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Research article

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'Closing the Life Cycle' of *Andricus quercuslanigera* (Hymenoptera: Cynipidae)

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Abstract

While the alternation of asexually and sexually reproducing generations is common among the oak gall wasps (Hymenoptera: Cynipidae: Cynipini), it has been hypothesized that the diversity of taxa displaying this unique life cycle is underestimated because either 1) the alternative generation has not yet been described or 2) each generation is currently described as two distinct species and should be collapsed into one heterogonic organism (referred to as 'closing the life cycle').Through field observations, experimental rearing, morphological identification, laboratory behavioral assays, and genetic analysis, we demonstrate heterogony in the cynipid species *Andricus quercuslanigera* (Ashmead 1881) (Hymenoptera: Cynipidae), which was previously only described from the asexual generation. We confirm that the asexual generation, which develops in 'fuzzy' galls on the central vein on the underside of leaves on live oaks in southeast Texas, *Quercus virginiana*, represents only one generation in a bivoltine life cycle that alternates with a newly discovered sexual generation that develops in galls on catkins on the same host. Our study highlights the need for detailed inspections of the life cycles of unisexual gall wasp species and we discuss the closure of the *A. quercuslanigera* life cycle in light of recent advances in the study of the ecology and evolution of heterogony in the Cynipidae.

Key words: cyclic parthenogenesis, gall wasp, heterogony, *Quercus*

Oak gall wasps (Hymenoptera: Cynipidae: Cynipini) have long fascinated naturalists largely due to two unique biological features: 1) the spectacular diversity of complex gall structures that they induce on their host plants (i.e., galls) and 2) the complex life cycles they display—called cyclic parthenogenesis or heterogony—where species alternate between asexually and sexually reproducing generations (Stone et al. 2002, Stone and Schönrogge 2003, Price et al. 2004). Approximately 1,300 named species exist within the Cynipidae (Stone et al. 2002, Abe et al. 2007), while estimates of diversity range from 13,000 (Buhr 1965) to >210,000 worldwide (Espirito-Santo and Fernandes 2007). The majority of wasps in the tribe Cynipini lay their eggs into a specific tissue of a single host plant species (typically oaks in family Fagaceae) and manipulate the plant to form nutrient-rich plant outgrowths within which the insects feed and develop (Stone et al. 2002). Despite their incredible diversity, the knowledge of the biology, natural history, host association(s), and life cycles of gall forming cynipids is largely fragmented, particularly in North America, where many species or generations are likely undiscovered or undescribed, compared to European taxa (Dreger-Jauffret and Shorthouse 1992, Espirito-Santo and Fernandes 2007, Abe et al. 2007).

Cynipids exhibit one of three reproductive strategies: 1) sexual reproduction only, 2) asexual reproduction only (via parthenogenesis), or 3) heterogony (or cyclic parthenogenesis) where asexual (agamic) and sexual (gamic) generations alternate to complete a bivoltine life cycle (Stone et al. 2002). Heterogony has been clearly documented in approximately 85–100 cynipid species, but it is hypothesized that the diversity of species that exhibit the life cycle is underestimated because either 1) the alternate generation has not yet been described or 2) two separately described species unknowingly represent a single heterogonic species (Pujade-Villar et al. 2001). The process of either describing the alternate generation of a heterogonic species or synonymizing two previously described univoltine species is referred to as 'closing the life cycle' (e.g., Lyon 1959, Lund et al. 1998, Rokas et al. 2003, Ide and Abe 2016).

Documenting heterogony still remains difficult, however. During a heterogonic life cycle, the asexual generation produces eggs parthenogenetically. Typically, asexual females can produce both male (arrhenotoky) and female (thelytoky) offspring from eggs that are usually deposited into a specific host plant part (Stone et al. 2002). At or near the site of oviposition, galls are induced within which the sexual generation males and females will feed, develop, and

later emerge as adults. The emergent individuals will mate and the females will initiate galls usually on the same host plant species, and in rare cases, on a different host plant species (Askew 1984, Stone et al. 2002). The females of both generations can be morphologically dissimilar or near-identical, but most often induce galls on different host plant organs that differ drastically morphologically (e.g., Felt 1965, Lyon 1970, Meyer 1987, Lund et al. 1998, Stone et al. 2008). Thus, differences in the morphology of female wasps and galls between generations in combination with the lack of detailed knowledge about the natural history and life cycles of cynipids have made it challenging to link generations of a single cynipid species. However, advances in rearing and husbandry techniques, the accumulation of detailed morphological data for taxonomic identification, and the advent of DNA sequencing have generated potential solutions to the challenge of diagnosing heterogony.

Here, we use a series of analyses to demonstrate that the previously described univoltine parthenogenically reproducing 'fuzzy' gall wasps, *Andricus quercuslanigera* (Ashmead 1881) (Hymenoptera: Cynipidae), represents only one generation in a bivoltine life cycle. By combining multiple approaches, we describe the previously unknown sexual generation of *A. quercuslanigera* that emerges from galls on catkins of its host plant, the southern live oak, *Q. virginiana*, in southeast Texas and effectively close the gall wasps' life cycle. We also provide a description of the catkin galls produced by asexual females, detail the morphological differences between asexual and sexual generation females, and document the timing of life cycle events (oviposition and adult emergence) of the sexual generation. Our results highlight the likelihood that there are many taxa expressing heterogony, which are currently undiscovered, and emphasize the need for multidisciplinary approaches that include natural history, taxonomy, behavior, and genetics to better understand the ecology and evolution of gall forming cynipids.

Materials and Methods

Study System

The 'wool-bearing' or 'fuzzy' gall wasp, *A. quercuslanigera* (Ashmead 1881) [= *Andricus linaria* (Kinsey 1937) (Hymenoptera: Cynipidae)] has previously been described from the asexual (agamic) generation only (Fig. 1A–F). The gall wasp is native to four live oak species in the subsection *Virentes* along the Gulf Coast of the southern United States and Mexico: *Quercus virginiana*, *Quercus geminata*, *Quercus fusiformis*, and *Quercus oleoides*. There are two other live oak species from which we do not know if the gall wasp has been found: *Quercus sagraena* (native to Cuba) and *Quercus brandegeei* (native to southern Baja, Mexico) (Cavender-Bares et al. 2015). Finally,

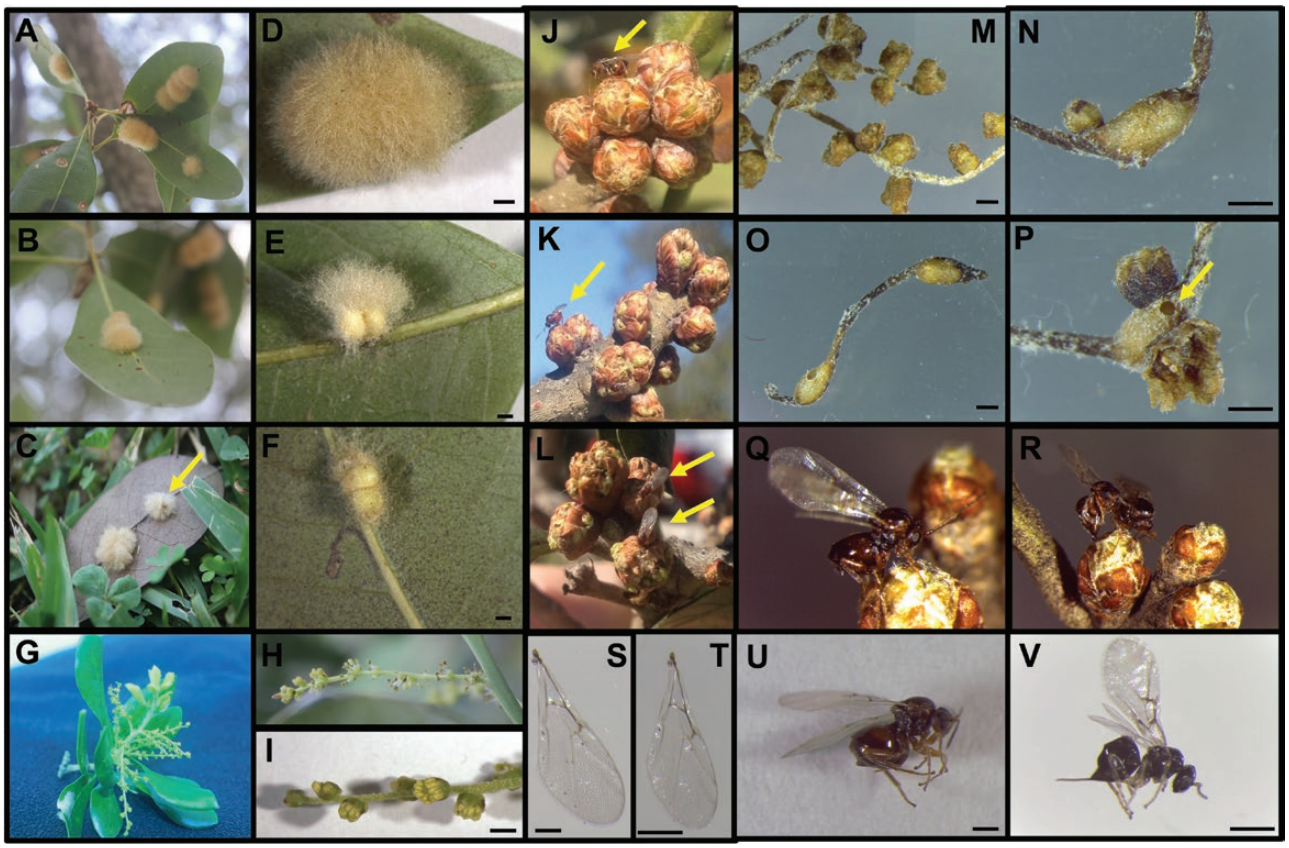

Fig. 1. (A and B) Fuzzy galls of the asexual generation of *A. quercuslanigera* developing on live oak, *Q. virginiana*, in Houston, TX. (C) An abscised galled leaf depicting *A. quercuslanigera* emergence holes (yellow arrow). (D) Fuzzy galls induced on the central vein of a live oak leaf. The hairs of neighboring galls have grown together, resembling a single large gall. (E and F) When the hairs are removed, the inner chamber of three distinct galls can be seen growing adjacent to each other. (G) Newly flushed leaves and catkin and (H) close-up of new catkin growth. (I) Fluorescents of male flowers (aments) of live oak catkins. (J, K, and L) Field observations of asexual generation *A. quercuslanigera* ovipositing into catkins buds at field sites in Houston, TX (yellow arrows). (M) Individual aments of live oak catkins and the catkin galls (N, O, and P) of the sexual generation of *A. quercuslanigera*. The galls form on the central stalk of the catkins as small swellings. In panel (P), sexual generation *A. quercuslanigera* emergence holes can be seen in the catkin galls (yellow arrow). (Q and R) Asexual generation *A. quercuslanigera* ovipositing into catkin buds in laboratory behavioral observations. The forewings of (S) asexual generation female and (T) sexual generation female and (U) asexual generation female and (V) sexual generation female. Scale bars (horizontal black lines) in panels D, E, F, I, L, M, N, O, S, T = 1 mm; U, V = 500 μm.

A. quercuslanigera has been documented on non-native, transplanted *Q. virginiana* in San Joaquin County, CA (Pujade-Villar et al. 2016) and the University of California–Davis Arboretum, Yolo County, CA (S.P.E., personal observation). There are other descriptions of specimens from various host associations that have been morphologically identified as *A. quercuslanigera* that may require further genetic analysis to solidify species identity. These include *A. quercuslanigera* potentially found on the netleaf oak, *Q. rugosa*, in a native portion of its range distributed throughout the temperate highlands of central Mexico (Serrano-Munoz et al. 2016). *Q. rugosa* also exists in small disjunct populations throughout south-central Arizona, southwestern New Mexico, and southwestern Texas (Little 1999), but the gall former has yet to be documented here. Furthermore, *A. quercuslanigera* has been reported to have shifted from native oak, *Q. oleoides*, to form galls on nursery live oak, *Q. virginiana* (native to southeastern United States and Texas), and pin oak, *Q. palustris* (native to the mid-western United States) in Tamaulipas and Nuevo Leon, Mexico, where it is classified as a pest species (Pujade-Villar et al. 2016). Our study is restricted to documenting alternative life cycles of *A. quercuslanigera* on the most wide-spread live oak species, *Q. virginiana*, in a native portion of its range in southeastern Texas, where we are currently working on various aspects of the evolutionary ecology of the entire community of gall wasp species and their natural enemies that reside on *Q. virginiana* (Egan et al. 2013, Forbes et al. 2015).

Many gall wasps can only form galls into newly formed meristematic tissues produced on an annual cycle (e.g., the yearly flush of new leaves on deciduous oak trees) (Harper et al. 2004). Once these tissues reach a certain age, galls may no longer be induced following oviposition (e.g., Hood and Ott 2010). Thus, it is critical for gall wasps to synchronize their life cycle (the timing of adult emergence and oviposition) to match the timing of new tissue production of their host plant and important to understand the timeline of production of new host plant tissues that can potentially form galls. We briefly describe the yearly phenology of the two tissues (leaves and catkins) used by the alternating generations of *A. quercuslanigera* on its host plant, *Q. virginiana*, that guided our experimental approach of field observations and to help better understand the timing of the gall wasp life cycle (see below). Live oaks in southeast Texas are a deciduous hardwood species whose buds (unopened flowers) are visible year-round but begin to swell and unfold or 'break' starting as early as December, but peak mid- to late-January through late-February. The briefly broken buds form catkins (male flowers) for approximately 2 wk. Several days before or immediately following catkins senescence, new leaves are beginning to flush in the spring, beginning in early-March continuing through late-April (Muller 1961, Hood and Ott 2010). Both catkin production and leaf flush can overlap significantly within and between individual host plants and vary across years (G.R.H., personal observation).

Very little is known about the biology and natural history of *A. quercuslanigera* in North America (Melika and Abrahamson 2002). While heterogony is common in European species of *Andricus* (Stone et al. 2008), approximately two-thirds of the estimated 300–400 *Andricus* species globally reside in North America (Cook et al. 2002, Melika and Abrahamson 2002, Liljeblad et al. 2008). Additionally, most, but not all (e.g., Melika et al. 2009), of these species are described from one generation only (Stone et al. 2002, Joseph et al. 2011). The previously described asexual generation of *A. quercuslanigera* develops within 'fuzzy' single chambered leaf galls located on the underside of new leaves on the midvein alone or in close proximity to other conspecific galls. The single chambered galls consist of a small inner capsule initially covered in short white hairs that eventually turn yellow to light brown and measure approximately 0.7–4.0 mm in final size but may often be interpreted as much larger due to the 'fuzziness' of a group of closely developing galls on the same leaf appearing as one large gall (Pujade-Villar et al. 2016; Fig. 1D–F). Galls are first visible beginning in late-August and continue to grow and mature by early-September to early-December (Pujade-Villar et al. 2016, Serrano-Munoz et al. 2016, S.P.E., personal observation). Asexual females will begin emerging from late-September and continue through early-March. Unlike many examples of life cycle closure in the Cynipidae, the alternative generation of *A. quercuslanigera*, to the best of our knowledge, has not yet been described as another species. A detailed literature search returned no occurrence of catkin galls on any of the seven species of live oaks in the subsection *Virentes* in southern United States and Mexico as well as the alternative aforementioned oak species that serve as non-native hosts of *A. quercuslanigera* in Mexico and California.

Study Design

We used a five-prong approach to test for the presence and location (tissue type) of the sexual generation of *A. quercuslanigera* that included the following: 1) field behavioral observation, 2) field collections and laboratory rearing, 3) morphological identification, 4) laboratory behavioral assays of adult asexual individuals, and 5) DNA barcoding and comparative phylogenetic analysis of known and unknown gall wasps. When combined, these approaches provide definitive evidence of the existence of a sexual generation of *A. quercuslanigera* on live oak, *Q. virginiana*, in southeast Texas. Each of the five approaches is described, in turn.

Field Behavioral Observations

To explore which tissues emerging individuals of the asexual generation oviposited into that would give rise to galls which the sexual generation developed within, beginning in mid-January through early-March 2013 (during peak asexual gall wasp emergence) on sunny afternoons, we surveyed for asexual generation females emerging from fuzzy leaf galls under five high gall density trees on the Rice University campus in Houston, Harris County, TX (29.717341, –95.404965). We observed groups or individuals for 2–4 h over the course of 5 d displaying a number of behaviors including flying near trees, sunning and cleaning themselves, and walking or perching on leaves, stems and buds. However, overwhelmingly we observed asexual *A. quercuslanigera* females landing on and appearing to oviposit into the newly engorged or recently broken buds of live oaks, which produce catkins (also referred to as 'aments' or an inflorescence of male flowers) several days later and then new leaves in the following weeks (Fig. 1G–I). Using these observations, we conducted a series of behavioral observations to test the hypothesis that the asexual generation oviposits into and produces galls on live oak catkins that give rise to the sexual generation of *A. quercuslanigera*.

To confirm that 1) live oak catkins were used as oviposition sites by the asexual generation, 2) galls were produced on catkin tissue, and 3) these catkin galls give rise to the sexual generation of *A. quercuslanigera*, we used a three-step behavioral approach. First, we made additional observations in nature on host plants of the asexual generation ovipositing into newly formed catkin buds (Fig. 1J–L). Typically, cynipids will oviposit into newly differentiating tissues at or prior to the onset of 'visible' growth when undifferentiated and reactive host plant material is almost unnoticeable. In the case of catkin gallers, this may include ovipositing inside an unopened bud just prior to catkin formation (e.g., Stone et al. 1995).

Second, following oviposition, we sampled and reared the contents of the galls induced in the field observations (Fig. 1M–P) in 2013 (see above) and again in 2014 (see below) for morphological analysis (Fig. 1S–V) and DNA barcoding and phylogenetic analysis (see below). Last, we performed host tissue choice tests of asexual individuals of field-caught adults in the laboratory to confirm the use of catkins compared to alternative oak tissues (Fig. 1Q and R).

Beginning in late-January 2014, we located and marked approximately 10 branches (using orange flagging tape) containing high densities of new buds with developing catkins (e.g., Fig. 1G–I) on each of the five trees that previously displayed high densities of asexual *A. quercuslanigera* galls in 2013 at Rice University. Then, in teams of two, beginning in early-February through early-March 2014 (during peak asexual emergence), we observed marked branches until asexual individuals were seen flying near or landing on catkin buds (Fig. 1J–L). Once seen, we followed individuals for a maximum of 10 min and noted if and where oviposition took place, and when possible, carefully aspirated individuals into 500-ml clear plastic cups to be used for laboratory-based host choice experiments or genetic analysis (see below). In addition, we carefully marked groups of catkin buds (again, using orange flagging tape) where oviposition was observed and collected the inflorescence after catkins had fully emerged from the bud and matured (usually 7–10 d) to be used in rearing experiments and genetic analysis. We placed groups of mature catkin clusters from each tree in a 500-ml clear plastic cup. The cups were checked daily for the following 6 mo, until well after gall former emergence had ceased. All emerging individuals identified to the genus level prior to morphological analysis, were preserved in 95% ethanol, and stored in a −80°C freezer. A subset of five individuals that emerged from catkins and morphologically identified as *Andricus* were DNA barcoded (see below).

Experimental Rearing

To sample the previously described asexual generation for morphological comparison and DNA barcoding (see below) and to measure the timing of adult emergence, we haphazardly collected leaves from eight high gall density live oak trees from November through January 2014. This collection included the same five trees used in the field observation study at Rice University and three additional trees located approximately two km from Rice at Hermann Park, Harris County, TX (29.718894, −95.391910). Galls were stored and individuals were reared and preserved following the methods outlined below.

In a general effort to locate potentially undescribed alternative generations of several species of gall formers on live oaks in southeast Texas, we systematically sampled plant tissues from different host plant organs over multiple years. During this process and independent of our field observations, we discovered a gall on the catkins of many individual *Q. virginiana*. Catkins are a likely location for finding undiscovered alternative generations because 1) the tissue is extremely ephemeral, lasting less than 2 wk from bud growth, through emergence, and to abscission, and 2) mature galls on this tissue are extremely small and inconspicuous. Our pilot rearing trials in 2013 led us to more thoroughly sample the catkin galls in late-March and early-April 2014 and 2015. Again, we haphazardly collected mature catkin clusters from the same eight live oak trees from Rice University and Hermann Park containing high densities of asexual generation leaf galls. Clusters of mature catkins from each tree were stored separately in 500-ml clear plastic cups and monitored daily for 6 mo in shade under field conditions, until well after gall former emergence had ceased. Upon emergence, gall wasps were stored in 95% ethanol, and placed in a −80°C freezer for comparative morphology and DNA sequencing.

To measure the timing of adult emergence for both the sexual and asexual generations, we noted the number of individuals that emerged on each day. To describe the general differences between generations, we pooled individual emergence times of males and females across trees, and calculated the mean emergence date ± SE for each generation. These estimates of the timing of adult emergence are used to construct a detailed description of the timing of life history (oviposition and emergence) for *A. quercuslanigera* across both generations of its life cycle.

Morphological Analysis

We confirmed that 1) individuals reared from the new catkin galls morphologically key to the genus *Andricus*, and 2) compared the previously described asexual generation of *A. quercuslanigera* to the potential sexual generation adults by describing the sexual generation males and females and redescribing the asexual generation females. In addition, we also compared individuals emerged from the newly discovered catkin galls to asexual individuals of *A. quercusfoliatus,* which is host specific to and forms galls on female flowers and newly growing acorns of *Q. virginiana* in southeastern Texas (Ashmead 1881). For many species of cynipid, the morphologically distinguishing characteristics are near-identical between generations (but they almost always differ significantly in body size), while in other heterogonic species the generations are morphologically distinct for many defining characteristics (e.g., Lund et al. 1998, Ide and Abe 2016). However, even morphologically diverged generations of the same heterogonic species share distinguishing morphological characteristics in common compared to different species. Thus, we also compared the distinguishing morphological features of the known asexual generations of *A. quercuslanigera* and *A. quercusfoliatus* to the newly discovered sexual generation emerging from the catkin galls.

We first used the recent taxonomic review of the world genera of oak cynipid wasps (Melika and Abrahamson 2002) to key individual live oak catkin gall formers to the genus level. Unfortunately, there is no species-level taxonomic key for North American *Andricus*. We, therefore, used the original description of *A. quercuslanigera* (Ashmead 1881) and a revised description of *A. quercuslanigera* by Kinsey (1937), as well as the original description of *A. quercusfoliatus* (Ashmead 1881), to determine if individuals that emerged from catkin galls closely morphologically resemble previously described asexual individuals emerging from *Andricus* galls on *Q. virginiana*.

In addition, we provide the first description of the sexual generation catkins galls. Taxonomic nomenclature follows the descriptions given in Ashmead (1881), Kinsey (1937), Melika and Abrahamson (2002), Wachi and Abe (2010), and Ide and Abe (2016). Following Wachi and Abe (2010) and Ide and Abe (2016), we have also provided detailed photographs of dorsal views of the mesosoma and anterior views of the head for descriptive purposes. All laboratory-based photographs of galls and gall wasps in Fig. 1 were made using a Leica M125 Steroscope, with lighting provided by a Leica LED5000 MCI and a Rotterman contrast TM Transmitted Light Base with Rotterman contrast TM, brightfield and two-sided darkfield. Field-based photographs in Fig. 1 were taken with a Cannon Rebel T2i. Images detailing the morphological variation between generations in Fig. 2 are comprised of 30–50 stacked photographs taken every 10–50 µm using a Cannon 5DSr fitted with a MP-E 65mm Cannon lens, which was mounted on Stackshot 3× Automated Focus Stacking Macro Rail (Cognisys Inc.). Images in Fig. 2 were processed using

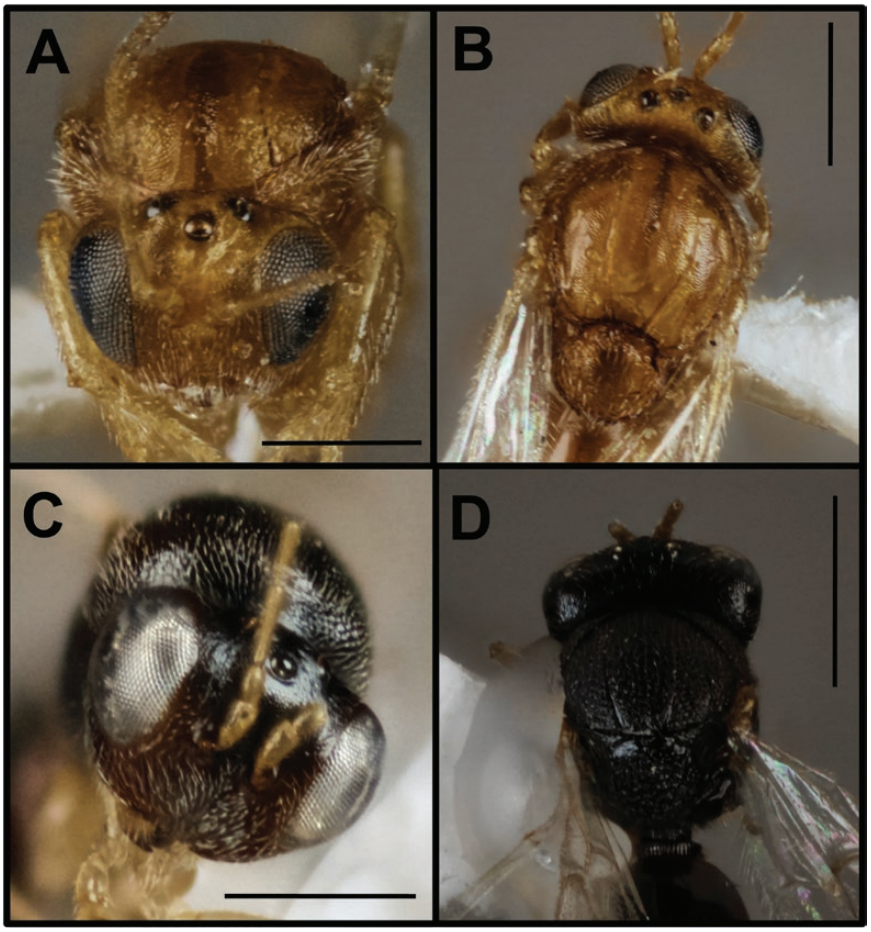

Fig. 2. (A) Head, anterior view, and (B) mesosoma, dorsal view, of an asexual generation female of *A. quercuslanigera*. (C) Head, anterior view, and (D) mesosoma, lateral view, of a sexual generation female of *A. quercuslanigera*. Horizontal (A and C) and vertical (B and D) scale bars = 500 μm.

Zerene Stacker (Zerene System LLC., Richland, VA) and images in both Figs. 1 and 2 were prepared for publication in Adobe Illustrator and Microsoft PowerPoint. A single lectotype and four paralectotype specimens were deposited in Invertebrate Zoology collections at the American Museum of Natural History.

Laboratory Behavioral Observations

We performed host tissue choice tests to determine which tissue field-caught asexual female wasps emerging from fuzzy leaf galls preferred in a laboratory setting. Based on our field observations, we hypothesized that catkins were the tissue galled by the purported asexual generation, in which the sexual generation developed. We used field-caught as opposed to naïve individuals to ensure that the sampling of the ephemeral catkin resource that decompose in the laboratory quickly coincided with 'oviposition ready' insects. In a 10-cm diameter Petri dish, we combined breaking buds containing clusters of newly formed live oak catkin tissue (Fig. 1Q–R) with the tissues galled by other cynipid species of live oaks in southeastern Texas (Egan et al. 2013), including new and old leaf tissue and a 5-cm clipping of a freshly growing stem collected from local *Q. virginiana*. In each cup, we aspirated one asexual female *A. quercuslanigera* and observed individuals for 30 min, noting the total amount of time each individual spent on each tissue type or on the inside surface of the cup, as well as their behavior (walking, stationary, or ovipositing). In total, we tested 25 individual asexual *A. quercuslanigera* females in tissue choice tests. Following the experiment, the field-caught wasps used in the host-choice assays were morphologically identified to confirm species identity. For analyses, the total amount of time spent on each tissue type is given as a mean percentage of the total time ± SE across replicates.

DNA Barcoding and Phylogenetic Analysis

We hypothesize that *A. quercuslanigera* mtDNA sequence divergence would be much lower between individuals reared from asexual generation fuzzy galls and the sexual generation catkin galls (i.e., between generations within a species) compared to sequence divergence between different *Andricus* species. To test this hypothesis, we compared DNA sequences from a 511-bp region of the mitochondrial barcoding gene, cytochrome oxidase subunit I (COI), from the following four groups: 1) the previously described asexual fuzzy gall generation (n = 25), and 2) the newly sampled and purported sexual generation galling catkins (n = 12 females; n = 3 males which were both reared directly from catkins or aspirated off of catkins in the field), with 3) sequences from asexual generation of a closely related species, *A. quercusfoliatus* occurring on the same individual host plants on Rice University (n = 2) and from *Q. virginiana* in southeastern Louisiana (in St. Mary Parish approximately 424 km east of Houston, TX; 29.83407, –91.56842; n = 3; see Supplemental File 1 for mtDNA COI sequences), and 4) eight individual sequences from seven different European, Middle Eastern, and Northern Africa *Andricus* taxa retrieved from GenBank (one individual each from *A. inflator* [GenBank DQ012623], *Andricus coriarius* [DQ012620],

Andricus caputmedusae [DQ012619], *Andricus kollari* [AF395176], *Andricus pictus* [DQ012625], *Andricus mayri* [DQ012624], and two individuals from *Andricus curvator* [DQ012621, AF395177]). The mtDNA COI gene is commonly used for barcoding and taxonomically distinguishing and genetically identifying cryptic insect species (e.g., Smith et al. 2008, 2013; Forbes and Funk 2013; Forbes et al. 2015).

Genomic DNA was extracted from individual *A. quercuslanigera* and *A. quercusfoliatus* from whole body tissue using DNeasy Blood and Tissue kits (Quigen Inc., Valencia, CA). We used a pair of degenerative primers developed by Simon et al. (1994), modified by Kaartinen et al. (2010), and recently used by Egan et al. (2017): COI pF2: 5′–ACC WGT AAT RAT AGG DGG DTT TGG–3′ and COI 2437d: 5′–GCT ART CAT CTA AAW AYT TTA ATW CCW G–3′. Amplified PCR fragments were purified using Exonuclease I (New England Biolabs, Ipswich, MA) and Shrimp Akaline Phosphatase (Fermentas Life Sciences, Glen Burnie, MD) and sequenced in the forward direction only at the University of Arizona using an ABI 3730XL DNA Analyzer (Applied Biosystems, Inc.).

To phylogenetically test the hypothesis that sequences from the asexual generation of *A. quercuslanigera* and the purported sexual generation are more closely related to each other than to different *Andricus* species, we constructed a maximum likelihood phylogenetic tree based on Tamura-Nei's genetic distance (Tamura and Nei 1993) calculated between individuals, with bootstrap support determined by 10,000 replicates using Geneious v.6.1.18 (Kearse et al. 2012). The tree is rooted with an outgroup individual, *Synergus thaumacerus* Dalman (GenBank EF486970), a cynipid species native to Europe from the tribe Synergini, paraphyletic with Cynipini (Ronquist et al. 2015).

Taxonomy

Type Material

LECTOTYPE. Sexual generation female, collected in Houston, Harris County, TX, on 3 March 2016, emerged 22 March 2016, dry-mounted, deposited in the Invertebrate Zoology collections at the American Museum of Natural History (Barcode number: AMNH_IZC 00332623). PARALECTOTYPES. Two each sexual generation males and females, collected in Houston, Harris County, TX, on 3 March 2016, emerged 25 March 2016, dry-mounted pointed and pinned, deposited in the Invertebrate Zoology collections at the American Museum of Natural History (Barcode number: AMNH_IZC 00332624-00332627). Additional material examined included five male and females from the sexual generation and five additional asexual generation females collected in Houston, Harris County, TX, on 4 March 2016, emerged 19 March 2016 through 2 April 2016, dry-mounted.

Redescription of Asexual Generation Female

OVERALL BODY

2–2.5 mm. Larger than newly described sexual generation females. Overall body red-brown, and much lighter than newly described sexual generation (Figs. 1U and 2A and B). Head. Broad behind eyes; eyes dark brown; antenna 13 (occasionally 14) segments, jointed, slightly yellowish brown; uniformly bright rufous (Fig. 2A). MESOSOMA. Mesonotum shagreened, nearly naked with parapsidal grooves discontinuous, indefinite anteriorly, or complete to the pronotum; arched mesoscutum, with notauli indistinct in the anterior 1/3 to 1/4, subquadrate scutum that is as long as it is broad, and scutellar foveae distinct (Fig. 2B; Melika and Abrahamson 2002). METASOMA. Darker along the sides; a projecting portion of the ventral spine of the hypopygium, which is long with short spare subapical setae that reach beyond the apex of the spine and are never dense, and do not form a truncate tuft (Melika and Abrahamson 2002). LEGS. Light reddish brown, with ends paler and the posterior femora and tibia brown. FOREWINGS. Normal, about 1.3 times body in length, hyaline, not ciliate on dorsal margins, short ciliate on apical and posterior margins; radial cell slender, elongate; veins pale, including radial and cubital veins very light; subcostal and basalis clearly defined, but not dark; no heavy infuscation on any vein and without spots or blotches in any cell (Fig. 1U).

GALL

Wool covering an inner capsule of mature galls on underside of leaf along mid-vein (rarely on topside) final size 1.0–7.0 mm in diameter and 2–3 mm high (including the hairs); Wool creamy white, yellowing slightly when older; individual hairs making up the wool are straight and 0.5–2.0 mm in length; inner capsule is light brown and can be solitary or in clusters of 3–6 along midvein of leaf (Fig. 1A–F).

Description of Sexual Generation Female

OVERALL BODY

Length = 1.4–2.1 mm. Smaller than previously described asexual females and much darker in overall body color, which is dark red-brown to black (Figs. 1U and V and 2C and D); with head and mesosoma slightly lighter and metasoma darker, especially on lateral sides. HEAD: Broad behind eyes; eyes dark brown; antennae 13 (occasionally 14) segments, jointed, dark red-brown to amber on the basal 3–8 segments, grading to darker brown on the terminal third of each antennae (Fig. 2D). MESOSOMA. Mesonotum leather-like (or finely shagreened), nearly naked with parapsidal grooves discontinuous, indefinite anteriorly, or complete to the pronotum; arched mesosoma, with notauli indistinct in the anterior 1/3 to 1/4, subquadrate scutum that is as long as it is broad, and scutellar foveae distinct (Melika and Abrahamson 2002); scutellum longer than wide, well rounded posteriorly, finely rugose, sparingly hairy (Fig. 2D). METASOMA. Darker along the sides; elongate and positioned dorasally, or higher than long; metasomal tergum II covers about two-third of whole metasoma; surface entirely smooth and naked except for small and sparse patches of setae laterobasally; posterior margins of some segments microspopically punctate; a projecting portion of the ventral spine of the hypopygium, which is long with short spare subapical setae that reach beyond the apex of the spine and are never dense, and do not form a truncate tuft (Melika and Abrahamson 2002). In general, a lower density of setae is present on the entire body of the sexual generation compared to the asexual generation. LEGS. Light reddish brown, with ends paler, sometimes yellow or light rufous, and the posterior femora and tibiae brownish; tarsal claws fine, weak, and toothed. FOREWINGS. Normal, about 1.3 times body in length, hyaline, not ciliate on dorsal margins, short ciliate on apical and posterior margins; radial cell slender, elongate; veins pale, including radial and cubital veins very light; subcostal clearly defined, but not dark; no heavy infuscation on any vein and without spots or blotches in any cell (Fig. 1V).

GALL

Formed on catkins in the spring during host plant bud break (Fig. 1J–L). Small (~1 mm) swelling of the central stem of the catkin, with gall shape similar to a grain of rice, or ovate. Surface of

gall indistinct from the rest of the central stem of catkin except for swelling (Fig. 1M–P).

Description of Sexual Male

Length 1.2–1.6 mm. Smaller than either sexual or asexual female. Metasoma ovate; shorter than head and mesosoma combined. Other characters similar to sexual female.

Results

Field Behavioral Observations

During approximately 10 h (2 h/d for five different days) of field observations made on five live oak trees heavily infested with the asexual generation of *A. quercuslanigera*, we observed 112 asexual *A. quercuslanigera* flying near, landing on and/or ovipositing into new buds containing live oak catkins. While individuals walked, or flew between different live oak tissues, oviposition was observed 57 times, and in each case, into newly breaking catkin buds. A subset of five *A. quercuslanigera* that were observed ovipositing into catkin buds were aspirated directly off the tree for later morphological and genetic analyses (Fig. 1J–L). Groups of catkin buds on which oviposition was directly observed were marked with flagging tape and labeled with permanent markers for collections after galls matured on catkin tissue.

Experimental Rearing and General Morphological Analysis

We reared over 150 of the putative sexual generation gall wasps from catkin collections at Rice University and Hermann Park, Houston, TX, from eight live oak trees (Fig. 1M–P), as well as thousands of asexual generation females (Fig. 1A–F). Using the genus-level descriptions of *Andricus* given in Melika and Abrahamson (2002), all sexual generation males and females emerging from catkin galls contained the following defining morphological characteristics: the presence of 1) a projecting portion of the ventral spine of the hypopygium which is long with short spare subapical setae which reach beyond the apex of the spine and are never dense, and do not form a truncate tuft, and 2) an arched mesosoma, with notauli indistinct in the anterior 1/3 to 1/4, subquadrate scutum that is as long as it is broad, and scutellar foveae distinct. See Fig. 1S and T for detailed photographs of forewings and Fig. 1U and V for photographs of females of the asexual and sexual generations, respectively.

The sexual individuals that emerged from the catkin galls are smaller than asexual individuals that emerged from leaf galls (note the scale bar difference in Fig. 1S–V), an observation that has been documented for many *Andricus* species. However, the sexual generation shares defining morphologically similarities to the previously described asexual generation of *A. quercuslanigera* (see Fig. 1S and T for similarities in wing venation). The apparent morphological similarity at the species level between a previously described generation and an undescribed generation has often served as a starting point for taxonomic species diagnosis (e.g., Benson 1953). We, therefore, take this approach and used the original description of the asexual generation in Ashmead (1881) and the revised description by Kinsey (1937) of the asexual generation to corroborate our genus level classification and taxonomically identify the individuals to the species level. In this case, we found the following defining morphological characteristics: 1) legs light reddish brown or sometimes yellow rufous, femur and tibia brownish, 2) antennae are bright rufous on the basal three to eight segments, grading to darker brown on the terminal third of each antannae, and 3) head and mesosoma uniformly rufous. For a more detailed description of the taxonomy and a redescription of the asexual generation and description of the sexual generation, see *Taxonomy* section.

Description Life History Timing and of Sexual Generation Galls

The mean date of emergence for the asexual generation was 18 January (±1 d), with the range of emergence spanning a 5-mo period from 9 September to 24 February (n = 1083) (Fig. 3). The mean date of emergence for the sexual generation was 26 March (±1 d), with the range of emergence spanning a 2-wk period from 23 March to 9 April (n = 94) (Fig. 3). The difference in emergence times results in 10-fold difference in the length of the emergence periods between the asexual and sexual generations of *A. quercuslanigera*.

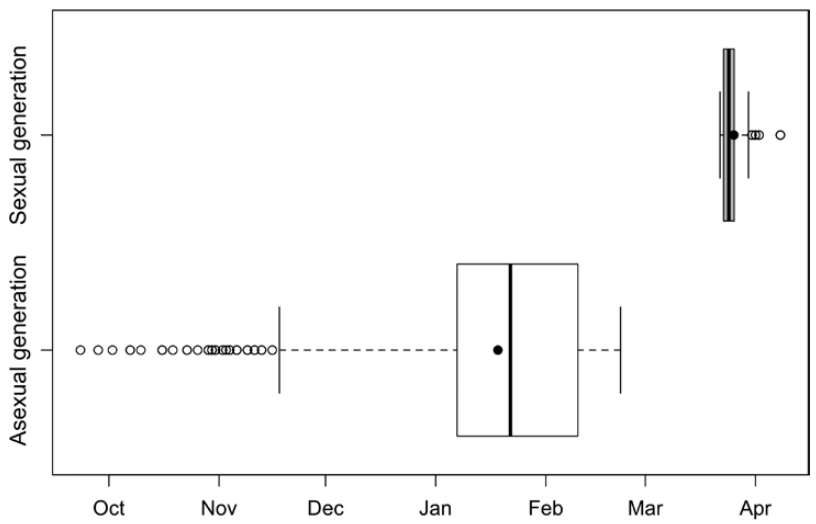

Fig. 3. Boxplot of the timing of adult emergence (day of the year) of the asexual (white) and sexual (gray) *A. quercuslanigera* from live oaks, *Q. virginiana,* in Houston, TX. The dark circles within the box represent the mean, dark vertical lines are the median, the left and right vertical edges of the box plots are the 25th and 75th quantiles, respectively, the left and right error bars around the plots represent the minimum and maximum values excluding outliers, and the white dots represent outliers.

By combining our field observations and laboratory rearing and our analysis of adult emergence, we were able to generate a timeline of adult emergence and oviposition of the bivoltine lifecycle of *A. quercuslanigera* on *Q. virginiana* in southeastern Texas (Fig. 4). Coinciding with spring leaf flush in mid-March to early April, the sexual generation, emerging from catkin galls (Fig. 4A), lay eggs into the lateral veins on the underside of newly flushed leaves (Fig. 4B). The fuzzy galls develop from mid- to late summer through the fall and winter (Fig. 4C and D), and asexual females emerge as adults beginning as early as September continuing through late February the following year (Fig. 4E) coinciding with catkin bud production. At that time, females oviposit into the unopened or newly opened bud of catkins (Fig. 4F). Galls on catkins grow rapidly in the spring, just as the catkins themselves do, with sexual adults emerging just weeks after asexual females initiate gall growth. Immediately following sexual generation adult emergence, males and females mate, and females oviposit into the newly growing leaves (Fig. 4E). The life history timing of each generation differs drastically (approximately 11 to 11(1/2) mo for the asexual generation and 2–4 wk for the sexual generation from oviposition to adult emergence).

The galls induced by asexually reproducing generation form on the central stalk in between or adjacent to the individual aments (inflorescent) on each catkin (Fig. 1M–P). When mature, the single chambered galls, which appear as swellings, are a golden-brown color and range from 0.5–2 mm in size. Upon close inspection, an emergence hole can often be seen on the side of galls (see yellow arrow in Fig. 1P). Typically, multiple galls can form on each catkin with as few as one but as many as four individual galls per catkin stem.

Lab Behavioral Observations

We performed 25 host tissue choice tests, where we exposed individual asexual females to different host plant tissues simultaneously for 30 continuous minutes and noted their location and behavior. On average, *A. quercuslanigera* spent significantly more time (68 ± 3% SE) of on catkin tissue compared to on all other tissues combined (32 ± 3%). Only 2 of the 25 asexual females (8%) spent less than half of the 30 min in contact with plant material on catkin buds. Moreover, *A. quercuslanigera* females were observed ovipositing into catkins in 13 of the 25 assays (52%) and all 13 (100%) of these oviposition events occurred into a catkin bud.

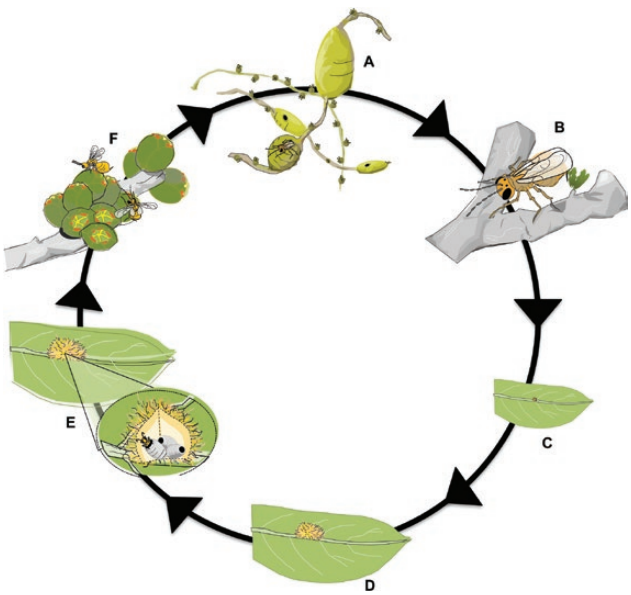

Fig. 4. The life cycle of the cyclically parthenogenic gall wasp, *Andricus quercuslanigera*, on its host plant, *Quercus virginiana*, in southeastern Texas. See text in *Description Life History Timing and of Sexual Generation Galls* section for a detailed description of the life cycle.

Phylogenetic Analysis

A maximum likelihood tree supported the existence of three distinct clades of *Andricus*, with the *A. quercusfoliatus* and *A. quercuslanigera* clade containing > 90% bootstrap support: 1) a Palearctic clade consisting of the eight individuals downloaded from GenBank, 2) a clade of five *A. quercusfoliatus* sampled from live oaks in southeastern Texas and southeastern Louisiana, and 3) a clade composed of all 25 asexual generation *A. quercuslanigera* females and all 15 of the potential sexual generation *A. quercuslanigera* (3 sexual generation males and 12 sexual generation females) that was sister to the *A. quercusfoliatus* clade (Fig. 5). The average nucleotide divergence within the *A. quercuslanigera* clade across all individuals (5.68 ± 0.06% SE; range = 0–10.85%) and between asexual and sexual individuals (6.14 ± 0.11%; range = 1.42–10.85%) is significantly less than the average difference between the *A. quercuslanigera* and *A. quercusfoliatus* clades (13.58 ± 0.22%; range = 8.40–20.62%) and the *A. quercuslanigera* and the Palearctic *Andricus* clade (10.27 ± 0.11%; range = 5.95–15.18%). Within the *A. quercuslanigera* clade, distinct clusters exist composed of all sexual individuals or all asexual individuals, but the largest single cluster consists of a mixture of asexual *A. quercuslanigera* and both sexual male and female *A. quercuslanigera* (Fig. 5). Interestingly, while genetic variation within the *A. quercuslanigera* was quite large, nucleotide divergence of this magnitude is common in cynipids (e.g., Hayward and Stone 2006). This genetic data provides the clearest evidence in support of closing the life cycle.

Discussion

Here, we provide evidence through field observations, natural history, field collections, laboratory rearing, morphological analysis, laboratory behavioral assays, and phylogenetic analysis via DNA barcoding that the previously described asexual generation of *A. quercuslanigera* developing inside galls induced on live oak leaves alternates with a sexually reproducing generation developing inside galls induced on live oak catkins to complete a bivoltine heterogonic lifecycle. Our results suggest, as others have hypothesized (Pujade-Villar et al. 2001, Stone et al. 2002), that heterogony is underestimated in Cynipidae and highlight the need for detailed inspection of different host plant tissues as hosts of gall formers. Additionally, our results indicate that one generation of the life cycles of heterogonic cynipids can be exceptionally brief and the galls produced can be visible for a short period of time. Thus, it is critical to consider the phenology of different host plant tissues. We hypothesize that because catkins are an abundantly reliable resource in early spring, they may be an underappreciated resource for alternative generations of gall formers on oaks (particularly in the United States), but due to their extremely ephemeral nature (on live oak they are in great abundance for approximately 2 wk only) they are often overlooked when searching for galls (see Schönrogge et al. 1996, Melika et al. 2000, Cook et al. 2002, Bailey et al. 2009, e.g., in Palearctic taxa). In fact, gall formation on ephemeral tissue such as catkins has been known to extend the life span of the structures likely to prolong the period available for oviposition and development (Csoka 1997, Stone et al. 2002).

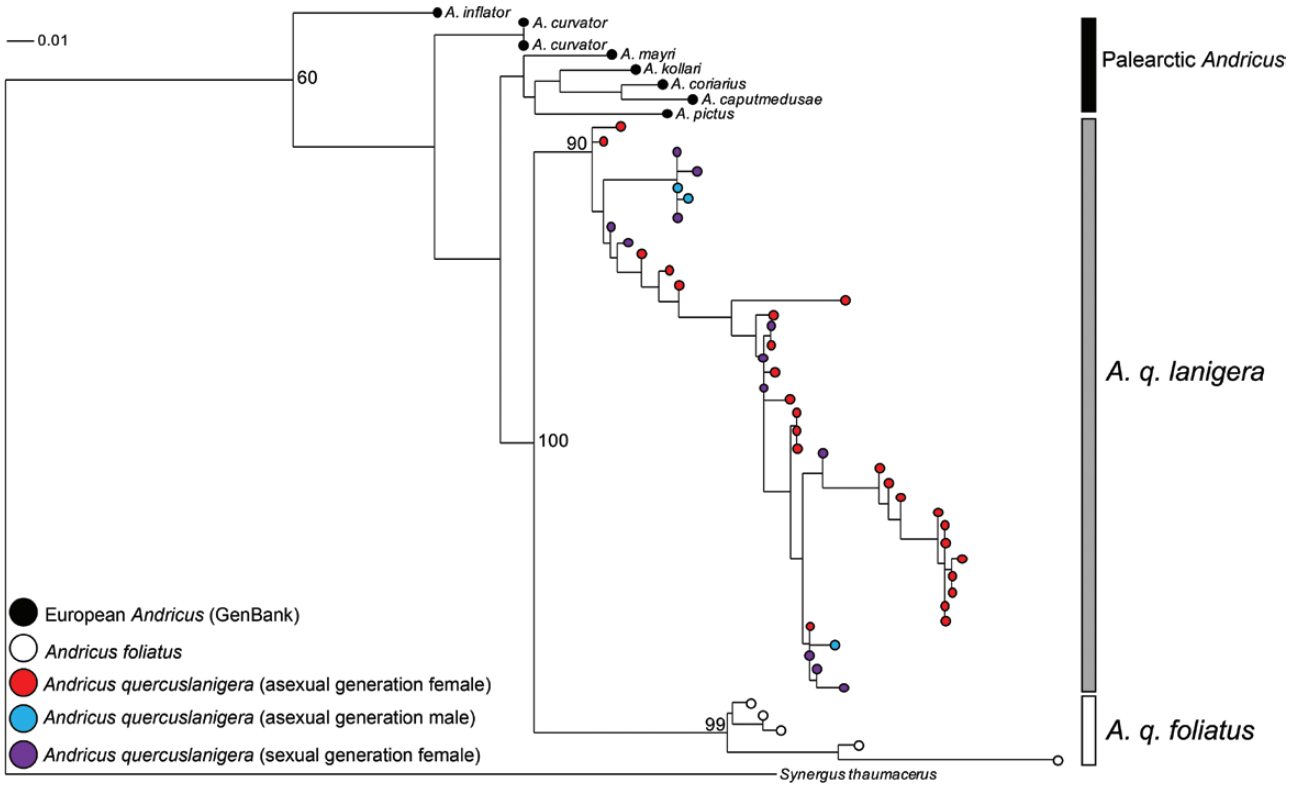

Fig. 5. Maximum parsimony mtDNA COI gene tree of sexual generation male (blue) and female (purple) *A. quercuslanigera*, asexual generation *A. quercuslanigera* (red), *Andricus quercusfoliatus* living on the same live oak species (white), eight Palearctic *Andricus* species (black), and the outgroup taxon, *Synergus thaumacerus* based on 10,000 bootstrap replicates. All individuals group into distinct clades (Palearctic *Andricus* = black vertical bar; *A. foliatus* = white vertical bar) including male and females of the sexual and asexual generation *A. quercuslanigera* sequenced form a distinct monophyletic group with ≥90% bootstrap support with individuals (gray vertical bar), indicating that the previously described asexual generation represents only one generation in a bivoltine life cycle that alternates with a newly discovered sexual generation.

Approximately 95% of the described biodiversity of Cynipidae exists from a single generation and most named species are from the asexual generation only (Pujade-Villar et al. 2001). Yet, despite the evidence presented herein, the loss of sexuality is hypothesized to be common in Cynipidae and has been documented experimentally in at least eight taxa (Stone et al. 2008). However, Stone et al. (2008) used DNA sequence data to determine if and how often the sexual generation of different European *Andricus* is taxonomically misidentified. Despite extensive prior study of European *Andricus*, the authors found that morphologically indistinguishable individuals of a single purported species (*A. burgundus*) represented the sexual generation of at least six cryptic species. We postulate that much of the diversity of gall wasps and more specifically *Andricus* may represent cryptic heterogony. The diversity of the Cynipidae peaks in Nearctic and an estimated two-thirds of the world's approximately 300 *Andricus* species are hypothesized to occur in North America and northern Mexico (Melika and Abrahamson 2002, Stone et al. 2002, Stone et al. 2008, Pujade-Villar and Ferrer-Suay 2015). Thus, there is a world of opportunity for revision and description of North American *Andricus* specifically and Cynipini more generally.

The 10-fold difference in the length of the emergence period between the asexual (5 mo) and sexual (2 wk) generations is likely related to the timing of tissue availability that each generation can use at different times of the season. The asexual generation begins emerging in late-September, a full 3–4 mo before the onset of mass catkin growth, suggesting that these early emerging asexual generation outliers will have no tissues available for oviposition. However, a small number of asynchronous catkins are available throughout the year (G.R.H., L.Z., and S.P.E., personal observation). Therefore, the relatively few number of asexuals that emerge earlier in the season will potentially have available catkin tissue to oviposit into. Alternatively, these individuals may lay into buds and deposited eggs may not hatch until the onset of mass availability of catkin tissue in February and March when the majority of oviposition takes place. In contrast, the comparably short emergence period of the sexual generation is likely due to 1) the ephemeral nature of the catkin tissue they develop on and 2) the short 2-wk time period between when catkins buds break and mature catkins abscise from the host plant, and 3) how quickly new leaves flush after catkins mature (a week or less). Therefore, the sexual generation may be highly restricted to a narrow emergence window to coincide with the timing of new leaf flush. For other species of galls developing on live oak leaves, we know that leaf tissue is only susceptible to gall formation for less than a week, well before new leaves are fully mature (Hood and Ott 2010, Zhang et al. 2017), potentially contributing to the brief period of emergence and tight synchrony between new leaf flush and sexual generation emergence.

What remains to be seen is if the heterogonic life cycle of *A. quercuslanigera* is maintained across its geographic and host ranges. The gall wasp occurs on at least four different live oak species in the subsection *Virentes*: *Q. virginiana*, *Q. fusiformis*, *Q. geminate*, and *Q. oeloides* distributed across the southern and southeast United States, south to central Mexico, and potentially includes an additional white oak, *Q. rugosa*, and a red oak, *Q. palustris* (Pujade-Villar et al. 2016). Given that within-species variation in life cycle exists where asexual, sexual and heterogonic forms are present

dependent on geographic location and/or host plant affiliation (e.g., Stone et al. 2001), and many species show plasticity in response to environmental fluctuations (Schönrogge et al. 1999, Stone et al. 2002), this open question in the *A. quercuslanigera* system is an interesting avenue for future research. In this regard, future research in this system will also include more broad and intense sampling across the species range to document and compare the natural enemy community (typically composed of inquilines and parasitoids) associated with each generation to determine if and how tritrophic interaction may play a role in the evolution of heterogonic life cycles (Forbes et al. 2015, Hood and Ott 2017).

Supplementary Data

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

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