UNIVERSIDAD DE COSTA RICA
SISTEMA DE ESTUDIOS DE POSGRADO

# ESTRUCTURAS FLORALES Y SU FUNCIÓN EN LA POLINIZACIÓN DE ORQUÍDEAS DEL GÉNERO *DRACULA* (ORCHIDACEAE: PLEUROTHALLIDINAE)

Tesis sometida a la consideración de la Comisión del Programa de Estudios de Posgrado en Biología para optar al grado y título de Maestría Académica en Biología

KAREN SOFÍA GIL AMAYA

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Dr. Erick Fuchs Castillo
Decano/Representante del Decano, Sistema de Estudios de Posgrado

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Director de Tesis

Dr. Mario Blanco Coto
Lector

Dr. Alfredo Cascante Marín
Lector

Dr. Diego Bogarín Chaves
Director, Representante del Director, Programa de Posgrado en Biología

Karen Sofía Gil Amaya
Sustentante

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## RESUMEN

Pleurothallidinae (Epidendreae) es la subtribu de orquídeas neotropicales más diversa. Comprende más de 5480 especies, la mayoría de las cuales se creé que son polinizadas por insectos del orden Diptera. Las flores del género *Dracula* (Pleurothallidinae) son conocidas por presentar una estrategia de polinización que gira en torno al mimetismo de hongos Agaricales. Las flores de *Dracula* producen compuestos volátiles que simulan aquellos producidos por los hongos (imitación química) y los labelos asemejan las lamelas de un hongo agarical (imitación morfológica). A pesar de que se ha sugerido que la polinización por engaño podría representar la estrategia usada por casi la mitad de las especies de orquídeas, las recompensas florales no evidentes como fragancias, sustancias ricas en lípidos, pequeñas cantidades de néctar, proteínas o carbohidratos, podrían llevar a una sobreestimación del número de casos en que no se ofrece ningún tipo de recompensa. En Orchidaceae, el conocimiento de los sistemas de polinización de géneros ricos en especies, incluidos los de Pleurothallidinae, todavía es escaso y varios de estos no se han estudiado detalladamente. Para contribuir al conocimiento sobre los mecanismos de atracción y polinización del género *Dracula*, este proyecto de tesis se ha enfocado en documentar las funciones de las estructuras florales en el proceso de polinización a través del estudio de su morfología, la detección de glándulas secretoras florales y el efecto de la manipulación de partes florales sobre el éxito reproductivo.

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# INTRODUCCIÓN

El estudio de la polinización de las plantas con flor por animales contribuye al entendimiento de complejas adaptaciones y relaciones evolutivas. Los polinizadores son agentes de selección de rasgos florales y esta selección ha sido considerada una importante fuerza evolutiva que impulsa la diversificación de las Angiospermas (Darwin, 1862; Stebbins, 1970; Fenster, 2004). Sin embargo, factores antropogénicos (entre ellos la deforestación, la contaminación química y lumínica, la urbanización y el cambio climático global) amenazan los sistemas de polinización, disminuyen las poblaciones de plantas y polinizadores, además de aumentar los desajustes fenológicos entre las especies (Pyke, 2016). Por lo tanto, los esfuerzos para estudiar aspectos básicos como la historia natural de las plantas y de los polinizadores se hacen necesarios en la toma de decisiones de manejo óptimas para la conservación de las especies e interacciones planta-animal en los ecosistemas naturales (Kearns et al., 1998).

La familia de las orquídeas, con aproximadamente 880 géneros y 27.800 especies (Givnish et al., 2016), es reconocida por su enorme diversidad de mecanismos de polinización y por la abundancia de especies con flores que aparentemente no ofrecen recompensa a sus polinizadores (Jersáková et al., 2006). En Orchidaceae se utilizan dos grandes categorías de estrategias para atraer polinizadores: la recompensa y el engaño. Como recompensas florales se han encontrado fragancias (Dressler, 1968; Hetherington-Rauth & Ramírez, 2016), aceites (Pauw, 2006), néctar (Ackerman et al., 1994; Stpiczyńska et al., 2004), polen (Kocyan & Endress, 2001) y pseudopolen (Davies et al., 2000). Las estrategias de polinización por engaño se basan en el ofrecimiento de algo que busca el polinizador relacionado con su biología (por ejemplo, alimento, una pareja potencial, el sustrato para oviposición, etc.).

Estas estrategias de polinización por engaño, ya sea de alimentación (Pansarin et al., 2008; Stpiczyńska & Davies, 2008; Pansarin & Pansarin, 2011; Vale et al., 2011) o sexual (Ayasse et al., 2003; Schiestl et al., 2003; Singer et al., 2004; Blanco & Barboza, 2005; Martel et al., 2016), están bien documentadas entre las orquídeas y han evolucionado independientemente en diferentes linajes dentro de la familia (van der Pijl & Dodson, 1966; Cozzolino & Widmer, 2005). De hecho, la polinización por engaño parece haber contribuido al aumento de la diversidad de orquídeas ya que pequeños cambios en los rasgos florales

pueden causar la atracción de diferentes polinizadores y así el aislamiento reproductivo de las poblaciones (Smith, 2010; Givnish et al., 2015).

Se estima que el engaño es empleado como estrategia de polinización por cerca de un tercio de las especies de las orquídeas (van der Pijl & Dodson, 1966; Ackerman, 1986; Jersáková et al., 2006; Shrestha et al., 2020). Mimetismo es un término propio de la ecología animal que originalmente hace referencia a un mecanismo de defensa. Mientras que en las plantas, usualmente implica un engaño, o puede ser parte de un sistema mülleriano en el que se ofrecen recompensas similares (Castro et al. 2022) para atraer visitantes y favorecer su reproducción (Dafni, 1984). En este sistema de engaño (siempre dirigido a insectos) el imitador debe ocurrir en simpatría con el modelo, sus tiempos de fenología deben coincidir y el imitador debe parecerse al modelo en la medida en que el receptor de las señales sea incapaz de distinguir entre el imitador y el modelo moviéndose libremente entre ellos (Dafni, 1984; Jersáková et al., 2016).

Se pueden dar varios tipos de mimetismo al engañar al polinizador. Por ejemplo, atrayéndolo con aromas que suelen combinarse con señales visuales esperando obtener una recompensa alimenticia (Kunze & Gumbert, 2001; Salzmann et al., 2007), lugares de encuentro ("rendezvous pollination"; Nilsson, 1983; Johnson & Steiner, 1994; Steiner, 1998) o imitación del sustrato para oviposición (Jin et al., 2014; Díaz-Morales, 2017). Las flores que atraen moscas que ovipositan en hongos imitan el olor y aspecto morfológico de los cuerpos fructíferos de estos últimos (Vogel, 1978; Kaiser, 2006; Stökl et al., 2010). Se han reportado tres familias de plantas que imitan hongos para engañar a las moscas que los buscan: Aristolochiaceae (Vogel, 1978; Sugawara, 1988; Mesler & Lu, 1993) Araceae (Vogel, 1978; Vogel & Martens, 2000) y Orchidaceae (Vogel, 1978; Proctor et al., 1996; Dentinger & Roy, 2010; Policha et al., 2016; Kelly et al., 2013; Kuiter & Findlater-Smith, 2017).

El género *Dracula* Luer es uno de los pocos géneros dentro de la familia Orchidaceae en los que se ha reportado la polinización por mimetismo de hongos (Vogel, 1978; Dentinger & Roy, 2010; Endara et al., 2010; Policha et al., 2016). El mimetismo de hongos en *Dracula* consta de tres componentes: (1) los modelos: esporocarpos carnosos de hongos del orden Agaricales, (2) los imitadores: flores de *Dracula* y (3) los receptores de señales: moscas de la familia Drosophilidae (Dentinger & Roy, 2010; Endara et al., 2010; Policha, 2014). Las

flores de *Dracula* aparentemente no imitan especies específicas de hongos, pero explotan las preferencias innatas de moscas drosofílidas para los tipos de hongos carnosos que crecen en el mismo hábitat (Dentinger & Roy, 2010). Muchos taxones dentro de Drosophilidae ovipositan en hongos Agaricales y utilizan sus cuerpos fructíferos para el alimento de sus larvas (Courtney et al., 1990).

Muchos estudios de polinización en orquídeas no reportan recompensas medibles u obvias, incluyendo aromas, resinas, sustancias ricas en lípidos y pequeñas cantidades de néctar. Debido a la falta de una recompensa visible de néctar, la ausencia de azúcares (Endara et al., 2010) y la supuesta incapacidad de que las larvas de las moscas se desarrollen hasta la adultez, son criterios para considerar que las flores de *Dracula* son imitadores batesianos (Vogel, 1978). Sin embargo, la evidencia de guías de néctar, nectarios o papilas secretoras de proteínas, se encuentran con frecuencia en estudios de polinización más detallados en la subtribu Pleurothallidinae (Barbosa et al., 2009; de Melo et al., 2010; Duque-Buitrago et al., 2014; Karremans et al*.,* 2015; Bogarín et al., 2018a, b).

La mayoría de las papilas que se ven en las flores de las orquídeas, contienen pigmentos o actúan como glándulas secretoras de aroma (osmóforos) y probablemente representan señales visuales o táctiles que guían a los polinizadores hacia las flores (Jersáková et al., 2006; Karremans et al., 2015). Estas glándulas secretoras florales se ubican generalmente en los sépalos, pétalos y labelo de las orquídeas (Vogel, 1990; Dressler, 1981). En Pleurothallidinae se ha demostrado que los osmóforos por lo regular se encuentran en los sépalos (de Melo et al., 2010). En algunos géneros, como *Restrepia* Kunth y *Scaphosepalum* Pfitzer, los osmóforos se concentran en estructuras morfológicamente bien definidas (Pridgeon & Stern, 1983; Pridgeon & Stern, 1985; Millner & Baldwin, 2016). Pero otros como *Gongora* Ruiz & Pav. (Stanhopeinae) tienen osmóforos crípticos que requieren de técnicas histoquímicas para poder ser localizados (Stern et al., 1986).

Vogel (1990) mencionó que examinó las caudas sepalinas de *D. erythrochaete* (mencionada como *Masdevallia erythrochaete* Rchb.f.) y no encontró ninguna indicación de que funcionaran como osmóforos. Sobre el aroma a hongos en las flores, mencionó que no era aparentemente perceptible para *D. bella* (Rchb.f.) Luer ni para la sección *Chimaeroideae* ([*Dracula*], d. Ref.), con excepción de *D. chimaera* (Rchb.f.) Luer y *D. chestertonii* (Rchb.f.)

Luer, Vogel (1978: 383–384). Sin embargo, en el género *Dracula*, hasta la fecha no hay estudios de detección o caracterización de glándulas secretoras florales.

Se ha formulado la hipótesis de que las moscas drosofílidas polinizan accidentalmente las flores de *Dracula* mientras inspeccionan el labelo como sitio potencial de oviposición (Vogel, 1978; Kaiser, 2006). A pesar de que se han observado comportamientos de las moscas asociados a la reproducción, como cortejo y apareamiento en las flores, no se ha documentado la presencia de huevos en las flores (Endara et al., 2010; Policha et al., 2016; Policha et al., 2019). El mecanismo de polinización reportado para la especie *Dracula lafleurii* Luer & Dalström, sugiere que la transferencia ocurre cuando el tórax del polinizador queda atrapado por los lóbulos laterales incurvados del rostelo (Endara et al., 2010).

Por otra parte, las flores de *Dracula* no producen néctar, al menos no en cantidades detectables (Endara et al., 2010). No obstante, se han aislado compuestos de olores fungosos en las flores de *D. chestertonii* dominados por constituyentes típicos de hongos, que contienen más del 70% de los volátiles: oct-1-en-3-ol (1), oct-1-en-3-ona (2), octan-3-ol (3), y 3-octanona (4) (Kaiser, 2006; Policha et al., 2016). Además, se ha reportado un recubrimiento lustroso en el labelo de *D. lafleurii* sólo durante el día de apertura de la flor (Endara et al., 2010). Se ha hipotetizado que este recubrimiento sirve como medio de crecimiento para levaduras, que hacen parte de la dieta de las moscas micófilas de la familia Drosophilidae, y que incluso podrían ser dispersadas por ellas mismas (Endara et al., 2010).

McAlpine (2013) aisló siete especies de levaduras creciendo en el labelo y sépalos de *Dracula felix* (Luer) Luer y *D. lafleurii.* El autor encontró más especies de levaduras creciendo en el labelo que en los sépalos de *D. felix* y propone que éstas constituyen una recompensa alimenticia para las moscas drosofílidas y que además podrían modificar el aroma que producen las flores (McAlpine, 2013). También, propone que estas levaduras podrían llegar a otras flores de *Dracula* a través de las moscas visitantes iniciales o a través del viento por dispersión de las esporas.

En la subtribu Pleurothallidinae, se ha documentado la polinización en ocho de los 44 géneros, lo que representa aproximadamente una quinta parte y en 30 de las 5481 especies descritas, cerca del 2% de las especies (Karremans, 2016; Karremans & Díaz-Morales, 2019; Karremans & Vieira-Uribe, 2020). En el género *Dracula*, la polinización solo se ha estudiado detalladamente en dos de las 138 especies descritas, *D. lafleuri* y *D. felix* (Endara et al., 2010;

Policha et al., 2016; Policha et al., 2019). Dado que las especies del género *Dracula* exhiben una diversidad de colores y especializaciones en estructura floral, es muy probable que existan variaciones en el mecanismo de polinización descrito por Endara et al. (2010).

Con todo esto, la pregunta de investigación es: ¿Cuál es el papel que juegan los sépalos, pétalos, el labelo y la columna de *Dracula* en la atracción de polinizadores y en el mecanismo de polinización? No están claramente entendidas las funciones que cumplen las caudas, papilas y patrones de coloración de los sépalos, los pétalos, el labelo y el rostelo (estructura que normalmente impide la autofecundación en las orquídeas). Contestar la pregunta de este trabajo comprende un aporte importante al conocimiento de la historia natural de *Dracula* y de sus polinizadores, que ayudará en un futuro a tomar decisiones a favor de su conservación.

## EL GÉNERO *DRACULA* (ORCHIDACEAE: PLEUROTHALLIDINAE)

Las plantas de *Dracula* son epífitas que crecen principalmente en bosques maduros, con preferencia en sitios sombreados y bien drenados (Escobar et al., 1991; Luer, 1993). El Latín *dracula* es un diminutivo de *dracus*, y literalmente significa pequeño dragón, que describe el aspecto grotesco de las pequeñas flores parecidas a dragones colgantes en el ápice de las inflorescencias de las *Dracula* (Pupulin et al., 2009). La primera especie descrita de *Dracula* fue recolectada en la Cordillera Occidental de los Andes de Colombia en 1871 por Benedict Roezl, y fue publicada por el Profesor Heinrich Gustav Reichenbach (1872), bajo el nombre *Masdevallia chimaera*. En 1978, Carlyle Luer la transfirió a su género actual como *Dracula chimaera*, designándola como especie tipo. El epíteto específico hace alusión a una figura de la mitología griega, Quimera, un dragón que libera fuego, compuesto por las partes de varios animales (Luer, 1993; Pupulin 2005; Pupulin et al., 2009). En la actualidad, se siguen nombrando a las especies con otros nombres relativos a murciélagos, vampiros, y dragones.

El género *Dracula* se separó del género *Masdevallia* Ruiz & Pav. debido a caracteres que incluían el tipo de inflorescencia, morfología de ovario, sépalos, pétalos y labelo (Luer, 1993). De acuerdo con análisis moleculares de ADN (Pridgeon et al., 2001) se indicó una posición filogenética de *Dracula* como un grupo monofilético, hermano del clado que incluye *Porroglossum* Schltr. y *Masdevallia*. Algunas especies son estables

morfológicamente, mientras que otras son tan variables que no hay dos especímenes idénticos (Pupulin, 2005). Al parecer, las especies de *Dracula* se hibridan fácilmente como se ha observado en condiciones de invernadero (Luer & Escobar, 1989; Escobar et al., 1991) y aunque los híbridos naturales también existen, han hecho que el reconocimiento de nuevas especies sea controversial (Pupulin, 2005).

Las especies de *Dracula* se distinguen morfológicamente por tener hojas suaves y generalmente delgadas, provistas en el envés por una carina o vena media bien definida, como una quilla longitudinal. Las plantas son cespitosas, con un tallo (ramicaule) mucho más corto que la hoja; las inflorescencias son filamentosas, generalmente descendentes (Fig. 1A–H), raramente erectas [como en *D. sodiroi* (Schltr.) Luer] (Fig. 1I), con flores secuenciales, dirigidas hacia abajo. Los sépalos están unidos en la base, son pubescentes con tricomas y papilas en la superficie adaxial, raramente glabros [como en *D. gigas* (Luer) Luer], con el ápice contraído en delgadas colas sepalinas, que pueden llegar a medir hasta 25 cm de largo [como en *D. chimaera*]. Los pétalos generalmente son muy reducidos, muy raramente alargados [como en *D. andreettae* (Luer) Luer], apicalmente gruesos, verrucosos. El labelo es generalmente móvil, articulado al pie de la columna, raramente rígido [como en *D. chestertonii*], compuesto por un hipoquilo con una hendidura central y un epiquilo cóncavo con laminillas radiales que se asemejan a las de los macrohongos agaricales. La columna es semiterete, con la antera incumbente y el estigma ventral, el rostelo es conspicuo y con viscarium, que es la sustancia pegajosa que secreta la superficie abaxial; y dos polinios con caudículas, pero sin viscidio, con la forma típica de "cola de ballena" (Luer, 1993; Pupulin et al., 2009; Karremans & Vieira-Uribe, 2020).

*Dracula* es un grupo de orquídeas exclusivo del Neotrópico perteneciente a la subtribu Pleurothallidinae. Comprende 138 especies, algunas tienen rangos de distribución geográfica amplia [como *D. vespertilio* (Rchb.f.) Luer], mientras que muchas son conocidas solamente de áreas muy definidas. Su distribución altitudinal principalmente está entre 1500 y 2500 m. *Dracula* se distribuye desde México hasta Perú, con la mayoría de las especies creciendo en Colombia (74 especies; Peláez et al., 2020) y Ecuador (ca. 60 especies; Pupulin et al., 2009). No se conocen registros para Bolivia, Brasil o Venezuela (Luer, 1993). Un estudio reciente indica que *Dracula* es un grupo relativamente joven, de aproximadamente

7–10 Ma, con una tasa de diversificación alta, perteneciente a un clado de especiación rápida (Pérez-Escobar et al., 2017).

## EL GÉNERO *DRACULA* EN COSTA RICA

Al norte de la región del Chocó y a través del Istmo Centroamericano, la diversidad de orquídeas del género *Dracula* disminuye rápidamente, con una especie [*Dracula pusilla* (Rolfe) Luer], que llega a las regiones meridionales de México, y otra que va hasta Nicaragua (*D. vespertilio*) (Luer, 1993; Pupulin & Strigari, 2016). En Costa Rica las especies de *Dracula* se han documentado creciendo en elevaciones entre 700 y 2000 m (Dressler, 2003), y se han registrado hasta ocho especies diferentes (Luer, 1993; Pupulin, 2001; Pupulin, 2002; Dressler, 2003). Aquí, se confirma la presencia de estas ocho especies creciendo en un rango de elevación de 714–2691 m (Fig. 2). Cuatro de ellas (50%) son endémicas: *D. astuta* (Rchb.f.) Luer, *D. carlueri* Hermans & P.J. Cribb, *D. inexperata* Pupulin y *D. ripleyana* Luer. El registro de menor elevación es para la especie *Dracula vespertilio, Dressler s.n.* [Herbario del Jardín Botánico de Missouri (MO)-5477353], y el de mayor elevación para la especie *Dracula pusilla, Bogarín 12271* [Herbario del Jardín Botánico Lankester (JBL)]. Aunque *Dracula* es un género de pocas especies en Costa Rica, nunca se ha realizado un estudio sistemático en el país para esclarecer su taxonomía.

De acuerdo con el mapa de distribución de *Dracula* (Fig. 2), se puede inferir la presencia de dos especies abundantes en Costa Rica: el complejo de especies de *Dracula erythrochaete* (Rchb.f.) Luer y la muy persistente *D. pusilla*. El área de distribución de estas dos especies traslapa, siendo probablemente simpátricas en la localidad de Monteverde en la Cordillera de Tilarán. Otro punto de coincidencia de estas dos especies, es la región de la Cordillera Volcánica Central, en Alajuela, Heredia, y una parte de la Cordillera de Talamanca, entre los límites de San José y Cartago. Sin embargo, *D. erythrochaete* presenta una distribución concentrada en tierras más altas de la Cordillera Volcánica Central, mientras, que *D. pusilla* tiene un rango de distribución con preferencia de tierras más bajas, desde la Cordillera de Guanacaste, hasta la Cordillera de Talamanca.

La especie *D. carlueri* vive en simpatría con *D. ripleyana* en dos localidades: en la Cordillera de Tilarán, en Monteverde (Puntarenas) junto a *D. vespertilio*, y, en la Zona Protectora Cerros de Escazú, en la Cordillera Volcánica Central**.** Igualmente, es simpátrica

con *D. astuta* en las Provincias de Alajuela y San José en un rango de elevación de 1450–2000 m. *D. carlueri* también traslapa su distribución con *D. erythrochaete* y *D. pusilla* en la Cordillera Volcánica Central en las Provincias de Alajuela, Heredia y San José.

La especie *D. astuta* tiene un rango de distribución restringido a la Cordillera Volcánica Central en las Provincias de Alajuela (1450 m) y San José (1600 m) y es probablemente simpátrica con *D. erythrochaete* y *D. pusilla* en estas localidades. En la región geográfica de Cartago, entre la Cordillera Volcánica Central y la Cordillera de Talamanca, hay un traslape en una parte de la distribución de *D. erythrochaete* con la distribución de *D. inexperata*, *D. ripleyana* y *D. vespertilio*. Sin embargo, varias especies difieren en el rango de elevación dentro de estas localidades. Por último, el área de distribución de *D. vespertilio* y *D. pusilla*, traslapa en dos localidades de la Cordillera de Talamanca, siendo simpátricas en el área específica de Pérez Zeledón en Montecarlo de Quizarra (700 m) y Coto Brus, Puntarenas (1100–1300 m).

A continuación, se presentan cada una de las especies reportadas para Costa Rica, con los registros de distribución, incluyendo datos de altitud y fenología. Las especies indicadas con un asterisco, son endémicas de Costa Rica.

1. *Dracula astuta** (Fig. 3), endémica de Costa Rica, es reportada en Bosque pluvial (Dressler, 2003) y se distribuye en un rango de elevación de 1400–1600 m. Es comúnmente tratada como una forma de *D. erytrhochaete*, distinguida por hojas ligeramente más anchas, flores más grandes, sépalos con caudas más largas, más coloridos, ampliamente extendidos, y un labelo más grande (Luer, 1993). Sin embargo, aún hay dudas sobre su taxonomía, y se necesita un estudio más detallado basándose en el holotipo. Se ha reportado florecida en los meses de marzo, junio, setiembre y noviembre.

2. La especie *Dracula carlueri** (Figs 4 y 5), es endémica de Costa Rica y está reportada en Bosque nuboso (Dressler, 2003) en un rango de elevación de 1000–2000 m. Esta especie difiere de *D. erythrochaete* por los márgenes incurvados de los sépalos. Los sépalos son densamente más largos pubescentes en el interior. El epiquilo del labelo, es proporcionalmente más pequeño que el de *D. erythrochaete*. En el Jardín Botánico Lankester, están en cultivo varios individuos, sin embargo, hay diferencias morfológicas evidentes,

sobre todo en poblaciones de diferentes localidades. Tiene registros de floración entre abril y setiembre.

3. El complejo de especies de *Dracula erythrochaete* (Figs 6 y 7) muestra una gran variabilidad morfológica entre y dentro de sus poblaciones, especialmente en los sépalos (Fig. 8), pétalos (Fig. 9) y labelos (Fig. 10). Se ha colectado en Panamá y en Costa Rica, en Bosque pluvial y nuboso (Luer, 1993; Dressler, 2003). Sin embargo, es posible que la especie también se encuentre en Nicaragua; dos fotografías de morfotipos afines a *D. erythrochaete* fueron identificadas como *D. pusilla* (A37) y *D. vespertilio* (A38) respectivamente, por van den Berghe & Gurdian (2008). En Costa Rica, está presente en un amplio rango de elevación de 1100–2250 m, siendo la especie más abundante. Tiene registros de floración durante todo el año, con dos picos de floración que van desde abril hasta junio y de agosto hasta octubre.

4. La especie *Dracula inexperata** (Fig. 11) endémica de Costa Rica, se encuentra en Bosque pluvial (Dressler, 2003) y está distribuida en un rango de elevación de 1150–1650 m. Es una especie rara (Pupulin & Strigari, 2016), siendo el único miembro centroamericano de un grupo de especies de *Dracula* sudamericanas (Dressler, 2003). Se reconoce por un labelo muy pequeño, color rosa y sépalos triangulares. Tiene un período de floración que va desde noviembre a diciembre, extendiéndose a veces hasta febrero.

5. *Dracula maduroi* Luer (Fig. 12) es una especie descrita originalmente de Panamá y representada por sólo 2 ejemplares (1,3%) de Costa Rica en los herbarios nacionales. Esta especie ha sido colectada en las provincias de Heredia y Puntarenas, en un rango de elevación de 1150–2500 m. Recientemente se encontró también en Alajuela, en la Reserva Biológica Bosque de Paz, a una altitud de 1500 m (N. Belfort & K. Gil-Amaya, obs. pers., julio 2019), del cual se preparó el ejemplar testigo *Belfort 380* (JBL-spirit). Tiene registros de floración en abril y octubre.

6. La especie *Dracula pusilla* (Figs 13 y 14), es ampliamente distribuida y bien conocida en gran parte de Centroamérica, desde Chiapas en México hasta Chiriquí en Panamá (Luer, 1993). En Costa Rica se reporta en Bosque pluvial y nuboso (Dressler, 2003). Se distribuye en un amplio rango de elevación de 850–2650 m y probablemente haya más de una especie críptica que necesita ser revelada en este complejo de especies. Se han colectado ejemplares florecidos durante todo el año con dos picos de floración, el primero entre abril y mayo, el segundo entre agosto y setiembre.

7. La especie *Dracula ripleyana** (Fig. 15) es endémica de Costa Rica y está estrechamente relacionada a *D. erythrochaete,* distinguiéndose por las pequeñas flores blancas, con un centro púrpura oscuro y una corta pubescencia blanca en el interior de los sépalos. El ápice del epiquilo del labelo es inflado, obtuso en lugar de suborbicular, y finamente verrugoso en la superficie abaxial (Luer, 1979; Luer, 1993). Se reporta en un rango de elevación de 800–2200 m. Tiene registros de floración entre febrero y mayo.

8. *Dracula vespertilio* (Fig. 16) tiene la distribución geográfica más amplia de todo el género; está en Centroamérica, en la Cordillera Occidental de Colombia y en ambos lados de los Andes del Ecuador (Luer, 1993). En Costa Rica ha sido reportada en Bosque muy húmedo (Dressler, 2003) en un rango de elevación de 700–1400 m. Tiene registros de floración en abril, mayo, julio, noviembre y diciembre.

## ***DRACULA ERYTHROCHAETE*** **COMO CASO DE ESTUDIO**

El nombre *Dracula erythrochaete* (Fig. 6), proviene del griego *erythrochaete*, "*una cerda o pelo largo y rojo*", en referencia a las colas sepalinas delgadas y rojas (Luer, 1993). La especie florece durante todo el año con dos picos de floración en Costa Rica desde abril hasta junio y de agosto a octubre, coincidiendo con la temporada de lluvias. Es la especie de más amplia distribución en el país (Fig. 2), y cuenta con las poblaciones más grandes, siendo la más abundante del género en Costa Rica, lo que la convierte en el taxón ideal para desarrollar los experimentos sobre manipulación floral y polinización de esta tesis.

Esta especie se reconoce por sus hojas delgadas y angostas, flores de tamaño pequeño a mediano que generalmente emiten un aroma similar al de los hongos, sépalos de color blanco a amarillo pálido teñidos de rojo púrpura o marrón con tricomas y papilas en la superficie adaxial, ápices contraídos en delgadas colas rojas, un labelo móvil blanco, rosado o marrón claro con tres lamelas primarias y pocas venas elevadas radiales. Sin embargo, *D. erythrochaete* es difícil de circunscribir taxonómicamente basándose únicamente en la morfología floral debido a que varía ampliamente entre diferentes poblaciones.

La primera observación reportada de polinización en *Dracula* fue la de Dodson (1965), quien notó que la especie *D. erythrochaete*, bajo el nombre *Masdevallia erythrochaete*, fue visitada por varias moscas similares a *Drosophila* (Fallén) que

deambulaban por el epiquilo antes de subir a la base del labelo (hipoquilo) en busca (supuestamente) de néctar (van der Pijl & Dodson, 1966). Con estos antecedentes, esta especie es perfecta como modelo de estudio, no solo por su disponibilidad en el campo para obtener datos, sino para apoyar o no la hipótesis de que las moscas buscan néctar como recompensa en las flores de *Dracula*, sugerida por Calaway Dodson†, pionero en el estudio de la polinización de las orquídeas. Los resultados de este trabajo serán una contribución muy importante para entender la influencia de los polinizadores en la diversificación y evolución de los rasgos florales del complejo de especies de *D. erythrochaete*, y para comprender el papel de los polinizadores como impulsores de la diversidad de especies en el género *Dracula*.

# CONCLUSIÓN GENERAL

Este estudio es el más completo sobre la polinización de *Dracula* basado en la evidencia morfológica, anatómica, histoquímica, y de comportamiento animal, comparado con el número de especies estudiadas de este género hasta ahora. Es el primero en realizarse en una especie del género *Dracula* en Centroamérica, lo que permite la discusión de los resultados con las especies Sudamericanas. También ofrece amplias oportunidades para explorar y ahondar en temas como la evolución floral y las relaciones filogenéticas en los sistemas de polinización por engaño en la subtribu Pleurothallidinae. Aunque el engaño en la biología de la polinización se relaciona con la falta de recompensa, es posible que las flores utilicen una estrategia combinando engaños y recompensas que explotan las preferencias innatas de los insectos. La identificación de la composición química de los excretados glandulares de las flores de *Dracula* puede revelar preferencias alimenticias particulares de las especies de moscas involucradas, y profundizar en el grado de especificidad ecológica entre *Dracula* y *Zygothrica*.

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Figura 1. Variación en el hábito de las especies de *Dracula*. A. *D. pusilla* (*Bogarín 12426*). B. *D.* cf. *erythrochaete* (*Bogarín 13017*). C. *D. carlueri* (*JBL-38253*). D. *D. vespertilio* (*JBL-39984*). E. *D. pusilla* (*K. Gil 243*). F. *D. erythrochaete* (*K. Gil 145*). G. *D. vespertilio* (*Bogarín 12550*). H. *D. inexperata* (*Bogarín 12558*). I. *D. sodiroi* (*K. Gil s.n.*). Escala = 10 cm.

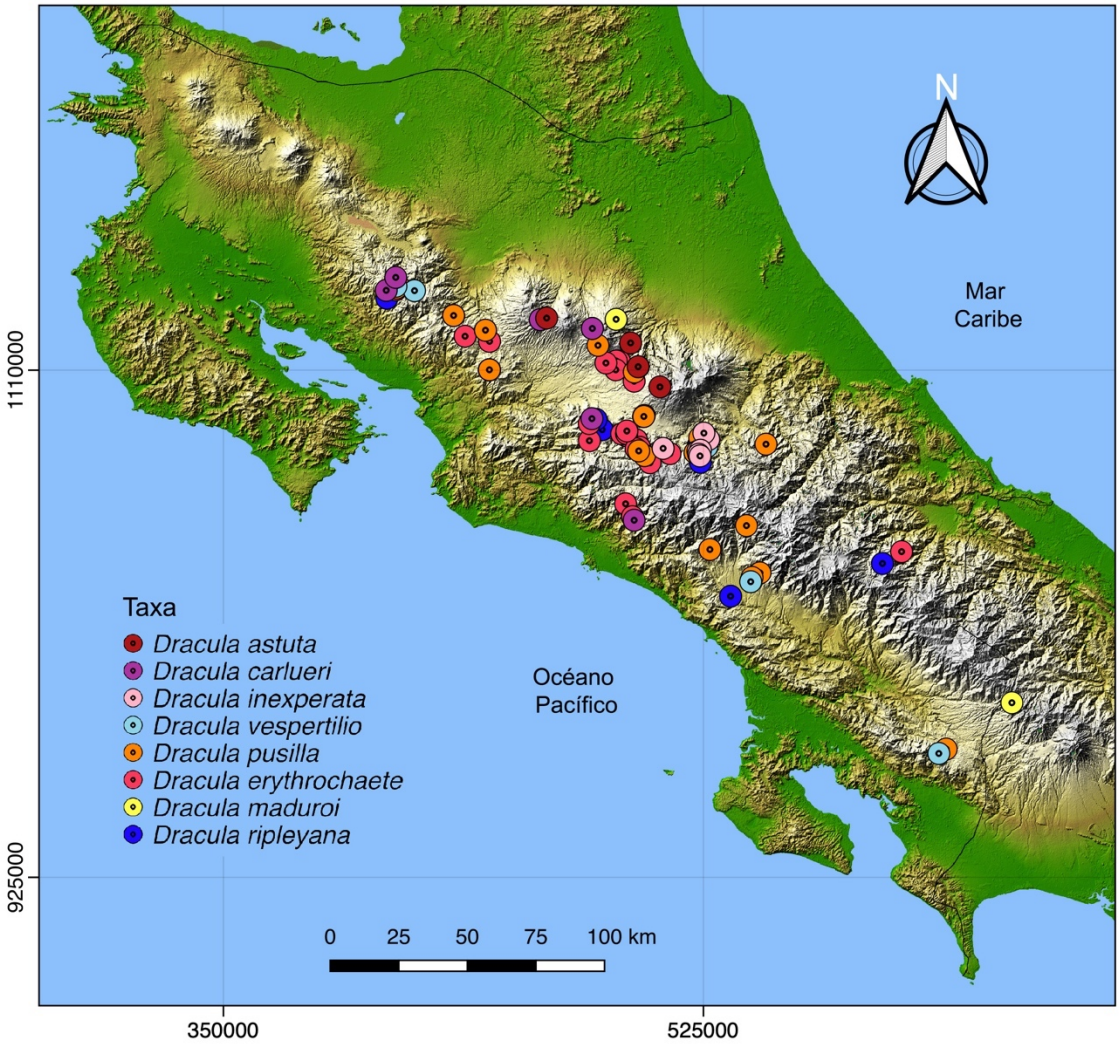


Figura 2. Distribución geográfica del género *Dracula* en Costa Rica, con base en los especímenes de herbario depositados en el Herbario Nacional de Costa Rica (CR), Herbario del Instituto Nacional de Biodiversidad INBio (INB), Herbario del Jardín Botánico Lankester (JBL), Herbario del Jardín Botánico de Missouri (MO) y el Herbario Luis Fournier Origgi (USJ). Fuente de la imagen satelital: NASA Jet Propulsion Laboratory, the National Imagery and Mapping Agency of the U.S. (NASA/JPL/NIMA), 2000.

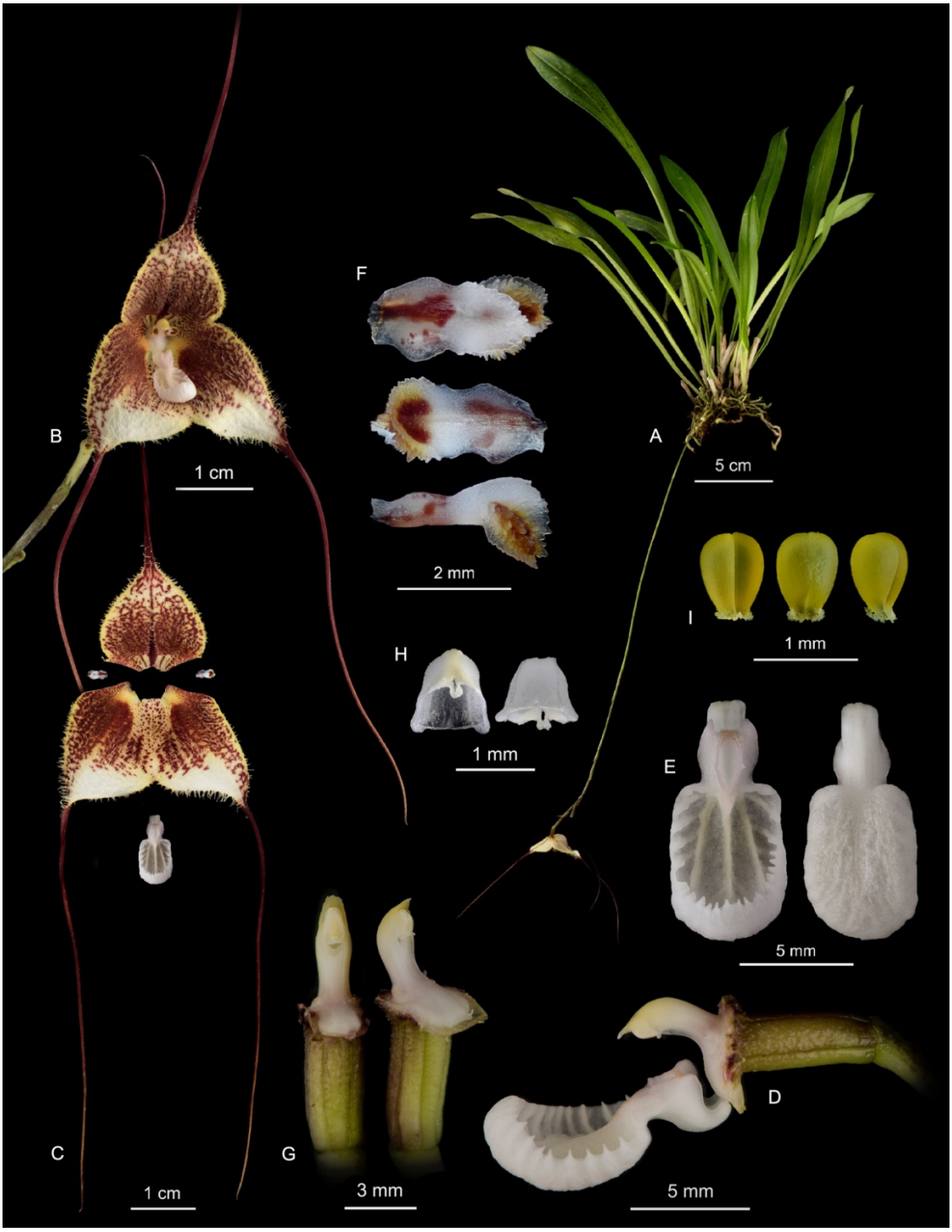


Figura 3. Lámina de Disección Compuesta Lankester (LCDP) de *Dracula* cf. *astuta.* A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas ventral y de tres cuartos. H. Antera, dos vistas. I. Polinario, tres vistas. Basado en *K. Gil 241* (JBL-spirit).

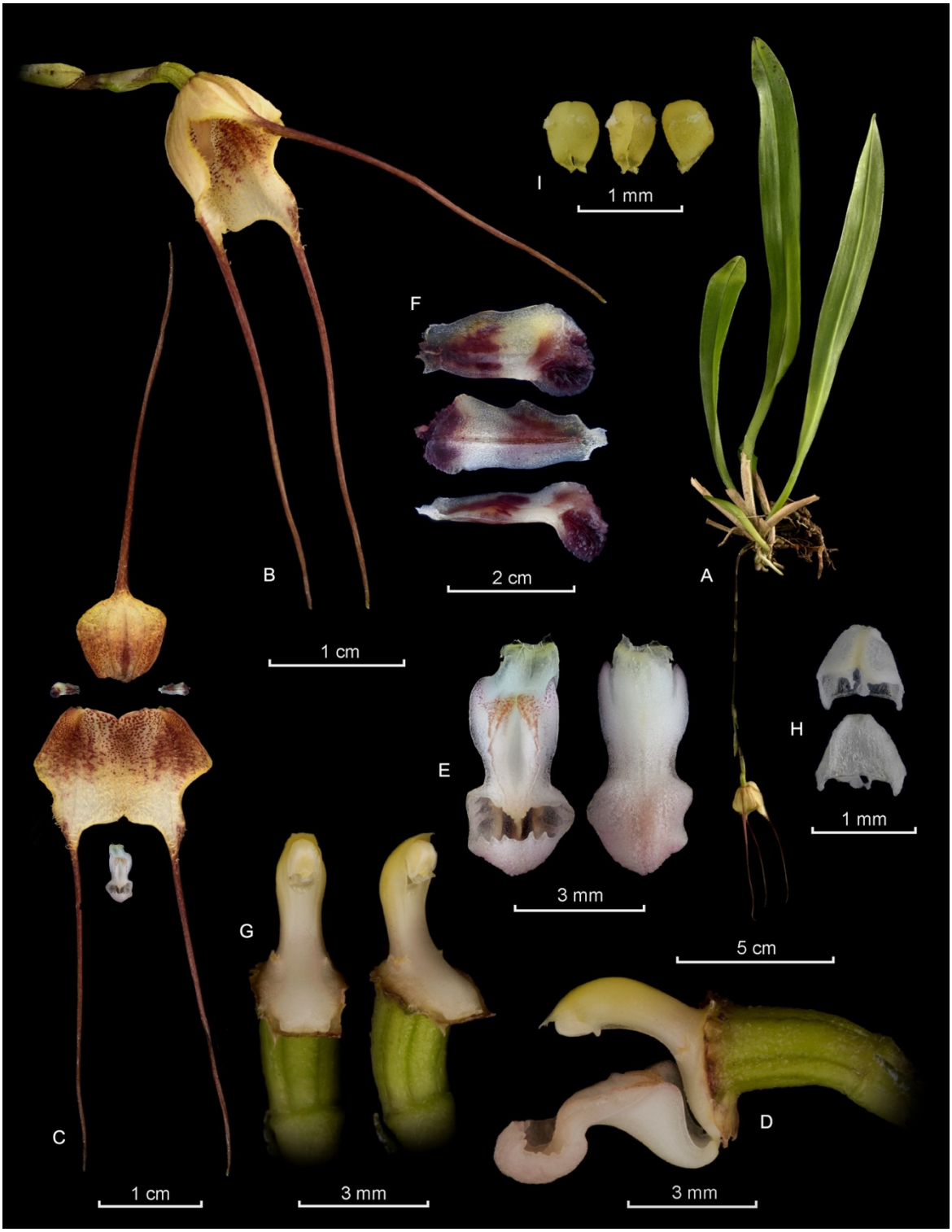


Figura 4. LCDP de *Dracula carlueri.* A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas ventral y de tres cuartos. H. Antera, dos vistas. I. Polinario, tres vistas. Basado en *JBL-38253* (JBL-spirit).

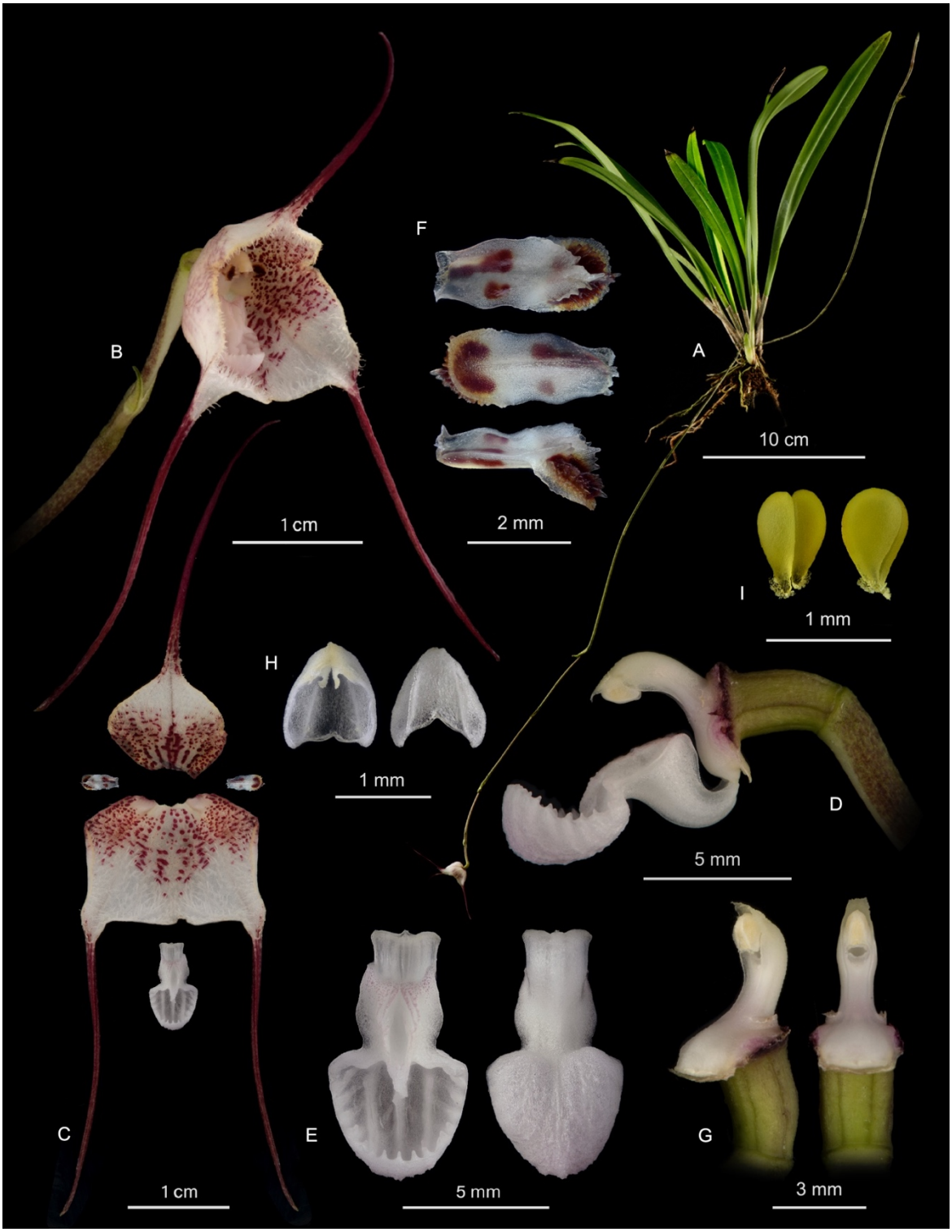


Figura 5. LCDP de *Dracula carlueri*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas de tres cuartos y ventral. H. Antera, dos vistas. I. Polinario, dos vistas. Basado en *JBL-09768* (JBL-spirit).

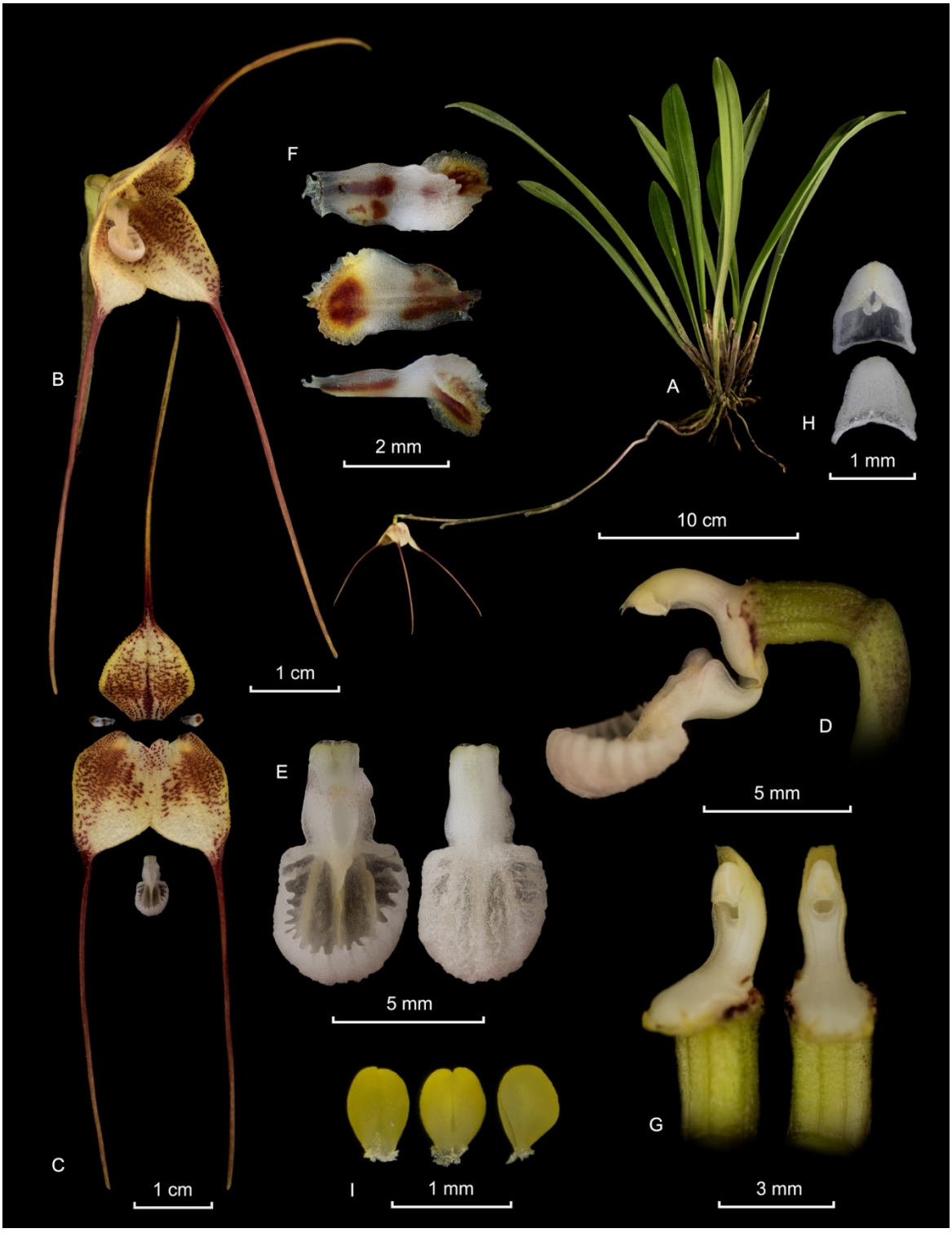


Figura 6. LCDP de *Dracula erythrochaeate.* A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas de tres cuartos y ventral. H. Antera, dos vistas. I. Polinario, tres vistas. Basado en *K. Gil 145* (JBL-spirit).

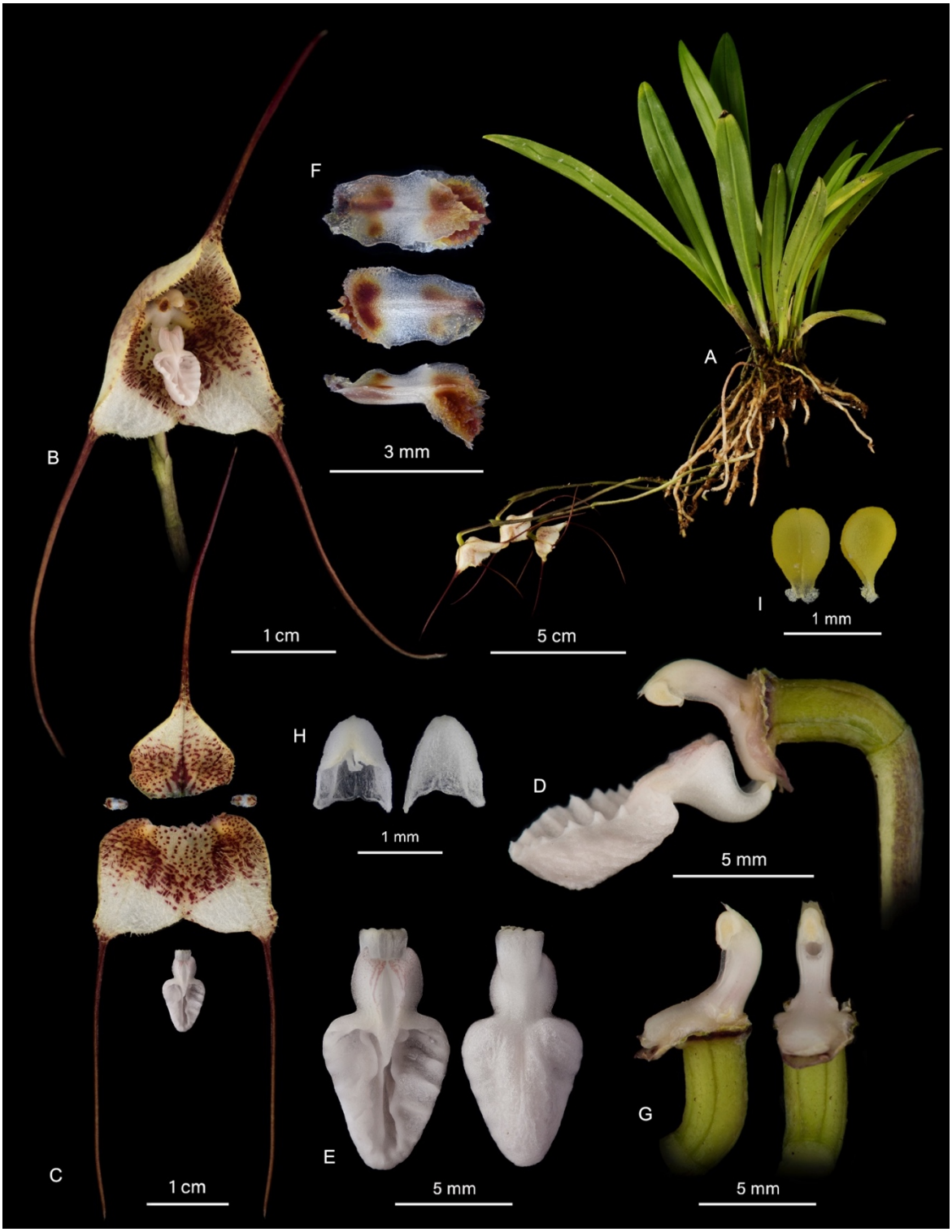


Figura 7. LCDP de *Dracula* cf. *erythrochaete*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas lateral y ventral. H. Antera, dos vistas. I. Polinario, dos vistas. Basado en *Bogarín 13017* (JBL-spirit).

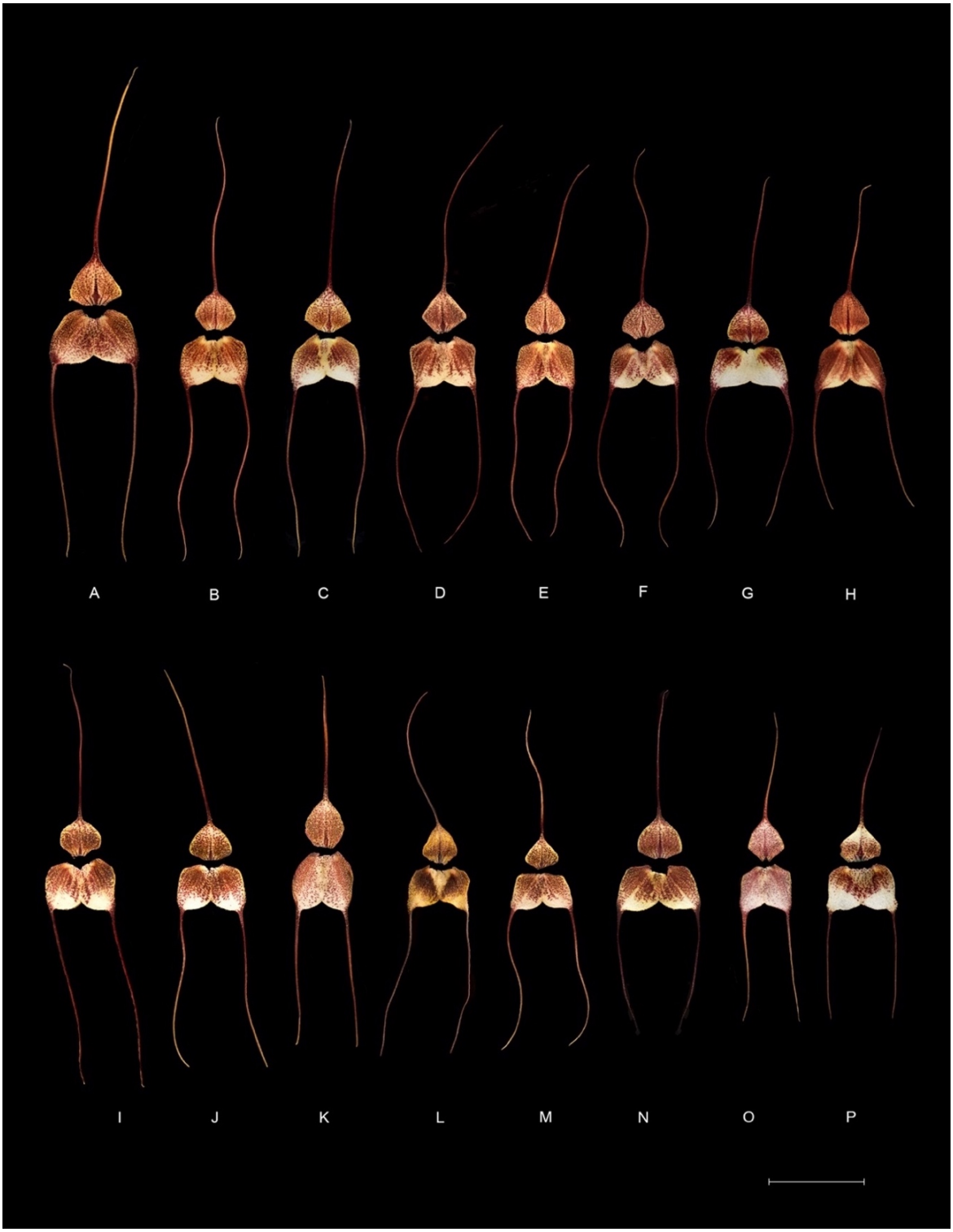


Figura 8. Variación en los sépalos de *Dracula erythrochaete*. A. *K. Gil 171*. B. *K. Gil 136*. C. *K. Gil 184*. D. *K. Gil 114*. E. *K. Gil 132*. F. *K. Gil 121*. G. *K. Gil 143*. H. *K. Gil 126*. I. *Karremans 8118*. J. *Karremans 8115*. K. *Valverde s.n.* L. *Rojas-Alvarado 119*. M. *Bogarín 11966*. N. *Rojas-Alvarado 323*. O. *K. Gil 245*. P. *Bogarín 13017*. Escala = 3 cm.

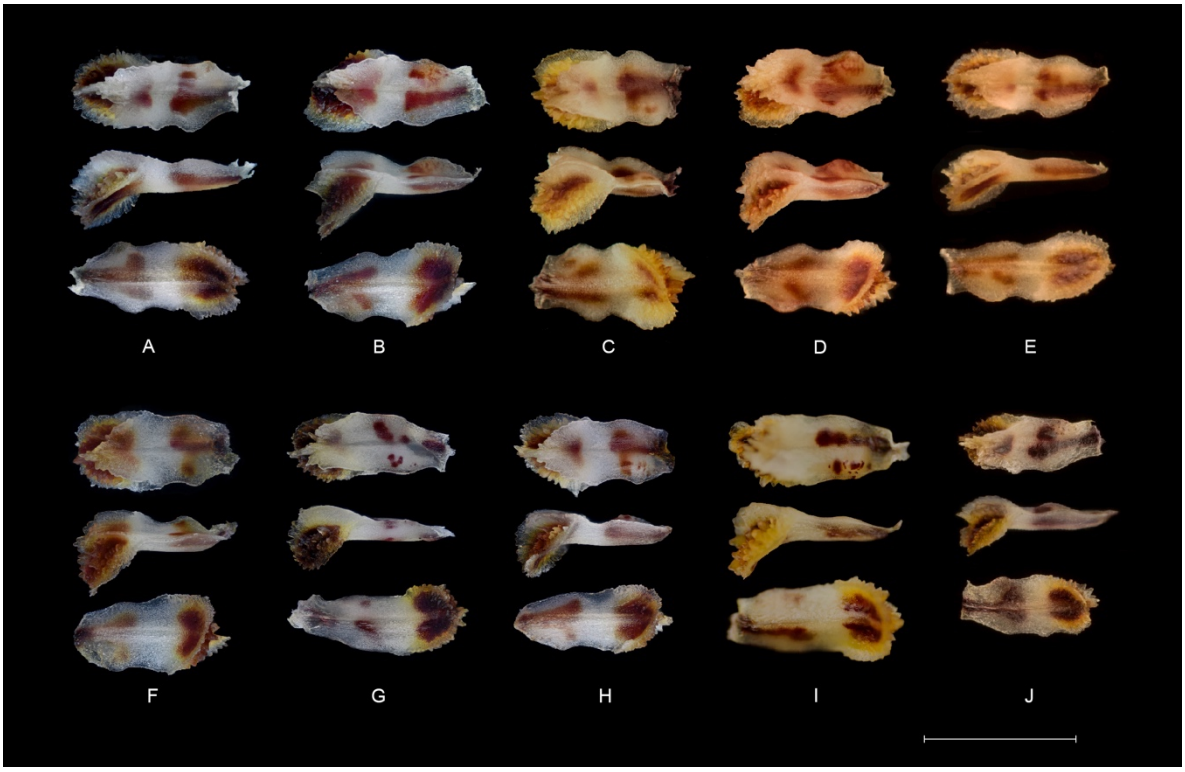


Figura 9. Variación en los pétalos de *Dracula erythrochaete*. A. *K. Gil 184*. B. *K. Gil 143*. C. *K. Gil 171*. D. *K. Gil 126*. E. *K. Gil 132*. F. *Bogarín 13017*. G. *K. Gil 245*. H. *Rojas-Alvarado 323*. I. *Valverde s.n.* J. *Rojas-Alvarado 119*. Escala = 3 mm.

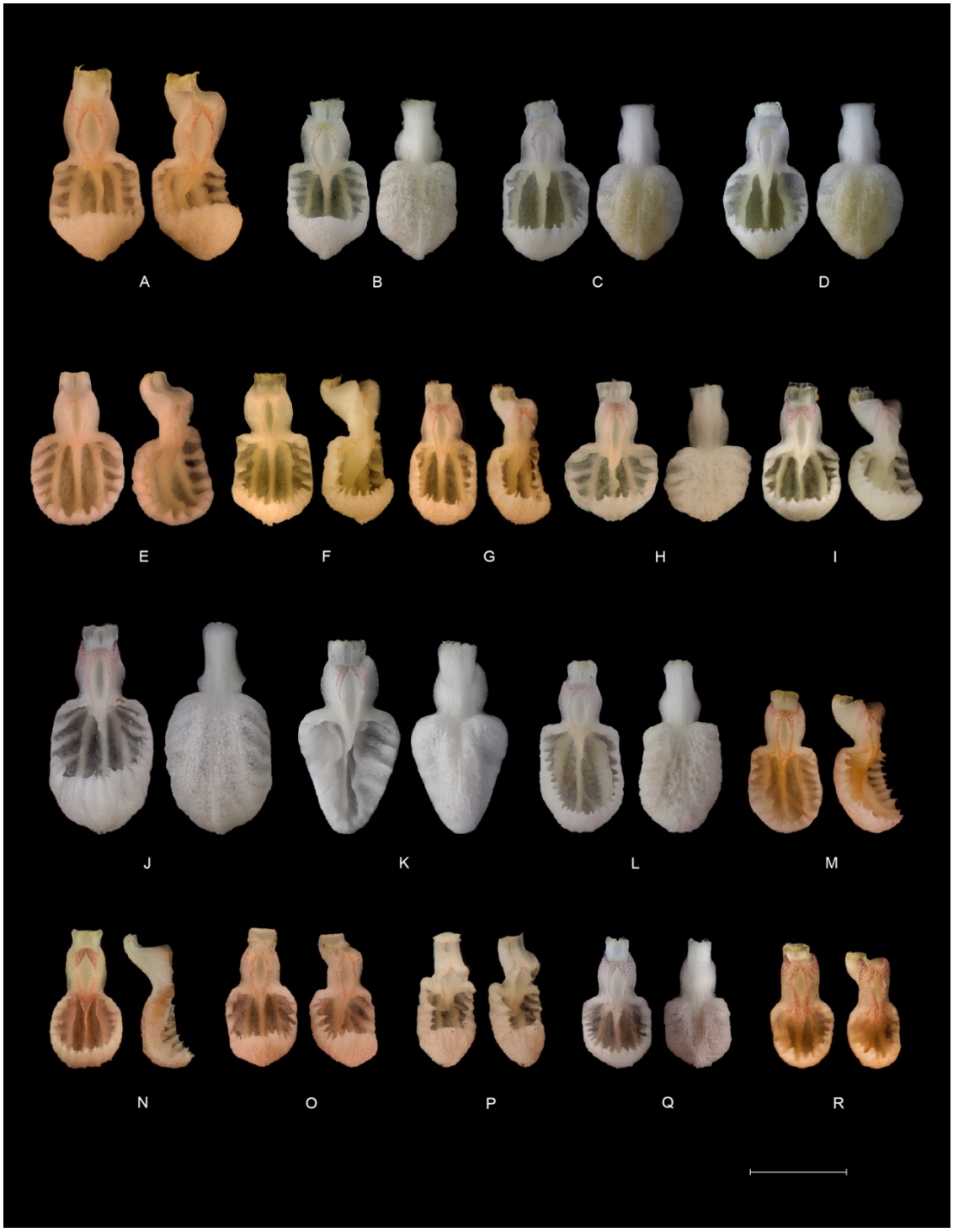


Figura 10. Variación en los labelos de *Dracula erythrochaete*. A. *K. Gil 132*. B. *K. Gil 184*. C. *K. Gil 160*. D. *K. Gil 143*. E. *K. Gil 126*. F. *K. Gil 136*. G. *K. Gil 114*. H. *K. Gil 171*. I. *K. Gil 121*. J. *Belfort s.n.* K. *Bogarín 13017*. L. *Rojas-Alvarado 323*. M. *Karremans 8115*. N. *Rojas-Alvarado 119*. O. *Bogarín 11966*. P. *Pupulin 5502*. Q. *K. Gil 245*. R. *Valverde s.n.* Escala = 5 mm.

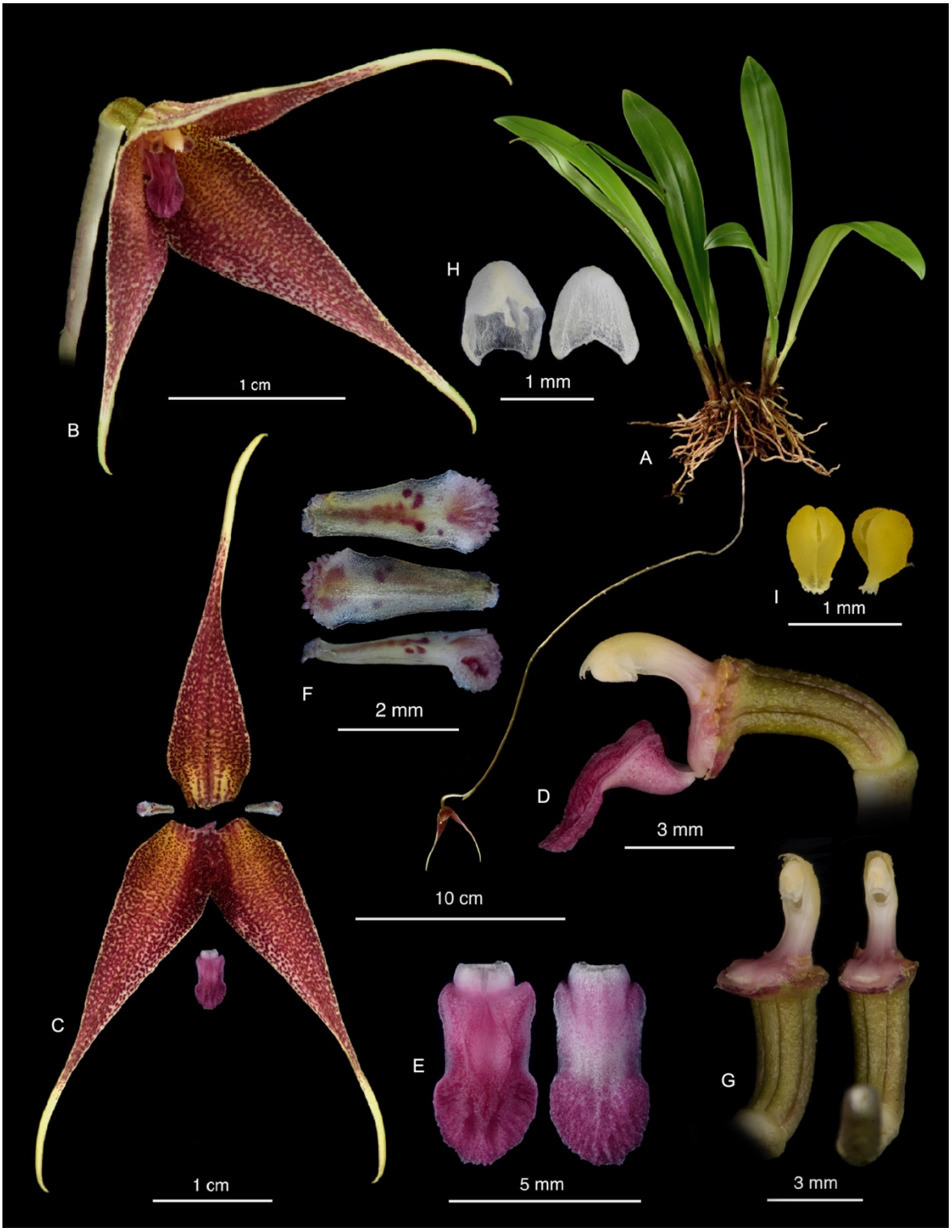


Figura 11. LCDP de *Dracula inexperata*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas de tres cuartos y ventral. H. Antera, dos vistas. I. Polinario, dos vistas. Basado en *Bogarín 12558* (JBL-spirit).

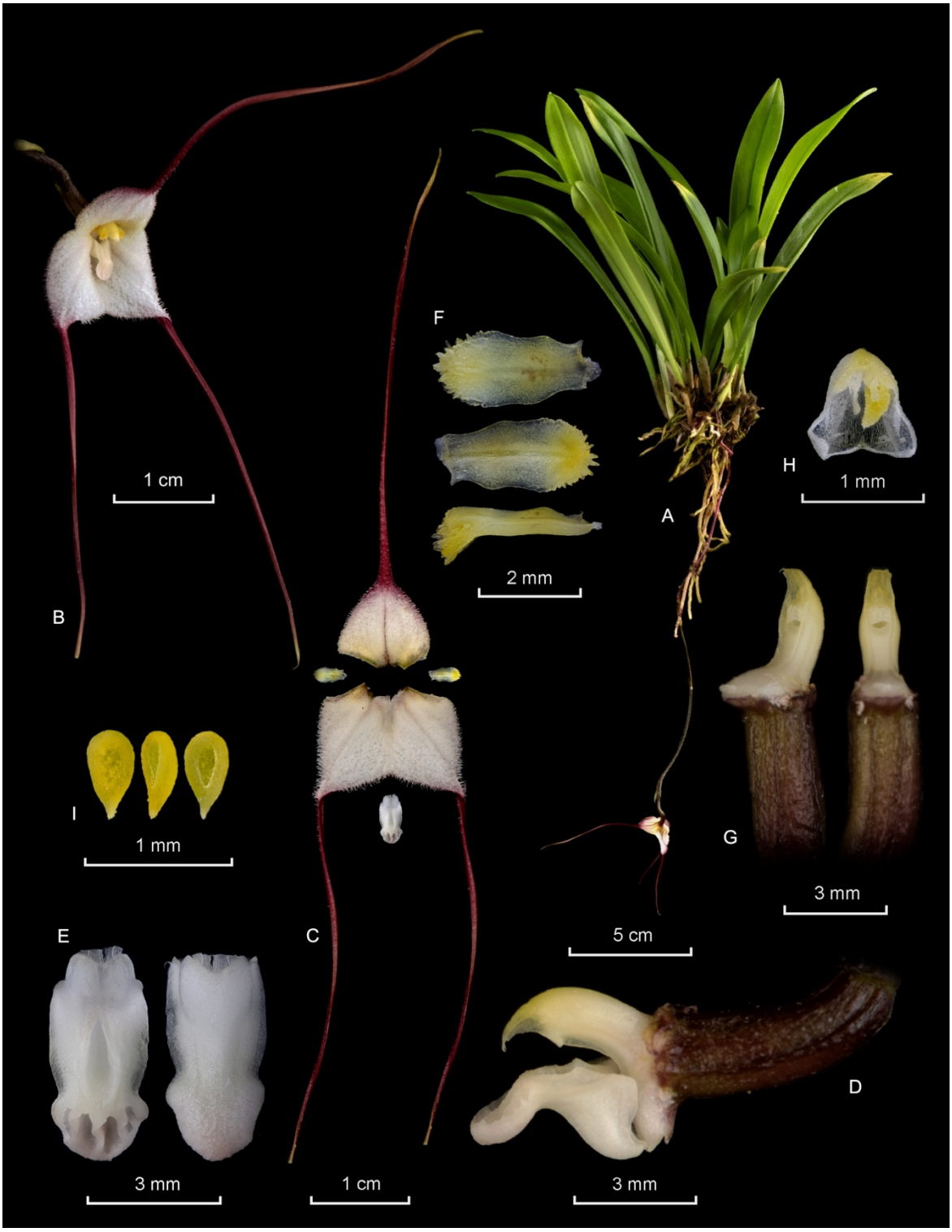


Figura 12. LCDP de *Dracula maduroi*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas de tres cuartos y ventral. H. Antera. I. Polinio, tres vistas. Basado en *Belfort 380* (JBL-spirit).

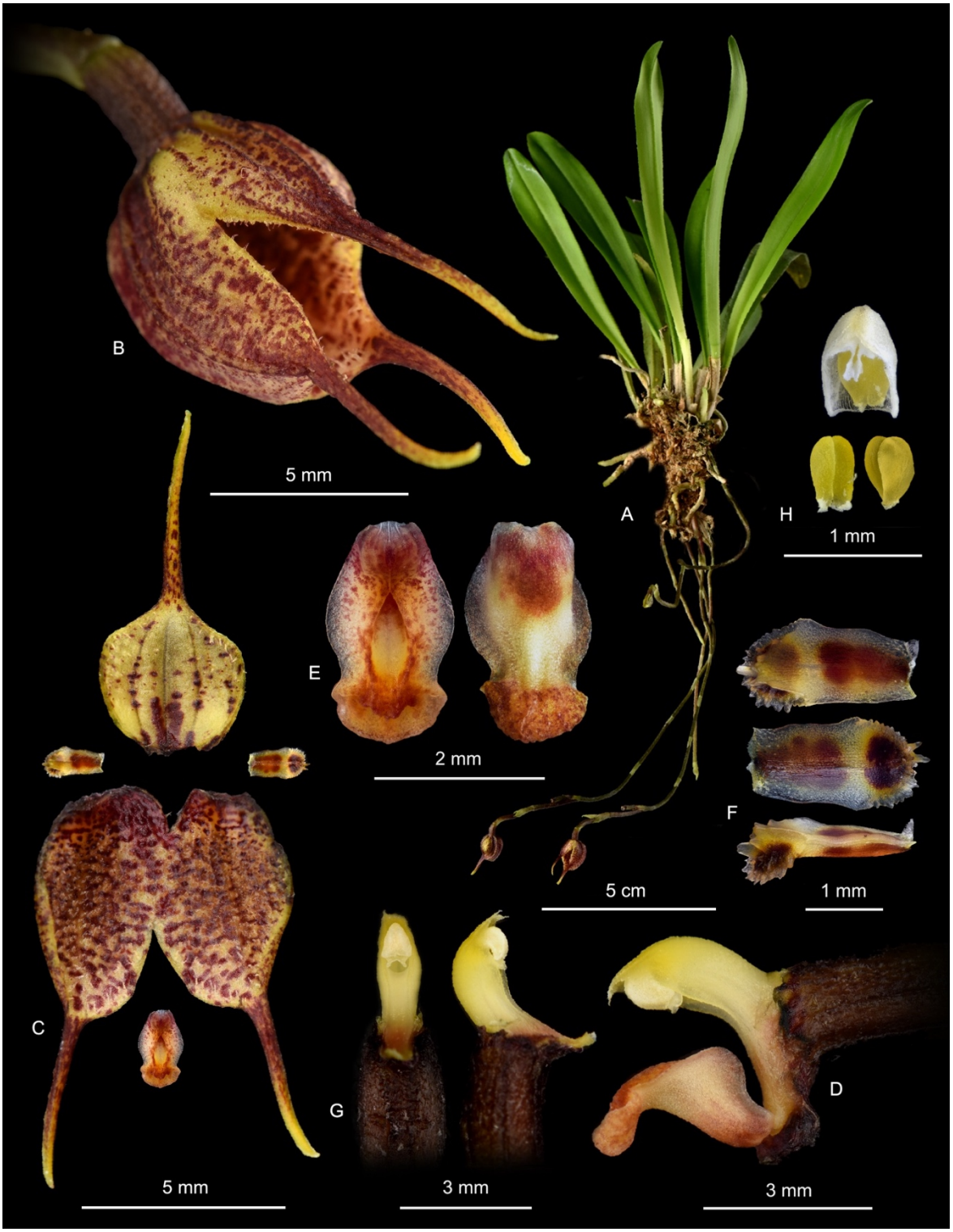


Figura 13. LCDP de *Dracula pusilla*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas ventral y lateral. H. Antera, vista ventral y polinario, dos vistas. Basado en *Bogarín 12426* (JBL-spirit).

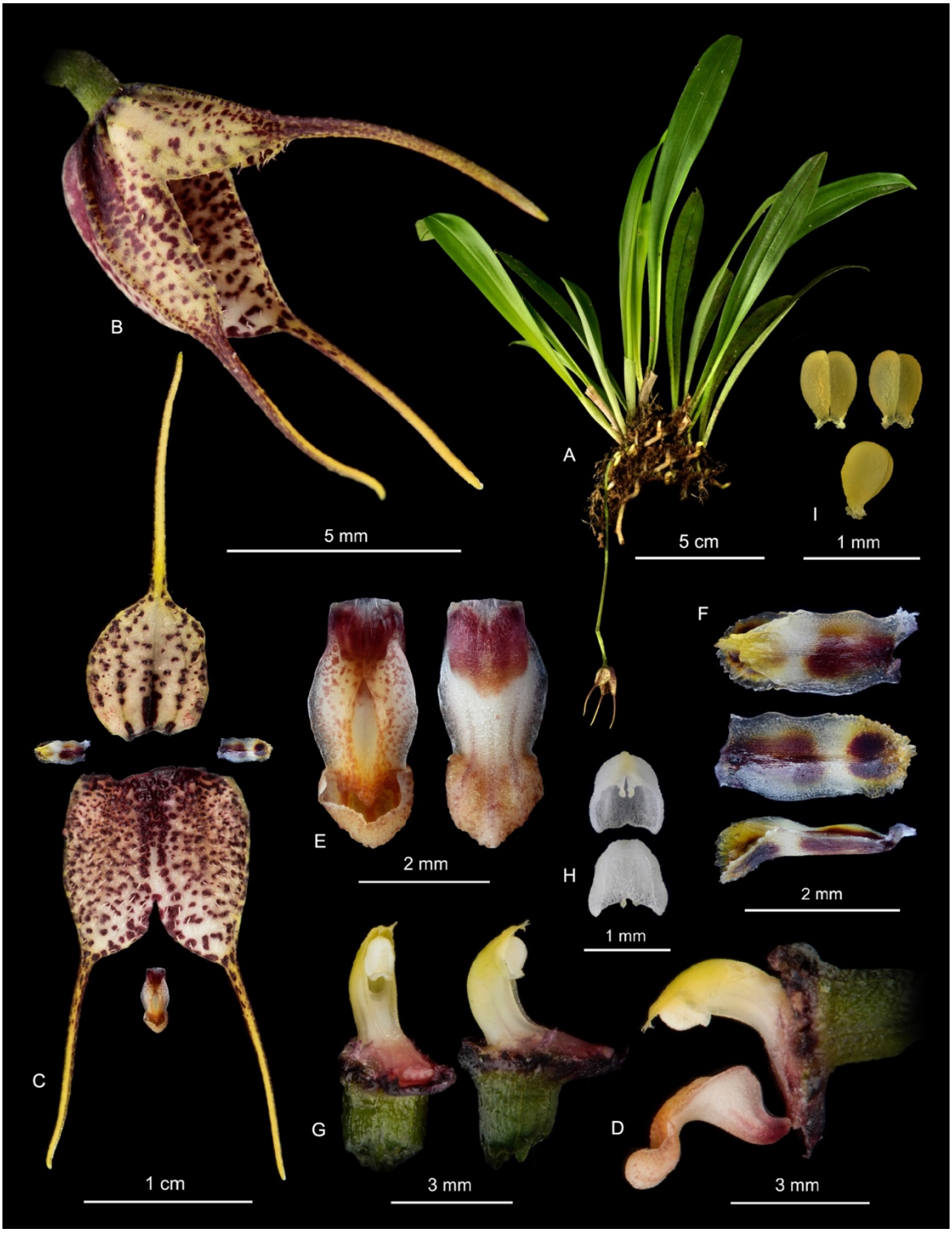


Figura 14. LCDP de *Dracula pusilla*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna y labelo, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas de tres cuartos y lateral. H. Antera, dos vistas. I. Polinario, tres vistas. Basado en *K. Gil 243* (JBL-spirit).

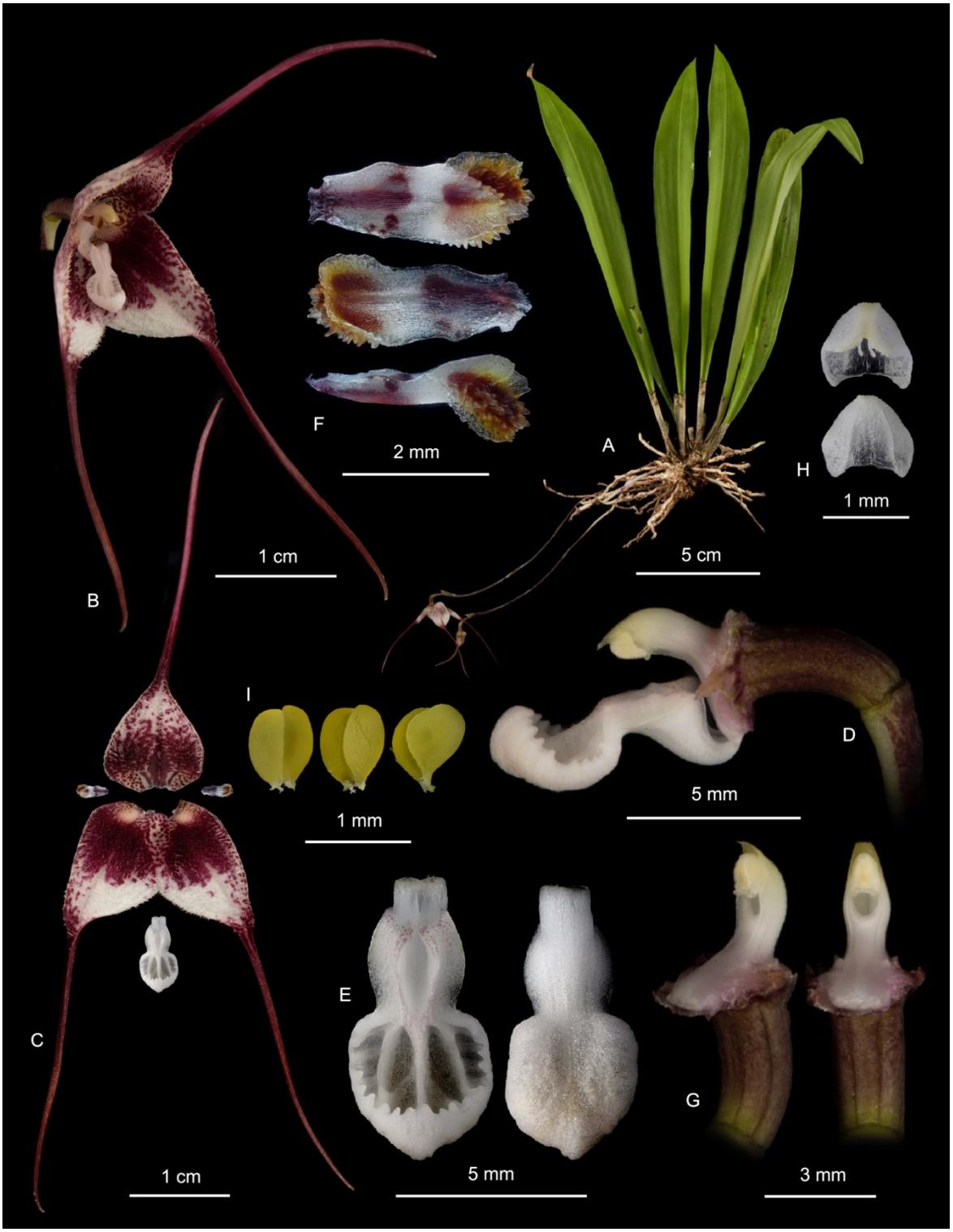


Figura 15. LCDP de *Dracula ripleyana*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas de tres cuartos y ventral. H. Antera, dos vistas. I. Polinario, tres vistas. Basado en *Bogarín 10128* (JBL-spirit).

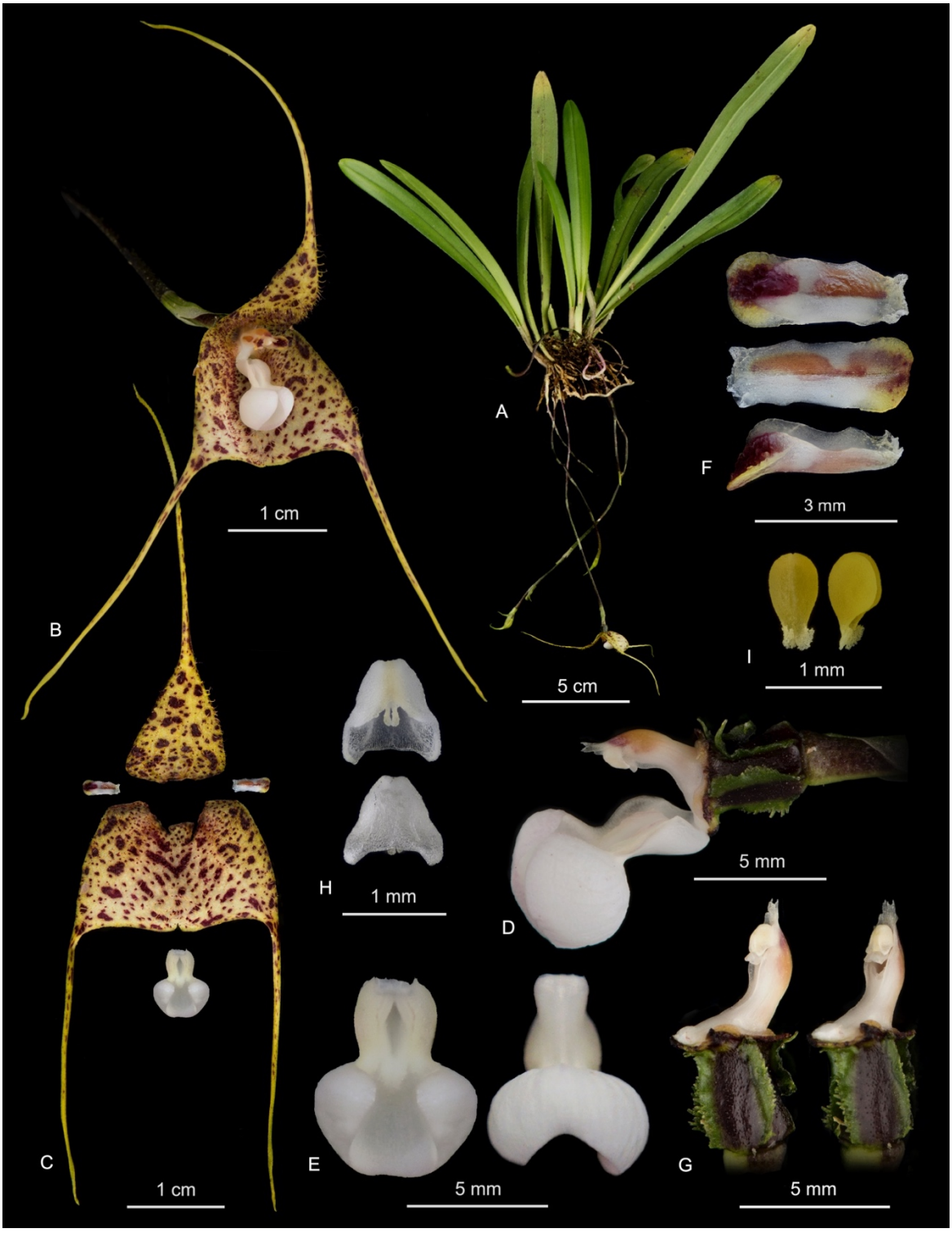


Figura 16. LCDP de *Dracula vespertilio*. A. Hábito. B. Flor. C. Perianto disectado. D. Columna, labelo y ovario, vista lateral. E. Labelo, vistas adaxial y abaxial. F. Pétalos, vistas abaxial, adaxial y lateral. G. Columna, vistas lateral y de tres cuartos. H. Antera, dos vistas. I. Polinario, dos vistas. Basado en *Bogarín 12550* (JBL-spirit).

**ARTÍCULO: Pollination ecology of *Dracula* (Pleurothallidinae): *D. erythrochaete* as a case of food deception in a fungal mimicry system**

Karen Gil-Amaya[1,2*], Melania Fernández[1,3], Miguel Benavides[4], Lizbeth Oses[1], Alfredo Cascante-Marin[5], Mario A. Blanco[1,5,6], Adam P. Karremans[1,7]

[1] *Jardín Botánico Lankester, Universidad de Costa Rica, Cartago, Costa Rica.*

[2] *Programa de Posgrado en Biología, Universidad de Costa Rica, San José, Costa Rica.*

[3] *Department of Plant and Soil Science, Texas Tech University, Lubbock, Texas, USA.*

[4] *Centro de Investigación en Granos y Semillas (CIGRAS), Universidad de Costa Rica, San José, Costa Rica.*

[5] *Escuela de Biología, Universidad de Costa Rica, San José, Costa Rica.*

[6] *Centro de Investigación en Biodiversidad y Ecología Tropical, Universidad de Costa Rica, San José, Costa Rica.*

[7] *Naturalis Biodiversity Center, Leiden, The Netherlands.*

[*] *Corresponding author: karen.gil@ucr.ac.cr*

## ABSTRACT

Pleurothallidinae is the most diverse Neotropical subtribe in Orchidaceae and is almost exclusively pollinated by insects of the order Diptera. *Dracula*, a genus of 138 species in the Pleurothallidinae, is known to attract flies of the genus *Zygothrica* (Drosophilidae), which are common visitors of macrofungi, by imitating fungal volatile compounds and lamellae. However, knowledge on the pollination of *Dracula* species is incomplete. No species of *Dracula* is known to offer nectar, and pollination by brood-site imitation has been hypothesized. In this study, we document the pollination mechanism of the Central American species *Dracula erythrochaete*, and characterize the floral structures involved in the mechanism by morphological, anatomical and footage evidence. Flowers of *D. erythrochaete* shared the same group of visitors with nearby macrofungi (orders Agaricales and Russulales), including different *Zygothrica* species, five of which were determined as effective pollinators. Male and female flies were attracted to the flowers from the first day of anthesis, and displayed a feeding behavior. Accordingly, proteins were detected in high concentrations on the papillae at the base of the movable lip and in papillary trichomes of the sepals, near the column, presumably to guide flies towards the reproductive organs of the flower. Osmophoric tissue was documented on sepals, petals, and lip, indicating three possible centers of fragrance synthesis. Brood-site imitation is debated as no oviposition events were observed nor were eggs found on the flowers, and an alternative for pollination by food deception is proposed.


**KEYWORDS:** Drosophilidae – floral biology – histochemistry – movable lip –orchid pollination – osmophoric tissue – protein secretion – *Russula* – *Zygothrica*.

## INTRODUCTION

The orchid family, with over 880 genera and 27,800 species (Givnish et al., 2016), is recognized for a remarkable diversity of pollinators and pollination systems that include both rewarding and deceptive pollination (Darwin, 1877; van der Pijl & Dodson, 1966; Dressler, 1981; Ackerman, 1985). Fragrances (Dressler, 1968; Hetherington-Rauth & Ramírez, 2016), oils (Pauw, 2006; Blanco et al., 2013), nectar (Ackerman et al., 1994; Stpiczyńska et al., 2003), resins and waxes (Stpiczyńska & Davies, 2009; Davies & Stpiczyńska, 2012), pollen (Kocyan & Endress, 2001) and pseudopollen (Davies et al., 2000; Davies et al., 2004), have been identified as floral rewards. On the contrary, deceptive pollination strategies involve the imitation of a resource required by the pollinator (e.g., food, a potential mate, the substrate for oviposition) with no compensation for the visit.

Flowers of a third or more of the Orchidaceae are assumed to be deceptive (van der Pijl & Dodson, 1966; Ackerman, 1986; Shrestha et al., 2020). The most common deceptive pollination strategies in orchids involve generalized food deception (Pansarin et al., 2008; Pansarin et al., 2014; Vale et al., 2011; Caballero-Villalobos et al., 2017; Bogarín et al., 2018a; Naczk et al., 2018; Lipińska et al., 2020) and sexual deception (Ayasse et al., 2003; Schiestl et al., 2003; Singer et al., 2004; Blanco & Barboza, 2005; Martel et al., 2016). These strategies are well documented among orchids and have independently evolved in different lineages within the family (van der Pijl & Dodson, 1966; Cozzolino & Widmer, 2005). Deceptive pollination, in particular, appears to have contributed to the increase in orchid diversity through small shifts in floral traits that may attract a different set of pollinators and promote reproductive isolation (Smith, 2010; Givnish et al., 2015).

In plants, visual and chemical mimicry are involved in the attraction of visitors that favor reproduction (Dafni, 1984; Jersáková et al., 2006). There is a type of mimicry namely fungal mimicry, in which flowers attract flies that oviposit or feed on fungi by imitating the odor and morphological appearance of the fruiting bodies of the latter. Three plant families have been reported with this strategy to attract flies for pollination: Aristolochiaceae, Araceae, and Orchidaceae (Vogel, 1978; Vogel, 1990; Mesler & Lu, 1993). At least three genera of orchids are reported to be fungal mimics: *Corybas* Salisb. (Kelly et al., 2013), *Cypripedium* L. (only for a few species; Proctor et al., 1996) and *Dracula* (Dentinger & Roy, 2010; Endara et al., 2010; Policha et al., 2016).

Specifically, genus *Dracula* bears descendent inflorescences with flowers directed downwards. These flowers are characterized by a concave lip that is usually movable and composed of an epichile with radiating lamellae that imitate the fleshy sporocarps of macrofungi of the order Agaricales, exploiting the innate preferences and feeding behavior of Drosophilidae flies that look for fungi growing in the same habitat (Endara et al., 2010; Policha et al., 2019). Numerous taxa within Drosophilidae oviposit on macrofungi and use their fruiting bodies to feed their larvae (Courtney et al., 1990).

*Dracula* flowers do not produce nectar in detectable amounts (Endara et al., 2010) but may produces specific volatile compounds. In *Dracula chestertonii*, composition of floral volatiles is dominated by typical fungi constituents, which contain more than 70% of the volatiles: oct-1-en-3-ol, oct-1-en-3-one, octan-3-ol, and octan-3-one (Kaiser, 2006). The emission of oct-1-en-3-ol was also found in *Dracula lafleurii* Luer & Dalström, in which production is mainly restricted to the lip (Policha et al., 2016). Even though chemical aromatic profiles have been carried out in species of *Dracula* pollinated by Drosophilidae flies, none of these include anatomical studies that elucidate the secretory structures responsible for the production of these scent glands (osmophores) and neither report anatomical structures that produce rewards for pollinators. Furthermore, a lustrous film was observed on the lip of *D. lafleurii* during the first day of anthesis, and it was proposed to serve as growth medium for yeasts (Endara et al., 2010). McAlpine (2013) found yeast species growing on the lip and sepals of *D. felix* (Luer) Luer and *D. lafleurii* and suggested that these yeasts could constitute a food reward or modify the scent produced by the flowers.

Drosophilidae flies have been suggested to accidentally pollinate *Dracula* flowers while inspecting the lip as a potential oviposition site (Vogel, 1978; Kaiser, 2006). Policha et al. (2019) attempted rearing flies from diverse substrates (including flowers of *Dracula* and other plants), but very few flies emerged from the *Dracula* flowers and there was no overlap in the reared insect species and the pollinators of *Dracula*.

In the genus *Dracula*, pollination has only been studied in detail in two of the 138 described species, *D. lafleurii* and *D. felix*, both from Ecuador. Those were visited and pollinated mostly by flies of the genera *Zygothrica* (Wiedemann) and *Hirtodrosophila* (Duda) (Drosophilidae) (Endara et al., 2010; Policha et al., 2014; Policha et al., 2016). Pollination in *D. lafelurii* occurs when the fly's thoraces are trapped by the incurved flaps of

the rostellum which lodges in the space between the scutellum and the abdomen of the insect. Eventually the fly escapes, removing the viscarium together with the pollinia (Endara et al., 2010). However, not only does the pollination mechanism of most *Dracula* species remain undocumented, but in general terms, the functions of the various floral structures and traits (e.g., sepaline tails, trichomes, papillae, patterns of coloration of the sepals, petals) are not clearly established.

In this study, we address three specific questions: 1) Who are the visitors and pollinators of *D. erythrochaete*? 2) What is the behavior of the pollinators in flowers and macrofungi? and 3) Which are the anatomical structures involved in the production of scents and possible rewards in *D. erythrochaete*? By answering these questions, we hope to better understand the pollination mechanism of *D. erythrochaete*. Based on the characteristics of the cells and their contents, we hope to predict a possible function that certain organs play in the pollination system of *D. erythrochaete*.

## MATERIALS AND METHODS

### STUDY SITE AND SPECIES

We studied the pollination of *Dracula erythrochaete* from a wild population occurring in Tablón, Quebradilla, Cartago, Costa Rica (9°49'38.3"N 84°01'45.4"W), in a lower montane tropical forest dominated by *Quercus* spp. and plantations of *Pinus*, at 1890 m. The roots, trunks and branches of these trees are colonized by a large array of epiphytes, and are surrounded by macrofungi growing on decaying oak wood, mosses, and organic matter (Table 1).

*Dracula erythrochaete* (Rchb.f.) Luer is distributed from Panama to Costa Rica, it is the most abundant species of the genus *Dracula* in Costa Rica, and blooms throughout the year with two flowering peaks from April to June and August to October, coinciding with the rainy season. This species is recognized by its thin, narrow leaves, small to medium-sized flowers that usually emit a fungus-like scent, sepals white to pale yellow suffused with red purple or brown with trichomes and papillae on the adaxial surface, apices contracted into slender red tails, a movable lip white, pink or light brown with 3-primary lamellae and few radiating elevated veins.

## REMOVAL OF FLORAL STRUCTURES

To identify the possible role of floral structures and their relative importance in the mechanism of pollinator attraction to flowers, pollinia removal and deposition on the stigma, six treatments were applied to *D. erythrochaete* flowers under natural conditions, which consisted of the subtraction of different floral parts: a) removal of the sepaline tails, b) removal of the trichomes and papillae of the sepals, c) complete removal of sepals, d) removal of petals e) removal of the lip, and f) complete flowers (control). Fourteen replicates (14 flowers) were made for each treatment, from different plants and inflorescences, during June and July of 2019, for a total of 140 flowers (n=140). Each treatment was applied at the same time to flowers, depending on availability of flowers at each visit. Only flowers in early anthesis, with both the pollinia and stigma intact, were used to rule out the influence of any previous visits.

## POLLINATION MECHANISM IN *D. ERYTHROCHAETE*

Specimens of *D. erythrochaete* were collected at the study site and cultivated in the greenhouses at Lankester Botanical Garden (JBL), University of Costa Rica, from 2018 to 2021. Plant voucher specimens were prepared from cultivated material and deposited in the JBL (spirit) and USJ herbaria. Flies were observed, filmed, and photographed in situ and in the open-air greenhouses at JBL. Observations and collections of flies from floral structures and macrofungi were carried out with a pooter, between 6:00 and 18:00 h for a total of ca. 35 days of observation. In addition, examination of the flowers after visits and pollination events to look for eggs or larvae of Drosophilidae flies were carried out. Flies were identified by a combination of morphological characteristics following Grimaldi (1987; 2010) and non-invasive (sample rescue after lysis) DNA barcoding of the 660-bp COI (mitochondrial cytochrome c oxidase subunit COI marker). The DNA extraction and sequencing service were performed in Naturalis Biodiversity Center, Leiden, The Netherlands.

Macrofungi were identified to the genus level by Milagro Mata Hidalgo at School of Biology, University of Costa Rica based on photographs and videos of details of the fertile surface (hymenophore) as well as the substrate where they were attached. Only visitors that lingered on flowers and were interactive with sepals and the movable lip (for > 30 min) were considered potential pollinators. The flies that carried pollinia were considered effective

pollinators. Additionally, the sex of the pollinators was determined based on the presence of female terminalia (ovipositor) or male terminalia (epandrium), to understand their implications within the mechanism of pollination. Vouchers for the insects were deposited in the Pollinator Collection at JBL and the Insect Museum at the University of Costa Rica (San José, Costa Rica).

## PHOTOGRAPHY, VIDEO AND DIGITAL IMAGING

Photographs were taken with Nikon D7100 and D810 digital cameras, coupled with 105 mm macro lenses or a bellows-mounted 40 mm Zeiss Luminar lens. Images were stacked with Zerene Stacker (Zerene Systems LLC, Richland, WA) and edited using Adobe Photoshop CS6. Videos were taken using the continuous video option on the Nikon D3200, D7100 digital cameras and iPhone 6s, and then digitally processed. Final digital images and composite figures were processed in Adobe® Photoshop CC 2018 and videos with Final Cut Pro. Digital images of light microscopy (LM) were taken using a Leica DM500 microscope with a Leica ICC50 camera and the Leica Application Suite (LAS) v4.12 microscope software.

## INSECT BEHAVIOUR

Data from 392 minutes (6.5 hours) of video recordings were obtained and analyzed, in 77 events of insect visits to flowers of *D. erythrochaete* and macrofungi. Each event was freely recorded in the period of observation between 6:00 and 18:00 h, and more than one behavior per individual could be recorded in the visitation events. We determined and described from observations and recordings: 1) possible interactions between insects and flowers or macrofungi; 2) the duration of visiting and pollination events; 3) the events of removal and deposition of pollinia.

We classified the behaviors of the flies as described by Grimaldi (1987) in: a) feeding, when the flies have the proboscis extend, b) resting, when flies simply standing without movement, c) cleaning, when flies touch their own body with their legs before feeding or pollinated d) walking, when the flies passively move on the substrate, e) courting, when male displays wing movements (semaphoring) or abdominal curling to females, f) ovipositing,

when females approach their ovipositor on the substrate, g) copulating, when males mount on females.

## LIGHT MICROSCOPY (LM) AND HISTOCHEMISTRY

To identify and characterize anatomical structures involved in the production of scent molecules, lipids, polysaccharides and protein synthesis, fresh and paraffin-embedded flowers were subjected to a series of staining and imaging protocols. Positive and negative controls were included for each staining protocol, while glycerin-mounted fresh unstained flowers were used to observe natural pigmentation. In fresh flowers, heavily-pigmented tissues were cleared for 2 hours (sepaline tails for 24 h) in 10% (v/v) commercial solution of sodium hypochlorite and rinsed in 30% ethanol for 1 hour, to avoid the interference of tissue coloration in staining results (Ruzin, 1999). In the case of not process the flowers the same day, samples were fixed in 30% ethanol for subsequent staining.

Areas of scent emission, lipids and secretory tissue, were detected by submerging the samples in a solution of aqueous Neutral Red 0.1% (NR) (w/v, tap water) for ~1 hour, and differentiated with tap water (Ruzin, 1999). To identify possible lipids contents (fats, oils and waxes), we used Sudan Black B (SBB) 0.07% (w/v, ethanol 70%) and Sudan IV (SIV) 0.5% (w/v, 70% ethanol), while osmium tetraoxide ($OsO_4$) was used for unsaturated fats (Bronner, 1975; Ruzin, 1999). The samples were stained for 1 hour, rinsed briefly in water, and mounted on slides in glycerin (Brundrett et al., 1991).

Insoluble polysaccharides and starch were detected with a periodic acid–Schiff reaction (PAS) by oxidizing the samples in aqueous solution of periodic acid (HIO4) 5% (w/v) for 10 minutes, rinsing 3 times in distilled water for 2 minutes and submerging for 15 minutes in Schiff's reagent, and finally submerging in tap water at 50-60ºC for 5 minutes (Ruzin, 1999). Proteins were detected with Coomassie brilliant blue R-250 (CBB), submerging the samples in a solution of CBB 0.25% (w/v) for 10 minutes, rinsing three 3 times in 50% ethanol, then in 7% acetic acid for 10 minutes, and rinsed in tap water (Jensen, 1962; Fisher, 1968).

To prepare paraffin-embedded flower tissues for staining, sections of ca. 1 cm of the sepals, petals, and lips were fixed in FAA (ethanol 70%, formalin 10%, acetic acid 5%, v / v), for 48 hours and separated in histological cassettes. Dehydration was performed through

a graded series of ethanol (70%, 80%, 90%, 95%, 100%, 100%) and xylene, then they were infiltrated with Paraplast Plus® for tissue embedding (Sigma-Aldrich, USA) in a laboratory oven at 60ºC, the tissue was embedded in stainless-steel base molds, and allowed to solidify at room temperature. Paraffin blocks were trimmed and sliced to obtain sections of 7µm thick with a Leica RM2125 RTS rotary microtome.

To prepare for staining, the paraffin sections mounted in microscope slides were immersed in xylene two times, then rehydrated with a series of ethanol (100%, 100%, 95%, 70%) and distilled water for 2 minutes in each step using a manual staining rack. The paraffin sections were stained by submersion in Toluidine blue (TB), Coomassie brilliant blue (CBB), and Periodic acid–Schiff (PAS), for 20 minutes, then dehydrated with ethanol (95%, 100%, 100%), and xylene for 2 minutes. Sections were mounted in a Leica CV Mount® mounting medium and dried overnight at room temperature.

## SCANNING ELECTRON MICROSCOPY (SEM)

With the aim of characterized the morphology of indument (trichomes and papillae) on the surface of sepals, petals and lips, dissected fresh flowers of *D. erythrochaete* were fixed for 24 hours in a 2.5% glutaraldehyde and 2% paraformaldehyde solution in 0.1 M sodium phosphate (Karnovsky, 1965). The samples were degassed with a vacuum system and then rinsed three times for 15 minutes each in 0.1 M sodium phosphate buffer (pH 7.4) prior to embedding. Post fixation was performed for 2 hours in 2% osmium tetroxide (OsO4) and rinsed three times for 15 minutes in distilled water.

Fixed samples were dehydrated in a series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, 100%, 100%), then were submersed in absolute ethanol and amyl acetate solution 1:1 and amyl acetate for 20 minutes, as a transition liquid before drying to the critical point. The samples were desiccated using the LEICA® EM CPD300 automated critical point dryer (Leica Byosystems, Germany). The samples were arranged on aluminum bases and covered with a layer of 20nm of gold, in an EMS 150R S rotary-pumped coating system (Electron Microscopy Sciences, Pennsylvania) and observed with a HITACHI 3700-N scanning microscope at the Research Center for Microscopic Structures (CIEMIC) of the University of Costa Rica**.**

# RESULTS

## FUNCTIONAL IMPORTANCE OF FLORAL STRUCTURES

None of the flowers whose sepaline tails, sepals and lip were removed produced fruit, all other treatments produced fruit, but in a lower percentage than the control treatment. By removing the petals, 2% of the flowers produced fruit. When removed the trichomes and papillae of the sepals, only 1% of the flowers produced fruit. The 5% of the flowers in the control treatment produced fruits (Fig. 1).

## POLLINATION BIOLOGY AND INSECT BEHAVIOR

Flies are attracted to the flowers of *D. erythrochaete* from the first day of anthesis, even before the sepals have finished extending. The opening of the flower begins from the apex of the sepaline tails downwards, until the sepals extend fully. At this point, the flies can access the lip directly. The insects were observed attempting to feed with the extended proboscis and walking on the lip and sepals (Supporting Information, Video S1). A fungus-like scent was perceived from flowers in the mornings and afternoons, becoming stronger and citrusy in the late afternoons, during or after rain. In two cases at JBL greenhouses, the strong scent was still felt on the flowers until 18:40 h. Visitation can happen any time during the day, mostly from 7:00 to 17:30 h. No visitation event was observed at night. Groups of up to 24 flies were observed on a single flower. However, there were also solitary visits or in smaller groups of two or three individuals. The amount of time that individual flies spent on the flowers ranged from a few minutes to more than 3 hours per visit. We observed flies trapped between the column and movable lip of the flower, occasionally carrying pollinaria attached to the scutellum.

We collected a total of 197 insects, 75% from flowers and 25% from macrofungi at two locations in the *D. erythrochaete* population and in the open-air greenhouses at JBL. The majority of specimens caught (182) belonged to the order Diptera (92%). The remaining identified specimens included 10 Hymenoptera (5%), four Hemiptera (2%) and one Coleoptera (0.5%) (Supporting Information, Table S1). Within Diptera, the most representative family was Drosophilidae, with 164 (90%) specimens caught, other fly families included Dolichopodidae, Lauxaniidae, and Sphaeroceridae. The flies of the genus *Zygothrica* (Drosophilidae) were the most common visitors to the flowers and macrofungi

(Figs. 2A-J, 2M-V; Supporting Information, Table S1). Furthermore, the wasps Platygastridae (Hymenoptera) (Fig. 2K) and *Spilomena* (Crabronidae: Hymenoptera) (Fig. 2L) were found on the flowers, while Braconidae (Hymenoptera) (Fig. 2W) and Eucoilinae (Figitidae: Hymenoptera) (Fig. 2X) were found on the macrofungi. Close inspection of 35 flowers using a stereoscope after visits and pollen removal or deposition revealed the presence of aphids (Aphididae: Hemiptera) and yellow slug eggs (Gastropoda). However, Diptera eggs or larvae were never located on any flowers both in the field and greenhouses. Scanning electron microscopy (SEM) also did not reveal any (trace of) Diptera eggs or larvae.

Forty-one flies with pollinaria of *D. erythrochaete* were collected (Table 2), 39 from flowers, and two from macrofungi, one was captured on *Russula* Pers. (Russulales) (Fig. 3; Supporting information, Video S2), and the other in *Gymnopus* (Pers.) Roussel (Agaricales). Of the 41 pollinators caught, 27 (65%) were sequenced (Fig. 4), and of these 21 (77%) belonged to one species (Figs 4, 5A–R), while six (23%) belonged to four different species (Figs 4, 5S–U). In total, five species of *Zygothrica* remove pollinaria of *D. erythrochaete* (Figs 4, 5). All of the sequenced flies (visitors and pollinators) belong at least seven species of the genus *Zygothrica* (Fig. 4). Among the pollinators, we found the same sex ratio, 16 males and 16 females with the orchid pollinaria (Fig. 5; Table 2). It was not possible to determine the sex of nine individuals because their genitalia were not preserved or they were collapsed, however, in most of the pollinators we observed the female terminalia (Fig. 6A) and the male terminalia (Fig. 6B).

## FLY BEHAVIOUR ON MACROFUNGI

We identified 29 macrofungi taxa coexisting with the flowering plants of *D. erythrochaete* at three localities, one at JBL, 9°50’21.018”N, 83°53’23.058”W, and two on the wild population in Tablón, Quebradilla, Cartago, 9°49’38.3”N, 84°01’45.4”W, and 9°49’46.2”N, 84°01’38.6”W. They belong different orders, 18 of them were Agaricales (62%), 3 were Boletales (10%), one Polyporales (3%) and seven Russulales (24%). The most diverse macrofungi found in this study were Agaricales represented in 10 genera, with six morphospecies of the genus *Gymnopus*, and the order Russulales represented by a single genus *Russula*, with seven morphospecies observed. In a range of 1 to 5 meters distance to the closest flowering plant, the most visited macrofungi by flies were the genera *Russula*

(Russulales) (ca. 119 individuals), *Oudemansiella* Speg. (Agaricales) (ca. 30), and *Gymnopus* (Agaricales) (ca. 20) (Table 1). All of them with lamellae on the hymenophore. The same groups of flies were observed moving freely between *D. erythrochaete* and different macrofungi (Supporting Information, Video S3). At times, when the macrofungi were completely full of flies, any interruption made the flies take off and fly to the flowers. When the flowers were not nearby, the flies would perch on the leaves, moss and hepatics.

While visiting the macrofungi, the flies spent most of their time feeding (31%) (Figs 7A, 8A; Supporting Information, Video S4) and resting (22%), we also documented other behaviors in the pileus and lamellae of the macrofungi such as walking (19%), cleaning (19%), courting (5%), ovipositing (3%), and copulating (1%) (Fig. 7A). Males of the genus *Zygothrica* were observed performing courtship behaviors, such as wing movements "semaphoring", and "curling" which is when the male curves his abdomen to show the coloration of the apex of his wings to the female (Supporting Information, Video S5). This behavior was documented in *Oudemansiella canarii* (Jungh.) Höhn (Agaricales), and in two morphospecies of the genus *Russula*, where oviposition was also seen in the pileus of macrofungi (Supporting Information, Video S6). A wasp of the subfamily Eucoilinae, which could be parasitoid of the larvae of flies, was observed and collected in these macrofungi (Fig. 2X). We also found eggs and larvae of flies growing on lamellae of macrofungi of the genera *Lepista* (Fr.) WGSm. (Agaricales) at 1-10 meters of flowering plants (Fig. 8B).

## FLY BEHAVIOUR ON *D. ERYTHROCHAETE*

The interactions between flies and flowers of *D. erythrochaete* were recorded in 318 minutes (5.3 hours), during 58 visits. The flies approached the flowers in direct flight and landed on the sepals or epichile of the movable lip, very rarely on the sepaline tails. They immediately walked and began to inspect the trichomes on the adaxial surface of the sepals and the papillose surface of the lip, usually from the apex to the base for about 30 to 60 minutes before leaving. They inspected the denticulate margins and lamellae of the epichile more intensely (Fig. 8C), as well as the tips of the longest trichomes and papillary trichomes of the sepals where they tasted and sucked exudates from the cuticular surface using the labial palpi of their articulated jutting proboscis (Supporting information, Video S7). The flies showed no interest in the petals, sometimes they were observed walking on the papillae, but

unlike the behavior documented in the sepals and lip, they were not observed sucking with their proboscis extended, attempting to feed.

During visits, recorded flies spent most of their time trapped between column-lip cavity (55%), struggling to get free (Figs 7B, 8D), while the remaining time (45%) the flies remained free on the sepals, petals, and lip, performing behaviors such as feeding (40%), walking (23%), and cleaning themselves (23%), rarely just resting (10%) (Fig. 7C). When fly aggregations grazed on the lip or sepals, we observed very occasional courting (4%) behaviors. At the wild population in Cartago, we recorded flies of the genus *Zygothrica* displaying repetitive wing movements with wings spread around 45º at various amplitudes and speeds. On one occasion, a fly approached another laterally with a wing extended about 90º, then began pawing over the fly's thorax with the forelegs extended very rapidly and several times (Supporting Information, Video S5). In groups of five or more individuals, there were competition for space in the lip and sepals of the flowers, but even in these situations they didn't stop sucking the surface of these structures. Copulation was observed only once occurring on the leaves. No oviposition or copulation were ever observed on *D. erythrochaete* flowers.

## POLLINATION MECHANISM IN *D. ERYTHROCHAETE*

The flies first inspect the lamellae and elevated veins of the epichile (Fig. 9B), then they walking to the concave base of the lip with the extended proboscis (Fig. 9C), sucking and attempting to feed on the clavate papillae of the hypochile, detailed in (Fig. 9A). The flies are pressed between the movable lip (which can be assisted by the wind) and the column. The natural position of the lip hanging like a pendulum moving in the wind (Fig. 9C), facilitates the movement of the lip and probably helps to push the fly against the column (Fig. 9D). Figures 9C–D shows this change in lip position. The small petals help to support the fly (Fig. 9E). During visits of up to 90 minutes or even more, we found flies trapped between column and lip, stuck to the viscid fluid (viscarium) on the rostellum (Figs 9D–F, 10). In the process of pollinia removal and deposition, the pollinators can be assisted by the wind to reach the movable lip with their hind legs and use it as a platform to exit (Supporting information; Video S8).

The flies rested at times, if there was no wind, then continued forcing themselves until they managed to grab the 3-primary lamellae of the epichile with their legs (Fig. 9F), sometimes turning from side to side in the direction of the petals, but these keep the flies in a horizontal position and prevent them from coming out of the sides and leaving without the pollinaria. As the fly exits backwards its scutellum is smeared with sticky viscid fluid that produced by the rostellum, as detailed in (Fig. 9F). Then, it brushes against the whale-tail-like caudicles of the pollinia, ensuring they are removed or deposited. When the pollinator gets free, falls into the epichile (Fig. 9G), and move to the lateral sepals for rest, then begins to clean exhaustively their head, proboscis, body, legs, and wings. Flies may or not remove the pollinaria with the anther cap (Fig. 9G–H), if they do, additional time is needed to remove it, usually with their hind legs. When flies remove the pollinia without the anther, they left the flower faster.

## FLORAL MICROMORPHOLOGY AND HISTOCHEMISTRY

The patterns of coloration of the sepaline tails, sepals, petals, and lip of *D. erythrochaete* are made of carotenoid pigments (yellow and red), flavonoids (colorless, white or pale yellow), and anthocyanins (red or purple) (Fig. 11). We found yellow carotenoids and red/purple anthocyanins at the apex of the glandular trichomes (Fig. 11C–D), papillary trichomes in sepals (Fig. 11E–F), and papillae of the petals (Fig. 11I). The flowers become darker as the amount of pigment increases; at the population, we observed both individuals with very intense red-purplish flowers and less colorful ones, with predominantly yellow flowers.

Stomata, trichome-like colleters, multicellular glandular trichomes, papillary trichomes, papillae and secretory materials were identified on the surface of the floral organs in *D. erythrochaete* (Table 3). The epidermal surface of the sepaline tails has rectangular flattened cells, smooth at the apex and covered with glandular trichomes at the base (Fig. 12D). The cuticle was ornamented with a striated pattern (Fig. 12C, L) and a thick epicuticular wax recognizable by the formation of fissures (Fig. 12A, C, L).

Both surfaces of the sepaline tails carried stomata. The stomata have pores formed by two occlusive cells and 4-6 subsidiary cells (Fig. 12A, C). A higher density of stomata was observed near the midrib where the tail folds inward and close to the margins (Fig. 12C),

some in a lateral position. Open and closed stomata were found, with secretory activity detected (NR) (Fig. 12B), and reserves of insoluble polysaccharides (PAS) accumulated in cell walls (Fig. 12E). Lipid droplets were identified with (SBB) and (SIV) and concentrated in the guard cells and subsidiary cells of the stomata (Fig. 12F–G), multicellular glandular trichomes (Fig. 12D), and trichome-like colleters (Fig. 12H). Proteins (CBB) were not detected at the apex or in the median region of the sepaline tails, except for the trichomes at the base. Osmiophilic bodies were revealed by ($OsO_4$) in the cytoplasm and cell walls, with a concentration on the epidermal cells (Fig. 12I) and trichomes.

Secretory trichome-like colleters were detected in the sepaline tails (Fig. 12H, J–L) and the sepals (Fig. 13A–B). The colleters are cylindrical at the base and globose at the apex. We find them scattered, solitary, or in groups (Fig. 13A) located close to the margins, on both surfaces of the sepals (Fig. 13B). These trichomes reacted positively with NR, PAS (Fig. 12J–K), SBB (Fig. 12H), and SIV (Fig. 13A), showing the concentration of lipids and insoluble polysaccharides at the base and some at the apex. The trichome-like colleters were visible only under staining, including in paraffin sections with (TBO) and ($OsO_4$) (Fig. 13B), none reacted positively for proteins (CBB).

Numerous glandular trichomes were identified on the adaxial surface of the entire blade of sepals (Fig. 13C–H) and at the base of the tails (Fig. 12D). The glandular trichomes are multicellular, multiseriate, with 1-3 elongated secretory terminal heads (Fig. 13C–D), a stalk composed of several cells, and a base of 8-12 cells, covered by regular striated cuticle and epicuticular secretions (Fig. 13D). The trichomes are different in size and arrangement; they are longest near the margins, as well as more concentrated and shorter in the middle region towards the center of the flower.

At the base of the sepals, we identify papillary trichomes without a developed apex, like elevated cells that form papillae on a glandular epidermis, with epicuticular striation (Fig. 13I). Histochemical tests revelated the content of lipids (NR, SIV, SBB), food reserves of proteins (CBB) and polysaccharides (PAS) on the apices of trichomes (Fig. 13E–H, J, K) and polyhedral starch grains of parenchyma (Fig. 13L). Trichomes and secretory tissue at the base and middle region of the sepals show a higher concentration of proteins than those at the margins.

The petals have elongated conical papillae apically (Fig. 14A) and a papillose surface in the median and basal region (Fig. 14G–H). They are covered by a conspicuously thickened reticulated cuticle (Fig. 14B) that forms irregular and asymmetrical celds with its cell wall, of five or six sides. We observed a large concentration of bundles of raphides in cells close to the margins (Fig. 14J) at the base and at the apex, these are visible with or without staining (Fig. 11H). Areas of scent emission and secretory activity were detected with (NR) and insoluble polysaccharides (PAS) on the elongated papillae (Fig. 14C–D) and the papillose surface (Fig. 17I), with starch grains stored in the parenchyma cells at the apex region (Fig. 14K). Proteins did not react positively to CBB (Fig. 14F), whereas lipophilic compounds (NR, SBB, SIV) and osmiophilic contents ($OsO_4$) were identified in large quantities within and on the apices of the papillae (Fig. 14E, L).

A papillose surface completely covers the adaxial (Fig. 15A) and abaxial (Fig. 15B) epidermis of the lip, including the raised lamellae in the epichile. We found elongated clavate papillae on the hypochile (Fig. 15F–J), both parts of the lip showing slightly striated cuticles with epicuticular secretions (Fig. 15E, H), and prismatic crystals (Fig. 15L), indicating a secretory function. Also, bundles of raphides were observed inside the cells in the papillae of the hypochile and papillose surface of the epichile. Although the lip reacted to the presence of carbohydrates (PAS) (Fig. 15D, F), lipids (SBB, SIV) (Fig. 15C), unsaturated fats ($OsO_4$) (Fig. 15K), proteins (CBB), and scents (NR), a higher protein concentration was found in the apices of the papillae on the hypochile (Fig. 15I–J). Concentrations of lipids (NR, SBB, SIV) and unsaturated fats ($OsO_4$) were detected in the papillae and epidermal surface, in addition to insoluble polysaccharides and starch grains (PAS) in the epichile (Fig. 15D), indicating the presence of scents and stored food materials, respectively.

## DISCUSSION

### FUNCTIONAL IMPORTANCE OF FLORAL STRUCTURES

The removal of floral structures significantly affects the fruit production of *D. erythrochaete*. Removal of the sepaline tails, sepals and lip, significantly influenced the proportion of flowers that produced fruit (0%). These structures contribute to the attraction of potential pollinators, probably because they have both a chemical and a visual role in the flowers of *D. erythrochaete*.

The fruit production of 2% in flowers with petals removed (Fig. 1), probably indicates a secondary or reinforcing function. Petals keeps the fly in the column-lip cavity, preventing them to exit from the sides, so they can play a complementary role in retaining the fly in a certain position, but they do not seem to be essential in attracting visitors or in the pollination mechanism.

According to our results, only about 5% of the complete flowers (control) produced fruits (Fig. 1), a very low percentage but in agreement with was reported for orchids that have a deceptive pollination strategy with low visitation and pollination rates (Neiland and Wilcock, 1998; Tremblay et al. 2005). Nevertheless, low reproductive success of orchids, are also frequent in tropical orchids dependent on a pollinator (regardless of the mechanism used to attract them), which may help explain unique pollination mechanisms and their extreme diversity.

## POLLINATION BIOLOGY AND INSECT BEHAVIOR

Our findings show that the sepaline tails up to 3-7 cm long, are regions composed of secretory tissue detected by Neutral Red (Table 3), with stomata and glandular trichomes, with concentrations of lipids (Fig. 12B, D, F–H, J), unsaturated fats (Fig. 12I), and insoluble polysaccharides (Fig. 12K), which are probably involved in the production of scents that could serve for long distance attraction. The attracted flies walk from the sepals to the lip and attempt to feed by following the trichomes and papillae guides, which also reacted positively to (NR, PAS, SBB, SIV, and $OsO_4$), indicating high secretory activity and showing epicuticular secretions with SEM images (Figs. 13D, I, 15E). The behavior suggests feeding, and although the reward is not visible, the papillae contain proteins (Figs. 13K, 15I–J). We could not identify the proteins synthesized by *D. erythrochaete*, nor the amount secreted, however, the stainings with Coomassie brilliant blue (CBB) showed a high concentration of proteins in the papillary trichomes of the sepals and papillae along the hypochile, probably to keep the flies longer and bring them to the correct position for pollination.

The movable lip (sometimes assisted by the wind) tilts towards the column, when it reaches with its extended proboscis the secretory papillae of the hypochile, closing the gap between the lip and column (Fig. 9C–D). In *D. erythrochaete*, the petals keep the fly in a horizontal position by pressing the wings against the column and mechanically block the exit

of the insect from the sides (Figs 8D; 9E). While the fly exits backwards, it removes the pollinia by first touching the sticky, viscarium-rich rostellar flap (Figs 9F; 10), and then the pair of caudicles with its scutellum; or deposits the pollinia on the stigma if it already carried them. The effective pollinator has to struggle to get free from the flower, and the wind can assist by bringing the movable lip closer to the fly.

This mechanism is a variation of the pollination mechanism generalized in Pleurothallidinae, defined by Karremans & Díaz-Morales (2019) as "masdevalliform" where pollinators enter the column-lip cavity, and are pressed by the hinged lip against the column, removing the whale-tail pollinia with their scutellum while exiting. Observations of flies trapped between column-lip cavity have been made in other *Dracula* spp. (Fig. 16), and sometimes dead flies with pollinia attached to the scutellum have also been found (Fig. 16A, D, E). However, these flies serve as a reference, they are important because they are similar insects to potential pollinators. The masdevalliform mechanism has also been reported with variations in the genera *Acianthera*, *Anathallis*, *Lankesteriana*, *Masdevallia*, *Muscarella*, *Myoxanthus*, *Octomeria*, *Scaphosepalum*, *Specklinia*, *Stelis* (*Effusiella* and *Unciferia*), and *Trichosalpinx* (Karremans and Díaz-Morales, 2019; Karremans & Vieira-Uribe, 2020), and it is also to be expected with variations in the sister groups of the "Masdevallia clade", *Trisetella* Luer and *Porroglossum*.

Drosophilidae is a cosmopolitan family with about 4400 species (Toda, 2021), it is reported as the single family that pollinates the genus *Dracula* with flies from different but closely related genera: *Drosophila* Fallén, *Hirtodrosophila* and *Zygothrica* (Policha et al., 2019; Karremans and Díaz-Morales, 2019). Central American species represent most of the ecological diversity throughout the Neotropical Region, and Costa Rica, with approximately 600 species, is the best studied country in Central America for drosophilids (Grimaldi, 2010); nevertheless, the field ecology is largely unexplored because the breeding sites of many species are inaccessible, difficult to find, or simply unknown (Courtney et al., 1990). Drosophilids feed and/or breed on various substrates such as fermenting fruits, sap fluxes, fungi, decaying plant materials, fresh plant leaves/stems, flowers, and cacti (Zhang et al., 2021). Mycophagy evolved independently in different lineages of the family Drosophilidae (Throckmorton, 1975; Zhang et al., 2021), and the genus *Zygothrica* with nearly 40 species described for Central America comprises mostly mycophagous specialists (Grimaldi, 1987;

2010). In mycophagous Drosophilidae, adults and larvae usually feed at the same fruiting bodies, adults usually feed (and lay eggs) on host material that is in a fresher state than the larvae feed on. Some other studies show adult drosophilids feeding on different materials than do their larvae (Courtney et al., 1990).

Flowers of *Dracula erythrochaete* and the nearby Agaricales and Russulales macrofungi share the same group of visitors, including Diptera and Hymenoptera. The flowering phenology overlaps with macrofungi that occur in high-density populations in the habitat, increasing the visit frequency of insects and allowing free movements between them (Supporting information, Video S3), attracting females and males of at least seven species of the genus *Zygothrica* (Fig. 4), five of these species removed the pollinaria (Figs 4; 5). We found that the two flies collected with pollinaria in the macrofungi belong to a single species of *Zygothrica*, being the most collected species among both substrates (Fig. 5A–R). Furthermore, and individual collected visiting the flowers of *D. chimaera*, which also belongs to the genus *Zygothrica*, is closely related to the flies of macrofungi that visit the flowers of *D. erythrochaete* in Costa Rica (Fig. 4). The flowers also attracted wasps, which are probably the most common non-Dipteran visitors of Pleurothallidinae in search of nectar (Karremans and Díaz-Morales, 2019). The small wasps of the genus *Spilomena* observed are reported as predators of adults and nymphs of Aphioidea, Thysanoptera, Psylloidea, Coccoidea and Eulophidae (Vardy, 1987; Antropov & Cambra, 2004), while the tiny wasps of the family Platygastridae are reported as parasitoids of the egg-larval or egg-pupal of Diptera: Cecidomyiidae, Orthoptera: Acrididae, and Hemiptera: Pseudococcidae (Buhl, 2001; Asadi-Farfar, 2021).

Endara et al. (2010) observed courtship and mating behaviors of flies in *D. lafleurii* and courtship behaviors in *D. felix*, but they never recorded oviposition or discovered eggs in flowers. Policha et al. (2019) tested the brood-site mimicry hypothesis by examining rearing success, although they found Drosophilidae larvae emerging from flowers, these were not from the pollinators, nor they did not observe any flies ovipositing. In contrast, the time the flies spent walking and sucking with their proboscis extended was significantly greater than time spent on mating behaviors. These courtship behaviors were also very rare in *D. erythrochaete* (Fig. 7C), and we also found no evidence for the laying of eggs in flowers. Oviposition normally requires an interplay between olfactory, taste, and mechanosensory

input to be released. However, in plants that use brood-site imitation pollination strategy, this releasing combination of signals is, in most instances, missing in the flower, with some exceptions, such as in some species of the genus *Stapelia* (Apocynaceae) (Jürgens et al., 2006), where the development of carrion fly larvae is commonly observed (Urru et al., 2011).

Flies use their sense of smell to locate a possible oviposition substrate from a distance, some odors cause oviposition while others inhibit it. Thus, there are odors that attract females because they signal an oviposition substrate and there are odors that stimulate oviposition over shorter distances (Cury et al., 2019). In *Drosophila melanogaster* Meigen, ethanol, and acetic acid elicit egg-laying, while olfactory cues that inhibit oviposition include deterrent odors such as geosmin, phenol, and parasitoid wasp pheromones (Ebrahim et al., 2015). A possible explanation for the absence of drosophilids eggs in *Dracula erythrochaete* may be associated with the presence of parasitoid wasps (Platygastridae) in flowers. It has been found that parasitoid wasp odors inhibit *Drosophila* egg-laying behavior (Cury et al., 2019). Also, visual detection of wasps is sufficient to alter the oviposition behavior of females that are actively seeking a safe environment for their eggs (Kacsoh et al., 2013). Drosophilidae females exposed to wasps can signal danger to unexposed flies with wing movements; observer flies receiving this visual signal, without seeing the wasps, will retain and destroy their eggs (Kacsoh et al., 2015).

Although *Zygothrica* flies may be attracted to *D. erythrochaete* flowers as a potential oviposition site, these flies are not being fully stimulated. Scents that are being volatilized to attract flies over long distances are present in the flowers, but odors that retain flies to stimulate oviposition fail to provide sufficient stimulation for egg laying. The fungal compound 1-octen-3-ol, obtained from flowers of some *Dracula* species (Kaiser, 2006; Policha et al., 2016), and released by many fungal genera such as *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Mucor* and *Ulocladium*; is responsible for the characteristic scent of mold and can be considered an indicator of fungal growth indoors (Inamdar et al., 2012; Bennett & Inamdar, 2015). However, drosophilids that were exposed by inhalation of 1-octen-3-ol for 24 h, showed a decrease in the survival and locomotor ability of flies (Macedo et al., 2020); this volatile organic compound induces mitochondrial morphological alterations and respiration dysfunctions in *Drosophila melanogaster* (Macedo et al., 2020). This volatile compound, instead of being an attractant, as proposed Policha et al. (2016), correlated with

events such as sporulation and spore germination of fungi, may actually have the function of inhibiting the stimulation of fly oviposition.

Diptera is characterized by presenting a larval stage very different from the adult, this type of development allows juvenile stages to use different food sources and habitats than adults, which eliminates the possibility of competition between the different stages of their life cycle and allows adults and larvae to coexist with higher population densities (Nadia & Machado, 2014). In mycophagous species, there is a spatial component of niche division between adults and larvae, the adults restrict feeding to the surface of the pileus while the larvae tunnel through the fungal material (Lacy, 1984; Courtney et al., 1990). Diptera do not have parental care, therefore, they look for food for their own consumption, being less active in the search for food compared to other insects that care for their offspring (Faegri & van der Pijl, 1979). These food habit (mycophagy) preferences in the *Zygothrica* genus can be exploited by the flowers of *Dracula erythrochaete.*

## FLORAL MORPHOLOGY AND HISTOCHEMISTRY

The osmophoric glandular trichomes of the sepals and the osmophoric papillae of the lip indicate that two types of olfactory signals might be used for long-range attraction, while the osmophoric papillae on the petals probably indicate one olfactory signal for close-range attraction, as in *Bulbophyllum ornatissimum* (Rchb.f.) J.J.Sm. (Vogel, 1990) and *Trichosalpinx* (Bogarín et al., 2018a) in which two heterogeneous centers of fragrance synthesis were reported on the sepals and lip. Odors can attract pollinators from a distance or may direct them to certain points of the flower (Woodcook et al., 2014). The direct flights observed in *Dracula* flowers, regardless of wind direction, probably signify visual orientation caused by pigments in the trichomes, papillae, and epidermal surface. At close-range, odors can similarly act as guides, directing flies to resources (proteins and polysaccharides) and positioning them to effecting pollination. Physical vibration of flowers or flower parts as they move in the wind can also serve as an attractant, as in the case of *Bullbophyllum* (Orchidaceae), *Ceropegia* (Asclepiadaceae), or *Herrania* (Malvaceae) that accentuate their movement with motile appendages (Vogel, 1990; Woodcock et al., 2014).

The papillose surface of the lip shows slightly striated cuticles and various secretions such as lipids (SBB, SIV), insoluble polysaccharides (PAS), but most notably proteins

(CBB), especially on the clavate papillae of the hypochile (Fig. 15I–J). Our histological tests showed that there was a polarization in protein detection on the lip; the hypochile which is the concave base articulated at the column-foot, and therefore closest to the column, has the highest concentration of proteins in relation to the secretory papillae of the epichile, which is consistent with the description of Luer (1993) in which proposes that this deep central cleft of the hypochile in the genus *Dracula* is possibly a nectary or fragrance source. The feeding behavior of flies suggests that pollinators are guided towards this point of the lip to obtain the proteins as a possible floral reward. In Pleurothallidinae, detailed investigations as in *Anathallis, Lankesteriana, Trichosalpinx, Lepanthopsis* (Bogarín et al., 2018b), and species of *Bulbophyllum* (Davies & Stpiczyńska, 2014; Kowalkowska et al., 2015), demonstrated by histochemical tests protein-secreting papillae on the lip, however, only in *Trichosalpinx* these results were supported by the feeding behavior of the flies and as stated by Bogarín et al., (2018a) these proteins were not enough to be considered a reward.

The presence of starch grains accumulated in the epichile and restricted to the parenchymatic cells surrounding the vascular bundles (Fig. 15D), suggests that they are probably reserves used as a source of energy to produce volatile compounds. Furthermore, positive reaction with (NR), the presence of lipophilic compounds (Fig. 15C), and accumulation of lipid-rich substances (SBB, SIV), probably precursors or the fragrance itself, indicate scent synthesis and secretion through the papillose surface. The starch grains in the osmophoric tissue are a common feature of scent glands as documented in osmophores of other orchids (Wiemer et al., 2009; Antoń et al., 2012; Pansarin et al., 2014; Casique et al., 2021; Pansarin et al., 2021) and these starch agglomerations decrease as volatile evaporation occurs since starch is used as an energy source for the production of nectar or scent (Vogel, 1990; Effmert et al., 2006).

The non-positive reaction for proteins in the petals of *D. erythrochaete* (Fig. 14F; Table 3), together with the low interaction of the flies, walking without the extended proboscis, suggest that no reward is produced there. In contrast, the high secretory activity detected (NR), starch grains stored in parenchyma cells (PAS), osmiophilic droplets ($OsO_4$), and lipid compounds (SBB, SIV), suggest scent synthesis in the conical papillae of the apex and emission by diffusion through the conspicuously thickened reticulated cuticle (Fig. 14B). This reticulated cuticle could be linked to a mechanism of light diffraction, producing more

intense colors, and thus acting as visual cues (Antoniou Kourounioti et al., 2013). The parallel position of the petals with respect to the column and the lack of rewards or osmophoric tissue at the petal base (Table 3), suggests the function to keep the potential pollinators direct towards the base of the lip and preventing them to exit from the sides. We found many cells filled with bundles of long acicular crystals known as raphides (Figs 11H; 14J) probably associated with maintaining the rigidity of the petal structure. Large crystals may fill the cells and then determine the interior contour of the wall (Eames & MacDaniels, 1925).

Oxalate crystals are also present in the epichile and hypochile of the lip (Fig. 15L). We find them solitary in the cytoplasm, in a variety of very small cuboidal or prismatic forms, concentrated in areas with strong metabolism and high secretory activity of scents and proteins, which suggests that they are probably waste products, the result of metabolic processes, or crystalline deposits of stored calcium (Arnott & Pautard, 1970; Beck, 2010). However, in other species of orchids crystals occur in the sepals, petals and lip (de Melo et al., 2010; Bogarín et al., 2018a; Lipińska et al., 2020; Pansarin et al., 2021). In *Stelis* Swartz, Chase and Peacor (1987), suggest that refractile properties of crystals founded might mimic nectar droplets, which act as visual attractants that lure pollinators.

## CONCLUSIONS

The sepaline tails, sepals, and lip are essential structures for the attraction and pollination mechanism of *D. erythrochaete*. For the first time, it is confirmed that *Dracula* flowers effectively share the same group of visitors with nearby macrofungi (orders Agaricales and Russulales). Fly behavior on *D. erythrochaeate* flowers and the macrofungi was mainly related to feeding. *Dracula erythrochaete* flowers offer a food reward through secretion of proteins from the papillae of the sepals and lip. There is no evidence of Drosophilidae egg-laying on *Dracula* flowers. Based on our behavioral and histological data, we propose that this is not a deception system, but rather a reward system, with the offer of proteins as a food reward. Most of the 138 *Dracula* spp. show similar floral traits and therefore we hypothesize that other *Dracula* spp. are pollinated via a similar system. Future studies of *Dracula* orchid pollination may reveal many species of *Zygothrica* that are attracted to flowers and that remain undescribed.

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## SUPPORTING INFORMATION

Table S1. Voucher information of specimens caught on flowers of *Dracula* and macrofungi.

Video S1. *Zygothrica* (Drosophilidae) spp. attracted from the first day of anthesis of *D. erythrochaete* flowers.

Video S2. *Zygothrica* with pollinia of *D. erythrochaete* visiting the macrofungi *Russula* at the wild population in Cartago.

Video S3. Drosophilidae flies moving freely between *D. erythrochaete* and *Russula* (Russulales) in a range of 1 to 5 meters distance at the wild population in Cartago.

Video S4. Flies feeding on macrofungi coexisting with *D. erythrochaete*.

Video S5. Courtship behaviors in *Russula* and *D. erythrochaete* flowers performed by male *Zygothrica*.

Video S6. Oviposition behavior of female *Zygothrica* in *Russula* macrofungi.

Video S7. Flies feeding on flowers of *D. erythrochaete*.

Video S8. Pollinia removal and deposition events in *D. erythrochaete*.

Table 1. Macrofungi coexisting with *Dracula erythrochaete* in Cartago, Costa Rica.

| **Fungal order** | **Fungal genus** | **No. Visitors** | **Distance to closest flowering plant (meters)** | **Substrate** |
|---|---|---|---|---|
| Russulales | *Russula* | 119 | 0 - 5 | Organic matter, moss, soil |
| Agaricales | *Oudemansiella* | 30 | 0 - 1 | Soil |
| Agaricales | *Gymnopus* | 20 | 0 - 5 | Organic matter, moss, Wood |
| Agaricales | *Hypholoma* | 14 | 0 - 5 | Wood |
| Agaricales | *Gerronema* | 10 | 0 - 1 | Moss |
| Agaricales | *Lepista* | 4 | 0 - 5 | Organic matter |
| Agaricales | unknown | 1 | 0 - 1 | Wood |
| Agaricales | *Mycena* | 1 | 0 - 5 | Moss |
| Boletales | *Aureoboletus* | 0 | 0 - 1 | Moss |
| Agaricales | *Cortinarius* | 0 | 0 - 10 | Organic matter |
| Agaricales | *Crepidotus* | 0 | 0 - 5 | Moss, wood |
| Polyporales | *Ganoderma* | 0 | 0 - 10 | Wood |
| Agaricales | *Inocephalus* | 0 | 0 - 1 | Soil |
| Agaricales | *Pleurotus* | 0 | 0 - 5 | Moss |
| Boletales | *Scleroderma* | 0 | 0 - 5 | Organic matter |
| Boletales | *Strobilomycetes* | 0 | 0 - 1 | Wood |

Table 2. *Zygothrica* specimens caught with pollinia of *Dracula erythrochaete*.

| Insect ID | Plant species / Fungal species | Genus | Species | Sex | Locality and coordinates | Insect voucher |
|---|---|---|---|---|---|---|
| 001 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Jardín Botánico Lankester, Cartago, 9°50'21.018"N 83°53'23.058"W | (JBL) |
| 003 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 020 | *Dracula erythrochaete* | *Zygothrica* | unknown | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 022 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 026 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 035 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 036 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 037 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 041 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | Insect Museum (UCR) |
| 046 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 053 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL) |

| | | | | | | |
|---|---|---|---|---|---|---|
| 054 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | Insect Museum (UCR) |
| 055 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | Insect Museum (UCR) |
| 056 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 057 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 058 | *Dracula erythrochaete* | *Zygothrica* | sp. 4 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 068 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 075 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | Insect Museum (UCR) |
| 078 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 084 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | Insect Museum (UCR) |
| 086 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 103 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 104 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 113 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ? | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 116 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 117 | *Dracula erythrochaete* | *Zygothrica* | sp. 5 | ♀ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 118 | *Dracula erythrochaete* | *Zygothrica* | unknown | ? | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 119 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'46.2"N 84°01'38.6"W | (JBL)* |
| 143 | *Dracula erythrochaete* | *Zygothrica* | sp. 2 | ♂ | Jardín Botánico Lankester, Cartago, 9°50'21.018"N 83°53'23.058"W | (JBL)* |
| 150 | *Dracula erythrochaete* | *Zygothrica* | sp. 4 | ♀ | Jardín Botánico Lankester, Cartago, 9°50'19.29"N 83°53'18.34"W | (JBL)* |
| 152 | *Dracula erythrochaete* | *Zygothrica* | sp. 3 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 154 | *Dracula erythrochaete* | *Zygothrica* | sp. 4 | ♀ | Jardín Botánico Lankester, Cartago, 9°50'19.29"N 83°53'18.34"W | (JBL)* |
| 155 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♀ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 156 | *Dracula erythrochaete* | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 157 | *Gymnopus* (Agaricales) | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |

| | | | | | | |
|---|---|---|---|---|---|---|
| 173 | *Russula* (Russulales) | *Zygothrica* | sp. 1 | ♂ | Tablón, Quebradilla, Cartago, 9°49'38.3"N 84°01'45.4"W | (JBL)* |
| 181 | *Dracula erythrochaete* | *Zygothrica* | unknown | ♀ | Jardín Botánico Lankester, Cartago, 9°50'21.018"N 83°53'23.058"W | (JBL)* |
| 192 | *Dracula erythrochaete* | *Zygothrica* | unknown | ♀ | Jardín Botánico Lankester, Cartago, 9°50'19.29"N 83°53'18.34"W | (JBL) |
| 193 | *Dracula erythrochaete* | *Zygothrica* | unknown | ♀ | Jardín Botánico Lankester, Cartago, 9°50'19.29"N 83°53'18.34"W | (JBL) |
| 195 | *Dracula erythrochaete* | *Zygothrica* | unknown | ♂ | Jardín Botánico Lankester, Cartago, 9°50'19.29"N 83°53'18.34"W | (JBL) |
| 196 | *Dracula erythrochaete* | *Zygothrica* | unknown | ♂ | Jardín Botánico Lankester, Cartago, 9°50'19.29"N 83°53'18.34"W | (JBL) |

(JBL) Jardín Botánico Lankester

(JBL)* Jardín Botánico Lankester, Sequencing

Insect Museum (UCR) Universidad de Costa Rica

Table 3**.** Metabolites identified in stomata, trichomes and papillae of *Dracula erythrochaete* flowers by histochemical tests.

| **Target compounds** | **Staining** | **Sepaline tail stomata** | **Sepaline tail trichome-like colleters** | **Sepaline tail glandular trichomes** | **Sepal blade trichome-like colleters** | **Sepal blade glandular trichomes** | **Sepal blade papillary trichomes** | **Petal base papillose surface** | **Petal apex papillae** | **Lip Hypochile papillae** | **Lip Epichile papillose surface** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Lipids, areas of fragrance emission, secretory tissue (red) | Neutral Red (NR) | + | + | + | + | + | + | + | + | + | + |
| Insoluble polysaccharides and starch (pink) | Periodic acid-Schiff (PAS) | + | + | + | + | + | + | + | + | + | + |
| Proteins (blue) | Coomassie brilliant blue (CBB) | - | - | + | - | + | + | - | - | + | + |
| Total lipids (fats, oils and waxes) (black) | Sudan Black B (SBB) | + | + | + | + | + | + | - | + | + | + |
| Total lipids (fats, oils and waxes) (red) | Sudan IV (SIV) | + | + | + | + | + | + | - | + | + | + |
| Unsaturated fats (black) | Osmium tetraoxide ($OsO_4$) | + | + | + | + | + | + | + | + | + | + |

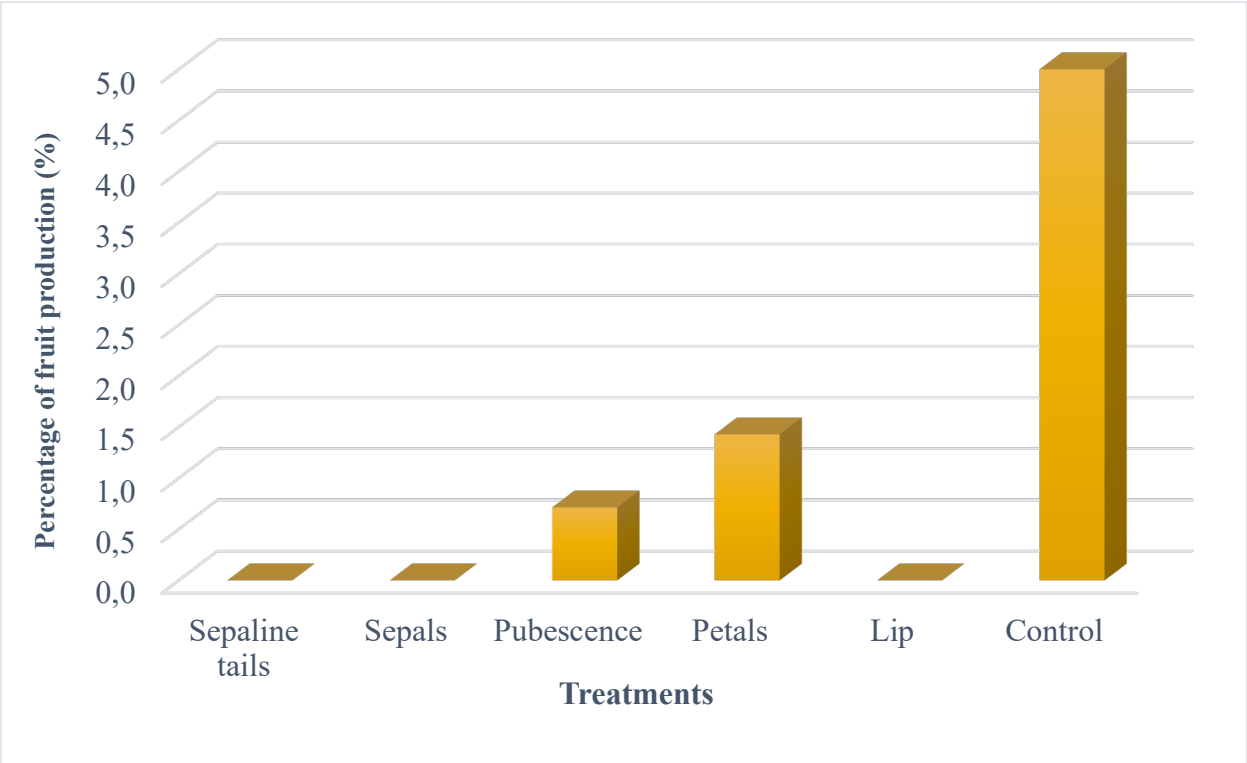



Figure 1. Effect of the removal of floral parts on the success in fruit production of *D. erythrochaete* at the wild population of Quebradilla, Cartago, Costa Rica.

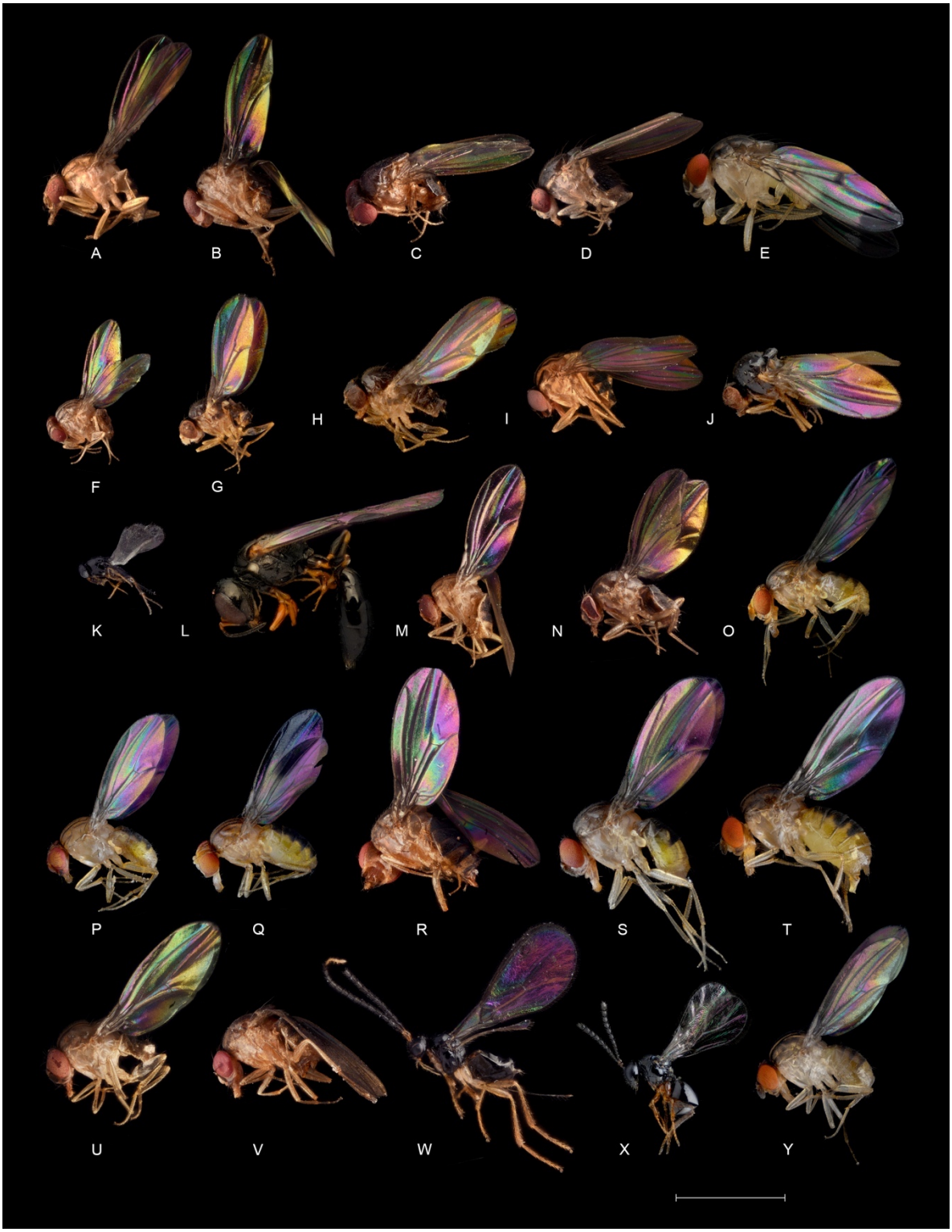



Figure 2. Insect visitors of *Dracula* spp. and macrofungi. Specimens caught on flowers of *Dracula erythrochaete*: A. *Zygothrica* (ID 050). C. *Zygothrica* (ID 145). D. *Zygothrica* (ID 016). E. *Zygothrica* (ID 180). F. *Zygothrica* (ID 044). G. *Zygothrica* (ID 130). H. *Zygothrica*

(ID 130). I. *Zygothrica* (ID 087). J. *Zygothrica* (ID 051). K. Hymenoptera: Platygastridae (ID 034). L. Hymenoptera: *Spilomena* (ID 158). Specimens caught on flowers of *Dracula chimaera*: B. *Zygothrica* (ID 149). Specimens caught on macrofungi: M. *Zygothrica* (ID 122). N. *Zygothrica* (ID 070). O. *Zygothrica* (ID 175). P. *Zygothrica* (ID 171). Q. *Zygothrica* (ID 165). R. *Zygothrica* (ID 101). S. *Zygothrica* (ID 172). T. *Zygothrica* (ID 169). U. *Zygothrica* (ID 008). V. *Zygothrica* (ID 073). W. Hymenoptera: Braconidae (ID 124). X. Hymenoptera: Eucoilinae (ID 168). Y. Drosophilidae (ID 166). Scale bar = 2 mm. Photographs by K.G.


Figure 3. A. *Russula* and *D. erythrochaete* growing in a range of 1 meter distance, both visited by *Zygothrica* flies. B. *Zygothrica* with pollinia walking on the pileus of *Russula*. C. ♂ *Zygothrica* sp. 1 (ID 173) with pollinia on the scutellum. Scale bar = 1 mm. Photographs by K.G.

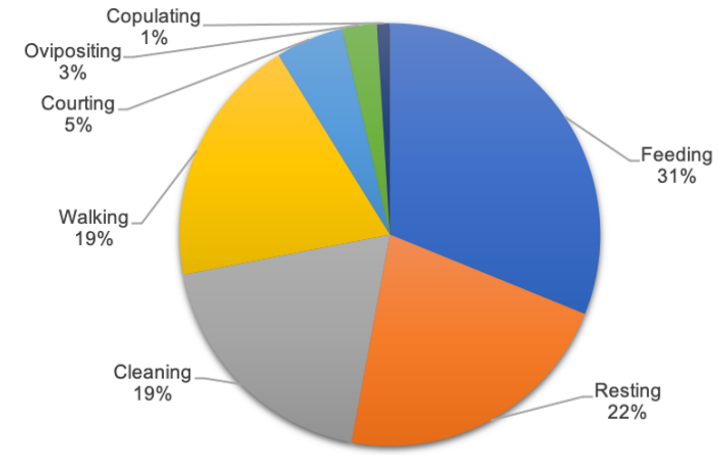

Figure 4. Phylogenetic relationship amongst the collected fly specimens. The trees were produced by analysis of the COI dataset using BEAST v1.6.0. Edited by A.P.K. using FigTree v.1.3.1


Figure 5. *Zygothrica* flies with pollinia of *D. erythrochaete* on the scutellum. A–R, *Zygothrica* sp. 1; A. ♂ (ID 156). B. ♂ (ID 173) Caught on *Russula* (Russulales). C. ♀ (ID 155). D. ♀ (ID 057). E. ♂ (ID 157) Caught on *Gymnopus* (Agaricales). F. ♀ (ID 036). G. ♂

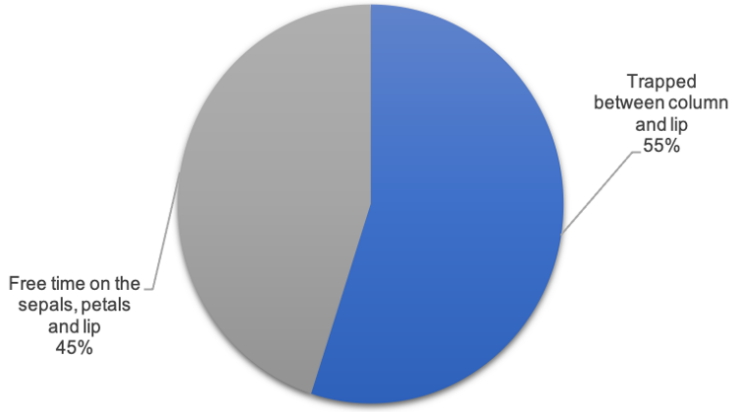
(ID 078). H. ♂ (ID 056). I. ♂ (ID 022). J. ♂ (ID 046). K. ♂ (ID 104). L. ♂ (ID 026). M. ♀ (ID 103). N. ♀ (ID 119). O. ♀ (ID 068). P. ♂ (ID 116). Q. ♂ (ID 035). R. ♀ (ID 086). S. *Zygothrica* sp. 3: ♀ (ID 152). T–U, *Zygothrica* sp. 4: T. ♀ (ID 150). U. ♀ (ID 154). V–Y, *Zygothrica* unknown species: V. ♂ (ID 195). W. ♂ (ID 020). X. ♂ (ID 196). Y. ♀ (ID 181). Scale bar = 2 mm. Photographs by: K.G.


Figure 6. Sexual dimorphism in *Zygothrica* flies. A. ♀ Female. The arrow indicates the female terminalia with the visible ovipositor in a lateral view. The circle indicates the ovipositor in a ventral view. B. ♂ Male. The arrow indicates the male terminalia with the epandrium in a lateral view. The circle indicates the epandrium in a ventral view with the claspers, a structure used to hold the female during copulation. Scale bar = 1 mm. Photographs by: K.G.

A




B

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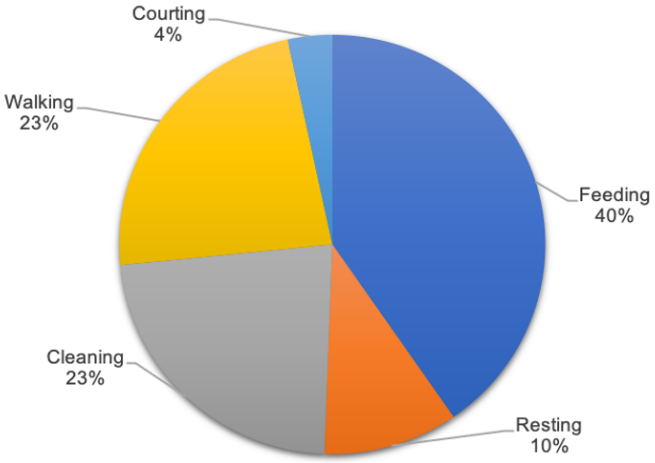


Figure 7. Fly behavior on macrofungi and *D. erythrochaete* flowers in Cartago, Costa Rica. A. Percentage of visits relative to behavior in macrofungi coexisting with *D. erythrochaete* flowers. B. Total percentage of time flies spend on flowers. C. Percentage of visits relative to behavior in flowers of *D. erythrochaete.*


Figure 8. Interactions between Drosophilidae flies, macrofungi, and *D. erythrochaete* flowers. A. Adult fly with extended proboscis, feeding on the lamellae of macrofungi (Agaricales). B. Larvae of Drosophilidae feeding on macrofungi *Lepista* (Agaricales). C. Adults flies with extended proboscis, feeding on the margins and the lamellae of the epichile of *D. erythrochaete*. D. Fly trapped between column-lip cavity of *D. erythrochaete*. Photographs by: K.G.

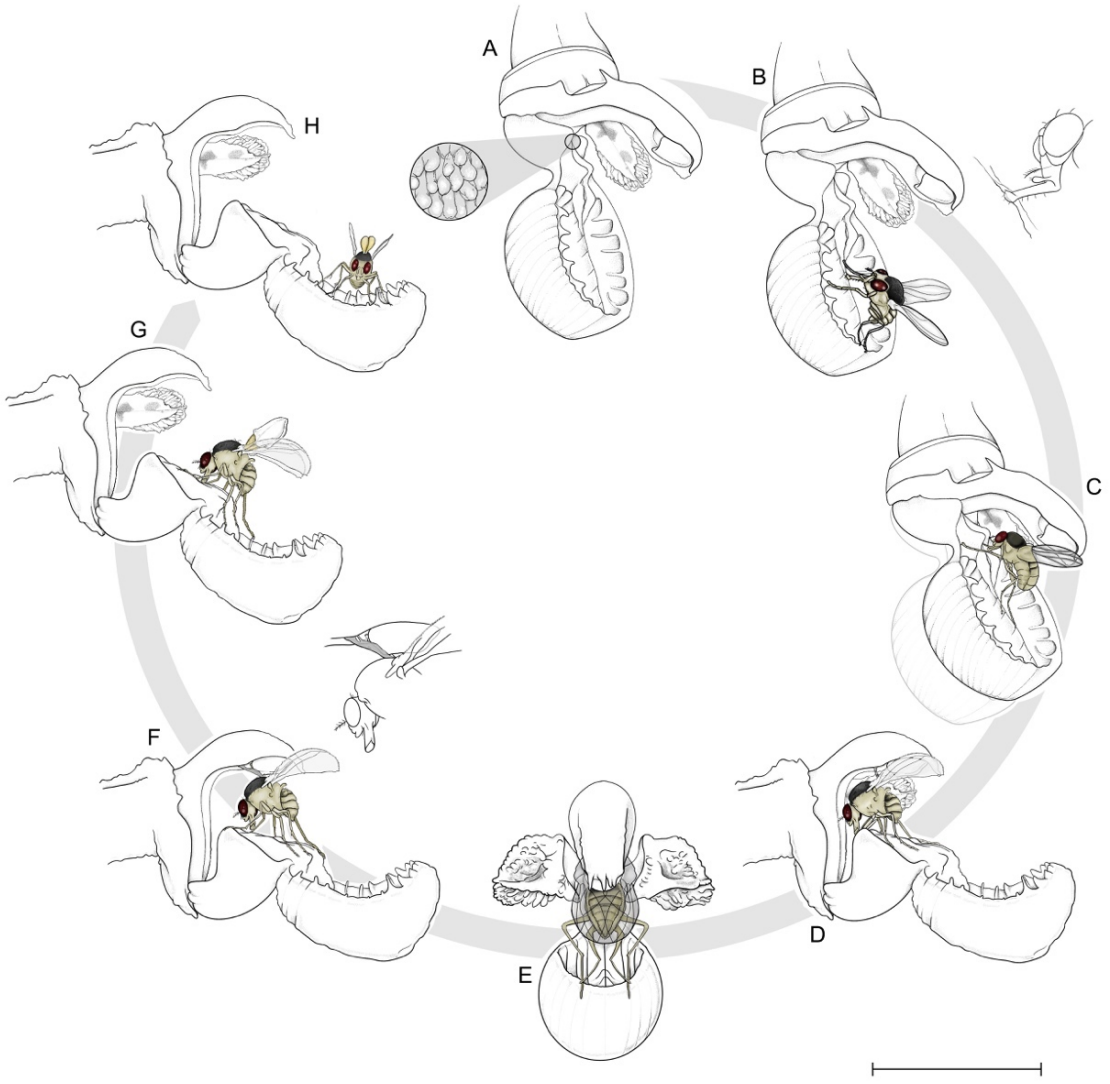


Figure 9. Pollination mechanism of *D. erythrochaete*. A. View of the lip in natural position, close-up of the clavate papillae in the hypochile. B. The fly attracted, attempts to feed on the papillose surface, detail of the proboscis extended on the epichile of the lip. C. The movement of the lip possibly assisted by the wind pressing the fly against the column. D. The fly is trapped between the column and lip, attached to the viscarium of the rostellum. E. Front view of the petals, and lip with the pollinator in the removal position. F. The pollinator struggling to get free. Detail of the viscarium. G. The pollinator removes the pollinaria and falls into the epichile. H. The pollinator removes the anther with its legs. Detail of the pollinia in the scutellum of the pollinator. Scale bar = 5 mm. Illustrations by Lizbeth Oses.

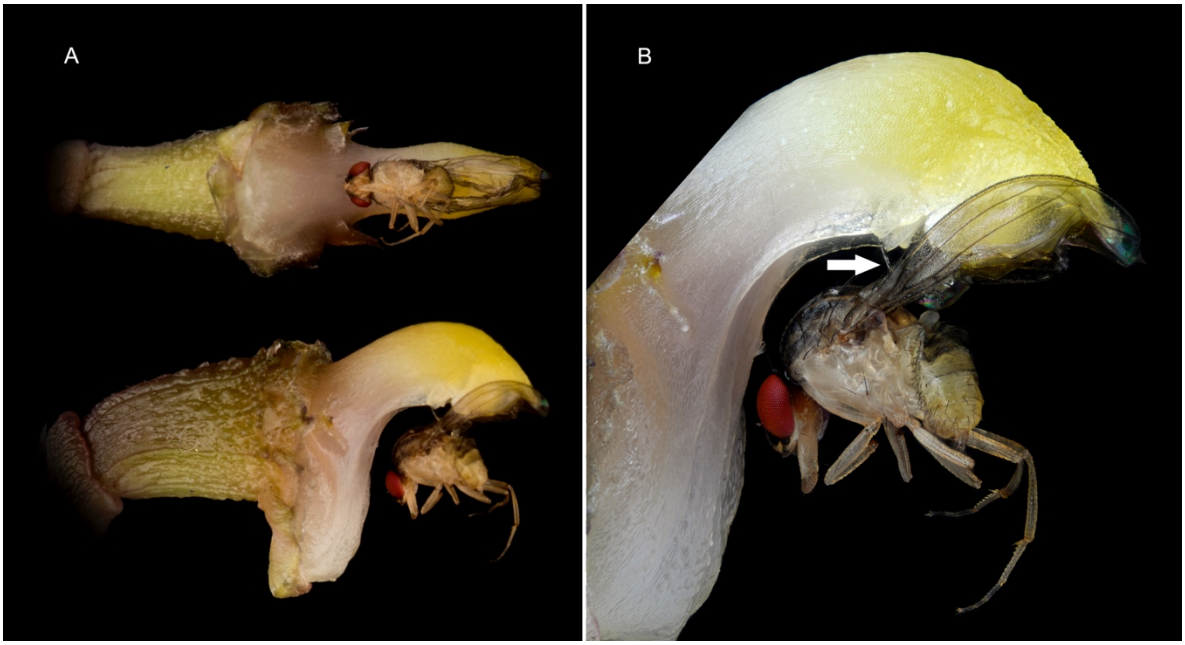


Figure 10. Column of *D. erythrochaete* with *Zygothrica* attached to the viscarium of the rostellum. A. Ventral and lateral view of the column and the trapped fly. B. Detail of the lateral view with the sticky viscarium visible on the scutellum of the fly indicated by the arrow. Photographs by: K.G.

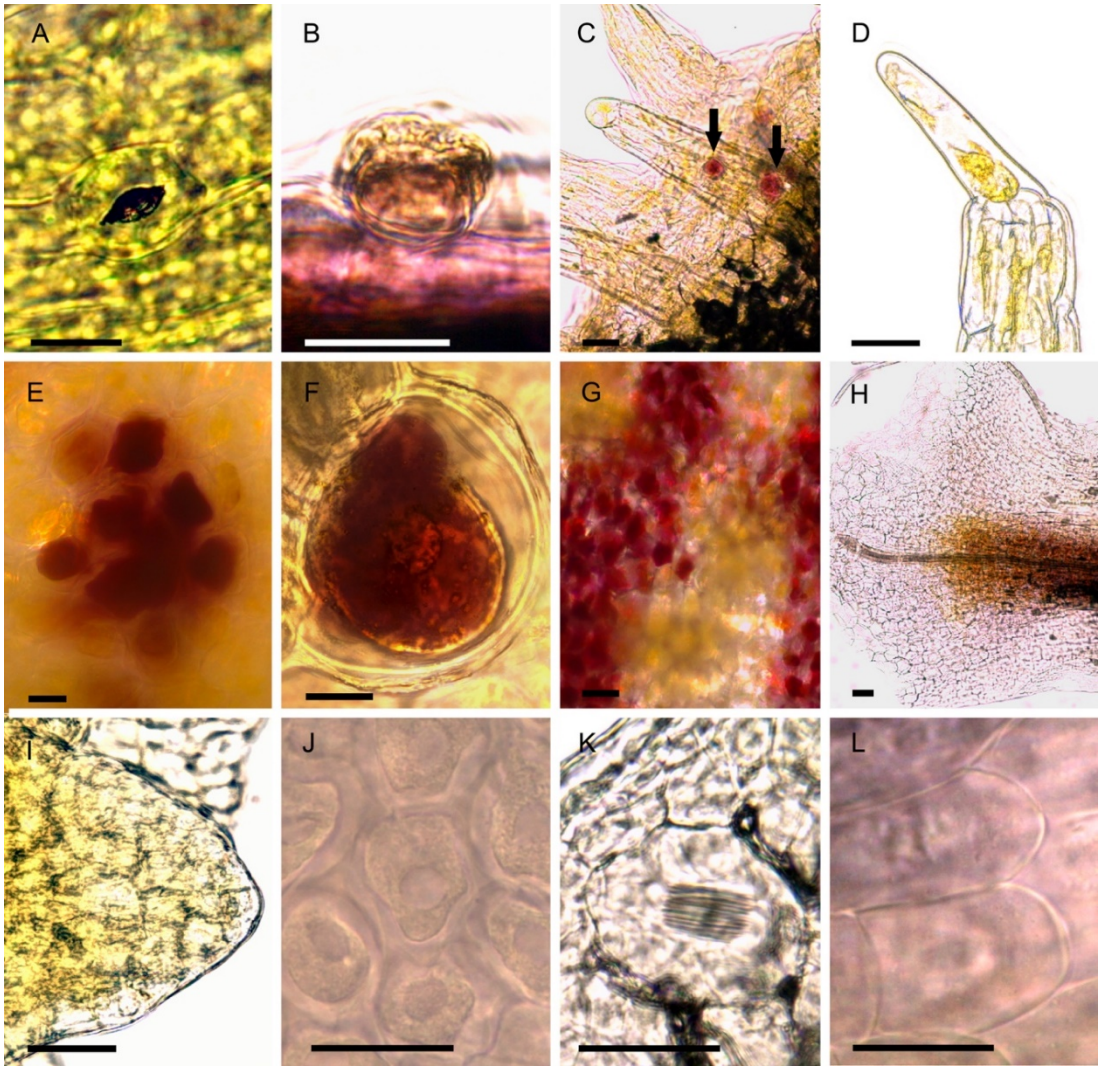


Figure 11. Micromorphology of the sepals, petals, and lip of *D. erythrochaete* showing natural pigmentation. A. Stomata on the sepaline tails with yellow epidermal cells containing carotenoids. B. Stomata at the margin of the sepaline tails with yellow (carotenoids) and red/purple (anthocyanins) epidermal cells. C. Glandular trichomes from the base of sepaline tails, with stomata indicated by the arrows. D. Multicellular glandular trichome of the sepals, showing carotenoids. E. Papillary trichomes at the base of the sepals, with anthocyanins and carotenoids. F. Papillary trichome with anthocyanins at the apex. G. Epidermal cells of the sepals. H. Petal base showing colorless epidermal cells on the margins and flavonoids in the center, also raphides in some cells. I. Papillae at the apex of the petals with carotenoids. J. Papillose surface of the epichile showing epidermal cells (flavonoids). K. Epidermal cell of epichile with raphides. L. Papillae of the hypochile showing flavonoids. Scale bars = 20, 20, 50, 50, 20, 50, 50, 50, 50, 20, 50 and 20 µm, respectively. Photographs by K.G.

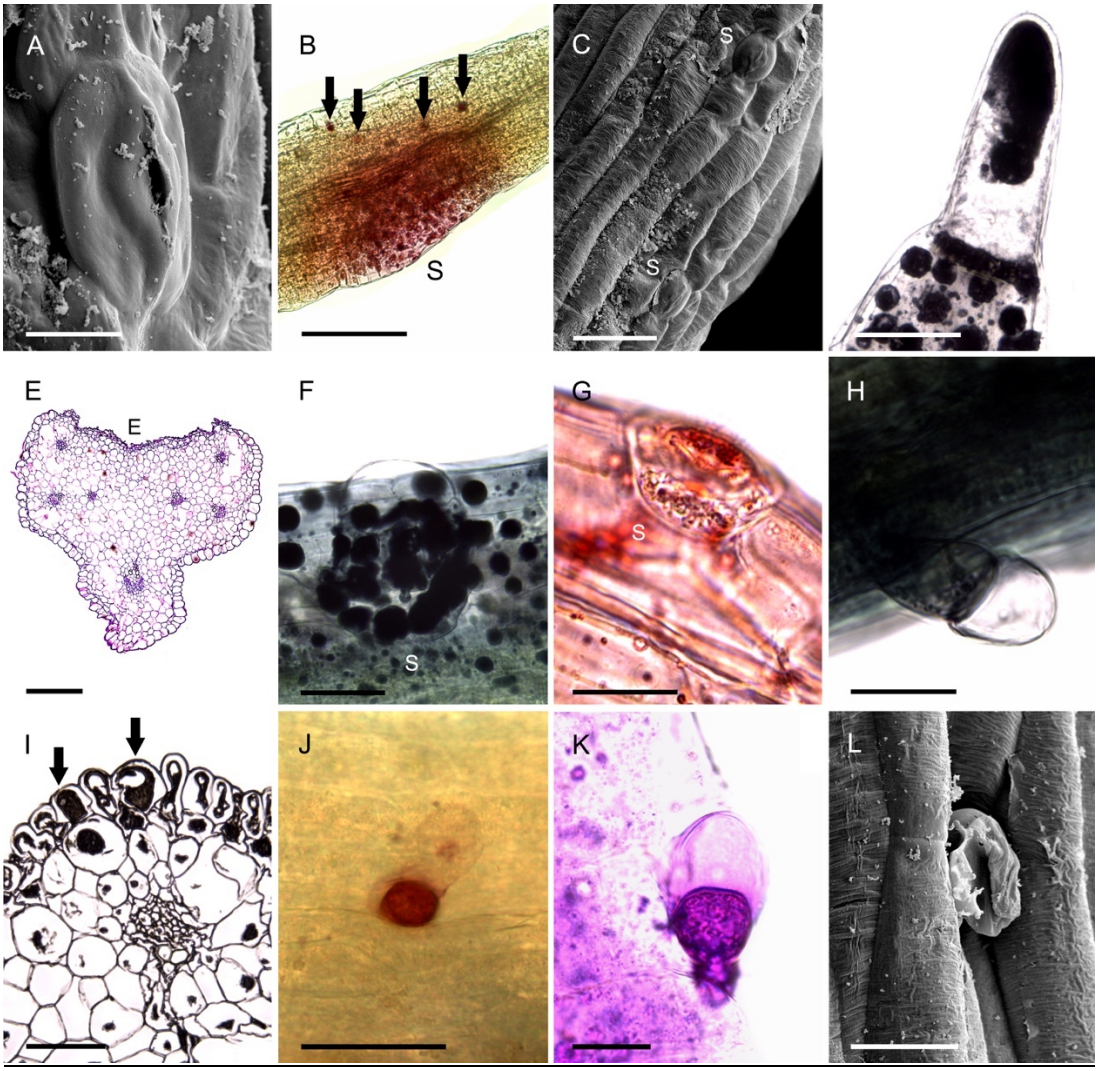


Figure 12. Micromorphology and histochemistry of the sepaline tails of *D. erythrochaete*. A. SEM of guard cells of a stoma with secretory materials on the surface. B. Secretory activity detected with (NR) in stomata (S) and colleters, indicated by the arrows. C. SEM of two stomata (S) close to the margins. D. Lipid concentration (SBB) on the multicellular glandular trichome. E. LM transverse section showing polysaccharides (PAS) in the epidermal cells (E). F. LM of open stoma (S) with lipid droplets (SBB). G. Lipid concentration (SIV) in the guard cells. H. Colleter close to the margins with lipid concentration at the base (SBB). I. Transverse section showing osmiophilic bodies in the epidermal cells, indicate by the arrows. J. LM of the colleter showing lipid concentration at the base and some at the apex detected with NR (red). K. Colleter showing insoluble polysaccharides at the base (PAS). L. SEM of the colleter with a collapsed globose apex. Scale bars = 10, 50, 50, 50, 200, 20, 20, 20, 50, 50, 20, and 20 µm, respectively. Photographs by K.G.

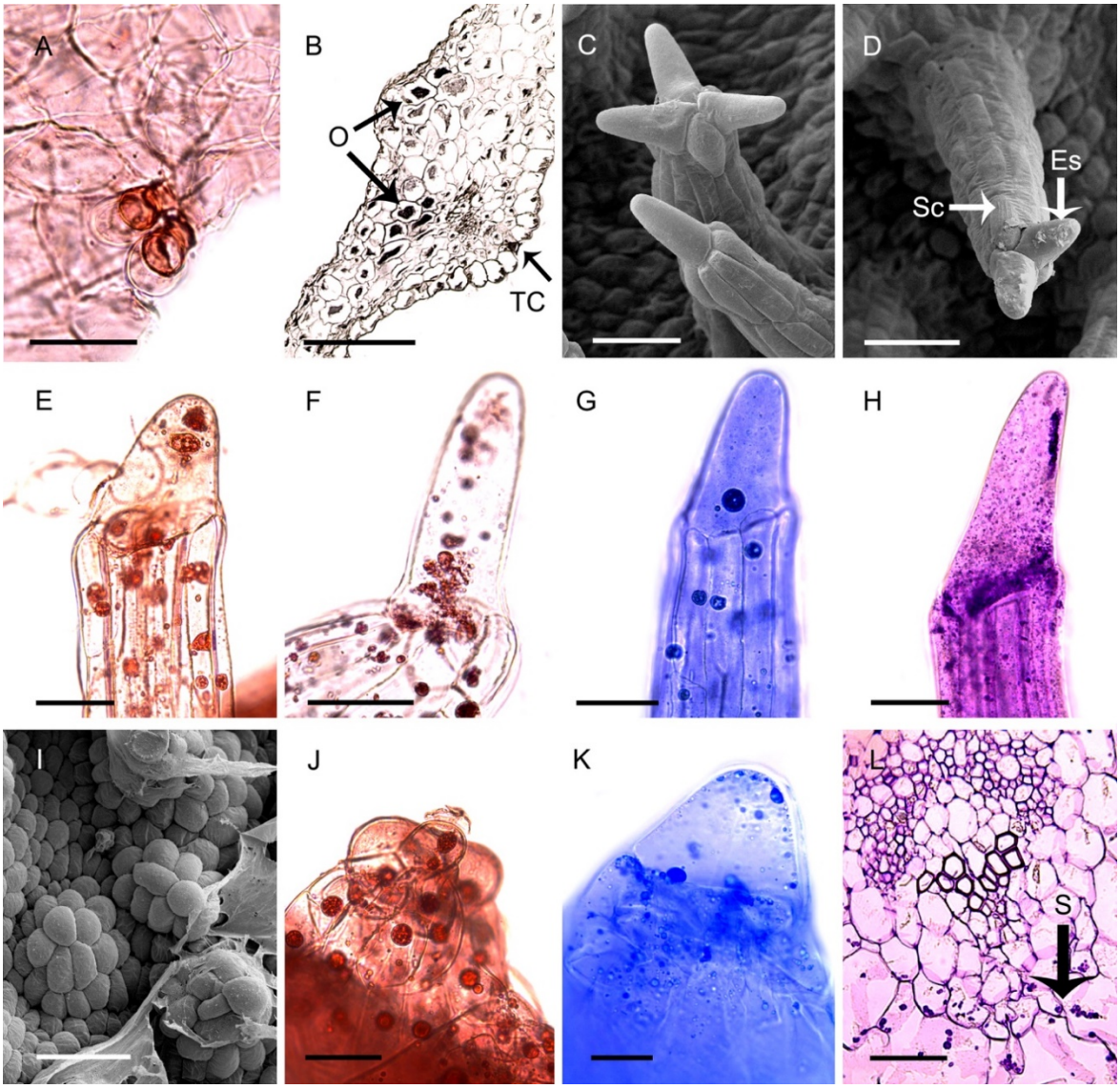


Figure 13. Micromorphology and histochemistry of the sepals of *D. erythrochaete*. A. Colleters in groups close to the margins showing lipids (SIV) concentrated at the base. B. LM transverse section stained with ($OsO_4$) showing unsaturated fats in the epidermal cells (O) and trichome-like colleter (TC) on the abaxial surface. C. SEM of multicellular glandular trichomes with 1 and 3 secretory terminal heads. D. SEM of multicellular glandular trichome with 2 secretory heads. Arrows indicate the striated cuticle (Sc) and epicuticular secretions (Es). E. Multiceullular glandular trichome showing lipids (NR). F. Multicellular glandular trichome showing lipids (SIV). G. Proteins detected with (CBB) in multicellular glandular trichome. H. Multicelullar glandular trichome showing polysaccharides detected with PAS (purple). I. SEM of papillary trichomes and papillae at the base of sepals with striated cuticle

and epicuticular secretions. J. LM of papillae stained with (NR) showing lipids. K. LM of papillae with proteins concentrated at the apex detected with CBB (blue). L. Transverse section stained with PAS showing starch grains (S) on the ground parenchyma. Scale bars = 50, 200, 90, 100, 50, 50, 50, 50, 120, 50, 20 and 50 μm, respectively. Photographs by K.G.

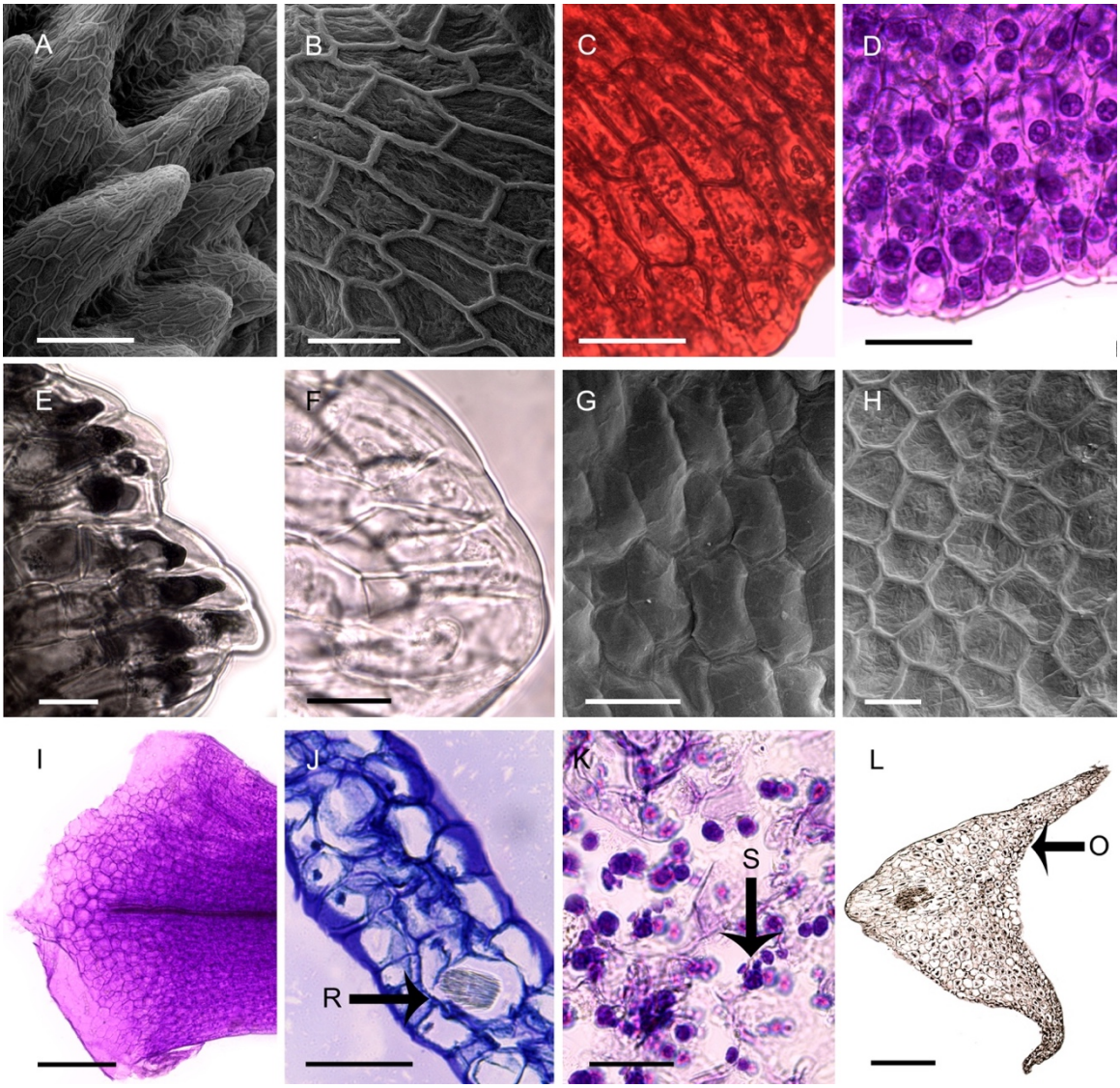


Figure 14. Micromorphology and histochemistry of the petals of *D. erythrochaete*. A. SEM of the conical papillae at the apex. B. Detail of the reticulated and striated cuticle of the papillae. C. LM of the papillae stained with (NR) showing secretory activity. D. Insoluble polysaccharides detected with (PAS) on the papillae. E. LM of papillae showing lipids (SBB) concentrated at the apex. F. LM of the apex papillae stained with (CBB) showing the negative reaction to proteins. G. SEM of the papillose surface at base of the petal. H. SEM of the papillose surface at the median region showing striated cuticle. I. LM of the petal base stained with (PAS). J. Transverse section of petal base stained with (TBO) showing bundles of raphides (R). K. Starch grains (S) detected with PAS (purple). L. LM transverse section of petal base showing osmiophilic contents (O) in epidermal cells. Scale bars = 100, 30, 50, 50, 20, 20, 60, 20, 50, 200, 20 and 200 µm, respectively. Photographs by K.G.

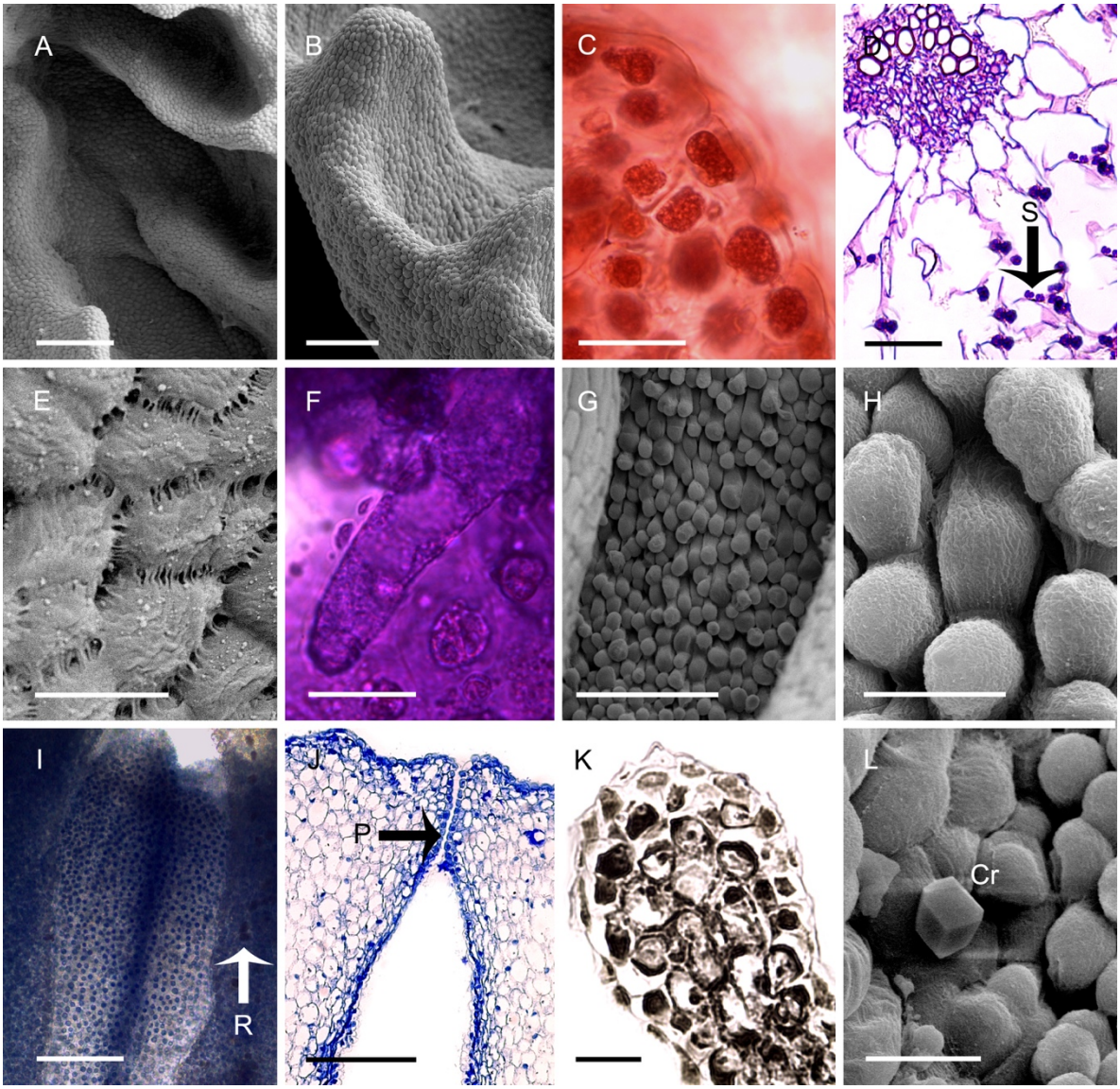


Figure 15. Micromorphology and histochemistry of the lip of *D. erythrochaete*. A. SEM of the papillose surface on the raised lamellae of the epichile. B. SEM of the abaxial surface of the epichile with a papillose surface. C. Papillose surface of the epichile stained with (SIV) showing lipid concentration. D. LM of transverse section of epichile stained with PAS showing starch grains (S) on the parenchyma. E. SEM of the epidermal surface of the epichile showing epicuticular secretions. F. LM of the papillae on the hypochile stained with (PAS). G. SEM of the hypochile showing elongate clavate papillae. H. SEM detail of the papillae on the hypochile with striated cuticles and epicuticular secretions. I. LM of the hypochile stained with CBB showing proteins concentrated at the apex of the papillae (blue tips). The arrow indicates raphides (R). J. Transverse section of the hypochile showing proteins (CBB)

concentrated in the papillae (P) indicate by the arrow. K. LM transverse section of epichile showing osmiophilic bodies in the epidermal surface papillae. L. SEM of the hypochile showing a prismatic crystal (Cr). Scale bars = 200, 120, 20, 50, 20, 20, 120, 20, 200, 200, 20 and 20 μm, respectively. Photographs by K.G.

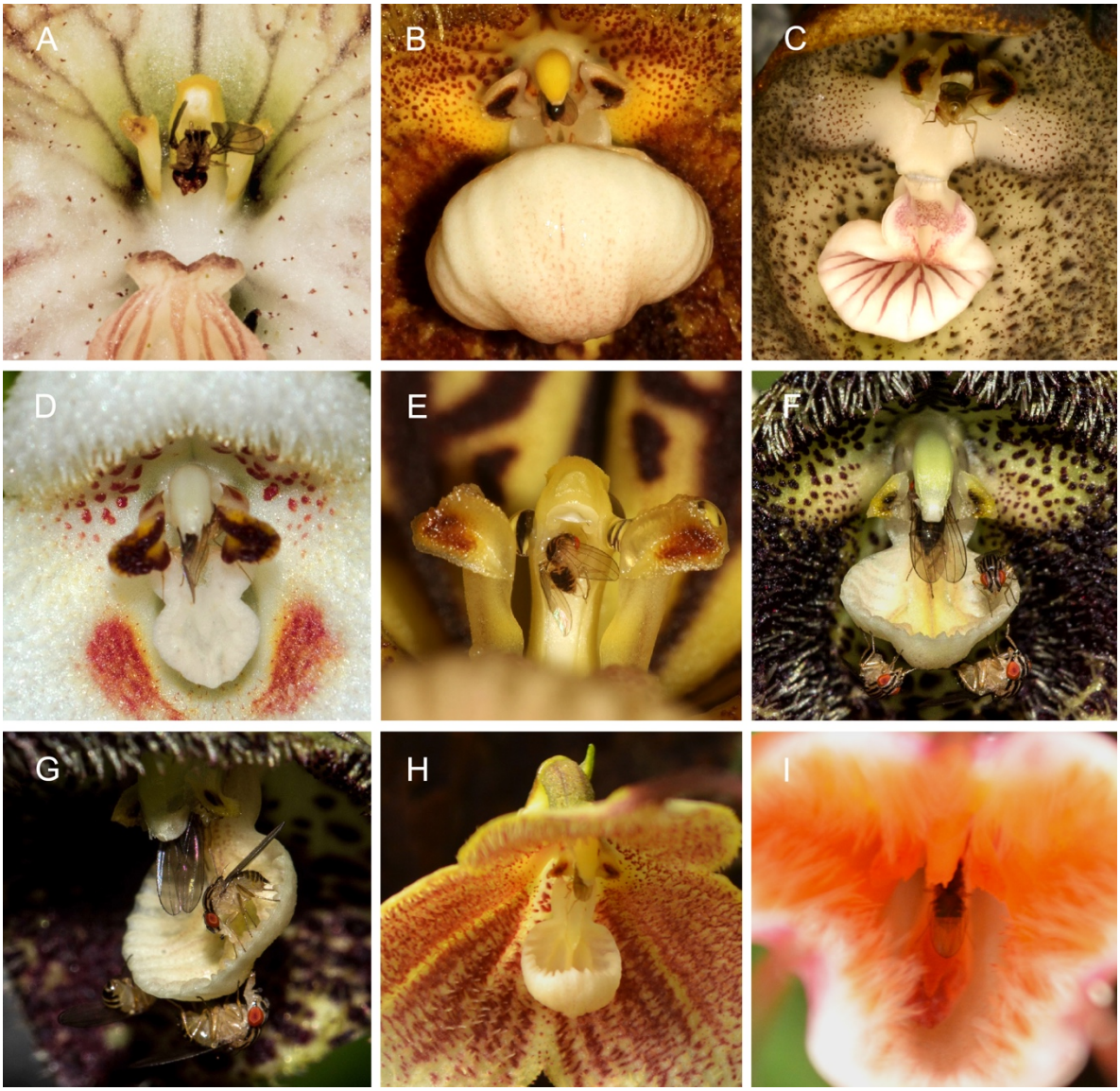


Figure 16. Drosophilidae flies trapped between column-lip cavity in *Dracula* spp. A. *D. psyche* (Luer & Andreetta) Luer. B. *D. chimaera* (Rchb.f.) Luer. C. *D. radiella* Luer. D. *D. lotax* (Luer) Luer. E. *D. vampira* (Luer) Luer. F–G. *D. minax* Luer & R. Escobar. H. *D. erythrochaete* (Rchb.f.) Luer. I. *D. sodiroi* (Schltr.) Luer. Photographs by: Carlos Augusto Mesa (A, B, D, E, F, G). Henry Oakeley (C). Karen Gil (H, I).