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CARACTERIZACIÓN MOLECULAR, BIOLOGÍA Y HÁBITOS DE NINFAS DE *Ocoaxo assimilis* (Walker) (HEMÍPTERA: CERCOPIDAE)

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RAQUEL CID MUÑOZ

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DR. DAVID CIBRIÁN TOVAR

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CARACTERIZACIÓN MOLECULAR, BIOLOGÍA Y HÁBITOS DE NINFAS DE *Ocoaxo assimilis* (Walker) (Hemíptera: Cercopidae)

Tesis realizada por **Raquel Cid Muñoz** bajo la supervisión del comité Asesor indicado, aprobada por el mismo y aceptada como requisito parcial para obtener el grado de:

Maestro en Ciencias en Ciencias Forestales

Director:

M. C. David Cibrián Tovar

Asesor:

Dra. Ernestina Valadez Moctezuma

Asesor:

M. C. Francisco Armendáriz Toledano

Asesor:

M. C. Antonio Villanueva Morales

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DEDICATORIA

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"Si he visto más lejos es porque estoy sentado sobre los **hombros de gigantes**"

Isaac Newton

DATOS BIOGRÁFICOS

Raquel Cid Muñoz nació el 27 de marzo de 1987 en Nicolás Bravo, Puebla. Estudio su bachillerato en la Preparatoria Federal por Cooperación “Gilberto Martínez Gutiérrez” Área Ciencias Biológicas y de la Salud, en 2009 ingresa a la Universidad Tecnológica de Tehuacán en donde obtiene los títulos de Técnico Superior Universitario en Agrobiotecnología y el de Ingeniero en Biotecnología.

En 2012 colaboró en el proyecto de investigación “El potencial biotecnológico de la diversidad microbiana en la agricultura y alimentos” en la Universidad Tecnológica de la Selva, realizando la colecta, aislamiento, purificación y pruebas fisicoquímicas a bacterias de los géneros *Burkholdelia* spp., *Azospirillum* spp, y algunas bacterias acido lácticas, así como la inoculación y evaluación de los beneficios de *Azospirillum* spp. en maíces criollos de la región Selva del estado de Chiapas, del cual derivó un Artículo presentado en el 7° Congreso Bioalimentario realizado en Pachuca, Hgo. 20, 21, 22 de marzo de 2013 y con registro ISBN: 978-607-9166-04-5.

En el año 2013 ingresa a trabajar a la Universidad Tecnológica de Tehuacán como profesor de asignatura B en el Programa Educativo de Agricultura Sustentable y Protegida, donde laboró hasta el año 2017, desempeñándose como encargada de los laboratorios de Biología Agrícola, Química de suelos y Fitopatología, realizo actividades relacionadas a análisis de suelos y agua, apoyando en cátedra e impartición de prácticas de las materias de Química, Matemáticas y Fitopatología. En 2016 participó en el equipamiento de los laboratorios antes mencionados y la acreditación CACEI del programa educativo.

RESUMEN GENERAL

Caracterización molecular, biología y hábitos de ninfas de *Ocoaxo assimilis* (Walker) (Hemíptera: Cercopidae)[1]

En 2008, en Nicolás Bravo, Puebla se reportó un amarillamiento en los pinos, dicho fenómeno fue denominado como “declinación de los pinos” y se asoció a la presencia de adultos de *Ocoaxo assimilis*. Al ser una “nueva plaga” se desconocían aspectos importantes de su biología; la morfología de sus etapas juveniles, los hábitos que estas presentan, su voltinismo, entre otras. El objetivo de la presente investigación fue caracterizar molecularmente las ninfas encontradas para asociarlas a adultos de *O. assimilis* y mediante muestreos y recolectas de especímenes vegetales y de ninfas brindar conocimientos sobre su biología y hábitos. La toma de datos se realizó sobreponiendo una capa con cuadrantes de 500 m^2 sobre el mapa de la zona de estudio y seleccionando aleatoriamente el lugar de colecta, se muestrearon un total de treinta y cuatro cuadrantes a lo largo de un año, donde se recolectaron plantas asociadas a masas de saliva de ninfas y las ninfas encontradas dentro de las salivas. Con ayuda del gen que codifica para la Citrocromo Oxidasa I (COI) se verificó que las ninfas recolectadas pertenecen a *O. assimilis*, y mediante el análisis de 19 caracteres (15 continuos y 4 discretos) se determinó que *O. assimilis* presenta 5 ínstares ninfales. Mediante Microscopia Electrónica de Barrido (MEB) se verificó la modificación de algunas estructuras con respecto a la edad. También se comprobó que la presencia de ninfas está íntimamente relacionada con el inicio de la temporada de lluvias y que la especie es univoltina. Se confirma que las ninfas tienen el mismo hospedante que el adulto y que las plantas pertenecientes a familias diferentes a la Pinaceae, solo pueden considerarse como plantas alimenticias debido a que en ellas las ninfas no completan su metamorfosis en este caso *Pinus pseudostrobus* var. *apulcensis.*

Palabras clave: *Ocoaxo assimilis,* estadios ninfales, Citrocromo Oxidasa I (COI), Microscopia Electrónica de Barrido (MEB), plantas hospedantes.

[1] Tesis de Maestría en Ciencias en Ciencias Forestales. Universidad Autónoma Chapingo.
Autor: Cid Muñoz Raquel.
Director: Dr. David Cibrián Tovar.

ABSTRACT

Molecular characterization, biology and habits of nymphs of *Ocoaxo assimilis* (Walker) (Hemíptera: Cercopidae) [2]

In 2008, in Nicolás Bravo, Puebla state, Mexico, a yellowish was reported in the pines, this phenomenon was denominated as "pine decline" and was associated with the presence of adults of *Ocoaxo assimilis*. Considered as a "new plague", important aspects of its biology are unknown; the morphology of its youthful stages, the habits they present, their voltism among others. The objective of the present investigation was to molecularly characterize the nymphs found to associate them with adults of *O. assimilis* and through samplings and collections of plant and nymph specimens to provide knowledge about their biology and habits. The data collection was done by superposing a layer with quadrants of 500 m on the map of the study area and randomly selecting the collection site, a total of thirty-four quadrants were sampled over a year, were collected plants associated to saliva masses of nymphs and nymphs found inside the saliva With the help of a gene that codes for Citrochrome Oxidase I (COI) it was verified that the nymphs collected belong to *O. assimilis*, and through of the analysis of 19 characters (15 continuous and 4 discrete) it was determined that *O. assimilis* presents five nymph instars. Through scanning electron microscopy (SEM) the modification of some structures with respect to age was verified. It was also found that the presence of nymphs is intimately related to the beginning of the rainy season and that the species is univoltine. It is corroborated that the plants belonging to families other than the Pinaceae, can only be considered as food plants because in them the nymphs do not complete their metamorphosis, and it is confirmed that the nymphs have the same host as the adult, *Pinus pseudostrobus* var. *apulcensis.*

Key words: *Ocoaxo assimilis*, nymphal stages, Citrochrome Oxidase I (COI), Scanning Electron Microscopy (SEM) and host plant.

[2] Thesis of Maestría en Ciencias en Ciencias Forestales. Universidad Autónoma Chapingo.
Author: Raquel Cid Muñoz.
Advisor: Dr. David Cibrián Tovar

CAPÍTULO 1

INTRODUCCIÓN GENERAL

La familia Cercopidae está conformada por 62 géneros distribuidos en la región neotropical (Carvalho & Webb, 2005; Castro-Valderrama, Carvalho, Peck, Valdez-Carrasco, & Romero Nápoles, 2019; Paladini, Takiya, Cavichioli, & Carvalho, 2015).Los cercopidos son polífagos que se alimentan en general de monocotiledóneas, herbáceas, árboles y arbustos. Su importancia radica en que tanto las ninfas como los adultos extraen agua y nutrientes de la planta, lo que activa mecanismos de respuesta como el oxidativo (Especies reactivas de Oxigeno ERO o ROS por sus siglas en inglés) (Pacheco Coeto, Cardenas Torres, Hernandez Rosas, Hidalgo Contreras, & Aquino Perez, 2019), así como también son vectores de patógenos como *Xylella fastidiosa*, entre otros. Esto provoca importantes pérdidas económicas en cultivos de caña y pasturas principalmente (Bolwell, 1999; Carvalho & Webb, 2005; Lapointe, 1993; Orozco Restrepo et al., 2017; Overall & Rebek, 2017; Pires, Sujii, Fontes, Tauber, & Tauber, 2000; Rivera Cabrera, Buentello Volante, Díaz de León Sánchez, & Pérez Flores, 2008).

Uno de los principales factores que influye notablemente en la duración del periodo de incubación es la humedad relativa; se conoce que en condiciones de humedad del 80 o 90% los huevos incuban en un periodo de 15 días, con un rango de 12 a 18 días; y en condiciones de baja humedad relativa el periodo de incubación puede prolongarse de 20 o 30 días. En ocasiones esta incubación se inhibe y los huevos entran en diapausa, esta puede durar varios meses, hasta que las condiciones sean favorables. La mayoría de los huevos ovipositados al final del periodo de lluvias permanecen en el suelo en estado de diapausa hasta el inicio del siguiente periodo lluvioso, razón por la cual la primera generación de salivazo generalmente coincide con el inicio del periodo de lluvia (CIAT, 1982; Biedermann, 1998; Carvalho & Webb, 2005).

Las ninfas recién eclosionadas están desprovistas de zonas quitinizadas, son sumamente activas e inmediatamente buscan refugio en las partes sombreadas,

húmedas, cubiertas con hojarasca o en grietas, de 3-10 cm de profundidad e inician su alimentación, situándose generalmente en las raíces secundarias, terciarias o tallos de la planta hospedera (CIAT, 1982; Ulises Castro Valderrama, 2017). Durante el desarrollo de las ninfas se pueden identificar cinco instares o estadíos (CIAT, 1982; Fagan & Kuitert, 1969; Peck, 1998, 2003; Peck, Perez, & Medina, 2002; Rodríguez, Ch., Castro, V., Morales, R., & Peck, 2003). Al final de cada instar la ninfa sufre una muda y aumento de tamaño, desarrollando progresivamente las estructuras alares y reproductivas, con excepción del quinto instar, que se divide en Va y Vb, y no existe una muda entre ellas, pero se pueden distinguir entre ellas por características morfológicas relacionadas con las alas y las espinas de la corona distal (Fagan & Kuitert, 1969; Peck, 1998, 2003).La longitud del adulto varía entre 4 mm hasta 17 mm dependiendo el género y la especie.

Los individuos pertenecientes a esta familia son comúnmente conocidos como salivazos debido a que en etapas juveniles se cubren de un exudado espumoso formado por derivados de ácidos grasos, alcoholes, ϒ-lactonas y un solo 1-monoacilglicerol, así como el poliol pinitol, el polihidroxialcanoato y el poli-3-hidroxibutirato. Dicho exudado no es irritante pero si le ayuda a repeler depredadores como las hormigas, a evitar parasitismo, crear un microclima y la protege de condiciones climáticas adversas (Carvalho & Webb, 2005; Del Campo, King, & Gronquist, 2011; Hamilton Andrew, 1982; Tonelli et al., 2018).

Dentro de la familia Cercopidae se encuentran los *Ocoaxos*, conocidos como “salivazos de los pinos” u “Ocoaxos de los pinos” (Castro Valderrama, 2017) sus principales características morfológicas son: la cabeza es más estrecha que el ancho del pronoto, el margen anterior del pronoto es recto, los ojos son globulares, el postclípeo está muy desarrollado, tibias posteriores tienen dos espinas laterales y las patas traseras son fuertes para dar grandes saltos (Burrows & Picker, 2010; Castro Valderrama, 2017). La longitud de las especies de *Ocoaxo* spp. se encuentra entre 10-12 mm (Castro Valderrama, 2017), sus

daños consisten en anillos de coloración amarillenta o parduzca en acículas que pueden derivar en muerte de las acículas y defoliación del árbol .

Se tienen reportes en Europa de la existencia de *Haematoloma dorsarum* en (España y países bajos) atacando *Pinus ponderosa, P. jeffreyi, P. brutia, P. sylvestris, P. nigra, P. pinaster y Pinus* sp. (Cobos, 1995; Hernández Alonso, Martín Bernal, & Pérez Fortea, 1992; Moraal, 1996; Notario, Catresana, & Baragaño, 1981), en Norte America se reporta a *Aphrophora saratogensis* (Fitch) afectando al Pino Rojo (*Pinus resinosa* Aiton) y al Pino Jack (*Pinus banksiana* Lamb) en Estados Unidos y Canadá (Ewan, 1961).

En México se reportaron daños por *Ocoaxo cardonai* (Castro-Valderrama et al., 2019), *Ocoaxo assimilis (Walker)* y *Ocoaxo varians* (Stål) en *P. pseudostrobus* Lindl, *P. patula* Schiede Ex Schltdl & Cham, *Pinus* sp., *P. pseudostrobus* var*. apulcensis* (Lindley) Shaw, *P. chiapensis* (Castro-Valderrama et al., 2017; Pichardo Segura, Pérez Miranda, Ramírez Huerta, Arriola Padilla, & Ramírez García, 2017).

La clasificación de individuos pertenecientes a la familia Cercopidae sigue siendo problemática, el esquema de clasificación más aceptado es el de Fennah (1968) que divide a los cercopidos en dos subfamilias: Cercopinae del Viejo Mundo e Ischnorhininae del Nuevo Mundo (anteriormente conocida como Tomaspidinae) (Carvalho & Webb, 2005). Debido a esta problemática los genes mitocondriales son típicamente conocidos por tener una alta velocidad comparativa de substitución de nucleótidos y son comúnmente utilizados como fuentes de información filogenética para divergencias más recientes. Gracias a los análisis filogenéticos basados en secuencias de nucleótidos de ADN, entre ellos dos fragmentos no continuos del gen Citocromo Oxidasa I (COI) de aproximadamente 970 pb, (Cryan & Svenson, 2010), se concluyó que Cercopinae representa un conjunto parafilético de linajes, mientras que Ischnorhininae es monofilética.

Más allá de la clasificación morfológica del adulto de *O. assimilis* se desconocen aspectos básicos de su biología como la morfología de las ninfas, los hábitos alimenticios de estas, si es una especie uni o multivoltina, las condiciones en las

que se rompe la diapausa, entre muchos otros importantes para su entendimiento y control.

Objetivo general

- Asociar los adultos de *Ocoaxo assimilis* con las ninfas encontradas en Nicolás Bravo, Puebla e identificar las ninfas morfológicamente, describir su biología y hábitos.

Objetivos específicos

- Reconocer morfológicamente a los adultos y asociar a las ninfas con el adulto mediante el uso de un fragmento del gen COI (Barcode).
- Describir el número de instares ninfales mediante caracterización morfológica.
- Definir los periodos de ocurrencia de los estadios y asociarlos con cambios ambientales, a partir de muestreos continuos.
- Conocer el voltinismo de la especie de interés.
- Determinar el espectro trófico de huéspedes que presentan las ninfas.
- Identificar la preferencia de las ninfas a los diferentes tipos de huéspedes.
- Precisar cuál es el hospedante en el que las ninfas completan su metamorfosis.

CAPÍTULO 2
MARCO CONCEPTUAL

2.1 Antecedentes

Los Bosques de Puebla en especial los bosques de pino se ven amenazados por gran variedad de factores bióticos, abióticos y antrópicos (Ruíz et al., 1998, FAO 2007, CONABIO 2011, Pichardo et al. 2017).

Pichardo et al. (2017) detectaron defoliaciones en diferentes especies de Pino en la Sierra Norte de Puebla. Según el mismo estudio para 2015 la afectación alcanzaba 3000, observando la presencia de micromicetos del género *Lophodermium s*p. (Rhytismataceae), *Pestalotiopsis* spp. (Melaconiales: Melaconiaceae), y *Coleosporium sp.* (Uredinales: Coleosporiaceae) e insectos del género *Ocoaxo* sp.

Castro et al. (2017) reportaron que desde 2008 se observó daño causado por adultos de salivazos en los Bosques de Puebla y Oaxaca, las especies en las que se observó la afectación fuerón: *P. oaxacana* Mirov cerca de Nicolás Bravo, Puebla y de *P. chiapensis* (Martínez) Andresen en Santa Ana Cuauhtémoc (región perteneciente a la Cañada, estado de Oaxaca, México).

De acuerdo al Plan de Contingencia Fitosanitaria en varios municipios del estado de Puebla de 2016, en el Municipio de Nicolás Bravo el área afectada por *Ocoaxo assimilis* en ese año ascendió a más de 431.03 ha (Unión Agroforestal de Puebla A.C., 2016), motivo por el cual se declaró una Contingencia Sanitaria para este agente causal el mismo año. Dentro de las acciones realizadas en este proyecto destacan la formación de brigadas fitosanitarias las cuales tuvieron como objetivo el monitoreo y saneamiento de las áreas afectadas. Se realizó la aplicación de tratamientos químicos con: acefato, bifentrina y bifentrina + imidacloprid, biológicos con: *Metarhizium anisopliae* solo o en combinación con *Beauveria bassiana y Paecilomyces fumosoroseus* además también se realizó una combinación del producto químico con los biológicos–y se realizó el barrido y

quema de la materia orgánica para ayudar en la penetración de los productos químicos y biológicos (Cibrián Tovar et al., 2016). Derivado de esto se concluyó que el acefato fue la manera más rápida de controlar la población de ninfas de *Ocoaxo* y que se debe crear un producto biológico efectivo para el combate de esta plaga y que el barrido de la materia orgánica trae más problemas ecológicos que beneficios por tal motivo se debe evitar.

Otra parte importante que se realizó fue el monitoreo de *O. cardonai* en los municipios de zona norte lo que dio pauta también para conocer un poco de la biología de las ninfas de esta especie, diseñando tipos de muestreo y probando la eficiencia de trampas de luz, resultado de esto se desprende que el monitoreo diseñado requirió muchas horas hombre de esfuerzo por el tamaño de la superficie (400 m^2) y que las trampas de luz no funcionan para colectar adultos de *O. cardonai. In vitro* se probaron los tratamientos mencionados y se pusieron a copular adultos de *O. cardonai*, los resultados *In vitro*, determinaron que la forma de los huevos es elipsoidal, más anchos al centro y ahusados hacia los extremos, con las puntas redondeadas. Al principio, cuando tienen poco tiempo de ovipositados, son de color blanquecino y posteriormente se tornan color amarillo cremoso y que los huevos entran en hibernación varios días después de

ovipositados requiriendo permanecer en un ambiente húmedo para sobrevivir (Cibrián Tovar et al., 2016).

Figura 1 Huevos de *O. cardonai.* Fuente Cibrián Tovar et al.,(2016).

2.2 Zona de Estudio

El municipio de Nicolás Bravo se localiza en la parte sureste del estado de Puebla. Sus coordenadas geográficas son los paralelos 18º 32´ 12" y 18º 40´ 24" de latitud norte y los meridianos 97º 18´ 06" y 97º 24´ 54" de longitud occidental. Colinda al norte con el Estado de Veracruz, al sur con Tehuacán, al este con Vicente Guerrero y al oeste con Chapulco. Tiene una superficie de 108.92 kilómetros cuadrados que lo ubica en el lugar 47 con respecto a los demás municipios y se ubica dentro de la región morfológica de la sierra de Zongolica, que es considerada dentro de las subregiones de prioridad critica según la

Comisión Nacional para el Conocimiento y Uso de la Biodiversidad (CONABIO, 2011).

Figura 2 Mapa de localización.

Pertenece a la subprovincia de las Sierras Orientales que se encuentra dentro de la provincia Sierra Madre del Sur, esta subprovincia desciende desde la región de Orizaba, Veracruz, hasta Salina Cruz, Oaxaca. Se extiende al noreste de la cuenca de Tehuacán y se conforma por un sistema de topoformas de sierras bajas, sierras altas y valles (CONABIO, 2011).

De acuerdo a datos proporcionados por el INAFED (2017), el municipio tiene una superficie de 108.92 kilómetros cuadrados, se ubica dentro de la región morfológica de la sierra de Zongolica, estribación de la Sierra Madre Oriental que se caracteriza por su rápido descenso hacia la planicie Costera del Golfo. Su territorio es atravesado de norte a sur por el parteaguas que marca el declive de la sierra hacia la planicie costera al oriente y hacia el valle de Tehuacán al poniente. Su mayor altura es de 2,800 metros sobre el nivel del mar.

Pertenece, en su mayor parte, a la cuenca del Río Blanco. Es recorrido por varios arroyos de norte a sur, provenientes de las cumbres de Acultzingo, destacando el río Huertilla, formador del Tehuacán, el cual recorre el

Valle del mismo nombre y es uno de los principales formadores del Papaloapan. El municipio es también recorrido al noroeste por arroyos que desembocan en el Xoxocotla, afluente del Río Blanco, el cual desemboca en la Laguna de Alvarado, al igual que el Papaloapan (INAFED, 2017).

En este municipio se marca la transición entre los climas secos de la vertiente occidental de la Sierra de Zongolica, a los climas templados de las partes altas de la Sierra mencionada, así presenta un clima seco y tres templados que, conforme se avanza de poniente a oriente, marca descenso de temperatura y aumento de humedad. Clima semiseco templado con lluvias en verano y escasas a lo largo del año, es el clima que se presenta al poniente, en el declive occidental de la Sierra de Zongolica. Clima templado subhúmedo con lluvias en verano, se presenta en una ancha franja al centro del municipio y en la parte alta de la Sierra. Clima templado húmedo con abundantes lluvias en verano, se presenta en el extremo oriental.

El municipio presenta la transición vegetativa de las zonas de riego en el Valle de Tehuacán, a las áreas boscosas de la Sierra de la zona montañosa. Al centro del municipio, en las zonas francamente planas, se localizan áreas dedicadas a la Agricultura. En la zona montañosa del poniente y en la ladera occidental de la Sierra de Zongolica, se encuentra matorral desértico rosetófilo asociado a un matorral subinerme; también aparecen chaparrales con vegetación secundaria arbustiva. Por último, al extremo noreste, donde el relieve alcanza mayores alturas, se presenta una gran área de bosques de pinos.

El suelo predominante es Andosol, que cubre todo el centro del municipio, al oriente presenta suelo luvisol con fase cítica profunda y al sur y poniente litosol; al extremo poniente en la zona baja, cuenta con una pequeña área de suelo feozem.

2.3 Cercopidos

El salivazo es un insecto chupador que se alimenta exclusivamente de la savia que extraen del xilema de las plantas. las ninfas se alimentan de las raíces superficiales y de los tallos, en la base de la planta, por lo que cuando se presentan infestaciones altas causan estrés hídrico, retrasando el crecimiento de la planta y, por lo tanto, la producción de biomasa (Rodríguez, 1979, esta cita ya no tiene que ver con Ocoaxos). No mezclar información de Ocoaxos con otros salivazos, mejor separar ambos temas.

Las ninfas exudan una masa blanca que parece “saliva espumosa” de ahí su nombre (salivazo) conocida también como; salivita, cigarrilla, candelilla, salivazo, mosca pinta, chinche salivosa (Jaramillo 2014, Castro., 2017). La cual actúa como defensa de enemigos naturales, y protección a adversidades climáticas (Rodríguez, Castro, Morales & Peck, 2003).

Generalmente los salivazos presentan diferentes etapas de desarrollo, pasando por fases de huevo, ninfa y adultos (Peck, 2002). En las distintas etapas por las que pasa el salivazo se debe de alimentar de su hospedante, los insectos hibernan en forma de huevo, al eclosionar producen ninfas que se alimentan de la savia de tallos y hojas (Jaramillo, 2014).

Los adultos al igual que las ninfas se alimentan a través del tejido del tallo, pero regularmente optan partes aéreas de la planta (hojas), que tienen tejidos poco profundos (Peck, 2003). Aunque diferentes especies de salivazos actúan como herbívoros generalistas de algunas gramíneas, ciertas especies del género *Clastoptera*, sólo se registran en plantas ornamentales, como *Juniperus chinensis* L., y *Juniperus virginiana* L., en Estados Unidos y México (Martínez, Lara, Gaona, & Sánchez, 2012) y en *Casuarina equisetifolia* (L.) Fort et Forst., en Costa Rica (Martínez et al., 2012).

Las especies de salivazo se han propagado en las últimas décadas más de lo habitual, aumentando con ello sus hospederos, en mayo y junio del 2010 se tuvieron por primera vez 2 registros de una especie de *Clastoptera* asociada a

Harpalyce arborescens A. Gray (Fabaceae), en el bosque tropical deciduo de la sierra de Tamaulipas, donde esta comunidad presentó su distribución más septentrional (Martínez Ávalos et al., 2012).

La presencia de los salivazos y su comportamiento, tiene una relación muy alta con la precipitación y temperatura, debido a que es un factor detonante para la aparición de los primeros adultos de *Aeneolamia* spp. es la presencia de lluvia (De Yta, Cabrera & Villanueva Jiménez, 2002). En estudios sobre otros cercópidos se ha encontrado que las lluvias influyen en la abundancia y sincronización de las poblaciones del salivazo, aun sin conocer la correlación directa (Peck *et al*., 2002b).

La fluctuación del insecto puede estar o no relacionada con la abundancia de la población, sin embargo se puede esperar que el hábitat (suelos, drenaje, composición botánica) influyan en estos factores así como la fenología del salivazo (Peck et al., 2002b).

La determinación precisa de los estados de vida, variación en tamaño y duración del ciclo de vida es relevante en la determinación del impacto y manejo del insecto. Estos factores son importantes para el nivel de daño al hospedero y el periodo del insecto en el campo. Aumenta la resolución en la interpretación de muestreos y estudios sobre dinámica poblacional tanto como la aplicación y efectividad de las tácticas de manejo (Rodríguez et al., 2002).

2.3.1 Ocoaxos

Los Ocoaxos pertenecen a la Familia Cercopidae presentan una longitud entre 10-12 mm de longitud, sus principales características morfológicas son: La cabeza es más estrecha que el ancho del pronoto, el margen anterior del pronoto es recto, los ojos son globulares, el postclípeo está bien desarrollado, las tibias posteriores tienen dos espinas laterales y las patas traseras son fuertes para dar grandes saltos (Castro Valderrama, 2017).

Tabla 1 Clasificación Taxonómica de *O. asimilis.* (Paladini et. al., 2018)

CLASIFICACIÓN TAXONÓMICA	
Reino	Animalia
Phylum	Arthropoda
Subphylum	Hexapoda
Clase	Insecta
Orden	Hemiptera
Suborden	Auchenorrhyncha
Superfamilia	Cercopoidea
Familia	Cercopidae
Subfamilia	Ischnorhininae
Tribu	Tomaspidini
Genero	*Ocoaxo*
Especie	*assimilis*
Nombre binomial	*Ocoaxo assimilis*

De acuerdo a Castro (2017) los insectos adultos se reconocen por tener varios colores. El área ventral es rojiza, patas con fémur rojizo, pero tibias y tarsos oscuros, casi negros en la parte dorsal, la cabeza y parte del pronoto son rojizos.

La parte posterior del pronoto presenta una mancha blanca, la cual se continúa por los márgenes inferiores del primer par de alas, a manera de banda, y se curva hacia arriba en su porción más posterior, el resto de las alas es gris oscuro.

El escutelum también es rojizo no se conoce el estado ninfal y aparentemente sólo existe una generación por año, similar a como ocurre con otras especies que afectan coníferas en México.

Figura 3 Adulto de *Ocoaxo assimilis* alimentándose en acículas de *Pinus pseudostrobus* var*. apulcensis* (Lindley) Martínez. Fotografía Raquel Cid.

La presencia de adultos está basada en la temporada seca y la llegada de la temporada de lluvias (Hernández Rosas y Cibrián Tovar, 2016) y se prolonga entre el mes de octubre -noviembre; los machos y las hembras son de tamaño similar y no se reconocen a simple vista; aunque la hembra tiene un ovipositor característico; la cópula se ha observado en los meses de agosto y septiembre, pero es posible que continué en los siguientes meses.

Los daños en los árboles son producidos por los adultos los cuales se alimentan exclusivamente de la savia de las acículas, tras insertar su estilete en el tejido de la acícula, inyectan saliva dentro de la planta mediante la bomba salivar, para aspirar después la savia a través del canal de succión por la acción del cibarium, que funciona como una bomba activada por un grupo de músculos andados en la zona posterior del clípeo. Alrededor de las picaduras aparecen decoloraciones de los tejidos de la acícula en áreas anulares concéntricas. Estos anillos pasan por distintos tonos de color, desde el verde claro al principio hasta tonos rojizo y,

finalmente, café en los sitios de alimentación, esto debido quizá a la activación del mecanismo ERO o la introducción de algún patógeno (Overall & Rebek, 2017; Pacheco Coeto et al., 2019), dicho síntoma es más notorio en los meses de noviembre a febrero cuando se detecta el cambio más severo en el color.

2.4 Amplificación de ADN por PCR

La reacción en cadena de la polimerasa o PCR (Polymerase Chain Reaction), es una tecnología que se utiliza para sintetizar *in vitro* fragmentos específicos de ADN con la finalidad de detectar una secuencia o gen de interés en el genoma de un individuo.

Tiene varios requerimientos, entre los cuales es indispensable una secuencia consenso, templado o molde de ADN, moléculas iniciadoras llamadas “primers” o iniciadores, una enzima ADN polimerasa resistente a fluctuaciones de temperatura (Taq), una mezcla de desoxirribonucleósidos trifosfato denominados dNTPs (dATP, dCTP, dGTP, dTTP), un amortiguador apropiado y un equipo llamado termociclador que tiene la capacidad de cambiar las temperaturas dependiendo de los ciclos del programa.

La PCR consiste de tres pasos esenciales: el primero es la desnaturalización y sirve para separar mediante temperatura (94 °C) la molécula de doble cadena del ADN a cadenas sencillas, en el segundo se alinean las moléculas iniciadoras a la secuencia blanco del ADN molde a una temperatura que varía de 25 °C a 65 °C y el tercero, la extensión (síntesis) de la molécula iniciadora mediante la enzima Taq, es decir, la ADN polimerasa, a 72 °C. En el tercero, se lleva a cabo el alargamiento o extensión. Estos 3 pasos (ciclos) se repiten en el termociclador, permitiendo obtener de manera exponencial el fragmento o fragmentos discretos sintetizados a partir del molde de ADN (Valadez y Kahl, 2000).

2.4.1 Herramientas moleculares aplicadas al estudio de la diversidad

Los análisis basados en marcadores moleculares, mitocondriales y nucleares, son frecuentemente muy usados para responder cuestiones taxonómicas. El hecho que el ADN mitocondrial (mtADN) no sufre recombinación y que, al menos en animales, evoluciona más rápido que el ADN nuclear, hace que el mtADN sea

una herramienta importante en los estudios de genética de poblaciones, filogenias moleculares y sistemática (Pages & Holmes, 1998). Sin embargo, numerosos estudios sugieren que los arboles basados en mtADN son frecuentemente incongruentes con los arboles basados en datos morfológicos, citológicos o en marcadores nucleares. Esto se debe a la introgresión del mtADN vía eventos de hibridación y resulta de la combinación de herencia materna a diferentes tasas de evolución (Arnold et al., 1999). Por esta razón, generalmente se recomienda incluir uno o más marcadores nucleares para investigar la historia evolutiva de complejos de especies recientemente divergentes (Kawakami et al., 2007). La elección del marcador debe estar en concordancia con el nivel del problema taxonómico a resolver porque cada marcador molecular evoluciona a diferente tasa de evolución.

CAPÍTULO 3

Contribution to Biology of Pine Spittlebug *Ocoaxo assimilis* (Hemiptera: Cercopidae)

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Cid-Muñoz Raquel [1], Cibrián-Tovar David [1], Valadez-Moctezuma Ernestina[2], Estrada-Martínez Emma [1], Hernández-Rosas Francisco [3], Villanueva -Morales Antonio [1] and Armendáriz-Toledano Francisco[4.5].

[1]Universidad Autónoma Chapingo, División de Ciencias Forestales, Carretera Federal México-Texcoco Km 38.5, Texcoco, México C.P. 56230,

[2] Universidad Autónoma Chapingo, Departamento de Fitotecnia, Carretera Federal México-Texcoco Km 38.5, Texcoco, México C.P. 56230,

[3]Colegio de Posgraduados campus Córdoba, Km.348 Carretera Federal Córdoba-Veracruz, congregación Manuel León, Municipio de Amatlán de los Reyes, Veracruz, México. C.P.94946,

[4,5] Universidad Nacional Autónoma de México, Instituto de Biología, Departamento. De Zoología, Colección Nacional de Insectos, Cto. Zona Deportiva S/N, C.U., Ciudad de México, México C.P. 04510 and [5]Corresponding author, e-mail: farmendariztoledano@ib.unam.mx.

3.1 Abstract

In Nicolás Bravo, Puebla state, Mexico, a yellowish was reported in natural pine forest, this phenomenon was denominated as "pine decline" and was associated with the presence of adults of *Ocoaxo assimilis*. In the present study using morphological and molecular data we were able to associate the immature instars with the adults of this species, which in turn allowed to determine the number of nymphal stages of this species, to know its abundance over time and to describe the morphological changes it presents during its life cycle. Additionally, nymph and adult sampling, together with field and laboratory experiments, allowed to know the voltism, spectrum and host preferences of these stages. *O. assimilis* has a univoltine life cycle, with 6 stages in its life cycle, five nymphales and one adult. The emergence of the nymphs is associated with the beginning of the rainy season, the preference of hosts in the most juvenile stages (N1- N3) is very wide they can eat from herbaceous to pines passing through the bushes, from stage 4 the nymphs prefer pines, in which they complete their metamorphosis and eat when they are in adult stage.

Key words: Voltism, Barcode, Citrochrome Oxidase I (COI), Scanning Electron Microscopy (SEM) and Nymphal instars.

3.2 Introduction

The Cercopidae together with the Aphrophoridae and Clastopteridae families, also commonly named as spittlebugs, constitute the largest xylem-sap sucking insect group in the World (Carvalho and Webb 2005). The nymphal states of this family produce copious amounts of frothy excreta, constituted by a mixture of air and organic compounds that provides to the insects a protective covering that repels predators, avoid parasitism and protect from adverse weather conditions (Hamilton Andrew 1982, Tonelli et al. 2018), while they obtain sufficient nutrients from the dilute xylem sap of host (Rakitov 2002).

These insects feed on a great variety of plants (Carvalho and Webb 2005), most of them herbaceous monocots (Castro et al. 2005) , herbaceous dicots (De la Cruz Zapata et al. 2016), flowering trees (Peck 1998), shrubs (Ewan 1961) and few species of conifers (Notario et al. 1981). The feeding sites include leaves, stems, crowns and exposed roots, where both nymphs and adults produce water stress and chlorosis that spreads from the feeding site, among other consequences (Hamilton Andrew 1982, Del Campo et al. 2011).

In America, more than 400 valid spittlebugs species has been recorded (Carvalho and Webb 2005), however, the neotropical cercopids are best studied for the damage caused by direct feeding to forage grasses and sugarcane (Thompson and Carvalho 2016). As a consequence of the last, detailed biology of cercopid species is described in greater depth in taxa of economic importance, such as *Aeneolamia reducta* Lallemand (Peck et al. 2002) *Deois flavopicta* Stål. (Fontes et al. 1995), *Isozulia astralis* Distant (Peck 2001), *Kanaima nigra* Distant (Paladini and Carvalho 2008), *Mahanarva fimbriolata* Stål.(Orozco-Restrepo et al. 2017), *Maxantonia mimica* Paladini & Cryan (Paladini and Cryan 2012), *Notozulia entreriana* Berg (Foieri et al. 2016), *Prosapia bicinta* Say (Fagan and Kuitert

1969), *Sphenorhina pseudoboliviana* Paladini & Carvalho (Paladini and Carvalho 2013), *Tunaima brunneolutea* Carvalho (Carvalho 1990) and *Zulia carbonaria* Lallemand (Cardona et al. 2004); meanwhile in the most members this topic is poorly studied, particularly in those species with conifer feeding preferences (Castro-Valderrama et al. 2019).

Cercopids adults has been documented feeding on conifer species of Pinacea and Cupresaceae families; for example, *Haematoloma dorsatum* Ahrens is considered a generalist pest from Europe that promote needles drying of at least seven *Pinus* species and also members of *Abies* Mill., *Cedrus* Trew, *Cupressus* L., *Juniperus* L., *Picea* Link and *Pseudotsuga* Carriére (Notario et al. 1981, Covassi et al. 1989, Cobos 1995). The pine spittlebug *Aphrophora cribrata* (Walker et al. 1858) is other generalist species, native of North America that cause serious injury to conifers of all sizes, in nursery stock and mature forest trees (Wilson 1991) of at least 17 species of *Pinus* L., 12 of *Picea* (Link), three of *Abies* Mill., *Larix* Mill. *Pseudotsuga* Carriére and *Tsuga* Carriére. Both species *H. dorsatum* and *A. cribrata* are univoltine, display five nymphal states, which feed of new leaves and roots of different species of herbs and grasses preferentially, their adults feed of old or new pine needles and transmit microorganism that promote weakening of trees, which makes them susceptible to other pests and diseases (Wilson 1991, Paine and Lieutier 2016).

In the last ten years, the first records of spittlebug outbreaks were documented in Mexican pine forests from Puebla, Oaxaca, and Veracruz states (Castro-Valderrama et al. 2017, CONAFOR 2018); the symptomatology of insects was named “pine decline”, and it was similar to those caused by adults of pine spittlebug pest *H. dorsatum quinquemaculatum*,

H. dorsatum lugens and *Aphrophora cribrata,* which induces yellowish or brownish discoloration rings on the needles, resulting in drying and their early fall in summer (Wilson 1991, Paine and Lieutier 2016). The characteristic colors and other symptoms of tree damage by spittlebugs adults were evident during the rainy season and these disappeared when trees recover their health appearance at the beginning of the next rainy season; phenomena that occurs at different geographical scales every year until 2018 (Castro-Valderrama et al. 2017). The impact of damage of spittlebugs outbreaks ranging approximately 2500 ha in Veracruz State (CONAFOR 2018) to 3,000 ha in Puebla (CONAFOR 2017, Pichardo Segura et al. 2017), which has led to consider to these insects as pest of economic importance in these regions.

From several collecting events and the revision of specimens deposited in insect Collections, the associated agents with these damages were identified as almost three species of *Ocoaxo* genus (Fennah 1968): *O. assimilis* Walker, *O. varians* Stål and a new species *O. cardonai* Castro Valderrama, Carvalho & Valdez Carrasco (Castro-Valderrama et al. 2019). By their morphological similarities and habits, these taxa were clustered in a species complex with vestigial feeding habits within Cercopidae (Fennah 1968).

Of these species, outbreaks of *O. assimilis* was found in Nicolas Bravo municipality in Puebla (Castro-Valderrama et al. 2017) and in the Xoxocotla, Atlahuilco, Soledad Atzompa and Tequila municipalities in Veracruz (CONAFOR 2018); areas where *Pinus pseudostrobus* var (Lindl.) Martínez, *P. patula* Schiede ex Schltdl. & Cham. and *Quercus rugosa* Née, predomínate (INEGI 2000, CONABIO 2011). In both Puebla and Veracruz states, spittlebugs displayed the symptomatology “pine decline” and more than 2650.2 ha of pine forest presented foliar fall (Unión Agroforestal de Puebla 2016, CONAFOR 2018).

In addition to the information from these regions, the records in entomological collections and photographic records in the naturalist database support (Naturalista 2019) the presence of this species in Santa María Chilchotla, Puerto Escondido, Santa Cruz Acatepec and Guelatao, Oaxaca state (Castro Valderrama 2018).

Beyond the taxonomic and distribution information of these species promoted by recent sipitterbugs outbreaks on pine forest, the most basic aspects of its biology remain as a question to be answered. The nymphal stages of *O. assimilis* and other *Ocoaxo* species are undescribed and several basic aspects of their biology are unknown such as: number of generations per year (voltism), reproduction season, oviposition site, nymphal habitus, nymphal feeding spectrum, number of nymphal instars, etc. The absence of biological information of *Ocoaxo* species has led to reconsider their relationship with pine decline (Castro-Valderrama et al. 2017); therefore, its status as pest species of economic importance is questioned.

This study is aimed to describe some aspects of biology of *O. assimilis* in natural pine forest at Nicolás Bravo, Puebla, Mexico. It is focused to elucidate basic aspects of the habits and behavior relevant to its pest status, including association of nymphal stages with adults, description of nymphal instars and preferences of the life stages (nymphal stages and adults), voltism and life cycle biology. The results further our understanding of the variation in the biology and behavior of this group and will guide improvements in spittlebug management based on species and life-stage-specific understanding.

3.3 Methodology

*Study site***.** This investigation focused on a population of *Ocoaxo assimilis* located in the municipality of Nicolás Bravo, Puebla, Mexico, area where there are reports of damage to pine forests caused by this species since 2008 (Castro-Valderrama et al. 2017). This region presents the temperate subhumid climate with rains in summer of lower humidity, it is found between 1860 to 2800 m, where the Pine-Oak forest predominate (INEGI 2009).

The samplings were carried out in the "Las Majaditas" property, which is located northwest of the municipal capital; in this area, a polygon of 42 hectares was established, corresponding to an outbreak of *O. assimilis* reported in 2017 (CONAFOR 2017), where *Pinus pseudostrobus* (Lindley) Martínez and *Quercus rugosa* Née inhabits. To establish the monitoring zones, on the map of the study area, a layer with quadrants of 2500 m^2 was superimposed, the quadrants were numbered in ascending numbers for their subsequent selection by random numbers. Field inspections were conducted from two to eight per month; at each visit a random quadrant was selected, in which a smaller square of 9.0 m^2 was established in sites with pine trees that presented chlorotic spots, a most evident symptom of damage by *O. assimilis* (CONAFOR 2017). Within the square, two people performed the active search for saliva masses containing nymphs, in the soil and roots of herbaceous plants, grasses and trees; the adults were collected on pine needles. Putative nymphal and adults insects of *O. assimilis* were collected live and in absolute alcohol during one year between the months of December 2017 and 2018.

Specimen identification. The morphological identification of adults was carried out using the key of Castro-Valderrama et al. (2019) from male genital morphology. Male genitalia

were cleared by incubating it for 24 hrs at 60°C in 10% KOH. After incubation, structures were immersed in 20% acetic acid solution to neutralize the KOH and subsequently rinsed whith 70% etanol. Genital elements were mounted in temporal slides with glycerol. Because, adults of *O. assimilis* has not been associated with nymphal stages (Castro-Valderrama et al. 2017) a fragment of Cytochrome Oxidase subunit I mtDNA (COI) was amplified from two adults identified morphologically and specimens of the different nymphal stages, to evaluate their position in a phylogenetic tree including other *Ocoaxo* and Cercopid species.

The DNA extractions of the nymphs and adults were conducted using the standard protocol for Kit EZ-10 spin column genomic DNA (Bio Basic Inc.). Amplification of a 970 bp fragment of COI was carried out using the primers Ron (F), MidR (R), Midf (F) and Calvin (R) for Cercopids (Paladini et al. 2018). A Maxigene Axigen TM termal cycler was used for double-stranded amplifications; the cycling profile began with one cycle of DNA denaturation at 94°C for 2 min and followed by 38 cycles of sequence amplification (DNA denaturation at 94°C for 30 s, primer annealing at 50 °C for 30 s and sequence extension at 72°C for 1 min) and final sequence extension at 72°C for 10 min. Amplified DNA was visualized using 1% agarose gel electrophoresis. Sequences were generated in the Laboratorio Nacional de Biodiversidad (LANABIO) at Instituto de Biología, Universidad Nacional Autónoma de México (UNAM).

Sequences were edited manually, concatenated and aligned using Seaview 2. 2 (Galtier et al. 1996) and ClustalX 1.83 (Thompson et al. 1997). GenBank accession numbers of the resulting haplotypes are (in process). All obtained sequences correspond to positions between 1667 and 2639 in the mitochondrion genomes of Cercopid species *Cosmoscarta*

bispecularis White (KP064511.1). The sequences of *Ocoaxo assimilis* (KX239960.1), *O. lineatus* Walker (KX239961.1), *O. ornatipenis* Stål (GU446994.1), *O panamensis* Nast (KX239962.1), *O. tucurricae* Lallemand (GU447030.1), *Hybosgarta melichari* Lallemand (KX239946.1), *Sphenorhina distinguenda* Walker (KX239967.1), *S. coronata* Lallemand (KX239966.1), *S. parambae* Jacobi (GU4444.1), *S. rubra* Linné (GU447015.1), *S. prosiherpina* Distant (GU447011.1) from Paladini et al. (2018) were included in the analyses; those of *H. melichari* and *Sphenorhina* spp. were used as external groups.

The maximum-likelihood (ML) algorithm implemented in the program aLRT-PHYML (Guindon and Gascuel 2003) was used to infer the phylogenetic relationships among mitochondrial DNA sequences. Previous to phylogenetic analysis, an appropriate model of DNA evolution and model parameters using the Akaike Information Criterion (AIC) tests was determinate, as implemented in Modeltest v3.7 (Posada and Crandall 1998). This tests supported the TIM2+I+G model (—LnL= 3937.7517; AIC= 7962.5, K=42) with gamma distributed rate variation among sites (G =0.224) and fraction of sites evolutionarily invariable (I< 0.00). These values, along with an optimized base frequency, were used in aLRT-PHYML. To estimate nodal support, the approximate likelihood ratio test (aLRT; Anisimova and Gascuel 2006) with the Shimodaira-Hasegawa-like procedure option was used. The genetic distances of Nei, between and within *Ocoaxo* species, and between the *Ocoaxo* spp. and the external groups were calculated in MEGA 5.2.2 (Tamura et al. 2011).

Number of instars. Representative specimens of entire sampling period were randomly selected for determinate the number of nymphal instars. Discrimination among instars of

spittle bugs has been performed from frequency distribution of continuous characters and the respective recognition of additional discrete attributes of body (Peck et al. 2002, Rodríguez, Ch. et al. 2003, Rodríguez Ch. and Peck C. 2007, Foieri et al. 2016). Therefore, to determinate the number of instars in *O. assimilis,* 15 continuous and four discrete characters from external morphology were evaluated on 116 nymphal specimens. For this, specimens were placed in 80% glycerin solution for three hours, to avoid dehydration and allow mobility. Appendages (legs and depending of nymphal instar wing pads or wings) were retired from all specimens to correct positioning and measuring. Images of body (dorsal and ventral view), right wing pad or wings (I) and right legs (I-III) from all specimens were obtained with an Olympus C- 5060 camera mounted on stereomicroscope Nikon SMZ800. A subset of specimens was dehydrated with a series of five solutions, four corresponding to alcohol of ascending concentration from 70% to 100% and the last to acetone 98%; after, the structures were critical point-dried, mounted on aluminum stubs and gold-coated for examination with a Hitachi S-2469N scanning electron microscope (SEM) for further refinement of initial observations. Discrete characters were observed directly on specimens and SEM micrographs; meanwhile, the continuous ones were measured from the images using discrete homologous anatomical loci or landmarks (lm); which were located in a two-dimensional space by Cartesian coordinates in program tpsDig ver. 2.31 (Rohlf 2017). Landmarks were placed to define the length and width of appendages and different parts of body. Measurements were obtained in micrometers from landmarks configurations, measuring the distance of the respective lm´s in PAST ver. 1.95 (Hammer et al. 2001)

Character states for each of the following attributes were documented for all individuals:

1.- Body Color (BC) (1) White to cream, (2) reddish-orange. Nymphal states display conspicuous changes in color body trough development (Foieri et al. 2016, Castro Valderrama 2017).

2.- Number of flagellomeres (NF). Multiannulated flagellum of antenna is divided in different number of "segments" called flagellomeres; tlhe number of these segments increase trough develops in cercopid species (Foieri et al. 2016).

3.- Development state of wings (SWD). In dorsal view. (0) without wing appendages, (1) length of wing pads do not exceed thorax, (2) length of wing pads do not exceed second abdominal segment, (3) length of wing pads exceed third abdominal segment, (4) wings larger than abdomen. The encased undeveloped wings in the hemimetabola nymphs are called wing pads (Nichols and Schuh 1989). The presence and relative length of these appendages varies through development (Foieri et al. 2016).

4.- Number of metatarsi spines (NMS). The tarsomeres in some cercopids adults display cuticular ornamentations like spines, the number of these elements increase trough development (Foieri et al. 2016).

5 – 19. The next continuous characters were measured: 5 body length (BL), 6 length of cephalic capsule (LCC), 7 width of cephalic capsule (WCC), 8 distance between eyes (DE), 9 stylet length (SL), 10 thorax length (TL), 11 abdomen width (AW), 12 - 14 femur length from first (FI), second (FII) and third (FIII) pair of legs, 15 – 17 tibia length from first (TI), second (TII) and third (TIII) pair of legs, 18 - 19 length of right wing pad or wing from anterior (LRWa) and width of right wing pad or wing from anterior (WRWa).

Statistical Analyses. Normality of the distribution of each continuous character was tested independently by the Shapiro-Wilkinson test (Shapiro and Wilk 1965). To evaluate if the variation of these characters display discontinuities that allow identify discrete groups corresponding to instars, distribution histograms were plotted to each one of them. Also to explore multidimensional patterns of morphological variation of both continuous and discrete characters and their relative contribution to recognize the nymphal instars, both Principal Component Analysis (PCA) and Principal Coordinates Analysis (PCoA) were used (Legendre and Legendre 1998). In both analyses, each specimen was considered an operational taxonomic unit (OTU); PCA was performed with a variance-covariance matrix computed from 15 continuous character, meanwhile, PCoA from distance matrix previously computed with the Gower index using the 15 continuous plus four discrete characters define in this study.

Individual contribution of continuous characters to discriminate among nymphal instars and adults was evaluated calculating minimum, maximum, mean and standard error for each continuous attributes and by mean Kruskal Wallis test, with respective Tukey´s comparisons.

Voltism and life cycle. The period of occurrence of *O. assimilis* was evaluated from the field inspections; at each visit, the number of saliva masses was recorded within the quadrants. All saliva masses found within the quadrant were collected for the posterior determination and quantification of nymphal instars containing within them.

To determine the host species of *O. assimilis*, when the saliva masses were detected on the roots, they were followed until finding the aerial part of the plant, which was collected

for later identification. Nymphs were stored in Vacutainer® tubes with 96% alcohol for preservation and analysis. With the data obtained in the field, a matrix was constructed in which for each quadrant sampled the absence-presence of instar stages and the host plants in which they were found, were documented.

Host preferences. Saliva masses with nymphal instars inside of *O. assimilis* were found in eight host plants: a grass, four herbaceous, a bush and two arboreal species (see results); thus, to determine the feeding preferences of the nymphs among these plants, the formation of protective frothy excreta on the plants as a signal of host selection and the respective development of the first nymph stages of *O. assimilis* species as signal of the establishment of them for feeding were evaluated. To do this, under controlled conditions, two individuals of each host plant species ("two replicas") were selected; the plants, with exception of *Quercus rugossa* Neé (in total seven host species were analyzed), were transplanted to plastic containers with substrate from the same site, the containers with the plants were kept in a conditioned place near the sampling site, to monitor them. In each plastic container (treatment), a host plant and four insects in early nymphal states were put inside of the box (in total 56 insect specimens). Spittle bugs using in the experiment were collected during the first half of July, period in which nymphs were observed in early stages (N2-N3, see result). Every third day the containers were sprinkled with water to maintain humidity and the number of saliva masses present as an indicator of live nymphs was recorded, after one month the percentage of survival of the nymphs in each host species including both replicates was counted. Host specimens were deposited in the herbarium of the Forest Sciences division of the Chapingo Autonomous University (Numbers: 69559 to 69565).

To determine if the arboreal species where *O. assimilis* saliva masses were found correspond to hosts in which the insect can develop its metamorphosis (nymph-adult), as well as to evaluate the preference of nymphal to different arboreal species, walking tests to paired selection of host and the respective development of the last nymphal stages was followed under controlled conditions. Thus, young plants of *Quercus rugosa* Née, *Pinus pseudostrobus var. apulcensis* and *P. patula* Schiede Ex Schltdl & Cham were placed in plastic containers (90 x 45 x 20 cm). In each box, two trees were placed vertically with the same sustrate from the field and they separated by 15 cm of distance between them (Fig. S8). Two specimens corresponding to the same host species and inter-specific combinations of the three hosts were placed in each plastic container, quantifying a total of six treatments: (T1) *Q. rugosa-Q. rugosa*, (T2) *P. pseudostrobus var. apulcensis - P. pseudostrobus var. apulcensis*, (T3) *P. patula - P. patula*, (T4) *Q. rugosa - P. pseudostrobus var. apulcensis*, (T5) *Q. rugosa - P. patula* and (T6) *P. pseudostrobus var. apulcensis - P. patula*; each treatment was performed in duplicate; although *P. patula* was not registered in the study site, this species was included in the experiment because it is distributed around study area.

Within each box, roots of both trees were carefully washed until that their surface was uncovered from 30% to 50% of its surface (Fig. S8), later in each box, eight specimens of nymphal stage four (N4) were placed (four individuals per tree). In each host specimen, the insects were placed 10 cm away from the root.

The boxes corresponding to each treatment were placed in a Prendo CB-20 bioclimatic chamber, controlling the humidity and temperature of the soil with the help of the KC-300 4 in 1 Soil Survey Instrument sensor, as well as the internal temperature of the chamber,

programming it to a temperature range that oscillated between 18 and 21 ° C. The treatments were monitored daily and the number of saliva mases and adults present was counted, the experiment was followed until the last adult emerged.

3.4 Results

Identification of specimens. The adults showed a dark brown to black spot in the union of the postclipeo and in the mandibular plate that is born in the antennal fossa; tegmina with a cream-yellow basal spot joined to a cream yellow longitudinal line, ending as a poorly delineated "tajamata", dark brown wing background (Figure 4); male genitalia displayed parameres strongly curved, forming a right angle before the tip; subgenital plate broad and short, dilated apically; aedeagus shaft not straight, its distal third slightly curved in lateral view, slightly thinner toward the base in ventral view; dorsolateral spines of aedeagus straight and away to the shaft (Figures 5 and 6). These characters correspond with those described as diagnostic from *Ocoaxo assimilis* (Castro-Valderrama et al. 2019). Eight partial sequences, one of them corresponding to an adults identified morphologically as *O. assimilis* and seven to different nymphal states of 970 bp of CO1 were obtained. Double peaks, nonsynonymous mutations, indels, frameshifts, or additional stop codons did not observe, which suggest a low probability of having sequenced nuclear copies (nuclear mitochondrial DNA [NUMTs]). After the manual edition of the sequences, a fragment of 970 bp was used in the analysis (GenBank in process).

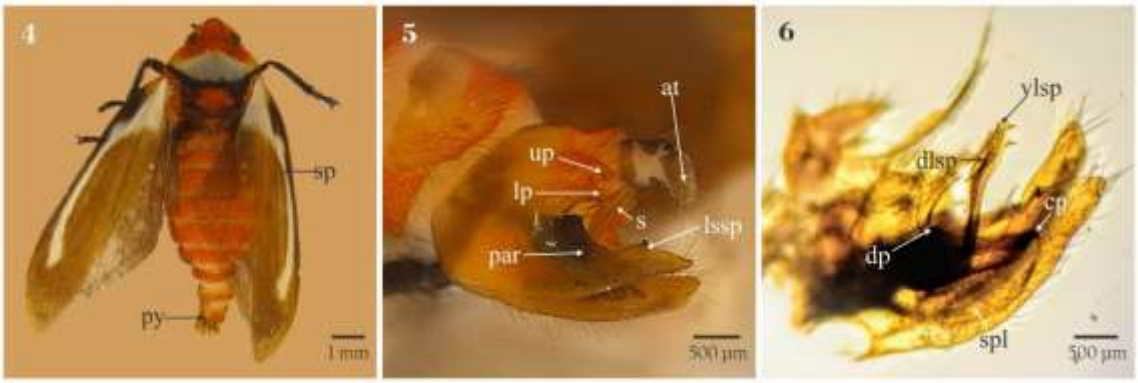


Figure 4-6 Male of *O. assimilis*. (4) Male adult in dorsal view; (5,6) Parts of male pygofer in lateral view: aedeagus and paramere. **sp** cream-yellow basal spot, ending as a poorly delineated "tajamata"; **py**, pygofer; Parts of aedeagus and paramere: **pb**, phalobase; **at**, anal tube; **up**, upper process of pygofer; **lp**, lower process of pygofer; **lssp**, lateral spine of the subgenital plate; **par**, paramere; **spl**, subgenital plate; **dlsp**, dorsolateral spines of aedeagus; **vlsp**, ventrolateral spines of aedeagus; **s**, shaft **cp**, claw of the paramere and **dp**, dorsal process.

The recovered phylogenetic tree showed a clear separation among the specimens studied and other *Ocoaxo* species (Fig. 7). All nymphal sequences were clustered together with the sequence from adult previously identified, within a single group with high nodal support (100%); the average genetic distance among sequences of this cluster was 0.00305; which support that nymphal specimens correspond to the same species that the adults identified as *O. assimilis* (Fig. 4). However, the target sequences of *O assimilis* of Nicolas Bravo of this study displayed considerable difference resect to sequence of *O. assimilis* from Guatemala previously deposited in the NCBI (Paladini et al., 2018). Our sequences were not clustered together with specimen from Guatemala rather with *O. ornatipenis* and *O. lineatus*, respectively; the average genetic distances among target *O assimilis* sequences respect to its conspecific from Guatemala was greater (0.08467), respect others species *O. ornatipenis* (0.04858) and *O. lineatus* (0.0766).

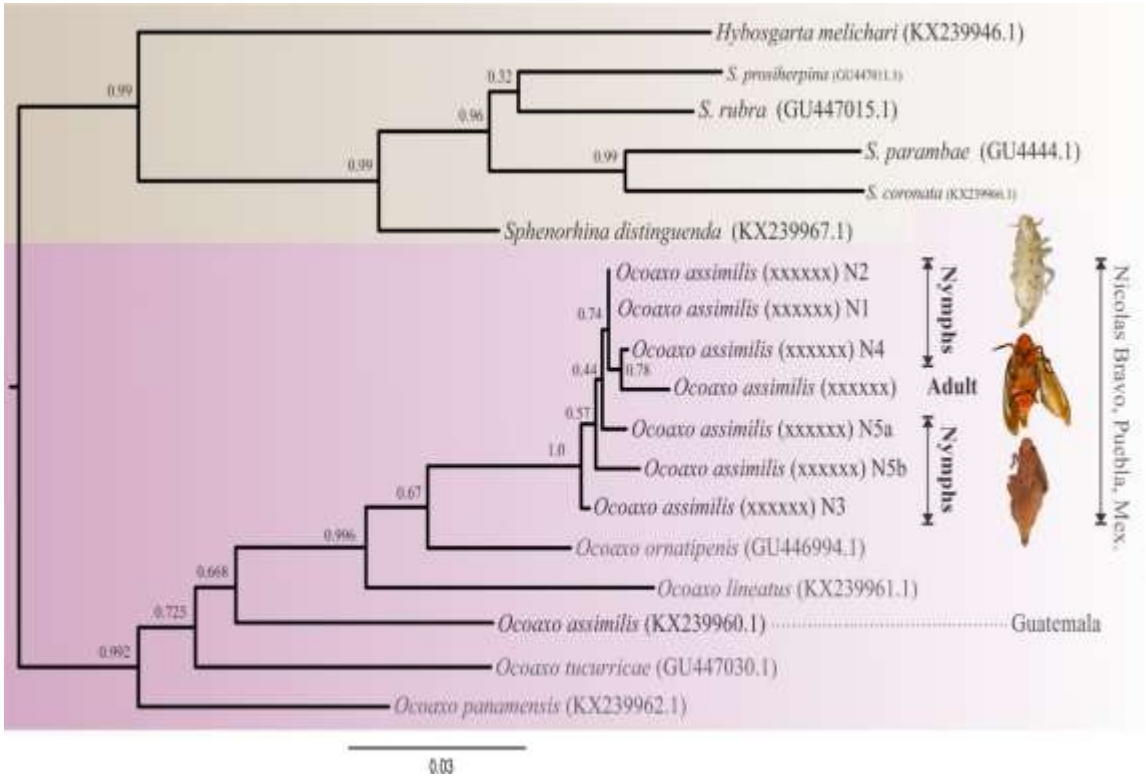
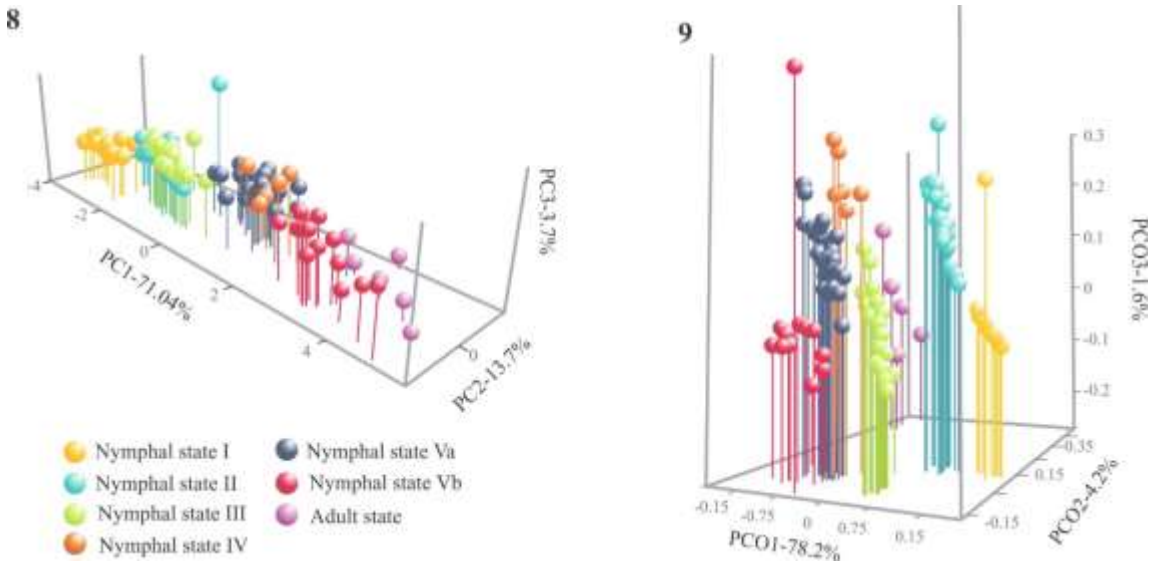


Figure 7 Phylogenetic tree resulting from ML analysis of cytochrome oxidase I (COI). Bootstrap values after 1,000 pseudoreplicates are indicated at the nodes.

Number of instars. Four outliers from the total data were excluded of the analyses in each of the 15 continuous morphological characters (n=121). Minimum, maximum, mean and standard error of 15 continuous, characters variables are showed in table 1. Without outliers, only two variables were normally distributed (LCC, AW) and 13 were not (BL, WCC, DE, SL, TL, FI, TI, F2, TII, FIII, TIII, LRWa, WRWa) (Table 1). Distance between eyes (DE), thorax length (TL), femur length from third pair of legs (FIII) and tibia length from third pair of legs (TIII) were skew to the right; meanwhile body length (BL), stylet length (SL) to left. Width of cephalic capsule (WCC) and femur, tibia and wing measurements (FI, FII, TII, TII, LRWa, WRWa) showed bimodal distribution.

The first three components of the PCA considering the 15 continuous characters (BL, LCC, WCC, DE, SL, TL, AW, FI-FIII, TI-TIII, LRWa, WRWa) from both nymphal and adult specimens quantified 88. 8% of total variation (PC1-71.4%, PC 2-13.7%, PC 3-3.7%). The scatter plot of the two first components (PC 1 vs. PC 2) showed segregation of the specimens into five recognizable discrete phenotypic groups (Fig. 8), one of them corresponding to adult specimens and the remainder to nymphal ones. The adults were segregated in PC2, meanwhile the nymphal specimens along of PC1. Biplots of relative contribution of each character in both PC´s supported that characters T3, T2, WCC and F2 contributed in higher proportion to explain the differences in PC1 (among nymphal groups) and LRWa, WRWa, T2 and T1 in PC 2 (among nymphal and adult specimens).The first three components of the PCoA considering the four discrete characters (BC, FN, SWD and SMN) and 15 continuous characters (BL, LCC, WCC, DE, SL, TL, AW, FI-FIII, TI-TIII, LRWa, WRWa) from both nymphal and adult specimens quantified 84% of total variation (PCo1-78.2%, PCo 2-4.2%, PCo 3-1.6%). The scatter plot of the two first coordinates (PCo 1 vs. PCo 2) showed segregation of the specimens into six recognizable discrete phenotypic groups (Fig. 9), five of them corresponding to nymphal specimens and one more to adults. The nymphal specimens were segregated in PCo1, meanwhile adults along of PCo2.



Figures 8-9 Scatter plots from principal components (**PC**) and principal coordinates (**PCO**). (**8**) **PC** of 15 morphological continuous characters of nymphal specimens of *Ocoaxo assimilis* and (**9**) **PCO** of 15 continuous and five discrete characters of nymphal specimens of *Ocoaxo assimilis*.

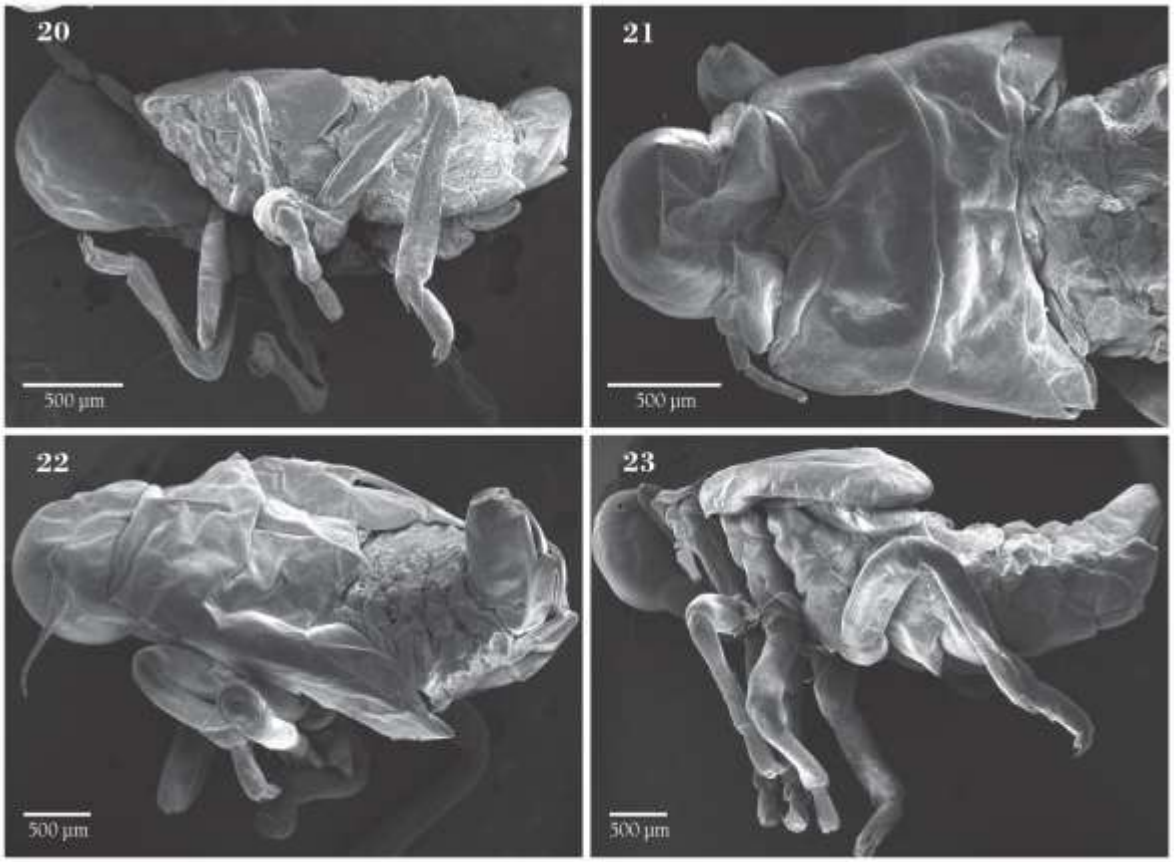
Together, the results of both PCA and PCoA supported that *O. assimilis* displayed six development stages, which can be recognized using both continuous and discrete characters of external morphology, five of them corresponding to nymphal stages and one more to adult.

Relative contribution of characters. The four qualitative attributes showed exclusive character states among different instars. All specimens from nymphal state one (N1) to four (N4) displayed body color (BC) hyaline yellowish, meanwhile, those from five nymphal state (N5) present two body color patterns a hyaline yellowish color and the other redish-orange body and adults showed redish-orange body (Fig. 10-14). Development state of wings (SWD) also was different among nymphal states, in all specimens from instars N1 and N2, the wing appendages were absent, in insects from N3 the length of

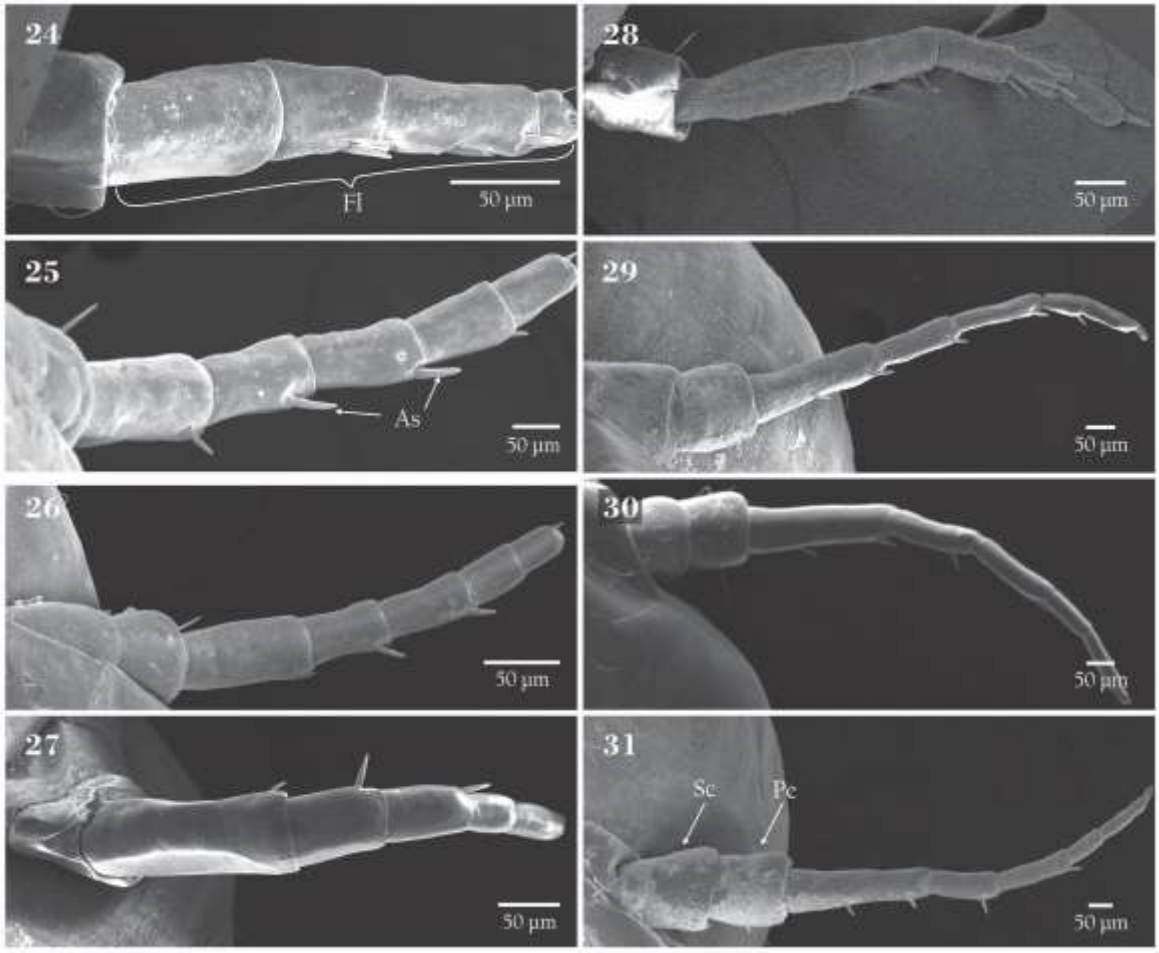
wing pads do not exceed thorax, in N4 these appendages do not exceed second abdominal segment, in N5 the length exceed third abdominal segment, and in adults wings were larger than abdomen (Figs. 15-23). Number of antenal flagellomeres (NF) and Number of metatarsi spines (NMS) increase trough of nymphal states, two flagellomeres were present in N1, four in N2, six in N6, and nine in N4, N5 and adult specimens (Fig. 24-31); meanwhile two metatarsi two apical spines were quantified in N1, four two apical spines in N2, six in N3 and nine in N4 and 11 to 14 in N5 (Fig. 32-35).

Figs. 10-14 Color patterns ventral view. (**10**) Nymph stage 2; (**11**) Nymph stage 4; (**12**) Nymph stage 5a;(**13**) Nymph stage 5b and (**14**) Adult.

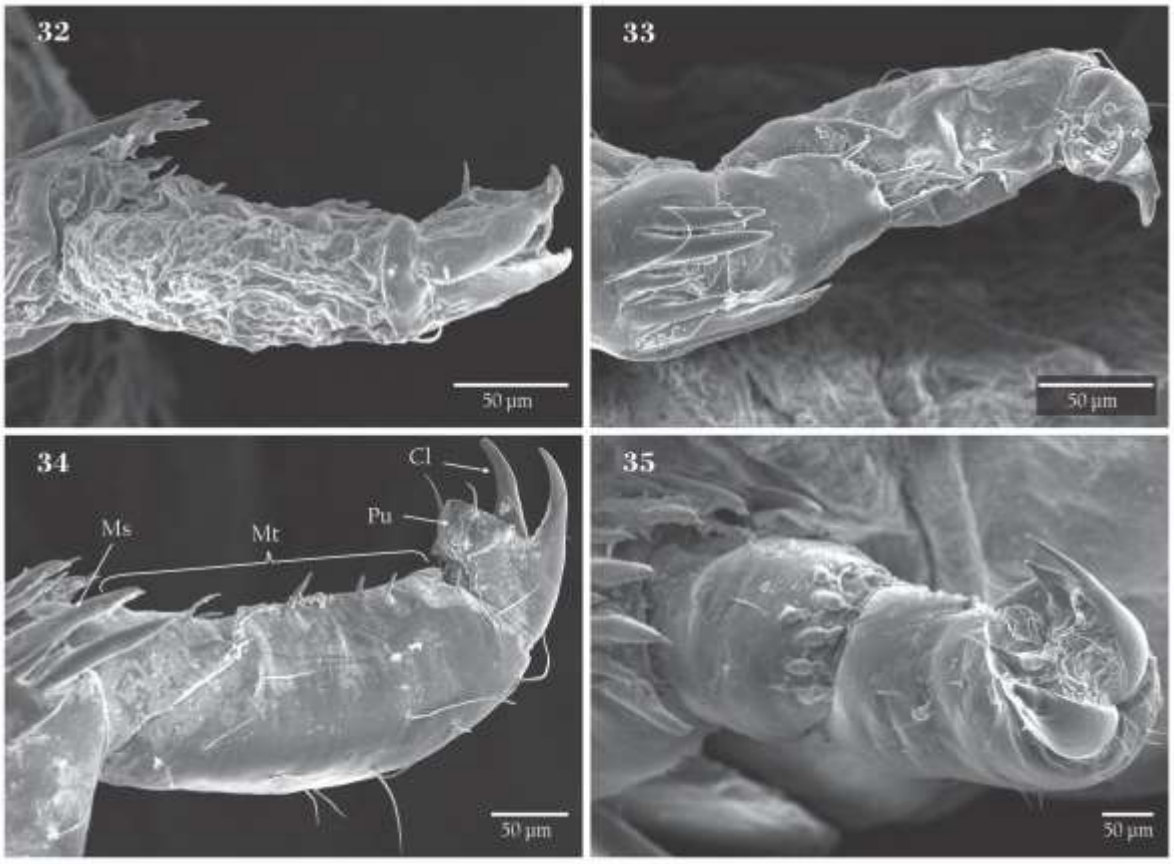
Figs. 15-19 Development wings, lateral and dorsal views. (**15**) Nymph stage 2; (**16**) Nymph stage 4; (**17**) Nymph stage 5a;(**18**) Nymph stage 5b and (**19**) Adult.



Figs. 20-23 Development wings, lateral and dorsal views by SEM. (**20**) Nymph stage 3; (**21**) Nymph stage 4; (**22**) Nymph stage 5a and (**23**) Nymph stage 5b.



Figs. 24-31 Antennal development by SEM. **As,** Antennal sensilla; **Fl,** Flagellomeres; **Sc,** Scapus and **Pe,** Pedicellus.



Figs. 32-35 Development of: Metatarsi, tarsomeres and basal spines of tarsomeres. **Ms,** basal spines of metatarsi**; Mt**, metatarsi; **Pu**, Pulvillo and **Cl**, Claw.

Discrete frequency distributions were not found in any continuous characters among five nymphal instars recovered by multivariate analysis, maximum and minimum values were overlapped between contiguous nymphal states (Table 2). However, the Kruskal - Wallis and respective Tukey´s tests supported that 15 continuous characters display differences among groups of at least three or four nymphal states, however these were not always presented in the same groups (Table 2). The specimens of nymphal state one (N1) showed lower average values in 15 characters (BL, LCC, WCC, DE, SL, TL, AW, FI, TI, FII, TII, FII) than the specimens of other instars and adult state (At); the first three instars (N1-N3)

displayed differences among them in FII, TII, FIII and TIII attributes, average values of this instars were lower than N4, N5 and At; N2 and N3 were did not show differences in ten attributes (BL, LCC, WCC, DE, SL, TL, AW, FI, TI, TIII), average values in these instars were higher than N1 and lower than N4, N5 and At; nymphal states N4, N5 and At presented similar average values in ten measurements (WCC, DE, SL, TL, AW, FI, TI, FII, TII, FIII, TIII), which were higher than N1, N2 and N3. The last two instars were differentiated by their higher averages values in LRWa and WRWa.

Instar description. Minimum, maximum, median and standard error of 15 continuous characters among nymphal instars and adults (averaging, male and female together) are presented in table 2.

Table 2 Morphometric characters of nymphs of *O. assimilis*. Data distribution: (*) Normal, (L) Left, (R) Right and (B) bimodal.

Character	N1 (18)	N2 (25)	N3 (20)	N4 (7)	N5 (46)	Adults (5)
	(Range);Mean± SE	(Range);Mean± SE	(Range);Mean± SE	(Range);Mean± SE	(Range);Mean± SE	(Range);Mean± SE
BL[L]	(2.1,3.6);2.8a ± 0.1	(3.0,5.8);4.2b ± 0.1	(3.3,5.7);4.3b ± 0.2	(4.5,9.6);5.9c ± 0.2	(4.5,7.2);6.5c ± 0.2	(7.1,9.4);8.1d ±0.4
LCC[*]	(0.4,0.7);0.5a ± 0.0	(0.6,1.0);0.7b ± 0.0	(0.6,1.0);0.8b ± 0.0	(0.8,1.3);1.0c ± 0.1	(0.7,1.2);0.9c ± 0.0	(0.7,0.9);0.8bc ±0.0
WCC[B]	(0.5,0.9);0.7a ± 0.0	(0.7,1.3);1.0b ± 0.0	(0.8,1.3);1.0b ± 0.0	(1.2,1.7);1.5c ± 0.1	(1.1,1.7);1.5c ± 0.0	(1.5,1.7);1.6c ±0.0
DE[R]	(0.3,0.6);0.5a ± 0.0	(0.7,0.9);0.8b ± 0.0	(0.6,0.9);0.7b ± 0.0	(0.8,1.2);1.0c ± 0.0	(0.7,1.1);1.0d ± 0.0	(0.6,0.9);0.7b ±0.0
SL[L]	(0.3,0.7);0.6a ± 0.0	(0.3,1.2);0.7b ± 0.0	(0.5,1.0);0.8b ± 0.0	(0.8,1.4);1.2c ± 0.1	(0.7,1.7);1.2c ± 0.0	(1.1,1.5);1.4c ±0.2
TL[R]	(0.4,1.0);0.7a ± 0.0	(0.8,1.5);1.1b ± 0.0	(1.0,1.4);1.2b ± 0.0	(1.3,2.2);1.9c ± 0.1	(1.3,3.2);2.1c ± 0.1	(2.7,3.5);3.1c ±0.2
AW[*]	(0.6,1.3);1.0a ± 0.0	(0.9,2.2);1.5b ± 0.1	(1.1,2.1);1.5b ± 0.1	(1.7,2.2);2.0c ± 0.1	(1.6,3.0);2.2c ± 0.0	(2.4,3.0);2.9c ±0.1
FI[B]	(0.2,0.8);0.5a ± 0.0	(0.6,0.9);0.8b ± 0.0	(0.4,1.0);0.8b ± 0.0	(1.0,1.5);1.2c ± 0.1	(0.8,1.5);1.2c ± 0.0	(1.3,1.6);1.5c ±0.0
TI[B]	(0.3,0.5);0.4a ± 0.0	(0.5,0.9);0.7b ± 0.0	(0.6,1.5);0.7b ± 0.0	(1.0,1.1);1.0c ± 0.0	(0.8,1.3);1.0c ± 0.0	(1.4,1.7);1.6c ±0.0
F2[B]	(0.4,0.6);0.5a ± 0.0	(0.7,1.0);0.8b ± 0.0	(0.7,1.3);0.9c ± 0.0	(1.0,1.4);1.3d ± 0.1	(1.0,1.5);1.3d ± 0.0	(1.3,1.6);1.4d ±0.1
T2[B]	(0.3,0.6);0.4a ± 0.0	(0.6,1.0);0.7b ± 0.0	(0.6,1.1);0.7b ± 0.0	(1.0,1.3);1.1c ± 0.0	(0.9,1.6);1.1c ± 0.0	(1.6,1.8);1.7c ±0.0
F3[R]	(0.5,0.8);0.6a ± 0.0	(0.8,3.2);1.0b ± 0.1	(0.8,1.4);1.0c ± 0.0	(1.2,1.7);1.4d ± 0.1	(0.9,1.8);1.4d ± 0.0	(1.3,1.6);1.4d ±0.1
T3[R]	(0.3,0.7);0.6a ± 0.0	(0.8,1.2);1.0b ± 0.0	(0.8,1.5);1.0b ± 0.0	(1.5,1.7);1.6c ± 0.0	(1.1,1.8);1.5c ± 0.0	(1.8,2.2);2.1c ±0.1
LRWa[B]			(0.7,1.7);1.0a ± 0.1	(1.9,2.6);2.3b ± 0.1	(1.5,3.2);2.6b ± 0.1	(8.4,10.6);9.4c ±0.4
WRWa[B]			(0.3,0.8);0.5a ± 0.0	(0.8,1.1);0.9b ± 0.0	(0.6,1.1);0.8b ± 0.0	(2.8,3.2);2.8c ±0.1

Nymphal instar one (N1). Color of body hyaline yellowish, multiannulated flagellum of antenna divided in four “flagellomeres”, without alar appendages and with two apical spines on metatarsi.

Nymphal instar two (N2). Color of body hyaline yellowish, multiannulated flagellum of antenna divided in four “flagellomeres”, without alar appendages and with four apical spines on metatarsi.

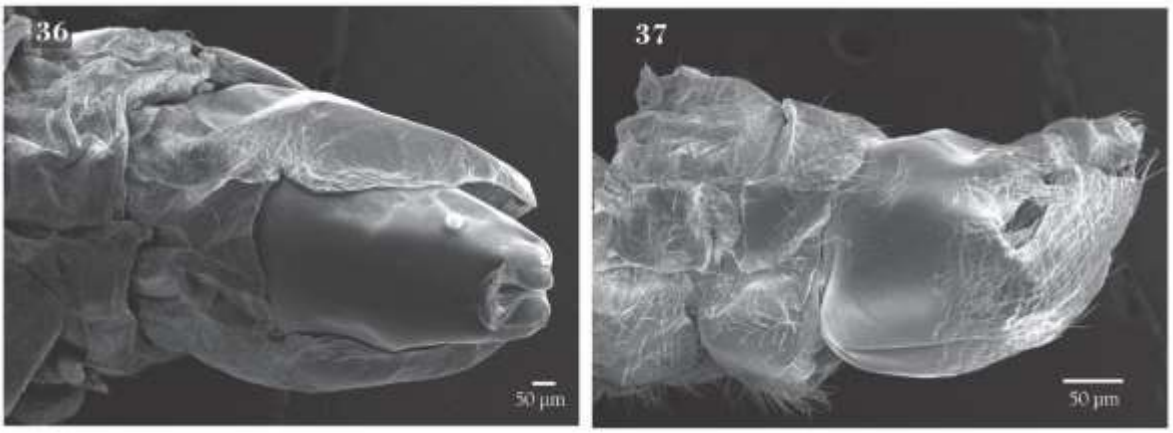
Nymphal instar three (N3). Body color equal to N2, multiannulated flagellum of antenna divided in five flagellomeres (Fig. 28), wing appendages present as wing pads, which do not exceed thorax in dorsal view; with six apical spines on metatarsi.

Nymphal instar four (N4). Body color and number of flagellomeres on antenna equal to N3, wing appendages present as wing pads, which do not exceed second abdominal segment in dorsal view, with nine apical spines on metatarsi.

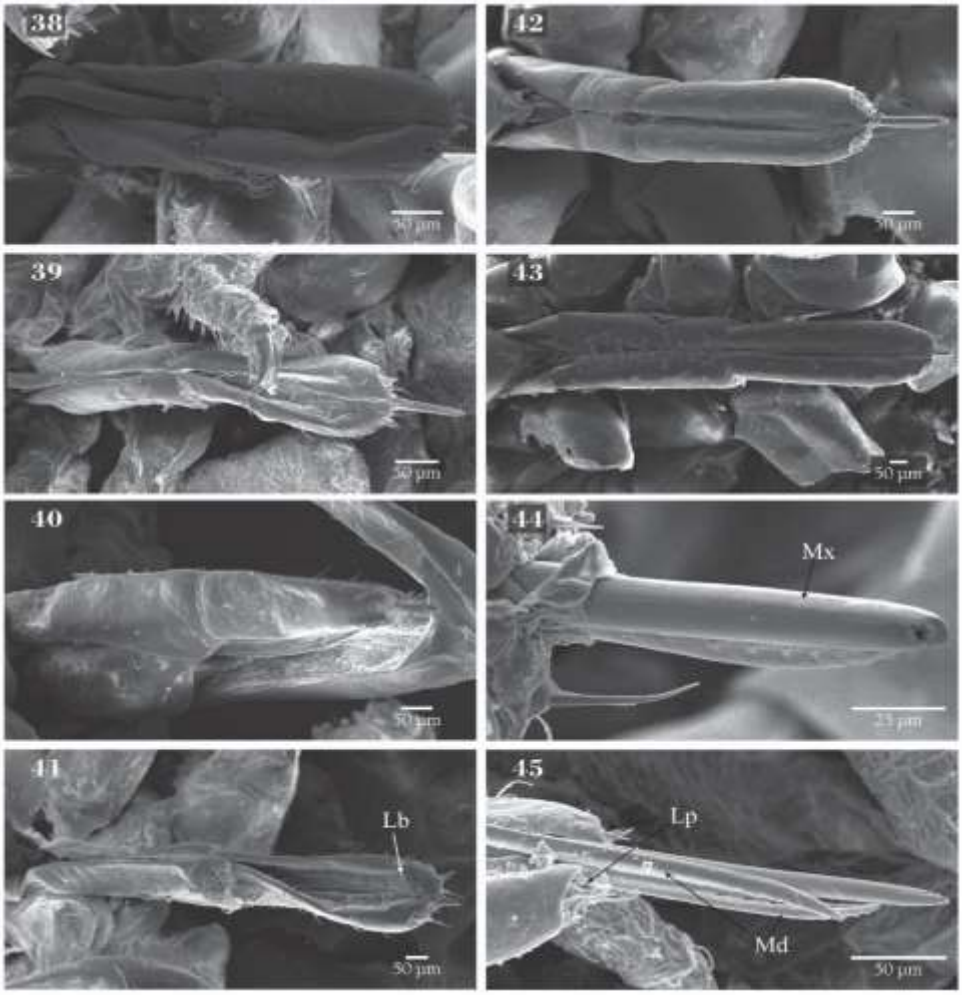
Nymphal instar five (N5). Multiannulated flagellum of antenna divided in six flagellomeres (Figs. 30 and 31), wing appendages present as wing pads, which exceed third abdominal segment in dorsal view; with eleven to fourteen apical spines on metatarsi, abdomen similar to earlier instars with genital plates on the eighth and ninth segment more developed, allowing differentiation of the sexes (Figs. 36 and 37). In this instar the specimens displayed conspicuous morphological differences, thus two subgroups Nymphal state 5a and 5b can be recognized. Specimens of N5a presented hyaline yellowish body, wing pads larger than third abdominal segment and same color as the body, BL they are smaller than N5b; meanwhile 5b specimens show color of body orange-

red, brown wing pads that exceed third abdominal segment, tegmina with a cream-yellow basal spot, typical of the specie.

Adult. Body color reddish-orange, multiannulated flagellum of antenna divided in six flagellomeres, buccal apparatus conformed by maxillary stylets, saw mandibles, labium and palpus; these elements can be recognized from the nymphal stage one from which they increase their size in each of the instars (Figures 38-45); wings well developed, larger than abdomen, with eleven to fourteen apical spines on metatarsi; wings display the diagnostic tegmina with a cream-yellow basal spot joined to a cream yellow longitudinal line, ending as a poorly delineated "tajamata".



Figs. 36-37 Genital plates. (**36)** Female and (**37**) Male.



Figs. 38-45 Mouthparts development. **Mx**, Maxilla; **Lp**, Labial palps and **Md**, saw Mandibles.

Voltism and life cycle. Presence of *O. assimilis* was evaluated from the las week of December 2017 to first week of January 2019; in these period, in total 34 quadrants were analyzed (Fig. 46), in which 1612 saliva masses contained the same number of specimens were recorded. From these records, the occurrence period of *Ocoaxo assimilis* in the Pine.

Forest of Nicolas Bravo was documented, which showed a marked seasonality from the beginning of the rains in summer, in the second half of June, until to autumn in the second half of October. In these period, eggs were not found in the area and in total 1612

specimens of five nymphal instars (N1-N5) and adults corresponding to only one brood were recorded and collected.

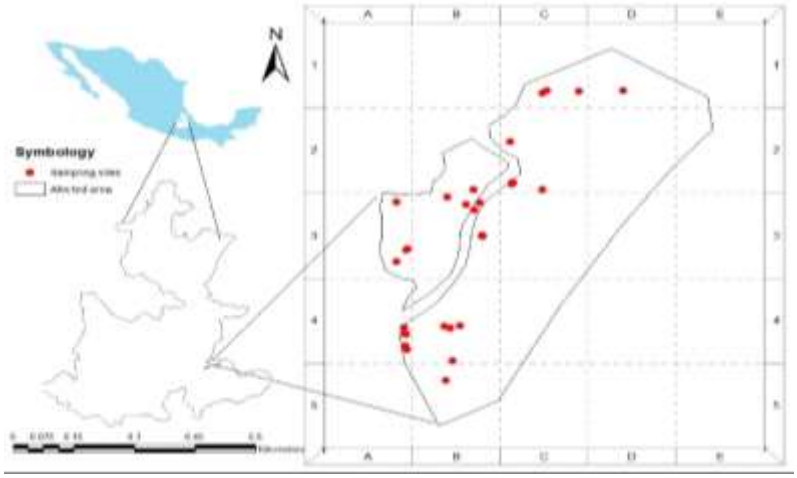


Figure 46 Location map.

The presences of the nymphs were synchronized with the beginning of the rainy season; the nymphal instars, N1 and N2 were recorded from the end of June until the first week of July; which was associate with changes in the average monthly rainfall (avg R) from 72. 1 mm to 260 mm and monthly average minimum temperature (avg min temp) from 12.4 to 14 °C; the higher abundances of N1(n=26) and N2 (n=36) specimens were recorded in the first and second weeks of July, respectively. Nymphal instar three (N3) was observed during the entire period of July until the first week of august, overlapping with N2, this period presented 76.9 mm average monthly rainfall and 11.9°C monthly average minimum temperature; the higher abundances of N3 specimens were recorded in the last week of July (n=152). Nymphal state four (N4) was recorded from the second week of July, overlapping with N3, to third week of August, this period presented increases from July to August in both AVR from (76. 9 mm to 238.8 mm) and AVmT (12.4 to 14 °C); the higher abundance of N4 was quantified in de second week of August (n=34). The last nymphal instar (N5) was collected from the second week of July to the

second week of October, overlapping with N4, period in which there was environmental changes quantified in AVR (238.8 mm in August to 173.1 mm in September) and AVmT (14 °C in August to 12.1°C in September). Adults were found from the second week of July to the third week of October, overlapping with N5; the higher abundance of adults was recorded in the last week of august (N=35) (Table 3).

Table 3 Weather conditions related to the appearance of nymphs and adults of *O. assimilis* during the year 2018.

Date	avg R (mm)	avg min temp (°C)	avg mean temp (°C)	avg max temp (°C)	Eggs	N1	N2	N3	N4	N5	Adults
22 May	72.1	12.4	20.1	27.8	✗	✗	✗	✗	✗	✗	✗
04 June	260.1	14	20.4	26.8	✗	✗	✗	✗	✗	✗	✗
27 June	260.1	14	20.4	26.8	✗	✔	✔	✗	✗	✗	✗
03 July	76.9	11.9	19.5	27.1	✗	✔	✔	✔	✗	✗	✗
04 July	76.9	11.9	19.5	27.1	✗	✗	✔	✔	✗	✗	✗
05 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✗	✗	✗
10 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✔	✔	✗
14 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✔	✔	✔
23 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✔	✔	✔
25 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✔	✔	✔
29 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✔	✔	✔
30 July	76.9	11.9	19.5	27.1	✗	✗	✗	✔	✔	✔	✔
01 Aug	238.8	12.4	19	25.7	✗	✗	✗	✔	✔	✔	✔
06 Aug	238.8	12.4	19	25.7	✗	✗	✗	✗	✔	✔	✔
10 Aug	238.8	12.4	19	25.7	✗	✗	✗	✗	✔	✔	✔
12 Aug	238.8	12.4	19	25.7	✗	✗	✗	✗	✔	✔	✔
18 Aug	238.8	12.4	19	25.7	✗	✗	✗	✗	✔	✔	✔
22 Aug	238.8	12.4	19	25.7	✗	✗	✗	✗	✗	✔	✔
06 Sept	173.1	12.9	19.6	26.3	✗	✗	✗	✗	✗	✔	✔
17 Sept	173.1	12.9	19.6	26.3	✗	✗	✗	✗	✗	✔	✔
04 Oct	167.3	12.1	18.7	25.2	✗	✗	✗	✗	✗	✔	✔
19 Oct	167.3	12.1	18.7	25.2	✗	✗	✗	✗	✗	✗	✔
03 Nov	84.8	9.6	16.8	24	✗	✗	✗	✗	✗	✗	✗

Specimens of five nymphal instars (N1-N5) were detected on the roots of eight species of plants: a grass (*Jarava ichu* Ruiz & Pav), four herbaceous (*Bidens odorata* Cav., *Penstemon barbatus* (Cav.), *Tagetes lucida* Cav., *Bouvardia ternifolia* (Cav.) Schltdl), a bush (*Symphoricarpos microphyllus*) and two trees (*Pinus pseudostrobus* var. *apulcensis*, *Quercus rugosa* Née). Of them 30 % (n= 486) of nymphal specimens were found on *Pinus*

pseudostrobus var. *apulcensis* (Lindl.) Martínez, 22.2 % (n= 358) on *Quercus rugosa* Née, 14.0% (n= 230) on *Symphoricarpos microphyllus* with, 11.1% (179) *Bidens odorata* Cav. with, 9.0% (n= 153) *Penstemon barbatus* (Cav.), 6. 3 % (n=102) *Jarava ichu* Ruiz & Pav, 3.0% (n=51) on *Tagetes lucida* Cav. and 3.0% (n=51), *Bouvardia ternifolia* (Cav.) Schltdl.

Host preferences. The feeding preferences among herbaceous, bush and arboreal species of *O. assimilis* nymphs under controlled conditions were evaluated through July and August from N2 to N5 instars. In both replicas of seven hosts species, 100% of insects (n=56) walked toward the roots of plants, on it secreted protective frothy excreta to feeding and continued their development. However, the specimens were not capable to continue their development in all host species, in total only 23% of them (n=13) survived until the end of the period.

The hosts in which higher survival rates were present corresponded to species *Pinus pseudostrobus* var. *apulcensis* (Lindl.) Martínez with an average of 62.5%, followed of the b *Penstemon barbatus* with 50%, the bush *Symphoricarpos microphyllus* with 37.5%, *Bidens odorata* Cav. with 25%, *Jarava ichu* Ruiz & Pav. with 12.5%; in the treatments corresponding to *Tagetes lucida* Cav. and *Bouvardia ternifolia* (Cav.) Schltdl no nymph survived.

The walking tests and the respective development tracking were carried out during 23 days, period in which the last nymphal specimen reached the adult stage from S4. In this period, 60.41% of specimens (n= 29) complete the metamorphosis before 14 days. In the six treatments, the 100% of specimens (n=48) walked toward the roots of plants, on it

secreted protective frothy excreta to feeding and continued their development; however only 68.7% of them (n=33) could complete their metamorphosis and 21.3% died (n=15).

Among homospecific treatments, T1 (*Q. rugosa- Q. rugosa*) displayed lowest survival rate (25%), because six of eight insects died during the first week, before complete the metamorphosis (Figure 50); meanwhile T2 (*P. pseudostrobus - Pspeudostrobus*) and T3 (*P. patutla - P. patula*) showed higher survival rate, because seven (87.5%) and six (75%) specimens, respectively, complete their development until adult (Fig. 47).

In the treatments with hetero specific combinations (T4 - T6) all nymphs selected the nearest roots from where they were placed, however, during first week of experiment some of them changed host to contiguous plant. In T4 (*Q. rugosa-P. psudostrobus var. apulsensis*) two specimens died and six complete their metamorphosis, of these last, two specimens change the tree host from oak to pine; in T5 (*Q. rugosa- P. patula*), three nymphs died and five reached adult stage, of them, two nymphal specimens change host from *Q. rugosa* to *P. patula*; in T6 (*P. pseudostrobus var. apulcensis - P. patula*) only one insect died and seven complete metamorphosis, of which only one change from *P. patula* to *P. pseudostrobus* var. *apulsensis* (Fig. 47).

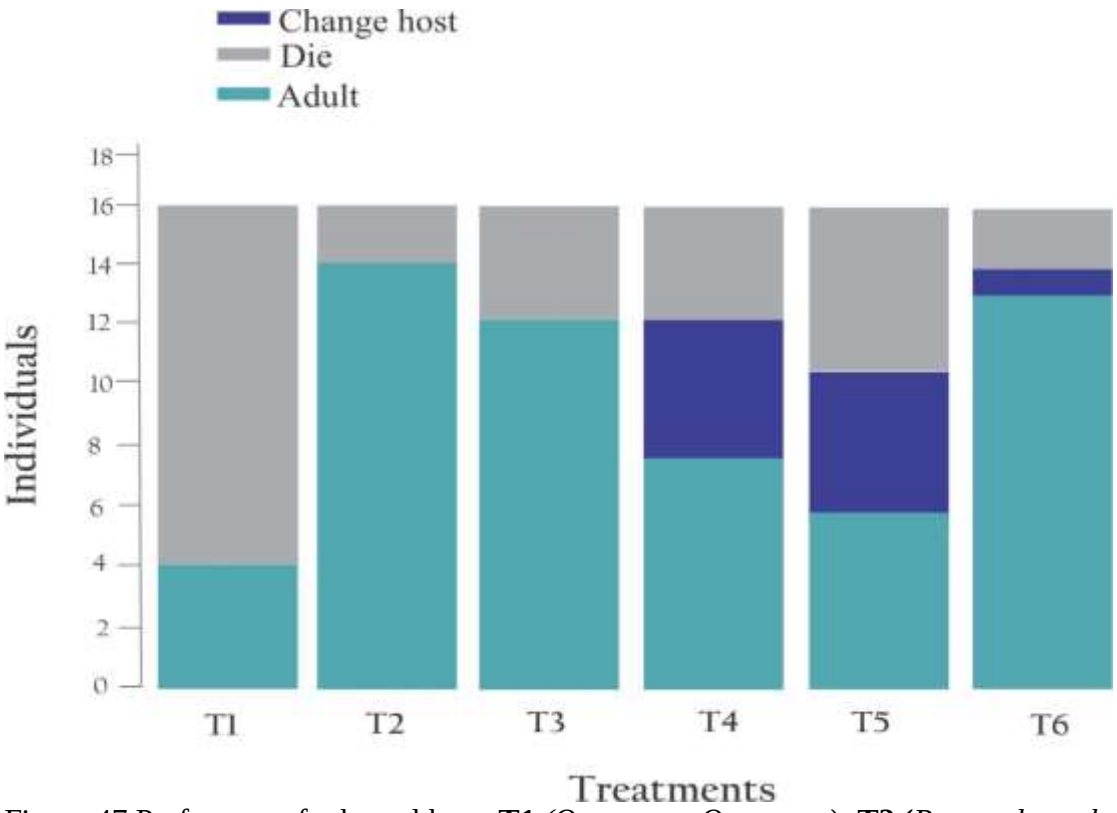


Figure 47 Preference of arboreal host. **T1** *(Q. rugosa- Q. rugosa);* **T2 (***P. pseudostrobus - Pspeudostrobus);* **T3** *(P. patutla - P. patula);* **T4** *(Q. rugosa-P. psudostrobus var. apulsensis);* **T5** *(Q. rugosa- P. patula) and* **T6** *(P. pseudostrobus var. apulcensis - P. patula).*

3.5 Discussion

In the present study using morphological and molecular data we were able to associate the immature instars with adults of *O. assimilis*, which in turn allowed to determine the number of nymphal stages of this species, to know its abundance over time and to describe the morphological changes that it presents during its life cycle. Additionally, nymphs and adults, together with field and laboratory, allowed to know the voltism, the spectrum and preferences of these stages.

Identification of nymphal specimens. Traditionally in cercopid species, the association of nymphal stages with adults has been done by laboratory reproduction of adult specimens

and F1 development monitoring (Rodriguez-Chaires and Peck 2007). In other groups of insects and species of cercopids, the Barcode has constituted a reliable tool for this purpose (Hebert et al. 2003, Miller 2007, Seabra et al. 2010, Wang et al. 2015) allowing the identification of specimens in nymphal state and their respective association with the adult state, as is the case of the specimens of *O. assimilis* analyzed in the present study.

Recently, in some localities of Mexico, the habitat and host plants associated with pine salivazos species of the genus Ocoaxo (*O. assimilis*, *O. cardonai* and *O. varians*) have been described and associated with the phenomenon known as pine decline (Pichardo-Segura et al.2017). However, the biology and ecology of these insects was poorly described and the nymphs and their respective hosts were unknown (Castro-Valderrama 2017).

The results of phylogenetic analysis and calculation of genetic distances using Cytochrome oxidase subunit 1 gene as DNA barcode, supported that nymphs collected in Nicolas Bravo, corresponded to the same species that the adults identified morphologically, because they conformed a monophyletic group and the average genetic distance among them (0.00305) was much less than those calculated among other *Ocoaxo* spp. (0.09109) included in the Genebank (Paladini et al. 2018).

The morphological characterization of collected adults in this study agree with the keys provided by Castro Valderrama et al. (2019) to identify *O. assimilis*; adult insects presented diagnostic tegmina of this species with a cream-yellow basal spot joined to a cream yellow longitudinal line, ending as a poorly delineated “tajamata” and aedeagus shaft with its distal third slightly curved, displaying straight dorsolateral spines. Based on these characters, specimens from Nicolas Bravo were determinate as *O. assimilis*.

An outstanding result of phylogenetic analysis was the position of the sequences of O assimilis from Nicolas Bravo, Puebla respect to the especific sequence from Guatemala, previously deposited in Genebank (Paladini et al. 2018); which were paraphyletic and far phylogenetically among them. Pine spittlebugs from Nicolas Bravo were closer to *O. ornatipens* and *O. linneatus*, respectively than *O. assimilis* from Guatemala and displayed 8.2 % of divergence among them. Genetic differences observed between Guatemala and Nicolas Bravo Sequences are similar to the 9.1% divergence in the COI sequence data found among other Ocoaxo species and 7.1% in other species of Cercopidae (e. g. *Sphenorhina* spp.) (Paladini et al. 2018). The strong genetic differences in COI observed between Ocoaxo assimilis are equivalent or higher to that found between full species *Ocoaxo*. For example, the species *O. ornatipens* and *O. lineatus*. are 7.0% divergent in COI (Paladini et al. 2018). These results suggest that specimens from Guatemala and Nicolas Bravo could be different species; more work needs to be done to distinguishable morphological traits that could be used to separate and identify the potential species within *Ocoaxo*.

Instar Number. Instar number in Cercopidae members has been determinate by mean univariate analysis using morphometric measurements, such as body length, body width, length of wings pads, length of wings and width of cephalic capsule; of them, the most informative characters has been the body length and width of cephalic capsule, because they allow recognize discrete groups corresponding to nymphal states (Peck et al. 2004, Rodríguez Chaires and Peck 2007). In the present study, the variation of body measurements was wide and overlapped among nymphal stages, which did not allow to separate them. With these characters, PCA only allow recognize at least five groups

corresponding to NI, N2, N3, N4, NIV-NVa-NVb and adults. Because specimens from nymphal state four and five ("a" and "b") did not segregate in discrete groups.

However, the multivariate analysis of both continuous and discrete characters together, support the existence of five nymphal stages in *O. assimilis* as it has been documented in the cercopids *Aphrophora cribrata* and *Aphrophora saratogensis* (Ewan 1961, Wilson 1991); but different from *Haematoloma dorsarum* that presents four nymphal stages (Roversi and Baccetti 1994). A very marked difference among *O, assimilis* and the aforementioned pine spittlebugs is that *O assimils* specimens corresponding to nymphal state five presented morphological differences in discrete and continuous characters; which were recovered in scatter plot of PCoA as two subgroups in this Nymphal instar (Fig. 9). This pattern has also been identified in the grass and leave cercopids *Mahanarva andigena*, *Zulia carbonaria*, *Aeneolamia reducta* and *Prosapia simulans*, in which, the specimens of nymphal instar five are classified as "5a" and "5b" based on differences in body color and size measurements (Rodríguez- Chaires and Peck 2007). According with these spittlebugs, the most notable differences between N5a and N5b in *O. assimilis* are body color and the presence of tegmina with a cream-yellow basal spot joined to a cream yellow longitudinal line, ending as a poorly delineated "tajamata" on the wing pads in 5b, absents in 5a.

Notable changes were also distinguished throughout its development, such as development of the wing packs or stumps, which helps to differentiate the first two instars from the others, this facilitates the classification in the field of individuals per instar.

Life cycle. The presence of nymphal state one was closely associated with the increase in humidity in the environment, which is why nymphs appear when the rainy season begins,

nymphs appear gradually just like adults, but only one generation per year of both is observed. Nymphs are present from the beginning of the rainy season in the region, mid-June to early September. But the first stages, N1 and N2, were only found the last week of June and the first week of July. The nymphs of the last moments were found from the third week of July until the first week of October. Adults met in the field from the third week of July until the third week of October. The results with respect to voltism confirm that the species is univoltin as well as *Aphrophora cribrata*, *Aphrophora saratogensis* and *Haematoloma dorsarum* (Ewan 1961, Wilson 1991, Roversi and Baccetti 1994), cercoids that also damage pines and that their appearance is closely related to the time high humidity

It is shown how polyphagous the individuals of this species are, because they feed on plants of different families, from asteraceae to pines, but they cannot be considered as host plants because they do not complete their metamorphosis. It is corroborated that plants belonging to families other than pinaceae can only be considered as food plants as discussed by Burckhardt et al. (2014) since they are used to feed on their way to their host plant, *Pinus psudostrobus* var. *apulcensis* (Lindl.) Martínez.

After the Pines, the plant where more nymphs were found was *Symphoricarpos microphyllus* Kunth, a common woody shrub in association with Pinos in Mexico (Rzedowski 2006) followed by herbaceous plants such as *Bidens odorata* Cav. and *Penstemon barbatus* (Cav.) Roth, in accordance with that described by Ewan (1961), which mentions the preference for the first instants of Saratoga spittlebug (*Aphrophora saratogensis*) for herbaceous plants and woody stems. This perhaps due to the greater

presence of these species in the forests and the need not to starve in search of the host plant.

The survival of the nymphs in the first experiment was perhaps linked to that described by Ewan (1961) which says that the major stages (nymph three onwards) prefer woody plants and perform a differentiated migration and not a mortality.

Regarding preferences, the preference of *Ocoaxo assimilis* for *Pinus psudostrobus* var. *apulensis* (Lindl.) Martínez, which ratifies it as a host plant since in it the nymphs fulfill their metamorphosis and in the nymphal stage as in the adult stage they feed on it. But it is also shown that they can feed on *Pinus patula* as mentioned by Castro Valderrama (2018) and the reports of the National Forestry Commission (CONAFOR 2018), in turn agreeing with that described by Ewan (1961), Notario et al. (1981), Covassi et al. (1989) and Cobos 1995 the preference they have for woody plants, also denoting with this experiment the serious danger that pine trees run if this pest cannot be controlled.

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3.8 Supplementary Data

S1-S8. S1-S2 Damage by *O. assimilis*; **S3** teneral of *O. assimilis*; **S4** search of nymphs; **S5** nymphs of *O. assimilis* and **S6-S8** insect breeding protocols.

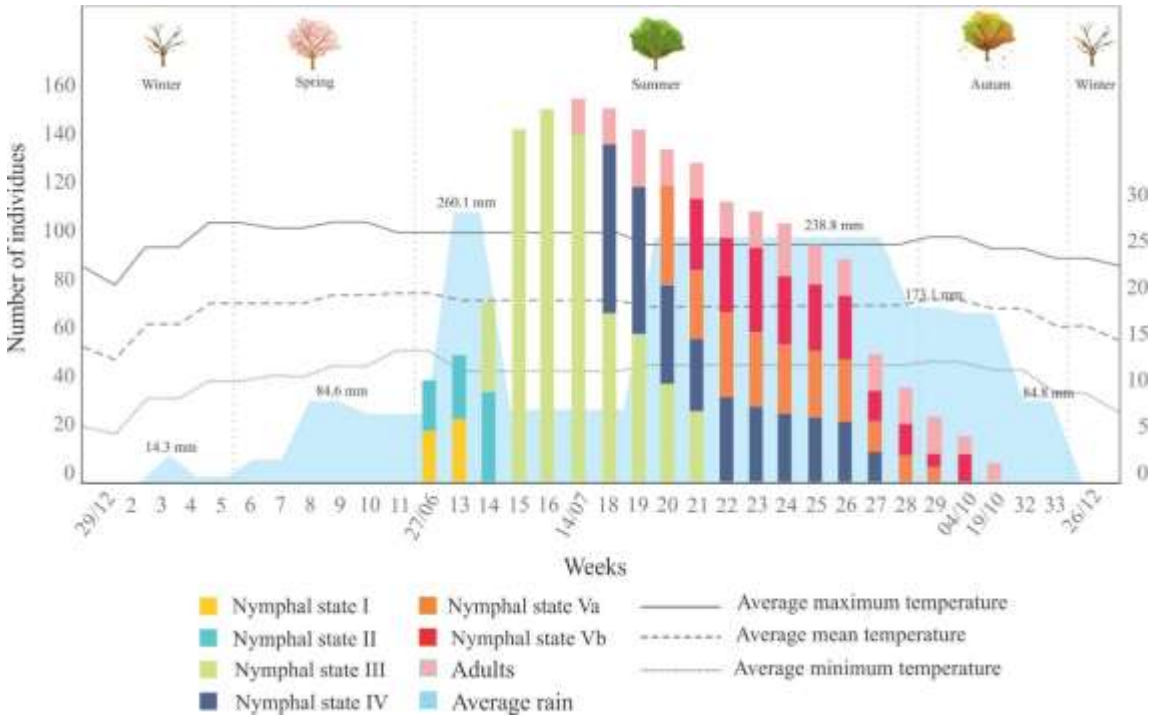

Figure S9 Lifecycle of *O. assimilis.*

CONCLUSIONES GENERALES

Este estudio proporciona información importante respecto a aspectos desconocidos de la biología de *O. assimilis* como lo son: las características morfológicas que poseen las ninfas, sus plantas hospedantes asociadas en esta etapa y el número de generaciones por año que presenta esta especie de salivazo de los pinos.

Mediante el apoyo de claves taxonómicas proporcionadas por Castro-Valderrama et al. (2019) se verifico que los adultos encontrados en el municipio de Nicolás Bravo, Puebla, pertenecen a *O. assimilis* y molecularmente con ayuda del Gen mitocondrial que codifica para la Citocromo Oxidasa I (COI) se comprueba que las ninfas si están relacionadas con los adultos.

Al comparar las secuencias obtenidas con las registradas en el Genebank para *O. assimilis* se concluye que estas no son similares, lo que abre una posibilidad para el estudio mediante morfología geométrica de características morfológicas para así actualizar las claves taxonómica y apoyar a una correcta clasificación de los especímenes.

Los individuos de esta especie presentan una generación por año por lo tanto es una especie univoltina y la aparición de sus ninfas está íntimamente relacionado a la época de lluvias y la temperatura mínima que existe en el medio ambiente.

Se ratifica la existencia de cinco estadios ninfales para esta especie como lo menciona Castro Valderrama (2017), al igual que se comprueba la existencia de un instar 5a y 5b. Mediante caracteres discretos se diferencian casi todos los instares, lo que facilitaría su clasificación y separación en campo.

Se evidencia lo polífagas que son las ninfas de *O. assimilis* debido a que en sus primeros instares (N1-N3) se alimentan tanto de herbáceas, arbustos y árboles, pero también se comprueba que una vez que encuentran a su hospedante, en este caso *Pinus psudostrobus* var. *apulensis* (Lindl.) Martínez o *Pinus patula*

Schiede Ex Schltdl & Cham, en el completan su metamorfosis Burckhardt et al. (2014).

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