly the Palearctic species sampled, *C. erythrocephala* (Giraud), grouped with the Nearctic *C. tumifica* (Osten-Sacken). This makes *Callirhytis* the only genus to have a Nearctic and Palearctic species in the same lineage. They were sister to the Nearctic *Neuroterus*. The Nearctic, *C. perfoveata* (Kinsey) and *C. serricornis* (Kinsey), were a separate monophyletic lineage more deeply nested in the tree. These two species were regarded by Melika and Abrahamson (2002) as belonging in *Andricus* but they did not make a formal new combination. Our results show that they do not group with any subset of *Andricus*, thus,

a new genus will likely need to be described for them. These two taxa were also sister to *Amphibolips*. It was considered whether *C. perfoveata* and *C. serricornis* should also be transferred to *Amphibolips* as well in order to make *Callirhytis* monophyletic, however, they do not belong in *Amphibolips* morphologically based on Melika and Abrahamson's (2002) characterization of that genus.

Cerroneuroterus Melika and Pujade-Villar

Cerroneuroterus was represented by only one taxon. It was found sister to *Trichagalma serratae* and nested within the core *Pseudoneuroterus*-group (Fig. 2).

Cynips Linnaeus

In our analysis this genus consists of at least 3 lineages (Fig. 7), one Palearctic and two Nearctic. The European *Cynips* were represented by *C. quercusfolii* which came out sister to the European *Trigonaspis* + *Biorhiza*. The Nearctic taxa sampled were also polyphyletic with one lineage represented by *C. mirabilis* Kinsey and the other by *C. douglasii* (Ashmead) and *C. quercusechinus* Osten-Sacken. All three of these Nearctic species were transferred to *Cynips* from *Besbicus* Kinsey and *Antron* Kinsey which were both synonymized with *Cynips* by Melika and Abrahamson (2002). It is likely that *Antron* and *Besbicus* will need to be revived to define a monophyletic *Cynips*.

Disholcaspis Dalla Torre and Kieffer

A polyphyletic *Disholcaspis* sensu lato was supported by our data (Fig. 7). However, as previously reported in Nicholls et al. (2017, Fig. 6) the morphologically "typical" *Disholcaspis* (synapomorphies include incomplete notali, scutellar foveae generally absent, propodeal carinae curved, propodeal carinae broken, gradually tapering hypopygial spine that is at most 4.5 times as long as wide, hypopygial spine with long

subapical setae reaching beyond the apex of the spine, and hypopygial spine setae not forming a tuft.) are monophyletic (100% bootstrap support, Fig. 7). This is *Disholcaspis* sensu stricto (Fig. 7). There were at least 3 other lineages representing the other species currently in *Disholcaspis* but known to differ from the rest of the genus morphologically (Fig. 9). These three lineages of the "nontypical" *Disholcaspis* are: *D. corallina* which was sister to *Acraspis*, *D. chysolepidis* (Beutenmüller) plus *D. lasia*, and *D. spectabilis*. *Acraspis* and *D. corallina* were found to be sister to *Disholcaspis* sensu stricto.

The unidentified *D.* sp. E65 and *D.* sp. E64 likely represent undescribed species. They were both from nipple-less globular stem galls similar to *D. quercusglobulus* but they were found on *Q. stellata* Wangenheim which is not a known host for *D. quercusglobulus*. These two species probably would have been identified as *D. quercusglobulus* based on the galls alone but only *Disholcaspis* sp. E65 is particularly closely related to *D. quercusglobulus*. This in addition to *D. quercusglobulus* appearing to be a paraphyletic complex based on cytb and ITS2 data (Chapter III) suggest that this species and any species with similar galls needs to be revised. This may be a good starting place for revising that genus.

Dryocosmus Giraud

Dryocosmus was polyphyletic in our tree (Figs. 7, 9). More species should be sampled but there are at least two separate lineages currently in this genus, Nearctic and Palearctic. The Nearctic lineage is sister to *Heteroecus* and the Palearctic lineage was sister to *Plagiotrochus/Aphelonyx/Trichagalma/Neuroterus*. One or the other of these lineages will need to comprise a new genus, or, each could be folded into other genera.

Heteroecus Kinsey.

Only *H. pacificus* was sampled. It was placed sister group to the Nearctic *Dryocosmus* (Figs. 7, 9).

Neuroterus Hartig

Neuroterus is extensively polyphyletic (Figs. 7, 9). The two Nearctic species are grouped with each other and then to the Palearctic *Callirhytis*. However, they are only distantly related to their Palearctic congener. The genus will require extensive reclassification.

Phylloteras Ashmead

Phylloteras was represented by only one taxon. It was found sister to the third lineage of Nearctic *Andricus* (Fig., 9).

Plagiotrochus Mayr

Plagiotrochus was represented by only one taxon. It was sister to *Aphelonyx/Trichagalma/Cerroneuroterus vonkuenburgi* (Figs. 7, 9).

Trichagalma Mayr

Trichagalma was represented by only one taxon. It was found to be sister to *Cerroneuroterus vonkuenburgi* (Fig. 7).

Trigonaspis Hartig

Trigonaspis was represented by only one taxon (Figs. 7). It was found to be sister to *Biorhiza eburneum* (Bassett).

Relationships within *Disholcaspis*.—

Disholcaspis was already known to be polyphyletic (Liljeblad et al. 2008 and Nicholls et al. 2017). Melika and Abrahamson (2002) discussed a subset of species genus that doesn't match *Disholcaspis* sensu stricto morphologically but they were placed into or left in the genus provisionally. This subset includes *D. chrysolepidis* (Beutenmuller), *conalis* Weld, *corallina* (Bassett), *lasia* (Ashmead), *plumbella* Kinsey, *reniformis* McCracken and Egbert, *spectabilis*, *sulcata* (Ashmead), and *washingtonensis* (Gillette). Of the species used in the UCE phylogeny, only *D. lasia* and *D. chrysolepidis* grouped together (Fig. 8, node 46, BP = 100). *Disholcaspis coralina* was found to be sister to *Acraspis* (node 48, BP = 100) and *D. spectabilis*, as mentioned earlier, was found to be at the base of the Cynipini tree.

Within *Disholcaspis* sensu stricto, the unidentified *Disholcaspis* sp. E64, which was collected from *Q. stellata* Wangenheim in Mississippi, was found to be sister to the rest of the species sampled (node 51, BP = 100). The next two branches were both Californian taxa, *D. eldoradensis* (Beutenmüller) and *D. simulata* Kinsey. The remaining taxa seem to group together based on geographical location. First, *D. quercusvirens* and *D. cinerosa*, which are both from hosts isolated to the Southern U.S.A., grouped together (node 55, BP = 100). Next, the species from the Southwestern states (Colorado, Utah, Arizona, New Mexico, and Western Texas) grouped together (node 58, BP = 100). The third geographic grouping of species was from the broad range of the Eastern U.S.A. including Eastern Texas (node 65, BP=100). The species *D. perniciosa* (Bassett) is the exception to these geographical groupings since it is a Southwestern species but it was sister to the Eastern U.S.A. group.

The sister group to *Disholcaspis* sensu stricto has also been debated. The morphological phylogenetic analysis in Liljeblad et al. (2008) concluded that the *Disholcaspis* sensu stricto (represented by the type species *D. quercusglobulus* [Fitch]) were closely allied with *Aphelonyx cerricola* (Giraud). The UCE analysis (Fig. 7) instead puts *A. cerricola* with the *Cerroneuroterus vonkuenburgi* (Dettmer), *Trichagalma serratae* (Ashmead), *Plagiotrochus quercusilicis* (Fabricius), and *Dryocosmus kuriphilus* which are all distant to the *Disholcaspis* sensu stricto. Nicholls et al (2017, Fig. 6) had the sister group to *Disholcaspis* sensu stricto as a polytomy including *Acraspis*, the "nontypical" *Disholcaspis*, *Andricus truckeensis* (Ashmead) which used to be in *Disholcaspis* (Dailey and Menke 1980), and *Andricus fullawayi* Beutenmüller. The UCE data resolved some of these relationships putting *Acraspis* + *Disholcaspis corallina* sister to *Disholcaspis* sensu stricto (Fig. 7).

Distance analyses.—

Proportional pairwise distances excluding missing/ambiguous sites were examined between specimens of the same species to get an idea of how much the UCE loci vary at this level. The largest pairwise distance seen within a species was seen between the two *Heteroecus pacificus* specimens at 0.5682% different across 318,903 characters. The pairwise distance seen between the two *Disholcaspis cinerosa* (Bassett) specimens was 0.0920% different across 379,398 characters. These two specimens came from two different species of host plant, *Q. virginiana* Miller and *Q. fusiformis* Small. The pairwise distances seen within *D. rubens* (Gillette) ranged from 0.1452–0.1589% different across 328,595 characters. The specimen *Disholcaspis*_nr._*rubens* was a Utah

specimen that matches *D. rubens* morphologically but had conical instead of globular shaped galls. It was found on the same host plant as *D. rubens*, *Q. gambelii* Nuttall and Liebmann. This specimen was shown to be 0.1421% different from *Disholcaspis_rubens*_E56 which was a typical *D. rubens* specimen from Colorado. It was also 0.1716–0.1926% different from the two typical *D. rubens* specimens from Arizona. The phylogeny grouped the Colorado and Utah specimens together and the Arizona specimens together suggesting that this method can determine population or species complex relationships.

Figure 7. Maximum Likelihood best tree for Cynipini UCE data with Diplolepis rosae as outgroup. Nodes are numbered (left of slash) for discussion purposes. Only bootstrap values (100 reps) below 100% are listed (to the right of slash). Biogeographical realm is mapped on the tree with blue being Palearctic and black Nearctic. Host plant data is mapped at the tips; host plant data that is split between two colors indicates species that alternate host plants between generations. Taxon names reflect taxonomic changes in text. Posterior probabilities for individual nodes in the character state reconstruction that were under 0.75 are graphed on those nodes as pie graphs.

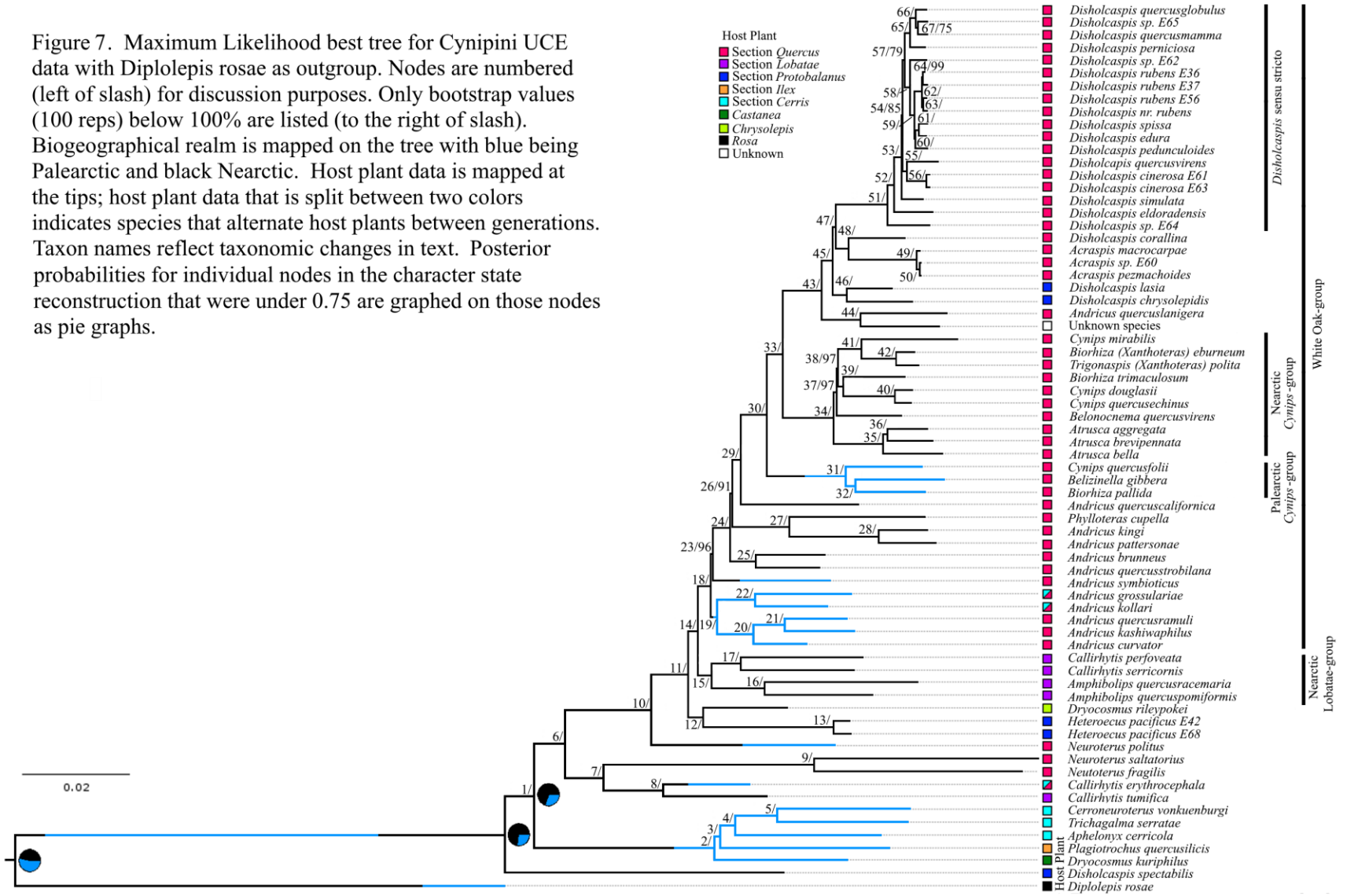


Fig. 8. Cladogram based on Maximum Likelihood analysis of Cynipini UCE data with *Diplolepis rosae* as outgroup. Numbers above branches are bootstrap values (100 reps). Nodes within Cynipini are numbered below branches for discussion purposes. Binomial names reflect taxonomic changes made in text.

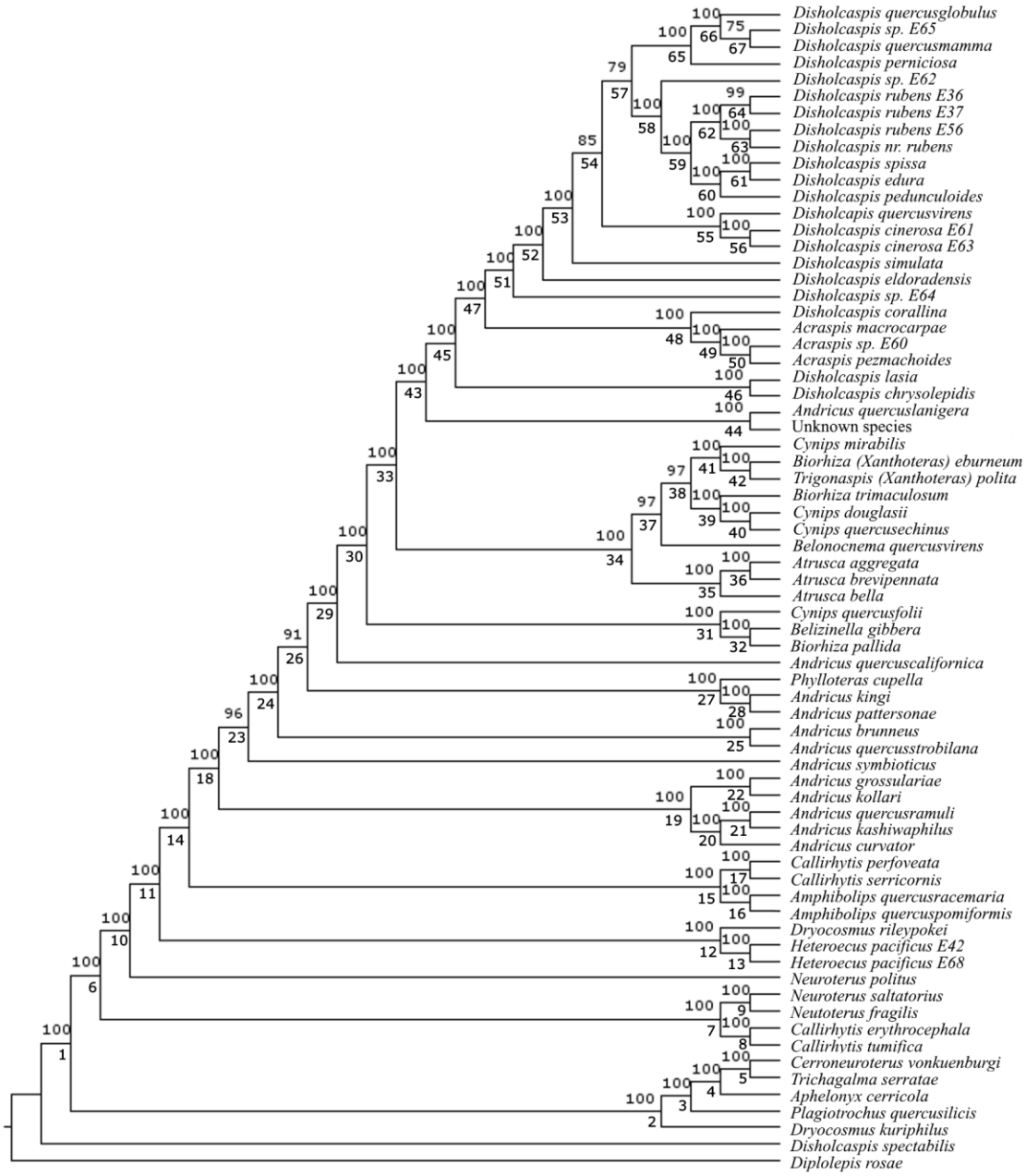
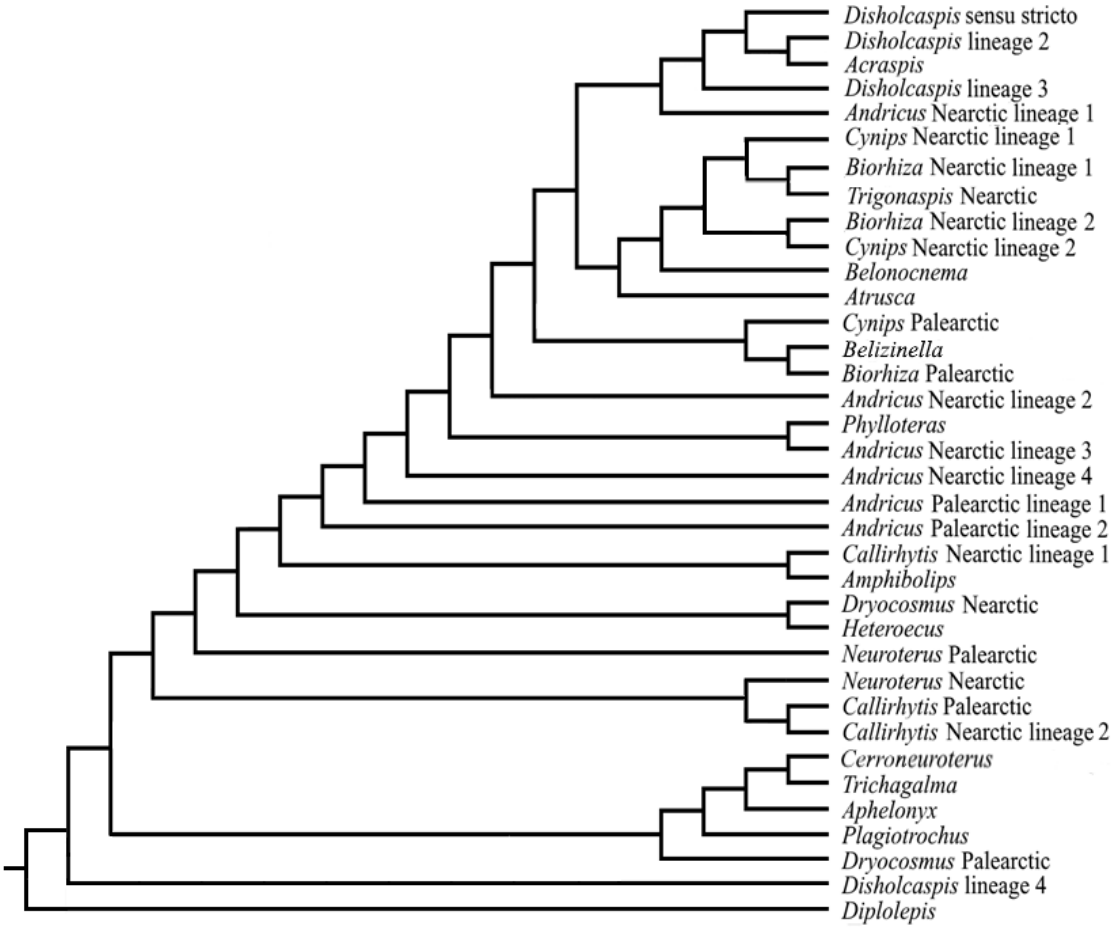


Fig. 9. Cladogram of the genera in Cynipini based on UCE data.



Concluding Remarks

The UCE data provide remarkably strong resolution in a group in which morphology has been exceptionally confusing. Our data show that *Andricus*, *Biorhiza*, *Callirhytis*, *Cynips*, *Disholcaspis*, *Dryocosmus*, and *Neuroterus* are all polyphyletic. Some of these relationships may be due to recent taxonomic movement between genera but it is obvious that morphology based taxonomy in Cynipini is problematic. The genera with Holarctic distributions were polyphyletic with the Nearctic and Palearctic species being distantly related. Revisions of these groups are needed. The combination of UCEs and greater taxon sampling in the future would result in a clearer picture of how many new genera need to be established to correct the polyphyletic genera.

Using ultraconserved elements and their flanking regions as phylogenetic markers within Cynipini has been shown to recover relationships that past studies have found while also resolving other relationships that were previously uncertain. This method can be used in its entirety or perhaps particularly variable regions can be filtered as loci that can be used for revision purposes. One concern that may need to be addressed is that the UCE method has been shown in amniotes to recover almost the entire mitochondrial genome (Amaral et al. 2015). This can be concerning since mitochondrial loci have been shown to have problems delimiting species in Cynipini due to hybridization between species (Nicholls et al. 2012). Whether mitochondrial loci are abundant and how these effect the topology of UCE data may need to be explored, though the large amount of nuclear data should be numerically overwhelming the analysis.

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Chapter 3: Contributions to the genus *Disholcaspis* Dalla Torre and Kieffer

Abstract

The genus *Disholcaspis* (Hymenoptera: Cynipidae: Cynipini) is of interest for future revision because it is polyphyletic. This study sought to begin the imaging and research needed to support the revision. During this process three biological and taxonomic contributions were discovered and a large image-based dataset was established. The first two contributions are the descriptions of the sexual generations for *D. mamillana* and *D. prehensa*. These generations are identified using a combination of molecular and host plant data. The third contribution is the new species *Disholcaspis erugomamma* Cooke **n. sp**. from Texas. Lastly, the locations for the various type specimens and image data for a majority of the species in the genus are presented with discussion about the overall morphology of the genus.

Introduction

Oak gall wasps (Hymenoptera: Cynipidae: Cynipini) are obligate parasites on oaks (Fagaceae: *Quercus*) that induce tumor-like growths called galls. The gall is thought to provide nutrition and protection for the developing gall wasp larva until it completes its development and leaves the gall as an adult. There is a wide diversity of gall morphologies across the group as a whole, and the gall morphologies are often species-specific or even generation-specific (Stone et al. 2002).

The Nearctic Cynipidae were largely studied before 1950. This means that most taxonomic studies in this group were performed prior to the conception of what we know as phylogenetic systematics (Hennig 1965). These genera and species were

described with minimal morphological descriptions, without modern imaging, based on only half of the lifecycle, and relationships were often inferred based on gall morphology rather than wasp morphology. This created monstrous polyphyletic genera, namely *Andricus*, *Callirhytis*, and *Neuroterus* (Melika and Abrahamson 2002). These genera have become chaotic and will likely need to be revised in portions. Therefore I propose starting with comparatively smaller genera that are known to have need for revision. *Disholcaspis* fits these criteria and has biological characteristics that make it an appealing group to research.

As with most gall-inducing insects, oak gall wasps have a high level of host specificity, generally only attacking a single or few closely related species of host plant (Stone et al. 2002). Some gall wasp species alternate hosts between generations (heteroecy) as well, but this is limited to two Western Palearctic groups in the genera *Andricus* Hartig and *Callirhytis* Förster (Askew 1984, Folliot 1964, Nieves-Aldrey 1992, Pujade-Villar et al. 2005, Stone et al. 2002, Stone et al. 2009).

Most oak gall wasps are heterogonous, with a strict alternation between an asexually reproducing generation followed by a sexually reproducing generation (Stone et al. 2002). Not only are the gall morphologies generally different between generations but also the wasp morphologies are different between generations as well which has probably led to generations having been described as separate species (Pujade-Villar et al. 2001, Stone et al. 2002). Accurate association of the two generations can be a complicated process traditionally done by experimental rearing.

Disholcaspis. —

Disholcaspis is a relatively large genus of oak gall wasps with 54 species (Table 3) (Burks 1979, Medianero and Nieves-Aldrey 2011, Melika and Abrahamson 2002, Melika et al. 2011, Pujade-Villar et al. 2009, Pujade-Villar et al. 2010) ranging from Canada to Panama. The genus was first established under the name *Holcaspis* Mayr (Mayr 1881). Though *Disholcaspis* has never been fully revised, Beutenmüller (1909) produced a summary of the species with notes on taxonomic histories, wasp morphology, gall morphologies, host plants, and plates depicting galls. His publication listed 23 species, of which 16 remain valid. Dalla Torre and Kieffer (1910) discovered subsequently that the name *Holcaspis* was preoccupied in Coleoptera and proposed *Disholcaspis* as a replacement.

The most prolific contributor to the genus was Alfred Kinsey. From 1920–1938, Kinsey described 16 species currently within *Disholcaspis* (Kinsey 1920, 1922a, 1922b, 1937, 1938) and organized 17 manuscript names that were found in the Kinsey collection at the American Museum of Natural History (AMNH). Some of these manuscript names were mentioned in publications (Kinsey 1942) as manuscript names and assigned species complexes. However, while these species complexes were discussed, correlating morphological traits were not defined. The second most prolific contributor to the genus was Lewis H. Weld. Between 1921 and 1957 he described 12 species (Weld 1921, Weld 1926, and Weld 1957).

The most recent morphological treatment of the genus came from Melika and Abrahamson (2002) who gave a morphological overview of how *Disholcaspis* sensu stricto is diagnosed from related genera and moved *arizonicus* Cockerell, *lasius*

Ashmead, *reniformis* McCracken and Egbert, *spectabilis* Kinsey, and *lapiei* Kieffer into *Disholcapsis*. All but *lapiei* (Pujade-Villar et al. 2010), are still in the genus and all except *arizonicus* were included by Melika and Abrahamson (2002) in a subset of species that differ morphologicially from *Disholcaspis* sensu stricto but are kept in the genus provisionally. Some of the species in this subset have been shown to make the genus polyphyletic using either morphological (Liljeblad et al. 2008) or molecular data (Nicholls et al. 2017, Chapter II). Melika et al. (2013) later suggested that there are actually 3 lineages within the genus including a separate group for the species *D. spectabilis* (Kinsey).

Disholcaspis sensu stricto attack white oaks in the section *Quercus.* A few of the species in the subset that are thought to be different from the rest of *Disholcaspis* are known to attack oaks in the section *Protobalanus* (Burks 1979, Melika and Abrahamson 2002). The only other exception is that *D. persimilis* (Ashmead) has "blackjack oak" written on its label which is the common name for the red oak *Q. marilandica* Münchhausen in the section Lobatae (Nixon 1997). Some species are only known from a single host species while others are apparently capable of attacking numerous hosts. So far, the sexual generations within *Disholcaspis* have always been found on the same host plants as the asexual generation (Bird et al. 2013, Evans 1972, Frankie et al. 1977, Melika et al. 2013, McEwen et al. 2015).

The sexual generations within *Disholcaspis* have mostly remained undescribed apart from a few relatively recent updates. The first species to have the sexual generation discovered and described was *D. eldoradensis* (Beutenmüller) (Evans 1972). The sexual generation of *D. cinerosa* (Bassett) was then discovered and its

biology published in 1977 (Frankie et al. 1977), though a morphological description was never published for this species. The morphological and biological descriptions for *D. quercusvirens* (Ashmead) and *D. quercusmamma* (Walsh and Riley) were published over 35 years later (Bird et al. 2013, Melika et al. 2013, McEwen et al. 2015). They used both traditional rearing experiments as well as genetic data to associate the sexual generations with their corresponding asexual generations (Bird et al. 2013, Melika et al. 2013, McEwen et al. 2015). A large amount of genetic data has recently been released for other *Disholcaspis* species (Nicholls et al. 2017) and offers help in determining species identifications for sexual generations. This study seeks to use this data to infer identifications without the need for time consuming rearing experiments.

Molecular Species Identifications.—

Delimiting species using DNA data is often done with 'DNA barcodes' (Hebert et al. 2003). Nicholls et al. (2012) determined that mitochondrial barcodes don't delimit morphospecies in European *Andricus* due to hybridization with extensive backcrossing. They also showed that a multilocus approach including nuclear loci could successfully delimit those same species. Since then research within *Disholcaspis* has used the ribosomal internal transcribed spacer region 2 (ITS2) as a nuclear locus accompanied by mitochondrial cytochrome b (cytb) to infer conspecific relationships between sexual generations and asexual generations (Melika et al. 2013, McEwen et al. 2015).

In preparation for eventual revision, field work and lab work were undertaken in an effort to increase the current knowledge of the genus. Contributions to the genus occurred in two ways; first by adding to our understanding of the morphology of the sexual generations and by adding a new species. The second way that this study

contributes to the genus is that the current locations of all the type specimens were examined and the majority of the species were imaged. This increases the foundation of data surrounding this genus, bringing it one step closer to being ready for revision.

Methods

Specimen collecting. —

Galls were collected from across the United States of America during 2013–2017 and, when possible, were stored individually until the wasps emerged. Wasps were transferred to either ethanol or RNAlater until they could have their DNA extracted and be preserved.

Taxon sampling. —

Various sexual generation specimens and one asexual generation specimen were reared and needed molecular identification. Identified taxa from GenBank (Table 2) were chosen to compare to the unidentified specimens based on whether they shared at least 97% sequence identity using BLAST and individual sequences were chosen based on whether a single specimen had both cytb and ITS2. Some comparison sequences were excluded because they were identical to other comparison sequences being used. All comparison sequences used were from Nicholls et al. (2017).

For the preparation of the genus for eventual revision, the majority of species were chosen for imaging (Table 3). The United States National Museum (USNM) and the American museum of Natural History (AMNH) contained the most types for the genus and imaging efforts were concentrated on those specimens. The newer Central American taxa were not imaged since their descriptions already provided modern imaging.

Molecular analysis. —

Eighteen sexual generation specimens and one asexual generation specimen were sequenced for this study. Total DNA was extracted from whole specimens soaked for a minimum of 2 hours in proteinase K and extraction buffer. The DNeasy ® tissue kit spin column protocol (QIAGEN 2006) was followed except the last elution was not repeated to maintain higher DNA concentration in the extract. Two genes, cytb and ITS2 (following McEwen et al. 2015 and Melika et al. 2013) were amplified using the primers and thermal cycling conditions listed in Table 1. Two different thermal cycling programs were used for ITS2 and both were successful. The second ITS2 thermal cycling program was a touchdown program that decreased the annealing temperature by 1° C each cycle. Polymerase chain reaction (PCR) product was processed and sequenced at either the Beltsville Agricultural Research Center or at the Smithsonian Laboratory of Analytical Biology Washington, DC. There were four specimens that only had ITS2 data (USNMENT00961702, USNMENT00961954, USNMENT00961465, and USNMENT00961466).

Sequences were trimmed and forward and reverse consensus sequences were generated and aligned, using Geneious ® v. 9.1.5. Comparison sequences were obtained through NCBI GenBank (Table 2). Genes were aligned and then concatenated using Geneious. Data was run through RAxML v.8 (Stamatakis 2014) on the CIPRES Science Gateway (Miller et al. 2010) with the settings GTRGAMMA, rapid bootstrapping, partitioned by locus and codon position, with 100 bootstrap replicates, and the outgroup was set to *D. spongiosa* Karsch following Nicholls et al. (2017). All specimens were included in a proportional pairwise distance analysis, excluding ambiguous sites, using

PAUP* v. 4.0b10 (Swofford 2002) for each individual gene and the concatenated alignment.

The sequence data was evaluated using three criteria. First, the phylogeny (Fig. 1) was used to determine which sexual generations were forming a monophyletic group with a single species. Second, the observed maximum intraspecific distance was determined and used as a relatedness cutoff for determining species identifications. This criterion was met if there was only one species within the cutoff. In cases where there are more than one species found within that cutoff, host plant data was used to help determine to which species the sexual generation belongs. Specimens that share a host plant with another species that has not yet been sequenced required a more conservative approach. For these cases, a third criterion was used in which specimens had to have zero distance between sequences to be considered conspecific.

Table 1. Primers used for PCR amplifications of cytochrome b (cytb) and the ribosomal internal transcribed spacer 2 (ITS2) in various *Disholcaspis* wasps.

	Source	Cycling Conditions		
Primer	Sequence	Denaturation	Annealing	Extension
cytb	Jermiin and Crozier (1994)	94° C	46° C	72° C
CB1	5’ TATGTACTACCATGAGGACAAATATC 3’	(30 sec)	(30 sec)	(40 sec)
CB2	5’ ATTACACCTCCTAATTTATTAGGAAT 3’			x35 cycles
ITS2	Campbell et al. (1994)	94° C	55°C	72°C
ITS2f	5’ TGTGAACTGCAGGACACATG 3’	(45 sec)	(45 sec)	(90 sec)
ITS2r	5’ AATGCTTAAATTTAGGGGGTA 3’	-or-		x29 cycles
		92° C	58° C	72° C
		(10 sec)	(15 sec)	(1.5 min)
			-1° C ea. cycle	x13 cycles

Table 2. *Disholcaspis* specimens analyzed and their GenBank accession numbers for cytb and ITS2. Asterisks denote specimens previously identified and sequenced by Nicholls et al. (2017). Remaining specimens were newly collected and sequenced for this study.

Specimen	**Accession Number**
D_edura_Disedu3*	KX683528, KX683718
D_edura_Disedu9*	KX683530, KX683719
D_edura_Disedu16*	KX683525, KX683716
D_eldoradensis_Diseld2*	KX683639, KX683720
D_eldoradensis_Diseld4*	KX683533, KX683721
D_eldoradensis_Diseld7*	KX683534, KX683722
D_insulana_Disins1*	KX683535, KX683723
D_insulana_Disins2*	KX683536, KX683724
D_mamillana_Dismam3*	KX683541, KX683729
D_mamillana_Dismam6*	KX683542, KX683730
D_mamillana_Dismam8*	KX683544, KX683732
D_mamillana_Dismam11*	KX683539, KX683727
D_mellifica_Dismel1*	KX683547, KX683735
D_mellifica_Dismel8*	KX683549, KX683736
D_mellifica_Dismel12*	KX683546, KX683734
D_pedunculoides_Disped4*	KX683553, KX683739
D_pedunculoides_Disped5*	KX683554, KX683740
D_pedunculoides_Disped15*	KX683550, KX683737
D_pedunculoides_Disped18*	KX683551, KX683738
D_perniciosa_Disper1*	KX683555, KX683741
D_perniciosa_Disper3*	KX683557, KX683743
D_prehensa_Dispre1*	KX683559, KX683745
D_prehensa_Dispre3*	KX683560, KX683746
D_pruniformis_Dispru1*	KX683561, KX683748
D_quercusglobulus_Disqgl1*	KF554466, KF554484
D_quercusglobulus_Disqgl2*	KF554467, KF554485
D_quercusglobulus_Disqgl3*	KX683562, KX683749
D_quercusglobulus_Disqgl4*	KX683563, KX683750
D_quercusmamma_Disqma2*	KF554459, KF554475
D_quercusmamma_Disqma4*	KF554460, KF554477
D_quercusmamma_Disqma6*	KF554455, KF554469
D_quercusmamma_Disqma7*	KF554462, KF554479
D_quercusmamma_Disqma9*	KF554464, KF554482
D_quercusmamma_Disqma10*	KF554465, KF554483
D_quercusmamma_Disqma11*	KF554456, KF554470
D_quercusmamma_Disqma16*	KF554457, KF554472

Table 2 continued. *Disholcaspis* specimens analyzed and their GenBank accession numbers for cytb and ITS2. Asterisks denote specimens previously identified and sequenced by Nicholls et al. (2017). Remaining specimens were newly collected and sequenced for this study.

Specimen	**Accession Number**
D_quercusomnivora_Disqom2*	KX683565, KX683751
D_quercusomnivora_Disqom3*	KX683566, KX683752
D_quercusvirens_Disqsu1*	KF039986, KF040003
D_quercusvirens_Disqsu4*	KF039988, KF040004
D_quercusvirens_Disqsu6*	KF039989, KF040005
D_quercusvirens_Disqvi8*	KF040001, KF040009
D_quercusvirens_Disqvi10*	KF039992, KF040007
D_quercusvirens_Disqvi24*	KF039997, KF040008
D_rubens_Disrub2*	KX683570, KX683756
D_rubens_Disrub6*	KX683574, KX683760
D_rubens_Disrub24*	KX683569, KX683755
D_rubens_Disrub40*	KX683571, KX683757
D_simulata_Dissim6*	KX683578, KX683764
D_simulata_Dissim10*	KX683576, KX683762
D_spissa_Disspi8*	KX683586, KX683772
D_spissa_Disspi15*	KX683583, KX683769
D_spissa_Disspi21*	KX683584, KX683770
D_spongiosa_Disspo3*	KX683589, KX683775
101_010_370_USNMENT00961656	MG190055, MG192111
101_010_379_USNMENT00961866	MG190056, MG192112
101_010_389_USNMENT00961522	MG190057, MG192113
101_010_409_USNMENT00961819	MG190058, MG192114
101_010_458_USNMENT00961943	MG190059, MG192115
101_010_636_USNMENT00961763	MG190060, MG192116
101_010_637_USNMENT00961458	MG190061, MG192117
101_010_642_USNMENT00961924	MG190062, MG192118
101_010_704_USNMENT00961568	MG190063, MG192119
101_010_706_USNMENT00961761	MG190064, MG192120
101_010_758_USNMENT00961772	MG190065, MG192121
101_010_784_USNMENT00961547	MG190066, MG192122
101_010_786_USNMENT00961957	MG190054, MG192110
101_010_369_USNMENT00961702	MG190067
101_010_405_USNMENT00961954	MG190068
101_010_601_USNMENT00961465	MG190069
101_010_608_USNMENT00961466	MG190070
USNMENT00961585_JLM0570	MG190071, MG192123
USNMENT01231547_EI20	MG190072, MG192124

Morphological Analysis. —

Insect specimens were point mounted or card mounted using shellac gel. Sexual generation specimens and one asexual generation specimen went through DNA extraction before being mounted for preservation. Color photos were taken using an EntoVision micro-imaging system with a Leica M16 zoom lens and JVC KY-75U 3-CCD digital video camera attached to a M16 column on a Wild M-5 stereo microscope. Whole, point mounted, uncoated specimens were imaged using a Hitachi TM3000 desktop scanning electron microscope (SEM) set to charge up reduction and used in Analy mode. Images will be deposited on morphbank prior to publishing and new specimens were deposited at USNM with the barcodes listed in Table 2.

Type specimens were in most cases tracked down and requested. The most recently described Central American species were not requested since they were already imaged. Museum codes listed in Table 3 are based on the original descriptions or if needed, updated to modern museum codes following Evenhuis (2017).

Host plant vouchers were attempted for all collected galls. However, many of the plant samples were delicate young spring growth that didn't fare well during transportation and were too badly damaged to be preserved. Samples that survived were pressed and mounted on 11-1/2 x 16-1/2 inch herbarium paper using water-soluble glue. Identifications were made in the field and confirmed in the lab using the key in Nixon (1997). Host plant vouchers were deposited in the Norton Brown Herbarium at the University of Maryland (MARY).

Terminology for morphological features follows Melika (2006) and the Hymenoptera Anatomy Ontology Portal

(http://portal.hymao.org/projects/32/public/ontology/). Antennal segment length ratios are presented as scape:pedicel:F1:F2:F3:F4:F5:F6:F7:F8:F9:F10:F11:F12. Lateral ocellus width was measured by its longest width. Mesosoma length was measured laterally from the anterior margin of the pronotum collar to the posterior-most tip of the scutellum, and height measured perpendicular to that from the top of the mesoscutum through the anterior 1/3 of the tegula to the lower mesopleuron. Mesoscutal width was measured by the longest width which is just anterior to the tegula.

Results

Molecular analyses. —

There was no barcode gap seen in either gene or in the combined matrix. There are numerous species that have interspecific distances smaller than the maximum intraspecific variation. The observed maximum intraspecific distance seen in the ITS2 matrix was 1.0152% and the interspecific variation was 0–6.0914%. The observed maximum intraspecific distance seen in the cytb matrix was 3.6145% and the interspecific variation was 0–9.1566%. The observed maximum intraspecific distance seen in the combined matrix was 2.1014% and the interspecific variation was 0.1236–7.1693%. The cutoff used for both genes combined was 2.1014% and the cases where only ITS2 was available 1.0152% was used as the cutoff for that gene. It should be noted that within ITS2 *D. edura* + *D. spissa* were the only species with zero distance between them and this may be indicative of taxonomy problems between these two species. The galls for these two species can be very difficult to tell apart (Figs. 2–3; pers. comm. J. Nicholls) and the only morphological difference is in coloration (Weld 1957). These same two species as well as *D. quercusmamma* (Walsh and Riley) + *D.*

quercusglobulus (Fitch) had zero distance between their cytb sequences which could be indicative of mitochondrial introgression between these species (Nicholls et al. 2012). There is no evidence of this kind of relationship between the other species in the analysis.

Following the first criterion, the maximum likelihood (ML) tree showed a subset of five sexual generation specimens that were sister to *D. mamillana* Weld (Fig. 1) forming a monophyletic group (100% bootstrap support). Meeting the second criterion, specimens USNMENT00961866, USNMENT00961522, USNMENT00961943, and USNMENT00961819 were at most 0.3399% different from *D. mamillana* and none had another identified asexual specimen within 2.1014% difference. The other sexual generation specimen that form the monophyletic clade with *D. mamillana* was USNMENT00961954 (only had ITS2 data) and it also met the second criterion with 0.6980% difference from *D. mamillana* and no other taxa within the ITS2 cutoff. All specimens mentioned came from the same host plant, *Q. douglasii* Hooker and Arnott which is a known host plant for *D. mamillana* (Weld 1957). There is another *Disholcaspis* species on *Q. douglasii* (Burks 1979) that was not in the analysis because it has not yet been sequenced, *D. canescens* Bassett. Specimens USNMENT00961866, USNMENT00961522, and USNMENT00961943 all had zero distance between their sequences and Dismam6 for both genes, meeting the third criterion. Specimen USNMENT00961819 also met the third criterion since it had identical cytb sequences to both Dismam6 and Dismam8. These specimens are determined to be *D. mamillana*. Specimen USNMENT00961954 is left unidentified at this time since it has another

unsequenced species on the same host plant species and while it was genetically very close to *D. mamillana* it does not identically match the sequence data.

Seven sexual generation specimens were grouped together and were nested within *D. prehensa* Weld (80% bootstrap support; Fig. 1) meeting the first criterion. Specimens USNMENT00961656, USNMENT00961763, USNMENT00961924, USNMENT00961568, and USNMENT00961761 all had more than one species within the cutoff values. These sexual generation specimens were an average of 1.2114 % dissimilar to *D. prehensa* but also 1.9530% different from *D. mellifica* and 2.0354 % different from *D. eldoradensis*. Three of the specimens came from *Q. berberidifolia* Liebmann but the others lack host data. *Quercus berberidifiolia* is a documented host for *D. prehensa* (Burks 1979, Gross 2007 and 2012, and Weld 1957) though it used to be called *Q. dumosa* (Burks 1979, Nixon 1997, and Weld 1957). The other two specimens that monophyletically grouped with *D. prehensa* only had ITS2 and were an average of 0.2538% different from *D. prehensa.* There were no other species within the cut off so these specimens meet the second criterion. However, the only documented host plant for any of these sexual generation specimens, *Q. berberidifolia*, is also a known host plant for the non-sequenced *D. canescens* (Burks 1979). Therefore, the third criterion is warranted in this case. The specimen USNMENT00961761 had identical ITS2 sequence compared to both Dispre1 and Dispre3, meeting the third criterion. It is determined to be *D. prehensa*. Specimens USNMENT00961656, USNMENT00961763, USNMENT00961924, and USNMENT00961568 all had zero distance between cytb sequences compared to USNMENT00961761 so they should get the same identification as that specimen based on the third criterion. It should be noted

that the ITS2 of these specimens was only one base pair different from that of Dispre1 and Dispre3. These sexual generation specimens are determined to be *D. prehensa*. Specimens USNMENT00961702 and USNMENT00961465 had identical ITS2 to the previous subset of specimens suggesting that they too are conspecific and should get the same identification under the third criterion. These sexual generation specimens are determined to be *D. prehensa*.

The sexual generation specimen USNMENT00961585 was the only specimen sequenced out of a set of six specimens reared from the same host plant. This specimen came out nested within a polyphyletic *D. quercusglobulus* + *D. quercusomnivora* (Ashmead) + *D. quercusmamma* group so it doesn't meet the first criterion. These three species are noted to show less than 2.1014% interspecific difference. This sexual generation specimen is on average 1.3906% different from *D. quercusglobulus*, 1.1897% different from *D. quercusmamma*, and 1.4833% different from *D. quercusomnivora;* all within the cutoff. The combined data suggest that *D. quercusmamma* is the more closely related species. However, when just ITS2 is examined it shows that the distance to both *D. quercusmamma* and *D. quercusglobulus* is 0.5711% but the closest specimen was D_quercusglobulus_Disqgl3 at 0.2538% different (two base pairs). In this case, the genetic evidence has narrowed it down to three species but cannot differentiate between them using these genes alone. This sexual generation specimen was collected from *Q. alba* Linnaeus, which is only known as a host plant for *D. quercusglobulus* and not the other two species. Asexual *D. quercusglobulus* galls were also noted on the host plant at the time of collection. Since the sexual generation of *D. quercusmamma* has already been described, all six of the

reared specimens were compared and morphological differences were found between the sexual generation *D. quercusmamma* and this set of specimens. It is not likely that this sexual generation specimen is *D. quercusomnivora*, which comes from a host plant in Florida (Weld 1959), because this specimen was collected from Maryland. Based on the genetic evidence alone this specimen could not be identified, however including host plant data, collection locality, and the morphological differences observed compared to *D. quercusmamma* this specimen and its cohorts are thought to mostly likely be *D. quercusglobulus*. However, *D. terrestris* Weld can also be found on *Q. alba* and has not been sequenced. Since the sequences for ITS and cytb are not identical to an identified asexual generation specimen this specimen does not meet the third criterion. Therefore, these sexual generation specimens are left unidentified until a closer genetic match can be found or until *D. terrestris* is sequenced.

Two more sexual generation specimens had multiple species with some but not all specimens meeting the percent difference cutoff and are left unidentified until more sequence data can confirm identifications. USNMENT00961458 was nested within *D. eldoradensis*, (81% bootstrap support) which also has *D. mellifica* and *D. prehensa* nested within. All three were within the cutoff at 0.9477% different from *D. eldoradensis,* 1.4421 % different from *D. mellifica*, and 1.4833% different from *D. prehensa*. No host data is availble for this specimen to help narrow down the potential species from the second criterion. There are numerous species within *Disholcaspis* that are not sequenced and this specimen is not genetically identical to any other specimen in this analysis. While this specimen's sequences were closer to *D. eldoradensis* it cannot be determined at this time. It is left unidentified until a better asexual generation

sequence match can be found. USNMENT00961547 was nested within *D. spissa* Weld and *D. edura* Weld with low support. Its sequences were 0.7417% different from *D. spissa* and 0.7417% different from *D. edura*. It should also be noted that it was within 2.1014% different from *D. insulana* Kinsey, *D. pedunculoides* Weld, *D. perniciosa* (Bassett), and *D. rubens* (Gillette) though it was not phylogenetically grouped with those species in the ML tree. There is no host data for this specimen. While its sequences were closer to *D. spissa*, it is being left unidentified until closer sequences from additional asexual specimens are found.

The sexual generation specimens USNMENT00961466 and USNMENT00961772 were grouped together (100% bootstrap support) though it should be noted that USNMENT00961466 only had sequence data for ITS2. There were no other species closely grouped with these specimens and it is possible that their species is not sequenced yet. They both remain unidentified at this time and their host plant voucher specimen, identified as *Q. turbinella* Greene, is deposited at MARY. Sexual generation specimen USNMENT00961957 was not nested within a species and was not sister to a single species. This sexual generation specimen is left unidentified at this time but the host plant is identified as *Q. gambelii* Nuttall and deposited at MARY.

The specimen USNMENT01231547 is an asexual generation specimen representing the newly described species. It came out grouped with *D. pruniformis* + *quercusglobulus* + *quercusomnivora* + *quercusmamma* with 86% bootstrap support. However, it is shown to be at least 3.0902% different from those species and at least 3.7083% different from every other specimen in this analysis demonstrating that it is genetically distinct from the represented species. The ITS2 distances for this specimen

showed slightly greater variation with it being at least 5.5838% different from the species it was grouped with and at least 4.8223% different from the other represented species. The biology of this specimen will be discussed later.

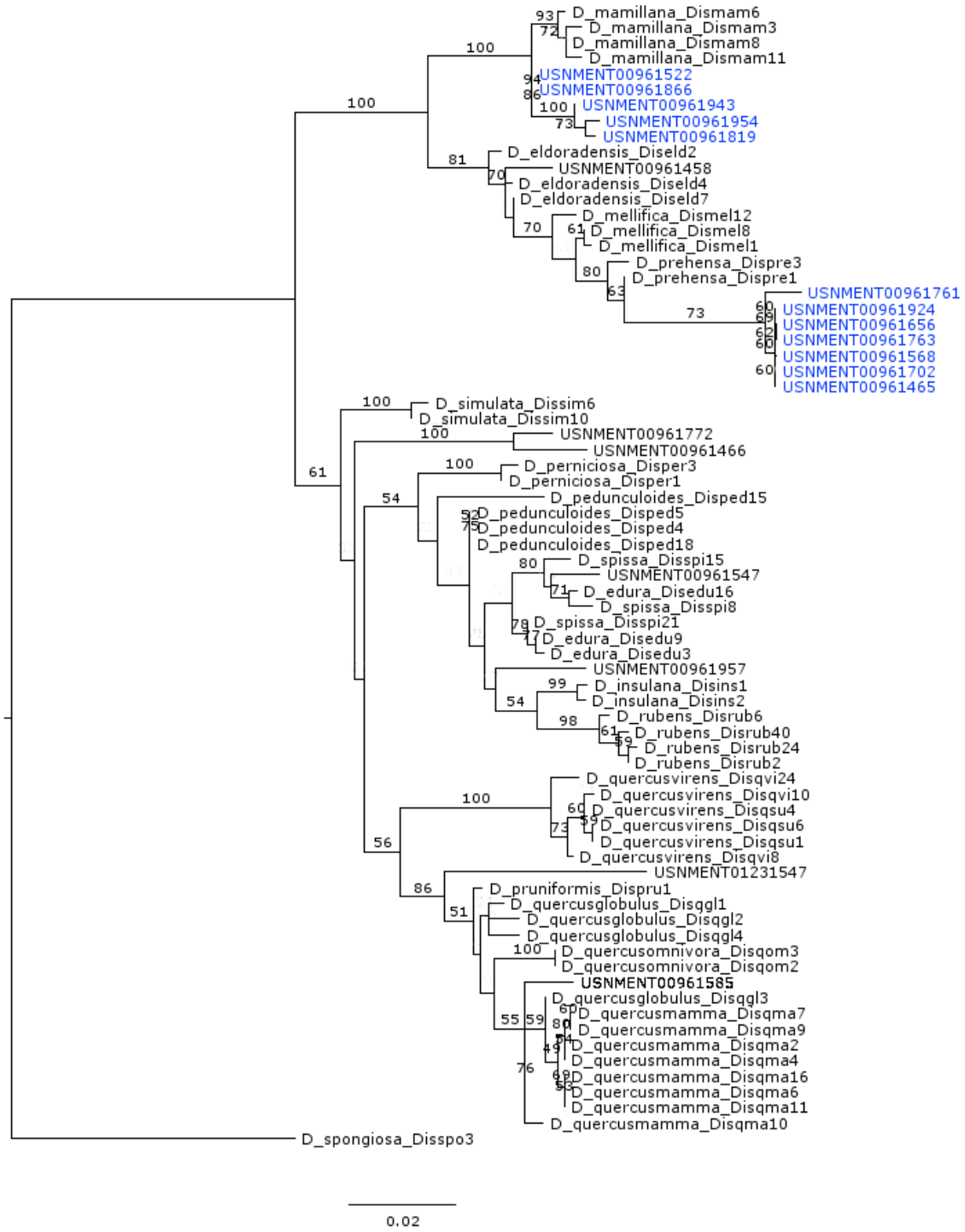


Figure 1. Maximum likelihood tree for cytb and ITS2, for known species of *Disholcaspis* and unidentified specimens. Names starting with "D_" represent specimens from Nicholls et al. (2017). Names starting with USNM are newly collected; those in blue (only) can be assigned to previously-described species based on phylogenetic placement, genetic distances, and host plant data. Bootstrap values above 50% are shown to the left of the nodes.

Morphology of *Disholcaspis*.—

Asexual generation wasps.

The traits that differentiate *Disholcaspis* sensu stricto from other genera are as follows: Mesosoma (Fig. 4–5). Notauli incomplete anteriorly; scutellar foveae generally absent or not apparent, scutellum sometimes with anterior transverse impression in the place of foveae; propodeal carinae fragmented and curved or absent. Metasoma (Fig. 6–7). Setation on metasoma generally limited to patches laterally on the second tergum; extended portion of the hypopygial spine tapering and is at most 4.5 times as long as wide; hypopygial spine with long subapical setae that reach beyond the apex of the spine but never form a dense, truncate tuft.

Variation and exceptions.

Disholcaspis corallina (Bassett), *D. plumbella* Kinsey, *D. washingtonensis* (Gillette), and *D. arizonicus* (Cockerell) are exceptions to the setation on the metasoma with all terga setose. As noted by Melika and Abrahamson (2002) there was a subset of species that differ from the rest of the genus morphologically. This subset included *D. chrysolepidis* (Beutenmuller), *D. conalis* Weld, *D. corallina*, *D. lasia* (Ashmead), *D. plumbella*, *D. reniformis* (McCracken and Egbert), *D. spectabilis* (Kinsey), *D. sulcata* (Ashmead), and *D. washingtonensis*. *Disholcaspis arizonicus* morphologically agrees with some of the species in this subset and should also be included. Melika and Abrahamson thought that this subset all had the hypopygial spine as broad throughout its entire length and the length of the extended portion of the spine appearing equal to or less than its width. The shape of the hypopygial spine in some of these taxa were examined using SEM imaging and the shape of some was found to be more complicated

than originally thought. The species *D. corallina*, *plumbella*, and *washingtonensis* have the hypopygial spine laterally bulbous basally followed by a very short parallel-sided thinner section (Fig. 8). The species *D. sulcata,* lasia, and *arizonica* may also have similarly shaped hypopygial spines but they were difficult to see and the lateral sections weren't as bulbous as in the previously described spines. The species *D. chrysolepidis*, *conalis,* and *spectabilis* have convexly tapering spines that are about as long as wide (Fig. 9). The shape of the hypopygial spine of *D. reniformis* has not been examined. The two species *D. lasia* and *chrysolepidis* were grouped together in the UCE phylogeny (See chapter 2) but differed in the shape of the hypopygial spine.

Sexual generation.

In comparison to the asexual generation, sexual generation wasps are less pubescent, with a smoother mesosoma, are smaller, and tend to be black or dark brown (*D. quercusvirens* is an exception) with brown to golden legs (Evans 1972, Platt 2009, Bird et al. 2013, Melika et al. 2013, McEwen et al. 2015).

Gall Morphology.

Disholcaspis asexual generation galls are generally characterized as being monothalamus, detachable, woody, twig or root galls. As with most gall wasps, the morphology of *Disholcaspis* galls is generally species specific on a given host plant and is often times the only diagnostic trait for identifying species. In some species many galls develop close together while in others the galls are distributed more sparsely. Some of the wasps in this group are known to induce the host to secrete nectar or honeydew from the outer gall surface, in asexual galls, which is thought to be involved in a mutualistic relationship with ants that tend the galls as a result (Inouye and Agrawal

2004, Nicholls et al. 2017, Seibert 1993). The nectar secretions can also attract stinging wasps which can be problematic in domestic horticultural settings (Eckberg and Cranshaw 1994). The galls basically become extrafloral nectaries in a group that generally don't have floral or extrafloral nectaries (Bernardello 2007, Taylor et al. 2012, Weber and Keeler 2013). The induction of nectar secretions and the galls themselves are thought to be part of the extended phenotype of the gall wasp (Nicholls et al. 2017, Stone et al. 2002).

Unlike the asexual generation galls, the sexual generation galls have been found to be very similar between species with the galls looking similar to buff colored grains of rice that are located in or around the meristem of the leafing buds in the early spring. The spring galls often times disfigure the newly developing stem making it shorter and the new leaf petioles will often curl around the gall making it difficult to see the gall without spreading or removing the leaves (Fig. 10).

Descriptions. —

Disholcaspis mamillana Weld, sexual generation

Diagnosis. Body dark brown, F2 0.75–0.9x shorter than F1, wing veins dark brown, extended portion of ventral hypopygial spine tapering evenly to tip, and extended portion of ventral hypopygial spine 1.7–2.2x as long as broad basally. Found on *Q. douglasii* (Weld 1957).

Sexual female (Fig. 11–19). Total body length 2.1–2.5mm. Color. Antennae and mouth palps brown, sometimes F1–4 slightly lighter brown; head, mesosoma, and coxae dark

brown to black; legs light brown to golden; metasoma dark brown; wings hyaline with dark brown veins.

Head (Fig. 12–14). Anteriorly 1.3x as broad as high, finely coriaceous with malar space more delicately coriaceous; rounded in anterior view with ocelli raised; lower face with sparse setae. Gena not broadened behind compound eye in anterior view; lateral width of the gena 0.34x as wide as the compound eye height. Compound eye 0.7x as high as frontal head height, transfacial line 0.9x as long as compound eye height; length of malar space 0.2x height of compound eye; compound eyes parallel. LOL 0.82–1x as long as the lateral ocellus; OOL 0.86x length of lateral ocellus; POL 2.3–2.6x as long as OOL and 2–2.4x as long as length of lateral ocellus. Diameter of torulus subequal to distance between them; distance between torulus and compound eye margin subequal to the diameter of torulus. Clypeus rectangular, 0.9x as broad as high, ventral margin convex; tentorial pits present; clypeo-pleurostomal line indistinct; epistomal sulcus broad, shallow, smooth, and bare. Occiput rounded and without carina. Labial palpus 3-segmented, maxillary palpus 4-segmented. Antennae 14–segmented; pedicel 1.3x as long as broad; antennal segment length ratio 19:11:30:22:22:21:18:16:13:12:11:12:11:16; placoid sensilla present on F3–12.

Mesosoma (Fig. 15–18). Laterally 1.1x as long as high. Pronotum setose medially, setose dorsally, ventrally with sparse setae, margin along mesopleuron with fine striations; impressed along propleuron. Propleuron striate along margins, flat medially. Mesoscutum delicately coriaceous lateral to the notauli and along anterior margin,

sometimes very delicately coriaceous between notauli; 1.1x as wide as long. Notauli present in posterior 2/3 fading to coriaceous texture anteriorly, incomplete and converging posteriorly; anterior parallel lines not visible; parapsidal lines broad and smooth, extending just over 1/3 length of mesoscutum; median mesoscutal line absent; setae along notauli and lateral margin of mesoscutum. Mesoscutellum slightly longer than broad, rugose, overhanging metanotum; scutellar foveae indistinct sometimes smooth and sometimes with rugose texture intruding. Mesopleuron mostly smooth with several indistinct transverse striations, with sparse setae in the striated area and near mesocoxa foramen.; mesopleural triangle setose and with transverse striations; acetabular carina distinct. Dorsoaxillar area smooth but setose, slightly convex; axillar carina with longitudinal striae; axillula setose and smooth; subaxillular bar smooth; metapleural sulcus reaching mesopleuron just over half its height. Metascutellum delicately rugose; metanotal trough smooth and with setae; ventral impressed rim smooth. Lateral propodeal carinae bent outward at the middle sometimes nearly merging dorsally forming a ring, medial propodeal area with medial carina extending over half way to the nucha; lateral propodeal area setose; nucha short with longitudinal carinae. Forewing extending beyond apex of metasoma; radial cell 3.8x as long as wide; Rs curved slightly, nearly reaching wing margin; areolet closed and indistinct; Rs+M reaching or nearly reaching M. Tarsal claws with basal lobe.

Metasoma. Tergum 2 with setae laterally, following 4 tergites without setae; all tergites smooth and shiny. Ventral hypopygial spine (Fig. 19) tapering evenly to tip, extended

portion 1.7–2.2x as long as broad basally, with subapical setae reaching beyond spine apex.

Male. Unknown.

Gall (Fig. 20). Length 2.4–2.9mm, buff colored, oblong grain-shaped, bluntly pointed, thin walled, and glabrous. Arising from the buds at the base of new leaves during spring flush. Emergence holes are rough edged and near the apex.

Host plant remarks. The recorded host for the asexual generation wasps is *Q. douglasii* (Weld 1957). All sexual generation specimens here identified as *D. mamillana* were taken from a field identified *Q. douglasii*. No host voucher is available for these specimens.

Disholcaspis prehensa Weld, sexual generation

Diagnosis. Body dark brown, F1 shorter than scape+pedicel, F2 subequal to F1, wing veins brown, extended portion of ventral hypopygial spine parallel near base then tapering to tip, and extended portion of ventral hypopygial spine 1.1–1.2x as long as broad basally.

Sexual female (Fig. 21–30). Total body length 2.2–2.4mm. Color. Antennae and mouth palps light brown to brown, sometimes getting gradually darker towards apex; head, mesosoma, and coxae dark brown to black; legs light brown to golden; metasoma dark brown; wings hyaline with dark brown veins.

Head (Fig. 22–24). Anteriorly 1.1x as broad as high, finely coriaceous with malar space more delicately coriaceous; rounded in anterior view with ocelli raised; lower face with sparse setae. Gena not broadened behind compound eye in anterior view; lateral width of the gena 0.35x as wide as the compound eye height. Compound eye 0.6x as high as frontal head height, transfacial line 1.1x as long as compound eye height; length of malar space 0.6x height of compound eye; compound eyes slightly converging ventrally. LOL 0.99–1.4x the length of the lateral ocellus; OOL 1.5–1.8x length of lateral ocellus; POL 1.3–1.5x as long as OOL and 2.2–2.6x as long as length of lateral ocellus. Diameter of torulus subequal to distance between them; distance between torulus and compound eye margin 1.2x the diameter of torulus. Clypeus rectangular, 1.3x as broad as high, ventral margin convex; tentorial pits present; clypeo-pleurostomal line indistinct; epistomal sulcus broad, shallow, smooth, and bare. Occiput rounded and without carina. Labial palpus 3–segmented, maxillary palpus 4–segmented. Antennae 14–segmented; pedicel as long as broad; antennal segment length ratio 16:7:21:19:18:17:14:14:11:12:10:11:8:14; placoid sensilla present on F3–12.

Mesosoma (Fig. 25–29). Laterally 1.2x as long as high. Pronotum with delicate longitudinal striations medially, setose dorsally, ventrally with sparse setae, margin along mesopleuron with fine striations; impressed along propleuron. Propleuron striate along margins, flat medially. Mesoscutum delicately coriaceous around notauli and along anterior and anterolateral margins, sometimes very delicately coriaceous between notauli and on anterior third of mesoscutum; 1.1x as wide as long. Notauli present in

posterior 2/3 fading to coriaceous texture anteriorly, incomplete and converging posteriorly; anterior parallel lines either not visible or only visible when the anterior third of mesoscutum is delicately coriaceous; parapsidal broad and smooth, extending just over 1/3 length of mesoscutum; median mesoscutal line absent; setae along notauli and lateral margin of mesoscutum. Mesoscutellum slightly longer than broad, rugose, overhanging metanotum; scutellar foveae indistinct with rugose texture intruding. Mesopleuron mostly smooth with several indistinct transverse striations, setae near mesocoxa foramen; mesopleural triangle setose and with transverse striations; acetabular carina distinct. Dorsoaxillar area smooth but setose, slightly convex; axillar carina with longitudinal striae; axillula setose and delicately coriaceous; subaxillular bar smooth; metapleural sulcus reaching mesopleuron just over half its height. Metascutellum delicately rugose; metanotal trough smooth and with setae; ventral impressed rim smooth. Lateral propodeal carinae bent outward at the middle sometimes nearly merging dorsally forming a ring, medial propodeal area with varying numbers of carinae extending from the lateral propodeal carinae towards the nucha; lateral propodeal area setose; nucha short with longitudinal carinae. Forewing extending beyond apex of metasoma; radial cell 3.9x as long as wide; M, Rs+M, and Cu1a barely visible; Rs curved slightly, nearly reaching wing margin; areolet closed and indistinct. Tarsal claws with basal lobe.

Metasoma. Tergite two with setae laterally, following four tergites without setae; all tergites smooth and shiny. Extended portion of ventral hypopygial spine (Fig. 30)

parallel basally then tapering to tip, extended portion 1.1–1.2x as long as broad basally, with subapical setae reaching beyond spine apex.

Male (Fig. 31–34). Similar to females in coloration and size; however antennae generally a brown to dark brown, antennae 15-segmented, basal half of femora are brown, and propodeum with lateral propodeal carinae not nearly merging dorsally.

Gall (Fig. 35). Length 2.2–3.5mm, buff to light brown colored, oblong grain-shaped, bluntly or sharply pointed, thin walled, and glabrous. Arising from the buds at the base of new leaves and flowers when they flush in the spring. Emergence holes are rough edged and near the apex.

Host plant remarks. *Quercus dumosa* is the host plant listed in the original description of *D. prehensa* (Weld 1957). However, the name *Q. dumosa* has been applied to almost all species of scrub oak in central and southern California. Asexual generation *D. prehensa* have also been photo-documented (Gross J. 2007 and 2012) and reared from *Q. berberidifolia* (listed as host for specimen D_prehensa_Dispre3). The host plant voucher for the sexual generation specimens USNMENT00961761, USNMENT00961924, and USNMENT00961763 is deposited at MARY and identified as *Q. berberidifolia*. This shows that the sexual generation of *D. prehensa* is found on the same host species as the asexual generation. There is no host plant identification or voucher specimen corresponding to USNMENT00961702, USNMENT00961656, USNMENT00961568 and USNMENT00961465.

Disholcaspis erugomamma Cooke, **sp. nov.**

Diagnosis. Special emphasis was placed on comparing *D. erugomamma* with other species that have been found on the same host plant, those that could be collected in Texas, and species that had similar galls. The only other species of *Disholcaspis* documented on *Q. havardii* Rydberg is *D. spissa* (Weld 1957). *Disholcaspis erugomamma* differs from *D. spissa* in that it has the scape + pedicel shorter than F1 rather than slightly longer, the notauli black instead of brown, mesoscutellum mostly black instead of mostly brown, and the extended portion of the hypopygial spine 1.3–1.9x as long as wide instead of 2.3x as long as wide. *Disholcaspis erugomamma* differs from most of the Texas inhabiting species in that the head has black markings around the toruli, ocellar triangle, and malar margins; antennae are black; notauli black; mesoscutellum mostly dark brown to black; and metasoma mostly black. Lastly, most species in Texas differ from *D. erugomamma* in their gall morphologies. No specimen was examined for *D. fungiformis* Kinsey but this species has dramatically different gall morphology with an expanded skirt around the base of the gall and a mushroom-shaped top portion of the gall (Fig. 48). *Disholcaspis fungiformis* is also only known from *Q. virginiana* Miller (Kinsey 1920 and Weld 1959). The type for *D. cinerosa* Bassett and *D. spongiosa* could not be found for comparison so general collection specimens were used. *Disholcaspis erugomamma* is morphologically most similar to *D. quercusmamma, D. quercusglobulus, D. bassetti* (Gillette), *D. globosa* Weld, *D. persimilis* (Ashmead), *D. colorado* (Gillette), and *D. lacuna* Weld. However, it should be noted that this species was determined in this study to be genetically distant from *D. quercusmamma*

and *D. quercusglobulus*. The diagnostic combination of traits for *D. erugomamma* is antennae black with some specimens having first 4 segments dark brown apically; head trapezoidal in anterior view; a black patch at the upper corner of the gena; notauli black; mesoscutellum black at least along anterior and lateral margins; metasoma mostly dark brown to black; extended portion of hypopygial spine 1.3–1.9x as long as wide; and galls 1.2–1.5mm smooth surfaced, rounded, and globular shaped galls with a short pointed nipple. For the examined types and other specimens representing the other *Disholcaspis* species see Table 3.

Asexual female (Fig. 38–46). Total body length 3.9mm. Color. Antennae, area around and below toruli, clypeus, malar space margin, area around ocelli, patch at upper corner of gena, back of head black; rest of head brown to dark brown; mouth palps dark brown at base and fading to brown distally; mandibles brown medially and black along the margins; compound eyes and ocelli silver. Pronotum medially, along posterior margin, and along anteroventral margin black, remainder brown to dark brown; mesoscutum, with thick area around anterior parallel lines, parapsidal lines, and notauli black, rest of mesoscutum dark brown; scutellum black except posterior margin dark brown; mesopleural triangle, mesopleural margins, and patch extending diagonally across mesopleuron from mesopleural triangle black, remainder brown to dark brown; propodeum and metanotum black. Legs dark brown, coxae brown. Metasoma mostly black with dark brown in small patch laterally on the second tergum and along some tergite margins.

Head (Fig 38–40). Anteriorly 1.3–1.4x as broad as high, dorsally 0.38x as long as broad, coriaceous with lower face, malar space, and gena more finely coriaceous, coriaceous to finely rugose around ocellar triangle; near-trapezoidal in anterior view with ocelli raised; face with white setae throughout but more dense on lower face. Gena broadened behind compound eye in anterior view; lateral width of the gena 0.57x as wide as the compound eye height. Compound eye 0.50x as high as frontal head height; transfacial line 1.3x as long as compound eye height¸ diameter of torulus 1.1x distance between them; distance between torulus and compound eye margin 1.2x diameter of torulus; length of malar space 0.44x height of compound eye, without striae and sulcus; compound eyes nearly parallel. LOL 1.5x as long as the lateral ocellus; OOL 2.1x length of lateral ocellus; POL 1.5x as long as OOL and 3.1x as long as length of lateral ocellus. Clypeus trapezoidal with ventral margin longer than dorsal margin, 1.6x as broad as high, ventral margin convex; anterior tentorial pits present; clypeo-pleurostomal and epistomal sulcus distinct, deeply depressed, and smooth. Occiput rounded, setose, and without carina; postocciput smooth; postgena setose, otherwise smooth; height of occipital foramen 0.78x as long as postgenal bridge and 1.1x as long as broad; hypostomal sulci separated at oral foramen. Labial palpus 3-segmented, maxillary palpus 4-segmented. Antennae 14-segmented; pedicel as long as broad; antennal segment length ratio 24:11:39:36:30:28:22:19:13:11:11:9:8:13; placoid sensilla on F4–F12 conspicuous, absent on F1–F3.

Mesosoma (Fig. 41–44). Laterally 1.2x as long as high. Pronotum with white setae, smooth laterally; impressed along propleuron. Propleuron setose but smooth otherwise.

Mesoscutum delicately coriaceous except smooth between notauli, setose; 1.2x as wide as long. Notauli present in posterior 1/2 fading to coriaceous texture anteriorly, incomplete and converging posteriorly; anterior parallel distinct, extending 0.47x as long as mescutum length; parapsidal lines broad and smooth, extending just over 1/2 length of mesoscutum; median mesoscutal line absent. Mesoscutellum slightly broader than long, smooth and depressed along anterior margin, coriaceous otherwise, overhanging metanotum, with white setae, more setose on posterior half; scutellar foveae absent. Mesopleuron smooth, setosee throughout; mesopleural triangle densely setose and with transverse striations; acetabular carina only visible ventrally. Dorsoaxillar area setose; axillar carina with longitudinal striae; axillula densely setose; subaxillular bar smooth; metapleural sulcus reaching mesopleuron just over half its height. Metascutellum delicately rugose; metanotal trough smooth and with setae; ventral impressed rim smooth. Lateral propodeal carinae bent outward and broken, medial propodeal area smooth; lateral propodeal area setose; nucha short and rugose. Forewing extending beyond apex of metasoma; radial cell 3.5x as long as wide; M, Rs+M, and Cu1a visible; Rs curved, nearly reaching wing margin; areolet closed Rs+M reaching half way to M. Tarsal claws with basal lobe.

Metasoma (Fig. 45–46). Length 0.83x as long as mesosoma and head. Second tergite 0.70x length of metasoma; tergite 2 with setae anteromedially to laterally, following 4 tergites without setae; all tergites smooth and shiny. Hypopygial spine ventrally tapering evenly to tip, extended portion 1.9x as long as broad basally, with subapical setae reaching beyond spine apex.

Variation. Some specimens have more black coloration on the upper face between the toruli and the ocelli; in a few specimens the basal 4 antennal segments are dark brown apically while the remainding segments are black; notauli in some specimens with a black patch along the medial margin; in a few specimens the mesoscutellum is dark brown medially as well as along the posterior margin. The variation in the various ocellar lengths compared to the lateral ocellus widest width is as follows: LOL 1.5–2.0x as long as the lateral ocellus; OOL 1.8–2.7x length of lateral ocellus; and POL 3.1–4.3x as long as length of lateral ocellus. There is variation in the ratios of the antennal segments but F1 is always longer than scape+pedicel and F2; radial cell 3.3–4.0x as long as wide; extended portion of the hypopygial spine 1.3–1.9x as long as wide. Radial cell 3.2–3.8x as long as wide.

Gall (Fig. 47). Reddish brown, globular with a short obtusely pointed nipple apically. Dispersed or more commonly tightly clustered and deforming around each other. Internally with dense, rather hard, corky texture; larval cell not free in galls examined. Gall is most similar to that of *D. quercusmamma* but the surface is wrinkled in that species and relatively smooth in *D. erugomamma*. It is uncertain if the galls are nectar secreting, but the black sooty mold that is typical for mature nectar secreting galls was lacking. The galls were covered in dust when found but it is uncertain if that dust was a trait of the gall itself as opposed to its dusty arid environment near a road.

Type Material. Holotype: first label “USA: TX: Crane Co. East bound I-20 frontage road, 31.650381, -102.745986 9.Nov.2013 C.Cooke”, second label “Ex. *Q. havardii*,

Emg. 25.Dec.2013, Assoc. gall USNMENT00961500", and third label "USNMENT 00961981". Paratypes: first label for all specimens "USA: TX: Crane Co. East bound I-20 frontage road, 31.650381, -102.745986 9.Nov.2013 C.Cooke"; one specimen with second and third labels "Ex. *Q. havardii*, Emg. 25.Dec.2013, Assoc. gall USNMENT00961500" and "USNMENT00961260"; five specimens with second label "Ex. Q. havardii, Emg. 26.Dec.2013, Assoc. gall USNMENT00961973" and third labels "USNMENT00961825", "USNMENT00961685", "USNMENT00961485", "USNMENT00961768", and "USNMENT00961455"; one specimen with second and third labels "Ex. Q. havardii, Emg. 30.Dec.2013, Assoc. gall USNMENT00961863" and "USNMENT00961882"; one specimen with second and third labels " Ex. Q. havardii, Emg. 12.Jan.2013, Assoc. gall USNMENT00961846" and "USNMENT00961488"; one specimen with second and third labels "Ex. Q. havardii, Emg. 25.Dec.2013, Assoc. gall USNMENT00961262" and "USNMENT00961932"; one specimens with second and third labels "Ex. Q. havardii, Emg. 30.Dec.2013, Assoc. gall USNMENT00961959" and "USNMENT00961641"; and three specimens with second labels "USNMENT00961734", "USNMENT00961798", and "USNMENT00961984".

Biology. Only the asexually reproducing generation is known at this time. All wasps reared for this study emerged toward the end of December or early January.

Etymology. Named for the gall morphology. From Latin, erugo meaning to free of wrinkles and mamma for referring to the mammiform gall shape (Brown 1956).

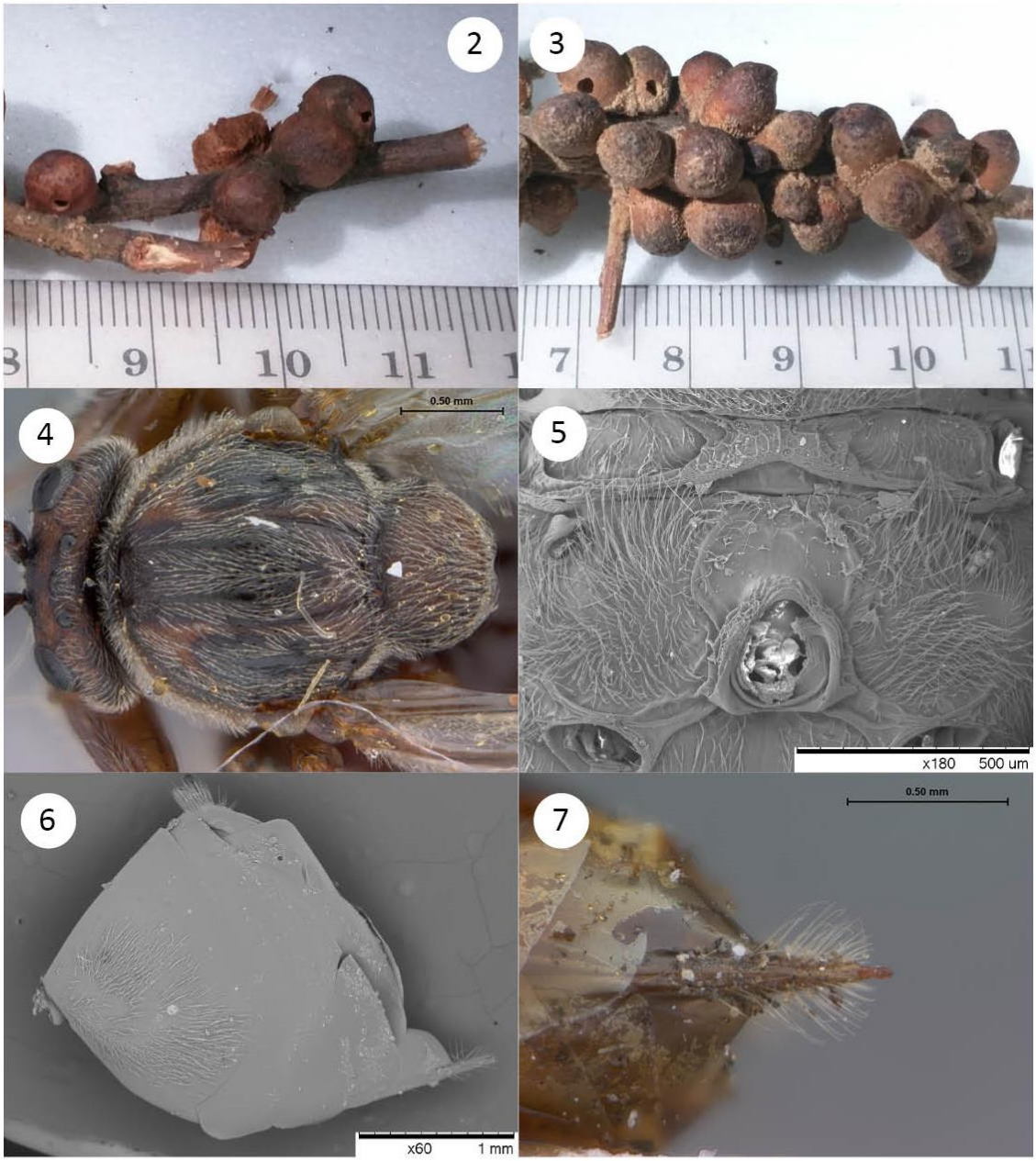
Distribution. Known only from the single collecting event in western Texas.

Host plant remarks. The host plant voucher is deposited at MARY and identified as *Q. havardii*. The host plant *Q. havardii* is has a small range from northwestern Texas to New Mexico and Oklahoma, with disjunct populations in northern Arizona and southern Utah (Nixon, 1997).

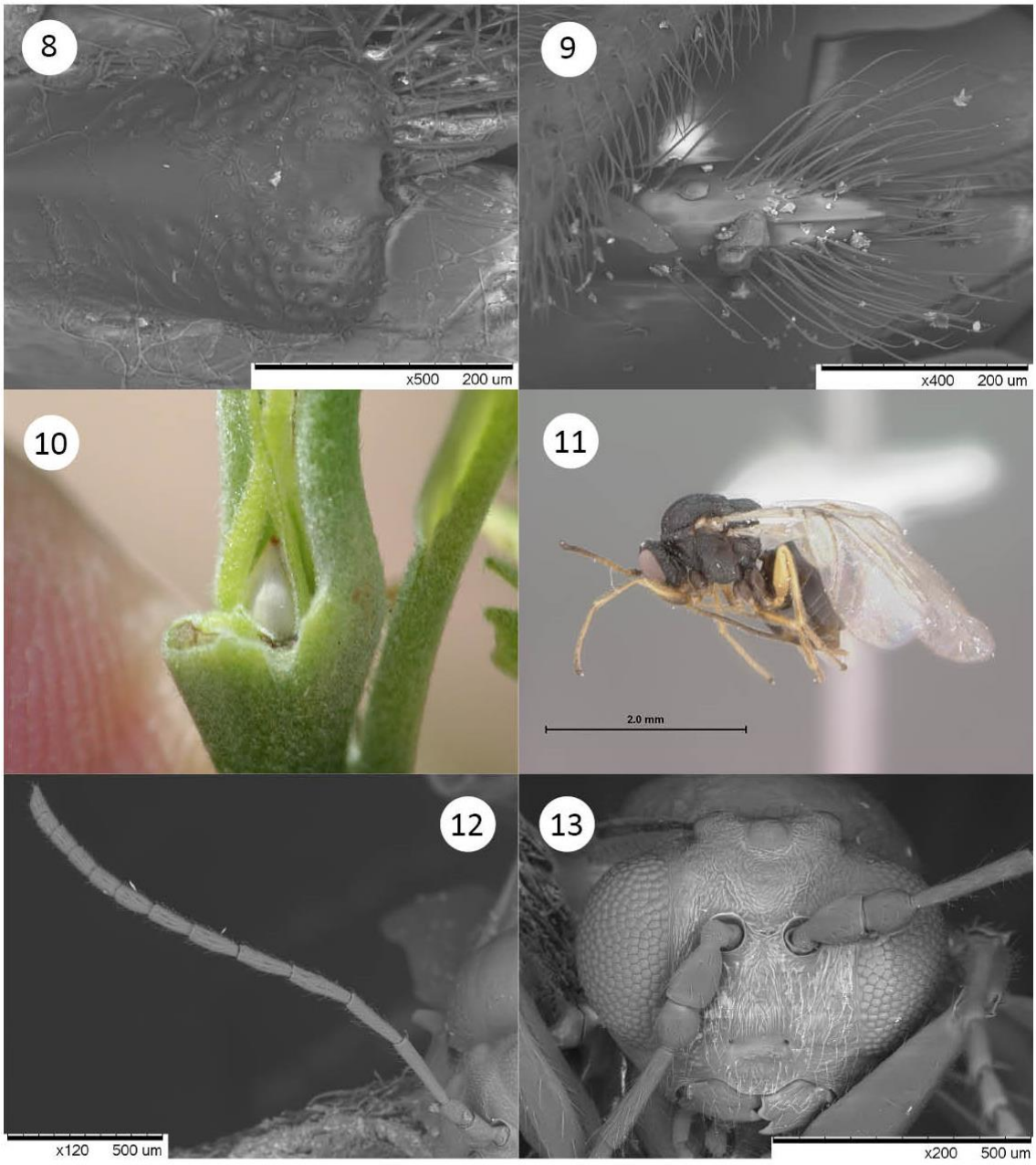
Key to the known sexual generations. —

1.–	Female and male main body color brown to light brown	*D. quercuvirens*
–	Female and male main body color dark brown to black	2
2.–	Female and male main body color dark brown, male and female ocelli similar	3
–	Female and male main body color black, males ocelli enlarged	*D. quercusmamma*
3.–	F1 as long as scape+pedicel	*D. mamillana*
–	F1 slightly shorter than scape+pedicel	*4*
4.–	Wing veins brown, host plant *Q. douglasii*	*D. prehensa*
–	Wing veins dark brown, host plant *Q. lobata* or *Q. garryana*	*D. eldoradensis*

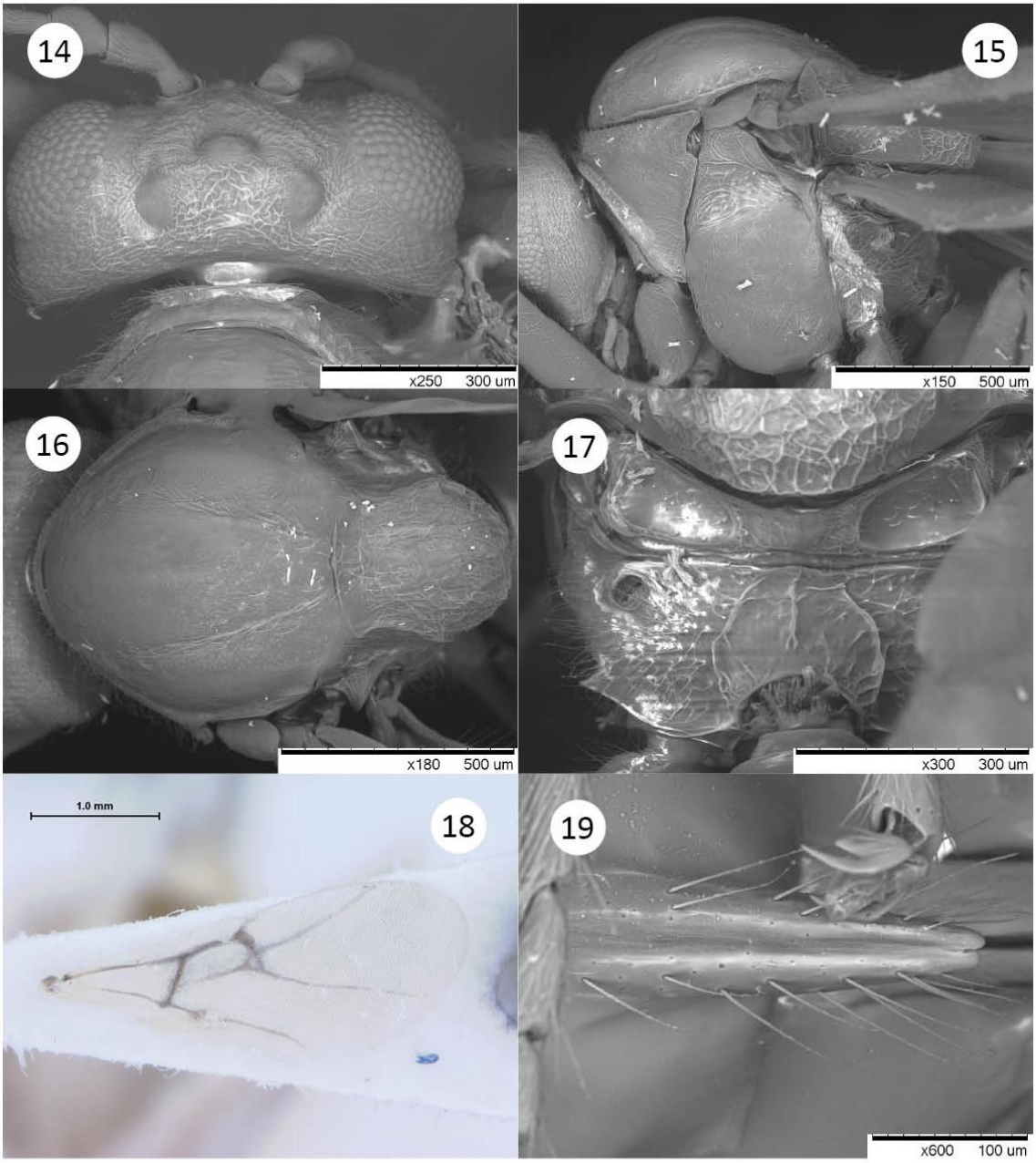
*Description (McEwen et al. 2015) said radial cell was 2.8x as long as wide but when measured again it was 3.8x as long as wide.



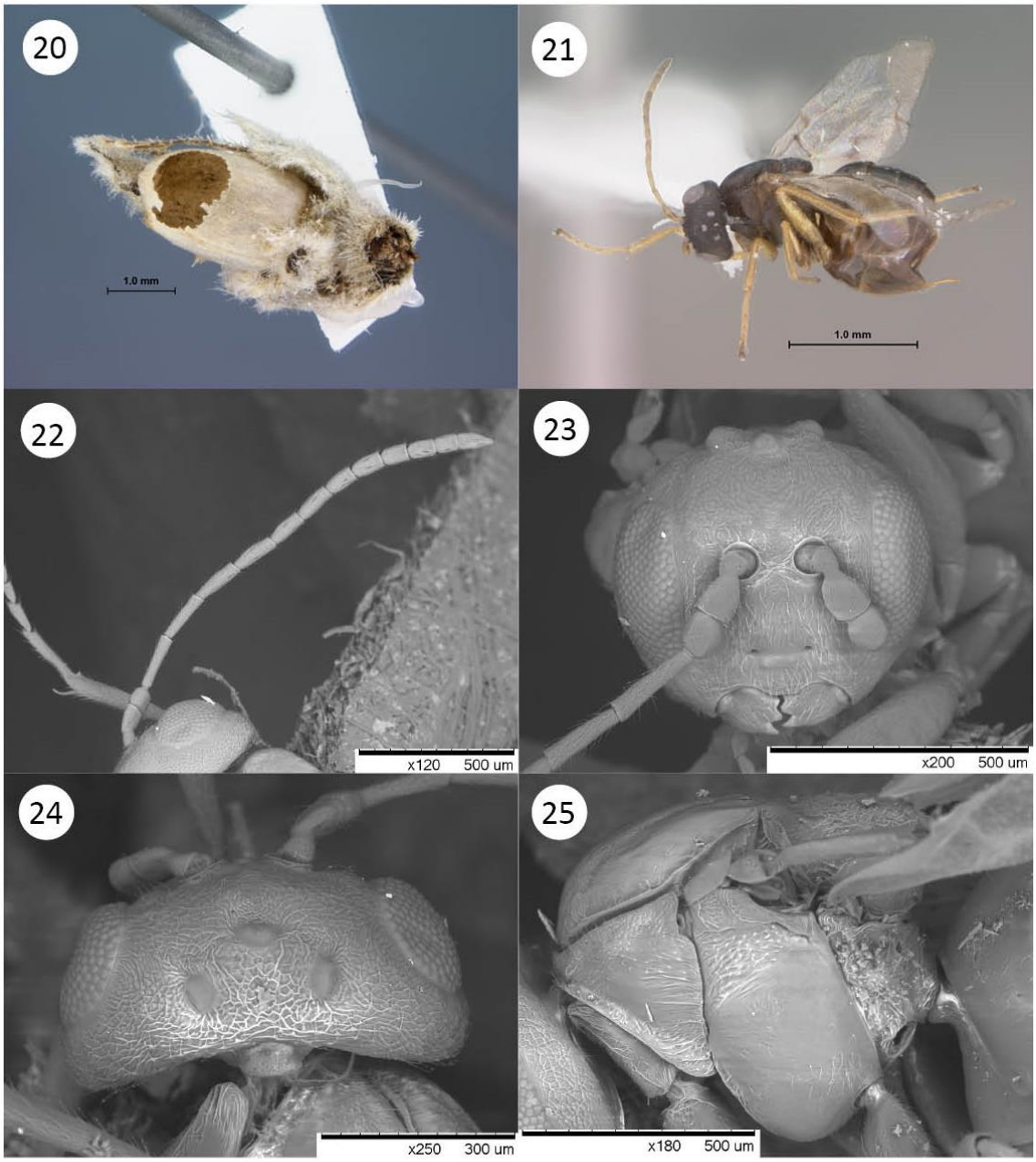
Figures 2-7. (2) Gall asexual generation *Disholcaspis edura*; (3) Gall asexual generation *D. spissa*; 4-7 asexual generation *D. quercusglobulus* (4) dorsal mesosoma; (5) propodeum; (6) lateral metasoma; (7) ventral hypopygial spine.



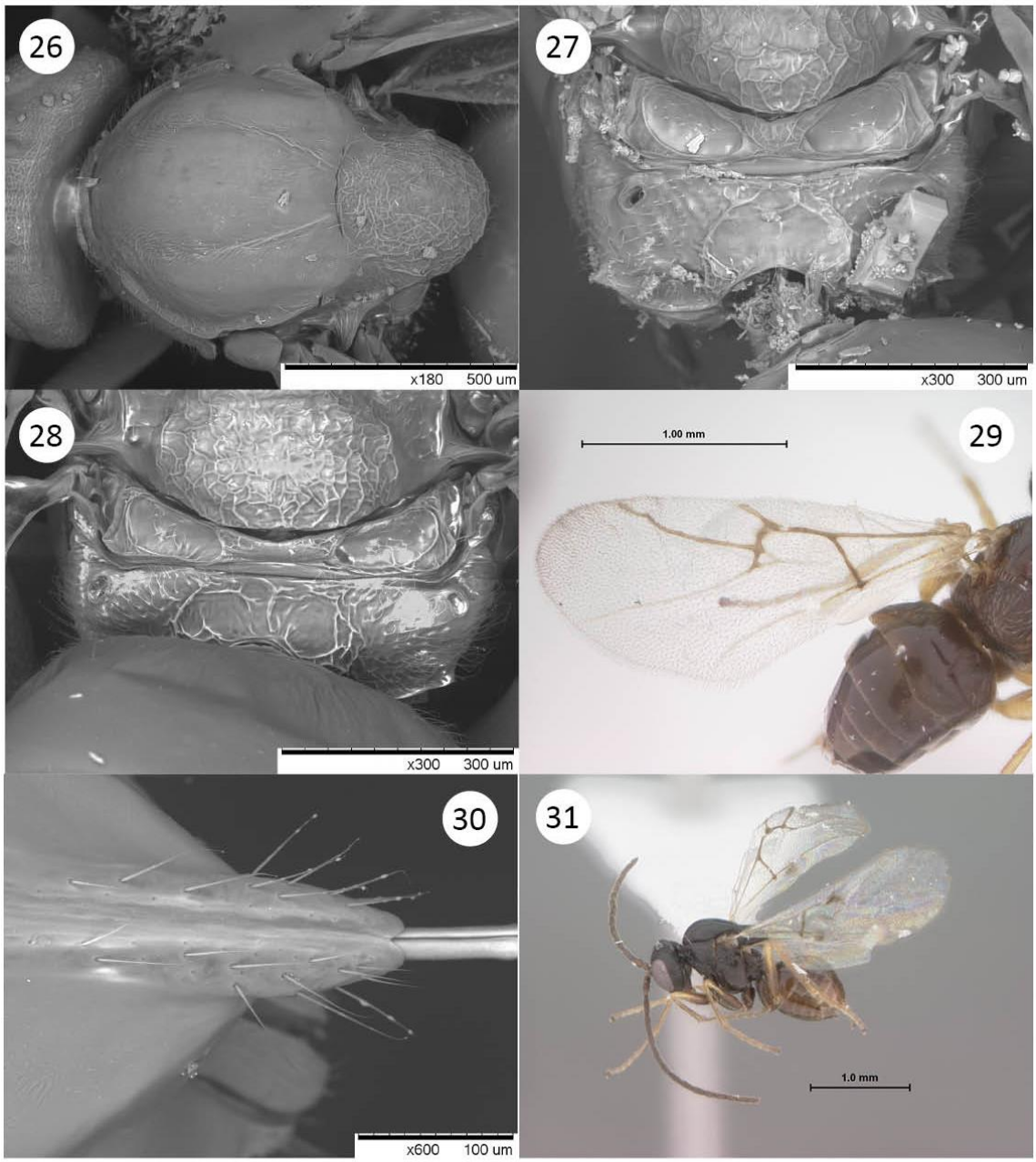
Figures 8-13. (8) Ventral hypopygial spine, *Disholcaspis corallina*; (9) ventral hypopygial spine, *D. chrysolepidis*; (10) *Quercus* sp. leaf petioles curling around a sexual generation gall with one leaf removed so that you can see the gall; 11-13 Sexual female *Disholcaspis mamillana*, (11) lateral habitus; (12) antenna; (13) anterior head.



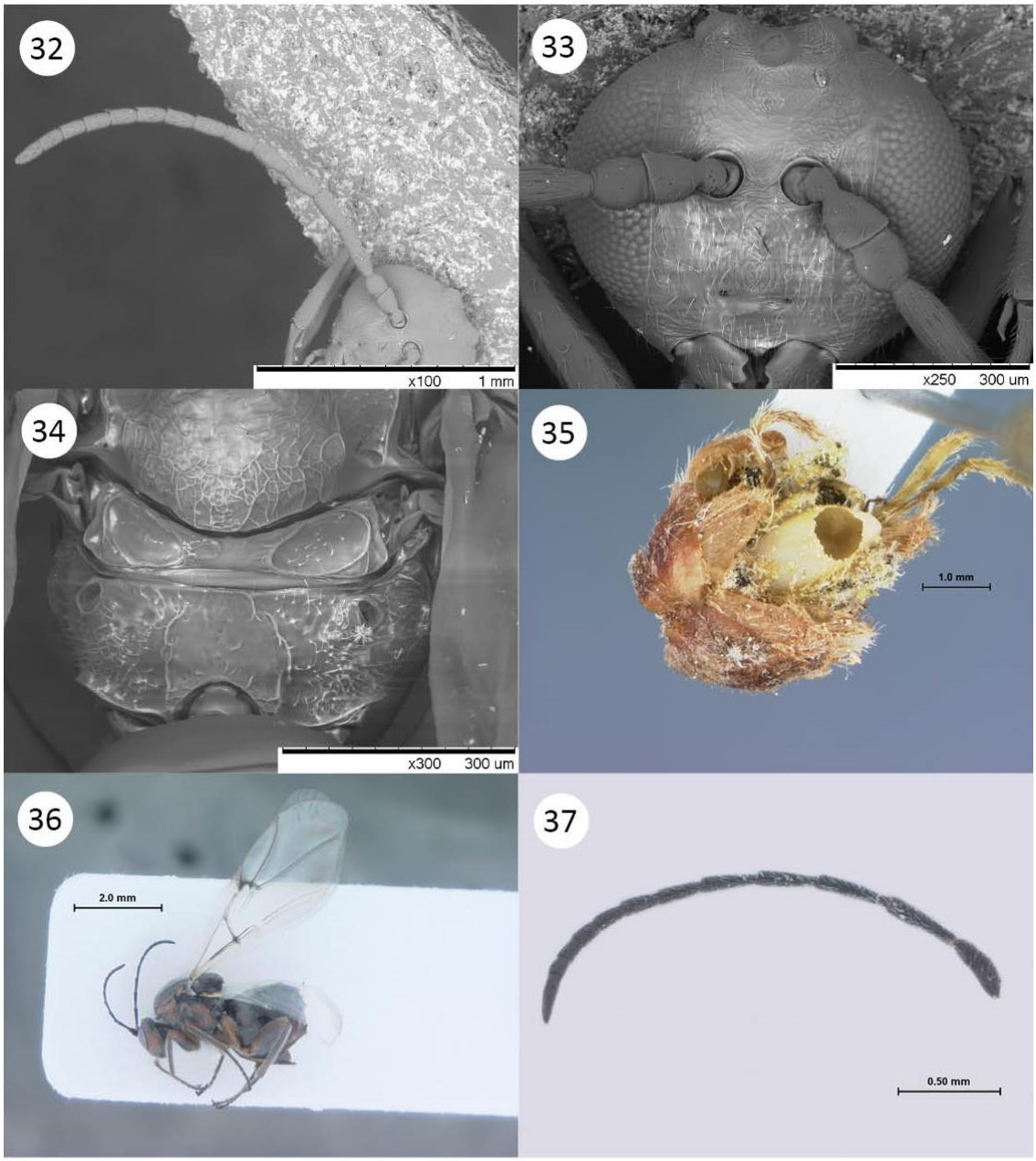
Figures 14-19. Sexual female *Disholcaspis mamillana*, (14) dorsal head; (15) lateral mesosoma, (16) dorsal mesosoma, (17) propodeum; (18) wing; (19) ventral hypopygial spine.



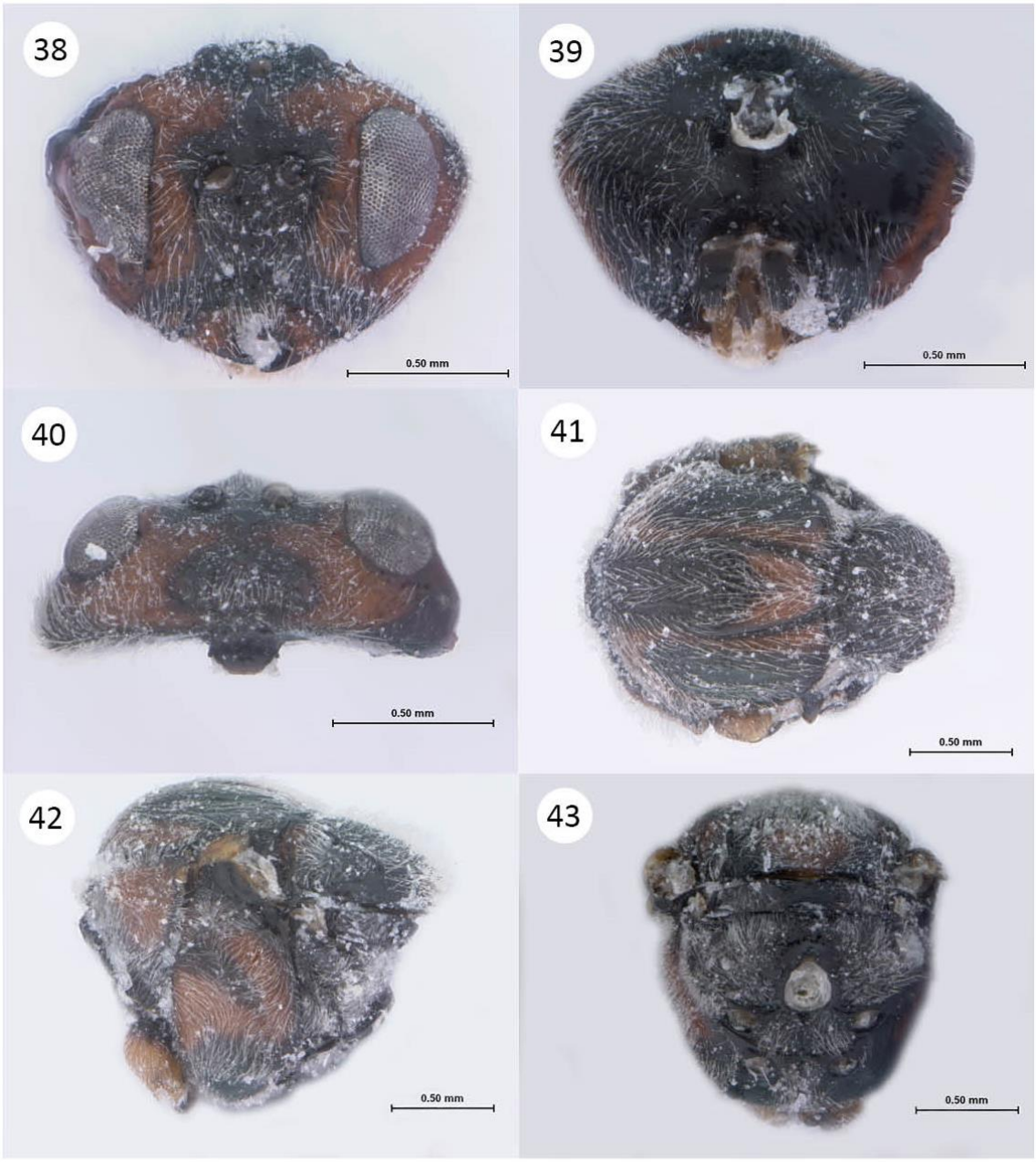
Figures 20-25. Sexual generation *Disholcaspis*. (20) Sexual generation gall, *D. mamillana;* 21-25 Sexual females *D prehensa*, (21) lateral habitus, (22) antenna, (23) anterior head, (24) dorsal head, (25) lateral mesosoma.



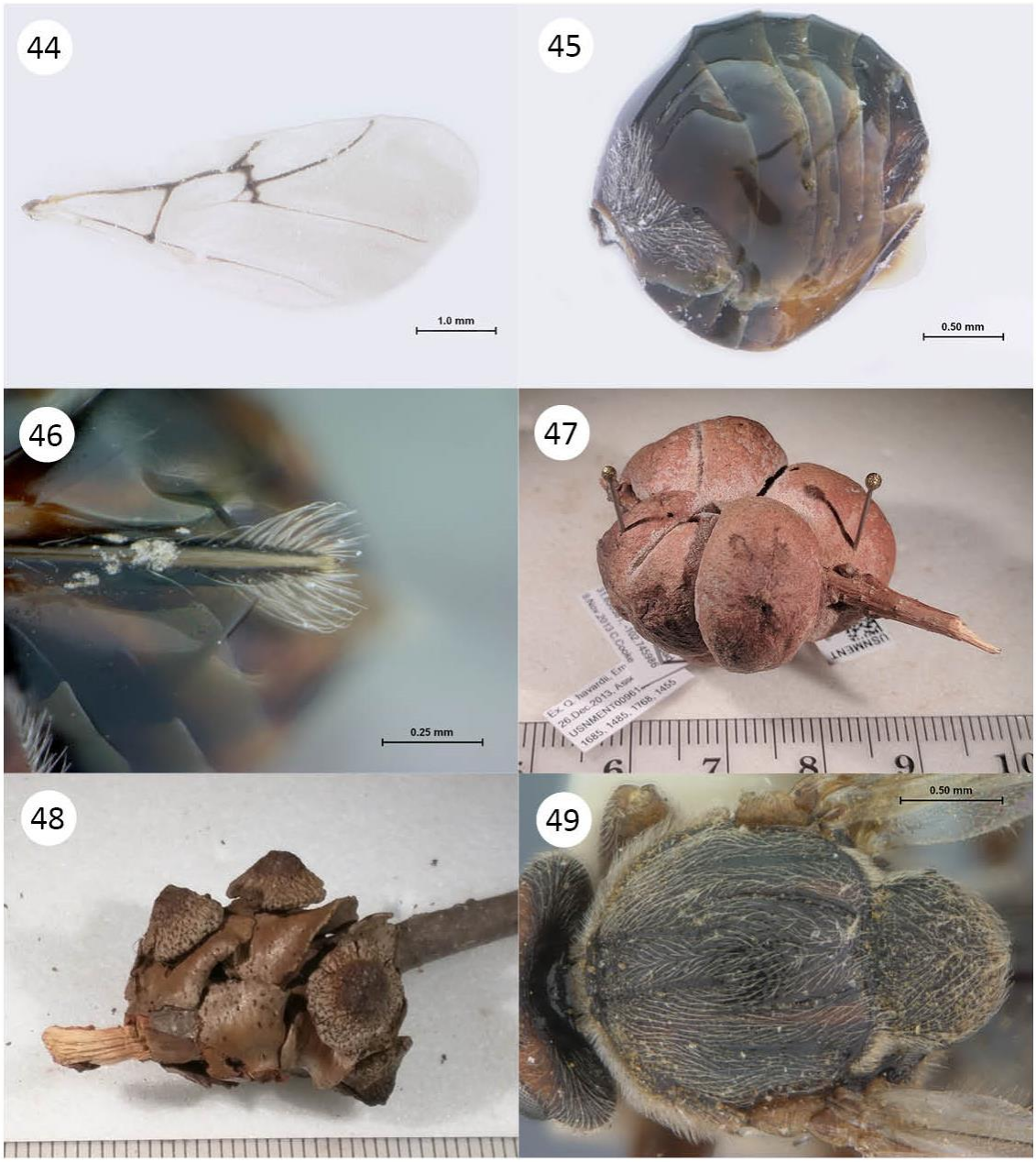
Figures 26-31. 26-30 Sexual *Disholcaspis prehensa*, (26) dorsal mesosoma, female, (27) propodeum, female, (28) propodeum, female, (29) wing, female, (30) ventral hypopygial spine; (31) lateral habitus, sexual male *D. prehensa*.



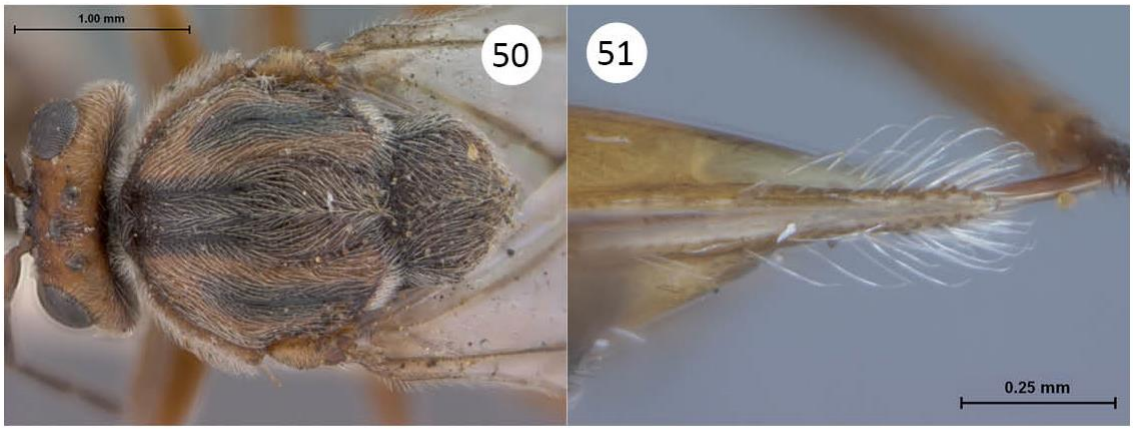
Figures 32-37. *Disholcaspis*. 32-34 sexual male *D. prehensa*, (32) antenna, (33) anterior head, (34) propodeum, (35) gall of sexual generation *D. prehensa;* 36-37 Asexual female *D. erugomamma* n. sp. (36) lateral habitus, (37) antenna.



Figures 38-43. Asexual female *Disholcaspis erugomamma* n. sp., (38) anterior head, (39) posterior head, (40) dorsal head, (41) dorsal mesosoma, (42) lateral mesosoma, (43) posterior mesosoma.



Figures 44-49. *Disholcaspis*. 44-46 Asexual female *Disholcaspis erugomamma* n. sp. . (44) wing; (45) lateral metasoma, (46) ventral hypopygial spine, (47) galls of *Disholcaspis erugomamma* n. sp., (48) galls of asexual *D. fungiformis*, (49) dorsal mesosoma, asexual *D. quercusglobulus*.



Figures 50-51. *Disholcaspis*. (50) dorsal mesosoma, asexual *D. quercusglobulus*, (51) ventral hypopygial spine, asexual *D. mamillana*.

Disholcaspis Type Specimen Notes. —

During the course of this study type and some non-type specimens were imaged for a majority of *Disholcaspis* species. Table 3 lists all the species in the genus *Disholcaspis*, their type location (if determined during this study), and which ones were imaged. There has also been a considerable amount of imaging performed on some of Kinsey's manuscript names. These specimens are listed as *Disholcaspis* sp. 1–17 in Table 3. Consistent diagnostic differences could not be determined between the manuscript name specimens and their closely related species so none are described at this time.

The type specimen for *D. cinerosa* is considered missing. It was supposed to be deposited at the American Entomological Society collection (ANSP) with the rest of Bassett's types but was documented in 1922 as missing at the time of the collection relocation (Cresson 1922). The collection at ANSP was checked again during this study and this specimen was determined to still be missing. Cresson (1922) mentioned that the type collection was divided with the intention of sending some specimens to Yale or Cornell. This side collection was said to have been evidently destroyed by Mrs. Bassett after her husband's death (Cresson 1922). It is possible the *D. cinerosa* type was with those ill-fated specimens but just in case the intended destinations for that side collection was checked as well. Yale looked for the specimen and did not have it in their holdings. Cornell's collection has their types digitized and there were no specimens for this species listed in their collection ("Browse the Collection", 2012). Cresson (1922) also made note that some of the Bassett types were found at the American Museum of Natural History (AMNH) and "other collections". All the *Disholcaspis* types were

requested from AMNH and this type was not found during their search for specimens. It is possible that the *D. cinerosa* type may be at another collection. Between 1881 when Bassett first described the species and 1902 when he died, the only two gall wasp workers to publish anything dealing with *Disholcaspis* were Gillette and Ashmead (Table 3). The Colorado State University C.P. Gillette Museum of Arthropod Diversity (CSUC) did not contain the specimen so it does not appear that Gillette had the specimen on loan. Ashmead's Hymenoptera collection ended up at USNM ("USNM Hymenoptera collections" 2017, para. 3) but the *D. cinerosa* type was not found in that collection either. The curator at USNM mentioned that some of Ashmead's material may have ended up at the British museum and their curator was not able to find the *D. cinerosa* type . The Illinois Natural History Survey was also mentioned by the USNM curator as a good place to search for early gall worker material so they were also contacted about this type specimen with no luck in finding it. This specimen is currently considered lost.

The location of the *D. mexicana* Beutenmüller type specimen is also unknown. It should probably be located at the AMNH with the rest of Beutenmüller's specimens but they did not find this specimen in their search for types. However, AMNH did have some general collection specimens for that species. Another specimen that was expected to be at AMNH but whose location is currently unknown is the type for *D. lasia sublasius* (Kinsey). The type for the other subspecies described in that same paper was found at AMNH but not this particular specimen.

It is unclear whether a type specimen exists for *D. quercusmamma*. There was no clearly designated specimen in the original description (Walsh and Riley 1869).

During the various searches for *Disholcaspis* types (Table 3) a specimen for this species was never found. Even the collection at the Illinois Natural History Survey was photographed and searched for this specimen. Unfortunately there are no other clues or mentions of a type to aid in the search.

As for the *D. spongiosa* type specimen, Beutenmüller (1909) mentioned that it might be at the Berlin museum. The Museum für Naturkunde (ZMHB) in Berlin was contacted and the type could not be found despite their collection including much of Karsch's material. The original description (Karsch 1880) did not specify that a type was designated so it is possible one does not exist.

Table 3. List of species or subspecies in *Disholcaspis*, with notes on location of type and imaging performed. The sp.1–17 refer to Alfred Kinsey's manuscript names.

Species	**Original genus**	**Author**	**Year**	**Type location**	**Images**
acetabula	*Disholcaspis*	Weld	1921	USNM	Type
arizonicus	*Holcaspis*	Cockerell	1902	USNM	Type
bassetti	*Holcaspis*	Gillette	1889	USNM	Type
bettyannae	*Disholcaspis*	Medianero and Neives-Aldrey	2011	MNCN	--
bisethiae	*Disholcaspis*	Medianero and Neives-Aldrey	2011	MNCN	--
brevinota	*Disholcaspis*	Weld	1921	USNM	Type
canescens	*Holcaspis*	Bassett	1890	ANSP	Type
chrysolepidis	*Holcaspis*	Beutenmüller	1911	USNM	Type
cinerosa	*Cynips*	Bassett	1881	missing	Other
colorado	*Holcaspis*	Gillette	1893	USNM	Type
conalis	*Disholcaspis*	Weld	1926	USNM	Type
corallina	*Holcaspis*	Bassett	1890	ANSP	Other
costaricensis	*Disholcaspis*	Melika and Pujade-Villar	2011	UB, Spain	--
edura	*Disholcaspis*	Weld	1957	USNM	Type
eldoradensis	*Holcaspis*	Beutenmüller	1909	AMNH	Type, Other
erugomamma	*Disholcaspis*	Cooke-McEwen	2018	USNM	Type
fungiformis	*Disholcaspis*	Kinsey	1920	MCZ	--
globosa	*Disholcaspis*	Weld	1921	USNM	Type, Other
heynei	*Disholcaspis*	Kieffer	1910	ZMHB	Type
insulana	*Disholcaspis*	Kinsey	1938	AMNH	Type, Other
lacuna	*Disholcaspis*	Weld	1921	USNM	Type, Other
laetae	*Disholcaspis*	Kinsey	1937	AMNH	Type, Other
largior	*Disholcaspis*	Kinsey	1938	ANMH	Type, Other
lasia	*Callirhytis*	Ashmead	1896	USNM	Type, Other
lasia sublasius	*Andricus*	Kinsey	1922	AMNH	--
lasia areolaris	*Andricus*	Kinsey	1922	unknown	--
mamillana	*Disholcaspis*	Weld	1957	USNM	Type
mellifica	*Disholcaspis*	Weld	1957	USNM	Type
mexicana	*Holcaspis*	Beutenmüller	1911	unknown	Other
pallens	*Disholcaspis*	Kinsey	1938	AMNH	Type, Other
pattersoni	*Disholcaspis*	Kinsey	1922	AMNH	Type
pedunculoides	*Disholcaspis*	Weld	1926	USNM	Type, Other
perniciosa	*Holcaspis*	Bassett	1890	ANSP	Type, Other
persimilis	*Holcaspis*	Ashmead	1896	USNM	Type

Table 3 Continued. List of species or subspecies in *Disholcaspis*, with notes on location of type and imaging performed. The sp.1–17 refer to Alfred Kinsey's manuscript names.

Species	**Original genus**	**Author**	**Year**	**Type location**	**Images**
plumbella	*Disholcaspis*	Kinsey	1920	MCZ	Other
potosina	*Disholcaspis*	Kinsey	1937	AMNH	Type, Other
prehensa	*Disholcaspis*	Weld	1957	USNM	Type
pruniformis	*Disholcaspis*	Kinsey	1920	MCZ	Paratype, Other
pulla	*Disholcaspis*	Kinsey	1937	AMNH	Type, Other
purlans	*Disholcaspis*	Kinsey	1937	AMNH	Type
purpurea	*Disholcaspis*	Kinsey	1937	AMNH	Type, Other
quercusglobulus	*Callaspidia*	Fitch	1859	USNM	Type, Other
quercusmamma	*Cynips*	Walsh and Riley	1869	unknown	Other
quercusomnivora	*Cynips*	Ashmead	1885	USNM	Type, Other
quercusvirens	*Cynips*	Ashmead	1881	USNM	Type, Other
regina	*Disholcaspis*	Kinsey	1937	AMNH	Type, Other
reniformis	*Andricus*	McCracken and Egbert	1922	CAS	--
rubens	*Holcaspis*	Gillette	1893	USNM	Type
sileri	*Holcaspis*	Bassett	1890	ANSP	--
simulata	*Disholcaspis*	Kinsey	1922	AMNH	Type
simulata vancouverensis	*Disholcaspis*	Kinsey	1922	AMNH	Type
spectabilis	*Andricus*	Kinsey	1922	AMNH	Type, Other
spectabilis incisus	*Andricus*	Kinsey	1922	AMNH	Type
spectabilis ukiahensis	*Andricus*	Kinsey	1922	AMNH	Type
spissa	*Disholcaspis*	Weld	1957	USNM	Type
spongiosa	*Diplolepis*	Karsch	1880	unknown	Other
sulcata	*Cynips*	Ashmead	1896	USNM	Type, Other
terrestris	*Disholcaspis*	Weld	1921	USNM	Type, Other
unicolor	*Disholcaspis*	Kinsey	1920	MCZ	--
washingtonensis	*Cynips*	Gillette	1894	USNM	Type, Other
sp. 1	--	--	--	AMNH	"Type"
sp. 2	--	--	--	AMNH	"Type"
sp. 3	--	--	--	AMNH	"Type"
sp. 4	--	--	--	AMNH	"Type"
sp. 5	--	--	--	AMNH	"Type"
sp. 6	--	--	--	AMNH	"Type"
sp. 7	--	--	--	AMNH	"Type"
sp. 8	--	--	--	AMNH	"Type"
sp. 9	--	--	--	AMNH	"Type"

Table 3 Continued. List of species or subspecies in *Disholcaspis*, with notes on location of type and imaging performed. The sp.1–17 refer to Alfred Kinsey's manuscript names.

Species	Original genus	Author	Year	Type location	Images
sp. 10	--	--	--	AMNH	"Type"
sp. 11	--	--	--	AMNH	"Type"
sp. 12	--	--	--	AMNH	"Type"
sp. 13	--	--	--	AMNH	"Type"
sp. 14	--	--	--	AMNH	"Type"
sp. 15	--	--	--	AMNH	"Type"
sp. 16	--	--	--	AMNH	"Type"
sp. 17	--	--	--	AMNH	"Type"

Discussion

This is the first time within *Disholcaspis* that a sexual generation is associated with the asexual generation without the use of rearing experiments. The findings from Nicholls et al. (2012) suggest that caution should be used when relying on mitochondrial loci, such as cytb, which is why it is generally only used in addition to ITS2. The other nuclear loci recently made available include long wave length opsin and ribosomal 28S. However, Nicholls (pers. comm.) found that there was little to no variation in those genes between *Disholcaspis* species, suggesting that they will be of little help in species delimitation. More loci need to be developed in this group.

At this time neither a review nor revision are attempted for this genus. The morphology of *Disholcaspis* species is relatively uniform with few, if any, unique and consistent traits between species. Diagnosis for many species relies almost solely on host plant data and gall morphology. Traits that may have diagnostic potential for at least some species include coloration, radial cell length to width ratio, and length to width ratio of the extended portion of the hypopygial spine. While these traits don't

diagnose all the species of *Disholcaspis*, they can be helpful when trying to differentiate between two or three closely related species.

There is some interspecific variation in coloration or dark markings that may help in diagnosing certain species. There are very few species of *Disholcaspis* without dark markings and few with as much dark coloration as *D. globosa* Weld. However, some species have also been shown to have a great amount of intraspecific variation in coloration making it a dubious method for diagnosing some species. For example, the type species *D. quercusglobulus* has variable degrees of dark patterns on the mesoscutum ranging from being nearly all dark brown with little differentiation between the dark markings and surrounding areas (Fig. 49) to being lighter brown with black markings around the anterior parallel lines, paraspidal lines, and toruli (Fig. 50) (one of the most common coloration patterns observed across *Disholcaspis*). There are multiple species that have demonstrated intraspecific variation in coloration but this has not been formally examined in most species.

In the species imaged, the ratio of length to width of the radial cell varies from 3.0–4.7x as long as wide. This is the only part of the wing that is commonly measured in gall wasps. Wing venation has been shown in other Hymenoptera to have diagnostic abilities (Kozmus et al. 2011, Oleska and Tofilski 2015, Schwarzfeld and Sperling 2014, and Villemant et al. 2007) and morphometric analysis might reveal other informative wing venation traits. Intraspecific variation in length to width ratio of the radial cell has not been examined across *Disholcaspis* though variation was noted in *D. erugomamma*.

The current study found variation in hypopygial spine length to width ratios but it is not clear whether this variation will prevent the trait from being diagnostic. One

complication to this trait is that it is measured ventrally but it can be hard to determine ventrally exactly where the spine diverges from the rest of the hypopygium since it does so dorsally. When viewing the spine ventrally the base is generally determined by where the sides of the hypopygium converge and meet the spine margin. Figure 51 shows a hypopygial spine and depending on which side of the spine you look at the base of the spine would appear at two different points along the spine. Another factor that may affect the utility of this trait is that the width of the spine may vary depending on whether the specimen died with the metasoma contracted vs. distended since this affects the overall thickness of the metasoma and therefore the spread of the hypopygium sides. The rigidity of the spine and its ability to resist flexion along its length has not been confirmed. If the spine does flex, this may increase the level of variation seen in this trait and affect its diagnostic ability. These difficulties with being able to consistently measure the full width of the base of the hypopygial spine may complicate the use of this trait in morphometric analyses.

Further confounding current morphological efforts are the existence of Kinsey's 17 manuscript names. As mentioned before imaging has been done on some of these manuscript names to try to determine if they can be diagnosed from current species and from each other. At this time, diagnostic traits are not known so they remain undescribed. It would be beneficial if DNA could be extracted from these specimens but so far the ones attempted were not successful even after a 24 hour soak in extraction buffer.

Towards the end of his Entomological career, Kinsey started discussing species complexes within *Disholcaspis* (Kinsey 1942) and these complexes seem to have been

united by gall morphology. There do not appear to be any morphological characters of the wasps that differentiate the groupings. However, they may prove useful for dividing up the genus for revising in sections.

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Part II. Wasps potentially defending walnut trees

Chapter 4: A new species of *Theocolax* Westwood (Hymenoptera: Pteromalidae: Cerocephalinae) reared from *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae)

Preface

The following article entitled "A new species of *Theocolax* Westwood (Hymenoptera: Pteromalidae: Cerocephalinae) reared from *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae)" which was published in the Proceedings of the Entomological Society of Washington in volume 117, issue 2 is reproduced in its entirety with permission from the editors, Matthew Buffington and Tom Henry, of the Proceedings of the Entomological Society of Washington.

Proceedings of the Entomological Society of Washington

17 November 2017

Ms Crystal Cooke-McEwen
Department of Entomology
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Dear Ms Cooke-McEwen,

With this letter, the Entomological Society of Washington gives you permission to use your paper titled "A new species of *Theocolax* Westwood (Hymenoptera: Pteromalidae: Cerocephalinae) reared from *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), previously published in the Proceedings (2015. 117(2): 162-178), as a chapter in your forthcoming Ph.D. dissertation. We ask only that you acknowledge the Society for giving this permission.

Sincerely yours,

Thomas J. Henry
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A NEW SPECIES OF *THEOCOLAX* WESTWOOD (HYMENOPTERA: PTEROMALIDAE: CEROCEPHALINAE) REARED FROM *PITYOPHTHORUS JUGLANDIS* BLACKMAN (COLEOPTERA: CURCULIONIDAE: SCOLYTINAE)

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Abstract.—The new species ***Theocolax americanus*** **McEwen, n. sp.** (Hymenoptera, Pteromalidae, Cerocephalinae), is described from the United States of America. A description is provided with a diagnosis and comparison to similar species. A key to differentiate the *Theocolax* species recorded in the USA is included and notes given on new morphological features. *Theocolax americanus* is thought to attack the walnut twig beetle (*Pityophthorus juglandis* Blackman), which transmits thousand cankers disease. Sequencing of CO1 for the new species, other *Theocolax* species found in the U.S.A., *Neocalosoter pityphthori* Ashmead (Hymenoptera, Pteromalidae, Cerocephalinae), and *P. juglandis* is reported.

Key Words: cytochrome oxidase subunit 1, thousand cankers disease, walnut twig beetle, *Pityophthorus juglandis*, *Theocolax americanus*; *Theocolax* spp., key, parasitoid, hosts, distribution.

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The morphology and occurrence of species of *Theocolax* Westwood in the Nearctic Region were previously discussed by Grissell and Hevel (2005). They treated two species: *T. elegans* (Westwood), a cosmopolitan species that attacks beetles in stored grain; and *T. ingens* Xiao and Huang, which was described from China and subsequently collected in Maryland from trees infested with various scolytids (Grissell and Hevel 2005). Here, a new species of *Theocolax* is described from Colorado that was reared from wood infested with the walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera, Curculionidae, Scolytinae).

The walnut twig beetle normally bores in twigs of Arizona walnut (*Juglans major* (Torr.) A. Heller) in New Mexico, Arizona, Southern California, and Mexico (Grant et al. 2011, Seybold et al. 2013, Tisserat et al. 2009, Tisserat et al. 2011). The beetle is also known to attack black walnut, *Juglans nigra* L., a new and non-native host of horticultural value in the

western United States. On *J. nigra* the beetles not only attack twigs and branches but also infest the trunk (Tisserat et al. 2009, Tisserat et al. 2011). They carry the fungus, *Geosmitha morbida* Kolařík, Freeland, Utley, & Tisserat (2011), the causative agent of thousand cankers disease which can kill black walnut trees (Grant et al. 2011, Seybold et al. 2013, Tisserat et al. 2009, Tisserat et al. 2011). *Pityophthorus juglandis* has now been documented in the native range of *J. nigra* (Grant et al. 2011, Seybold et al. 2013) and Europe (Montecchio and Faccoli 2014) where the trees are grown for timber production. With black walnut trees being economically important, it is imperative to investigate the parasitoids attacking these invasive beetles.

Specimens of the new *Theocolax* species were reared from *P. juglandis* infested wood during 2010–2013 by colleagues (the Cranshaw/Tisserat labs, Colorado State University) investigating the biology and natural enemies associated with the beetle in the western United States. Herein, the new *Theocolax* species is described and compared with other species of *Theocolax*, with notes on new diagnostic traits for the species groups.

Materials and Methods

Newly collected specimens were collected and stored in 70–90% ethanol. Specimens used for morphological analysis were dehydrated following the hexamethyldisilazane (HMDS) protocol described in Heraty and Hawks (1998) prior to point or stub mounting. Scanning electron microscope (SEM) images were taken with a Hitachi TM3000 in compo mode and charge–up reduction mode. Some specimens were point–mounted, uncoated specimens, whereas others were disarticulated and sputter coated with gold–palladium (~20nm coating, Cressington 108 Auto). Color images were taken using the EntoVision Imaging Suite including a firewire JVC KY–75 3CCD digital camera mounted on a Leica M16 zoom lens with a Leica z–step microscope stand. Composite images were created in Cartograph version 5.6.0, and edited in Archmed version 6.1.4 (Microvision Instruments, France). Black backgrounds and scales were added to SEM images using Photoshop and plates were assembled using InDesign (Creative Suite 6, Adobe systems Incorporated, U.S.A.). Images from plates were deposited at MorphBank (Image 835240, 835243–57, 835259–64, 835266, 835269–75, 835277–78, 835280–82, 835284, 835286, and 835288).

Measurements were taken using SEM and Entovision images in combination with ImageJ software version 1.47 (W. Rasband, National Institutes of Health, USA). Hymenoptera terminology for surface sculpture follows Harris (1979) and morphology follows the Hymenoptera Anatomy Ontology project (Yoder et al. 2010) and Gibson (1997). Abbreviations are as follows: F# = funicular segment, OOL = ocular ocellar line, POL = posterior ocellar line. Measurement explanations are as follows: body length– in lateral view from the anterior margin of the face to the posterior margin of the metasoma excluding the ovipositor sheath and without correcting for hunched posture; head height– in frontal view from the vertex to an imaginary line between the ventral margins of the gena; POL– the shortest distance between the posterior ocelli; OOL–the shortest distance between the lateral margin of a posterior ocellus and the eye margin; submarginal vein length– measured from just past the humeral plate to the point where the vein meets the wing margin; marginal vein length– measured from where it meets the wing margin to the point of junction between the stigma and postmarginal veins; stigmal vein– measured along its anterior

margin from the point of junction with the postmarginal vein to its apical margin; postmarginal vein length– measured along its posterior margin from its point of junction with the stigmal vein to its apex; gastral terga lenth–measured dorsomedially from basal to apical margins. Male antennae either have an additional funicular segment or a segmented clava compared to females. In females, the funicular segments have a single ring of sensilla whereas the unsegmented clava has the sensilla in multiple rows, which sometimes become more irregularly spaced towards the tip. The extra antennal segment of the males is therefore considered an additional, sixth funicular segment because it has a single row of sensilla (Fig. 15).

Abbreviations for collections are as follows: USNM (National Museum of Natural History, Smithsonian Institution, Washington, DC, U.S.A), CNC (Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa, Canada), and CSUC (C.P. Gillette Museum of Arthropod Diversity, Colorado State University, Fort Collins, Colorado, U.S.A.).

A single female paratype of *Theocolax phloeosini* Yang was borrowed from the CNC for comparison and imaging. This specimen was damaged during imaging and no other specimens could be located from. Female specimens of *T. bakeri* (Crawford), *T. elegans*, *T. formiciformis* Westwood, and *T. frater* (Girault) in USNM were also used for comparison and imaging. Female *T. oblonga* (Delucchi) type specimens were obtained from the Royal Museum for Central Africa in Tervuren, Belgium. The types and the series of *T. ingens* formerly collected in Maryland, U.S.A. could not be located so a new specimen was collected from the same in Maryland where this species was documented by Grissell and Hevel (2005). Due to exportation difficulties, *T. radhakrishnani* specimens could not be acquired and communications for morphological assistance failed. Comparisons between *T. americanus* and *T. radhakrishnani* Sureshan & Narendran (2005) are based on the original description.

DNA Extraction, Sequencing, and Analysis.—Two adult *T. americanus* specimens (collected in Colorado) were used for molecular comparison as well as one specimen of *T. elegans* (intercepted at a port in Minneapolis, MN, flight origin was India), one *T. ingens* (collected in Maryland), and two *Neocalosoter pityophthori* Ashmead (collected in Tennessee). Three *T. americanus* larvae (collected in Colorado) were also used for attempted extractions and the two host larvae (*P. juglandis* from Colorado) that the parasitoid larvae were attached to.

Total DNA was extracted from whole adults and larvae soaked in extraction buffer and proteinase K overnight to pull DNA from the specimens without destroying the specimen. The standard DNeasy® spin column protocol (QIAGEN 2006) was followed. Due to difficulties obtaining sequences in this group of wasps, the cytochrome c oxidase subunit 1 (CO1) was amplified using a variety of primer combinations (individual primers listed in Table 1). Single amplifications did not work with any of the primer combinations for *Theocolax* specimens. Product from the polymerase chain reaction (PCR), using the LepCoF–LepCoR primers, was used as the template sample for a second round of PCR involving various combinations of primers. Some of the successful reamplifications used heminested primers (MLep primers paired with another primer), whereas others were not nested (HymLepCo primers and LepCo primers). The following primers were successful as secondary amplifications: for *T. americanus* MLepF–HymLepCo2198R; for *T. ingens*: HymLepCo1490F – HymLepCo2198R, LepCo1490F –MLepR,

and MLepF–LepCo2198R; for *T. elegans* MLepF1–LepCo2198R, HymLepCo1490F –HymLepCo2198R, MLepF–HymLepCo2198R, and HymLepCo1490F –MLepR. The other taxa were amplified with a single PCR reaction using the LepCo1490F / LepCo2198R primers (for *P. juglandis* larvae) and LCO1490F / HCO2198R primers (for *N. pityophthori* adults).

Initial PCR amplifications of CO1 were performed in 25 µl reaction volumes consisting of 14.3 µl water, 2.5 µl 10x PCR buffer, 1 µl forward primer, 1 µl reverse primer, 2 µl dNTPs, 0.2µl Taq, and 4 µl DNA extract. The PCR reamplifications used the previous protocol with the exception of 16.3 µl water and 2 µl PCR template in the place of DNA extract. The touchdown PCR used a thermal cycling program as follows: 92° C for 2 minutes; 58° C for 15 seconds decreasing by 1° C every cycle; 72° C for 1.5 minutes; 12 cycles of 92° C for 10 seconds, and 58° C for 15 seconds, decreasing by 1° C every cycle; 30 cycles of 92° C for 10 seconds, 45° C for 10 seconds, and 72° C for 1.5 minutes; 72° C for 7 minutes; and a final incubation at 4° C once finished. The final PCR product was cleaned using a shrimp alkaline phosphatase and Affymetrix USB's *Exo I* with the ExoSAP-IT protocol, and then sequenced using an ABI 3730xl Genetic Analyzer at the Beltsville Agricultural Research Center (USDA, Beltsville, Maryland).

Basecalling was checked by eye using FinchTV version 1.4 (Geospiza Inc.). Sequences were aligned using MAFFT v.7 (Katoh and Standley 2013). *Asaphes suspensus* Nees (accession number JX507454.1) was used as an outgroup. The substitution model was selected using jModelTest 2.1.4 (Darriba et al. 2012) and both Akaike information criterion and Bayesian information criterion resulted in a transitional model with gamma distributed rate variation among sites (TIM+G) with a shape parameter of 0.4520. The total pairwise distances and the corrected (TIM+G) distances were calculated using PAUP* (Swofford 2003). All new sequences were deposited into GenBank (accession numbers KJ451419–21, KJ451423, KP119775–6). These data represent the only CO1 sequences for any species in the subfamily Cerocephalinae on GenBank. While the host DNA was not used in analysis, a fragment of CO1 was sequenced and the *P. juglandis* sequence was deposited in GenBank (accession number KJ451422).

Results

Theocolax americanus McEwen, n. sp.

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(Figs. 1–17, 20–21, 25, 29, 31–32, 37)

Table 1. Primers successfully used for PCR amplification, reamplification, and sequencing of CO1 in select *Theocolax* species and *Neocalosoter pityophthori*.

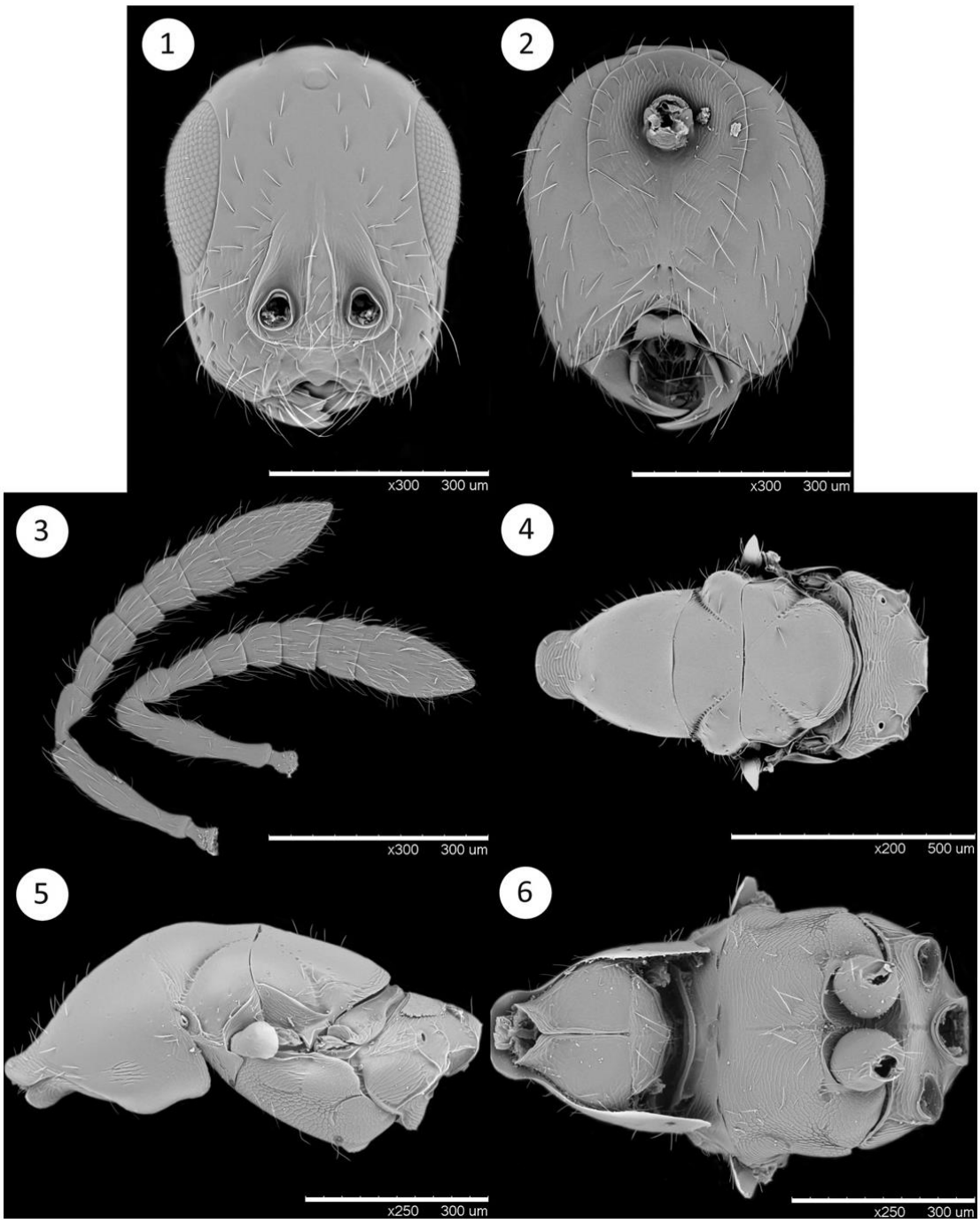
Primer	Sequence (5' 3')	Reference
LepCo1490F	ATTCAACCAATCATAAAGATATTGG	Herbert et al. 2004
LepCo2198R	TAAACTTCTGGATGTCCAAAAAATCA	Herbert et al. 2004
HymLepCo1490F	ATTCAACCAATCATAAAGATATGGTAT	Designed by Gary Oullette
HymLepCo2198R	TAAACTTCTGGATGTCCAAAAATCAAAATAA	Designed by Gary Oullette
MLepF1 (nested)	GCTTTCCCACGAATAAATAATA	Hajibabaei et al. 2006
MLepR1 (nested)	CCTGTTCCAGCTCCATTTTC	Hajibabaei et al. 2006
LCO1490F	GGTCAACAAATCATAAAGATATTGG	Folmer et al. 1994
HCO2198R	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. 1994

Description.—Female holotype (Fig. 37). Body length: 2.2 mm. Color: dark brown fading to lighter brown on ventral margin of head and metasoma; scape–F3 yellow to tan; anterior side of metacoxae and metafemora yellow; tibiae and tarsi mostly yellow with metatibiae light brown at middle; ovipositor sheath yellow at base and dark brown at tip; wings (Fig. 13) clear, with brown veins, brown macula extending from stigmal vein to hind margin, and brown macula under dark brown setal tuft at base of marginal vein.

Head (Figs. 1, 2): 0.84 X as broad as high, overall smooth, setae sparse on upper face and denser on lower face; clypeus trapezoidal with medial tooth in marginal depression; malar space moderately setate, lightly imbricate ventrally and near the scrobal depression, 0.8 X eye height; malar sulcus absent; gena in anterior view straight laterally and convex ventrally. Antennae (Fig. 3) with ratio scape:pedicel:F1:F2:F3:F4:F5:clava as 26:9:7:6:6:6:7:20; sensilla arranged in single longitudinal rows around F2–5 and in multiple rows longitudinally along clava; clava fused, as long as previous three segments. Ratio of posterior ocellus maximum diameter:OOL:POL as 5:12:13; distance between inner margins of toruli 1.7 X torulus diameter; interantennal lamella extending 3/5 distance to median ocellus. Occipital carina present; postgena imbricate and sparsely setose, imbrications widely spaced near tentorial pits and denser around occipital foramen; postgenal bridge smooth, slightly lighter in color than anterior area of head; occipital foramen located on upper quarter of head; tentorial pits nearer mouthparts than occipital foramen; hypostomal carina with dorsal and lateral margins concave.

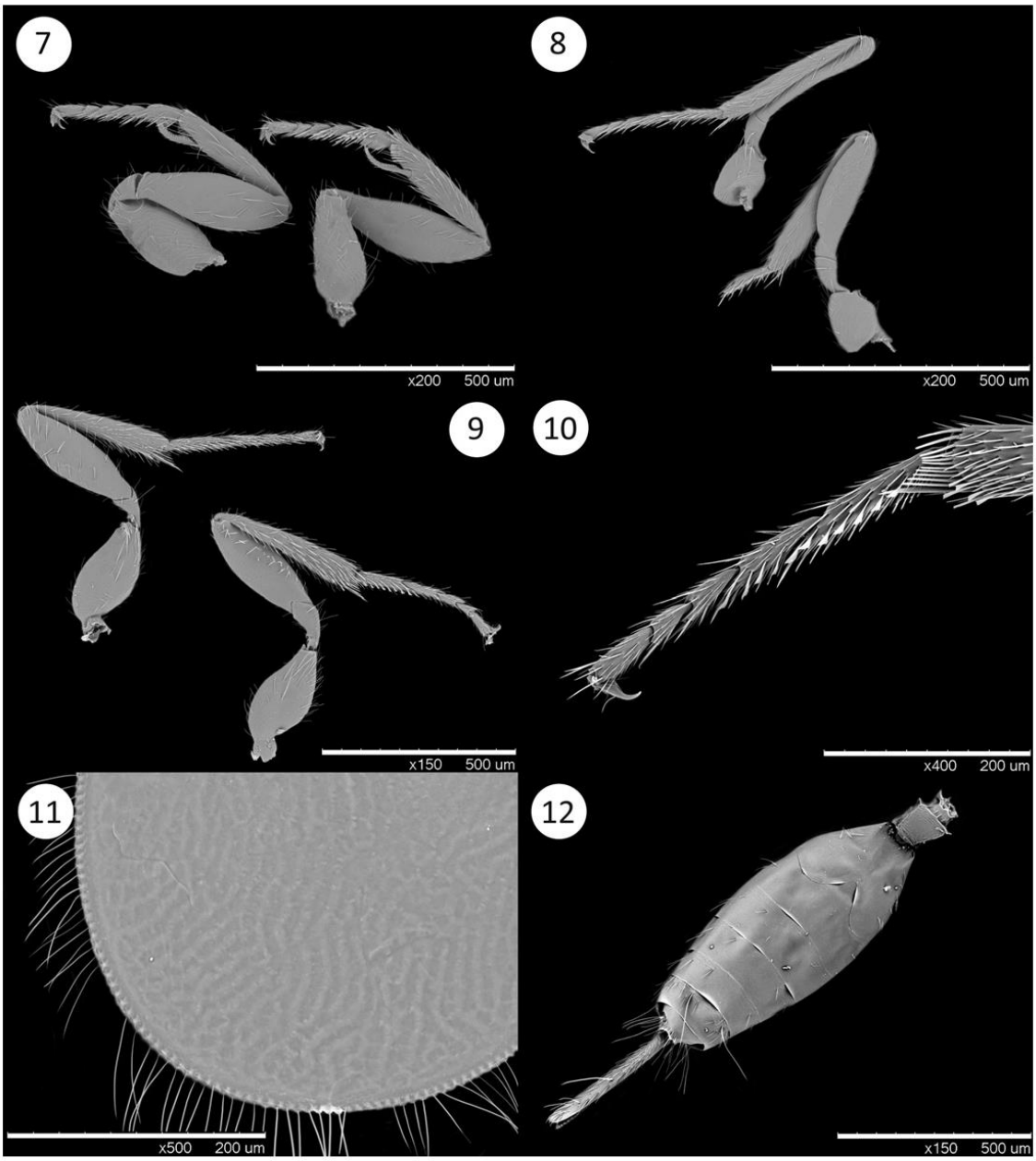
Mesosoma: Dorsally (Fig. 4) 2.1 X as long as wide; pronotum 0.7 X as long as wide at its widest point, neck 0.4 X as wide as collar; midlobe of mesoscutum 0.6 X as long as wide, with three adnotaular setae; notauli complete, deeper anteriorly; lateral lobe of mesoscutum rounded, with row of setae along notalus; transscutal articulation straight; axilla bulging less than lateral lobe of mesoscutum, sparsely setose posteriorly, scutoscutellar suture as an indent; scutellum mostly bare with a few setae along scutoscutellar suture, finely imbricate along posterior margin; metanotum with dorsellum narrow and with transverse carinae, lateral panel rounded and expanded and obscurely rugose to finely carinate. Propodeum (Fig. 25) lacking median longitudinal carina, anteriorly with transverse striae with slight medial interruption, posteriorly with subparallel median longitudinal carinae diverging near propodeal foramen, posteromedially imbricate to smooth, and with sublateral carinations posteromedial to spiracles; propodeal areola finely imbricate, posterior propodeal carina usually with two setae; callus imbricate, with few scattered setae. Pronotal side (Figs. 5, 31) with patch of horizontal striations anterad spiracle; prepectus finely imbricate; mesoplueron mostly shagreen with acropleural area more imbricate, pleural sulcus absent or only represented by more dense texture, transepimeral sulcus present; metapleuron elongate–imbricate. Propleura lightly imbricate laterally and anteriorly, medially with longitudinal striations. Prosternum (Fig. 6) imbricate with scattered setae; mesosternum anteromedially elongate–imbricate, posteriorly shagreen, slight depression posteromedially; metasternum elongate–imbricate, medially with slight depression.

Legs: Procoxae (Fig. 7) imbricate laterally and finely imbricate dorsomedially; forefemur finely imbricate medially; protibia with setae denser medially, with 2–3 subapical paddle–shaped setae in a row medially (Fig. 29), with subapical comb of truncate and parallel–sided setae, calcar curved with comb of rounded to truncate spines; protarsus with



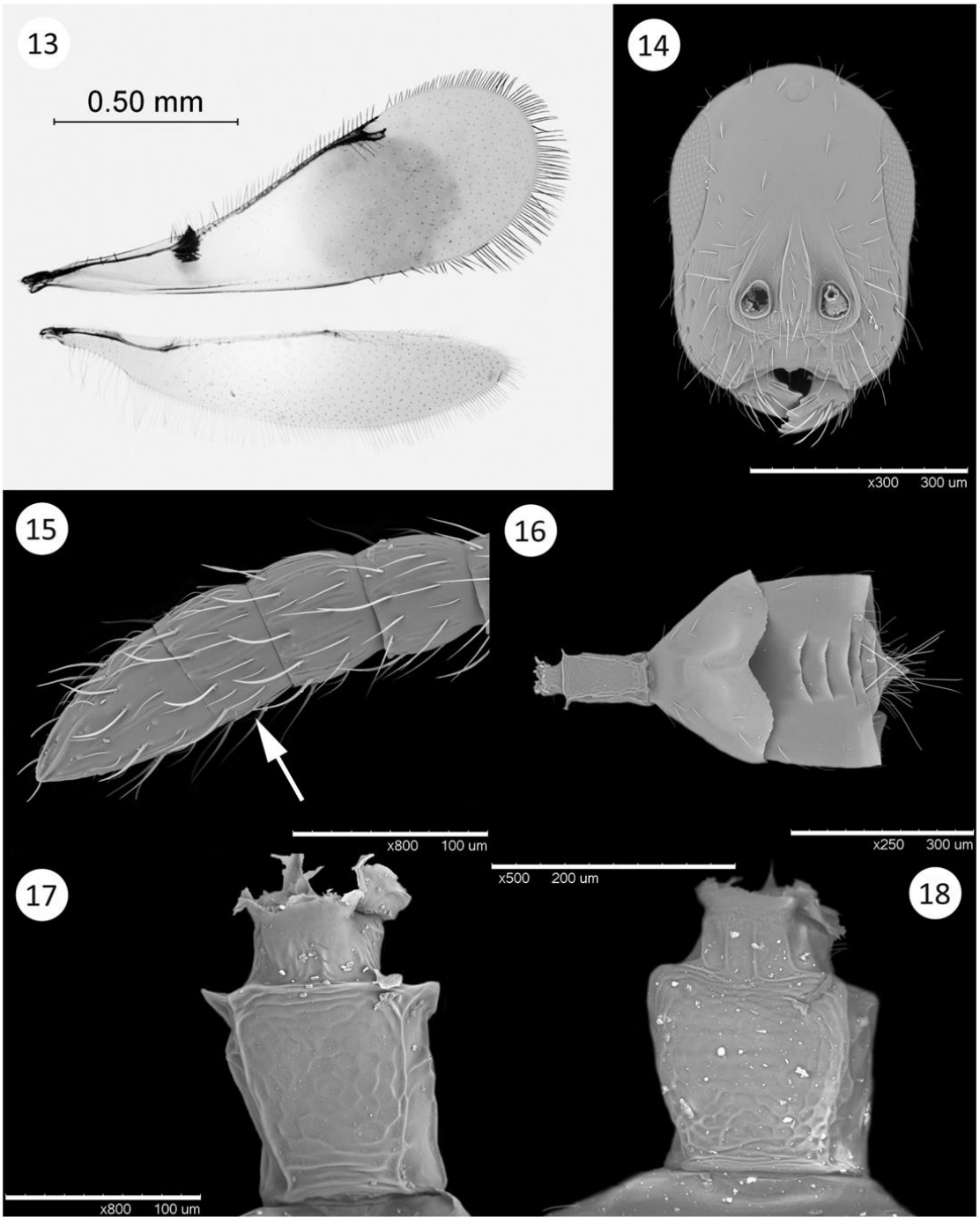
Figs. 1–6. 1. *T. americanus* female, anterior head; 2. *T. americanus* female, posterior head; 3. *T. americanus* female, antennae; 4. *T. americanus* female, dorsal mesosoma; 5. *T. americanus* female, lateral mesosoma; 6. *T. americanus* female, ventral mesosoma.

tapering setae laterally, ventrolaterally with long and tapering spines, ventromedially with comb of truncate and parallel–sided setae, first and second segments medially with paddle–shaped setae with pointed tips. Mesocoxae (Fig. 8)



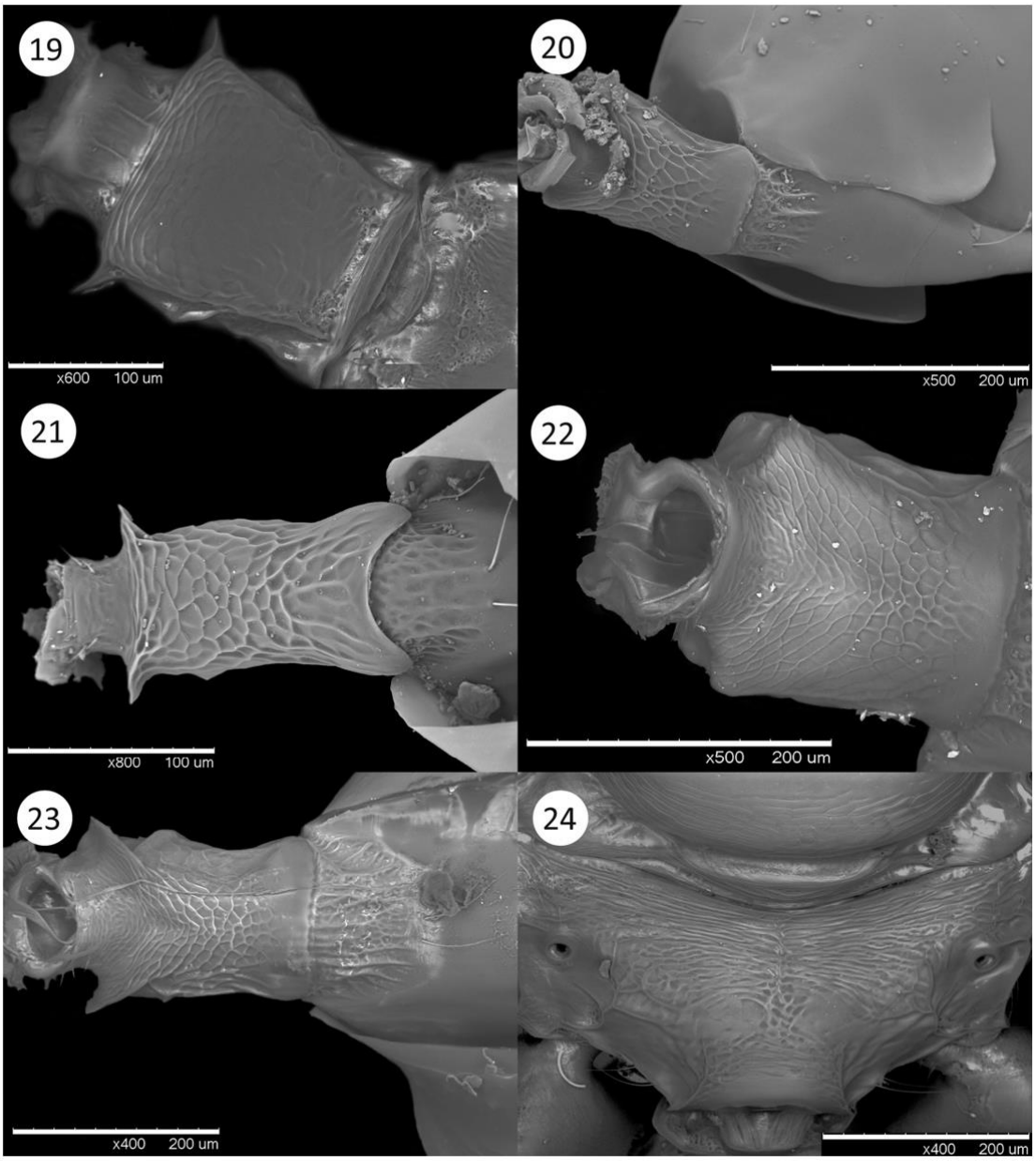
Figs. 7–12. 7. *T. americanus* female, forelegs; 8. *T. americanus* female, mesolegs; 9. *T. americanus* female, hindlegs; 10. *T. americanus* female, mesal hindtarsus; 11. *T. americanus* female, forewing surface; 12. *T. americanus* female, dorsal metasoma.

short and rounded, imbricate; mesofemur and tibia with scattered setae and finely imbricate; mesotarsus with tapering setae. Metacoxae (Fig. 9) finely imbricate, with scattered setae; metafemur finely imbricate, setae scattered laterally and ventromedially; metatibia with setae denser than rest of leg, posteroapically with row parallel–sided setae that are rounded at the tip; metatarsus with tapering setae on all segments, spatulate setae on first and second segments (Fig. 10).



Figs. 13–18. 13. *T. americanus* female, wings; 14. *T. americanus* male, anterior head; 15. *T. americanus* male, antenna, arrow= sixth funicular segment; 16. *T. americanus* male, dorsal metasoma; 17. *T. americanus* famale, dorsal petiole; 18. *T. phloeosini* female, dorsal petiole.

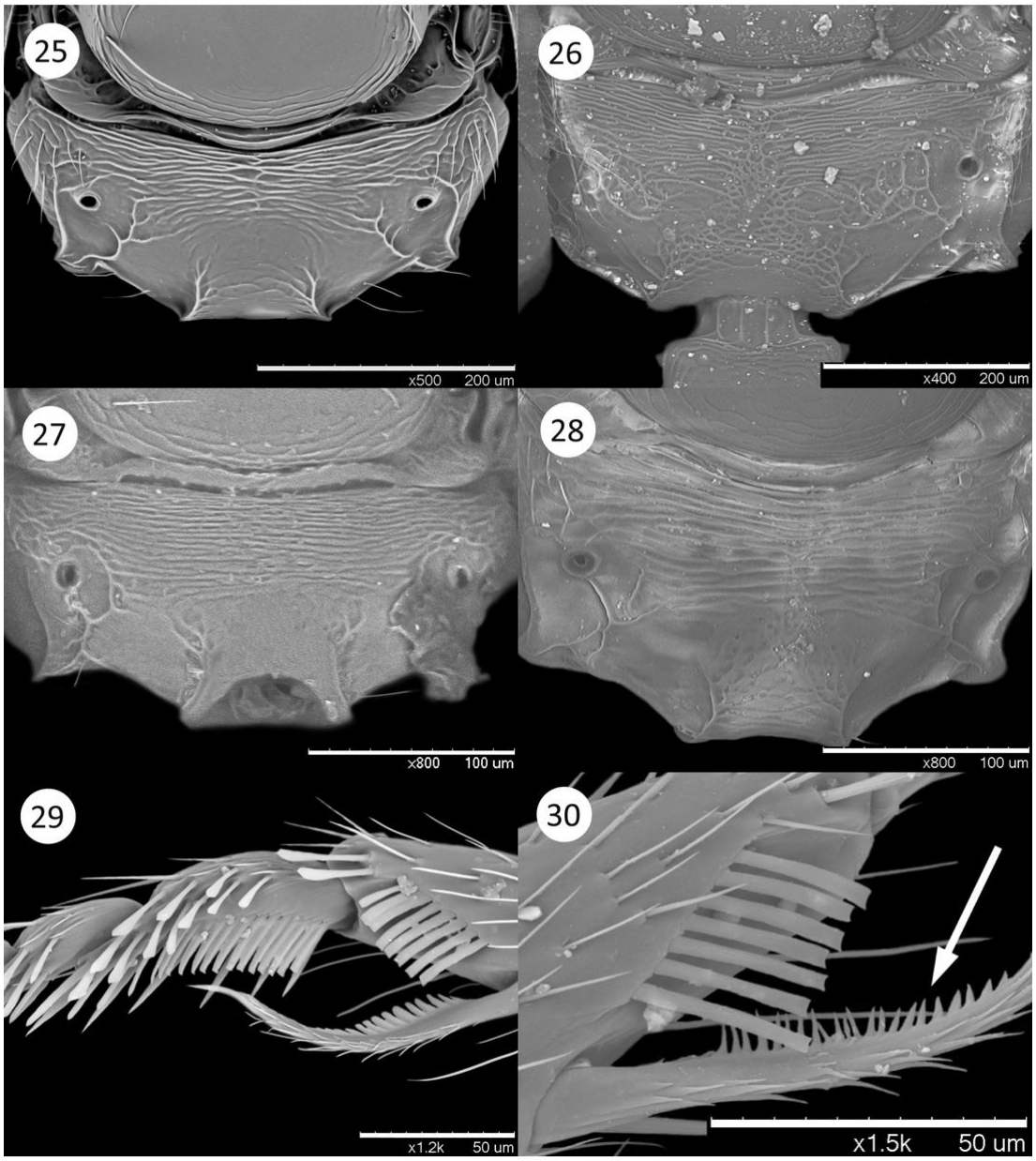
Wings (Fig. 13)*:* Forewing with ratio of submarginal: marginal: postmarginal: stigmal veins as 46:55:9:8; rounded macula extending from stigma vein to posterior wing margin, smaller macula at base of setal tuft, larger macula nearly as



Figs. 19–24. 19. *T. ingens* female, dorsal petiole; 20. *T. americanus* female, ventral petiole; 21. *T. americanus* male, ventral petiole; 22. *T. phloeosini* female, ventral petiole; 23. *T. ingens* female, ventral petiole; 24. *T. ingens* female, propodeum.

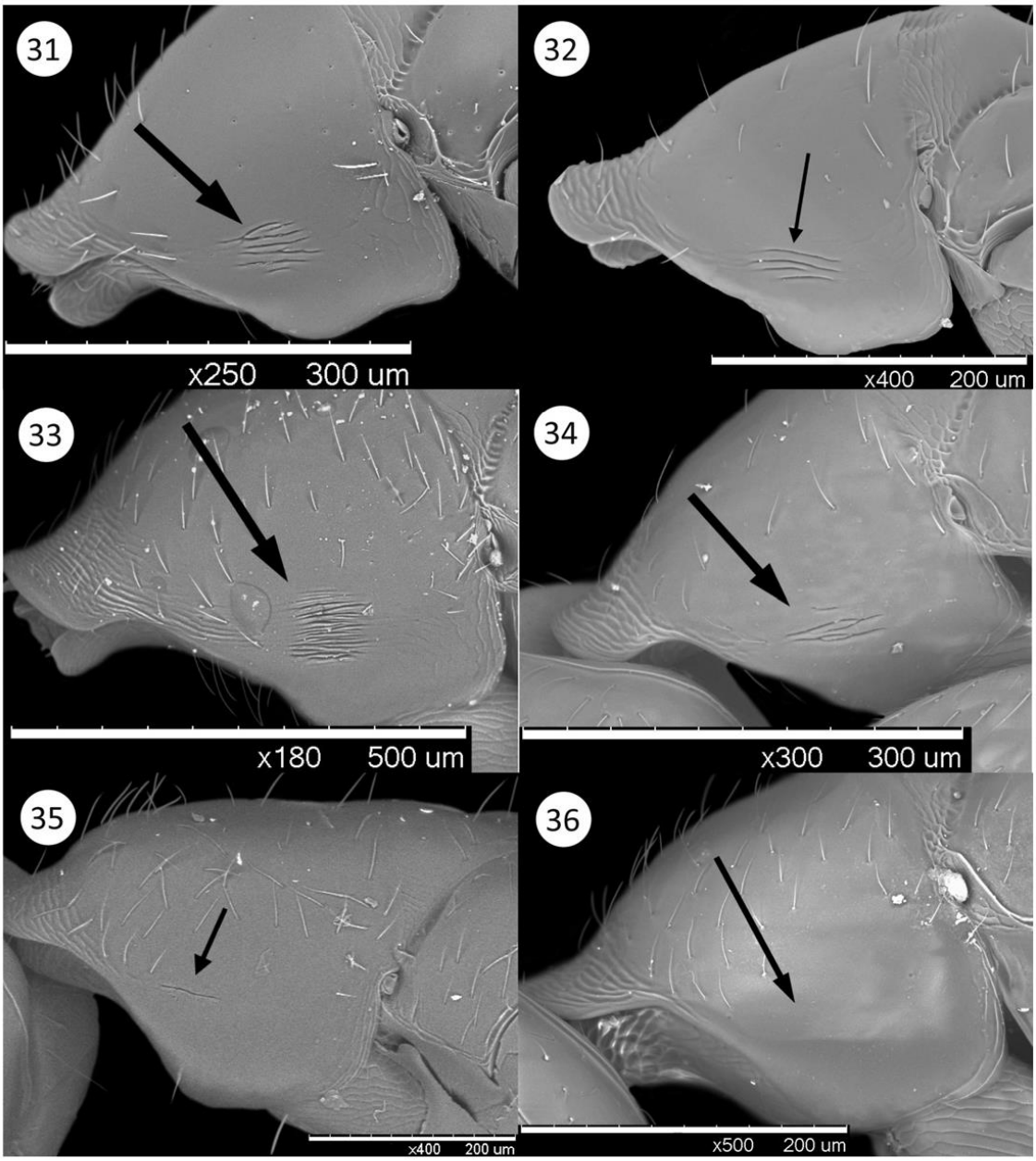
long as wide; setae around wing margin except basally, wing surface with spots or setal sockets but lacking setae, surface texture appearing finely rugose (Fig. 11). Hindwing also with spots or setal sockets that lack setae, marginal setae posteriorly and apically, few scattered setae along wing veins.

Metasoma: Petiole (Figs. 17, 20) 1.3 X as long as wide dorsally, lightly areolate dorsally, laterally with few crenulations, ventrally imbricate to areolate,



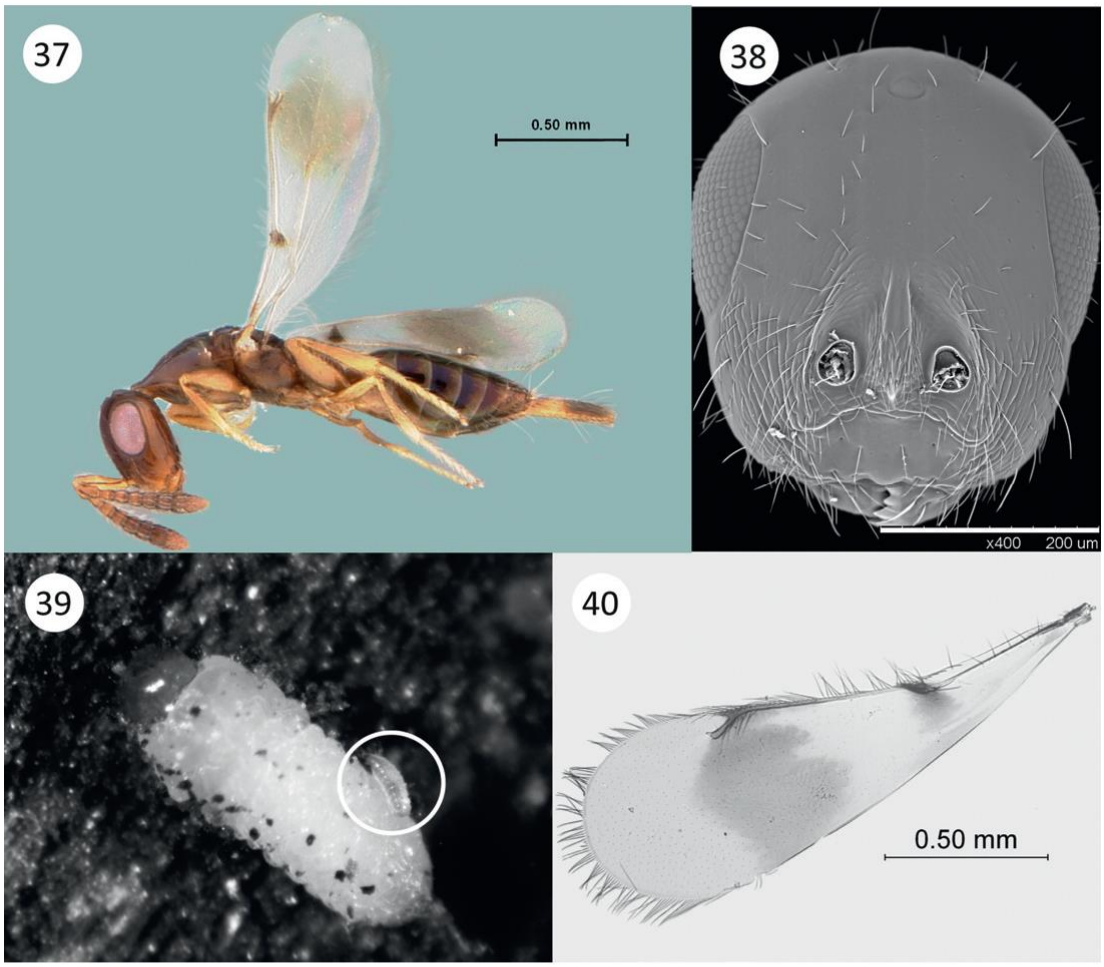
Figs. 25–30. 25. *T. americanus* female, propodeum; 26. *T. phloeosini* female, propodeum; 27. *T. frater* (USNMENT00892017) female, propodeum; 28. *T. frater* (USNMENT00892018) female, propodeum; 29. *T. americanus* female, antennal cleaner; 30. *T. elegans* female, antennal cleaner, arrow= pointed spines of calcar comb.

anterolateral angles acute beyond peduncle, ventrolaterally with subparallel carinae; gaster (Fig. 12) smooth with few transverse striations anteriorly on each segment, each segment with at least one row of sparse setae, first tergum with dorsomedial invagination extending 35% the tergum length at longest point, measure of gaster terga anterior to posterior as 8:9:6:5:4:3:3; ovipositor sheath imbricate basally, setose, half as long as gaster, 6 X longer than width laterally.



Figs. 31–36. 31. *T. americanus* female, lateral pronotum, arrow= patch of horizontal striations; 32. *T. americanus* male, lateral pronotum, arrow=patch of horizontal straiations; 33. *T. phloeosini* female, lateral pronotum, arrow= patch of horizontal striations; 34. *T. frater* female, lateral pronotum, arrow= patch of horizontal striations; 35. *T. oblonga* female, lateral pronotum, arrow= patch of horizontal striations; 36. *T. elegans* female, lateral pronotum, arrow= patch of horizontal striations.

Males (Figs. 14–16, 21, 32)*:* Differ from females in following characters: Head width 0.78 X the height, malar space subequal to eye height. Antennae with six funicular segments plus clava; with ratio scape:pedicel:F1:F2:F3:F4:F5: F6:clava as 16:6:5:4:5:4:4:4:8. Patch on pronotal side with fewer striations than females, varying between two to six. Petiole 2.1 X as long as wide dorsally,



Figs. 37–40. 37. *T. americanus* female, Holotype, lateral habitus; 38. *T. elegans* female, anterior head; 39. Small parasitoid larva (circled) attached to *Pityophthorus juglandis* larva, photo from Emily Peachy and the Cranshaw Lab, Colorado State University; 40. *T. oblonga*, forewing.

ventrally with more sculpturing than females. Overall, metasomal terga retracted distally, phallus often protracted, invagination of the first gaster tergum extends 20% the tergum length at longest point.

Variation: Total body length varies from 0.6 mm to 2.5mm; leg coloration from yellow to dark brown; midlobe of mesoscutum sometimes with two adnotaular setae on each side but usually three. Patch of striations on pronotal side with six to twelve lines.

Type Material.—*Holotype female*: "USA: CO: Jefferson Co.: Wheat Ridge: tree removed from Prospect Park Sep.–Nov.2009, 39.77479, -105.126991, E. Peachy" [first label]; "Ex *Juglans nigra* wood infested with *Pityophthorus juglandis* Blackman, Reared at CSU Ft. Collins 8.Jun.2011" [second label]; "*Theocolax americanus* McEwen, Det. C. L. McEwen 2013" [third label]; "Holotype *Theocolax americanus* McEwen ♀" [fourth label] (USNM). Paratypes (8♀, 5♂): 4 (2♀, 2♂) with "USA: CO: Larimer Co.: Ft. Collins: 1821 W. Drake Rd, tree felled 19.Jul.2012, 40.550579, -105.110976 R. Fithian" [first label], "Ex.

Juglans nigra wood infested with Pityophthorus juglandis Blackman, Reared in CSU Insectary Bldg. em. 10.Sep.2012" [second label] (USNM); 1♀ same as previous 4 paratypes except emerging date 27.Aug.2012 (USNM); 1♀ and 1♂ "USA: CO: Boulder Co.: Boulder, tree removed from Boulder Co. Sept.-Nov.2010, Wood coll. 40.025775, -105.230184 E. Peachy" [first label], "Ex. Juglans nigra wood infested with Pityophthorus juglandis Blackman Emerged 19.Apr.2011" [second label] (USNM); 1♀ and 1♂ "USA: CO: Otero Co.: Rocky Ford: tree felled from 400 S. 10th St., 15.Aug.2011, 38.050476, -103.719438 R. Fithian" [first label], "Ex. Juglans nigra wood infested with Pityophthorus juglandis Blackman Reared at CSU Ft. Collins Em. 21. May.2012" [second label] (USNM); 3♀ and 1♂ "Jefferson Co., CO, Coll.26 June 2009, C. Utley, Wheatridge, Ex *Juglans nigra*" [first label], "Em. 4 Nov 2009 Infested with *Pityophthorus juglandis*" [second label] (CSUC).

Diagnosis.—*Theocolax americanus* can be diagnosed by the following combination of traits: interantennal lamella extending more than half way to median ocellus (Fig. 1), postmarginal vein subequal in length to the stigmal vein (Fig. 13), pronotal side with a patch of horizontal striations located anterior to spiracle (Fig. 31) with the females having more than four striations, large macula near midpoint of the forewing (Fig. 13), the anterior portion of propodeum with transverse striae that are slightly interrupted medially, and the posterior portion of the propodeum imbricate to smooth between subparallel median longitudinal carinae (Fig. 25).

Remarks.—*Theocolax americanus* is morphologically most similar to *T. ingens* (China), *T. phloeosini* (China), *T. radhakrishnani* (India), and *T. frater* (Australia, Hawaii, Japan, and the Philippines) (Grissell and Hevel 2005, Sureshan and Narendran 2005, Xiao and Huang 2001, and Yang 1989). The primary traits showing variation between these species are the propodeal sculpturing, the form of the petiole, and the number of striations located laterally on the pronotum.

Both *T. ingens* and *T. phloeosini* (Figs. 24 and 26) have similar propodeal sculpturing with a distinct medial interruption across the anterior transverse striae, the medial portion of the propodeum areolate–rugose, and posteriorly with areolate–rugose sculpturing between the medial subparallel carinae. The propodeum of *T. americanus* (Fig. 25) is most similar to that of *Theocolax frater* (Figs. 27–28). There are two specimens in the USNM collection identified as *T. frater* with variable propodeal sculpturing, though both are different from *T. americanus* in that there is no medial interruption in the transverse striae.

The petiole of *T. phloeosini* (Figs. 18 and 22) has the anterior lateral angles rounded rather than acute and the base with four longitudinal carinae dorsally. The petiole of *T. ingens* (Figs. 19 and 23) is similar to that of *T. americanus* (Figs. 17, 20) in that the anterior lateral angles are acute but *T. ingens* has carinae distinctly bulging laterally.

The patch of horizontal striations on the pronotal side in *T. ingens* is very similar to that of *T. phloeosini* (Fig. 33). The patch in females of *T. americanus* (Figs. 31–32) has fewer striations than that of *T. phloeosini* and *T. ingens* but more striations than that of *T. frater* (Figs. 34).

While no *T. radhakrishnani* specimens were available for study, several differences between the two species can be deduced from the original description. *Theocolax radhakrishnani* differs from

this new species in that the clava is shorter than the three preceding segments, the notauli are incomplete, the large wing maculation is thinner than long, and the second gaster tergite is slightly invaginated medially (Sureshan and Narendran 2005).

Biology.—Reared from walnut twig beetle, *Pityophthorus juglandis* (Fig. 38), infested wood in large numbers throughout Colorado. *Pityophthorus juglandis* larvae were observed with ectoparasitic wasp larvae and *T. americanus* is the only parasitoid reared from *P. juglandis* infested wood in this region. Colorado is outside of the host beetle and host tree's native range. *Pityophthorus juglandis* is native to the southwestern U. S.A. and jumped to the non-native host tree, *J. nigra*, that is planted horticulturally in this region. *Theocolax americanus* has not been reared from this host beetle in the host's native range, suggesting that the wasp did not move into the new region with the beetle and that this beetle represents a novel host for this parasitoid. It is unknown what insect *T. americanus* attacks other than *P. juglandis*, though other species of *Theocolax* are known to attack various Scolytinae (Grissell and Hevel 2005, Sureshan and Narendran 2005, Xiao and Huang 2001,and Zhongqi 1989).

Distribution.—Known only from Colorado.

Etymology.—Third declension, singular. Named "*americanus*" as the first *Theocolax* species to have been described from the Americas.

Key to the species of *Theocolax* recorded in the United States.

1. Interantennal lamella not extending more than half way to the median ocellus (Fig. 40); post marginal vein shorter than stigmal vein (Fig. 39); pronotal side without patch of horizontal striations located anterior to spiracle (Fig. 36). . . . . *T. elegans*
– Interantennal lamella extending more than half way to median ocellus (Fig. 1); postmarginal vein subequal to stigmal vein (Fig. 13); pronotal side with patch of horizontal striations located anterior to spiracle (Fig. 31). 2
2. Forewing without a distinct macula. Propodeal sculpturing with distinct medial interruption across the anterior transverse striae and medially to posteromedially areolate–rugose. . . . . *T. ingens*
– Forewing with one large macula near midpoint and a smaller macula near the tuft of bristles at the proximal end of marginal vein (Fig. 13). Propodeal sculpturing with slight medial interruption across the anterior transverse striae and posteromedially smooth to imbricate. . . . . *T. americanus*

Molecular Analysis.—The original purpose for the molecular work was to sequence the larvae and adults of *T. americanus* to confirm host-parasite relationship but none of the primer combinations produced visible bands in agarose gels with the *Theocolax* larvae. Correspondence with other researchers confirmed that Cerocephalinae has been a difficult group to sequence. Due to these difficulties, only distance measurements are reported.

The corrected distances observed within species were similar between *T. americanus* and *N. pityophthori*. The distances (Table 2) between *T. americanus* and *T. ingens* were 0.02140 and 0.01881. The distances between *T. elegans* and the other *Theocolax* were between 0.1794 and 0.2027. The distances observed between genera were between 0.2080 and 0.3168. More comprehensive sampling of individuals within each species is needed to establish any distance thresholds that may exist with CO1.

Table 2. Distance matrix for CO1 sequences. Above diagonal corrected distances (TIM+G); below diagonal total number of pairwise character difference.

Specimen	*Theocolax americanus* KJ451421.1	*Theocolax americanus* KJ451423.1	*Theocolax ingens* KJ451420.1	*Theocolax elegans* KJ451419.1	*Neocalosoter pityphthori* KP119775	*Neocalosoter pityophthori* KP119776	*Asaphes suspensus* JX507454.1
Theocolax americanus KJ451421.1	-	0.00323	0.02140	0.17937	0.28861	0.28861	0.26102
Theocolax americanus KJ451423.1	2	-	0.01881	0.18232	0.26555	0.26555	0.25460
Theocolax ingens KJ451420.1	9	8	-	0.20274	0.27933	0.27933	0.31683
Theocolax elegans KJ451419.1	74	74	57	-	0.20798	0.20798	0.21350
Neocalosoter pityphthori KP119775	106	94	67	79	-	0.00156	0.26975
Neocalosoter pityophthori KP119776	106	94	67	79	1	-	0.26636
Asaphes suspensus JX507454.1	100	90	71	78	101	100	

Discussion

Based on the observations of Grissell and Hevel (2005) there appears to be at least two subgroupings of species within *Theocolax*, the *T. ingens* group (*T. ingens*, *T. phloeosini*, and possibly *T. radhakrishnani*) and the *T. bakeri* group (*T. bakeri*, *T. elegans*, *T. formiciformis*, and *T. oblonga*). They separated these species groups based on the length of the interantennal lamella and the length of the postmarginal vein. Following their observations this new species lies within the *ingens* group due to the interantennal lamella extending more than half way to the ocelli and the postmarginal vein being equal to or longer than the stigmal vein. The observed distances in CO1 sequences also support that *T. americanus* is more similar to *T. ingens* than *T. elegans*.

During this study, two additional characters were noted to be concurrent with the traits proposed by Grissell and Hevel (2005). In the specimens where these traits were visible, the species of the *ingens* group were found to have the spines of the calcar comb truncate or rounded apically (Fig. 29), whereas the species of the *bakeri* group have the spines pointed apically (Fig. 30). Also, a patch of horizontal striations on the pronotal side (Fig. 31–33) is present in all the *ingens* group species observed, whereas this trait is lacking in the species of the *bakeri* group (Fig. 36) with the exception of *T. oblonga* which may be morphologically intermediate (Fig. 35) based on this trait. *Theocolax oblonga* has the short interantennal lamella and short postmarginal vein of the *bakeri* group, but has a single striation on the pronotal side. Unfortunately, the calcar comb was not visible in either *T. oblonga* type specimen examined. Grissell and Hevel (2005) discussed *T. frater* as being intermediate in characters between the two species groups but the new traits support a greater similarity to the *ingens* group. *Theocolax radhakrishnani* was described simultaneous to the Grissell and Hevel (2005) paper, but based on the original description it likely belongs to the *ingens* group since the postmarginal vein is longer than the stigmal vein (Sureshan and Narendran 2005). There was no mention of the state of the calcar or pronotal side in the *T. radhakrishnani* description.

The species that *T. americanus* most closely resembles morphologically have been described from Asia (Grissell and Hevel 2005, Sureshan and Narendran 2005, Xiao and Huang 2001, and Yang 1989). Colorado is not a major port of entry and therefore it is not very likely that this wasp was introduced to Colorado directly from Asia. However, the wasps have been reared from trees that

were planted horticulturally and their origins may be varied and unknown. This group is understudied and under collected, which may account for the seemingly irregular distribution.

It should be noted that *Theocolax* sp. specimens were reared from *P. juglandis* infested wood in Knoxville, Tennessee. These specimens were originally thought to be the same as *T. americanus* though they have been found to differ in coloration and propodeal sculpturing. Their propodeal sculpturing most closely resembles that of *Theocolax ingens* (Fig. 24), with densely spaced transverse striae with distinct interruption anteromedially, and areolate sculpturing medially that extends to the subparallel longitudinal carinae near the propodeal foramen. The coloration of the Tennessee specimens differs from *T. americanus* and *T. ingens* in that it is bicolored with the head, lateral mesosoma, and legs being a light brown to yellow brown color and the dorsal mesosoma and most of the gaster being black brown. Only two specimens of the Tennessee *Theocolax* are known.

Without the molecular confirmation that the parasitoid larvae found attached to the beetle larvae are conspecific to the adult wasps described as *T. americanus*, the evidence for host–parasite association is circumstantial, yet strong. In Colorado, *T. americanus* adults were the only parasitoids reared from *P. juglandis* infested logs and numerous ectoparasitic larvae were found attached to *P. juglandis* larvae (Fig. 38). Further molecular work or controlled rearing can confirm the association.

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Chapter 5: Contributions to *Cerocephala* Westwood

Abstract

Studies exploring thousand cankers disease have revealed another new species of cerocephaline (Hymenoptera: Pteromalidae: Cerocephaline) wasp. The new species of *Cerocephala* Westwood was reared from thousand cankers disease infested wood and is described with an updated description for *Cerocephala caelebs* (Masi). Sequence data (CO1) is provided with evidence that *Cerocephala* might be nested within *Theocolax*, though no taxonomic changes are made at this time. More comprehensive molecular work between cerocephaline genera is needed to determine their relationships.

Introduction

Thousand cankers disease (TCD) is a potentially deadly disease affecting walnuts but particularly affecting black walnut (*Juglans nigra* Linnaeus). It is the result of a phytopathogenic fungus, *Geosmithia morbida* Kolařík , Freeland, Utley, and Tisserat, and its insect vector the walnut twig beetle, *Pityophthorus juglandis* Blackman. This disease was first described in 2009 (Tisserat et al. 2009) in the Western United States of America (U.S.A) where it affected horticulturally grown trees outside of their native habitats. It was later found to have spread to the native range of *J. nigra* (Grant et al. 2011) in the Eastern U.S.A. Thousand cankers disease has also unfortunately found its way from the U.S.A. all the way to Italy (Montecchio and Faccoli 2014).

Cerocephalinae are primarily known to attack wood boring beetles. A few species of Cerocephalinae (Hymenoptera: Pteromalidae) wasps have been reared from *P. juglandis* infested walnut wood. *Neocalosoter* spp. have been documented to have

emerged from TCD wood in California U.S.A. (Graves et al. 2009), Tennessee U.S.A. (Lambdin et al 2015), and Italy (Bosio and Cooke-McEwen unpub.). *Theocolax americanus* McEwen was described from this disease system in Colorado U.S.A. (McEwen 2015) and later found in the Piemonte region of Northwestern Italy (Bosio and Cooke-McEwen unpub.). Another unidentified *Theocolax* species was reared from TCD wood in Tennessee U.S.A. (Lambdin et al. 2015.

Bosio and Cooke-McEwen (unpub.) recently documented the other insects found on TCD infested trees in the Piemonte region of Northwestern Italy. In their rearings they found a new species of *Cerocephala* that came from the TCD wood in small numbers. The new species is here described and updated description for the type specimen of *C. caelebs* is also given.

Methods

Specimen collecting.—

Trees showing TCD symptoms and having *P. juglandis* boring holes visible on the surface had stems cut and transported to a laboratory in Torino. Twenty eight logs, each about fifteen cm long by two to three cm in diameter, were stored in emergence traps. The emergence traps were made of paperboard boxes with holes containing plastic vials for catching emerged insects that are attracted to the daylight. Environmental conditions were held at around 20°C. Emergences were checked between 4 September and October 31, 2015.

Specimen Handling.—

Specimens were viewed and imaged under a light microscope and scanning electron microscope (SEM). Color photos were taken using an EntoVision micro-

imaging system with a Leica M16 zoom lens and JVC KY-75U 3-CCD digital video camera attached to a M16 column on a Wild M-5 stereo microscope. The SEM used was a Hitachi TM3000 used in 15kV compo mode with charge–up reduction. Specimens were compared to closely related species in the Smithsonian collection and specimens on loan from the Natural History Museum of The United Kingdom (London, U.K.; HNMUK).

Measurements were taken using both SEM and Entovision images along with ImageJ software version 1.47 (W. Rasband, National Institutes of Health, USA). Hymenoptera terminology morphology follows the Hymenoptera Anatomy Ontology project (Yoder et al. 2010) and Gibson (1997). Abbreviations are as follows: F# = funicular segment, OOL = ocular ocellar line, POL = posterior ocellar line. Measurement explanations are as follows: body length– in lateral view from the anterior margin of the face to the posterior margin of the metasoma excluding the ovipositor sheath and without correcting for hunched posture; POL– the shortest distance between the posterior ocelli; OOL–the shortest distance between the lateral margin of a posterior ocellus and the eye margin; submarginal vein length– measured from the basal most remaining edge of the vein to the point where the vein meets the anterior wing margin; marginal vein length– measured from where it meets the wing margin to the point of junction between the stigma and postmarginal veins; stigmal vein– measured along its posterior margin from the point of junction with the postmarginal vein to its apical margin; postmarginal vein length– measured along its anterior margin from its point of junction with the stigmal vein to its apex.

Sequencing.—

Total DNA from one female and one male specimen was extracted from whole adults that were soaked overnight in extraction buffer and proteinase K. The standard DNeasy® tissue kit spin column protocol (QIAGEN 2006) was used except the last step was not repeated to maintain higher DNA concentration in the extract. Polymerase chain reaction (PCR) amplifications used 10ul of Syd labs master mix, 4ul of DNA template, 1ul of each primer, and 9ul of water. Cytochrome oxidase I (COI) was amplified using the primers LCO1490F: 5' GGTCAACAAATCATAAAGATATTGG 3' and HCO2198R: 5' TAAACTTCAGGGTGACCAAAAAATCA 3' (Folmer et al. 1994). The thermal cycling program was as follows: 93° C for 3 minutes; 34 cycles of 93° C for 15 seconds, 46° C for 45 seconds, 68° C for 45 seconds; 72° C for 7 minutes; and a final incubation at 4° C. Unpurified PCR samples were sent to GeneWiz, Inc. for sequencing.

Basecalling was checked, sequences were aligned, and sequences trimmed using Geneious ® v. 9.1.5. Geneious was also used to produce the neighbor joining (NJ) tree using the HKY model and 1000 bootstrap replicates. The taxa included in the analysis were all other cerocephaline taxa with COI data and an unidentified Eulophidae as the outgroup based on Desjardins et al. (2007). Proportional pairwise distances, excluding ambiguous sites, were determined using PAUP* v. 4.0b10 (Swofford 2002). Newly determined COI sequences were deposited in GenBank with the accession numbers MG100830 and MG100831.

Results

Descriptions.—

Cerocephala flavus Cooke-McEwen **sp. nov**.

Diagnosis. The following combination of traits distinguish this species from all others in the genus: coloration yellow-tan; gena with fine striations extending from the malar margin nearly to compound eye; lower face with carinae slanting towards clypeus and reaching clypeus; interantennal horn short, not extending more than 0.25x as long as height of compound eye; in dorsal view the lower facial process is not visible beyond the upper facial process; posterior half of pronotum dorsally smooth; female petiole short, slightly wider than long; male petiole around 1.5x as long as wide.

Description. Female holotype (Fig. 1). Body length: 1.4mm. Color: Yellow-tan anteroventrally fading to dark brown dorsally on mesosoma and metasoma; pedicel and scape yellow, F1 yellow and gradually turning darker brown to clava; compound eye silver; legs yellow, fore and hind coxa white; metasoma with Gt1 dorsally dark brown to black, Gt2–6 dorsolaterally dark brown to black with metallic sheen, dark brown patch ventrally on Gt3-4, ovipositor sheaths basally yellow and distally dark brown; wing hyaline with small macula under the setal tuft and larger macula near stigmal vein base extending 4/5 to wing margin.

Head (Figs. 2–5): Anteriorly width and height subequal. Setae short and sparse on upper face, less sparse on lower face with setae longer than torulus width. Clypeus not well

defined, represented only by tentorial pits. Lower face with carinae slanting towards clypeus, medial carina extending from clypeal edge half way to toruli. Gena length 0.68x height of compound eye; with fine striations in malar space extending from malar margin stopping just before compound eye; malar sulcus absent; smooth behind compound eye with scattered setae. Interantennal lamella extending 0.4x way to median ocellus; in lateral view interantennal process extending from front of face 0.13x the height of compound eye; in dorsal view lower facial process not visible over upper facial process. Antennal segment length ratio as follows: scape: pedicel: F1: F2: F3: F4: F5: F6: clava, 16:7:3:4:4:5:5:4:11; F4-clava with placoid sensilla. Ratio of posterior ocellus maximum diameter:OOL:POL as 6:16:15. Occipital carina present; occiput imbricate dorsal and lateral to occipital foramen; postgena smooth; occipital foramen located in upper quarter of head as seen laterally.

Mesosoma (Figs. 6–9): Dorsally 1.8x as long as wide; pronotum 0.63x as long as wide at its widest point, neck 0.48x as wide as pronotum at its widest point, wrinkled anteriorly on neck, smooth posteriorly with sparse setae; notauli complete, deeper anteriorly; lateral lobes of the mesoscutum bulging, sparsely setose along margins; transscutal articulation straight; axilla bulging less than lateral lobe of mesoscutum, sparsely setose; scutellum as long as wide, with row of three setae along the anterolateral margin, smooth dorsally, stiate to wrinkled posteriorly; metanotum with dorsellum narrow and with a transverse carina, lateral panels rounded and expanded, lateral panels with few raised carinae. Propodeum without median longitudinal carina, areolate-rugose; callus laterally delimited by carina, anterolaterally with posterior facing

setae. Lateral pronotum with sparse setae and a minute crevice extending from the surface sculpturing of the collar toward the posterior margin of the pronotum; mesopleuron shagreen, imbricate near forewing base; metapleuron smooth medially with anterior edge indented with carinae spanning the indent. Profemur and protibia with sparse setae; Procoxae imbricate; protibia and femur lightly imbricate. Midleg sparsely setose, mesocoxa imbricate basally; mesofemur imbricate posteriorly. Hindleg sparsely setose; metacoxa lightly imbricate basally. Ratio of submarginal: marginal: postmarginal: stigmal veins as 28:27:4:5. Maculae under setal tuft and along the posterior edge of the marginal-stigmal vein junction faint. Setae along anterior wing margin distal to setal tuft, posterior wing margin lacking setae except distal 1/3; wing surface with spots or setal sockets but lacking setae.

Metasoma (Figs. 10–11): Petiole dorsally areolate-rugose, slightly wider than long; laterally rippled with some longitudinal carinations; ventrally smooth. Gaster 3.6x as long as ovipositor sheaths, smooth, sparsely setose; seventh gastral tergum more heavily setose posteriorly, some setae half as long as ovipositor sheaths. Ovipositor sheaths setose.

Male (Figs. 12–14): In lateral view interantennal process extending from front of face 0.21x the height of compound eye Antennal segment length ratio as follows: scape: pedicel: F1: F2: F3: F4: F5: F6: F7: clava, 19:8:6:7:6:6:6:6:5:10. Ratio of submarginal: marginal: postmarginal: stigmal veins as 39:35:5:7. Petiole 1.5x as long as wide.

Variation: Primary color varies from yellow to yellow-tan. Most specimens have a dark brown patch surrounding the ocelli; ratio of posterior ocellus maximum diameter:OOL:POL varies with ranges as 4-6 : 13-16 : 12-17. There is some variation in the dark coloring on the antennae, varying from just the clava being dark to F5-clava being dark; F3 sometimes with placoid sensilla. There is sometimes a dark band that connects the dorsal dark patch of the gaster to the ventral dark patch and one specimen have Gt3-7 laterally mostly dark brown.

Type Material. Holotype female: top label “Italy:Piemonte: Novara: Olengo 20.Sept-10.Oct.2015 G. Bosio”, middle label “Ex. Walnut wood with thousand cankers disease and infested with invasive *P. juglandis*”, and bottom label “*Cerocephala flavus* Cooke-McEwen Holotype, ♀”. Paratypes: Two specimens with top label " Italy:Piemonte: Novara: Olengo 20.Sept-10.Oct.2015 G. Bosio”, middle label “Ex. Walnut wood with thousand cankers disease and infested with invasive *P. juglandis*”, and bottom label “*Cerocephala flavus* Cooke-McEwen Paratype, ♀” or “*Cerocephala flavus* Cooke-McEwen Paratype, ♂”. Seven specimens with the top label “Italy:Piemonte: Novara: Olengo Sept-Oct.2017 G. Bosio”, middle label “Ex. Walnut wood with thousand cankers disease and infested with invasive *P. juglandis*”, and bottom label “*Cerocephala flavus* Cooke-McEwen Paratype, ♀”

Biology and Distribution. The host and geographical origin of this species is uncertain. It was collected from wood infested with the invasive *P. juglandis*, which is a suspected

host for other cerocephaline wasps found at the same location, but there were also other beetles present in the wood so the host is unknown. These wasps were collected in Italy but they came from a disease system that has already introduced two species of North American cerocephalines into Italy (Bosio and Cooke-McEwen unpub.). *Cercephala flavus* could be an Italian native attacking wood boring beetles or it could be another North American cerocephaline introduction.

Etymology. From Latin, flavus meaning yellow (Brown 1956). Named for the yellow coloration that is so far unique to the genus.

Remarks. The holotype and two of the paratypes are very fragile from initial transport and DNA extraction. During imaging the antennae and metasoma fell off the holotype and the antennae could not be recovered.

Specimens of *Cerocephala petiolata* Hedqvist were not needed to determine diagnostic characters since that species was said to have the female petiole 2.5x as long as wide (Hedqvist 1969) and the *C. flavus* had a short petiole that was slightly longer than wide. Specimens from *Cerocephala rotunda* Delucchi were also not requested on loan since photos of the type and paratype for that species showed those specimens to be so dark in coloration (black and dark reddish brown) that they were easily distinguished from *C. flavus*. Also, *C. rotunda* has longer ovipositor sheaths at just over half as long as the gaster. It has also only been documented in the Congo region (Delucchi 1956).

The type of *Cerocephala aquila* (Girault) wasn't examined, but all general collection specimens of this species had two areas of surface sculpture that were not

found in *C. flavus*. First, *C. aquila* has surface sculpture on the pronotum consisting of concentric carinae resembling half a finger print (Fig. 15). Second, *C. aquila* has longitudinal striations on the axillula and a few along the transscutal articulation (Fig. 15). The following traits also aid in differentiating this species from *C. flavus*: body, legs, and antenna brown with posterior part of metasoma dark brown; gena without fine striations in malar space; lower face with carinae slanting towards clypeus but clypeus and supraclypeal area free of carinae except medial carina (Fig. 16); antennae with F1-clava having placoid sensilla; and female petiole 1.8x as long as wide.

The primary trait that differentiates *Cercephala cornigera* Westwood from *C. flavus* is that the head looks tridentate in dorsal view with the interantennal process being very long and the lower facial processes being visible beyond the upper facial processes (Fig. 17). The type for this species was missing its head and since this is the most important body part for this species general collection specimens were instead requested from the Natural History Museum of the United Kingdom (NHMUK). The following traits were found to also aid in differentiating this species from *C. flavus*: lower half of body and legs brown with posterior part of metasoma and antenna dark brown; and pronotum dorsally with surface sculpture, not smooth. According to Russo (1938, Fig 106 #3) *C. cornigera* also has placoid sensilla on F2 and F3 unlike *C. flavus*.

The type for *Cerocephala dinoderi* Gahan was found to have a much longer petiole at nearly twice as long as wide and the body was dark brown and black. Once these distinctive differences were determined, further SEM work used a general collection specimen so as to not risk damaging the type specimen. Another trait that was

found to differ from *C. flavus* was that the gena was without any fine striations in the malar space.

The type specimen for *Cerocephala eccoptogastri* Masi was not requested on loan based on differences that could be determined from the work of Grissell (1981). That study showed *C. eccoptogastri* to have the lower facial processes pronounced enough to be visible dorsally. Since this is not the state found in *C. flavus*, other traits were explored but using general collection specimens. These species were found to be very similar morphologically. The light brown coloration in *C. eccoptogastri* is also somewhat close to the yellow coloration of *C. flavus*. However, these species still differ in the state of the lower facial processes.

A search was performed for the type for *Cerocephala rufa* (Walker) but it is found to be missing. The Yorkshire museum looked in their collection as well as the Rudd collection and the specimen could not be found. The last published work on this specimen was Grissell (1981) so the specimen went missing sometime afterwards. The USNM collection, where Grissell worked, was also checked thoroughly for this specimen. One other possibility is that it was taken out on loan by John Noyes at NHMUK during that period so that collection was also searched. There were also two individuals that had large loans from NHMUK that were asked check their materials for this specimen. The specimen was not found during any of these searches. General collection USNM specimens from the synonymized "*C. dubrae"* (synomymized by Grissell, 1981) as well as general collection specimens from NHMUK were used for comparisons to *C. flavus*. The main differences found other than coloration (brown vs. yellow) was that *C. rufa* has the striations on the malar space only reaching half way

towards the compound eye (Fig. 18) and the striations on the front of the face extending above the toruli.

Cercephala caelebs (Masi, 1917)

Cerocephala (*Parasciatheras*) *caelebs* Masi, 1917, Novitates Zoologicae 24: 189–190, fig. 45–48.

Diagnosis. According to Gahan (1946) *C. caelebs* differs from *C. dinoderi* in that the antennae are more setose and individual setae can be longer than the segment from which they arise. This trait is diagnostic for *C. caelebs* against most species. This trait as well as the darker brown coloration and petiole being 3.3x as long as wide differentiate *C. caelebs* from *C. flavus*. This species is found in the Seychelles.

Description. Male holotype (Figs. 19–20). Body length: 1.4mm without head. Color: Mesosoma mostly brown fading to dark brown dorsally on mesoscutum; legs brown, tarsi light brown; petiole brown, gaster with Gt2–6 dark brown to black; wing hyaline with small macula under the setal tuft and larger macula near stigmal vein base extending 3/4 to wing margin.

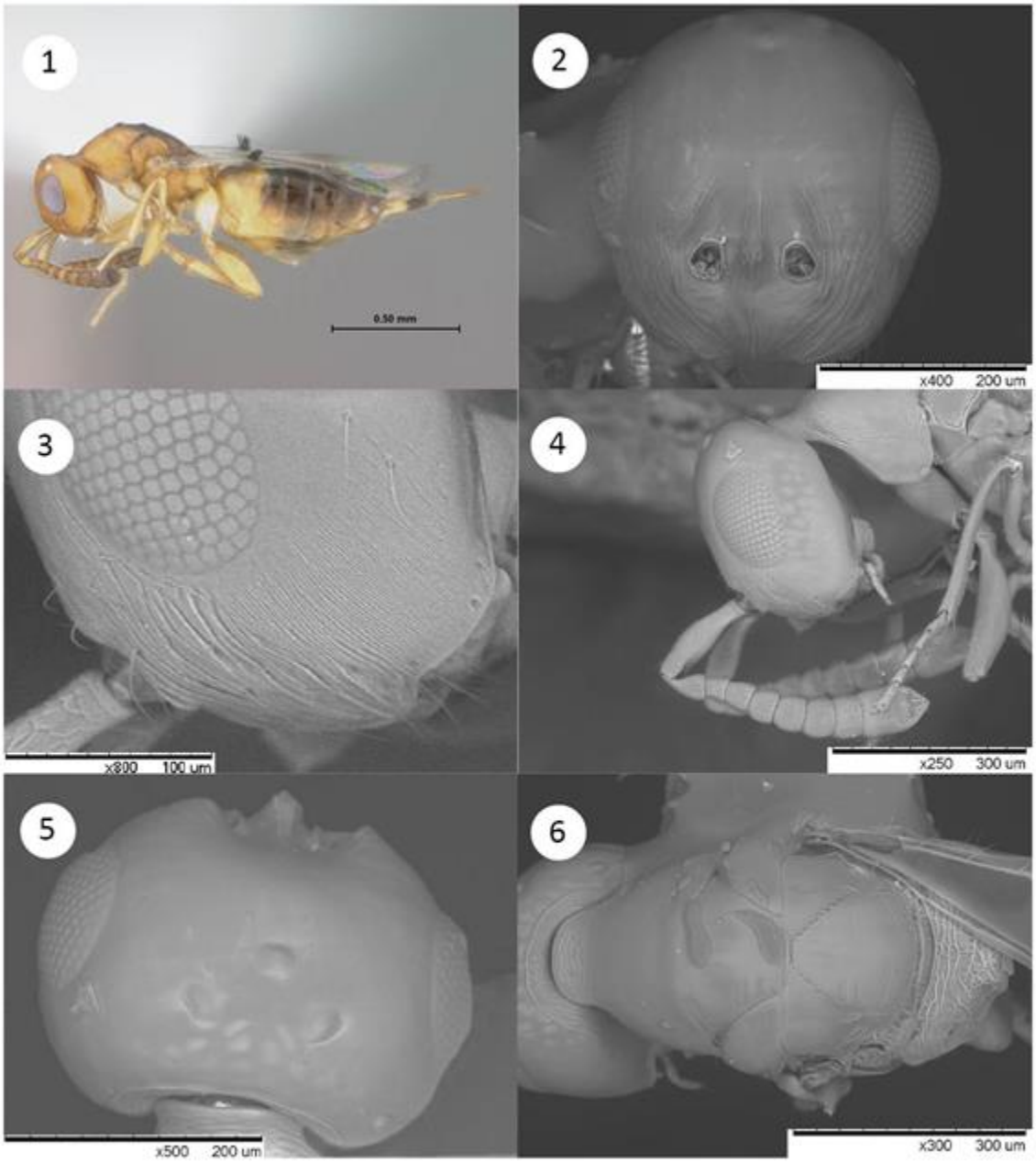
Head: Missing.

Mesosoma (Figs. 20–21): Positioned on point mount in way that prohibits direct dorsal view; dorsal measurements are approximate. Dorsally 2.5x as long as wide; pronotum nearly as long as wide at its widest point, wrinkled anteriorly on neck, smooth

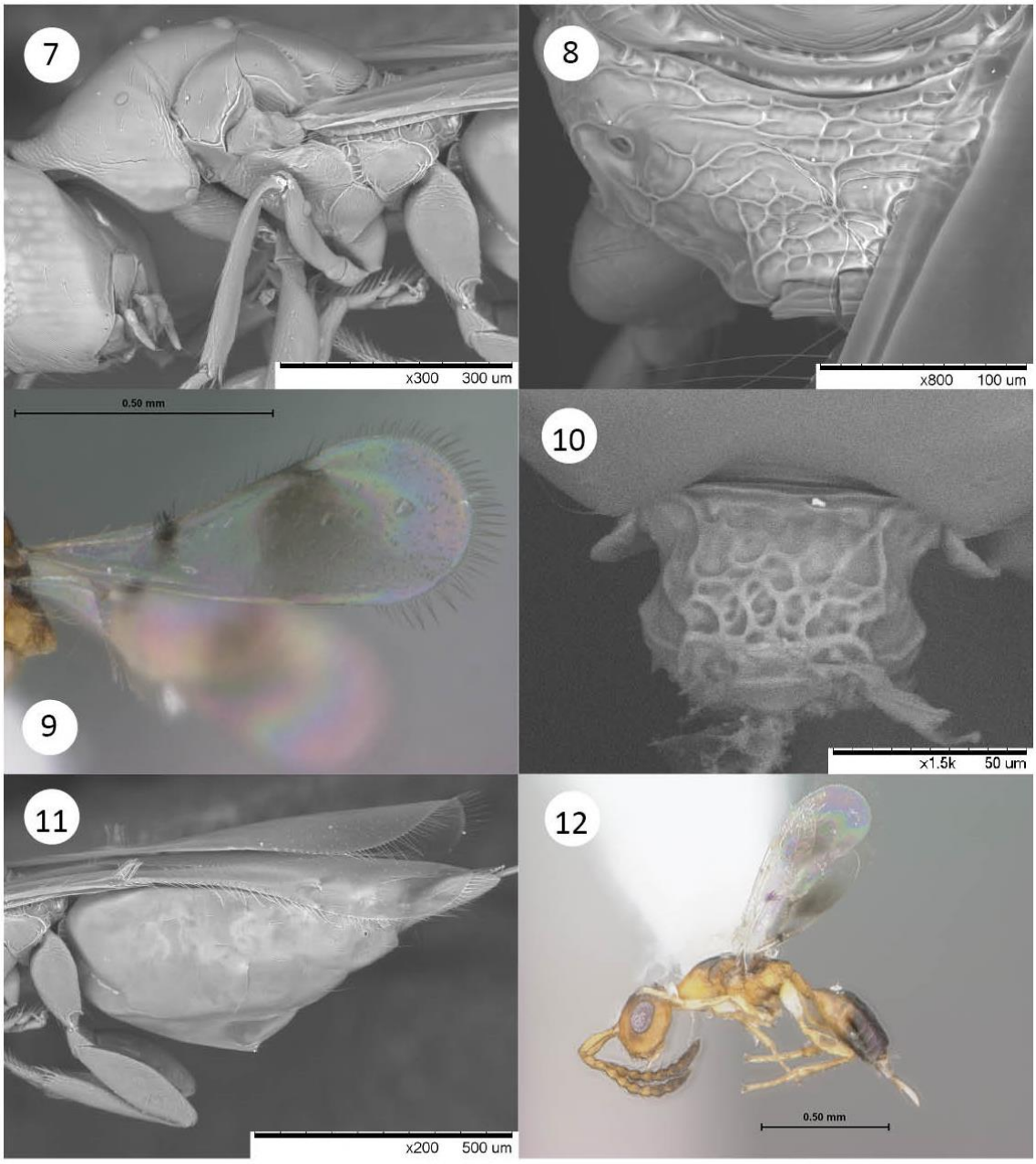
posteriorly with sparse setae; notauli complete, deeper anteriorly; lateral lobes of the mesoscutum bulging, sparsely setose along margins; transscutal articulation straight; axilla bulging less than lateral lobe of mesoscutum, sparsely setose; scutellum as long as wide, smooth dorsally, stiate to wrinkled posteriorly; metanotum with dorsellum narrow and with a transverse carina, lateral panels rounded and expanded; propodeum areolate-rugose. Lateral pronotum with sparse setae; mesopleuron shagreen, imbricate near forewing base; metapleuron smooth medially with carinae along anterior edge. Coxae imbricate basally; legs sparsely setose. Ratio of submarginal: marginal: postmarginal: stigmal veins as 21:22:3:4. Maculae under setal tuft and along the posterior edge of the marginal-stigmal vein junction. Setae along anterior wing margin distal to setal tuft, posterior wing margin lacking setae except distal 1/3; wing surface with spots or setal sockets but lacking setae.

Metasoma (Figs. 19–20, 22): Petiole dorsally lightly rugose, 3.3x as long as wide; laterally rippled with some longitudinal carinations. Gaster contracted, blunt, and smooth.

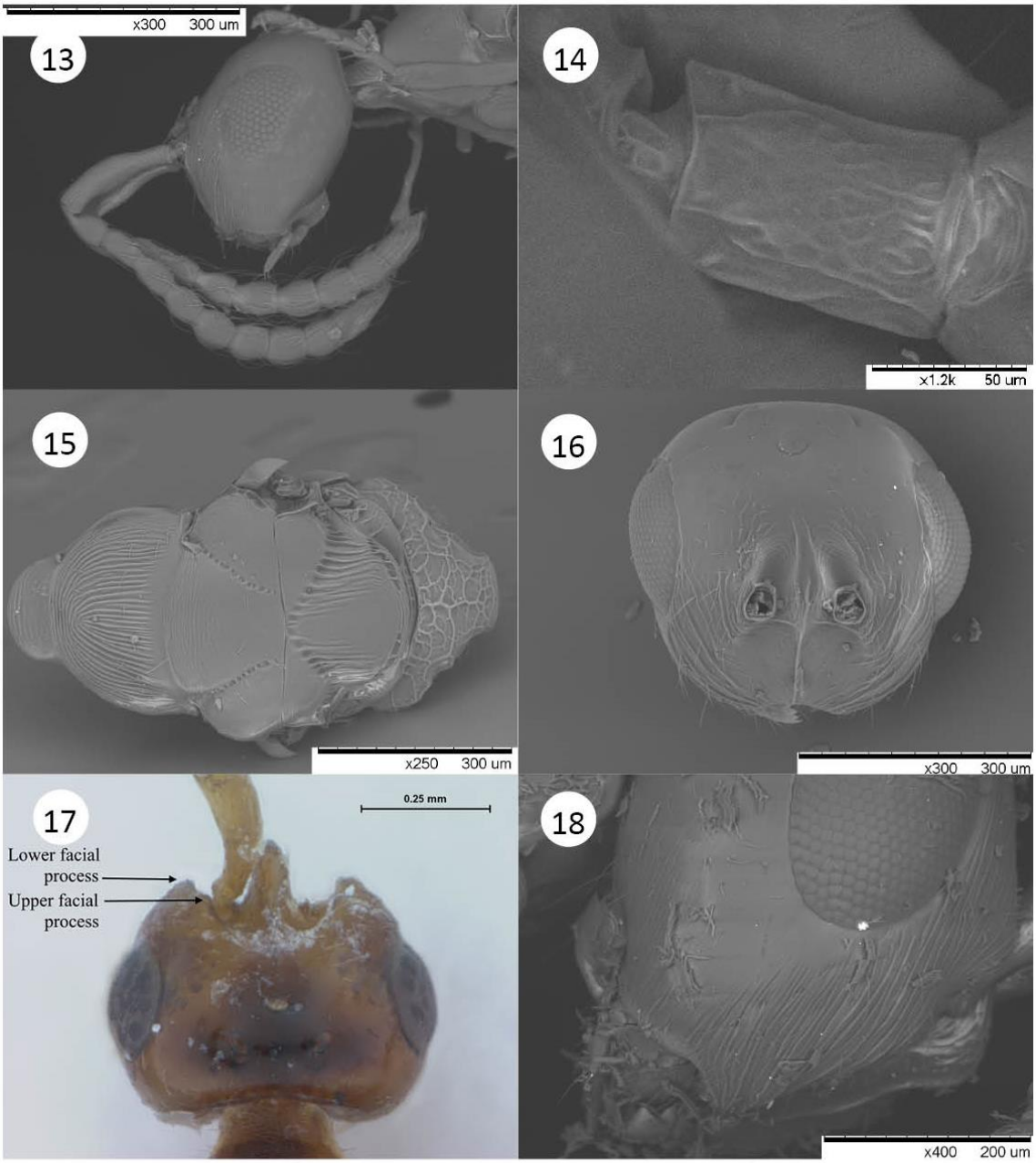
Remarks. This holotype was loaned from NHMUK.



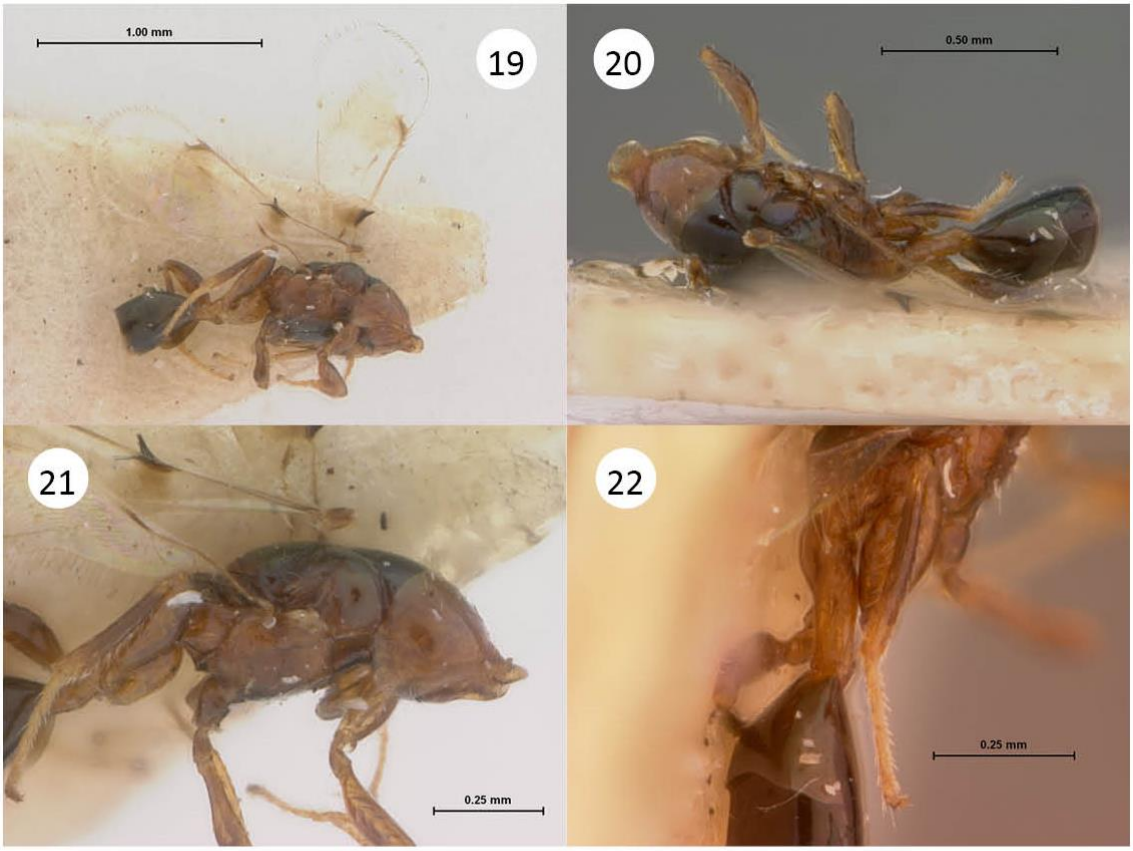
Figs. 1-6. *Cerocephala flavus* ♀ holotype. 1. Lateral habitus; 2. anterior head; 3. malar space; 4. lateral head and antenna; 5. dorsal head; 6. dorsal mesosoma.



Figs. 7-12. *Cerocephala flavus*. 7. lateral mesosoma, ♀ holotype; 8. propodeum, ♀ holotype; 9. wing, ♀ holotype; 10. dorsal petiole, ♀ holotype; 11. lateral metasoma, ♀ holotype; 12. lateral habitus, ♂ paratype.



Figs. 13-18. 13. lateral head, ♂ paratype *Cerocephala flavus*; 14. dorsal petiole, ♂ paratype *Cerocephala flavus*; 15. dorsal mesosoma, *Cerocephala aquila*; 16. anterior head, *Cerocephala aquila*; 17. dorsal head, ♀ *Cerocephala cornigera* from NHMUK; 18. malar space, *Cerocephala rufa* from NHMUK.



Figs. 19-21. *Cerocephala caelebs* from NHMUK, ♂ holotype. 19. Lateral habitus; 20. dorsal habitus; 21. lateral mesosoma; 22. dorsal petiole.

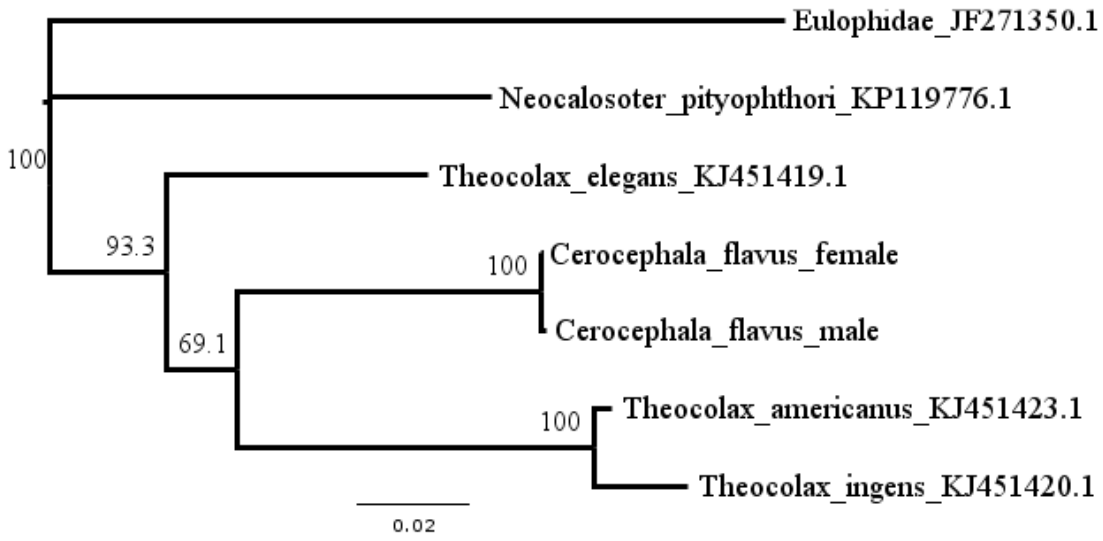
Sequencing of COI.—

The female and male *C. flavus* were grouped together on the Cerocephalinae phylogeny (Fig. 23) and the sequences showed zero pairwise distance between them supporting that they are conspecific. However, the phylogeny shows evidence for *C. flavus* being nested within *Theocolax* with low support (69.1% bootstrap value) even though it morphologically fits within *Cerocephala*. It is possible that all of *Cerocephala* is a lineage within *Theocolax* but no taxonomic changes are being made at this time.

Future sequencing of other *Cerocephala* species will aid in our understanding of how these genera are related.

Cerocephala flavus was closest in proportional pairwise distance to *T. elegans* (Westwood) at 9.4488% different. Next closest was *T. americanus* at 11.2861% different and then *T. ingens* Xiao and Huang at 12.8609% different. The distance between *Neocalosoter pityophthori* and *C. flavus* is 14.1732% different.

Fig. 23. Neighbor joining HKY consensus tree for Cerocephaline wasps with a Eulophidae the outgroup. Numbers by nodes indicate the bootstrap proportions.



Discussion

Not much is known about the biology of this new species other than it was found in TCD infested wood. The host is unknown; however, since the other cerocephalines in the TCD system are thought to attack *P. juglandis*, it is possible that this is also the host for *C. flavus*. Other insects have been noted to be taking advantage of TCD infested trees as they provide fungi and decaying wood (Bosio and Cooke-McEwen unpub.). It is possible that *C. flavus* is also taking advantage of a novel host in its environment.

However, there is also the possibility that these wasps came to Italy from the U.S.A. with the disease like the other cerocephalines reared from the Piemonte region.

The sequencing of COI for *C. flavus* represents the first sequences for this genus to be deposited on GenBank. Sequences showed that the female and male specimens that were suspected to correspond to each based on coloration other were actually conspecific. They also revealed a possible taxonomic problem with the *Theocolax* and *Cerocephala*. *Cerocephala flavus* being nested within *Theocolax* suggests that it is just another lineage within the genus. It should be noted that within *Theocolax, T. elgans* is in a different species group than the other two *Theocolax* species in this analysis (Grissell and Hevel 2005 and McEwen 2015) and *C. flavus* appears to be intermediate between these two species groups.

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