UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS
BIOLOGÍA EVOLUTIVA

Biología de la polinización y embriología del género *Bdallophytum* (Cytinaceae)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS BIOLÓGICAS

PRESENTA:

SANDRA RIOS CARRASCO

TUTORA PRINCIPAL DE TESIS: DRA. SONIA VÁZQUEZ SANTANA
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Ciudad Universitaria, Cd.Mx. 2022

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COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS
FACULTAD DE CIENCIAS
DIVISIÓN ACADÉMICA DE INVESTIGACIÓN Y POSGRADO
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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
Presente

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **02 de mayo de 2022** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la estudiante **RÍOS CARRASCO SANDRA** con número de cuenta **308270778** con la tesis titulada: **"Biología de la polinización y embriología del género *Bdallophytum* (Cytinaceae)"**, realizada bajo la dirección del(la**) DRA. SONIA VÁZQUEZ SANTANA:**

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Sin otro particular, me es grato enviarle un cordial saludo.

ATENTAMENTE
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 15 de junio de 2022

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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A mi familia

Índice

Resumen

El género *Bdallophytum* pertenece a la familia Cytinaceae, un grupo de plantas endoparásitas de raíz. El género tiene tres especies distribuidas desde México hasta Costa Rica parasitando raíces del género *Bursera* (Burseraceae). El estudio de su polinización, así como el estudio de su morfología floral y desarrollo, permiten conocer aspectos del ciclo de vida de las especies de *Bdallophytum* para tener un mejor entendimiento de su biología y reproducción. En el presente trabajo se abordaron diversos aspectos de la biología reproductiva de plantas holoparásitas, especialmente endoparásitas de Cytinaceae, entre ellos la polinización y desarrollo floral de las especies *B. americanum*, *B. andrieuxii* y *B. oxylepis*, la primera con poblaciones en Calvillo, Aguascalientes, la segunda en Santo Domingo Tonalá, Oaxaca y la tercera en San Fernando, Chiapas, México. Para el estudio de la polinización se monitorearon las poblaciones para registrar a los visitantes florales, así como para conocer aspectos de la biología floral como el tiempo de receptividad del estigma, el momento de apertura de las anteras y duración de la viabilidad de polen.

Los resultados muestran que *B. americanum* es polinizada principalmente por moscas, aunque algunos escarabajos pueden participar también en la polinización. Las flores masculinas de *B. americanum* se distinguen de las de *B. andrieuxii* y *B. oxylepis* por tener apéndices estaminales formados por el crecimiento apical del tejido conectivo que une a los microsporangios de las anteras. Dichos apéndices son usados por los polinizadores como plataforma de aterrizaje. Por otro lado, *B. andrieuxii* es polinizada por mariposas, las cuales toman néctar como recompensa y utilizan las flores como sitios de oviposición y sitios de cría.

Se demostró que *B. oxylepis* tiene una polinización especializada por abejas meliponinas, las cuales colectan polen como recompensa. Aunque no existe un patrón en la polinización de las especies de *Bdallophytum*, sí existe un patrón en el desarrollo floral de las especies estudiadas. Los resultados principales muestran que las flores de las tres especies presentan variación en tamaño, número de tépalos, estambres y lóbulos estigmáticos a lo largo de las inflorescencias. Por primera vez se describe a *B.* oxylepis como ginomonoica, con flores femeninas y bisexuales en la misma inflorescencia. Asimismo, en esta especie de describe el desarrollo de un ginostemio, estructura formada por la fusión de los órganos sexuales, siendo este el primer reporte de ginostemio en Cytinaceae. La esporogénesis y gametogénesis en androceo y gineceo se lleva a cabo de manera similar en las tres especies.

Abstract

The genus *Bdallophytum* belongs to the Cytinaceae family, a group of root endoparasitic plants. The genus has three species distributed from Mexico to Costa Rica, parasitizing roots of the genus *Bursera* (Burseraceae). The study of their pollination, as well as their floral morphology and development, allows knowing aspects about the life cycle of the species of *Bdallophytum* to understand their biology and reproduction better. In the present work, various aspects of the reproductive biology of parasitic plants, especially endoparasites of Cytinaceae, were addressed, among them the pollination and floral development of the species *B. americanum, B. andrieuxii*, and *B. oxylepis*, the first with populations in Calvillo, Aguascalientes, the second in Santo Domingo Tonalá, Oaxaca and the third in San Fernando, Chiapas. For the study of pollination, the populations were monitored to record floral visitors, as well as to learn about aspects of floral biology such as the time of receptivity of the stigma, the moment of opening of the anthers, and the duration of pollen viability. The results show that *B. americanum* is mainly pollinated by flies, although beetles can also participate. Male flowers of *B. americanum* are notable for having apical appendages formed by connective tissue growth. Pollinators use these appendages as a landing platform. On the other hand, *B. andrieuxii* is pollinated by butterflies, which take nectar as a reward and use the flowers as oviposition and breeding sites. Finally, *B. oxylepis* showed a highly specialized pollination by stingless bees that take the pollen as a reward. Although there is no pattern in the pollination of the *Bdallophytum* species, there is a pattern in the floral development of the studied species. The main results show that flowers of the three species present variation in size, number of tepals, stamens, and stigmatic lobes along the inflorescences. For the first time, *B. oxylepis* is described as gynomonoecious with female and bisexual flowers in the same inflorescence. In *B. oxylepis* the development of a gynostemium is described, a structure formed by the fusion of the sexual organs. This is the first report of a gynostemium in Cytinaceae. Sporogenesis and gametogenesis in androecium and gynoecium are carried out in a similar way in all three species.

Introducción general

Las plantas parásitas son aquellas que obtienen recursos de otras plantas (Musselman y Press 1995; Heide-Jørgensen 2008). El parasitismo ha surgido de 11 a 13 veces durante la evolución de las angiospermas, por lo que no se trata de un grupo monofilético, sino funcional (Těšitel 2016). Las plantas parásitas poseen atributos distintivos que permiten conjuntarlas en un grupo funcional dadas sus características ecológicas y sus interacciones con los hospederos (Heide-Jørgensen 2008; Těšitel 2016). Los grupos con mayor cantidad de representantes parásitos son el orden Santalales y la familia Orobanchaceae (Bennett y Mathews 2006), por lo que han sido objeto de estudio de diferentes atributos relacionados con su hábito parasítico, como la evolución de los genes del cloroplasto (Bungard 2004). Por otra parte, Westwood *et al*. (2010) mencionan que aquellas familias de angiospermas con menos representantes parásitos exhiben un mayor grado de especialización, sin embargo, han recibido menos atención (Heide-Jørgensen 2008).

La familia Cytinaceae es de las que presentan pocas especies (Fernández-Alonso y Cuadros-Villalobos 2012) y un alto grado de especialización (De Vega *et al.* 2007). Es una familia con especies endoparásitas, es decir, que la parte vegetativa de la planta, reconocida como endófito que es el tejido que se encuentra dentro del hospedero; mientras que el exófito es la parte que crece fuera del hospedero y está conformado únicamente por las flores e inflorescencias (Těšitel 2016). La familia comprende dos géneros, *Cytinus* con distribución en Europa, Asia y África, y *Bdallophytum*, género nativo de América (Alvarado-Cárdenas 2009; Fernández-Alonso y Cuadros-Villalobos 2012).

Varias especies del género *Cytinus* han sido objeto de estudio de su biología reproductiva (De Vega *et al.* 2009; Hobbhahn y Johnson 2015), sin embargo, *Bdallophytum* carece de estudios completos de este tipo. Sólo existe un antecedente en *Bdallophytum americanum* (antes *B. bambusarum*) en Veracruz (García-Franco y Rico-Gray 1997) en el cual se estudiaron los visitantes florales y las recompensas florales asociadas. En este estudio mencionan la presencia de moscas como principales polinizadores, haciendo énfasis en la producción de néctar como recompensa principal.

Para ampliar el panorama sobre las características reproductivas en las plantas endoparásitas y para conocer más sobre el grado de especialización de estas, son necesarios los estudios comparativos en especies de este tipo. Dichos estudios deben incluir aspectos de la biología reproductiva y estudios morfológicos que evidencien atributos florales involucrados en la perpetuación de las especies endoparásitas.

El género *Bdallophytum* es un grupo de plantas endoparásitas que está conformado por cuatro especies (Alvarado-Cárdenas 2009; Nickrent 2020). De las cuatro, tres especies se encuentran distribuidas en diferentes estados de la república mexicana. *Bdallophytum americanum*, *B. andrieuxii* y *B. oxylepis* son las especies con distribución en México y son parásitas de raíces de especies del género *Bursera*. Se caracterizan por tener inflorescencias unisexuales, aunque *B. oxylepis* también presenta flores bisexuales (Martínez-Camilo *et al.* 2012)

Objetivos

Objetivos generales

- Conocer la morfología floral de las especies de *Bdallophytum.*
- Estudiar la embriología en las tres especies del género *Bdallophytum.*
- Estudiar la biología de la polinización de *Bdallophytum andrieuxii* y *B. oxylepis*.

Objetivos particulares

- Conocer a los visitantes florales de las especies de *Bdallophytum*
- Explorar las recompensas florales
- Describir la biología floral de las tres especies
- Conocer la morfoanatomía de inflorescencias y flores de las tres especies de *Bdallophytum*
- Describir la morfogénesis de flores unisexuales y bisexuales
- Describir la micro y megasporogénesis y la micro y megagametogénesis de las especies de *Bdallophytum*

Organización de la tesis

Capítulo 1. Manuscrito que se enviará a la revista *Biological Reviews* para su publicación: Floral traits and pollination in holoparasitic angiosperms.

Se abordaron los atributos florales de las plantas holoparásitas en relación con la polinización. Entre los atributos florales estudiados se encuentran los sistemas sexuales, los atrayentes y las recompensas florales de las holoparásitas. Asimismo, mediante una revisión exhaustiva sobre los trabajos de polinización que existen hasta el momento para holoparásitas, se construyó una red de interacciones para conocer los patrones de polinización en este grupo de plantas.

Capítulo 2. Artículo publicado: Rios-Carrasco, S., de Jesús-Celestino, L., Ortega-González, P. F., Mandujano, M. C., Hernández-Najarro, F., y Vázquez-Santana, S. (2022). The pollination of the gynomonoecious *Bdallophytum oxylepis* (Cytinaceae, Malvales). *Plant Species Biology*, *37*(1), 66-77.

Se estudió la polinización de *B. oxylepis* analizando la funcionalidad de los diferentes morfos florales mediante tratamientos de polinización y observación del crecimiento de los tubos polínicos en las diferentes flores tratadas. También se realizaron observaciones directas a las inflorescencias para conocer a los polinizadores potenciales. Durante las observaciones se registró el comportamiento de los visitantes florales.

Capítulo 3. Artículo aceptado para publicación: Sandra Rios-Carrasco, César A. González-Martínez, Sonia Vázquez-Santana. Floral visitors of the holoparasite *Bdallophytum andrieuxii*: A new report of brood-site pollination and thermogenesis for Cytinaceae (Malvales). *Brazilian Journal of Botany* (aceptado el 13 de junio de 2022).

Se describió la biología floral y la polinización en una población de *B. andrieuxii*. Se estudió la duración de la receptividad estigmática y la viabilidad de polen. Asimismo, se exploró si las flores de *B. andrieuxii* exhiben termogénesis mediante la toma de temperatura de flores femeninas, masculinas y se comparó con la temperatura ambiente. También se realizaron observaciones directas para conocer los visitantes florales y los polinizadores potenciales.

Capítulo 4. Manuscrito sometido a la revista *Journal of Plant Research* para su publicación: Sandra Rios-Carrasco, Morayna F. Gutiérrez-Luna, Daniel Sánchez, Pactli F. Ortega-González, Manuel Edday

Farfán-Beltrán, María C. Mandujano and Sonia Vázquez-Santana. The floral biology and the role of staminal connective appendages during pollination of the endoparasite *Bdallophytum americanum*

Mediante observaciones directas y el monitoreo con cámaras trampa, se describió la polinización en una población de *B. americanum* cuyas flores no producen néctar. Se puso a prueba la función de los apéndices estaminales del tejido conectivo, característica única de dicha especie en el género. Se comparó la frecuencia de visita de los polinizadores en inflorescencias con flores mutiladas (sin apéndices estaminales) y en inflorescencias no manipuladas (control) para conocer el papel de los apéndices estaminales en la polinización.

Capítulo 5. Artículo publicado: Sandra Rios-Carrasco y Sonia Vázquez-Santana. 2021. Comparative morphology and ontogenetic patterns of *Bdallophytum* species (Cytinaceae, Malvales): insight into the biology of an endoparasitic genus. *Botany, 99*:221–238. Este manuscrito representa el artículo de requisito de acuerdo con los lineamientos del Posgrado en Ciencias Biológicas de la UNAM para la obtención de grado.

Se estudió el desarrollo floral de las tres especies mexicanas de *Bdallophytum*. Se abordó la morfoanatomía, la organogénesis y la embriología floral de las especies. Se utilizaron técnicas de microscopía electrónica de barrido y de histología vegetal.

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Capítulo 1

Biología reproductiva de plantas holoparásitas

(Floral traits and pollination in holoparasitic angiosperms)

Manuscrito para ser enviado a la revista *Biological Reviews*

Floral traits and pollination in holoparasitic angiosperms

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ABSTRACT

In holoparasitic angiosperms, individuals lack a visible vegetative body (except *Cuscuta*), thus, reproduction depends entirely on floral attraction patterns. Given the importance of flowers in holoparasites, this work is focused on holoparasites pollination in relation with their floral characteristics. The stemless holoparasites comprises 364 species distributed in 11 angiosperm families. The flowers are unisexual in seven holoparasitic groups. Besides, most of them exhibit multisensory signals as attractants for pollinators based on visual, olfactory, and thermal signals. The interaction network shows flies, beetles, and hymenopterans (bees and wasps) as the floral visitors with greater relevance in holoparasite pollination.

Key words: animal-plant interactions, brood-site pollination, dichogamy, endoparasites, floral scents, floral rewards, pollination networks, specialised pollination, thermogenesis

CONTENTS

I. INTRODUCTION

Parasitic plants are those that obtain their resources from host plants (Musselman & Press, 1995; Heide-Jørgensen, 2008; Westwood *et al*., 2010) and they are classified depending on some physiological characteristics, the organ that parasitizes either root or stem, and their photosynthetic capacity (Těšitel, 2016). Those that still photosynthesize are hemiparasites, while those that lack photosynthetic activity are holoparasites (Heide-Jørgensen, 2008; Westwood *et al*., 2010). In a general way, holoparasites have a reduced vegetative body that can be underground or as small as a cluster of cells that live inside the host known as endoparasites (Teixeira-Costa, Davis & Ceccantini, 2021). The exception is *Cuscuta* (Convolvulaceae) a genus with hemi- and holoparasitic species with a well-developed vegetative body with climbing growth stem and reduced leaves (McNeal *et al*., 2007). Leaving *Cuscuta* aside, the rest of the holoparasites emerge from their hosts only during the flowering stage and their vegetative body resides in the host or is buried; thus, aerial stems are not observed, but the flowers, later fruits, are the unique exposed structures (Heide-Jørgensen, 2008; Těšitel, 2016; Teixeira-Costa, Davis & Ceccantini, 2021). Although flowers or inflorescences can be recognized as the only aerial structures, some holoparasites have associated structures as peduncles, pedicels, bracts, and bracteoles (Nais, 2001; Medina & Cruz, 2016).

Flowers are the most important organs for the maintenance of populations through seed production and to introduce genetic variation within populations (Barrett, 2010; Harder & Barrett, 2006; Barrett & Harder, 2017). Thus, the floral traits play an important role in defining the way in which reproduction is carried out and is directly related to pollination (Barrett, 2002; Endress, 2010). The pollination might be mediated by strategies that some flowers exhibit to promote outcrossing and decreasing the self-pollination rate (Barrett, 1998, 2002, 2011). These strategies lie in the spatial (herkogamy, monoecy, dioecy) and temporal (dichogamy) separation of sexes and floral attractants like advertisements and rewards (Simpson & Neff, 1981; Barrett, 2002; Schiestl & Johnson, 2013). The set of floral traits that allows inferring the pollinator type is known as pollination syndrome (Faegri & van der Pijl, 1979; Fenster *et al*., 2004; Rosas-Guerrero *et al*., 2014). However, it is necessary to corroborate the plant-pollinator interactions by carrying out fieldwork.

Floral traits in holoparasites have received less attention (Heide-Jørgensen, 2008) and in most holoparasitic groups, the existence of ecological patterns related to pollination is unknown. Therefore, in this review, we gathered all the information which is known so far about holoparasites without an aerial vegetative body in relation to 1) floral traits, 2) floral strategies involved in holoparasite reproduction, and 3) pollination systems.

II. STEMLESS HOLOPARASITIC ANGIOSPERMS

In the present work a total of 365 holoparasitic angiosperms without aerial stem that belongs to 48 genera and 11 families are listed (Table 1, methods in appendix 1). Where most of the families are exclusively holoparasites, but Orobanchaceae have autotrophic, hemiparasite and holoparasite members (McNeal *et al*., 2013); while Loranthaceae have mainly hemiparasitic species except for *Tristerix aphyllus* (Medel *et al*., 2002).

Of all stemless holoparasites, 93.06% are root holoparasites, while the rest are stem parasites. Thus, the interactions must depend on the site of flower exposition like the ground level or more elevated above the ground, where flowers can be the target of different interacting agents (Faegri & Van Der Pijl, 1979). Stem holoparasites are mainly represented by the family Apodanthaceae (*Apodanthes* and *Pilostyles*) in which all their members are on the stems of their hosts (Bellot & Renner, 2014). Another stem holoparasitic species is *Cytinus baronii* (Cytinaceae), and some species of *Rafflesia* (Rafflesiaceae) have been found parasitizing the lianas of their hosts of the genus *Tetrastigma* (Vitaceae) (Lexington, 1997). Although the host attachment site provides a particular type of exposure to flowers for interacting agents, other floral traits such as the sexual systems, floral advertisements and rewards can determine the animal-plant interactions in these plants.

III. DIVERSITY OF SEXUAL STRATEGIES

(1) Diversity of sexual systems

The flowers of holoparasitic plants are predominantly bisexual (Fig. 1A). However, if the Orobanchaceae family (which represents more than half of species) is excluded, it turns out that the flowers of the remaining families are predominantly unisexual. This bias is also reflected in the sexual systems (Fig. 1B) where hermaphroditic systems appear to be the most predominant amongst holoparasitic plants (67.58%), however, this percentage is once again owed to the Orobanchaceae family that represents the 61.54% of all holoparasitic plants. This family belongs to the order Lamiales, of which all its members present hermaphroditic systems, and so far, have not registered any modification in sexual systems within the order (Bellot & Renner, 2013). Thus, despite the holoparasitic nature of Orobanchaceae, there is a resistance to maintaining the floral characteristics of the Lamiales given the phylogenetic history of the group (Hansen & Orzack, 2005).

Leaving Orobanchaceae aside, stemless holoparasites exhibit dioecy and monoecy with their variants, and the sexual organs or unisexual flowers are in a wide different arrangements Dioecy is the most frequent sexual system for the groups that are purely holoparasitic, followed by monoecy. Moreover, hermaphroditism is found in Hydnoraceae, Mitrastemonaceae, and in some populations of *Rhizanthes lowii* and *R. zippellii* of Rafflesiaceae (Bänziger, Lamb & Kocyan, 2007). There are also species that represent other uncommon sexual systems such as trimonoecy in Cynomoriaceae (Villar, 2007), gynomonoecy in *Bdallophytum oxylepis* (Cytinaceae) (Rios-Carrasco & Vázquez-Santana, 2022), and andromonoecy in *B. caesareum* (formerly *Sanguisuga caesarea*, Cytinaceae) (Fernández-Alonso & Cuadros-Villalobos, 2012; Christenhusz, Faym & Byng, 2018).

One of the possible explanations for the high incidence of dioecious holoparasites could be associated with the availability of pollinators which move the pollen from one plant to another, as well as with the perennial life cycle of the plants, given that dioecy is found to be mainly represented by perennial plants (Bellot & Renner, 2013). On the contrary, the low recurrence of sexual systems such as gynomonoecy and andromonoecy can indicate an evolutionary transition from one sexual system to another through the path of a monoecious system (Barrett, 2002). To corroborate both hypotheses, ecological and genetic studies on each group is required, as well as analysing them in robust phylogenies at the family level, in those groups that have different sexual systems.

(2) Dichogamy

There are mechanisms besides unisexuality that involve the separation of the sexes (Barrett, 2002). Dichogamy is a mechanism in which sexual separation is given in a temporary manner (Lloyd & Webb, 1986; Dai & Galloway, 2011). Protogyny, where the appearance of the receptive stigmas is in advance of the release of pollen, has been reported in Hydnoraceae and in some species of Balanophoraceae (*Ditepalanthus malagasicus*, *Helosis* spp. and *Lophophytum mirabile*) and Cytinaceae (*Cytinus hypocistis*) (Musselman & Visser, 1989; Seymour, Maas & Bolin, 2009; de Vega *et al*., 2009; Kuijt & Hansen, 2015). Protandry, where the grains of pollen are released before the stigmas are receptive, has been reported in the Mitrastemonaceae and in some Balanophoraceae species such as *Balanophora fungosa* (Meijer & Veldkamp, 1993). Generally, the information on the presence of dichogamy or herkogamy (spatial separation of sexes) in holoparasitic plants is unknown, thus, it cannot be generalised as a strategy for holoparasites.

(3) Cleistogamy

Cleistogamy is the most extreme case of self-pollination where the flowers never open giving place to formation of seeds by obligate self-fertilisation (Lord, 1981). So far there is only a record of three cleistogamous species within stemless holoparasites. Two of them are in Orobanchaceae, the first is *Epifagus virginiana*, where most of flowers within the inflorescence are cleistogamous (Abbate & Campbell, 2013). The second is *Orobanche lavandulacea* where cleistogamy is occasionally (Foley, 2001). The third is *Pilostyles maya*, a recently described species belonging to Apodanthaceae, a predominantly unisexual family; this species is the unique hermaphroditic species within the family and all flowers are cleistogamous (Ortega-González *et al*., 2020).

IV. FLORAL ADVERTISEMENTS

Floral attributes are of great importance to accomplish sexual reproduction (Willmer, 2011). As such, they present characteristics that allow pollination to be conducted, whether it be through biotic or abiotic vectors, which in turn are found under selection pressures exercised by pollinators that direct their evolution (van der Pijl, 1961; Galen, 1999; Harder & Barrett, 2006). Some floral characteristics are related to the attraction of visitors, and there are different mechanisms which depend on the sensorial capacity of the pollinators (Schiestl & Johnson, 2013). The most common attraction mechanisms are visual and olfactory signals, such as the size and shape of the flower, the floral spread, the colour, and the floral scents (Faegri & van der Pijl, 1979; Harder & Barrett, 2006; Leonard, Dornhaus & Papaj, 2011). The sexual reproduction of holoparasitic plants, given their vegetative limitations and the high incidence of unisexuality in the species, depend completely on these attraction mechanisms to attract floral visitors that transport the pollen from male to female flowers (Bellot & Renner, 2013) or, in the case of hermaphrodites, in order to promote outcrossing and increase variation within the populations (Barrett, 1998). The attraction mechanisms reported for holoparasitic plants are visual signals such as floral size, the colour and consistency of the perianth, nectar guides, landing platforms, olfactory signals such as floral scents, and thermal signals (Spaethe, Tautz & Chittka, 2001, López-Sáez, Catalán & Saez, 2002, Davis *et al*., 2007, Seymour, Maas & Bolin, 2009).

(1) Flower appearance and floral size

Flower appearance can be an important attractant regarding the floral spread. The majority of holoparasites exhibit their flowers in inflorescences (92%). In these cases, the visual attraction would depend not only on size, but also floral aggregation, colour, shape, and presence of other eye-catching structures such as the bracts or bracteoles of inflorescences (Leonard, Dornhaus & Papaj, 2011). In inflorescences, the fidelity of the vectors that transport pollen is maintained via a larger number of flowers which reap the rewards. Additionally, pollination and the subsequent seed formation through geitonogamy (pollen movement from one flower to another from the same plant) are encouraged (Schoen & Dubuc, 1990).

On the other hand, solitary flowers are present in Apodanthaceae, Hydnoraceae, Mitrastemonaceae, Rafflesiaceae and in *Aphyllon uniflorum* (Orobanchaceae) representing the 20% of all holoparasites here studied. Solitary flowers can have other strategies as visual attractants. Floral size, be it individual or joint, constitutes a visual attraction mechanism (Delph, Galloway & Stanton, 1996; Spaethe, Tautz & Chittka, 2001). The most extreme case in angiosperms, regarding the flower size found in holoparasites, are in Rafflesiaceae where the distinct example is *Rafflesia arnoldii* (Davis *et al*., 2007). Although evolution in said family has been studied, it is not known exactly whether a relationship exists between floral size and pollinators (Davis, 2008). One of the hypotheses regarding the evolution of floral size in

Rafflesiaceae suggests that the big flowers could be related to pollination by carrion flies (Davis, Endress & Baum, 2008, Bendiksby *et al*., 2010), given that these pollinate the biggest flowers or inflorescences within the angiosperms (Barkman *et al*., 2008).

In contrast to Rafflesiaceae, the Apodanthaceae family presents the smallest solitary flowers of holoparasites (González & Pabón-Mora, 2014a, b). Size has not been related to pollination in this family either. Although Apodanthaceae have solitary flowers, these grow gregariously (González & Pabón-Mora, 2014a), and the visual signal for the pollinators can be given by the floral spread.

(2) Floral colour

Colour of flowers is a visual signal for pollinators and is one of the attributes that are considered in the pollination syndromes (Faegri & van der Pijl, 1979; Fenster *et al*., 2004; Leonard, Dornhaus & Papaj, 2011). In the case of holoparasitic plants a tendency towards reddish colours is shown (Fig. 1C), that range from bright red (colour group I), typical of pollination by birds, and dark reds (colour group V), typical of flowers with sapromyophily (pollination by carrion flies). Amongst the families that exhibit flowers with a dark red colour are those of Rafflesiaceae. However, in this family olfactory signals play an important role in the attracting pollinators as discussed later.

Some floral colours that also stand out amongst holoparasites are yellow, bright, or pale colours, and those that have a purplish-pinkish-bluish colour scheme (colour group IV). The latter is only found in Orobanchaceae and Lennoaceae. There are some species whose flowers do not share any of these listed colour palettes which exhibit a combination of various colours. An example is Apodanthaceae whose flowers are mainly dark-coloured outside, while inside are white- or creamy-coloured. In these cases, the outer signals outstand, although both colours can attract different groups of pollinators depending on the behaviour of each one (Elam *et al.*, 1998).

Pollination by birds is given only to those flowers with a bright red colour such as *Cytinus sanguineus* (Cytinaceae) and *Hyobanche sanguinea* (Orobanchaceae), which allows the assumption that colour is an important visual stimulant to attract pollinators, at least for birds. Of the species pollinated by birds, *Cytinus sanguineus* presents flowers with diluted nectar and in great quantities, which is characteristic of the ornithophilous syndrome (Hobbhahn & Johnson, 2015).

(3) Nectar guides and landing platforms.

Another visual signal of great importance are the nectar guides and landing platforms. The first ones are a visual stimulus that indicate where the rewards are to pollinators, reduce the handling time, and increase the visitation rate (Leonard & Papaj, 2011). While the landing platforms are as they name said, are a space given by petals, tepals, bracts, or inflorescences that allow the pollinators to land and rest while foraging (Reyes, Sauquet & Nadot, 2016). In holoparasites, Lennoaceae species (pers. Obs.) and the majority of Orobanchaceae species have nectar guides (Piwowarczyk & Kasińska, 2017). However, there are no studies that explore the role of these visual signals in pollination.

Otherwise, she species *Cistanche pelyphaea*, *Orobanche pinorum* and *Harveya purpurea,* are examples of flowers that present a landing platform in addition to nectar guides (Ellis, Taylor & Harrod, 1999; Piwowarczyk *et al*., 2016). These characteristics are also related to a pollination syndrome of psicophily (pollination by butterflies), melittophily (pollination by bees) and myiophily (pollination by flies) (Faegri & van der Pijl, 1979), however, pollination studies that corroborate this, are required.

(4) Floral scents

Floral scents are of great importance as attraction mechanisms (Dudareva & Pichersky, 2006). Smell can be the first signal that pollinators sense, and once these animals detect where it is coming from, they direct themselves towards the visual stimulus (Leonard, Dornhaus & Papaj, 2011). The type of floral visitor that arrives to flowers depends on the type of scent emitted as an advertisement of the presence of floral rewards, a site to breed of feed (Dudareva & Pichersky, 2006). Among holoparasites, the most obvious case is in flowers with fetid scents whose pollination syndrome fits well with pollination by carrion flies (Faegri & van der Pijl, 1979). So far, the production of a scent as an olfactory stimulus has been reported in 30 species of holoparasitic plants, of which *Sarcophyte sanguinea* (Balanophoraceae) (López-Sáez, Catalán & Saez, 2002), *Bdallophytum americanum* (Cytinaceae) (García-Franco & Rico-Gray, 1997), *Hydnora africana*, *H. esculenta*, *Prosopanche americana* (Hydnoroideae) and Rafflesiaceae flowers have decaying-proteins fetid scents (Bänziger, 1991, 1996; Patiño, Grace & Bänziger, 2000; Bänziger, Lamb & Kocyan, 2007; Bolin, Maas & Musselman, 2009, Seymour, Maas & Bolin, 2009).

Otherwise, sweet scents are less common present in *Pilostyles* (Apodanthaceae) (Sipes *et al*., 2014), *Balanophora* (Balanophoraceae) (Vislobokov & Galinskaya, 2018), *Cytinus* (Cytinaceae) (Hobbhahn & Johnson, 2015) and in *Harveya* (Orobanchaceae) (Randle, 2006). Although fetid scents are the most frequent, other peculiar scents such as ‘spicy’ are reported in *Harveya capensis* (Randle, 2006), ‘musky’ (bitter, penetrating, and oily) in *Hyobanche* *atropurpurea* (Wolfe & Randle, 2001), ‘mothballs’ in *Lophophytum mirabile* (Kawakita & Kato, 2002) and ‘smell of dampness’ in *Balanophora fungosa* (Irvine & Armstrong, 1991).

(5) Thermal signals

The production of heat that certain flowers emit is considered a type of stimulus that pollinators are capable of detecting (Leonard, Dornhaus & Papaj, 2011). Some holoparasites produce this type of stimulus through the means of thermogenesis or homeothermy in flowers. This phenomenon has been described in species of the Hydnoraceae and Rafflesiaceae. In *Hydnora* and *Prosopanche*, thermogenesis stimulates the release of volatile compounds through osmophores, which means that in this family the flowers exhibit multisensory signals which increase the probability of attracting specific floral visitors that respond to olfactory, visual, and thermal stimuli (Bolin, Maas & Musselman, 2009, 2011; Seymour, Maas & Bolin, 2009; Bolin, Lupton & Musselman, 2018). In the case of the Rafflesiaceae species, in addition to being a signal to attract pollinators, homeothermy is also considered as a reward which provides thermal energy or heat to their pollinators (Patiño, Grace & Bänziger, 2000; Bänziger, Lamb & Kocyan, 2007). This can be reflected in prolonged visits, increasing the probability of accomplishing pollination in an efficient manner (Leonard, Dornhaus & Papaj, 2011).

V. FLORAL REWARDS

(1) Nectar

As another attraction mechanism, floral rewards are important to maintain consistency in the visits of pollinators (Grindeland, Sletvold & Ims, 2005; Molina-Montenegro & Cavieres, 2006). The rewards that are known for holoparasitic plants include nectar, pollen, and thermogenesis (discussed above). Nectar is presented as a main reward in the Balanophoraceae, Cytinaceae, Mitrastemonaceae, Mystropetalaceae, and Orobanchaceae families. The nectar presentation is different for each species, for example, in *Balanophora abbreviata* the nectar is only found in female flowers, whilst *B. harlandii* and *B. indica* present extrafloral nectar (Vislobokov & Galinskaya, 2018). Species such as *Cytinus sanguineus* and *C. visseri* produce nectar in great quantities, although with a low concentration of sugar (Johnson *et al*., 2011; Hobbhahn & Johnson, 2015). Contrary to *Cytinus*, in *Bdallophytum americanum* (Cytinaceae) the lack of pollen as a reward in female flowers is compensated for by a higher sugar concentration in nectar in comparison with the male flowers, whose main reward is pollen (García-Franco & Rico-Gray, 1997). In Apodanthaceae, the presence of liquid is described, and although it is presumed that this probably involves nectar, this has not been confirmed (Sipes *et al*., 2014).

In some cases, the nectar, in addition to being a reward, represent a defence mechanism against robbers, having the characteristic of being alkaline and repellent. This kind of nectar is produced by the flowers of *Lathraea clandestine* (Orobanchaceae) which reach a pH of up to 11.5 and is only tolerated by pollinating bumblebees of the genus *Bombus* (Prŷs-Jones & Willmer, 1992).

(2) Pollen

The pollen grain is the male gamete of angiosperms, and a reward potentially collectable and edible by almost any animal (Wilmer, 2011). In some holoparasites, the pollen is a reward in combination with nectar as in *Balanophora abbreviata* (Balanophoraceae), *Bdallophytum americanum*, *Cytinus hypocistis* (Cytinaceae), *Pilostyles aethiopica*, *P. haussknechtii*, and *P. thurberi* (Apodanthaceae). Otherwise, the pollen is the main reward in the absence of nectar in *B. oxylepis* (Cytinaceae) and *Conopholis alpina* (Orobanchaceae). In both cases pollination is carried out by bees, a specialised group of pollinators in collecting pollen (Willmer 2011).

The pollen of *B. abbreviata* has the particularity of being sticky (Govindappa & Shivamurthy, 1975; Suetsugu & Aoyama, 2014). This means that not only is it a resource that pollinators enjoy, but it also can easily adhere itself to the pollinator. In this way, although the pollinators consume great quantities of pollen, can stick to their bodies, and instantly upon visiting the female flowers and contacting the stigmas, pollination can take place.

(4) Brood sites

Another recurring phenomenon in Balanophoraceae and Rafflesiaceae is that pollination is carried out by beetles and flies that oviposit on flower or inflorescence structures (Beaman, Decker & Beaman, 1988). Whilst these insects perform the service of efficient pollination, the plants provide their pollinators with a site for oviposition and reliable food for their offspring (Kawakita & Kato, 2002; Sakai 2002; Goto, Yamakoshi & Matsuzawa, 2011; Vislobokov & Galinskaya, 2018). Although the species of the Balanophoraceae and Rafflesiaceae families provide breeding grounds and oviposition to pollinators (Bänziger, 1991; Bänziger, 1996; Nikolov & Davis, 2017), species of the Hydnoraceae family have imitation breeding grounds which favour pollination by deception (Bolin, Maas & Musselman, 2009). The brood-pollination is closely related to pollination by carrion insects, whose flowers mimic dead bodies or decaying materials (Urru, Stensmyr, & Hansson, 2011). The brood-site as reward is present in species with specialised pollination (Sakai 2002) and Hydnoraceae and Rafflesiaceae fit into this pattern of specialised pollination with flowers that emit multi-sensorial advertisements for beetles and flies respectively. Pollination patterns are discussed deeper in the following section.

VI. POLLINATION

Few studies on pollination exist regarding holoparasites and much of the knowledge about pollination is from sightings that have been carried out during the description of the species or during the carrying out of other work. The study of pollination among this kind of plant is complicated since finding its study populations depends completely on finding its flowers. In another way it is extremely difficult to locate them given that the vegetative part is found within the host in the case of endoparasites (Těšitel, 2016, Teixeira-Costa, Davis & Ceccantini, 2021), or buried in the case of the other holoparasites. With the search for floral visitors a network was constructed, composed of 17 functional groups of floral visitors, and 64 species of plants (studies of pollination until 2021), counting to a total of 135 interactions (Fig. 2). The connectance value of the network ($C = 0.124$) indicates high specialisation given that interactions are occurring between a reduced number of species or functional groups (Jordano, 1987). Combined with the connectance value in the force of interaction or dependency chart (Fig. 3), it is shown that most interactions are concentrated asymmetrically towards the low values of d^A (dependency of animals to plants). Consequently, it can be inferred that the dependency of the animals on the plants is low, whilst some of the interactions are found to have high values of d^P (dependency of plants to animals). Accordingly, in this case, the plants have a high dependency on the floral visitors, thus there is not a reciprocal dependency (Jordano 1987). This is reflected in the module matrix (Fig. 4) where the group in modules indicates that 45% of the interactions fall into specific modules ($Qb = 0.45$). Among the modules, three functional groups stand out indicating specialisation in pollination. For example, the fly module shows great relevance in the pollination of Rafflesiaceae, *Balanophora abbreviata, B. elongata* (Balanophoraceae) and *Pilostyles haussknechtii*. The beetle module shows up for 14 species, represented in their majority by species of Hydnoraceae. And finally, bees and wasps were grouped into a single module which contains 13 species of plants, mainly represented by Apodanthaceae. The rest of the modules are formed by two or more functional groups indicating a less specialised interaction.

VII. CONCLUSIONS

Unisexuality is highly represented in merely holoparasitic groups, which reflects the obliged dependency of pollinators. Holoparasitic plants exhibit floral traits which strengthen the attraction of visitors through visual, olfactory, and thermal stimuli. A combination of those advertisements makes flowers highly attractive to specialised pollinators of families Balanophoraceae, Hydnoraceae, and Rafflesiaceae. Although some holoparasites offered nectar and pollen as rewards, some species with specialised pollination offer brood sites to pollinators, and heat by floral thermogenesis. The pollination network for holoparasites suggests that Rafflesiaceae, Hydnoraceae and Apodanthaceae are groups with highly specialised pollination by flies, beetles, and hymenopterans like bees and wasps, respectively. Thus, there are rare species that have the potential to be at risk in case the pollination networks break. To strengthen the patterns found on the pollination of holoparasites, more studies of biological interactions are required to understand the maintenance of the populations of this limited group of angiosperms. With this revision, new research questions come out related to the specialised pollination at group level, the evolution of sexual systems, and the evolution of floral characteristics related to animal-plant interactions in holoparasites.

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Figures

Figure 1. Sexuality and colour of flowers in holoparasite angiosperms. A. Percentage of bisexual and unisexual flowers across holoparasites. B. Diversity of sexual systems in holoparasites. C. Diversity of floral colour in holoparasites by colour group (described in methods). Am = andromonoecy, D= dioecy, Gm= gynomonoecy, H= hermaphroditism, M= monoecy, Pol= polymorphic (with more than one sexual system), Tm= trimonoecy, U= unknown.

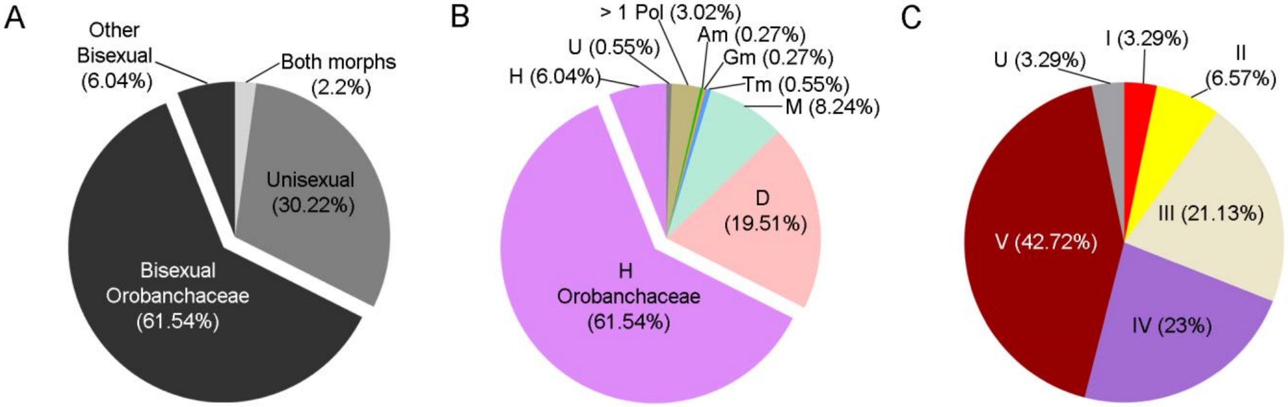

Figure 2. Pollination network in holoparasites.

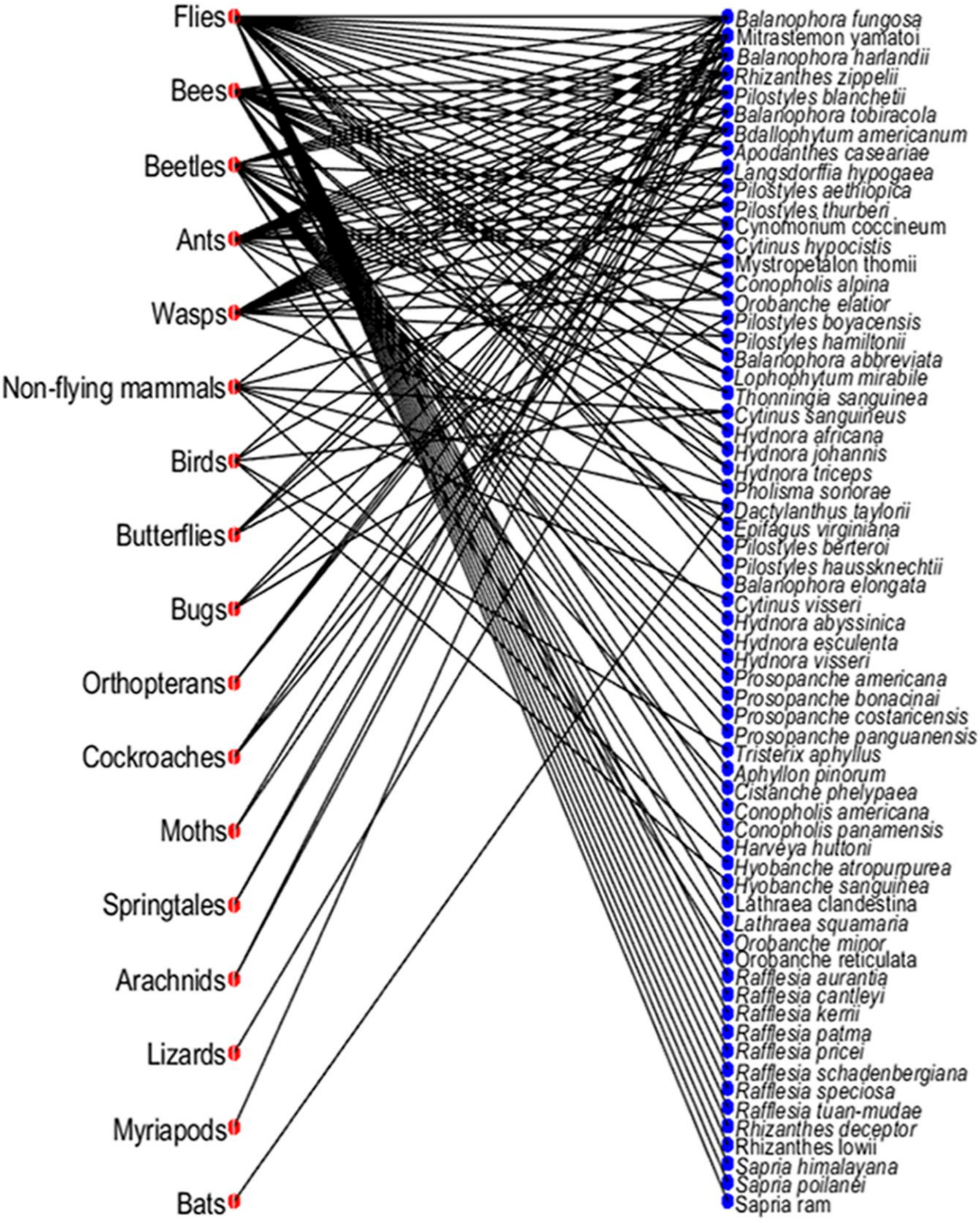

Figure 3. Force of interaction in the pollination of holoparasites.

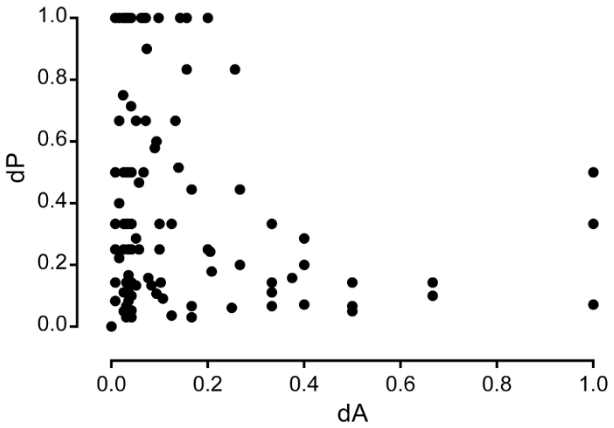

Figure 4. Module matrix showing specialised pollination by flies, beetles, bees, and wasps.

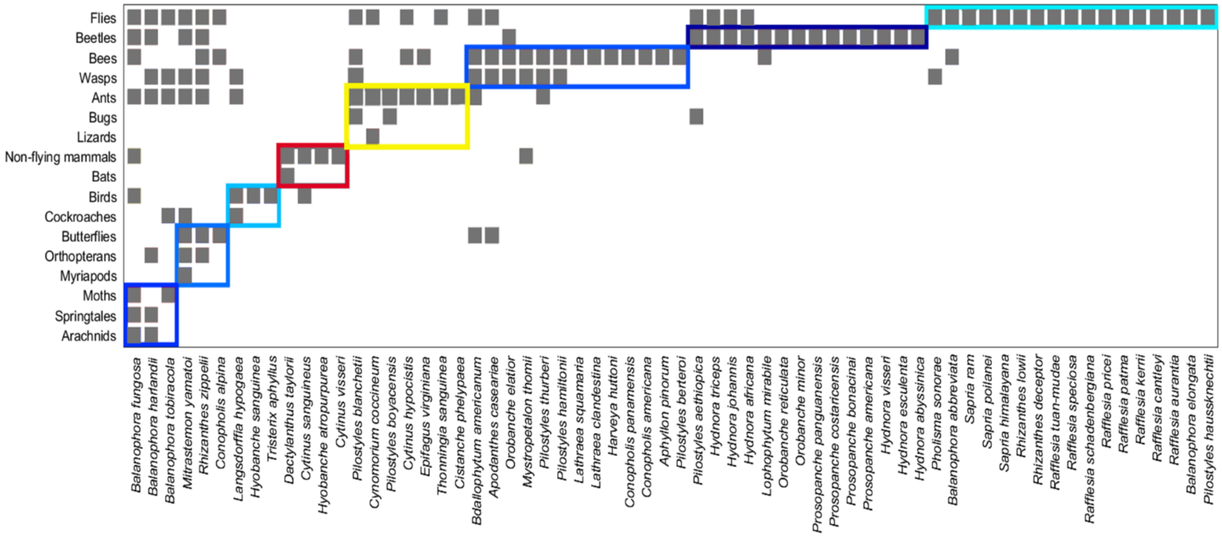

Table 1. Holoparasitic groups without aerial stems. Families in bold are those whose all members are holoparasitic.

Family (Order)	Genera	Number of Species
Apodanthaceae (Cucurbitales**)**	*Apodanthes*	1
	Pilostyles	11
Balanophoraceae (Santalales)	*Balanophora*	18
	Clamydophytum	1
	Corynaea	1
	Ditepalanthus	1
	Helosis	3
	Langsdorffia	4
	Lathrophytum	1
	Lophophytum	4
	Ombrophytum	6
	Rhopalocnemis	1
	Sarcophyte	1
	Scybalium	4
	Thonningia	1
Cynomoriaceae (Saxifragales)	*Cynomorium*	2
Cytinaceae (Malvales)	*Bdallophytum*	4
	Cytinus	8
Hydnoraceae (Piperales)	*Hydnora*	8
	Prosopanche	6
Lennoaceae (Boraginales)	*Lennoa*	1
	Pholisma	3
Loranthaceae (Santalales)	*Tristerix*	1
Mitrastemonaceae (Ericales)	*Mitrastemon*	2
Mystropetalaceae (Santalales)	*Dactylanthus*	1
	Hachettea	1
	Mystropetalon	1
Orobanchaceae (Lamiales)	*Aeginetia*	6
	Aphyllon	20
	Boschniakia	2
	Christisonia	22
	Cistanche	17
	Conopholis	3
	Epifagus	1
	Eremitilla	1 sp.
	Gleadovia	4 spp.
	Harveya	25 spp.
	Hyobanche	8 spp.
	Kopsiopsis	2 spp.
	Lathraea	4 spp.
	Mannagettaea	2 spp.
	Orobanche	88 spp.
	Phacellanthus	1 sp.
	Phelipanche	15 spp.
	Phelypaea	3 spp.
Rafflesiaceae (Malpighiaceae)	*Rafflesia*	36 spp.
	Rhizanthes	4 spp.
	Sapria	4 spp.

APPENDIX 1. METHODS

Database construction

We construct a database to gather the available information about the holoparasitic angiosperms known as far. Exhaustive research was carried out using information in local flora, specialized papers in taxonomy and ecology, and online resources (Supplementary material 1). We used Google Scholar using the following key words alone and combined: “holoparasites”, “pollination”, “parasitic plants”, “endoparasites” “flower ecology”. In addition, we also search by species name and its synonyms and family name to obtain all the necessary information for each group. The database has the following information for each species: order, family, name, parasite type (root or stem), floral sexuality (unisexual or bisexual), flower display (solitary flowers or inflorescences), sexual system monoecy, andromonoecy, dioecy, gynomonoecy, hermaphroditism, trimonoecy, and polymorphic), floral systems (herkogamy, dichogamy or cleistogamy), floral or inflorescence colour, floral scents and other advertisements, floral rewards, floral visitors. Floral and inflorescence colour section was completed with the help of original descriptions species and digital galleries as The Parasitic Plant Connection (https://parasiticplants.siu.edu), the “Botany Collections” from the Smithsonian National Museum of Natural History (https://collections.nmnh.si.edu/search/botany/) and the Global Biodiversity Information Facility (GBIF) digital gallery (https://www.gbif .org). The colours were clustered into five groups based on the pollination syndromes described by Faegri & van der Pijl (1979), I) orange and bright red, II) yellow, III) white to pale tones, IV) pink to bluish, V) burgundy to dark red tones (Fig. S1).

Finally, we did not consider *Cuscuta* (Convolvulaceae) because it has a conspicuous vegetative body in addition to the current debate if some species are truly holoparasitic as several species still are photosynthetic. The subspecies were omitted and to avoid synonymy and each species was corroborated with Tropicos (https://www.tropicos.org/), The Plant List (http://www.theplantlist.org/), and specialised taxonomic literature.

Data analysis

To evaluate the pollination in angiosperm holoparasites, a matrix was built with a total of 64 plant species (rows) and 17 functional groups of floral visitors (columns) classified according to their foraging behaviour (Faegri & Van der Pijl, 1979, Willmer, 2011). Each cell corresponds to the number of species per functional group interacting with a plant species. A bipartite network was constructed using the floral visitors and pollination information reported until now. The network consists of 64 species and 17 functional groups. To evaluate the network connectivity, and interaction modules we use the BiMat application for MATLAB R2019a software (Flores *et al.* 2016). To graph the interaction strength, we follow Jordano (1987).

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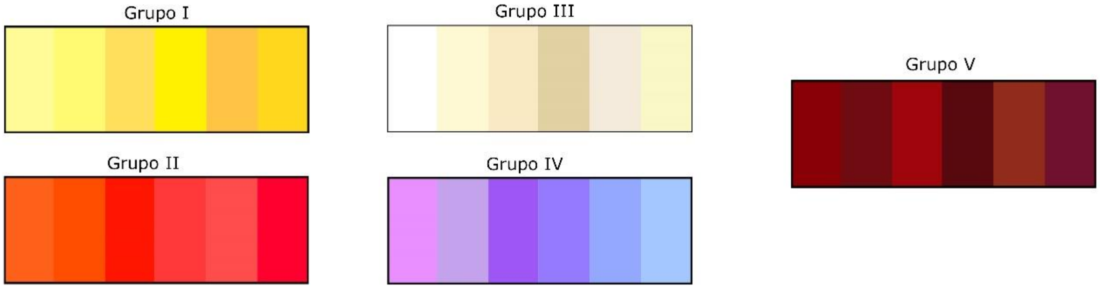

Fig. S1. Groups of colours of flowers present in holoparasites.

Capítulo 2

Biología de la polinización de *Bdallophytum oxylepis*

[The pollination of the gynomonoecious *Bdallophytum oxylepis* (Cytinaceae, Malvales)]

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ORIGINAL ARTICLE

PLANT SPECIES BIOLOGY WILEY

The pollination of the gynomonoecious *Bdallophytum oxylepis* (Cytinaceae, Malvales)

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Abstract

Bdallophytum oxylepis is a rare and endemic species belonging to the Cytinaceae family, a root holoparasitic plant in which most resources are allocated to attracting pollinators. This species is gynomonoecious with intra-individual variation in flower size and sex. Moreover, the flowers exhibit sapromyophilous traits, as do other species of *Bdallophytum*. Firstly, this study aimed to determine whether all floral morphs can form seeds and be pollen donors (in the case of bisexual flowers). Secondly, as this species has floral traits hypothesized to adapt to particular types of pollen vectors (carrion flies), we also studied the pollination of *B. oxylepis* to confirm whether the syndromes correspond to what occurs in nature. Through pollination treatments, we determined that all floral morphs are functional. By monitoring the inflorescences, we found that pollination is specialized in the studied population. Stingless bees performed pollination, as they have a high visitation rate, frequency, and constancy, and they are unique visitors that deposit pollen on the stigmas. Thus, they appear to be effective pollinators rather than carrion flies, as predicted by the syndrome. As shown here, animal–plant interaction studies can help establish a basis for conserving rare species such as holoparasites. Moreover, knowledge about the reproductive aspects of *B. oxylepis* reveals essential clues about its life cycle and role in maintaining native pollinators with economic and cultural value, such as stingless bees.

KEYWORDS
holoparasites, sapromyophily, specialized pollination, stingless bees, *Trigona fulviventris*

1 | INTRODUCTION

Holoparasites are parasitic plants that lack photosynthetic activity and have no aerial vegetative body, except for *Cuscuta* (Convolvulaceae), whose holoparasitic species have a pervasive external vegetative body (Heide-Jørgensen, 2008; McNeal et al., 2007). Holoparasites do not conform to a monophyletic group but have arisen independently 12 times along angiosperm evolution (Bromham et al., 2013; Westwood et al., 2010). Given the peculiar life cycle of holoparasites, it is of great interest to study the animal–plant interactions of these plants because their reproductive structures are their unique observable external characteristics (Blarer et al., 2004). Moreover, unisexuality is predominant in holoparasites (Bellot & Renner, 2013); thus, pollination is a crucial animal–plant interaction for maintaining holoparasite populations and their biodiversity (Ramos-Jiliberto et al., 2020).

Cytinaceae is a family whose members are all holoparasites (Těšitel, 2016). Cytinaceae, Apodanthaceae, and Mitrastemonaceae were formerly classified as Rafflesiaceae due to their parasitic lifestyle (Meijer, 1993). Nevertheless, phylogenetic studies have positioned the aforementioned families into different orders, leaving Cytinaceae as a member of Malvales (Nickrent, 2007). The family has approximately 12 species in two genera, *Cytinus* with eight species from Africa and the Mediterranean and *Bdallophytum* with four species from Mexico to Colombia (Alvarado-Cárdenas, 2009; Fernández-Alonso & Cuadros-Villalobos, 2012; Nickrent, 2020). In Cytinaceae, pollination has been studied mainly in *Cytinus*, where pollination is specialized, with different functional groups pollinating the different studied species, such as ants, birds, rodents, and shrews (de Vega et al., 2009; Hobbhahn & Johnson, 2015; Johnson et al., 2011).

Compared with *Cytinus*, *Bdallophytum* has received less attention. The genus *Bdallophytum* has four species: *B. americanum* from Mexico to Costa Rica (Alvarado-Cárdenas, 2009), *B. andrieuxii* and *B. oxylepis* endemic to Mexico (Villaseñor, 2016), and *B. caesareum* (formerly *Sanguisuga caesarea*) from Colombia (Fernández-Alonso & Cuadros-Villalobos, 2012). The method of pollination in this New World genus is only known for *B. americanum* (formerly *B. bambusarum*), where carrion flies accomplish pollination (García-Franco & Rico-Gray, 1997). The floral morphology is similar across the species of *Bdallophytum*. The perigone is dull to dark red and produces a fetid smell (Alvarado-Cárdenas, 2009). These traits fit a sapromyophilous pollination syndrome (Faegri & van der Pijl, 1979; Fenster et al., 2004). Other holoparasites belonging to the Balanophoraceae and Rafflesiaceae families, which also exhibit sapromyophilous traits, are also pollinated by carrion flies (García-Franco & Rico-Gray, 1997; Goto et al., 2012). However, in Balanophoraceae, there are sapromyophilous species whose floral visitors do not correspond to the syndrome, so it is important to make observations in natural populations to corroborate pollination (Quintero et al., 2017; Suetsugu & Aoyama, 2014).

As the sapromyophilous morphology is highly constant among the species of *Bdallophytum* (Alvarado-Cárdenas, 2009), we hypothesized that carrion flies could perform pollination in other *Bdallophytum* species. Of the three species of *Bdallophytum* found in Mexico, *B. oxylepis* is the least abundant, with few populations reported with records over 50 years old (Dirección General de Repositorios Universitarios, 2021). Moreover, many of these populations are threatened by intense habitat disturbance (Martínez-Camilo et al., 2012) caused by changes in land use and deforestation (S. Rios-Carrasco & P.F. Ortega-González, pers. obs.). *Bdallophytum oxylepis* was recently described as gynomonoecious, having bisexual and female flowers with intraindividual variation in flower size along the inflorescence axis (Rios-Carrasco & Vázquez-Santana, 2021). As populations of this endemic holoparasite are scarce, we seek to know whether all floral morphs are functional to inquire about their sexual system, incompatibility system, and pollination in a threatened natural population. Thus, the aims of this study are to (1) describe whether all floral morphs are functional, capable of forming seeds and being pollen donors (in the case of bisexual morphs); (2) describe the pollination system; and (3) identify the effective pollinator(s) in the studied population.

2 | MATERIALS AND METHODS

2.1 | Species and study site

B. oxylepis is a gynomonoecious root endoparasitic species of the *Bursera* (Burseraceae) trees. Inflorescences are spikes, and the flowers are arranged helically across the axis. The fieldwork was conducted in 2018 and 2019 during the short flowering period in May and fruiting from May to July. The study site is in Chiapas, Mexico, in the municipality of San Fernando at 16°55′45″ N, 93°10′50.6″ W; 1225 m asl. The study site corresponds to a remaining patch of a seasonally dry tropical forest of approximately 40 km^2 where Agavaceae, Anacardiaceae, Bromeliaceae, and Burseraceae species dominate. This location is near a crop field, a locality in which people routinely use fire to clear the vegetation and cultivate land. The species voucher was deposited at the Herbarium of the Instituto de Historia Natural de Chiapas (*Najarro 4684* CHIP).

Twelve parasitized *Bursera* trees were tagged and identified to species level. All the exophytes (inflorescences and infructescences) of *B. oxylepis* on *Bursera* roots were counted. We considered each exophyte as an individual of *B. oxylepis*. As *B. oxylepis* was recently described as gynomonoecious with bisexual and female flowers (Rios-Carrasco & Vázquez-Santana, 2021), we corroborated the sexual system in the studied population and counted and sexed all flowers of at least 50 randomly selected inflorescences of 10 different hosts. We monitored the population to determine the flowering and fruiting times, visiting once per week from April to August from 2017 to 2019, considering the presence or absence of inflorescences and infructescences. The obtained data were summarized in a graph, to which monthly rainfall records from 2017 in the studied area (CONAGUA: https://smn.conagua.gob.mx) were added to compare the parasite flowering time with the timing of the rainy season.

2.2 | Sexual functionality and mating system

As the inflorescences of *B. oxylepis* are gynomonoecious with intraindividual variation in flower size, we tested whether androecia and gynoecia from the different floral morphs are functional and capable of forming seeds. To standardize the use of flowers according to their sex and size, we used the classification of Rios-Carrasco and Vázquez-Santana (2021) to group the different floral morphs into four regions along the inflorescence: (I) undeveloped flowers (flowers at the base with tepals but with undeveloped sexual organs, 2–3 mm), (II) small bisexual flowers near the base (3–4 mm), (III) regular-sized bisexual flowers in the middle part (6–10 mm), and (IV) female flowers at the apex (2–4 mm). We performed 12 pollination treatments in May, during the flowering peak, crossing

TABLE 1 Male and female sexual functionality of *Bdallophytum oxylepis* in San Fernando, Chiapas during 2018 and 2019

Male functionality					**Female functionality**		
Type of mating (identifier) $n = 5$	**Pollen donor (floral region)**		**Receptive stigma (floral region)**	**PT style growth**	**Type of floral morph (floral region)**	**Total analyzed fruits ($n = 15$ infructescences)**	**Percentage of formed fruits**
Outcrossing (O1)	Small bisexual (II)	×	Small bisexual (II)	Yes	Small bisexual (II)	85	100%
Outcrossing (O2)	Small bisexual (II)	×	Regular bisexual (III)	Yes	Regular bisexual (III)	640	100%
Outcrossing (O3)	Small bisexual (II)	×	Female (IV)	Yes	Female (IV)	173	100%
Outcrossing (O4)	Regular bisexual (III)	×	Small bisexual (II)	Yes			
Outcrossing (O5)	Regular bisexual (III)	×	Regular bisexual (III)	Yes			
Outcrossing (O6)	Regular bisexual (III)	×	Female (IV)	Yes			
Geitonogamy (G1)	Small bisexual (II)	×	Regular bisexual (III)	Yes			
Geitonogamy (G2)	Small bisexual (II)	×	Female (IV)	Yes			
Geitonogamy (G3)	Regular bisexual (III)	×	Small bisexual (II)	Yes			
Geitonogamy (G4)	Regular bisexual (III)	×	Female (IV)	Yes			
Selfing (S1)	Small bisexual (II)	×	Small bisexual (II)	Yes			
Selfing (S2)	Regular bisexual (III)	×	Regular bisexual (III)	Yes			

Note: II, small bisexual flowers near the base (3–4 mm); III, regular-sized bisexual flowers in the middle part (6–10 mm); IV, female flowers at the apex (2–4 mm).

Abbreviation: PT, pollen tube.

TABLE 2 Visitation records, pollen loads on stigma, and pollen grains on visitors' body, by functional groups on flowers and inflorescences of *Bdallophytum oxylepis* in San Fernando, Chiapas during 2018 and 2019

Visitation traits	Bees	Ants	Flies
Visitation rate (per inflorescence/hour)	0.9 ± 0.34^{a}	0.09 ± 0.28^{b}	0.02 ± 0.03^{c}
Inflorescences visited per patch	2.74 ± 0.14	1	1.07 ± 0.26
Flowers visited per inflorescence	11.38 ± 1.15	Not registered	1.07 ± 0.07
Handling time per flower (seconds)	11.66 ± 1.13	Ephemeral	18.38 ± 4.26
Total number of records	1,046	107	21
Does the visitor touch both sexes of the flower?	Always	Rarely	Most of the times
Visitors captured (*n*)	23	3	2
Pollen grains on visitor body	14,410 ± 3093	6.66 ± 6.66	0
Stigmas collected after first visit (*n*)	11	5	4
Pollen load on stigma after first visit	842.5 ± 149.83	0	0

Note: Mean values ± *SEM*. Different superscripts denote the significance of the multiple comparison tests at $p < 0.05$.

different floral morphs from regions II, III, and IV to test whether pollen can germinate and advance from stigma to style. Simultaneously, the pollination treatments included outcrossing, geitonogamous, and selfing treatments that indicated the sexual system of *B. oxylepis* and whether this species exhibits an incompatibility system. The different pollination treatments are summarized in Table 1.

To perform the hand pollinations, we used 10 inflorescences and a total of 60 emasculated and labeled pre-anthetic flower buds. To perform outcrossing treatments, the pollen of flowers from different patches was mixed. For geitonogamous treatments, the pollen was collected from bisexual flowers of the same inflorescence, and for selfing treatments, the pollen was obtained from the same flower. After pollinations, the inflorescences were bagged to exclude foreign pollen (Kearns & Inouye, 1993). After 4 days, we collected and fixed all treated flowers in 70% ethanol for a minimum of 24 h. The styles and stigmas were softened with 10 M NaOH for 1 hour; the samples were then placed in decolorized aniline blue solution overnight. The samples were squashed on a slide to observe pollen tubes under fluorescence microscopy (LM microscope, Olympus Provis AX70 with a 5.1 MP digital camera, Evolution MP, Media Cybernetics).

To test whether female gametes can form seeds, we collected 15 mature infructescences to corroborate the development of fruits and the formation of seeds in all floral morphs. The seed number was discarded due to the small size of seeds, the concrescence of fruits, and the intraindividual variation.

2.3 | Visitation rate recording

We performed direct observations in four patches of 1 × 1 m, where each patch corresponded to one host with *B. oxylepis*. The observations were made during four consecutive days in May each year (2018 and 2019) for 48 inflorescences. Four direct observers registered the flower visitation rates in the diurnal schedule from 8:00

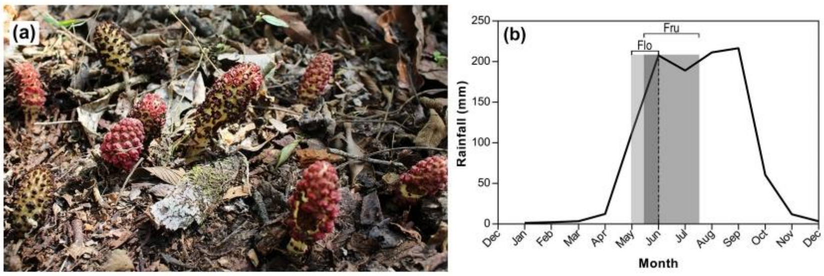

FIGURE 1 Flowering of *Bdallophytum oxylepis* at San Fernando, Chiapas. (a) Inflorescences of *B. oxylepis*. (b) Flowering (Flo) and fruiting (Fru) periods overlapping with the first rains in the studied area

a.m. to 4 p.m. (end time ranged from 3:30 p.m. to 4:30 p.m., depending on when rain appeared) in intervals of 15 min of observation and 15 min of rest. For security reasons, nighttime observations were made with four camera traps, one at each patch. The cameras were programmed to automatically detect movement in case of night visitors. Additionally, they were programmed to record 5 min every 15 min if the visitors were too small for the motion sensor to detect. The data obtained from direct observations and recordings were: the number of visitors per inflorescence, the number of flowers foraged per visit, the handling time in each flower and inflorescence, the number of inflorescences visited per visitor, and the foraging behavior of the visitors. Floral visitors were grouped in functional groups because their foraging behavior was similar (Fenster et al., 2004). These groups were stingless bees, flies, and ants. A generalized linear model (GLM) with a Poisson distribution and a log-linkage function was constructed using the package *stat* in the software R Core Team (2019) to determine differences in the frequency of visits among functional groups of flower visitors and between years of observation. The

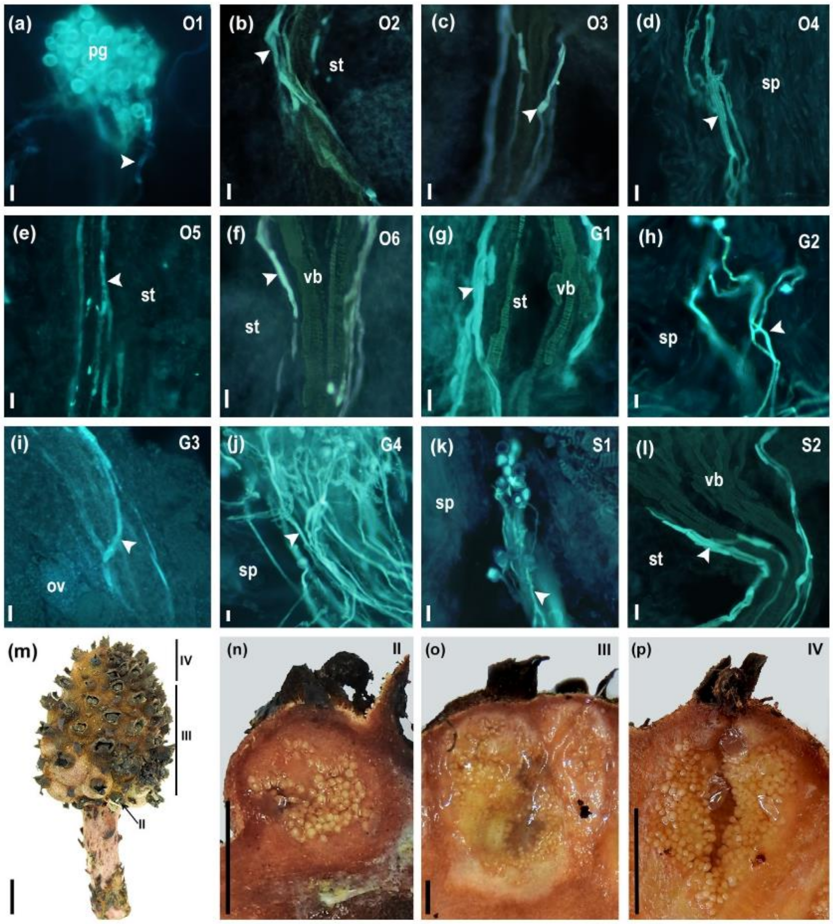

FIGURE 2 Male and female functionality of *Bdallophytum oxylepis* floral morphs. (a–l) Growing pollen tubes (arrowheads) from pollination treatments. (a–f) Outcrossing mates (O1–6). (g–j) Geitonogamous mates (G1–4). (k–l) Selfing mates (S1–2). (m) Mature infructescence showing the fruits from the different floral regions (roman numerals II-IV). (n) Fruit from small bisexual flower. (o) Fruit from regular-sized bisexual flower. (p) Fruit from female flower. The identifier on the upper right corner corresponds to the type of mate for pollination treatments (a–l), or to the region of flowers in the case of fruits (m–p). ov: ovule; pg: pollen grains; sp: stigmatic papillae; st: style; vb: vascular bundle. Bars a–l = 10 μm; m = 1 cm; n–p = 2 mm

frequency of visits was analyzed as the response variable, and the year of observation and the functional groups as predictor variables. The interaction between these two factors was analyzed. Differences in the visits between functional groups were determined by chi-square analysis. Multiple comparisons were made with the *ghlt* function (Hothorn et al., 2008) to determine the nature of these differences.

2.4 | Pollinator effectiveness and floral rewards

To evaluate the pollinator effectiveness, we considered the pollen loads and their placement on visitor bodies and stigmas (Gyan & Woodell, 1987; Ne'eman et al., 2010). Table 2 shows sample sizes of insects captured, and stigmas collected. During the observations, a sample of flower visitors was captured. These were sacrificed by using lethal chloroform chambers, they were then identified, and the pollen grains deposited on their bodies counted. Due to safety-related difficulties of accessing the site at night, the nocturnal visitors were not captured. The pollen was counted following the Gyan and Woodell (1987) method, with some modifications described as follows. Collected insects were immediately placed in 1.5-mL tubes and fixed in 70% ethanol to estimate the pollen load. In the laboratory, they were vortexed to release the pollen adhered to their bodies; the insects were then removed from the tube to identify them. Subsequently, the tubes were centrifuged at 10 000 rpm, the supernatant was discarded, and 100 μL of Schiff reagent was added to stain the pollen grains. Finally, 5 μL aliquots were taken to count the pollen grains in a Neubauer chamber. To evidence the placement of *B. oxylepis* pollen on visitor bodies, we used fluorescent dyes as pollen analogs on the androecia of five flowers in one inflorescence per patch. We used orange dye in one patch and green in another. Each collected insect was observed under a stereomicroscope with ultraviolet light to locate the areas of pollen adherence.

To measure the pollen grain load on stigmas, we bagged at least three inflorescences per patch with pre-anthetic buds to prevent pollination. When anthesis occurred, virgin flowers were labeled and observed. When the first visitor approached these flowers, the stigma was harvested and fixed in 70% ethanol to count the pollen deposited for that visitor, following the protocol described for insects. We compared these pollen samples with pollen from *B. oxylepis* anthers. As the number of ovules varies across flowers, and a single fruit is difficult to isolate, and due to the unpredictable presence of feeding larvae, it was difficult to count the seeds. Therefore, the efficiency was discarded as a parameter to compare the pollinators. Thus, pollen loads on stigmas were used to assess pollination success (Ne'eman et al., 2010).

3 | RESULTS

3.1 | *B. oxylepis* population

The inflorescences of *B. oxylepis* were found growing on the roots of *Bursera excelsa* (Figure 1a). Over the two sampling years, a total of 12 parasitized trees were found and labeled within a 10-km^2 area. The average number of exophytes of *B. oxylepis* per host was 16.43 ± 4.86 (mean ± *SEM*). During 2018 and 2019, *B. oxylepis* had a flowering peak in May. The flowering of *B. oxylepis* starts after the first rain (approximately in May), in the transition between the dry and rainy seasons (Figure 1b). The fruits were found from May until July. The fruits take 2–3 weeks to ripen, and infructescences can remain viable and attached to the host for up to 1 month after maturation. Inflorescences have approximately 50 ± 5.59 (mean ± *SD*) flowers, of which 43 ± 6.13 (mean ± *SD*) are bisexual, and 7.2 ± 2.12 (mean ± *SD*) are female; thus, this is a gynomonoecious population.

3.2 | Sexual functionality and mating system

The pollination treatments resulted in the germination of pollen grains and the growth of pollen tubes through the style in outcrossing (Figure 2a–f), geitonogamous (Figure 2g–j), and selfing (Figure 2k–l) matings (Table 1). Thus, the bisexual floral morphs have functional male

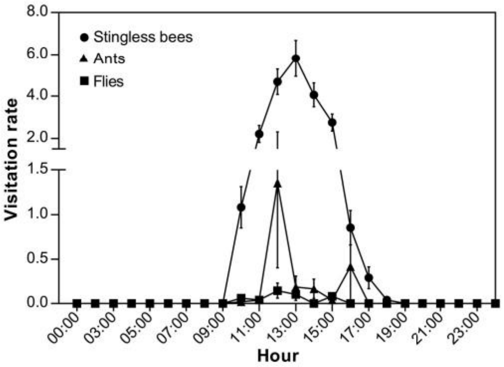

FIGURE 3 Visitation peaks of floral visitors per functional group in the 2 years of the study

FIGURE 4 Pollinators and floral visitors of *Bdallophytum oxylepis* during the flowering period of 2018 and 2019. (a–h) Stingless bee behavior. (a) *B. oxylepis* inflorescences visited by a stingless bee (arrow). (b) *Trigona fulviventris* (arrow) foraging female flowers at the apex of inflorescence. (c) *T. fulviventris* (arrow) visiting bisexual flowers. (d) Pollen baskets (arrows). (e) Pollen baskets with other floral tissues (arrows). (f) Florivory; bee destroying and depositing floral sexual organs on its pollen baskets (arrows). (g) Perigone and anthers (arrows) damaged. (h) Inflorescence with holes made by florivory (circle). (i) *Amiota* sp. (Drosophilidae) on the perigone. (j) *Camponotus sericeiventris* (Formicinae) touching flowers

gametes and functional stigmas, styles, and cell eggs in the female flowers. Additionally, gynoecia of all floral morphs are functional and capable of forming seeds; the collected infructescences developed all their fruits (Figure 2m, Table 1). Furthermore, fruits from female flowers (Figure 2n), regular-sized bisexual flowers (Figure 2o), and small bisexual flowers contained well-formed seeds (Figure 2p). Moreover, the flowers of *B. oxylepis* have no incompatibility system at the stigma or style level. Therefore, sexual reproduction of *B. oxylepis* can be performed by xenogamy, geitonogamy, or autogamy.

3.3 | Floral visitors of *B. oxylepis*

During diurnal observations, we recorded visits of stingless bees of the species *Trigona fulviventris* (Apidae: Meliponini), flies of the genera *Drosophila* sp. and *Amiota* sp. (Drosophilidae), and the ants *Camponotus sericeiventris* (Formicinae) and *Monomorium* sp. (Myrmicinae). The visitations were diurnal, between 9:00 a.m. and 5:00 p.m., and were highest at noon (Figure 3). No overnight visits were recorded. There were differences in the frequency of visits between years ($\chi^2 = 234.04$, $df = 1$, $p < 0.001$), with

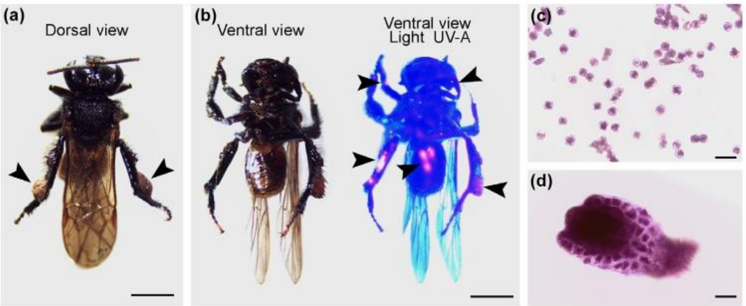

FIGURE 5 Sites of pollen deposition on *Trigona fulviventris* bodies. (a) Reward pollen on corbiculae (arrows). (b) Pollination pollen showed on a ventral view (arrows). (c) *Bdallophytum oxylepis* pollen on *T. fulviventris* removed from pollen baskets. (d) *B. oxylepis* ovule removed from pollen baskets. Bars a, b = 2 mm; c, d = 20 μm

more visits registered in 2019. We also found an interaction between years and functional groups ($\chi^2 = 249.78$, $df = 2$, $p < 0.001$) indicating that the frequency of visits among functional groups differed between years.

Floral visitors were classified into functional groups based on their behavior during foraging. These groups were stingless bees, flies, and ants, and their visitation rates differed ($\chi^2 = 1668.3$, $df = 2$, $p < 0.001$). The stingless bees were the most frequent visitors in both years (Table 2), although the visitation rate was higher in the second year of study (0.34 ± 0.02 in 2018 and 1.38 ± 0.09 in 2019; mean ± *SEM*). The foraging behavior of *T. fulviventris* stingless bees was characterized by landing directly at the anthers or stigma (Figure 4a). In one visit to an inflorescence, the stingless bees can visit female flowers (Figure 4b) and bisexual floral morphs (Figure 4c). They move among flowers, inflorescences, and patches, exhibiting high pollen-collecting activity (Figure 4d). In most visits, stingless bees consumed floral parts such as anthers, perigone, and inflorescence axes. Therefore, they exhibited florivorous behavior (Figure 4e–h). Both drosophilid fly species landed on the perigone and occasionally touched the sexual organs (Figure 4i); their visitation rate was less than that of bees (Table 2) and was similar during both years of study (0.008 ± 0.003 in 2018 and 0.02 ± 0.006 in 2019; mean ± *SEM*). Finally, the ant species roamed the inflorescences and accidentally touched the sexual organs in their path (Figure 4j). The frequency of ant visits decreased in the second year of observations (0.19 ± 0.06 in 2018 and 0.01 ± 0.003 in 2019; mean ± *SEM*), and no pollen was found on their bodies.

As the stingless bees maintain the highest visitation rate, invest more time per visit, carry more pollen on their bodies, and deposit large amounts of pollen on the stigma per visit, they are considered the most effective pollinator for *B. oxylepis* in the studied population (Table 2). Additionally, of the 23 stingless bees captured, 21 presented pollen exclusively from *B. oxylepis*, while the other two had pollen from different species in minimum amounts, demonstrating high fidelity to *B. oxylepis* flowers. The functional groups formed by flies and ants were discarded as effective pollinators in the studied populations because they did not deposit pollen on stigmas, and low numbers of visits were recorded in the 2 years of the study.

3.4 | Floral rewards

During anthesis, we perceived a protein-decaying smell from the open flowers. Moreover, we did not find nectar in the flowers. Therefore, the main reward was the pollen. We identified the deposition sites of reward and pollination pollen with the fluorescent dyes. Furthermore, finding both colors of fluorescent dyes on stingless bee bodies indicated an exchange of pollen from different patches at a maximum distance of 20 m. The pollen on corbiculae is mainly a reward (Figure 5a). The pollen on the femur and the ventral part of the abdomen is mostly for pollination (Figure 5b) because they are the body parts that primarily touch the sexual organs of the flowers. The pollen collected on corbiculae was almost exclusively from *B. oxylepis* (Figure 5c). Moreover, inflorescences and floral parts, such as ovules, were found in the pollen baskets (Figure 5d).

4 | DISCUSSION

4.1 | Sexual functionality and mating system

Intraindividual variation in flowers is a common trait in the Cytinaceae (Rios-Carrasco & Vázquez-Santana, 2021), and unique species with bisexual flowers are found in the genus *Bdallophytum*, such as *B. oxylepis* and *B. caesareum* (formerly *Sanguisuga caesarea*) (Fernández-Alonso &

Cuadros-Villalobos, 2012). So far, the absence of an incompatibility system in female and bisexual flowers has only been demonstrated in *B. oxylepis* (presented here). Despite intraindividual variation in *B. oxylepis* flowers, all floral morphs are functional and capable of forming seeds, just as all bisexual morphs can be pollen donors. Hence, the small flowers at the base and apex of inflorescences also contribute to the species' fitness. The absence of an incompatibility system may represent a reproductive assurance strategy (Parra-Tabla & Alonso, 2021) in this species with restricted distribution and threatened populations. In this case, the bees actively participate by moving pollen from one flower to another within the same inflorescence. Additionally, their florivorous behavior in breaking the anthers favors pollen deposition on the stigmas of the same flower. Otherwise, unisexual flowers represent an opportunity to introduce genetic variability if the pollen originates from different individuals (Barrett, 2002; Käfer et al., 2017). Stingless bees play an important role because they are highly active in foraging flowers from different inflorescences, even from different patches. Therefore, stingless bees can contribute to the production of seeds by autogamy, geitonogamy, and xenogamy in *B. oxylepis*, resulting in a mixed mating system (Goodwillie et al., 2005).

4.2 | Sapromyophily in *B. oxylepis*

The sapromyophilous syndrome is mainly characterized by the dark or dull color of flowers, enhanced by a fetid smell and the lack of nectar (Faegri & van der Pijl, 1979). Sapromyophilous flowers are reported in nonparasitic plants. Some examples are species of the families Araceae, Orchidaceae, and Apocynaceae (Chakraborty & Adhikary, 2018), where the pollinators are carrion flies of the families Calliphoridae, Muscidae, and Sarcophagidae (Moré et al., 2019). Holoparasites also include species with sapromyophilous traits, which are pollinated by carrion flies. These include the Rafflesiaceae species, pollinated mainly by calliphorid and sarcophagid flies (Bänziger, 1991, 1996; Bänziger & Pape, 2004) and *B. americanum* (Cytinaceae) that is pollinated by carrion flies of the family Sarcophagidae (García-Franco & Rico-Gray, 1997).

Our study shows that flies of the family Drosophilidae visit sapromyophilous flowers of *B. oxylepis*; however, they are not regarded as pollinators in the studied population, as they do not efficiently transport pollen and are not carrion flies. However, the flowers are also visited by stingless bees of *T. fulviventris*. Unlike flies, they are effective pollinators, as they carry more conspecific pollen of *B. oxylepis* on their bodies and are the unique functional group that deposits pollen on the stigmas. Moreover, the behavior of stingless bees differs from that of the flies because they are the only ones that show a constant foraging pattern. Thus, the pollination syndrome in the studied population of *B. oxylepis* does not reflect the pollinators as in other holoparasites mentioned previously.

As in *B. oxylepis*, the holoparasite species *Thonningia sanguinea* (Balanophoraceae) was described as sapromyophilous, and studies corroborated pollination by muscid and calliphorid flies (Goto et al., 2012). However, further work has demonstrated that sunbirds seem to play an even more essential role in pollination, and the flowers are not sapromyophilous as they produce diluted nectar and do not have a fetid smell (Quintero et al., 2017). Therefore, it is crucial to understand plant–pollinator interactions and know flower attributes before making predictions based on pollination syndromes. Balanophoraceae also has species that are visited by bees. In particular, it has been reported that stingless bees of the genus *Melipona* visit the flowers of *Balanophora abbreviata*, searching for pollen and nectar, but the flowers are not sapromyophilous (Govindappa & Shivamurthy, 1975). *Balanophora fungosa* ssp. *indica* (Balanophoraceae) is another example of a sapromyophilous holoparasite that is visited by bees, as in *B. oxylepis*. Nevertheless, the bees are not confirmed as pollinators because they are only observed to collect pollen from male flowers (Suetsugu & Aoyama, 2014).

4.3 | Pollination by stingless bees

It has been reported that *T. fulviventris* and other meliponines are attracted to fetid smells and even collect feces (Oliveira & Morato, 2000; Vit et al., 2013). Moreover, aggressive behavior while foraging on flowers has been reported (Roubik et al., 2018). *T. fulviventris* bees destroy the anthers of some Melastomataceae species to obtain pollen (Renner, 1983); this behavior was also observed in most visits to *B. oxylepis* flowers.

The main floral attractant could be the protein-decaying smell emitted by flowers. As the flowering peak is highly pronounced, the scent could be concentrated. The floral display could also function as a visual attractant (Leonard et al., 2011). Although nectar is reported in flowers of *B. americanum* (García-Franco & Rico-Gray, 1997), *B. oxylepis* flowers do not produce nectar. Instead, bees collect pollen and other floral tissues. A previous study found that trichomes present in the perigone and the inflorescence axis are the glandular type and contain insoluble polysaccharides (Rios-Carrasco & Vázquez-Santana, 2021). Therefore, given the sugar content, the perigone could be interpreted as an attractant with nutritional value for bees (Simpson & Neff, 1981).

T. fulviventris is listed as one of the species used more frequently in meliponiculture and builds its hives on the ground (Vit et al., 2004). Thus, the inflorescences of *B. oxylepis* growing at ground level are an available resource that implies less investment in flight distances. Other studies reported that *T. fulviventris* from Guatemala visits diverse flowers from different families (Vit et al., 2013). Thus, the pollination of *B. oxylepis* depends entirely on a generalist pollinator in the study site. Despite the extensive diet reported for *T. fulviventris*, stingless bees show high fidelity to *B. oxylepis* flowers. The temporary fidelity is related to the scarcity of resources (Real, 1980), and *B. oxylepis* have their flowering peak in May during the short time between dry and rainy seasons in the area. At that moment, most of the floral resources from the dry season are depleted, and the floral resources from the rainy season are about to sprout (Cortés-Flores et al., 2017). This temporary fidelity is consistent with the foraging behavior reported for the stingless bees of the genus *Trigona*, where the constancy is higher, as the floral resources are less available (Slaa et al., 1998).

Regarding pollination in Cytinaceae, there are no trends for pollination by certain functional groups within the family. Specialized pollination occurs by ants in *Cytinus hypocistis* (de Vega et al., 2009), birds in *C. sanguineus* (Hobbhahn & Johnson, 2015), rodents and shrews in *C. visseri* (Johnson et al., 2011), flies in *B. americanum* (García-Franco & Rico-Gray, 1997), and, contrary to expectations, by stingless bees in *B. oxylepis*, as described here. Although we reported the expected visits of flies, stingless bees are the effective pollinators for *B. oxylepis*, as they have more pollination success. Different criteria can be used to determine if a pollinator is effective; however, the criterion that is considered a direct measure of pollination success is pollen deposition on the stigmas (Ne'eman et al., 2010). Thus, the stingless bees are unique in transferring pollen effectively to accomplish pollination. Moreover, in both quality (duration of visits, pollen loads on visitor bodies, contacting both sexual organs) and quantity (number of visits by time and frequency) of pollination effectiveness (Ne'eman et al., 2010), the stingless bees are conspicuous among the other visitors.

Just as crop fields surround *B. oxylepis*, other holoparasites such as *T. sanguinea*, *B. fungosa* (Balanophoraceae), and *Dactylanthus taylorii* (Mystropetalaceae) are at risk because they grow in disturbed areas, which directly affects their hosts (Ecroyd, 1996; Goto et al., 2012; Pierce & Ogle, 2017). As holoparasites are completely host dependent, habitat disturbances can affect the host survival and consequently that of the holoparasites. Given that *B. oxylepis* appears to be a temporary resource for the stingless bees in the studied area, the loss of habitat would trigger the loss of several links in the food chain (Hadley & Betts, 2012), starting with the host, followed by holoparasites, and consequently the loss of pollinators. Ultimately, changes in land use and the fires, as for the *B. oxylepis* habitat, cause the loss of native biodiversity in the area that could harm animal–plant interactions (Ramos-Jiliberto et al., 2020). Therefore, it is essential to understand aspects of reproductive biology, such as pollination, to begin protecting native and rare species such as holoparasites.

5 | CONCLUSIONS

The sexual functionality and pollination biology of floral morphs of *B. oxylepis* are described here. The species does not present an incompatibility system; thus, the formation of seeds can occur by autogamy, geitonogamy, and xenogamy due to the foraging patterns of stingless bees. Although floral characteristics fit a sapromyophilous syndrome, pollinators are stingless bees that take pollen and other floral tissues as rewards. Stingless bees are effective pollinators as they carry large amounts of pollen on their bodies and are the unique visitors that deposit pollen on stigmas. Accordingly, this holoparasitic species is an essential resource for native bees in times of scarcity. Although some studies report specialized pollination at the family level, it is necessary to continue studying holoparasitic plants because they are rare, have small populations, and exhibit interactions that may be relevant to maintaining economic and cultural species of biological importance, such as stingless bees.

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Capítulo 3

Biología de la polinización de *Bdallophytum andrieuxii*

(Floral visitors of the holoparasite *Bdallophytum andrieuxii*: A new report of brood-site pollination and thermogenesis for Cytinaceae)

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Original Article

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Floral visitors of the holoparasite *Bdallophytum andrieuxii* Eichler: A new report of brood-site pollination and thermogenesis for Cytinaceae (Malvales)

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Abstract

Bdallophytum andrieuxii is an endemic dioecious holoparasitic species belonging to Cytinaceae. Given the background of specialized pollination for the family and the genus, we explored the floral visitors of *B. andrieuxii*, describing the flower ecology of this saprophilous species. We found that flowers display thermogenesis, as their temperature was above the air temperature. Flowers produce nectar only at noon, in similar quantities and concentrations among both female and male inflorescences. Floral visitors belong to four functional groups: ants, carrion flies, flies, and butterflies. Their visitation peaks coincide with the moment of nectar secretion. Butterflies were the most common visitors, foraging male and female inflorescences, always touching sexual organs. We observed oviposition behavior and found eggs on floral tissues after butterfly visits. Lepidopteran larvae were found in all analyzed infructescences, feeding on floral and fruit tissues. Despite the larvae appearing to develop parallel to fruits and seeds, these insects did not affect the fruit or seed development. We conclude that *B. andrieuxii* have brood-site pollination by butterflies of genus *Cissia* in the studied population. The flowers display traits related to this pollination type: dark-colored flowers, a rotten-fruit smell, and thermogenesis.

Keywords: Butterflies; Carrion pollination; *Cissia*; Endoparasites; Saprophily

1 Introduction

Carrion pollination is performed by various animals that are attracted to flowers that mimic decaying material (Faegri and Van der Pijl 1979; Jürgens et al. 2015). This pollination type has been associated with sapromyophily, a pollination syndrome in which carrion flies are the primary pollinators attracted by the carrion-mimicking flowers (Van der Pijl 1961; Faegri and Van der Pijl 1979). However, carrion pollination is not carried out exclusively by carrion flies but also by dung beetles and even stingless bees (Jürgens et al. 2015; Rios-Carrasco et al. 2022).

"Saprophily" instead of "sapromyophily" is a more general term that describes the syndrome in which flowers or inflorescences mimic dead bodies or decaying material as a strategy to attract copro-, necro-, or saprophagous insects (Jürgens et al. 2015). Saprophilous flowers have been described in 22 unrelated families of Angiosperms, indicating a convergent evolution of carrion pollination (García-Franco and Rico-Gray 1997; Jürgens et al. 2015; Rios-Carrasco et al. 2022). These flowers or inflorescences are usually dark-colored and characterized by unpleasant scents (Willmer 2011); some of these flowers also present rewards like nectar, heat (thermogenesis), and brood sites to pollinators (Jürgens et al. 2013; 2015).

Thermogenesis occurs when flowers increase their temperature above the air temperature (Johnson and Jürgens 2010; Zhu et al. 2011). Thermogenesis in flowers has been reported in cycads (Terry et al. 2004) and so far in 12 angiosperm families (Ito-Inaba et al. 2009; Seymour et al. 2009). The functional role of thermogenesis has been related to emanating volatile organic compounds (VOCs) that are linked to pollination (Terry et al.

2004). The unpleasant scent, the color of the flowers, and thermogenesis all maximize the signals to attract carrion pollinators by mimicking decaying food, rotting materials (including fruits), urine, dung, and brood sites (Kite et al. 1998; Küchmeister et al. 1998; Seymour et al. 2009; Johnson and Jürgens 2010; Jürgens et al. 2013).

Brood-site pollination is carried out by animals that oviposit on different floral parts, and their larvae develop on flowers after pollination occurs (Sakai 2002). This specialized pollination type is performed mainly by flies or beetles attracted to the strong fetid smell of flowers (Urru et al. 2011; Borges 2016). Although some authors consider brood-site pollination as a type of deceit pollination in which plants do not offer rewards (Urru et al. 2011), the breeding sites provided by floral tissues are rewards, in addition to the food and protection afforded to larvae (Sakai 2002).

Brood-site pollination has been documented for some holoparasites, a non-photosynthetic group of plants that obtain their resources from other plants (Heide-Jørgensen 2008). This type of pollination is registered in the holoparasites of the families Hydnoraceae (Bolin et al. 2009), Rafflesiaceae (Bänziger 1996; Patiño et al. 2000), and Balanophoraceae (Kawakita and Kato 2002; Goto et al. 2012). Flowers of those families are dark-colored and emit a decaying-protein smell, and species such as *Hydnora africana* Thunb. (Hydnoraceae) and *Rhizanthes lowii* (Becc.) Harms (Rafflesiaceae) incur carrion pollination by dung beetles and carrion flies, respectively (Patiño et al. 2000; Seymour et al. 2009).

Cytinaceae is an endoparasitic family. This term means that the vegetative body of the plant grows inside the host and is called an endophyte, while the exophyte, the outside part of the plant, is consists only of flowers or inflorescences (Thorogood et al. 2021). Cytinaceae include species with carrion flowers in the genus *Bdallophytum* Eichler (García-

Franco and Rico-Gray 1997; Rios-Carrasco et al. 2022). The genus *Bdallophytum* has four species in the American continent (Nickrent 2020), characterized as having dark-red flowers that emit a yeasty smell (García-Franco and Rico-Gray 1997; Meijer 1997; Rios-Carrasco et al. 2022). To date, the specialized pollination of *Bdallophytum americanum* (R.Br.) Eichler ex Solms and *B. oxylepis* (B.L.Rob.) Harms has been reported, with *B. americanum* being pollinated by carrion flies (García-Franco and Rico-Gray 1997) and *B. oxylepis* by stingless bees (Rios-Carrasco et al. 2022). Thermogenesis had not been explored for the family, despite the saprophilous traits of *Bdallophytum* species.

Given the background of the specialized carrion pollination of *Bdallophytum* species, we investigated the floral visitors of *B. andrieuxii*, a dioecious species endemic to Mexico. To date, there has been no information about the biotic interactions of *B. andrieuxii* or about the biology of its flowers. As the flowers have some saprophilous traits, we expected a carrion pollinator for the studied population. We also examined whether the flowers exhibited thermogenesis, as reported for other holoparasites with similar characteristics.

2 Material and methods

Study species and site – *Bdallophytum andrieuxii* is a dioecious species, in which flowers grow in spike inflorescences at ground level, given its root parasitic habit. The fieldwork was carried out in June 2021 in a population in the municipality of Santo Domingo Tonalá, Oaxaca, Mexico, within a protected area, namely the Flora and Fauna Protection Area "El Boquerón de Tonalá." The vegetation is a seasonally dry tropical forest, and *B. andrieuxii* parasitizes the roots of *Bursera xochipalensis* Rzed., a dominant species in the region.

***Bdallophytum andrieuxii* population** – We explored the El Boquerón de Tonalá area to register the number of hosts with parasites in an area of approximately 4 km^2, corresponding to the reachable area within the site. We marked the hosts, counted the exophytes (emerged inflorescences or infructescences), and sexed them. We report the mean and the standard error of the mean (SEM) of exophytes per host and the number of flowers per inflorescence. To determine whether the population is sex-biased, we sexed a total of 107 exophytes emerging from 11 hosts. We then compared the number of female and male exophytes through a generalized linear model (GLM) with a Poisson distribution and a log-linkage function, taking the number of exophytes as a response variable and sex of the inflorescences as an explanatory variable. We performed a chi-square test to determine the differences between the sexes, using the software R (R Core Team 2020).

Flower temperature – We measured the temperature of 30 *B. andrieuxii* male and female flowers every hour with an infrared thermometer (PC868 T-168) and a digital needle thermometer (1.5 V LR44). We pointed the infrared thermometer to the entire flower, and the needle thermometer was placed into the flowers (between the column and the perigone). We also measured the environmental temperature every hour. The data were transformed to a natural logarithmic scale to obtain equal variances to perform the Kruskar Wallis test; the aim was to determine if there were differences between the temperature of female and male flowers and the environment. A Wilcoxon post hoc multiple comparison test was performed to determine the nature of the differences, using R.

Nectar production – We monitored 30 flowers per sex, each hour from 9:00 to 16:00 h, seeking the presence of nectar. We used microcapillary tubes of 10 µl to collect and measure the amount of nectar when it was present. We also measured the nectar concentration using a hand refractometer. After corroborating the normality of data, we analyzed the differences in the amount and concentration of nectar between the sexes, using the *t* Student statistic in R.

Pollen viability and stigmatic receptivity – To determine when pollination occurred, we examined pollen viability and stigma receptivity over time. We used 90 male flowers to measure the pollen viability from the first until the sixth day of anthesis, at the rate of 15 flowers per day. Pollen samples were collected from different anthers with a dissecting needle; then, they were deposited on a slide with a drop of Alexander's reagent to detect if the pollen was viable. We calculated the percentage of viable pollen per sample (Alexander 1980).

To test stigmatic receptivity, we bagged 10 female inflorescences with preanthetic floral buds to avoid pollen contamination. We used 15 naïve flowers per day from days one to four. We placed a drop of hydrogen peroxide on the stigma to determine receptivity (Galen and Plowright 1987).

Floral visitors – We monitored two clusters per sex and five inflorescences per cluster, for four consecutive days. Direct observations were made at 15-minute intervals in a diurnal schedule from 9:00 to 16:00 h. In addition, we monitored the clusters during the night using camera traps, with the motion sensor activated from 16:00 to 9:00 h of the next day. We registered any visitors and recorded the frequency (total number) of visits per flower sex per hour. Visitors were classified into functional groups (ants, butterflies, carrion flies, and flies) according to their selection of specific floral traits (Fenster et al. 2004; Dellinger 2020).

Differences between the functional groups were analyzed under a GLM negative binomial, given the nature of the data, taking the frequency of visits as a response variable and the functional groups and sex of the inflorescences as explanatory variables. The MASS package and the *glm.bm* function were used for the analysis (Venables and Ripley 2002). The model and the binomial distribution are the right choice to analyze the data as the dispersion parameter is less than 1. Differences between the functional groups were obtained by chi-square analysis, and multiple post hoc comparisons were made with the *ghlt* function (Hothorn et al. 2008) in R. Detailed results about GLM and the formula are provided in Supplementary material S1.

We considered potential pollinators that visited both male and female flowers and touched sexual organs. To corroborate the pollination efficiency (Ne'eman et al. 2010), we chose 15 female inflorescences with preanthetic buds and bagged them to avoid pollen arrival. After the opening of flowers, we unbagged the inflorescences to expose them to visitors. We rebagged them after the first visit to count the formed fruits per inflorescence, per visitor type. We also collected 15 infructescences to determine the natural pollination efficiency. We compared the results with the number of flowers per female inflorescence (n = 30).

Brood-site pollination – During the fieldwork, we observed oviposition behavior by butterfly floral visitors. After a butterfly's visit, we explored the flowers to find eggs to determine whether these insects carried out pollination by brood-site. Additionally, we dissected 15 developing infructescences to look for larvae.

3 Results

Bdallophytum andrieuxii population – We registered 11 hosts of the species *Bursera xochipalensis* in the 4-km^2 study area. Six of them were infested only by *B. andrieuxii* female exophytes, and five hosts had only male exophytes. There was no difference between the number of female and male exophytes ($\chi^2 = 0.5$, $df = 1$, $p < 0.471$, Table 1).

Flower temperature and nectar production – The temperature differed between flowers and the environment ($\chi^2 = 112.25$, $df = 2$, $p < 0.001$), but it was similar between male and female flowers (Fig. 1a). Flowers produced nectar at noon (12:00–13:00 h). Early in the morning and after 13:00 h, the flowers of both sexes were observed without nectar. Both female and male flowers produced similar amounts of nectar (Student *t*-test = 0.1.33, df = 59, $p = 0.188$, Table 1). Regarding concentration, there were no differences between the sexes (Student *t*-test = 1.21, $df = 59$, $p = 0.23$, Table 1).

An additional advertisement detected in the *B. andrieuxii* population was the strong rotting-fruit smell. It was easily detectable to the human nose when approaching a cluster in male and female inflorescences. The smell was perceived in flowers throughout the direct observation schedule (9:00–16:00 h).

Pollen viability and stigmatic receptivity – The viability of pollen grains was maintained at a maximum in the first three days. However, viability fell sharply, almost by half, from the fourth day onward. On the sixth day, the pollen was no longer viable (Fig. 1b). The stigmatic receptivity lasted only three days.

Floral visitors – The visits were only diurnal, with a visitation peak at noon. Four functional groups were recorded: ants, carrion flies, flies, and butterflies (Fig. 1c, Table 2). There were no differences in the frequency of visits between floral sexes ($\chi^2 = 3.76$, $df = 1$, $p < 0.052$). We found differences in the frequency of visits between functional groups ($\chi^2 = 46.65$, $df = 3$, $p < 0.001$).

The most common visitors were butterflies of the genus *Cissia* Doubleday (Nymphalidae), and their visits were maintained throughout the day in both floral sexes (Fig. 1c). *Cissia* sp. always touched the sexual organs of male and female flowers and is capable to spend more than 15 minutes on a single inflorescence, foraging for different flowers (Fig. 2a, b, Table 2). Once butterflies land on flowers, they search for nectar (see video in Supplementary material S2).

Ants of the genus *Atta* Fabricius were registered only consuming floral parts (Fig. 2c, d). Carrion flies of the genus *Musca* Linnaeus (Muscidae) visited both floral sexes and occasionally touched the sexual organs; they spent less than one minute per inflorescence (Fig. 2e, f, Table 2). Flies (Drosophilidae) were more common visitors than were carrion flies (Fig. 2g) but spent more time resting on the perigone (> 30 minutes). Flies visited both male and female flowers but only accidentally touched the sexual organs. Hence, carrion flies and butterflies were potential pollinators in the studied population, but the latter were most frequent (Table 2).

Pollination success – *Cissia* sp. only visited the unbagged naïve inflorescences; they formed 11.9 ± 0.973 (mean ± SEM) fruits on a single visit, or approximately 18.46% of all flowers. In addition, we registered the visits of *Cissia* sp. in all observed clusters. Female inflorescences had 65 ± 1.83 (mean ± SEM) flowers, and the number of fruits formed naturally by each inflorescence was 26.5 ± 4.53 (mean ± SEM), representing approximately 40.7%.

Brood-site pollination – The foraging behavior of butterflies was characterized by always touching the sexual organs and ovipositing. By checking the inflorescences, we found eggs on reproductive structures (Fig. 3a) and frass around the perigone. Lepidopteran larvae were found in all dissected infructescences, with 5.93 ± 0.62 (mean ± SEM) larvae in each (Fig. 3b, c). In addition, we found well-developed fruits and seeds, which meant the larvae did not affect the seed or fruit development (Fig. 3d). We found holes in mature fruits, but not lepidopteran adults, and the holes were empty; therefore, the holes made by larvae could be the way out of adult butterflies.

4 Discussion

Thermogenesis and floral rewards – The temperature of flowers was higher than the environment, indicating thermogenesis in *B. andrieuxii* flowers (Zhu et al. 2011). Thermogenesis is reported for different angiosperm families (Ito-Inaba et al. 2009; Seymour et al. 2009) and even in cycads (Terry et al. 2004). Nevertheless, when thermogenesis is combined with other floral traits such as dark-colored flowers and the emitting of fetid smells, flowers are classified into the saprophily pollination syndrome (Faegri and Van der Pijl 1979; Willmer 2011; Jürgens et al. 2015). This syndrome is common in Araceae, Apocynaceae, Aristolochiaceae, and some holoparasites of the families Hydnoraceae and Rafflesiaceae (Van der Pijl 1961; Chakraborty and Adhikary 2018).

Thermogenesis is responsible for the emission of VOCs in flowers as an attraction mechanism (Zhu et al. 2011). In *Rhizanthes lowii* (Rafflesiaceae), the increased flower temperature allows the release of VOCs, which produce the fetid smell that attracts flies, the pollinators (Patiño et al. 2000). In *B. andrieuxii*, the flower temperature increases along with the environment, but the flowers always maintain their temperature as being higher than that of the air by at least a degree. Although the chemical characteristics of the floral smell of *B. oxylepis* are unknown, here we describe that it can be perceived throughout the day. Further studies are required to demonstrate a relationship between the flower temperature and the emission of the rotten-fruit scent.

Regarding floral rewards, thermogenesis is considered a type of reward for pollinators, as the heat of flowers provides the pollinators with the energy to continue flying around and foraging flowers (Seymour et al. 2003). When floral visitors take advantage of this heat as a resource, they often remain inside flowers for long periods while resting, feeding, or reproducing (Seymour and Matthews 2006). In *B. andrieuxii,* the butterflies spend long periods foraging for flowers. Fruit flies spend more time merely resting on the perigone of flowers, indicating that they can take advantage of the heat without pollinating.

Butterflies appear to benefit from the nectar, another reward observed in *B. andrieuxii* flowers. Nectar is not typically included in sapromyophilous species; however, some saprophilous species pollinated by carrion flies or dung beetles do offer nectar to pollinators (Jürgens et al. 2015). The nectar in *B. andrieuxii* was produced only briefly at noon, and this period matched the peak of pollination when butterflies were mainly observed foraging both floral morphs. In addition, butterflies were observed licking the nectar of flowers. The nectar, the heat, and the rotten-fruit smell render the flowers of *B. andrieuxii* a set of multisensory signals that favor pollination (Raguso 2004; Leonard et al. 2011). These characteristics play an essential role in attracting pollinators.

Floral visitors and pollination – Butterfly-pollinated flowers are reported here for the first time in a Cytinaceae species. So far, the pollination has been studied in six species of Cytinaceae (half of the total species), including *B. andrieuxii* here presented. All the studied species display specialized pollination. The three studied species of *Cytinus* L., *Cytinus hypocistis* (L.) L., *C. sanguineus* (Thunb.) Fourc., and *C. visseri* Burgoyne are pollinated by ants (de Vega et al. 2009), birds (Hobbhahn and Johnson 2015), and rodents and shrews (Johnson et al. 2011), respectively. In contrast, the studied *Bdallophytum* species are pollinated by carrion flies in *B. americanum* (formerly *B. bambusarum* (Liebm.) Harms) (García-Franco and Rico-Gray 1997), stingless bees in *B. oxylepis* (Rios-Carrasco et al. 2022), and butterflies in *B. andrieuxii* here presented.

Although *B. andrieuxii* is a saprophilous species characterized by dark-colored flowers and a strong rotten-fruit smell just as the other species of the genus (García-Franco and Rico-Gray 1997; Rios-Carrasco et al. 2022), in the study site it was pollinated by butterflies. However, butterflies of the genus *Cissia* have been reported to display carrion behavior as they are attracted by decaying material (Zacca et al. 2018). Thus, *B. andrieuxii* employ carrion pollination. This pattern is also observed in *B. oxylepis*, which is pollinated by stingless bees, an unexpected pollinator but with reports of carrion behavior (Jürgens et al. 2015; Rios-Carrasco et al. 2022). Although pollination occurs by unexpected functional groups of insects, saprophily in the species of *Bdallophytum* seems to take advantage of the behavior of carrion insects to perform pollination.

Pollination in the studied area was not as effective as in other species of the genus, such as *B. oxylepis,* where pollinators ensure the formation of all fruits within an inflorescence (Rios-Carrasco et al. 2022). The poor fruit production could be due to the florivorous behavior of ants, as they removed the perigone, the style, and stigma, thus preventing pollination. Despite that, butterflies appeared to be effective pollinators; they visited both inflorescences and their presence was registered in all studied patches. At each visit, they ensured the formation of one-fifth of the fruits of an inflorescence. Therefore, if the flowers received at least five visits, all the fruits could be formed.

Brood-site pollination – Brood-site pollination is a novel pollination type for Cytinaceae and the genus *Bdallophytum*. This pollination is reported for different angiosperms, and the most studied cases are the yucca-yucca mutualism (Althoff 2016) and the pollination of figs by wasps (Yu et al. 2018). In both cases, the oviposition occurs on flowers, and the larvae develop parallel to fruit formation. A similar phenomenon was observed in *B. andrieuxii,* where the most common visitors carried out pollination but oviposited on flowers. Afterwards, the larvae continue growing, feeding on flowers, and developing fruits to survive, without affecting the seed formation. In *B. americanum* (formerly *B. bambusarum*), moth larvae fed on floral tissue; however, they were reported as florivorous that affected fruit formation (García-Franco and Rico-Gray 1997).

Brood-site pollination has been reported for other holoparasites with carrion pollinators. In *H. africana*, the carrion beetles are attracted by the fetid smell and oviposit in the chambers of flowers (Bolin et al. 2009). In *Rhizanthes zippelii* (Blume) Spach*,* the pollination is performed by carrion flies, which oviposit on floral tissues where the larvae feed and develop (Bänziger 1996). *Lophophytum mirabile* Schott & Endl. (Balanophoraceae) is pollinated by pyralid moths, which leave their offspring on inflorescences (Borchsenius and Olesen 1990). In comparison, *Thonningia sanguinea* Vahl is pollinated by flies whose larvae feed on male inflorescences (Goto et al. 2012). Butterflies of the genus *Cissia* are added as holoparasite pollinators by brood-site, along with the aforementioned functional groups, beetles, flies, and moths.

Brood-site pollination is a specialized interaction with a strong plant–pollinator relationship (Urru et al. 2011). Although this interaction is mutualistic because the pollination service is exchanged for the breeding site (Sakai 2002), it can also negatively affect plants and insects. Plants with brood-site pollination where the oviposition is performed on gynoecium are at risk of losing their ovules or seeds (Sakai 2002). While developing, the larvae compete for food and space, causing a decrease in larval development (Althoff 2016). In *B. andrieuxii*, the butterfly larvae grow inside the infructescences, leaving channels between the fruits, but the fruits and seeds can nonetheless conclude their development.

Brood-site pollination in the studied population of *B. andrieuxii* was confirmed, as butterflies were the most efficient pollinators. In addition, all visited inflorescences had eggs on their flowers, and all infructescences displayed feeding larvae. It is evident that in *B. andrieuxii,* brood-site pollination favors seed formation and the feeding of *Cassia* sp. butterflies during this parasitic flowering period.

Bdallophytum andrieuxii is a rare endemic species to Mexico. This paper provides the first report of brood-site pollination for a holoparasite of the family Cytinaceae. Thermogenesis is also reported for the first time for the genus *Bdallophytum*. Although the flowers appear to be a saprophilous syndrome, the primary pollinators in the studied area were butterflies, which oviposited on the flowers. Their larvae developed parallel to the fruits without affecting seed development. The poor fruit formation in natural pollinated inflorescences is worrying, given that they are rare species but are valuable for several interactions. In addition, *B. andrieuxii* is growing in a preserved area, so the poor production of fruits indicates a weakness in the interaction networks in areas that are important for conserving local flora and fauna. Efforts must be increased to study rare, endemic species that offer great value for ecosystems, to lay the foundations for their preservation and to understand the interaction networks that maintain their populations.

Authors' contributions SRC and SVS authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SRC and CAGM. The first draft of the manuscript was written by SRC. Supervision, funding acquisition and resources were provided by SVS. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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Figure captions

Fig. 1 Flower behavior and pollination of *Bdallophytum andrieuxii* in Tonalá, Oaxaca, Mexico during 2021. **a** Changes in flower and environment temperature according to a diurnal schedule. Letters on the side of lines indicate differences in temperature as due to multiple comparisons at $p < 0.0001$. **b** Decreasing in pollen viability along anthesis time. **c** Total frequency of visits per functional group along with a diurnal schedule. Overlapping circles show potential pollinators and superscripts on functional groups shows results of *post hoc* multiple comparisons.

Fig. 2 Floral visitors of *B. andrieuxii* in the population of Santo Domingo Tonalá, Oaxaca, Mexico in 2021. **a** *Cissia* sp. foraging male flowers. **b** *Cissia* sp. foraging female flowers. **c** *Atta* sp. carrying a female flower. **d** Female inflorescence with removed flowers by *Atta* sp.

e *Musca* sp. foraging male flowers. **f** *Musca* sp. (arrow) foraging female flowers. **g** Drosophilid fly (arrow) visiting female flowers

Fig. 3 Oviposition and the parallel development of *B. andrieuxii* fruits and lepidopteran larvae. **a** Lepidopteran eggs (arrows) on male flowers after butterfly visit. **b** Lepidopteran larva emerging from a female flower. **c** Channels made by *Cissia* sp. larvae in a developing infructescence. **d** Mature fruits with well-developed seeds (detail of seeds on the corner). Scale: **b** = 2 mm; **c** = 5 mm; **d** = 1mm

Supplementary files

Supplementary file S1. Detailed results of GLM for floral visitors.

Supplementary file S2. Video of *Cissia* sp. butterfly foraging female flowers of *B. andrieuxii* in Santo Domingo Tonalá, Oaxaca, Mexico

Table 1. Attributes of *Bdallophytum andrieuxii* per sex.

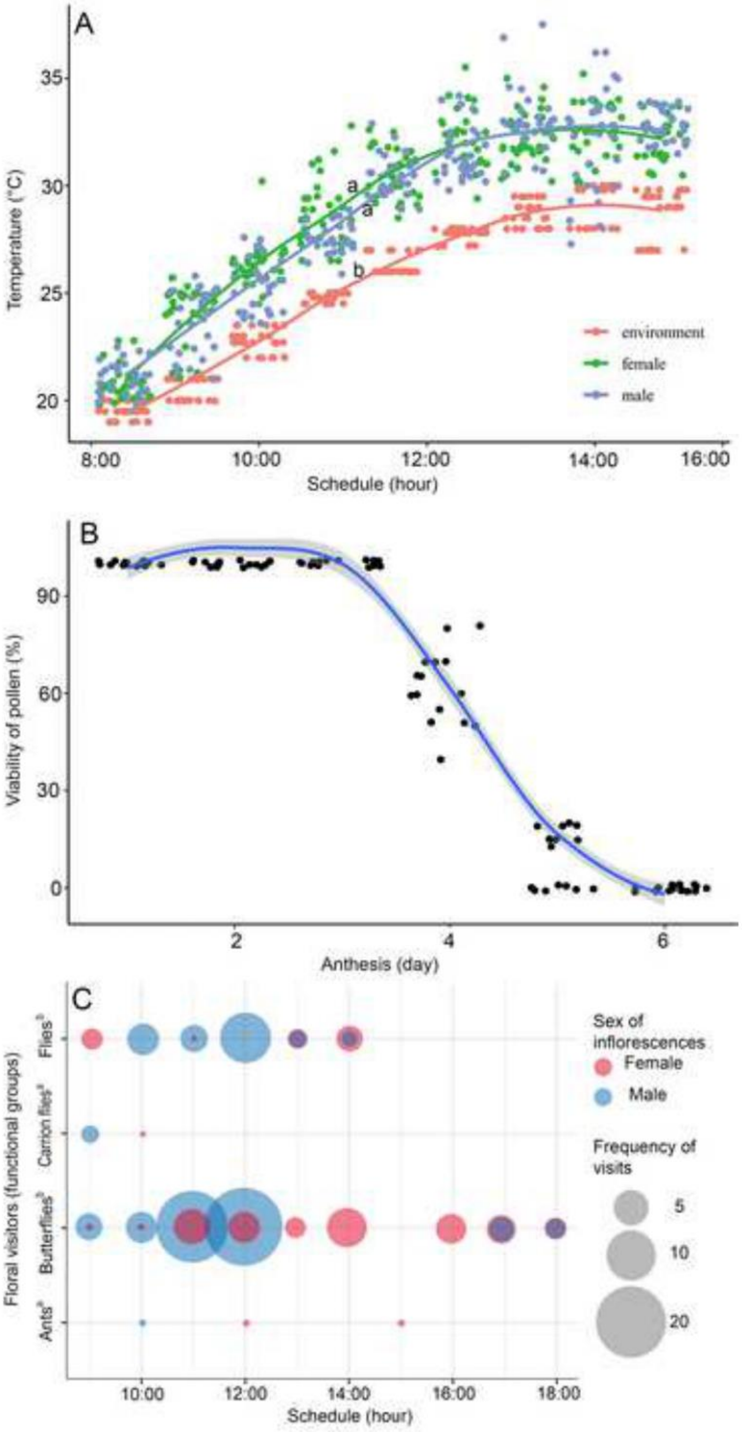
Trait		
Sex of inflorescences	Female	Male
Number of exophytes per host (mean ± SEM)	10.33 ± 1.78	9 ± 1.871
Flowers per inflorescence	65.7 ± 1.83	63 ± 1.52
Nectar production (µl)	3.93 ± 0.23	3.53 ± 0.19
Nectar concentration (°Bx)	23.9 ± 0.2	23.55 ± 0.18

Note: Mean values ± SEM.

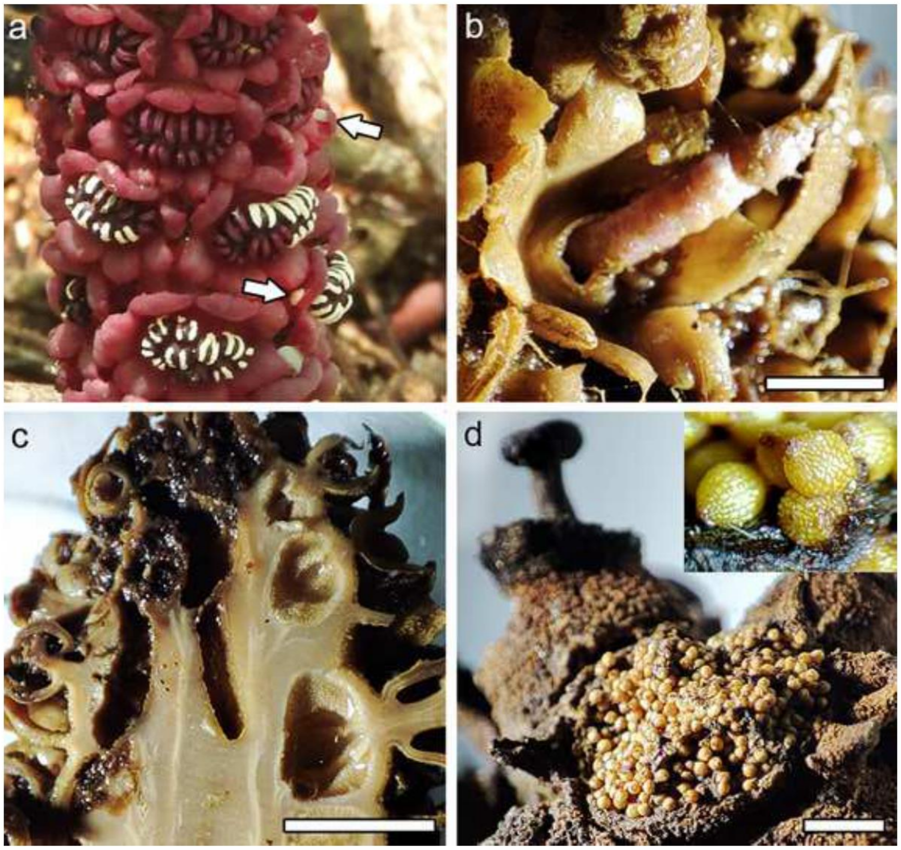
Table 2. Floral visitors of *Bdallophytum andrieuxii* in a population of Santo Domingo Tonalá, Oaxaca, Mexico during 2021.

Visitation traits	Ants		Carrion flies		Flies		Butterflies	
Sex of inflorescence	♀	♂	♀	♂	♀	♂	♀	♂
Total records (Frequency)	2	2	1	2	8	21	31	57
Inflorescences visited per patch	2	1.5 ± 0.5	1	1.5 ± 0.5	1.25 ± 0.16	1.28 ± 0.10	2.06 ±0.17	1.38 ± 0.8
Flowers visited per inflorescence	12.5 ± 4.5	13.5 ± 8.5	6	2.5 ± 0.5	1.25 ± 0.16	1.38 ± 0.14	4.55 ± 0.76	4.24 ± 0.39
Handling time per flower (seconds)	12.5 ± 7.5	5.5 ± 2.5	5	8 ± 2	354.61 ± 187.15	337.38 ± 114.2	19.77 ± 2.7	18.73 ± 2.01
Does the visitor touch the stigma/anthers?	Occasionally	Occasionally	Yes	Yes	Occasionally	Occasionally	Always	Always

Note: Mean values ± SEM.


A
Temperature (°C)
35
30
25
20
a
a
b
environment
female
male
8:00
10:00
12:00
14:00
16:00
Schedule (hour)
B
Viability of pollen (%)
90
60
30
0
2
4
6
Anthesis (day)
C
Floral visitors (functional groups)
Flies
Carrion flies
Butterflies
Ants
Sex of inflorescences
Female
Male
Frequency of visits
5
10
20
10:00
12:00
14:00
16:00
18:00
Schedule (hour)


a
b
c
d
e
f
g


a
b
c
d

Capítulo 4

Biología de la polinización de *Bdallophytum americanum*

(The floral biology and the role of staminal connective appendages during pollination of the endoparasite *Bdallophytum americanum*)

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The floral biology and the role of staminal connective appendages during pollination of the endoparasite *Bdallophytum americanum* (Cytinaceae)

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Abstract

Bdallophytum americanum (Cytinaceae) is an endoparasitic plant species, meaning only the flowers emerge from the host during the reproductive season. Reports on the pollination biology of this species state that its primary pollinators are carrion flies attracted by the smell of the flowers. However, the functional role of one of the most outstanding attributes of this species has been neglected. These are the staminal appendages formed by the apical overgrowth of connective tissue during anther development. To determine whether these staminal appendages play a role in reproduction, we monitored a nectarless population of *B. americanum*. We described the inflorescence emergence, floral movements, and pollination and performed field experiments to test whether the absence of the staminal connective appendages affected the visitation frequency. Male inflorescences emerge early, and both male and female flowers open during the day and do not close. Hoverflies are the most frequent visitors to both floral sexes and carry the most pollen. Moreover, the movement of staminal appendages matching the pollen viability changes is reported for the first time. The staminal appendages are the structures where the pollinators land before foraging. As a landing platform, the staminal connective appendages are crucial for pollinator positioning and pollination. The field experiments showed that the visitation frequency decreased sharply without staminal appendages.

Keywords

Copestylum, floral movements, hoverfly pollination, landing platforms, staminal appendages, visual advertisements

Introduction

Parasitic plants obtain their resources from other plants through a specialized structure called the haustorium and have evolved 12 times during angiosperm evolution (Nickrent 2020; Twyford 2018). The parasitic plants can be classified as hemiparasites or holoparasites depending on their photosynthetic capacity. The former retains photosynthetic activity while the latter has lost all photosynthetic functions (Heide-Jørgensen 2008). A subset of holoparasites is classified as endoparasites because their vegetative body grows inside the host, and only the flowers emerge from the host (Teixeira-Costa et al. 2021; Thorogood et al. 2021). The endophytic holoparasites comprise four families from different orders: Apodanthaceae (Cucurbitales), Cytinaceae (Malvales), Mitrastemonaceae (Ericales), and Rafflesiaceae (Malpighiales), the latter being the most notable species within the parasitic angiosperms (Nickrent 2020; Thorogood et al. 2021). Since the species of these families externally comprise only flowers or inflorescences, floral biology studies are essential to understand part of the life cycle of these peculiar species.

Floral biology ranges from the flowers' emergence, form, and function to advertisements and rewards concerning pollination (Gottsberger 1989; Willmer 2011). Although pollination has been studied in some endoparasites (Bänziger 1991; Beaman et al, 1988; De Vega et al. 2009; García-Franco and Rico-Gray 1997; Hobbhahn and Johnson 2015; Johnson et al. 2011; Sipes et al. 2014; Suetsugu 2019), the floral biology and discussions on the forms and functions of flowers are neglected. Within these endoparasites, the Rafflesiaceae species are the most studied, and their flowers have unique traits whose function has been related to pollination (Nikolov et al. 2014). An example is the presence of a barrier comprising floral acicular hairs allowing the entry of pollinators (Bänziger 1995). This prevents pollen robbery by non-pollinators, and the stigma form facilitates pollen deposition in Rafflesiaceae flowers (Beaman et al., 1988). Moreover, the flowers' colour pattern and foetid smell comprise a series of advertisements that attract carrion flies as pollinators (Faegri and van der Pijl 1979).

The strong foul smell, large or small flowers clustered in inflorescences, lack of nectar, dark colours resembling decaying meat, presence of additional filiform appendages, and the presence of hairy pads are traits characterizing the sapromyophilous pollination syndrome (Faegri and van der Pijl 1979; Willmer 2011).

In addition to Rafflesiaceae, other endophytic sapromyophilous species exist, such as those of the genus *Bdallophytum* of the Cytinaceae family. Pollination studies confirmed that flowers of *B. americanum* (formerly *B. bambusarum*) are pollinated by carrion flies (García-Franco and Rico-Gray 1997). Cytinaceae is the second species-rich endoparasite family, following Rafflesiaceae, with 12 species in two genera (Nickrent 2020). The genus *Cytinus* encompasses eight species from the Mediterranean, South Africa, and Madagascar (Sanjust and Rinaldi 2021); *Bdallophytum* includes four species from Mexico to Colombia (Nickrent 2020). Although little is known about the floral biology of the Cytinaceae species, the genus *Cytinus* has received the most attention in recent years (Sanjust and Rinaldi 2021). Regarding floral traits, the male flowers of *B. americanum* are noticeable because they have conspicuous staminal connective appendages resembling a multilobed stigma. The growth of the apical connective tissue forms these appendages during flower development, creating long extensions, one per anther, that are not seen in other species of the genus (Rios-Carrasco and Vázquez-Santana 2021). The form-function relationship of the connective appendages with pollination in Cytinaceae is unknown.

The first pollination study conducted with *B. americanum* was in a tropical semi-humid area (García-Franco and Rico-Gray 1997). However, this species is widely distributed in seasonal vegetation types characterized by marked dry and wet seasons (Alvarado-Cárdenas

2009). So far, the variation in pollination is unknown for other populations of *B. americanum* in different environments. Since the male flowers of *B. americanum* are noticeable within Cytinaceae due to their long appendages resembling filiform horns, this work aimed to study the floral biology of *B. americanum*, addressing the floral biology from the emergence of inflorescences to pollination. Notably, we especially focus on the functionality of the staminal connective appendages during pollination.

Materials and methods

Species and study site

Bdallophytum americanum is an endophytic holoparasite species belonging to Cytinaceae. This dioecious species is widely distributed, with populations from Mexico to Costa Rica (Alvarado-Cárdenas 2009). The species has the fewest flowers per inflorescence (12–18) and the largest ones within the genus (Rios-Carrasco and Vázquez-Santana 2021). Based on the floral morph, the flowers have a dark purple perigone contrasting with the bright yellow anthers or stigma. The genus *Bdallophytum* is a specialized parasite on the roots of *Bursera* (Burseraceae) trees.

The fieldwork was conducted in the municipality of Calvillo in the state of Aguascalientes, Mexico. The weather is predominantly semi-warm with an average annual temperature of 16.6°C–20.3°C and a total annual precipitation of 612.2 mm (Instituto Nacional de Estadística y Geografía 2017). The locality is in a remnant patch of a seasonally dry tropical forest where some species such as *Albizia plurijuga*, *Conzattia multiflora*, *Lysiloma* spp., *Leucaena* spp. (Fabaceae), *Bursera fagaroides* (Burseraceae), *Myrtillocactus geometrizans*, *Stenocereus queretaroensis*, and *S. drummondii* (Cactaceae) are dominant in the area (Siqueiros-Delgado et al. 2016). Within the municipality, the original vegetation is scarce since fields of guava cultivation have replaced it. This population of *B. americanum* is parasitizing *B. fagaroides.* The specimen vouchers were deposited at the Herbarium Luz María Villarreal de Puga of the Universidad de Guadalajara as follows: *Sánchez 681* IBUG (male inflorescences of *B. americanum*), *Sánchez 682* IBUG (female inflorescences of *B. americanum*), and *Sánchez 679* IBUG (host *Bursera fagaroides*).

***Bdallophytum americanum* population**

During three flowering seasons from 2018 to 2020, we counted the number of *B. americanum* hosts within 1 km^2 in a remnant patch of seasonally dry tropical forest between cultivated areas. We calculated the number of exophytes on roots for each host (equally to one cluster) and classified them according to sex. It is noteworthy that neither sexual morph showed any secretions or nectar during the fieldwork, at any moment or year of study. Thus, this is a nectarless population.

Floral movements, stigmatic receptivity, and pollen viability

We observed at least 50 pre-anthetic flowers per sex to detect any change or movement during opening, as well as the moment of anther dehiscence and movement in the connective appendages throughout the flower lifespan in male flowers. Additionally, we used hydrogen peroxide to determine the moment when stigmatic receptivity begins and its duration (Galen & Plowright, 1987). We tagged and bagged 10 female inflorescences to avoid pollen interference on stigmas. At least 15 flowers per age were used to measure stigmatic receptivity in newly opened flowers in 1-, 2-, 3-, and 4-day-old flowers; 30 µL syringes were used to place a few drops of hydrogen peroxide on the stigmas.

Regarding male flowers, we collected pollen samples from different anthers from each flower with a dissection needle. We placed them on glass slides to stain the pollen grains with Alexander's reagent (Alexander, 1980) to determine the duration of pollen viability over time. We classified flowers according to their age, and we calculated the percentage of viable pollen in flowers of different ages (1- to 6-day-old flowers). We used at least 15 flowers per age.

Floral visitors

We recorded the floral visitors in two flowering seasons during August and September of 2019 and 2020, respectively, for 4 days each year in six clusters (three clusters per sex). Each cluster had at least five flowering inflorescences of *B. americanum*. The records of diurnal floral visitors were produced through direct observations in five inflorescences per cluster from 9:00 to 16:00 h in 15 min observations followed by 15 min of rest. We also used trap cameras programmed to film during the night every 15 minutes and activated the motion sensor from 16:00 to 9:00 h. We obtained the number of visitors per inflorescence (male or female), the visited flowers, the foraging time, and the number of visited inflorescences per visitor. We used lethal chambers to capture samples of floral visitors. Mounted specimens were identified at least at the family level and to the finest level possible, using the following keys: de Carvalho et al. (2003), Elberg et al. (2009), Engel (2000), Goulet and Huber (1993), Hernández et al. (2013), Knutson and Orth (2001), Thompson (1999) and Triplehorn and Johnson (2005). When the species level was not reached, we used the morphospecies criterion.

We classified the floral visitors into functional groups according to their foraging behaviour (Fenster et al. 2004). Particularly, we split the fly functional group into two according to the foraging behaviour we observed in the field. The first group was treated as "flies" and the second as "hoverflies", given their pollinivorous and landing behaviour described in the results section. To determine differences in the visitation frequency among functional groups, we performed a generalized linear model (GLM) with a Poisson distribution and log-linkage function using the package *stat* in the software R (R Development Core Team 2019). We used the visitation frequency as the response variable and the functional groups, years of observation, and sexes as the explanatory variables. Additionally, we performed a multiple comparison analysis of the visitation frequency between functional groups. To differentiate pollinators from floral visitors or pollen robbers, we considered the visitation to female and male inflorescences, and pollen quantity on their bodies.

Role of connective appendages in pollination

We performed a field experiment to explore the role of the staminal connective appendages by evaluating whether their absence affected the visitation frequency to male inflorescences. The experiment comprised two male inflorescence-type treatments of two male clusters, namely pure clusters. The first was the mutilated cluster, where all flowers of 10 inflorescences were injured by cutting off and removing all the apical connective appendages from anthers. The second treatment was applied to a pure cluster with 10 male inflorescences that were not handled, so we used this as the control. Additionally, to discard the cluster effect and evaluate the decision-making by floral visitors, we performed the two treatments on one cluster, namely a mixed cluster. Of the 10 inflorescences in that patch, we randomly selected five to perform each of the two treatments described above. We compared the total visitation frequency (response variable) between treatments of pure and mixed clusters (mutilated *vs* control) and between cluster types (pure *vs* mixed). The comparisons were performed through a GLM with a Poisson distribution, log-linkage function, and chi-square to determine differences between treatments and clusters using the software R Development Core Team (2019). The analysis was conducted considering a daytime observation time from 9:00 to 19:00 h to avoid data overdispersion since the pollinators are diurnal. At night, only two non-pollinator visitors were registered in total for all treatments.

Results

Bdallophytum americanum population

The studied population of *Bdallophytum americanum* comprised nine clusters within approximately 1 km^2 in a seasonally dry tropical forest patch. During the first year of monitoring (2018), we found six clusters, of which three had exophytes from that season, while the other three had remains of exophytes apparently from the previous season. Moreover, the same clusters were found in the next 2 years and three more during the last year. Most exophytes had the same sex within a cluster, and this was maintained through the years (Table 1), indicating re-emergence.

The emergence of the inflorescences begins after the first rains in the area. The exophytes or young inflorescences can be observed from July, and as time passes, the number of exophytes increases. The first exophytes in the studied population corresponded to the male inflorescences, which predominated during the first month. The female inflorescences predominated from August to September, while only infructescences were present in October (Fig. 1). Despite the dimorphism at emergence, female and male inflorescences emit a strong yeasty smell when the flowers are open. This smell is easily detectable by the human nose when close to a patch.

Floral movements, stigmatic receptivity, and pollen viability

During the morning, the flowers are closed (Fig. 2a, i). The flower opening occurs around 8:00 to 9:00 h in the morning (Fig. 2b–c), reaching a full aperture at approximately noon (Fig. 2d, l). As the days pass, female flowers exhibit colour changes; they are initially pale red (Fig. 2b–d) and turn darker (Fig. 2e–g). The perigone remains open and withers around day 5 (Fig. 2h), indicating the start of fruit development if fertilization has occurred. In male flowers, the perigone is dark red from the beginning (Fig. 2i–k). Female ones reach the full aperture at noon (Fig. 2l); the flowers remain open until they wither on day 4 or 5 (Fig. 2m–o), later appearing whiter (Fig 2p). In the male flowers, the anthers and appendages of the connective tissue also showed movements and changes along the flower lifespan (Fig. 2q–x). The apical connective appendages are towards the centre when the flowers open, so the tips touch each other and protrude above the perigone (Fig. 2q–r). The appendages then extend out as the perigone unfolds to achieve complete opening (Fig. 2s–u). The anther dehiscence is extrorse. This occurs when the flowers are completely open, and the connective appendages are fully extended (Fig. 2t–u). The appendages remain extended during the first 2 days of anthesis (Fig. 2t–u). The appendages begin to darken and retract towards their initial position on the third day (Fig. 2v–x).

Regarding sexual functionality, the pollen grains are viable in male flowers during the first 4 or 5 days. Nevertheless, from the third day, viability decreases abruptly. The pollen grains are no longer viable on the sixth day (Fig. 3). The stigmas in female flowers start to be receptive as soon as the flowers start to open. The receptivity lasts 3 days. The stigmas are no longer receptive on day 4 (Fig. 3).

Floral visitors

We registered the visits of two species and 12 different morphospecies belonging to eight functional groups (Table 2). Most visits were registered in a diurnal schedule; however, two functional groups of visitors were rarely registered at night-time, rodents and moths (Fig. 4a). Male inflorescences had more frequent visits than female inflorescences ($\chi^2 = 6.166$, d.f. = 1, $p < 0.0001$). Additionally, we registered more visits in 2019 from more functional groups than in 2020 ($\chi^2 = 14.156$, d.f. = 1, $p < 0.0001$; see Fig. 4). Regarding the visitation frequency, differences occurred between the functional groups ($\chi^2 = 294.431$, d.f. = 7, $p < 0.0001$), the hoverflies being the most frequent in both years and both sexes (see Table 3).

Regarding the behaviour of visitors, butterflies, moths, orthopterans, and rodents were discarded as pollinators. Most of them only visited one type of inflorescence, or their visits were rare, and they spent a few seconds foraging (Table 3). Additionally, the orthopterans spent a long time eating floral parts. Thus, they were florivorous rather than pollinators. Nevertheless, *Augochlorini* sp 1 and sp 2 were the visitors carrying the most pollen grains of *B. americanum*. However, they spent more time collecting pollen than on their visits to female flowers. Moreover, the pollen on the bees' bodies was not exclusive to *B. americanum*, suggesting that this functional group mainly visits male flowers for pollen robbery. Beetles were registered to visit both floral sexes. Although beetles spend more time resting in the perigone than touching the sexual organs, they can pollinate as they arrive at stigmas with *B. americanum* pollen. Flies were more frequent than beetles, and we found that *Sepedon* sp 1 flies reached the female flowers with *B. americanum* pollen and sometimes touched the sexual organs. Therefore, they also contributed to pollination.

Finally, *Copestylum* sp 1 hoverflies were the most frequent visitors in both male and female inflorescences (Fig. 4a) during the studied years. They carried large amounts of pollen, exclusive to *B. americanum*, which was transported from male to female flowers (Table 3).

These floral visitors consistently touched the sexual organs. In male flowers, hoverflies landed on staminal connective appendages and used them as perches to move underneath the anthers and the rest of the flower (Fig. 5a). When they reached the female flowers, they landed on the stigma and began to move along the perigone of the flower (Fig. 5b). Once the hoverflies landed on stigmas or connective appendages according to sex, they moved to visit different flowers along the same inflorescence. However, they spent less time on female flowers than male ones. During most of their visits, we noticed that hoverflies could remain for more than 30 min resting on the perigone of the male flowers after foraging on the anthers. Therefore, given the amount of pollen on the body, the visitation frequency on both types of flowers, and the contact with sexual organs, hoverflies were pollinators of *B. americanum* in the study area.

Role of staminal appendages in pollination

The frequency of visits differed between mutilated and untouched (control) androecia ($\chi^2 = 114.56$, d.f. = 1, $p < 0.0001$). In the absence of connective appendages, pollinators do not approach the flowers. The visitation frequency was lower in mutilated flowers, which was observed in the mixed cluster. However, the mixed cluster was less visited than pure clusters ($\chi^2 = 46.79$, d.f. = 1, $p < 0.0001$). The inflorescences with mutilated androecia affected the visits to the rest of the untouched inflorescences within the cluster. Nevertheless, visitors discerned between mutilated and untouched flowers because the visits were more frequent in the control inflorescences within the same cluster. Visiting hours were mainly daytime, and the visits were consistent with the pollination observations where the flies and hoverflies were noticeable as the most frequent visitors (Fig. 4b).

Discussion

***Bdallophytum americanum* population**

Bdallophytum americanum growth showed re-emergence of same-sex inflorescences over the years. The inflorescence emergence occurs gradually during blooming, so the variation between the number of exophytes between years and between clusters depends on the moment they are observed. The same pattern of re-emergence according to sex was reported for other species of Cytinaceae, such as *Cytinus hypocistis* (De Vega et al. 2007) and *C. sanguineus* (Hobbhahn and Johnson 2015). This feature has also been observed in *Pilostyles thurberi,* another endoparasite of the family Apodanthaceae (P. F. Ortega-González et al., in prep.). In *C. hypocistis*, the re-emergence of exophytes has been followed over 5 years, indicating that this species has perennial plants (De Vega et al. 2007).

Bdallophytum americanum is a dioecious species whose male and female plants differ during emergence. The male inflorescences emerge before the female ones. This dimorphic emergence could affect reproduction. Female inflorescences take more time to appear as they need more resources to reproduce (Conn & Blum 1981). However, male inflorescences can be advantageous since they can compete for pollinators with other species growing on the same site and keep floral visitors close to the area, taking advantage of their learning skills and awareness of the resource offered by *B. americanum* flowers (Purrington and Schmitt 1998; Weiss 2001).

Floral movements and their implications in pollination

Floral whorl movements are essential to flower ecology because they are a functional trait related to pollinator behaviour favouring pollination (Henning et al. 2018; Sibaoka 1969). In *B. americanum,* the perigone movements occur only during flower opening in a diurnal schedule.

Nevertheless, once open, flowers do not close. Thus, the sexual organs of both female and male inflorescences remain exposed to visitors all day, favouring the reception or donation of pollen (van Doorn and van Meeteren 2003). All-day exposure to sexual organs allows a wide range of pollinators to arrive at flowers according to different schedules (Ganie et al. 2021), which can occur for 3 days on female flowers and up to 5 for male ones in *B. americanum*. However, this can also be a disadvantage since the reproductive organs are exposed to florivores, robbers, or damage (van Doorn and van Meeteren 2003).

Additionally, a novel floral movement is described here for the family. The apical staminal connective appendages move synchronously in all stamens and parallel with the direction of the perigone when it opens. However, this parallel movement of stamens and perianth is described for other angiosperms (Henning et al. 2018; Zhang et al. 2019). Nevertheless, in *B. americanum*, the movements are not provided by filaments or anthers when touched but by staminal connective appendages. In the first 2 days of anthesis, when the staminal appendages are completely extended or "open", the pollen is at its maximum viability, favouring pollen presentation. When the connective appendages begin to "close" or return to their initial position on the third day of anthesis, the pollen viability decreases abruptly. This match between pollen viability, the extended staminal appendages, and pollination is discussed in depth in the following sections.

Floral visitors and pollination

The pollination patterns can vary depending on the habitat composition, availability of resources, and surrounding communities of organisms (Evans et al. 2017). A previous study on the pollination biology of *B. americanum* (formerly *B. bambusarum*) described how both male and female flowers produce nectar; however, it is more concentrated in female flowers (García-

Franco and Rico-Gray 1997). The presence of floral rewards in both morphs explains the floral visits to male and female flowers. Nevertheless, in the studied population of *B. americanum*, all flowers lacked nectar. Thus, the pollination in this population appears to occur by deception as the female flowers do not offer a reward but expel a strong yeasty smell. Studies of the floral ecology of other sapromyophilous endoparasites, such as the Rafflesiaceae species, have described the floral scent as one of the main attractants to pollinators (Zain et al. 2020). The foetid smell of the sapromyophilous species is strongly related to attracting carrion flies (Chakraborty and Adhikary 2018). Although syrphids are known to feed on pollen and nectar, members of the subfamily Eristalinae (in which *Copestylum* is placed) have been reported as helpful in forensic entomology due to their consumption of carrion material (Martins et al. 2010). Thus, although the potential pollinators are hoverflies of the genus *Copestylum* rather than the expected carrion flies, the carrion pollination is maintained given the carrion foraging behaviour of *Copestylum* sp 1 in *B. americanum* flowers. Visits of *Copestylum* to female flowers are shorter and less frequent than to male ones. Still, the hoverflies carry pollen exclusively from *B. americanum* and consistently touch the stigma. Thus, pollination can occur, despite the lack of reward.

Despite the differences in the floral rewards offered by *B. americanum* to pollinators in different populations, the dipterans are maintained as pollinators (García-Franco and Rico-Gray 1997; Nunes et al. 2016). This pattern is also observed in other endoparasites such as *Sapria ram* (Rafflesiaceae) studied in different populations; however, the pollinators are consistently carrion flies, indicating a specialized interaction (Bänziger and Pape 2004; Pape and Bänziger 2000).

Sapromyophilous traits are exclusive of the genus *Bdallophytum* within the Cytinaceae. Nevertheless, the pollination by carrion flies has only been reported in one population of *B.*

americanum (García-Franco and Rico-Gray 1997). While hoverflies pollinate the studied population of *B. americanum* and *B. oxylepis* is pollinated by stingless bees (Rios-Carrasco et al., 2022). Otherwise, the genus *Cytinus* has non-sapromyophilous species such as *C. hypocistis*, *C. sanguineus*, and *C. visseri* but also has specialized pollination by ants, birds, rodents, and shrews, respectively (De Vega et al. 2009; Hobbhahn and Johnson 2015; Johnson et al. 2011). The specialized pollination evolves in close relationship with floral morphology and the development of specialized structures (Dellinger et al. 2019; Gavrutenko et al. 2020), which could apply to *B. americanum*, and the presence of long staminal connective appendages.

Staminal connective appendages as visual cues

Previous studies on *B. americanum* pollination did not address the role of apical connective appendages (García-Franco and Rico-Gray 1997). Thus, here, we describe for the first time their role in pollination. Flowers of *B. americanum* display a set of signals to attract pollinators, including the yeasty smell, the dark colour of the flowers, and the long and apical connective appendages demonstrated here. The connective appendages are conspicuous, moving structures with a functional role during pollination. Our results indicated that the visitation frequency decreases sharply without the connective appendages in all flowers within a cluster. Although the smell of inflorescences can primarily attract hoverflies, the absence of the appendages makes the pollinators avoid the flowers. It has been demonstrated that the scents are the primary attractants, and the visual attractants such as the colour of flowers or presence of particular structures being secondary (Weiss 2001). Thus, the yeasty smell of *B. americanum* flowers may function as a primary advertisement. Still, the large connective appendages can be a secondary visual cue based on which hoverflies decide whether to visit the flowers. Moreover, the connective appendages can serve as a landing platform that improves pollen presentation, collection, and transfer (Henning et al. 2018; Lawson and Rands 2018).

In a second scenario where untouched and mutilated androecia are on the same patch, the visitation frequency was less than in the control cluster. Generally, the visitation rate was affected by the presence of mutilated flowers in the patch, supporting the concept that connective appendages are an essential visual cue for pollinators to approach the patch. Despite the mixed cluster's low visitation frequency, almost all visits by hoverflies were made to flowers with untouched connective appendages. The preference for untouched flowers indicates a pollinator's decision-making, supporting the concept that specialized structures are maintained through selective pressures exerted by pollinators (Lawson and Rands 2018).

Additional floral appendages have been related to fly pollination (Faegri and van der Pijl 1979). Some fly-pollinated species have flowers with filiform appendages with different roles in pollination. For instance, in Rafflesiaceae, filiform structures resembling a filter to stamens allow the flies to traverse to the anthers but avoid the entry of other non-pollinators (Bänziger 1995). In *Mitella pauciflora* (Saxifragaceae), a fly-pollinated species, the petals have long filiform structures serving as a landing platform. In their absence, the visitation rate decreases (Katsuhara et al. 2017) as in *B. americanum*. As mentioned previously, the "opening" of connective appendages matches the maximum pollen viability and is the structure where the hoverflies land. Thus, the landing platform in *B. americanum* ensures the removal of viable pollen. Studies on stamen movements mentioned that they are adaptations facilitating pollen removal (Abdusalam et al. 2021). In *B. americanum*, apical connective appendages enhance the removal of viable pollen. These floral movements can also serve as advertisements to attract pollinators or even secure pollination (Tan and Tan 2018) once the appendages extend fully.

The pollination of a nectarless population of *Bdallophytum americanum* was studied, emphasizing the functional role of connective appendages. Our results show that specialized pollination by dipterans is maintained in *B. americanum* despite lacking nectar. We described a novel type of movement in these apical appendages of the androecium that coincides with the changes in pollen viability. Otherwise, the male flowers have specialized structures formed by a projection of the connective tissue resembling a multilobed stigma serving as a landing platform for hoverflies, the pollinators. This landing platform appears crucial for attracting pollinators. The form-function relationship of specialized structures must be studied in detail to better understand the specialized animal-plant interactions, particularly in the misunderstood endoparasites.

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Figure captions

Fig. 1 Percentage of emerged exophytes of *B. americanum* per sex through time in the population of Calvillo, Aguascalientes, Mexico. The withered male inflorescences were not counted.

Fig. 2 Changes and movements through time in *B. americanum* flowers. **a-h)** Female flowers showing their color change since their opening until their withering. **i-p)** Male flowers opening. **q-x)** Movements and changes of connective appendages of stamens.

Fig. 3 Decreasing in viability of pollen grains in male flowers of *B. americanum* over the days. The gray area indicates the duration of stigmatic receptivity in female flowers.

Fig. 4 Floral visitors to *B. americanum* inflorescences. **a)** Visitors by functional groups to see the frequency of visits to male and female inflorescences along the day in both years of study. Potential pollinators can be distinguished by overlapping different colored bubbles. **b)** Visits to untouched (control) and mutilated male flowers from mixed and pure patches resulted in the connective appendages treatments.

Fig. 5 Pollinators of *B. americanum* in the studied population. **a)** *Copestylum* sp 1 landing on the stigma of female flowers. **b)** *Copestylum* sp 1 landing on connective appendages of male flowers, using them as perch to move around the flower.

Figures

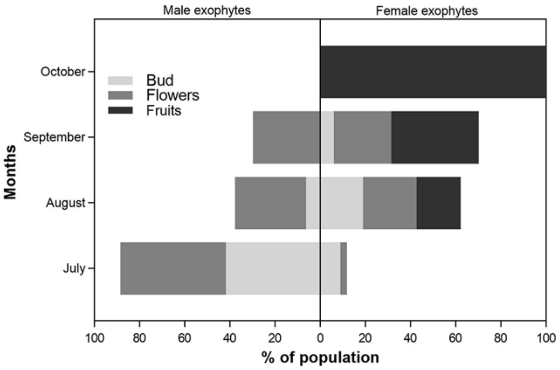

FIGURE 1

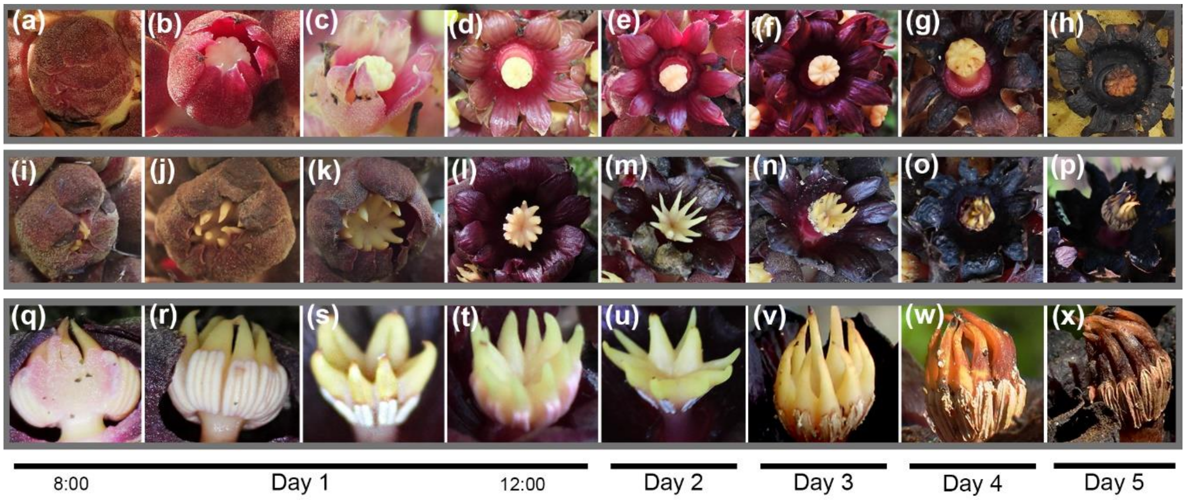

FIGURE 2

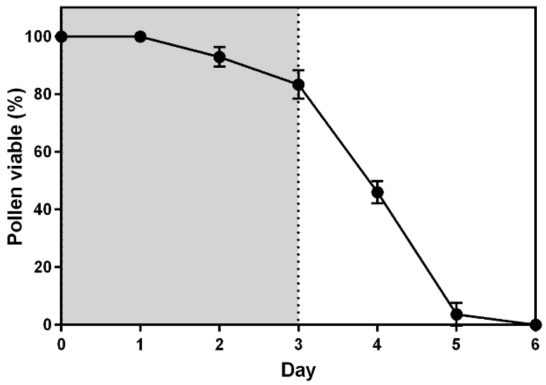

FIGURE 3

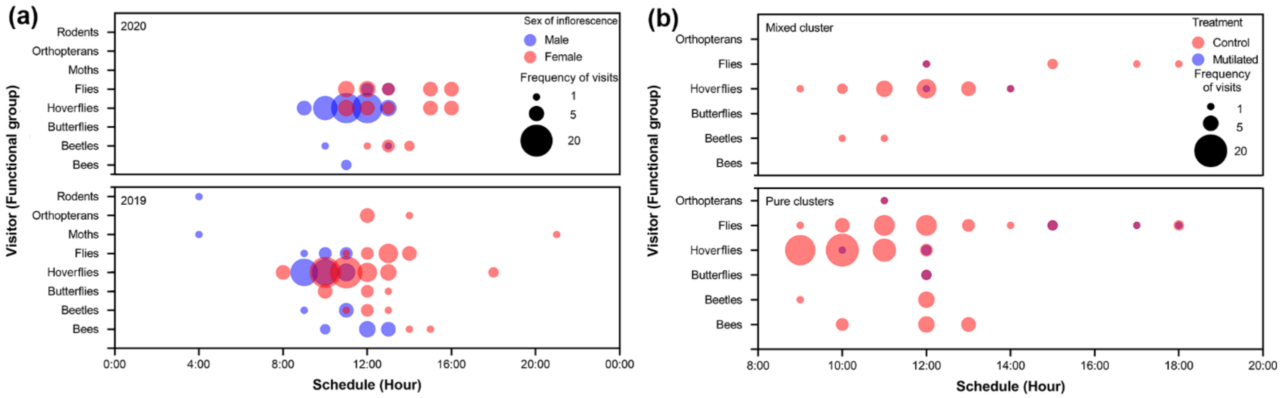

FIGURE 4

FIGURE 5

Tables

TABLE 1. Sex of the *B. americanum* exophytes per cluster indicating reemergence through years in the population of Calvillo, Aguascalientes.

Cluster	Sex		
	2018	2019	2020
1	♂	♂	♂
2	♀, ♂	♀, ♂	♀, ♂
3	♀	♀	♀
4	♀	♀	♀
5	♀	♀	♀
6	♂	♂	♂
7	-	-	♂
8	-	-	♀
9	-	-	♀

TABLE 2. Floral visitors in male and female inflorescences of *Bdallophytum americanum* in Calvillo, Aguascalientes, Mexico during 2019 and 2020. The visitors were classified into functional groups based on their behavior.

Visitors to female inflorescences			Visitors to male inflorescences		
Functional group	Taxonomical family	Visitor	Functional group	Taxonomical family	Visitor
Bees	Hallictidae	Augochlorini sp 1	Bees	Hallictidae	Augochlorini sp 1
Beetles	Curculionidae	*Epimechus adspersus*			Augochlorini sp 2
Butterflies	-	Butterfly 1	Beetles	Nitidulidae	Nitidulidae sp 1
Flies	Heleomyzidae	*Heleomyzidae* sp 1			Nitidulidae sp 2
	Sciomyzidae	*Sepedon* sp 1			Nitidulidaae sp 3
Hoverflies	Syrphidae	*Copestylum* sp 1	Flies	Fannidae	*Fannia canicularis*
Orthopterans	Acrididae	Acrididae sp 1		Sciomyzidae	*Sepedon* sp 1
			Hoverflies	Syrphidae	*Copestylum* sp1
			Rodents	-	Rodent 1

TABLE 3. Behavioral traits of floral visitors to female and male inflorescences of *B. americanum* in Calvillo, Aguascalientes, Mexico during both years of study. The n of the captured insects is in correspondence with the number of observations, the greater the number of visits, the greater the probability of capture.

Note: Superscripts in the functional groups indicates de results of comparison multiple analysis at $p < 0.001$.

	Female inflorescences					Male inflorescences				
Functional group	Inflorescences per cluster Mean ± SEM	Flowers per inflorescence Mean ± SEM	Handling time s	Pollen on their bodies Mean ± SEM	n	Inflorescences per cluster Mean ± SEM	Flowers per inflorescence Mean ± SEM	Handling time s	Pollen on their bodies Mean ± SEM	n
Bees^{ac}	1.5 ± 0.28	1	7.5 ± 2.5	5900 ± 900	2	2.12 ± 0.35	2.9 ± 0.64	19 ± 5.56	29100 ± 3900	2
Beetles^{bc}	1.4 ± 0.4	2.14 ± 0.4	17.44 ± 4.6	550	1	1.22 ± 0.22	1.42 ± 0.29	41 ± 23.61	325 ± 176	4
Butterflies^{ac}	1.33 ± 0.33	1.66 ± 0.67	6.55 ± 1.3	-	-	-	-	-	-	-
Flies^{c}	1.2 ± 0.13	2.27 ± 0.33	34.8 ± 9.6	375 ± 275	2	1	1.57 ± 0.29	132.5 ± 25.87	-	-
Hoverflies^{d}	2.12 ± 0.24	3.7 ± 0.48	25.7 ± 4.49	3442 ± 803	7	1.72 ± 0.23	3.91 ± 0.57	80.86 ± 16.4	3886 ± 974	8
Moths^{a}	1	1	2	-	-	1	1	4	-	-
Orthopterans^{ac}	1	1.5 ± 0.5	212 ± 66	0	2	-	-	-	-	-
Rodents^{ab}	-	-	-	-	-	2	1.5 ± 0.5	4.33 ± 0.3	-	-

Capítulo 5

Embriología de las especies del género *Bdallophytum*

[Comparative morphology and ontogenetic patterns of *Bdallophytum* species (Cytinaceae, Malvales): insight into the biology of an endoparasitic genus]

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ARTICLE

Comparative morphology and ontogenetic patterns of *Bdallophytum* species (Cytinaceae, Malvales): insight into the biology of an endoparasitic genus

Sandra Rios-Carrasco and Sonia Vázquez-Santana

Abstract: Cytinaceae are root endoparasitic plants with only three genera. Their biology is largely unknown, and most knowledge of the family is based on the Old World genus *Cytinus*. Here, we studied all three species of the New World *Bdallophytum* from Mexico. We describe their morphoanatomy, floral development, and embryology, highlighting the unique traits of *Bdallophytum* compared with two other genera of the family and members of Malvales. Both *B. americanum* and *B. andrieuxii* are dioecious, whereas *B. oxylepis* is gynomonoecious. The floral size and the number of floral organs vary within and among species, which appears to be common in Cytinaceae. The flowers of *Bdallophytum* exhibit synorganization in sexual organs, a synandrium in male flowers, and a gynostemium in bisexual flowers of *B. oxylepis*. Unisexual and bisexual flowers are zygomorphic at the early developmental stages. The unisexual flowers become actinomorphic in later development, while the bisexual flowers of *B. oxylepis* remain zygomorphic. The androecium of *Bdallophytum* has key traits shared with some Malvales, such as the fused filaments in Malvaceae and Sarcolaenaceae and the connective appendage shared with Dipterocarpaceae. Our results suggest that a unitegmic ovule is a unique trait for *Bdallophytum*. This is proposed here as a putative synapomorphy for *Bdallophytum*.

Key words: embryology, floral symmetry, gynomonoecy, gynostemium, synorganization unisexuality.

Résumé : Les Cytinaceae sont des plantes endoparasites des racines qui ne comportent que trois genres. Leur biologie est largement inconnue et la plupart des connaissances sur cette famille sont basées sur le genre de l'Ancien Monde, *Cytinus*. Les auteurs ont étudié ici les trois espèces de *Bdallophytum* du Nouveau Monde trouvées au Mexique. Ils décrivent leur morphoanatomie, leur développement floral et leur embryologie, en soulignant les caractéristiques uniques de *Bdallophytum* par rapport aux deux autres genres de la famille et membres des Malvales. *Bdallophytum americanum* et *B. andrieuxii* sont tous deux dioïques, tandis que *B. oxylepis* est gynomonoïque. La taille des fleurs et le nombre d'organes floraux varient au sein d'une même espèce et entre les espèces, ce qui semble commun chez les Cytinaceae. Les fleurs de *Bdallophytum* présentent une synorganisation dans les organes sexuels, un synandre dans les fleurs mâles et un gynostème dans les fleurs bisexuelles de *B. oxylepis*. Les fleurs unisexuelles et bisexuelles sont zygomorphes aux premiers stades de leur développement. Les fleurs unisexuelles deviennent actinomorphes au cours du développement ultérieur, tandis que les fleurs bisexuelles de *B. oxylepis* restent zygomorphes. L'androcée de *Bdallophytum* possède des caractéristiques clés partagées avec certaines Malvales, comme les filaments soudés chez les Malvaceae et des Sarcolaenaceae et l'appendice conjonctif partagé avec les Dipterocarpaceae. Ces résultats suggèrent qu'un ovule unitegmique est une caractéristique unique chez *Bdallophytum*. Ceci est proposé ici comme une possible synapomorphie chez *Bdallophytum*. [Traduit par la Rédaction]

Mots-clés : embryologie, symétrie florale, gynomonoécie, gynostème, synorganisation, unisexualité.

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Introduction

Cytinaceae are endoparasitic plants that have so far received minimal attention. The family is small and includes approximately 15 species in three genera (Nickrent 2007; Fernández-Alonso and Cuadros-Villalobos 2012; Alvarado-Cárdenas 2015; Těšitel 2016). *Cytinus* is distributed in Africa, Asia, and Europe (Smithies and Burgoyne 2010); *Sanguisuga* is found in South America (Fernández-Alonso and Cuadros-Villalobos 2012); and *Bdallophytum* is native to Central America (Alvarado-Cárdenas 2007). Endoparasites are parasitic plants that lack photosynthetic activity and thus acquire all their nutrients from their host. They lack a visible vegetative body and thus emerge from the host only as flowers or inflorescences (Heide-Jørgensen 2008; Těšitel 2016). Rafflesiaceae are probably the most well-known endoparasitic plants, owing to their large flowers (Heide-Jørgensen 2008). However, endoparasitism also occurs in Apodanthaceae, Cytinaceae, Mitrastemonaceae, and Loranthaceae (*Tristerix aphyllus*) (Mauseth et al. 1985; Rubiales and Heide-Jørgensen 2011; Těšitel 2016). Cytinaceae, together with the other three endoparasitic families, were formerly grouped into Rafflesiaceae s.l. due to their endoparasitic nature (Meijer 1993).

Studies based on molecular evidence have demonstrated that endoparasites belong to unrelated families and different orders, with Rafflesiaceae in Malpighiales (Davis and Wurdack 2004); Apodanthaceae in Cucurbitales (Filipowicz and Renner 2010); Mitrastemonaceae in Ericales (Barkman et al. 2004); and Cytinaceae in Malvales, as the sister group of Muntingiaceae (Nickrent 2007). However, another phylogenetic study showed recently that the position of Cytinaceae remains uncertain within the order (Hernández-Gutiérrez and Magallón 2019). In addition, the inclusion of Cytinaceae in Malvales is mainly supported on morphological traits of *Cytinus*, such as its floral sexuality and pubescence, which remain poorly known in the other genera (Nickrent 2007).

Bdallophytum has three species (Alvarado-Cárdenas 2009); *B. americanum* (R. Br.) Eichler ex Solms, *B. andrieuxii* Eichler, and *B. oxylepis* (B.L. Rob.) Harms, distributed from Mexico to Costa Rica (Alvarado-Cárdenas 2007; Nickrent 2007). They are root endoparasites specific to *Bursera* (Jacq. ex L.) trees. One of their most notable features is the predominance of unisexual flowers, *B. oxylepis* being the only species that also exhibits bisexual flowers (Alvarado-Cárdenas 2009; Martínez-Camilo et al. 2012). Currently, no studies have been published on the floral development in this genus or even the family. Morphological and embryological studies on this family are also scarce [e.g., *Cytinus hypocistis* (L.) L., Guzowska 1964, 1966].

Besides morphological studies, the embryological approach provides important information concerning the ecological and evolutionary aspects of plant biology (Endress 2005; Batygina 2019). In this work, we explored the biology of endoparasitic plants using comparative morphoanatomy and embryology. This study describes the inflorescence and flower morphology, and ontogenetic patterns during flower development and embryology across *Bdallophytum*. It focuses on (*i*) the initiation of floral organs; (*ii*) developmental patterns of the unisexual and bisexual flowers, (*iii*) micro- and megasporogenesis, and (*iv*) micro- and megagametogenesis. Finally, we discuss the morphological, anatomical, and embryological aspects of *Bdallophytum* with respect to Cytinaceae and other members of the order Malvales.

Materials and methods

Sample collection sites

Samples of each species were collected in different Mexican localities. *Bdallophytum americanum* [on *Bursera fagaroides* (Kunth) Engl. roots] samples were collected in Calvillo, Aguascalientes, in July 2018; *B. andrieuxii* [on *Bursera bicolor* (Willd. ex Schltdl.) Engl. roots] in Huaxtla, Morelos, in June 2017 and February 2018; and *B. oxylepis* [on *Bursera excelsa* (Kunth) Engl. roots] in Tuxtla Gutiérrez, Chiapas, in May 2018.

Floral morphology and development

Inflorescences of the three species at different developmental stages were sampled and photographed at the collection sites. We counted the number of flowers per inflorescence, and measured the total length of the inflorescence and peduncle for at least 15 inflorescences of each morph per species.

The collected samples were fixed in 70% ethanol. For sectioning, the material was dehydrated in a gradual ethanol series in a vacuum chamber and embedded in Paraplast (Sandoval-Zapotitla 2005) or LR White following standard protocols (Márquez et al. 2016). The Paraplast sections were stained with safranin and fast green or with periodic acid–Schiff (PAS) plus Naphthol Blue Black. Sections embedded in LR White were stained with toluidine blue O. Photomicrographs were taken using a light microscope (Olympus Provis AX70 with an Evolution MP 5.1 digital camera; Media Cybernetics, Inc.). For scanning electron microscopy (SEM), the samples were dehydrated in a gradual ethanol series, critical point dried with CO_2, and sputter-coated with gold. Observations were made with a JSM-5310LV JEOL scanning electron microscope. The pollen grain size was determined by measuring 50 pollen grains per species using Leica LAS EZ software, version 3.4.0. The Kolmogorov–Smirnov test was used to confirm the normality of the data. A one-way ANOVA and a Tukey test were then performed using the GraphPad Prism version 8.1.2 (332) software.

Results

Inflorescence morphology

Inflorescences are terminal and pedunculate spikes. Their size and peduncle length vary among the species (Table 1). The axis is pale yellow in the three species and covered with uniseriate trichomes. Bracts are arranged

Table 1. Morphological differences among the three *Bdallophytum* species.

Traits	*B. americanum*	*B. andrieuxii*	*B. oxylepis*
Inflorescence total length	♀ 8.05 ± 1.38 ♂ 13.1 ± 1.04	♀ 12.4 ± 2.3 ♂ 16.2 ± 4.59	8.2 ± 1.24
Peduncle length (cm)	♀ 6.4 ± 0.65 ♂ 6.3 ± 0.43	♀ 6.4 ± 0.81 ♂ 6.8 ± 1.49	4.1 ± 0.48
Inflorescence axis color	Pale yellow	Pale yellow	Pale yellow
Sexual system	Dioecious	Dioecious	Gynomonoecious
Flowers per inflorescence	♀ 15.06 ± 1.84 ♂ 15.93 ± 1.43	♀ 61.25 ± 8.75 ♂ 60.56 ± 8.49	♀ 7.46 ± 5.64 ⚥ 52.86 ± 5.51
Perigone lobes number	5–11	5–9	5–7
Perigone color	Dark purple	♀ Red to brown ♂ Reddish	Reddish
Stamen number	4–12	3–10	1–6
Connective appendage	Present	Present (only in the morph with non-parallel arrangement)	Absent
Anther attachment	Adnate	Adnate	Adnate
Anther color	Yellow	Reddish	Burgundy
Stigmatic lobes number	6–13	3–11	♀ 4–9 ⚥ 4–11
Stigma color	Yellow	Red to brown	Pale red

Note: The measurements are in centimetres (mean ± SD).

helically along the inflorescence axis; they are empty at the proximal end of the axis but subtend flowers further up. Like the axis, the bracts are covered with glandular uniseriate trichomes. The flower sizes vary according to their position within the inflorescence. The largest are located in the midregion, with smaller ones toward the base and apex (Fig. 1).

Bdallophytum americanum and *B. andrieuxii* are dioecious. However, the number of flowers varies for each species. *Bdallophytum americanum* has the largest flowers of the genus but the fewest flowers per inflorescence (ca. 12–19) (Figs. 1A–1C). By comparison, *B. andrieuxii* has more flowers, but they are smaller (ca. 42–84) and their inflorescences are dimorphic. The male inflorescences are longer and thinner than the female ones (Figs. 1D and 1E), and the perigone color is different for each floral morph (Fig. 1F). *Bdallophytum oxylepis* is a gynomonoecious species with approximately 41–62 flowers, of which 52 ± 5 are bisexual and 7 ± 5 are female (Figs. 1G and 1H). The floral morphs in this species vary according to their position along the inflorescence axis, and four regions are recognized based on their floral size and sexuality (Fig. 2A). Region I is in the most proximal portion of the axis; this contains small floral buds with well-developed perigone, but they do not develop sexual organs (Fig. 2B). Further up, region II contains smaller, bisexual flowers with well-developed sexual organs, although the ovary remains small, with only a few ovules (Fig. 1C). In region III, a large part of the inflorescence is covered with bisexual flowers, which have large ovaries and numerous ovules. The number of stamens varies according to the flower position along this region and decreases toward the apex (Fig. 2D). Region IV covers the distal portion of the inflorescence, with only female flowers. These flowers are small, with a reduced ovary and few ovules (Fig. 2E). The sterile structures in region I and the small bisexual flowers in region II develop after the other flowers. Despite the small size of the female flowers in region IV and bisexual flowers in region II, they have fully developed gynoecia (Figs. 2F–2I). Moreover, the pollen grains from region II flowers are well-developed (Fig. 2J) and were even observed at anthesis with dehiscent anthers (Fig. 2D). Therefore, all *B. oxylepis* flowers in regions II, III, and IV are functional. The development of female and bisexual flowers is described in detail below. Table 1 summarizes the inflorescence morphology of the *Bdallophytum* species.

Floral morphology

Bdallophytum flowers have an undifferentiated perianth, hereinafter referred to as a perigone, and formed by basally connate tepals (Figs. 1C, 1F, and 1H). The number of perigone lobes varies between the three species and among flowers of the same species (see Table 1). The perigone is dark-purple in both morphs of *B. americanum* (Fig. 1C). However, in *B. andrieuxii*, it is reddish in male floral morphs and brown to red in females (Fig. 1F). In *B. oxylepis*, it is reddish for both floral types (Fig. 1H). In all species, uniseriate glandular trichomes cover the dorsal side of the perigone (Figs. 3A and 3B). The trichomes are glandular, as they reacted positively to the PAS stain for detecting polysaccharides (Fig. 3C).

The stamens of male and bisexual flowers are completely connate along the filaments and bases of the anthers, forming a synandrium as a columnar androecium (Figs. 3D–3K). The number of stamens is not correlated to the number of tepals (see Table 1). Each stamen

Fig. 1. General morphology of *Bdallophytum*. *Bdallophytum americanum* (A–C). *Bdallophytum andrieuxii* (D–F). *Bdallophytum oxylepis* (G–H). (A) Male inflorescence. (B) Female inflorescence. (C) Male (up) and female (down) flowers. (D) Male inflorescence. (E) Female inflorescence. (F) Male (up) and female (down) flowers. (G) Gynomonoecious inflorescence. (H) Female (up) and bisexual (down) flowers. an, anther; ca, connective appendage; eb, empty bract; fb, floral bract; ff, fused filaments; ia, inflorescence axis; ov, ovary; p, peduncle; pe, perigone; pl, perigone lobe; st, stigma; sty, style; syn, synandrium. [Colour online.]

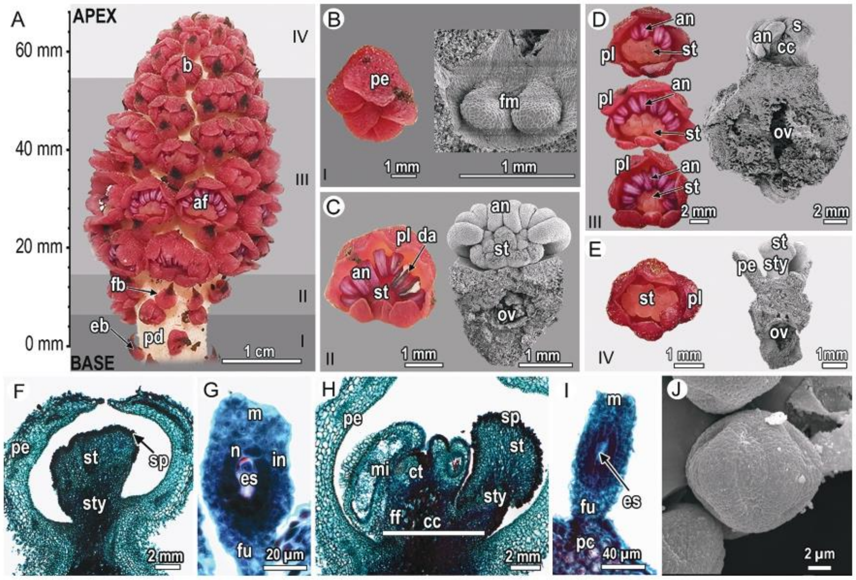
has one vascular bundle (Figs. 3E–3G). In *B. americanum* and *B. andrieuxii*, the anthers are apically free (Fig. 3F) with a connective appendage. The distal connective tissue of *B. americanum* is extended to form long acute tips (Fig. 3G) and is vascularized, whereas the connective appendages of *B. andrieuxii* are inconspicuous. The anthers in the three species are completely adnate to the filaments, and the dehiscence is extrorse (Figs. 3G–3J) by longitudinal slits. The anthers are yellow in *B. americanum* (Fig. 3H), reddish in *B. andrieuxii* (Fig. 3I), and burgundy in *B. oxylepis* (Fig. 3J).

The gynoecium of female and bisexual flowers has an inferior and unilocular ovary, an irregular intrusive parietal placentation (Figs. 3L–3N), and numerous ovules. Notably, in *B. oxylepis*, the stamens and style are fused, forming a gynostemium (Fig. 3N). The ovaries are concrescent in the three species (Fig. 3O). The ovary wall contains sclerenchyma formed by sets of cells with lignin thickenings (Fig. 3P) and calcium oxalate crystals (Fig. 3Q), which are also present in the stigmatic cells. The number of carpels is difficult to determine due to the concrescence of the ovaries and intrusive placentation. However, the number of stigmatic lobes in each flower is used to determine the number of carpels (Table 1). The number of carpels also varies within the same inflorescence along the axis. The style is cylindrical and hollow. In a cross-section, the vascular bundles apparently correspond to the number of carpels (Fig. 3R). The stigma is multilobed, comprising uniseriate stigmatic papillae with dense cytoplasm in their apical cells (Fig. 3S). The stigma is bright yellow in *B. americanum*, red to brown in *B. andrieuxii*, and pale red in *B. oxylepis*. Table 1 summarizes the floral morphological traits.

Early flower development

Development in *Bdallophytum* begins within the host root. During the early stages, only a bulge is seen from the outside (Fig. 4A). The young inflorescence (exophyte)

Fig. 2. Floral morphs of *Bdallophytum oxylepis* along the inflorescence axis. (A) Inflorescence of *B. oxylepis* divided into four regions (I–IV) (B) Floral bud (left) in the basal area whose sexual organs do not develop (right) from region I. (C) Bisexual flowers (left) near the base with a small ovary (right) from region II. (D) Bisexual flowers with variation in stamen number (left) from region III. These morphs have the largest ovaries of all flowers (right). (E) Female flowers from region IV with a small-sized and fully developed ovary (right) from region I. (F) Well developed female flower from region IV. (G) Ovules fully developed from region IV; female flower. (H) Fully developed bisexual flowers from region II. (I) Mature ovules from small basal bisexual flowers of region II. (J) Fully developed pollen grains in dehisced anthers from bisexual flowers of region II. af, anthetic flower; an, anther; b, floral bud; cc, central column; ct, connective tissue; da, dehisced anther; eb, empty bract; es, embryo sac; fb, floral bract; ff, fused filament; fm, floral meristem; fu, funiculus; in, integument; m, micropyle; mi, microsporangium; n, nucellus; ov, ovary; pc, placenta; pd, peduncle; pe, perigone; pl, perigone lobe; sp, stigmatic papillae; st, stigma; sty, style. [Colour online.]



emerges a few centimeters below the substrate surface (Fig. 4B), completely covered with bracts (Fig. 4C), and it progressively breaks the bark of the host root. The proximal bracts are empty, while the distal bracts subtend floral meristems (Fig. 4D). The inflorescence axis begins to vascularize, and mainly the empty bracts show high tannin contents (Fig. 4E). The floral bracts and subtended floral meristems grow perpendicular to the inflorescence axis. The floral meristems develop acropetally, with the youngest structures at the apex. Owing to their perpendicular position, the meristems have adaxial and abaxial sides. The abaxial side is proximal, and the adaxial side is distal to the floral bract (Fig. 4F). The floral development begins with the formation of an elliptical dome-shaped floral meristem (Fig. 4G). The floral meristem begins to form 5–11 unequally distributed primordia corresponding to the tepals (Fig. 4H). The primordia grow unilaterally and disordered, and the tepals on the adaxial side grow and differentiate (Fig. 4I) before those on the abaxial side (Figs. 4J and 4K). Differentiation of the adaxial and abaxial sides of the perigone produces zygomorphic floral symmetry, and the central zone of the floral bud invaginates (Figs. 4K and 4L). Before this stage, it is difficult to determine the sex of the flower, and the invagination marks the beginning of the reproductive organ formation. The bisexual flowers of *B. oxylepis* are recognizable at this stage, because the invagination occurs asymmetrically, leaving the adaxial side broader than the abaxial one (Fig. 4M).

Synandrium development

Stamen primordia appear after the initiation of all the tepals (Fig. 5A). The number of stamen primordia varies in all species (see Table 1) and depends on the flower position along the inflorescence axis. Initially, the developing flowers are similar in size along the axis. When the stamen primordia elongate and begin to differentiate, the filaments are not yet visible (Fig. 5B). As the stamens grow, the anthers remain apically free (Fig. 5C). Two thecae appear later on each anther. In *B. americanum*, an elongated apical connective appendage develops on

Fig. 3. Morphoanatomy of *Bdallophytum*. *Bdallophytum americanum* (B, D–I, L, and S). *Bdallophytum andrieuxii* (B–C, J, M, P–R, and T). *Bdallophytum oxylepis* (A, K, and N–O). (A) SEM micrograph of a floral bud and subtending bract with trichomes on its surface. (B) Detail of uniseriate trichomes, SEM micrograph. (C) Section of a perigone lobe showing the trichomes on the ventral side (micrography at the corner as reference for perigone position). (D) Transverse section showing the filaments forming a solid column. (E) Transverse section of synandrium stained with Safranin–Fast green at the base of anthers showing the attachment to filaments column and the basally united perigone. (F) Transverse section at more distal position showing the free portion of stamens and the perigone lobes. (G) Synandrium showing the connective appendages on the anthers. (H) Longitudinal section of connective appendage showing vascularization. (I and J) Longitudinal section of male flowers. (K) Longitudinal section of bisexual flower showing anther attachment. (L and M) Longitudinal sections of female flowers. (N) Longitudinal section of bisexual flower showing the ovary. (O) Concrescent ovaries (dotted lines) with irregular intrusive parietal placentation. (P) Ovary wall in transverse section showing sclerenchymatic cells. (Q) Calcium oxalate crystals (white arrowheads) on the ovary wall cells. (R) Transverse section of style showing the stylar canal and a vascular bundle per carpel. (S) Transverse section of stigmatic papillae (crystals on the corner). an, anther; c, connective tissue; cp, connective appendage; d, dorsal side; fb, floral bract; ff, fused filaments; gyn, gynostemium; o, ovules; ov, ovary; pc, placenta; pe, perigone; pl, perigone lobe; sc, stylar canal; scl, sclerenchymatic cell; sp, stigmatic papillae; st, sigma; sty, style; svb, stamen vascular bundle; syn, synandrium; tr, trichomes; v, ventral side; vb, vascular bundle. [Colour online.]

each anther (Fig. 5D). The fused filaments begin to grow into a solid column supporting the anthers to form the synandrium (Fig. 5E). Two microsporangia develop per theca, resulting in tetrasporangiate anthers (Fig. 5F). Finally, the longitudinal slits are conspicuous before anthesis; thus, the male flowers of *B. andrieuxii* and *B. americanum* have completed their development (Figs. 5G and 5H). The androecium of *B. andrieuxii* has two morphs, one with the thecae close and parallel (Fig. 5G) and the second with the thecae distant at a divergent angle, exposing the connective tissue (Fig. 5H). Furthermore, in this latter androecium type, the anthers have a short apical connective appendage. Both morphs are found in the same inflorescence of *B. andrieuxii* in no apparent pattern. Unlike *B. andrieuxii*, the connective appendages are longer in *B. americanum* (Fig. 5I). At anthesis, the anthers dehisce by longitudinal slits (Fig. 5J), releasing the pollen grains. *Bdallophytum americanum* has the largest pollen grains (Supplementary data, Fig. S1[1]); these are tetra- or pentacolpate, in the ratio 1:2, respectively, and their ornamentation is conspicuously rugose (Fig. 5K). The pollen grains of *B. andrieuxii* are tri- or tetracolpate in the ratio 1:1 with rugose ornamentation (Fig. 5L). Finally, the pollen grains of *B. oxylepis* are tetra- or pentacolpate in the ratio 2:1 with rugulate ornamentation (Fig. 5M). Both types of pollen grains are found for each taxon within the same microsporangium.

Gynoecium development

After perigone initiation, invagination occurs and the carpel edges begin to fold (Fig. 6A). The invagination of carpels penetrates deeply into the inflorescence axis, where ovary development begins (Fig. 6B). Subsequently, the carpel edges continue folding to form the stigmatic lobes; the style appears below these (Fig. 6C), and the placentae begin to protrude into the ovarian cavity (Fig. 6D). Stigmatic papillae then begin to form, while the style continues to elongate (Fig. 6E). Simultaneously, the ovary expands, and the ovule primordia emerge from the placentae (Figs. 6F and 6G). The integument primordium protrudes later, forming an annular structure around the nucellus (Fig. 6H). Ovules are unitegmic, as only one integument develops (Fig. 6I). When the nucellus is wholly covered with the integument, the ovules are fully developed. These are orthotropous with a short funiculus (Fig. 6J). The ovules are then completely covered with mucilage, with only the micropyles exposed (Fig. 6K). The mucilage is secreted by the inner epidermal cells of the ovary (Fig. 6L). Finally, the fully developed gynoecium of *Bdallophytum* comprises the stigma, style, and inferior ovary (Fig. 6M).

Gynostemium development

Bisexual flowers develop acropetally along the inflorescence axis of *B. oxylepis* (Fig. 7A). In the early stages, as mentioned above, flower invagination occurs asymmetrically after perigone formation and is more extensive adaxially than abaxially. The carpel edges begin to fold on both sides to form the stigma (Fig. 7B), and the invagination produces the ovary and inferior part of the style as in female flowers (Fig. 7C). However, while the stigma lobes further differentiate, the adaxial side of the developing stigma broadens (Fig. 7D). At this early stage, the young anthers are only distinguished in the longitudinal sections, where the microsporangia and connective tissue are recognizable and share vascularity with the gynoecium (Fig. 7E). After several folds and a size increase, the stigma begins to form the stigmatic papillae, and the anthers on the adaxial side are distinguished due to the emerging thecae.

Unlike the male flowers of the other species, the stamens of *B. oxylepis* develop asynchronously (Fig. 7F). As in the male flowers, the stamen filaments are connate. However, in bisexual flowers, the filaments are also adnate to the style, forming a gynostemium (Fig. 7G). As development progresses, the dorsiventral arrangement of the

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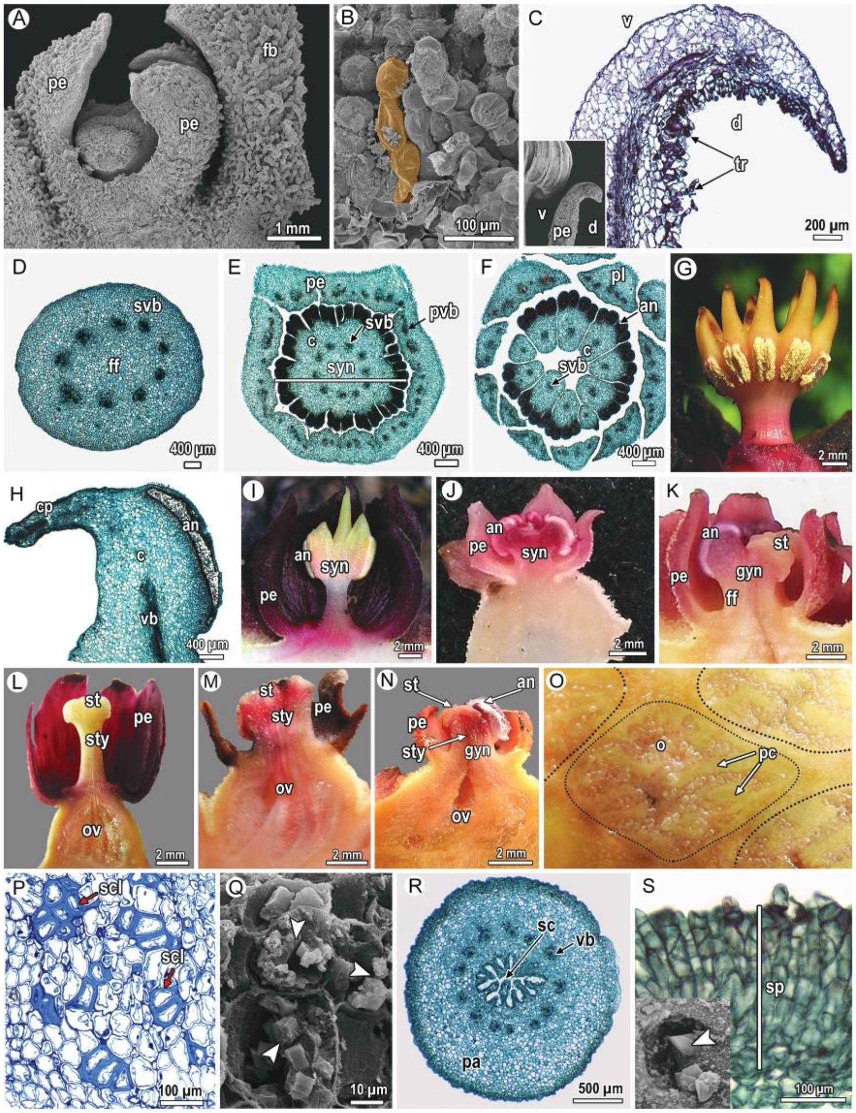
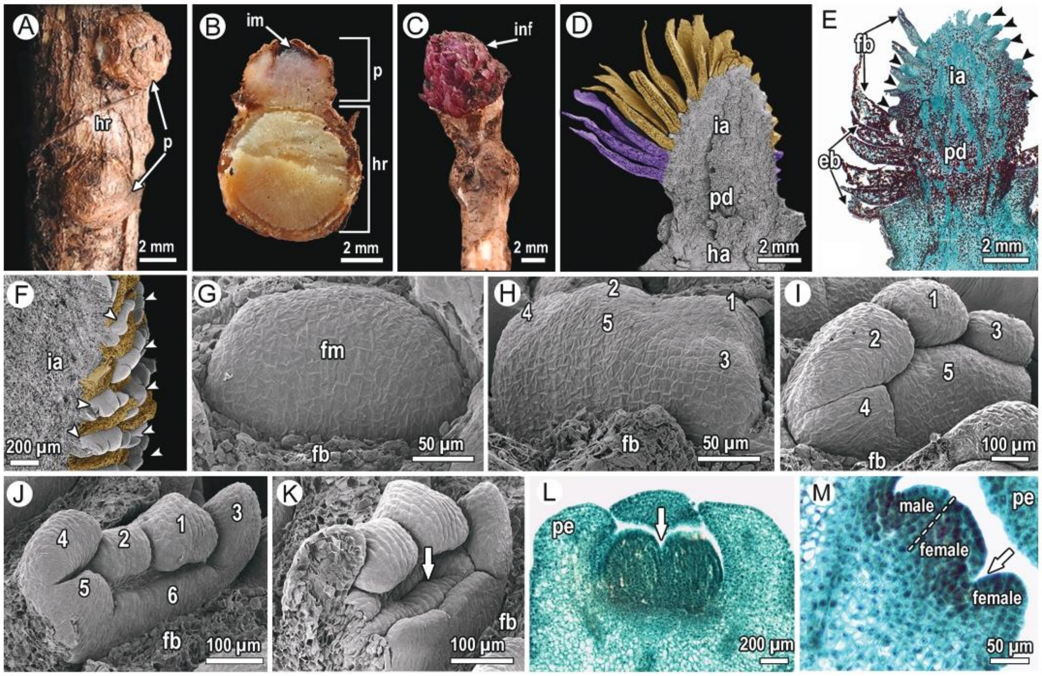
[1] Supplementary data are available with the article at https://doi.org/10.1139/cjb-2020-0025.



gynoecium and androecium is revealed. In the latter, the tetrasporangiate anthers are well-differentiated, while the stigma has differentiated papillae and noticeable folds (Fig. 7H). Additionally, the vascularization is prominent on the dorsal side, and the anthers elongate to partially cover the stigma (Fig. 7I). The longitudinal slits of the anthers are now visible (Fig. 7J). In the transverse sections during the microspore mother cell stage, the perigone and its vascularization are observed, together with the adnate androecium and gynoecium. The vascular bundles on the abaxial gynostemium serve the carpels, while those at the adaxial side are shared between the stamens and carpels (Fig. 7K). In the later stages, the gynostemium elongates producing a central column (Figs. 7L and 7M). Finally, at anthesis, the anthers dehisce by longitudinal slits, and their extrorse position and adnate attachment are well-distinguished, while the stigma is completely covered with stigmatic papillae (Fig. 7N).

Fig. 4. Early floral development. *Bdallophytum americanum* (L). *Bdallophytum andrieuxii* (C–K). *Bdallophytum oxylepis* (A, B, and M). (A) Parasite growing inside the host root (arrows). (B) Cross-section of the host root showing the parasite inflorescence meristem. (C) Emerging young inflorescence covered by bracts. (D) Longitudinal section of a young inflorescence covered by empty bracts (purple) at the base and floral bracts (yellow) in the distal area. (E) Same stage as D but in a longitudinal section stained with Safranin–Fast green, showing inflorescence axis vasculature, the high content of tannins mainly in empty the bracts, and the floral meristems (black arrowheads). (F) Floral meristems (white arrowheads) each subtended by its floral bract (in yellow), bracts were removed to expose the meristems. (G) Floral meristem in its elliptical dome-shape. (H) Floral meristem with five irregular protuberances (listed in order of appearance). (I) Tepals lobes differentiation at adaxial side. (J) Perigone differentiation at abaxial side. (K) Formation of carpels by invagination of central area (arrow) while abaxial perigone lobes differentiate. (L) Longitudinal section of developing floral bud showing the early invagination (arrow). (M) Asymmetrical invagination (arrow) of bisexual flower bud. eb, empty bract; fb, floral bract; fm, floral meristem; ha, haustorium; hr, host root; ia, inflorescence axis; im, inflorescence meristem; inf, inflorescence; p, parasite; pd, peduncle; pe, perigone. [Colour online.]

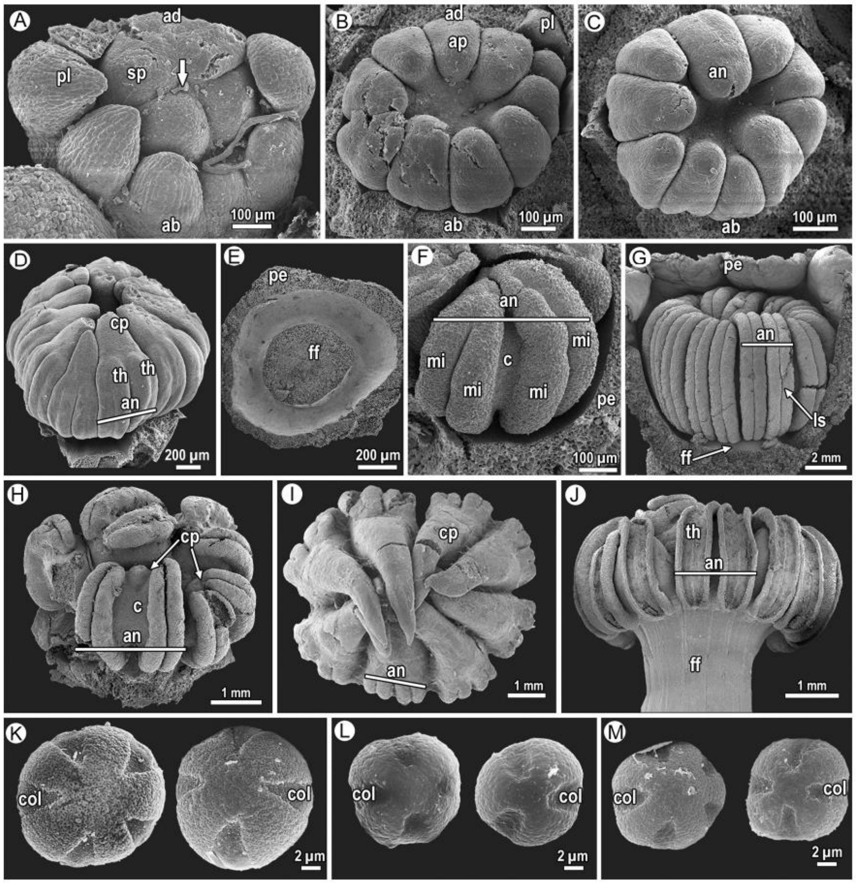


Embryology

Microsporogenesis and microgametogenesis

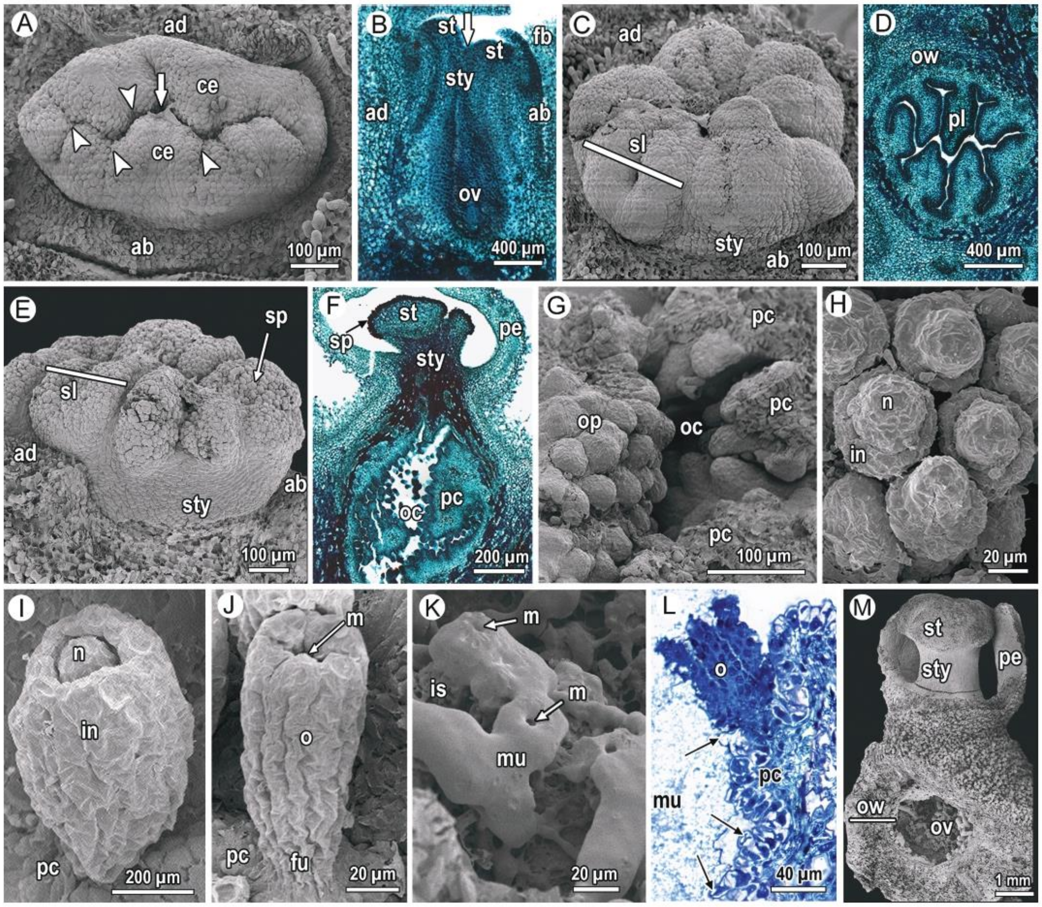
Microsporogenesis and microgametogenesis follow a similar pattern in the three species of *Bdallophytum*. Microsporogenesis begins in the stamen primordia, when the anthers begin to differentiate (Fig. 8A). In a close-up of microsporangia, the archesporial tissue is surrounded by the protodermis and two layers produced by the primary parietal layer: the outer and inner secondary parietal layers (Fig. 8B). Both secondary parietal layers then undergo a further periclinal division that produces the anther wall. The anther wall development belongs to basic type. The protodermis transforms into the epidermis, the outer parietal layer forms the endothecium and a middle layer, and the inner parietal layer divides periclinally to form another middle layer and the tapetum (Fig. 8C). When the anthers are conspicuously bithecal (Fig. 8D), the anther wall comprises the epidermis, endothecium, two middle layers, and a biseriate tapetum of irregularly shaped cells with dense cytoplasm surrounding the microspore mother cells (Fig. 8E). The microspore mother cells surrounded by callose undergo simultaneous meiosis; hence, tetranucleate microspore mother cells may be seen before cytokinesis occurs (Fig. 8F). After division, the callose layer surrounding the tetrads thickens. The secretory-type tapetum and its two layers are well-distinguished in some zones. The middle layers begin to degenerate (Fig. 8G). Tetrahedral (Fig. 8H) and rhomboidal (Fig. 8I) tetrads were found in all three species within the same microsporangium.

Fig. 5. Androecium development. *Bdallophytum americanum* (A–E, I, and K). *Bdallophytum andrieuxii* (F–H, J, and L). *Bdallophytum oxylepis* (M). (A) Stamen primordia initiation after invagination (arrow) surrounded by perigone lobes growing unilaterally. (B) Stamen primordia differentiation. (C) Elongating young anthers. (D) Anthers becoming bithecal; connective appendage elongating on *B. americanum*. (E) Transverse section of the central column formed by fused filaments. (F) Tetrasporangiate anther showing the connective between thecae (ventral view). (G) Synandrium with parallelly arrangement stamens. (H) Synandrium; arrangement of the stamens is non-parallel. (I) Apical view of synandrium showing the connective appendage of each anther. (J) Synandrium with dehisced anthers. (K) Penta- (left) and tetracolpate (right) pollen grains. (L) Tetra- (left) and tricolpate (right) pollen grains. (M) Penta- (left) and tetracolpate (right) pollen grains. ab, abaxial side; ad, adaxial side; an, anther; ap, anther primordium; c, connective tissue; col, colpi; cp, connective appendage; ff, fused filaments; ls, longitudinal slit; mi, microsporangium; pe, perigone; pl, perigone lobe; sp, stamen primordium; th, theca.



After the microspores are separated, the middle layers completely collapse, and the endothecium appears two or three-layered (Fig. 8J). When the pollen is mononucleated, the colpi begin to form, and microgametogenesis begins (Fig. 8K). The endothecium has fibrous thickenings and is multiseriate toward the connective tissue, while the septum narrows and the stomium differentiates (Fig. 8L). The anther wall then thins, since the secretory tapetum is almost completely degenerated, no middle layers are present, and the epidermal cells become thin (Fig. 8M). The pollen grains undergo the first mitotic division, producing two-celled pollen grains (Fig. 8N). Finally, at anthesis, the septum between the two microsporangia collapses, and the stomium disintegrates (Figs. 8O and 8P). The pollen grains are three-celled when released (Fig. 8Q), and germinating

Fig. 6. Gynoecium development. *Bdallophytum americanum* (G, I, and J). *Bdallophytum andrieuxii* (A, E, and K–M). *Bdallophytum oxylepis* (B–D, F, H, K, and L). (A) Carpels after invagination occurred (arrow) and the folding of carpel edges (arrowheads). (B) Longitudinal section of A showing the developing ovary. (C) Stigmatic lobe formation and elongation of style. (D) Transverse section of C showing the protruding of placentae. (E) Stigmatic papillae formation and elongation of style. (F) Longitudinal section of developing gynoecium showing the ovarian cavity and the start of ovule development. (G) Inferior ovary with developing placentae and ovule primordia. (H) Developing ovules with integument. (I) Integument covering the nucellus almost completely. (J) Mature orthotropous ovule with short funiculus. (K) Mature ovules covered by mucilage leaving the micropyle free. (L) Cells of the inner epidermis of the ovary wall secreting mucilage. (M) Fully developed gynoecium. (O) Transverse section of stigmatic papillae. (Q) Transverse section of the ovary; the placentae are irregularly intrusive. ab, abaxial side; ad, adaxial side; ce, carpel edge; fu, funiculus; in, integument; is, integument surface; m, micropyle; mu, mucilage; n, nucellus; o, ovule; oc, ovarian cavity; op, ovule primordia; ov, ovary; ow, ovary wall; pc, placenta; pe, perigone; sl, stigmatic lobe; sp, stigmatic papillae; st, stigma; sty, style. [Colour online.]



pollen grains were found on the anthers of *B. andrieuxii* (Fig. 8R).

Megasporogenesis and megagametogenesis

Megasporogenesis and megagametogenesis are identical for the three species of *Bdallophytum*. Megasporogenesis begins in the ovule primordium comprising an archesporial cell (Fig. 9A). At a later stage, the integument primordium arises to cover the unistratified nucellus, while the archesporial cell is maintained (Fig. 9B). As the integument grows, the archesporial cell expands and functions directly as a megaspore mother cell, as no parietal layers

Fig. 7. Bisexual flower development of *Bdallophytum oxylepis*. (A) Longitudinal section of the young inflorescence showing the acropetally development of flowers. (B) Carpel edges folding (arrowheads) after invagination (arrow); the asymmetry in adaxial and abaxial areas are shown (the perigone was removed). (C) Longitudinal section of floral bud showing the invagination that gives rise to the gynoecium. (D) Stigmatic lobe formation. The distal portion of the adaxial area without folds corresponds to staminal tissue (green). (E) Longitudinal section of D showing the staminal tissue differentiation at the distal portion of adaxial side (at left of dotted line). (F) Differentiation of stamens and stigmatic papillae formation. (G) Longitudinal section of F showing the adnate attachment of anthers and the filaments fused to style forming the gynostemium. (H) Tetrasporangiate anthers. (I) Longitudinal section of H showing the elongating anther covering a portion of stigma. (J) Stamens and stigma completely differentiated from each other. (K) Flower transverse section showing the connective tissue with one vascular bundle per carpel at abaxial side (arrowheads) but shared among more than one stamen and carpel at the adaxial side. The vasculature of perigone is also shown. (L) Longitudinal section of gynostemium showing the shared vascularization at adaxial side; together, stamen and style form a central column. (M) Lateral view of gynostemium. (N). Anthetic flower with dehisced anthers. a, androecium; ab, abaxial side; ad, adaxial side; an, anther; avb, axis vascular bundle; cc, central column; ce, carpel edges; ct, connective tissue; ei, early invagination; fb, floral bract; ff, fused filaments; fv, floral vascularization; gyn, gynostemium; ls, longitudinal slit; ov, ovary; pe, perigone; sa, stigmatic area; sc, stylar canal; shv, shared vascular bundle; sl, stigmatic lobe; sp, stigmatic papillae; st, stigma; sta, stylar area; sty, style; th, thecae; va, ventral area; vb, vascular bundle. SEM figures were artificially colored (blue, androecium; green, gynoecium). [Colour online.]

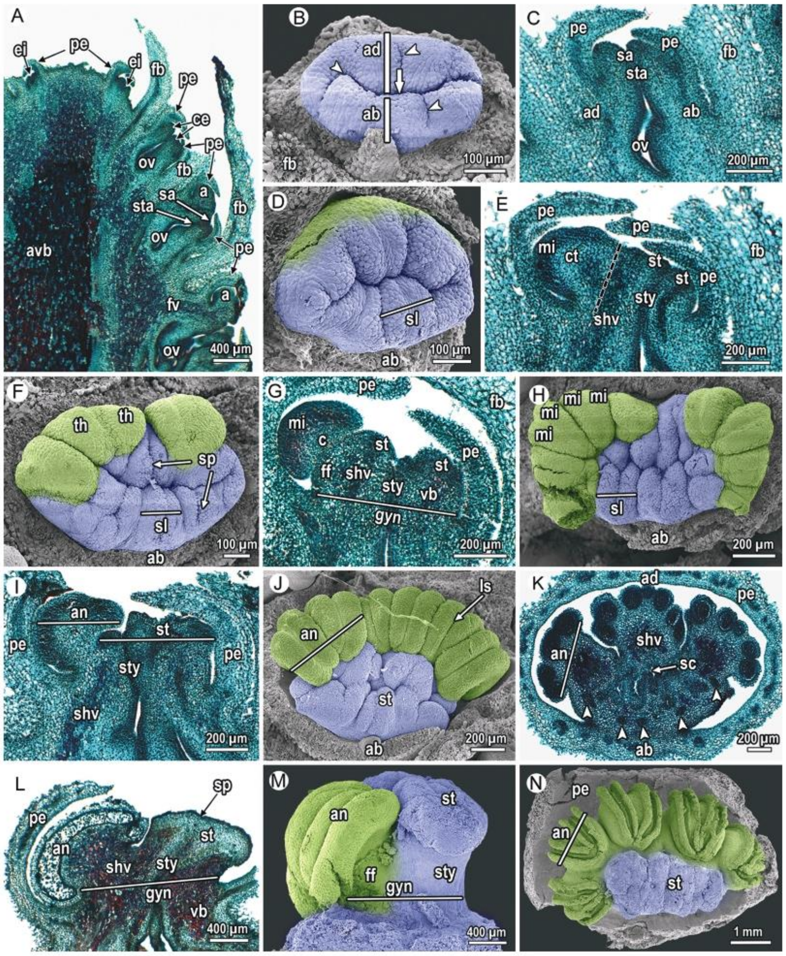


Fig. 8. Microsporogenesis and microgametogenesis. *Bdallophytum americanum* (A–B, L–O, and Q). *Bdallophytum andrieuxii* (D–E, G, P, and R). *Bdallophytum oxylepis* (C, F, and H–K). (A) Young tetrasporangiate anther. (B) Detail of one microsporangium from the latter stage showing the parietal layers. (C) Microsporangium with epidermis and differentiated sporogenous tissue; the two parietal layers undergo periclinal divisions (arrowheads) to form the anther wall. (D) Tetrasporangiate apically free anthers. (E) Detail of anther wall at microspore mother cell stage. (F) Microspore mother cell during simultaneous cytokinesis showing the nuclei (arrowheads) without cytokinesis. (G) Detail of anther wall at microspore tetrads stage. (H) Tetrahedral tetrad. (I) Rhomboidal tetrad. (J) Magnification of anther wall at free microspore stage. (K) Microspores. Start of gametogenesis and colpi formation (arrowheads). (L) Detail of theca at binucleate pollen grain stage. (M) Higher magnification of anther wall at same stage showing endothecium with fibrous thickenings. (N) Bicellular pollen grain. (O) Dehisced anther. (P) Detail of anther wall after releasing of pollen grains. (Q) Three-celled pollen grain. (R) Germinating pollen grain in *B. andrieuxii* microsporangium. an, anther; ar, archesporial tissue; aw, anther wall; ca, callose; ct, connective tissue; en, endothecium; ep, epidermis; fm, free microspore; ft, fibrous thickenings; gc, generative cell; ipl, inner secondary parietal layer; mi, microsporangium; ml, middle layers; mmc, microspore mother cell; ms, microspores; opl, outer secondary parietal layer; pd, protodermis; pg, pollen grain; pt, pollen tube; s, septum; spc, spermatic cells; st, sporogenous tissue; sto, stomium; t, tapetum; td, tetrad; th, thecae; vb, vascular bundle; vc, vegetative cell. [Colour online.]

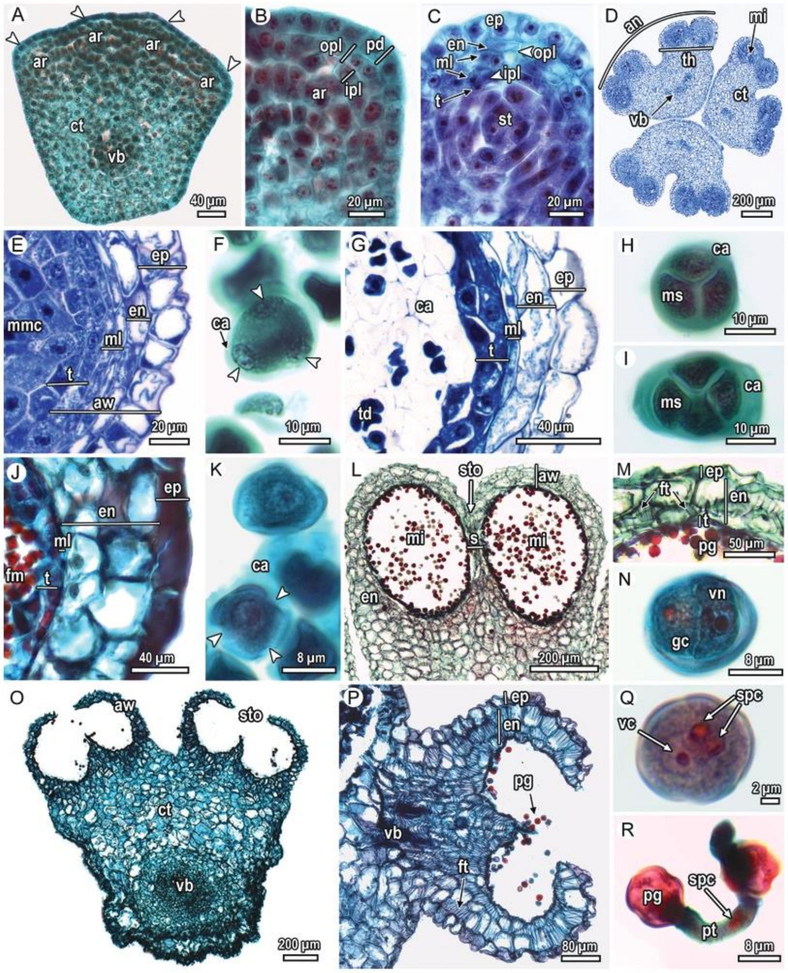
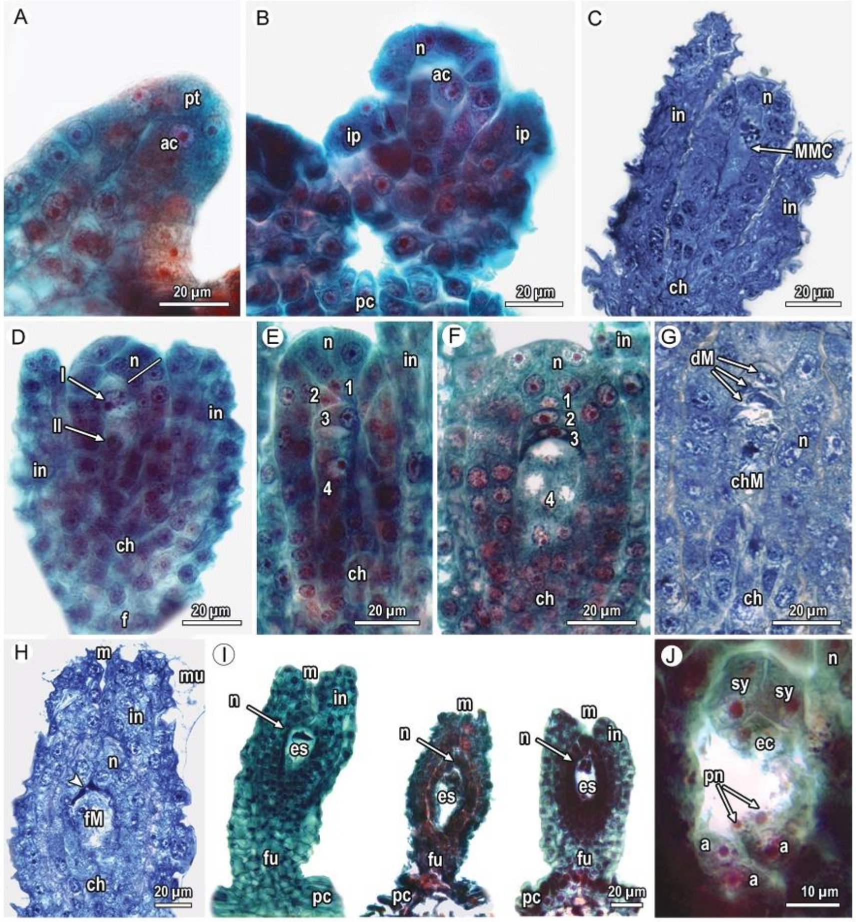


Fig. 9. Megasporogenesis and megagametogenesis. *Bdallophytum americanum* (B, E, F, and I). *Bdallophytum andrieuxii* (A, C, G, and H). *Bdallophytum oxylepis* (D, J, and I). (A) Young ovule with protodermis and archesporial cell. (B) Ovule with unistratified nucellus and integument primordium. (C) Ovule at megaspore mother cell stage. (D) Meiosis I of the megaspore mother cell resulting in two cells (I, II). (E) T-shaped megaspore tetrad (1–4). (F) Linear megaspore tetrad (1–4). (G) Micropylar megaspores in degeneration. (H) Chalazal functional megaspore with the remnants of the three micropylar megaspores (arrowhead); note ovule covered by mucilage. (I) Mature ovules of the three species. *Bdallophytum americanum* (left), *B. andrieuxii* (centre), *B. oxylepis* (right). (J) Polygonum-type embryo sac. a, antipodal cells; ac, archesporial cell; ch, chalaza; chM, chalazal megaspore; dM, degenerating megaspores; ec, egg cell; es, embryo sac; fu, funiculus; fM, functional megaspore; in, integument; ip, integument primordium; m, micropyle; MMC, megaspore mother cell; mu, mucilage; n, nucellus; pc, placenta; pn, polar nuclei; pt, protodermis; sy, synergid cell. [Colour online.]



are formed, and the nucellus still comprises a single layer (Fig. 9C). When the megaspore mother cell undergoes meiosis I to produce a dyad of megaspores, the nucellus divides periclinally, increasing the number of layers to two. Since the second stratum of the nucellus originates in the epidermis rather than the parietal layer, these are pseudocrassinucellate ovules (Fig. 9D). After meiosis II, a T-shaped (Fig. 9E) or linear (Fig. 9F) tetrad of megaspores is formed. The megaspore closest to the chalaza grows, while the other three begin to degenerate (Fig. 9G). Finally, the chalazal functional megaspore grows, displacing the remnants of the other megaspores toward the micropyle. At this stage, mucilage begins to cover the ovules, and megagametogenesis begins (Fig. 9H). After three mitotic divisions, the fully developed ovules (Fig. 9I) shelter the *Polygonum*-type embryo sac comprising an egg cell, two synergids, three antipodal cells, and a central cell with two polar nuclei (Fig. 9J).

Discussion

General morphology of inflorescences in *Bdallophytum* and Cytinaceae

This study describes and compares the inflorescence and floral morphology of the three species of *Bdallophytum*. The studied genus is morphologically similar to *Sanguisuga caesarea* in several aspects: it has the same type of inflorescence, and the flowers grow in identical arrangements (Fernández-Alonso and Cuadros-Villalobos 2012). Recently, *S. caesarea* was even transferred to *Bdallophytum* as *B. caesareum* (Fern. Alonso & H. Cuadros) Byng & Christenh. (Christenhusz et al. 2018). However, the authors did not include a morphological discussion justifying the new combination (Nickrent 2020). Our results support the inclusion of *Sanguisuga* in *Bdallophytum* based on mainly morphological traits. The color scheme of the inflorescences and flowers is identical, and *S. caesarea* is easily confused with *B. oxylepis* due to the pale-yellow inflorescence axis, burgundy perigone, and pubescence. Furthermore, *S. caesarea* and *B. oxylepis* also are the only species with bisexual flowers within Cytinaceae and share the same dorsiventral arrangement of the sexual organs. Although *S. caesarea* is distinguished by andromonoecy, it shares the floral variation along the inflorescence with the *Bdallophytum* species, as the male flowers are at the base, while the bisexual flowers are toward the apex (Fernández-Alonso and Cuadros-Villalobos 2012).

The morphology of *Bdallophytum* profoundly differs from that of *Cytinus*, the Old World genus (Smithies and Burgoyne 2010). Unlike *Bdallophytum*, *Cytinus* has inflorescences in racemes or short spikes where the flowers are growing at ground level (Villar 1997). The color scheme in *Bdallophytum* inflorescences is consistent among the species, while in *Cytinus*, the color scheme varies between white, yellow, bright red, and dark red in different species (Watanabe 1936; Villar 1997). Despite the different colors in *Cytinus* species, the morphological patterns of both genera of Cytinaceae appear to be constant within their distribution (Villar 1997; Alvarado-Cárdenas 2015). The morphology and biogeography facilitate recognition of the genera in the family Cytinaceae.

Intra-individual variation and sexual systems in Cytinaceae

Bdallophytum flowers were reported to have 5–9 perigone lobes (Nickrent 2007; Alvarado-Cárdenas 2009). However, in this study, flowers with up to 11 perigone lobes were found in *B. americanum*. The variation in the number of tepals, stamens, and carpels are the most variable characteristics along the inflorescence axes in all *Bdallophytum* species, including *Sanguisuga* (Fernández-Alonso and Cuadros-Villalobos 2012), and this has also been observed in *Cytinus* (Villar 1997; Smithies and Burgoyne 2010). The reduction in the number of organs is the most common form of intra-individual variation reported for angiosperms (Buide 2004; Zhao et al. 2010; Shakarishvili and Osishvili 2013); and it seems to be a common pattern in Cytinaceae. Moreover, the reduction in flower size is also highly variable in *Bdallophytum* species, and it depends on the position along the inflorescence axis (Barabé et al. 2000; Zhao et al. 2010). This has been related to the maternal resource investment, where the early developed flowers are the largest and the late ones are smaller (Buide 2004). This pattern is consistent with the gradient in flower size along *Bdallophytum* inflorescences, which matches the order of flower formation.

The intra-individual variation also involves changes in floral sex, observed in *B. oxylepis*, where the number of stamens decreases as the flowers approach the distal portion of the inflorescence. This sexual variation along the axis also occurs in *Sanguisuga* (Fernández-Alonso and Cuadros-Villalobos 2012) and monoecious species of *Cytinus* (Villar 1997). In *Cytinus*, the female flowers are in the most external portion of the inflorescence surrounding the male flowers in the center (Villar 1997). Hence, the change in floral sex along the axis is a typical characteristic of the monoecious species in Cytinaceae and appears to be a common pattern in other species with monoecious sexual systems (Ushimaru et al. 2003; Shakarishvili and Osishvili 2013).

In Cytinaceae, this intra-individual variation is related to the sexual system. Here, *B. oxylepis* was classified as gynomonoecious, while Alvarado-Cárdenas (2009) and Martínez-Camilo et al. (2012) describe it as polygamous. Thus, gynomonoecy in *B. oxylepis* and the gradual loss of stamens in bisexual flowers may be a transitory step in the evolution of sexual systems from bisexual flowers over monoecy toward dioecy (Barrett 2002). However, phylogenetic testing is required to confirm this. The variation concerning the gradual loss of organs left the bisexual flowers as a remnant that reflects the ancestral condition (González and Bello 2009). As in *Bdallophytum*, *Cytinus* has dioecious and monoecious species. However, no reports of bisexual flowers in the genus have yet been published (Villar 1997; Smithies and Burgoyne 2010). Thus, unisexuality is predominant in Cytinaceae flowers (Nickrent 2007). A lack of phylogenetic information at the family level hinders the determination of the ancestral condition of their sexual systems.

Floral morphology and synorganization

Another notable feature is the synorganization of sexual organs in *Bdallophytum* species. Male flowers have a synandrium and bisexual flowers a gynostemium. An androecium with fused filaments is a common feature within the order Malvales. This is at least basally united in Malvaceae, Dipterocarpaceae (Ashton 2003), and Sarcolaenaceae (Bayer 2003). Particularly, in Malvoideae and Bombacoideae (Malvatheca clade), the flowers form an androecial tube around the style (Van Heel 1966; von Balthazar et al. 2004) rather than forming a synandrium as in *Bdallophytum*. The androecium of *Bdallophytum* is more similar to those of the species of the clade Malvatheca, in which the androecial tube formed by the fused filaments has sessile monothecal anthers (von Balthazar et al. 2006). An additional trait in the androecium is the apical extension of the anther connective tissue in *B. americanum*, which is less conspicuous in *B. andrieuxii*. This characteristic is shared with its sister group, *Cytinus* (Burgoyne 2006; Nickrent 2007); however, its role in pollination has not been studied. An apical connective extension is also present in Dipterocarpaceae species, also from the order Malvales (Maury-Lechon and Curtet 1998).

As we discussed previously, the synorganization of stamens is common in Malvales; nevertheless, the gynostemium is an unusual feature in the order (Stevens 2001). The gynostemium involves the fusion of the androecium and gynoecium (Rudall and Bateman 2002) and is well-studied in Orchidaceae, Apocynaceae (Endress 2016), and Aristolochiaceae (González and Stevenson 2000; Shaiju and Omanakumari 2010). The fusion of both sexual organs is considered the most extreme case of synorganization and has been related to pollination (Endress 2016). In *B. oxylepis*, the synorganization is congenital as in Orchidaceae and Aristolochiaceae (González and Stevenson 2000; Endress 2016), as the gynoecium and androecium are fused from the beginning of their development.

Flower development and symmetry

Bdallophytum species develop unisexual flowers without the formation of the opposite sexual organs. Following the classification of unisexual flowers, male and female flowers of *Bdallophytum* species are of type I, that is, unisexual by inception (Mitchell and Diggle 2005; Diggle et al. 2011). Nevertheless, *B. oxylepis* also develops bisexual flowers with variable numbers of stamens in an acropetal gradient. The final step of this gradient produces female flowers at the apex. Our results show no evidence of male abortion; therefore, these are also type I unisexual flowers (Mitchell and Diggle 2005). Despite the flowers developing acropetally along the axis, the small bisexual flowers at the base (region II) of *B. oxylepis* inflorescences develop later. Thus, it would be of great interest to investigate the genetics of the delayed formation of new floral meristems.

Floral development in *Bdallophytum* begins with 5–11 tepal primordia. However, they are not equally distributed on the floral meristem as in other families of the order, such as Malvaceae (Van Heel 1966) or Bixaceae (Decraene 1989). The flower development of *Bdallophytum* is also not pentamerous, as the unilateral development allows the formation of more tepals at the abaxial side. Moreover, the number of tepals does not match the number of stamens or stigmatic lobes, as in most families within the order Malvales (Heinig 1951; Decraene 1989; Decraene and Smets 1996; Nandi 1998; Randrianasolo and Miller 1999; Ashton 2003; Horn 2004; von Balthazar et al. 2004; Nickrent 2007). Most of the species in Malvales are actinomorphic from the beginning of their development (Van Heel 1966; Decraene 1989; Nandi 1998; von Balthazar et al. 2004), unlike *Bdallophytum*, whose floral meristems are zygomorphic. The early zygomorphy in unisexual flowers of *Bdallophytum* is only expressed in the perigone, owing to its unilateral development. This zygomorphy transforms to actinomorphy when the sexual organs develop, and that symmetry is subsequently retained. Changes from zygomorphy to actinomorphy are reported for other angiosperms, mainly in flowers that grow in spikes, in which, during their development, the adaxial portion is more advanced than the abaxial (Endress 1999, 2012); as in *Bdallophytum*. Within Malvales, an example occurs in Thymelaeaceae, in the species *Wikstroemia delavayi* Lecomte, where the organ initiation occurs on the abaxial side, causing zygomorphy in the earlier developmental stages. However, at maturity, the flowers are almost actinomorphic (Yao-Dong and Yin-Zheng 2002), as in unisexual flowers of *Bdallophytum*.

In contrast to unisexual flowers, the bisexual flowers of *B. oxylepis* show no changes in floral symmetry, maintaining their zygomorphy. Zygomorphy is also present in the bisexual flowers of *S. caesarea*; nevertheless, its origin is unknown (Fernández-Alonso and Cuadros-Villalobos 2012). Thus, zygomorphy appears to be a common attribute in the bisexual flowers of Cytinaceae. In *B. oxylepis*, the zygomorphy is established in early floral development, indicated by the unilateral development of the perigone (only in the early stages) and the dorsiventral arrangement of sexual organs. The establishment of zygomorphy in the early stages is a common trait in flowers with bilateral symmetry (Endress 1987). The dorsiventral arrangement of the bisexual flowers of *B.oxylepis* resembles the chimeric flowers reported in other endoparasites such as *Pilostyles boyacensis* (Apodanthaceae), whose bisexual flowers are half-staminate and half-carpellate. However, this is a rare morph in the population (González and Pabón-Mora 2017). Given that the bisexual flowers of *B. oxylepis* have specific tissues for both sexual organs and are a constant morph in the population, we discard them as chimeric flowers. Additionally, these flowers have normal-sized ovaries like female flowers; thus, the dorsiventral arrangement does not indicate that the ovaries are incompletely developed.

Embryology

Bdallophytum species exhibit embryological traits that contrast with those described for *C. hypocistis*, the only other member of Cytinaceae studied to date (Guzowska 1964, 1966). As embryology provides a set of data that strengthens the description of *Bdallophytum* as a genus, it is necessary to expand the studies to *S. caesarea*, species with significant morphological similarity to *Bdallophytum* (discussed above), to understand the embryology of the

Table 2. Embryological traits of two genera of the Cytinaceae family.

Embryological trait	*Cytinus* (Guzowska 1964, 1966; Bouman and Meijer 1994)	*Bdallophytum* (present work)
♂		
Origin of tapetum	Sporogenous tissue	Parietal
Meiosis	Successive	Simultaneous
Tetrads	Isobilateral and decussate	Tetrahedral and rhomboidal
Middle layers	2	2
Endothecium strata	1	3
Colpi on the pollen grains	2	3–5
♀		
Ovule type	Orthotropous	Orthotropous
Integuments	2 (the outer integument does not develop completely)	1
Nucellus	Tenuinucellate	Pseudocrassinucellate
Tetrads	Linear	Linear, T-shaped
Embryo sac type	Polygonum	Polygonum

whole Cytinaceae family. Moreover, embryology can undoubtedly consolidate *S. caesarea* as a member of *Bdallophytum*.

Here, we found a cluster of embryological and micromorphological traits that are conserved in *Bdallophytum* but differ from *Cytinus* and would present a strong argument supporting the inclusion of *S. caesarea* into *Bdallophytum*, if both share these traits. These are the type of microspore tetrads, a multiseriate endothecium, more than three colpi on pollen grains, the presence of T-shaped megaspore tetrads, and unitegmic ovules (Table 2).

Bdallophytum has unitegmic ovules, unlike *C. hypocistis*, in which ovules develop two integuments. However, the outer integument of the latter is arrested in development and forms an annular structure covering only the most proximal parts of the orthotropous ovules (Guzowska 1964). Regarding the order Malvales, unitegmic ovules have not been reported for any other member (Stevens 2001); therefore, this could be a synapomorphy for *Bdallophytum*. Instead, the Polygonum-type embryo sac is most common in the order Malvales (Guzowska 1964; Nickrent 2007).

Previously, Cytinaceae species were included within Rafflesiaceae, together with Apodanthaceae and Mitrastemonaceae species; all endoparasitic plant families (Meijer 1993). This inclusion was supported by embryological characteristics, such as the absence of parietal cells, an archesporial cell that functions directly as a megaspore mother cell, and linear or T-shaped megaspore tetrads (Bouman and Meijer 1994). Nevertheless, Cytinaceae have been reclassified as a member of the order Malvales based on phylogenetic studies (Nickrent 2007), although its relationships remain uncertain (Hernández-Gutiérrez and Magallón 2019). It is expected that the data here obtained on morphology, development, and embryology of the genus *Bdallophytum*, will be useful to further phylogenetic and evolutionary studies on the family Cytinaceae.

Conclusions

This work provides information on the Cytinaceae family, detailing the inflorescence and flower morphology, floral development, and embryology of the three species of the genus *Bdallophytum*. All of the studied species show intra-individual variation along the inflorescence axis, which appears to be a common pattern within Cytinaceae. However, it is more evident in the gynomonoecious species *B. oxylepis*. The sexual systems are variable; nevertheless, the unisexual flowers are predominant in most of the species. All of the species show synorganization in sexual organs, a synandrium in male flowers, and a gynostemium in bisexual flowers of *B. oxylepis*. The unisexual flowers of *Bdallophytum* exhibit a change in their floral symmetry from zygomorphy to actinomorphy during their development, unlike the bisexual flowers, which retain their zygomorphy. The embryological traits show some dissimilarities from *Cytinus*, forming a unique combination of traits that define *Bdallophytum* within Malvales. The most important characteristic is the unitegmic ovules, proposed here as a genus synapomorphy. More species of Cytinaceae must be included in phylogenetic studies and additional morphological and embryological traits incorporated in analyses to clarify the position of Cytinaceae in the order Malvales. Finally, an increase in embryological studies on Cytinaceae or other parasites will elucidate ecologically and evolutionarily important characteristics in specialized groups such as endoparasites.

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Discusión general

Este es el primer trabajo que describe parte del ciclo de vida de las endoparásitas del género *Bdallophytum* (Cytinaceae) en México abarcando las interacciones planta-polinizador de las especies *B. americanum*, *B. andrieuxii* y *B. oxylepis* y su desarrollo floral. Los resultados muestran que la unisexualidad en las flores es una característica reproductiva sobresaliente en las tres especies estudiadas de *Bdallophytum*. Tanto *B. americanum* como *B. andrieuxii* son dioicas ya que tienen inflorescencias con flores unisexuales, por lo que deben tener una polinización cruzada obligada. Mientras tanto, en este estudio se demostró que *B. oxylepis* tiene un sistema sexual ginomonoico, antes descrito como poligamomonoico (Alvarado-Cárdenas 2009; Martínez-Camilo *et al.* 2012). En este caso *B. oxylepis* tiene flores bisexuales en la base de la inflorescencia y flores femeninas en la zona apical, por lo que estas últimas requieren el arribo de polen de otras flores para llevar a cabo la fecundación.

Polinización. Los resultados de polinización muestran que las especies estudiadas de *Bdallophytum* presentan polinización especialista. Las flores de *Bdallophytum* se caracterizan por ser de colores rojizo-oscuros y por emitir aromas fétidos que son típicos de la sapromiofilia, un síndrome de polinización asociado con moscas carroñeras (Faegri y van der Pijl, 1979; Willmer 2011). Por lo anterior, se esperaba que las moscas carroñeras fueran los polinizadores para las especies de *Bdallophytum*. Sin embargo, después del monitoreo de poblaciones de las tres especies, se demostró que la polinización de *B. americanum* se lleva a cabo por sírfidos de utilidad para estudios forenses, por lo que, a pesar de no ser moscas carroñeras, el comportamiento carroñero se mantiene y el síndrome de polinización se cumple para esta especie. La polinización por moscas carroñeras en dicha especie había sido reportada previamente por García-Franco y Rico-Gray (1997) en una población con flores que producen néctar, por lo que la polinización por moscas se mantiene con y sin dicha recompensa. Las flores de *B. americanum* destacan porque las flores masculinas presentan una proyección del tejido conectivo (apéndices estaminales) en la parte apical de las anteras, lo cual no se encuentra en las otras especies de *Bdallophytum*. En el presente trabajo se demuestra que esas proyecciones funcionan como señal visual y plataformas de aterrizaje de gran importancia para los polinizadores.

En cuanto a la polinización de *B. andrieuxii* y *B. oxylepis*, la polinización es especializada y se lleva a cabo por mariposas (*Cissia* sp.) y abejas (*Trigona fulviventris*), respectivamente. Aunque no se trata de los polinizadores esperados, se ha reportado que mariposas del género *Cissia* y abejas de la especie *T. fulviventris* pueden presentar comportamiento carroñero al forrajear materia en descomposición (Oliveira y Morato 2000; Vit *et al.* 2013; Zacca *et al.* 2018). Por lo tanto, la polinización carroñera se mantiene en el género, aunque no se trate exclusivamente de moscas. Con respecto a la polinización en Cytinaceae se describe como especializada, ya que se lleva a cabo por hormigas en *C. hypocistis* (de Vega *et al.* 2009), por aves en *C. sanguineus* (Hobbhahn y Johnson 2015), por roedores y musarañas en *C. visseri* (Johnson *et al.* 2011), por moscas carroñeras en *B. americanum*, por mariposas en *B. andrieuxii* y por abejas meliponinas en *B. oxylepis*, aquí descrito. Aunque se trate de polinización especialista, hasta el momento no se detecta una tendencia por algún grupo funcional para efectuar la polinización en las especies de Cytinaceae.

Morfología y desarrollo floral. El presente estudio describe y compara la morfología y desarrollo de las tres especies mexicanas del género *Bdallophytum*. Morfológicamente, el número de unidades del periogonio, el tamaño del ovario y el número de estambres son algunas de las características que varían en las flores insertas a lo largo del eje de las inflorescencias en cada una de las especies, además de los evidentes apéndices estaminales presentes en *B. americanum*. Por lo tanto, las especies estudiadas de *Bdallophytum* presentan variación intra-individual, lo cual también ha sido descrito para el género *Cytinus* (Villar 1997). La reducción en el número de órganos es el tipo de variación intra-individual más común en angiospermas (Buide 2004) y parece un tipo de variación común en las especies de Cytinaceae. Además del número de órganos, la variación intra-individual en la especie *B. oxylepis* se refleja en el sexo de las flores que también varía a lo largo del eje de la inflorescencia, es decir, a medida que las flores se acercan al ápice, el número de estambres disminuye hasta el grado de encontrar únicamente flores femeninas en la parte más distal de la inflorescencia. La variación sexual a lo largo del eje de las inflorescencias también se aprecia en *B. caesareum* (antes *Sanguisuga caesarea*) y en especies monoicas de *Cytinus* (Fernández-Alonso y Cuadros-Villalobos 2012; Villar 1997).

Además de la variación intra-individual, la sinorganización es otro atributo que se resalta en el género *Bdallophytum*. Ejemplo de ello es la presencia de sinandrio, es decir, la fusión de los estambres, en las flores masculinas de las tres especies. Sin embargo, en *B. oxylepis,* además de la fusión de los estambres, éstos también están unidos congénitamente al gineceo formando un ginostemio. La sinorganización es una característica común del orden Malvales (Stevens 2001), al cual pertenece la familia Cytinaceae, sin embargo, la presencia de ginostemio no es usual dentro del orden y es el caso más extremo de sinorganización (Endress 2016).

En términos del desarrollo floral, se aprecia que las flores femeninas, masculinas y bisexuales comienzan su desarrollo con una simetría bilateral, por lo que son zigomorfas. La simetría cambia conforme avanza el desarrollo de las flores unisexuales ya que éstas terminan su desarrollo siendo actinomorfas. Las flores bisexuales de *B. oxylepis* mantienen la zigomorfía durante todo su desarrollo hasta las flores maduras. El cambio en la simetría floral durante el desarrollo es común en angiospermas que se desarrollan en inflorescencias de tipo espiga (Endress 2012) tal como lo son las especies de *Bdallophytum*.

En cuanto a la embriología, el androceo y el gineceo se desarrollan de manera similar en las tres especies. No se encontraron diferencias en la gametogénesis ni en la esporogénesis entre las especies. Las únicas diferencias radican en el número de aperturas (colpos) de los granos de polen y que *B. americanum* tiene los granos de polen y los óvulos más grandes comparados con las otras dos especies. Las características embriológicas son particulares a nivel del género *Bdallophytum*, ya que en las tres especies el desarrollo floral, la esporogénesis y la gametogénesis se llevan a cabo del mismo modo y contrastan con algunas características de *Cytinus hypocistis*, tanto en el desarrollo de los granos de polen y de los óvulos (Guzowska 1964, 1966). Además, en el presente estudio proponemos a los óvulos unitégmicos de *Bdallophytum* como una sinapomorfía del género. Tales diferencias embriológicas entre *Bdallophytum* y *Cytinus*, así como la presencia de óvulos unitégmicos, pueden ser de utilidad para anidar a las especies a nivel de género.

Conclusiones

El género *Bdallophytum* (Cytinaceae) es un grupo de holoparásitas (plantas que no fotosintetizan) tipo endoparásitas, que se caracterizan porque su cuerpo vegetativo es reducido y crece dentro de las raíces de las plantas hospederas. El género *Bdallophytum* está conformado por cuatro especies, de las cuales *B. americanum*, *B. andrieuxii* y *B. oxylepis* se distribuyen en México y *B. caesareum* en sudamérica. Las especies de *Bdallophytum* parasitan las raíces de los árboles del género *Bursera*. En el presente trabajo se describen aspectos de la biología reproductiva de las especies mexicanas de *Bdallophytum*, en los cuales se pueden encontrar patrones a nivel de género. Las tres especies estudiadas presentan flores unisexuales, *B. americanum* y *B. andrieuxii* son dioicas, mientras que *B. oxylepis* por presentar además flores bisexuales conviviendo con flores femeninas se describe por primera vez como ginomonoica. Por otra parte, la floración de las especies comienza después de las primeras lluvias en las poblaciones estudiadas, por lo que las inflorescencias de *Bdallophytum* sólo se encuentran en una temporada corta del año menor a tres meses, en los cuales se pueden observar tanto flores como frutos.

Por otra parte, la flores de *Bdallophytum* son de color rojizo oscuro y emiten un aroma fétido similar a frutos en descomposición. Dichas características se ajustan al síndrome de polinización de sapromiofilia, donde las flores tienen características en relación con la polinización por moscas carroñeras. Después del monitoreo de las tres especies mexicanas de *Bdallophytum*¸ se observó que los polinizadores en las poblaciones estudiadas son sírfidos para *B. americanum*, mariposas para *B. andrieuxii* y abejas meliponinas para *B. oxylepis*. Aunque los polinizadores esperados eran moscas, sus polinizadores han sido reportados con comportamientos carroñeros. De las tres especies estudiadas, cada una presenta sus particularidades. Por ejemplo, sólo las flores de *B. andrieuxii* ofrecen néctar como recompensa, las flores masculinas de *B.*

americanum son las únicas que presentan apéndices apicales del tejido conectivo que los polinizadores utilizan como plataforma de aterrizaje y atrayente visual, y *B. oxylepis* es la única de las tres que presenta flores bisexuales.

Con respecto al desarrollo floral, las tres especies estudiadas presentan el mismo patrón de desarrollo. Las flores se desarrollan de manera acropétala y el desarrollo del gineceo y del androceo se desarrollan similar en las tres especies. Asimismo, todas las especies exhiben variación intra-individual, en donde las flores tienen diferente tamaño y número de tépalos, anteras y lóbulos estigmáticos, a lo largo de la inflorescencia. En cuanto a la biología floral, cada especie muestra una particularidad. Las flores de *B. americanum* son las más grandes del género, y las masculinas desarrollan apéndices estaminales apicales formados a partir del tejido conectivo. Estos apéndices también se presentan en *B. andrieuxii*, sin embargo, son inconspicuos. Además, esta especie puede desarrollar dos morfos masculinos, el primero con las anteras en un arreglo paralelo y el segundo con un arreglo desordenado. Por otra parte, las flores bisexuales de *B. oxylepis* se caracterizan por la presencia de ginostemio, una estructura compuesta por la fusión de los órganos femeninos y masculinos. El ginostemio se describe por primera vez para la familia Cytinaceae.

Finalmente, el presente trabajo aborda parte del ciclo de vida de un grupo de plantas parásitas poco estudiado. Con ello se espera un mejor entendimiento en la biología de estos organismos de difícil estudio. Asimismo, se abren nuevas preguntas de investigación en relación con la dispersión de las semillas y su germinación. También si el parasitismo afecta a los hospederos o funcionan como ingenieros ecosistémicos como se les ha considerado.

Perspectivas

Dado que las plantas endoparásitas son especies raras y de difícil estudio por su limitada presencia en la naturaleza y corta fenología, es preciso conocer el ciclo de vida de dichas especies para conocer su papel en las diferentes comunidades en las que habitan. En el presente trabajo se demuestra que las endoparásitas de la familia Cytinaceae tienen interacciones especialistas con sus polinizadores, los cuales son atraídos por señales multisensoriales emitidos por las flores. La polinización es un aspecto clave en el ciclo de vida de las endoparásitas, y para el caso de *Bdallophytum* ahora se conoce que las flores son un recurso importante en tiempos de escasez para algunos polinizadores. Aunque aquí se presentan resultados para algunas poblaciones, falta conocer y explorar la presencia de especies de *Bdallophytum* y sus interacciones planta-animal en diferentes poblaciones. También resta por conocer otras etapas del ciclo de vida de estas plantas como las interacciones relacionadas con la dispersión de frutos y semillas, el proceso de germinación en relación con la especificidad por el hospedero y la invasión al hospedero. Asimismo, la interacción planta-planta aún se desconoce, por lo que no se sabe si las plantas parásitas de *Bdallophytum* tienen algún efecto negativo en sus hospederos a corto o largo plazo.

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