PHYLOGENETIC RELATIONSHIPS OF PARANTHRENE (LEPIDOPTERA: SESIIDAE) USING MULTI-GENE ANALYSIS AND DETERMINATION OF MONOPHYLETIC RELATIONSHIPS ALONG COLOR FORM

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ABSTRACT

Paranthrene species are day flying moths that mimic wasps in both appearance and behavior. The genus has several species with diverse color patterns that are restricted geographically. Many of these color forms were originally described as species before becoming synonymized with each other due to similar behavior and morphology. To date, there have been no molecular multi-gene phylogenetic studies that investigate the monophyly of North American *Paranthrene* species boundaries. We reconstructed a phylogeny using partial DNA sequence from COI, EF-1alpha and wingless genes with 67 specimens representing all North American *Paranthrene* species, subspecies, and variants. Monophyly of any *P. simulans* color forms was not recovered in parsimony and Bayesian analyses. The results also suggest the synonymy of *P. simulans*, *P. pellucida*, and *P. hilairemontis*. Interestingly, the two-color forms of *P. tabaniformis* comprised two clades, one which was sister to a clade including *P. dolli*. Individuals of this clade also have a different flight period as *P. tabaniformis* which suggests the recognition of the clade as a species. *Paranthrene robiniae* presented as polyphyletic. Genitalic and genetic differences between *P. robiniae* populations suggest the recognition of two psuedocryptic species.

ACKNOWLEDGEMENTS

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INTRODUCTION

Sesiidae, or clearwing moths, are a family of over 1400 species worldwide within the order Lepidoptera. Of those species, 135 of them reside in North America and include many pests of agriculture and forestry (Lair and Herbert, 2018). The genera *Paranthrene* and *Synanthedon* are of significant importance to nursery trees and timber production in the United States (McKern and Szalanski, 2007). Due to their diurnal nature and mimicry of Hymenoptera, their presence and distribution can be challenging to detect, making pheromone traps one of the better ways to monitor these moths (McKern and Szalanski, 2007).

Pheromone traps provide an affordable way to monitor Sesiidae populations (McKern and Szalanski, 2007). The type of pheromone used in a trap can also help identify the species captured (Taft et al., 1991) although, there is some overlapping pheromone attraction among species (Taft et al., 1991). For example, both *Paranthrene simulans* and *Paranthrene pellucida* are attracted to the pheromone ZZA (3,13-octadecadien-1-o1 acetate) (Taft et al.,1991). This cross attraction, combined with the glue of sticky traps can often leave the moths damaged, making it difficult if not impossible to identify the specimen morphologically (McKern and Szalanski, 2007). To combat this identification problem researchers employ molecular identification, using the cytochrome oxidase I (COI) barcode region, to differentiate between species. (McKern and Szalanski, 2007).

The majority of phylogenetic studies on Sesiidae have been COI based and there have been no multi-gene phylogenetic studies of *Paranthrene*. Several North American *Paranthrene* are known to have various color morphs that vary with their distribution (Eichlin and Duckworth, 1988). Some of these morphs have been recorded with differing behaviors from the rest of their species (Eichlin and Duckworth, 1988). A multi-gene analysis of the genus would shed light on the species boundaries of these morpho-types.

Biology and Life History

Sesiidae life history

Members of Sesiidae share many common traits; nearly all adults are diurnal mimics of wasps and bees, in both their appearance and their behavior (Eichlin and Duckworth, 1988). Clearwing moths can hover in the same patterns and speeds as their hymenopteran models (Taft et al, 1991, Skowron Volponi et al, 2018).

Females oviposit single eggs on host plants, typically near natural or artificial openings, so to facilitate boring of newly hatched larvae into the plant (Eichlin and Duckworth, 1988). Most species have a narrow host plant range, often specializing on a single genus (Eichlin and Duckworth, 1988) or even a single species (Taft et al, 1991).

Clearwing moth larvae bore into a variety of economically important plants, and are considered pests (Solomon, 1995). For example, The lesser peachtree borer, *Synanthedon pictipes,* attacks peach trees and *Prunus* species (Cottrell et al., 2008). There are also pests of hardwoods and ornamentals like the banded ash clearwing, *Podosesia aureocincta* (Solomon, 1995). The moth larvae continue to expand the galleries throughout the larval instars until they exit and pupate in the pupal chamber (Eichlin and Duckworth, 1988). This tunneling in plant tissue can lead to death of part or the whole of the plant.

Even those species that typically restrict their damage to unmarketable wood, can exacerbate damage by providing opportunities for fungi and other insects to colonize the wood (Solomon and Morris, 1966). The boring of these subsequent insects into these trees increases the trees' susceptibility to structural weakness and rot (Johnson and Lyon, 1988).

Paranthrene life history

Eight currently recognized *Paranthrene* species occur in the United States, Canada, and Mexico (Baja California) (Eichlin, 1992; Handfield and Handfield, 2021).). Larvae feed on oaks (*P. asilipennis, p. pellucida, P. simulans*) and on willows and poplars (*P. dollii, P. robiniae, P. tabaniformis*) (Eichlin and Duckworth, 1988, Solomon, 1995). The remaining two species (*P. fenestrata* and *P. hilairemontis*) are believed to feed on oaks but documentation is lacking (Eichlin and Duckworth, 1988; Handfield and Handfield, 2021). Species typically have a 2-year

life cycle (Eichlin and Duckworth, 1988) but at least one species, *P. dollii*, has been observed with a shorter life cycle in select parts of its range (Solomon, 1995).

Phylogeny of *Paranthrene*

Phylogenetic studies of Sesiidae are few, with a majority of the work addressing the relationships among tribes and subfamilies (Cognato et al, 2023; Lait and Herbert, 2018; McKern et al, 2008). Current taxonomy places *Paranthrene* sister to *Euhagena/Vitacea* and together with *Albuna* they comprise Paranthrini which is supported by molecular data (Eichlin and Duckworth, 1988; McKern et al, 2008). A multi-gene phylogeny places Paranthrini sister to Synanthedonini as compared to a Paranthrini/Sesiini sister relationship in a previous COI based phylogeny (Lait and Herbert, 2018; Cognato et al., 2022). Phylogenies of North American sesiids only include *P. simulans* and *P. tabaniformis* (Cognato et al., 2023; Taft et al., 2016; McKern et al., 2008). There have been no molecular multi-gene phylogenetic studies conducted to determine the phylogenetic relationships of all North American *Paranthrene* species, nor has any study investigated species boundaries for species with vast ranges. Mitochrondrial DNA sequence (particularly the COI the barcode region) has been the primary data source for species identification and recognition for several sesiid species level studies (McKern et al., 2008; Taft et al., 2016; Taft and Cognato, 2017; Handfield and Handfield,2022). McKern and Szalanski (2007) report intraspecific mtDNA sequence variation among Arkansian *Paranthrene simulans* individuals and Cognato et al. (2023) show differences among populations from Minnesota and North Carolina. Handfield and Handfield, (2022) demonstrated monophyly of mt DNA haplotypes that were diagnostic for a population of *P. simulans* from Quebec and associated with a diurnal flight period asynchronous with other *P. simulans.* They described this group as a new species *P. hilairemontis*.

Research Objectives

Due to the lack of phylogenetic studies, the overall purpose of my research is to understand if there is a monophyletic relationship with color form in *Paranthrene.* I will attempt to accomplish this through two supporting objectives. The construction of a multi-gene phylogeny and the delineation of species boundaries along color forms.

Objective 1: Construct a phylogeny of North American *Paranthrene*

Despite Neighbor Joining COI analysis of most North American *Paranthrene* by Handfield and Handfield (2022), the phylogeny is far from settled. Multi-gene analysis can help elucidate these phylogenetic relationships (Cognato et al., 2023). I intend to construct both parsimony and Bayesian phylogeny of North American *Paranthrene* utilizing both nuclear and mitochondrial genes. This will be the first multi-gene analysis of the genus and the only COI analysis of all North American species.

Objective 2: Identify species boundaries of *Paranthrene* with diverse color morphologies

Paranthrene simulans is a polymorphic species with differing biologies among the color forms and evidence of geographic mDNA variations (Cognato et al., 2023). This demonstrates a need for further investigations into species boundaries of the different forms. I will attempt to delineate the species boundaries for the three forms currently recognized.

Several other species of *Parathrene* have multiple forms requiring a determination of species boundaries. *Parathrene robiniae* has three color morphologies within different localities of its range, *P. tabaniformis* has its typical and oslari forms along with an unnamed form investigated by this study and, *P. fenestrata* has two distinct color morphologies. I will conduct a multi-gene analysis of two of the P. robiniae color forms and all North American forms of *P. tabaniformis and P. fenestrtata* to identify molecular evidence of speciation.

CHAPTER 1: A NEW SPECIES OF *PARANTHRENE* (LEPIDOPTERA: SESIIDAE) FROM THE NORTHERN MIDWEST US

The dusky clearwing, *Paranthrene tabaniformis* Rottenburg 1775 (Sesiidae: Lepidoptera) (Fig. 1) is holarctic species (Solomon 1995). Like all other *Paranthrene* species found in North America, *P. tabaniformis* is a diurnal wasp mimic (Eichlin and Duckworth 1988). Its North American range includes the continental United States, Alaska, and Canada, with the exception of California (Taft et al. 1991). The larval host plants are poplars and willowsand the species can also be a major pest of nursery plants (Solomon 1995). Adults emerge from their 2-year cycle in June and July (Taft et al. 1991).

Several *Paranthrene* are known to have various color variants (Eichlin and Duckworth 1988). *Paranthrene tabaniformis* is no exception with three recognized color morphologies in North America (Eichlin 1989). This species is typically characterized by a blue-black head with a grey-black frons and white lateral margins, the thorax is blue-black with yellow on the anterior margins and beneath the wings, and the abdomen is blue-black with yellow bands on segments two, four, six and, seven (Eichlin 1989) (Fig. 1). The color forms “denotata” and “oslari” are largely similar with a few chromatic differences. Most notable is the form “oslari”, which is differentiated by a yellow band on all abdominal segments except segment one, with the bands on segments two and six wider than the rest (Eichlin 1989).

In 2022, near Bath township, Clinton County, Michigan, a specimen originally identified as *P. tabaniformis* was collected with a unique color morphology and large transparent areas in the forewings which suggested it was a distinct species. This specimen was similar to one photographed by Mr. James Sogaard, in Minnesota in 2007. We investigate this color variant’s phylogenetic placement among *Paranthrene* species and morphology variation. We demonstrate that it is distinct from the typical *P. tabaniformis* color variant and we conclude that it is a new species.

Material and Methods

Collection of specimens

In July 2022 along Stoll and State Roads in Clinton County (Rose Lake State Game Area), Michigan the second author collected three male sesiid moths using Multi-Pher #1® pheromone canister traps (Distributions Solida Inc) baited with Western Poplar Borer pheromone lure, a blend of (ZZ 3,13 OH and EZ 3,13 OH (50:50) (*Scentry* Biologicals, Inc).

Specimen preparation and imaging

One specimen of *P. tabaniformis* and the new form was selected for genital comparison. Abdomens were removed at the 4th or 5th segment. The removed segments were placed in separate vials filled with water and two 116mg tablets of potassium hydroxide and allowed to sit on a hot plate set just below boiling for 2 hours. Softened abdominal segments were removed from the vials and teased apart under a dissecting microscope with fine-tipped forceps until genitalia were revealed. Genitalia were preserved in glycerin and placed in 5mm glass micro vials and pinned under the associated specimen. The genitalia were not stained.

Specimens were photographed with a Visionary Digital Passport II system (Dun Inc., Palmyra, VA) using a Canon EOS 5D Mark II, 65.0-mm Canon Macro photo lens, two Dynalite (Union, NJ) MH2015 road flash heads, Dynalite RoadMax MP8 power pack and a Stack Shot (Cognisys, Inc, Traverse City, MI). Montage images were assembled using Zerene Stacker 1.04 and sized in Adobe Photoshop 2021 v. 22.5.1 (San Jose, CA).

DNA sequence data and phylogenetic analyses

DNA was extracted from a metathoracic leg from 25 frozen specimens representing *P. tabaniformis* and six other *Paranthrene* and outgroup species (Table 1) using a Qiagen DNeasy Blood and Tissue kit (Hilden, Germany) following the manufacturer's protocol. The remaining bodies were vouchered in the A. J. Cook Arthropod Research Collection. The purified DNA was used in PCR for mitochondrial cytochrome oxidase I and wingless (Table 2). EXO-SAP-IT (USB Corp., Cleveland, OH, USA) was used to ready the PCR products for sequencing at the Michigan State University Research Technology Support Facility using Big-Dye Terminator v 1.1 (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 Genetic Analyzer (Applied Biosystems). Sense and antisense strands were compiled using Sequencher (Ann Arbor, MI) to trim sequences of

primer sequences, align the sequences and to create consensus sequences. Final sequences were deposited in Genbank (Table 1) and assembled in a Nexus file for a total of 1060 nucleotides (639 from COI and 417 from Wingless) which included 205 parsimony-informative characters.

Phylogenetic parsimony analysis of the aligned sequences consisted of a branch and bound search using default options in PAUP v4.0a (build 168; Swofford 2002). Gaps were treated as missing data. Bootstrap values were determined with 500 pseudo-replicates each conducted by heuristic search with simple stepwise addition. Percent pairwise DNA difference was calculated as p-distance in PAUP*. In addition, Bayesian analysis under a likelihood optimality criterion was used to assess phylogenetic relationships using this dataset. Using Mr. Bayes 3.2.6 (Ronquist et al. 2012) two simultaneous analyses were conducted in which both genes were partitioned by codon position and a model of general time reversal + gamma + proportion of invariable sites was applied to each partition (unlinked parameters). Four Metropolis-coupled Markov chain Monte Carlo searches (one cold, three heated) were analyzed for 5 million generations. Each analysis was sampled every 100th iteration and all parameters reached stability. Bayesian posterior probabilities of clades were based on 75,000 trees—the total of both runs after a 25% burn-in.

Results

The PAUP* analysis found six most parsimonious trees that were mostly resolved in the strict consensus of those trees (Fig. 2). Intraspecific relationships for two clades and one interclade relationship were unresolved (Fig. 2). All clades of species had 100 bootstrap values and values for internal nodes varied but were mostly >85 (Fig. 2). The topology of the Bayesian tree was generally similar to the parsimony tree but differed in the placement the “sogaardi” clade as sister to *Vitacea* and the remaining *Parathrene* species. Posterior probabilities were >0.9 for all species clades except “sogaardi” at 0.7. The average interspecific pairwise “p” distance among sister species within the genus ranged between 0.062-0.101 for COI and 0.008-0.034 for Wingless. The average “p” distance between “sogaardi” and *P. tabaniformis* was 0.076 and 0.031 for COI and wingless, respectively, both of which are within interspecific ranges for other species within the genus. In comparison, intraspecific “p” distance amongst “sogaardi”

specimens was 0.005 and 0.003 while *P. tabaniformis* was 0.014 and 0.002. The genetic distance between "sogaardi" and *P. tabaniformis* is comparable to the interspecific distance between *P. tabaniformis* and *P. asilipennis* (Table 3). This genetic divergence, in addition to monophyly and morphological diagnostic characters, supports the recognition of a new species.

Taxonomy

Paranthrene **Hubner**

Paranthrene is characterized by the following: Males have an apical spine on the aedeagus (Eichlin & Duckworth 1988)

***Paranthrene sogaardi* Taft & Smith, new species**

Diagnosis

Paranthrene sogaardi n. sp. superficially resembles several other species of North American sesiidae, most notably *Paranthrene pellucida* and *Synanthedon rileyana*. *Paranthrene sogaardi* n. sp. differs from *P. tabaniformis* by its lack of scales in the discal cell of the forewing, a dark orange discal spot on the forewings, and uniform yellow bands along abdominal segments 2-7. Whereas the discal cell in the forewing of *P. tabaniformis* is covered in dark scales, without a discal spot, and has yellow bands only on abdominal segments 2, 4, 6 and 7. Concerning the male genitalia, the apical point of the valves of *P. sogaardi* n. sp. are more rounded than *P. tabaniformis* and the setae are denser at the base of the valves where setal density is relatively uniform along the valve margins in *P. tabaniformis* (Figs. 3 & 4). The distal end of the saccus in *P. sogaardi* n. sp. is flattened and squared off, while the saccus in *P. tabaniformis* ends in a point. Both aedeagi, both have a sclerotized, toothed ridge (Figs. 5 & 6). In contrast, this ridge is covered by a semi-translucent shingle like sclerite in *P. tabaniformis*, this feature is absent in *P. sogaardi*.

The following characters distinguish *P. sogaardi* n. sp. from *P. pellucida*. The cell below the Cu vein to the anal margin in the forewing of *P. sogaardi* n. sp. is covered in orange-brown scales, whereas the same cell in *P. pellucida* is without scales and transparent. The yellow bands on *P. pellucida's* abdomen are thicker distally from the thorax while all bands are roughly equal in thickness on *P. sogaardi* n. sp.

Scaling on the wings also differs between *P. sogaardi* n. sp. and *S. rileyana. Paranthrene sogaardi* n. sp. has brown-black scales on the apical tips of the forewings and brown-black scales along the M vein in the hind wings. *Synanthedon rileyana* lacks scales in both the aforementioned areas. In addition, *P. sogaardi* n. sp. has yellow tarsi while *S. rileyana* has black tarsi.

Description

Male (Fig. 7 & 8). Head: Vertex primarily flat black with fine light gray hair mixed near the base; Frons light translucent pearl with a blue sheen, white laterally (Fig. 9); occipital fringe straw yellow; labial palpus strongly roughened, pale yellow with brown-black scales mixed laterally; haustellum coiled, antenna orange with a narrow line of brown black dorsally; Thorax: Dorsum flat black with fine light gray hair-like setae mixed throughout, base of forewing surrounded by a yellow spot, mixed with pale white scales, a patch of yellow setae posterior to the cape (visible in lateral view (Fig. 8), yellow scales on the posterior and posterolateral margins of the metathorax. Legs with coxae flat black with bright yellow outer margins, femora dark brown-black; tibiae light orange-yellow with a small patch of brown-black setae medially covered by orange setae, tibial spurs, and tarsi light yellow-orange mixed with black setae. Forewing mostly hyaline with an outer brown-black margin, discal spot light orange with a light edging of brown scales around the margins; anal margin lined with orange and black scales; veins and fringe brown-black with pale yellow scales on outer margin between veins (often faded in older individuals). Hindwing hyaline with narrow margins; dark brown fringe transitions to pale yellow near the wing base. Abdomen: Brown black with yellow bands encircling segments two-seven (the band width appears variable across its range); anal tuft short with a mix of black and yellow scales. Male Genitalia: (Fig. 3) Valves rounded apically, with setal density thickest toward the base. Socci with dense light-colored setae. Saccus ending in a flat squared-off tip.

Female.

Unknown.

Host

Unknown but all *Paranthrene* moth species feed on trees and large shrubs (i.e., oak, poplar, aspen, and willow species).

Distribution

Known from Central Michigan and Minnesota. Additional images of specimens from western Quebec suggest that the range of *P. sogaardi* n.sp. may extend into Quebec (iNaturalist.com). Because of the scattered locations of both collected specimens and images we suspect their range may include parts of Wisconsin and Ontario.**Types**. Holotype: Male,

Michigan: Clinton Co., Bath Township, Rose Lake State Game Area, Lat/Long (42.7988, -84.40185), July 2, 2022, Coll. William H. Taft, MSUC_ARC_320053, deposited in the Albert J. Cook Arthropod Research Collection, Michigan State University, East Lansing (MSUC). Michigan paratypes (2 males) and Minnesota paratypes (1 male) at MSUC and (3) in James Sogaard's personal collection.

Etymology

The species is named after Mr. James Sogaard of Princeton, Minnesota who first captured and photographed a male specimen from the same area.

Remarks

In Michigan, the moth was collected in a wetland swale depression (Fig 10.) characterized by willow thickets and shrub wetlands surrounded by oak uplands. The common plant species of the type location were Quaking Aspen (*Populus tremuloides*), Northern Red Oak (*Quercus rubra*), Red Maple (*Acer rubrum*), Sand Bar Willow (*Salix interior*), Heart-leaved Willow (*Salix eriocephala*), Black Willow (*Salix nigra*), Elm (*Ulmus sp.)*, Gray Dogwood (*Cornus racemose*), and a non-native plant - Common Buckthorn (*Rhamnus cathartica*). The herbaceous plants were Joe-Pye Weed (*Eutrochium purpureum*), Elderberry (*Sambucus canadensis*), Raspberry (*Rubus* sp.), Boneset (*Eupatorium perfoliatum*), and Cattail (*Typha latifolia*).

The EPA ecoregion designation for the type location in Michigan is the Southern Michigan /North Indiana Drift Till Plains/ Lansing Loamy Plain with the mid-elevation forests and the foothills woodlands and shrub lands designation. In Minnesota, EPA ecoregion designation for the Princeton area is the Anoka Sand Plain and Mississippi Valley Outwash (White 2020). It is characterized by substantial agriculture, but much of the region is too wet and poorly drained to be cultivated, so is left as natural wetlands (Albert 1995).

Figures

Figure 1. *Paranthrene tabaniformis.*

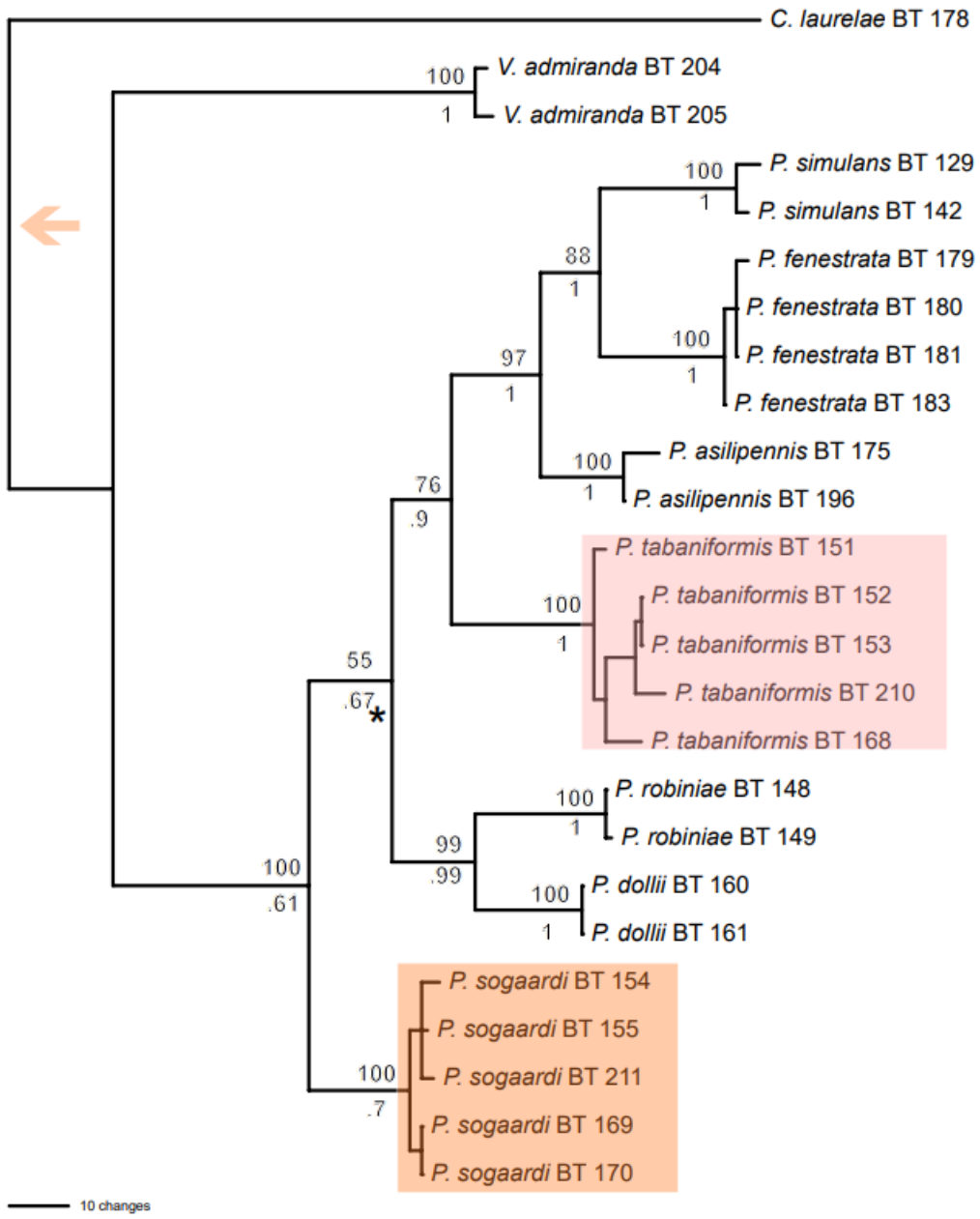

Figure 2. Strict consensus of six most parsimonious trees. Numbers above branches are bootstrap values and numbers below the branches are posterior probabilities. The arrow indicates the placement of *P. sogaardi* in Bayesian analysis.

Figure 3. Adult male *Paranthrene sogaardi* genitalia.

Figure 4. Adult male *Paranthrene tabaniformis* genitalia.

Figure 5. Adult male *Paranthrene sogaardi* aedeagus.

Figure 6. Adult male *Paranthrene tabaniformis* aedeagus.

Figure 7. *Paranthrene sogaardi* dorsal view.

Figure 8. *Paranthrene sogaardi* lateral view.

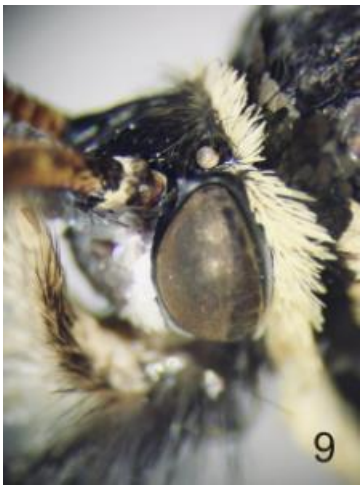

Figure 9. *Paranthrene sogaardi* frons.

Figure 10. Michigan collection site.

Tables

Table 1. Species, specimens, localities and Genbank numbers.

Species	DNA voucher #	Location	Genbank # COI	Genbank # Wg
C. laurelae	BT 178	Palmer Bridge, SC	PP333557	PP338690
V. admiranda	BT 204	Medicine Park, OK	PP333558	PP338691
V. admiranda	BT 205	Medicine Park, OK	PP333559	PP338692
P. simulans	BT 129	Jacksonville, NC	PP333560	PP338693
P. simulans	BT 142	Manistee County, MI	PP333561	PP338694
P. fenestrata	BT 179	Emory Pass, NM	PP333575	PP338706
P. fenestrata	BT 180	Emory Pass, NM	PP333576	PP338707
P. fenestrata	BT 181	Pinos Altos, NM	PP333577	PP338708
P. fenestrata	BT 183	Emory Pass, NM	PP333578	PP338709
P. asilipennis	BT 175	North Lima, OH	PP333574	PP338705
P. asilipennis	BT196	Swanton, OH	PP333579	PP338710
P. tabaniformis	BT 151	McClellanville, SC	PP333564	PP338697
P. tabaniformis	BT 152	Bath Township, MI	PP333565	PP338698
P. tabaniformis	BT 153	Bath Township, MI	PP333566	PP338699
P. tabaniformis	BT 210	Princeton, MN	PP333580	PP338711
P. tabaniformis	BT 168	Ravalli County, MT	PP333571	PP338702
P. robiniae	BT 148	Grant County, NM	PP333562	PP338695
P. robiniae	BT 149	Grant County, NM	PP333563	PP338696
P. dolli	BT 160	Clinton County, MI	PP333569	NA
P. dolli	BT 161	Clinton County, MI	PP333570	NA
P. sogaardi	BT 154	Bath Township, MI	PP333567	PP338700
P. sogaardi	BT 155	Bath Township, MI	PP333568	PP338701
P. sogaardi	BT 211	Princeton, MN	PP333581	PP338712
P. sogaardi	BT 169	Clinton County, MI	PP333572	PP338703
P. sogaardi	BT 170	Clinton County, MI	PP333573	PP338704

Table 2. PCR primers and cycling conditions.

PCR Primers						
Gene	Forward Primer	Primer Sequence	Reverse Primer	Primer Sequence	Annealing	Reference
COI	LCO1490	5' GGT CAA CAA ATC ATA AAG ATA TTG G 3'	HCO2198	5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'	95°C (15min); 5 cycles of 94°C (30s) / 45°C (90s) / 72°C (90 s); 30 cycles of 94°C (30s) / 50°C (90s) / 72°C (90 s); 72°C (5min)	Folmer et al. (1994)
Wg	LepWg1	5' GAR TGY AAR TGY CAY GGY ATG TCT GG 3'	LepWg2	5' ACT ICG CAR CAC CAR TGG AAT GTR CA 3'	95°C (15min); 36 cycles of 95°C (30s) / 55°C (45s) / 72°C (60 s); 72°C (10min)	Brower and DeSalle (1998)

Table 3. The intraspecific line is ordered as (Wg/COI). “P” distance values above the intraspecific line represent average interspecific Wg values and below the line are average interspecific COI values.

Wg and COI P-Distance									
	P. asilipennis	*P. dolli*	*P. fenestrata*	*P. robiniae*	*P. simulans*	*P. sogaardi*	*P. tabaniformis*	*C. laurelae*	*V. admiranda*
P. asilipennis	.002/.008	0	.008	.032	.013	.036	.022	.204	.075
P. dolli	.085	0/0	0	0	0	0	0	0	0
P. fenestrate	.072	.104	.001/.002	.028	.009	.031	.018	.205	.071
P. robiniae	.084	.062	.101	.002/0	.034	.023	.029	.202	.066
P.simulans	.07	.095	.071	.092	.005/.006	.037	.028	.206	.075
P. sogaardi	.073	.077	.098	.087	.09	.003/.005	**.031**	.201	.065
P.tabaniformis	.076	.08	.101	.09	.081	**.076**	.002/.014	.206	.068
C. laurelae	.149	.164	.163	.164	.168	.153	.155	NA/NA	.213
V. admiranda	.136	.149	.139	.149	.134	.133	.141	.174	.005/.005

CHAPTER 2: APOSEMATIC COLOR POLYMORPHISM IS A POOR INDICATOR OF SPECIES BOUNDARIES IN NORTH AMERICAN *PARANTHRENE* (LEPIDOPTERA: SESIIDAE) AS EVIDENCED BY A MULTI-GENE PHYLOGENY

Color polymorphism is a phenomenon among animals that has been implicated as a factor in speciation (Huxley 1955, Gray and McKinnon 2006). Selection and genetic drift act upon the color variants and the balance with gene flow among variants determines the probability that color forms will become associated with a speciation event (Gray and McKinnon 2006). Specific isolating mechanisms that drive genetic divergence among color variants include, allopatric isolation, assortative mating, and frequency- dependent selection. While sexual selection and natural selection in the context of heterogenous environment play an important part for several animals, each mechanism (alone or combined) can contribute to either the maintenance of polymorphism within a species or drive speciation (Gray and McKinnon 2006). For example, many studies of aposematic coloration, or defensive warning colors, in the context of Mullerian and Batesian mimicry demonstrate the importance of predation and the frequency of color forms (Mallet and Barton 1989, Sherratt 2008, Chouteau et al 2016). However, both positive frequency dependent selection and male mate-choice act upon aposematic *Heliconis* polymorphic species (Lepidoptera) to drive speciation (Mallet and Barton 1989, Joron and Iwasa 2005, Kronforst et al. 2006). Discovering the role that color polymorphism plays in speciation requires observation and experimentation (Gray and McKinnon 2006). Most important, a phylogeny provides an evolutionary pattern of species and associated color variants which can direct experimentation (e.g., Brower 1996).

Clearwing moths (Lepidoptera: Sesiidae) exhibit extensive aposematic color polymorphism within species (Eichlin and Duckworth 1988). All adults are diurnal mimics of wasps and bees, in both their appearance and their behavior (Eichlin and Duckworth, 1988). This exhaustive mimicry is demonstrated with some species mimicking their hymenopteran model's flight patterns and acoustics (Skowron Volponi et al 2018, Skowron Volponi et al 2021). As larvae, these moths bore into a variety of economically important plants, and are considered pests (Solomon, 1995). *Paranthrene* typically spend two years in the larval stage and feed on oaks or willows and poplars (Eichlin and Duckworth, 1988, Solomon, 1995). Two or three color

forms which mimics hymenopterans occur among adult *Paranthrene* species (Eichlin and Duckworth, 1988). These forms appear to converge their color morphology to match various locally abundant hymenopterans (Fig.11) Currently, there are nine recognized *Paranthrene* species occurring in the United States, Canada, and Mexico (Baja California) (Eichlin, 1992; Handfield and Handfield, 2021; Smith et al, 2024).

A few polymorphic species such as, *P. simulans* Grote 1881, *P. fenestrata* Barnes and Lindsey 1922, and *P. robiniae* Edwards 1880 have multiple color forms. The oak borer (*Paranthrene simulans*) has three unique color morphs, simulans, lugerii and, palmii forms (Fig. 12a-c). Its range includes the entire eastern United States and Canada, as far west as Texas and Minnesota (Solomon, 1995). The palmii form is found in the species' southern range and the *luggeri* form occurs in the western portion (Eichlin and Duckworth, 1988). In the areas where simulans and palmii overlap intermediates between the forms have been observed (Eichlin and Duckworth, 1988). Host plants for all populations are various oak species and the larvae feed on different parts of the tree depending on their range (Solomon, 1995). In the south, they feed on larger more mature trees, while in the north they are more likely to feed on saplings and small branches (Johnson and Lyon, 1995). Prior to 1988, *Paranthrene palmii* was considered a separate species. *Paranthrene palmii* was synonymized with *P. simulans* due to its similar morphology, host plants, and pheromone attraction but considered a form (Eichlin and Duckworth, 1988). Likewise, luggeri was originally described in 1891 and later synonymized (Beutenmüller 1896). Despite the similarities, some behavioral and life history details have been noted between the forms. While all forms feed and have a preference for red oaks, palmii has been recorded on black oak, and never on white oaks, with the inverse observed for simulans (Solomon and Morris, 1966). Even their emergence timing is slightly different. Palmii emerges between April to June, and simulans emerges between June and July (Solomon, 1995). These differences, while not enough to convince Eichlin and Duckworth to recognize the palmii form as a distinct species, prompted the researchers to suggest the need for additional study of the species boundaries (Eichlin and Duckworth, 1988). McKern and Szalanski (2007) reported intraspecific mtDNA sequence variation among Arkansian *P. simulans* individuals and Cognato et al. (2023) show differences among populations from Minnesota and North Carolina.

Handfield and Handfield, (2021) demonstrated monophyly of mtDNA haplotypes that were diagnostic for a population of *P. simulans* from Quebec and associated with a diurnal flight period asynchronous with other *P. simulans*. They described this group as a new species, *P. hilairemontis* Handfield and Handfield 2021.

Paranthrene fensetrata has two distinct sympatric color morphologies (Fig. 13a-b) with no evidence of hybrids. All specimens have been collected from high elevations in Arizona, New Mexico, Colorado, Utah, and the Mexican state of Hidalgo, otherwise, there is no life history available for this species (Eichlin and Duckworth, 1988).

The western poplar clearwing (*Paranthrene robiniae*) is found along the Pacific Coast of the United States, the Rocky Mountains, and the provinces of British Colombia and Alberta (Canada) (Johnson and Lyon, 1988). Its preferred host plants, poplars and willows, occur throughout western North America in isolated populations separated by other habitats non-condusive to their growth, like deserts (Solomon, 1995, Cooke and Rood 2007), There are three recognized forms, robinae, perlucida, and palescens (Fig. 14a-b). The perlucida form occurs in Canada (British Columbia and Alberta), and the United States (the Pacific Northwest to Montana) (Engelhardt 1946). The palescens form occurs in deserts of southern California (Engelhardt 1946) and Nevada (this study).

Phylogenetic studies of Sesiidae are few, with a majority of study addressing the relationships among tribes and subfamilies, and only *P. simulans* and *P. tabaniformis* have been included (Cognato et al, 2023; Taft and Cognato 2017, Taft et al. 2016 Lait and Herbert, 2018; McKern et al, 2008). There are no DNA-based phylogenies of North American *Paranthrene* species, nor has any study investigated species boundaries for these species including polymorphic individuals. In this study, we reconstructed a phylogeny using all known species of North American *Paranthrene* and examined the intraspecific relationships between known color forms. We hypothesize that monophyletic groups of different color forms associate with *Paranthrene* species which would suggest that polymorphism of aposematic coloration may have a role in *Paranthrene* speciation.

Materials and Methods

Collection and identification of specimens

All but two specimens were collected over a 5 year period using Multi-Pher #1® pheromone canister traps (Distributions Solida Inc) baited with various pheromone lures intended for different species (Table 4). Two *P. dollii* were collected in 1988. Most *Paranthrene* species identifications and color morphologies were confirmed using Eichlin and Duckworth 1988. The remaining species, having been described after that publication, were identified with Smith et al 2024 and Handfield and Handfield 2022

Specimen preparation and imaging

A single representative of most color forms for 8 species was picked for genital comparisons. *Paranthrene hilairemontis* genitalia comparisons were done using images in Handfield and Handfield 2022. Access to the genitals was gained by removing the abdomen at the 4th or 5th segment. The extracted segments were placed in individual vials filled with water and two 116mg tablets of potassium hydroxide and allowed to sit on a hot plate set just below boiling for 2 hours. Softened abdominal segments were removed from the vials and teased apart under a dissecting microscope with fine-tipped forceps until genitalia were revealed. Unstained genitalia was prepared for photography by being placed on a microscope slide with glycerin and spread open with fine-tipped forceps then held in place temporarily with a slide cover. After the images were taken genitalia were preserved in glycerin and placed in 5mm glass micro vials and pinned under the associated specimen

Specimens were photographed with a Visionary Digital Passport II system (Dun Inc., Palmyra, VA) using a Canon EOS 5D Mark II, 65.0-mm Canon Macro photo lens, two Dynalite (Union, NJ) MH2015 road flash heads, Dynalite RoadMax MP8 power pack and a Stack Shot (Cognisys, Inc, Traverse City, MI). Montage images were assembled using Zerene Stacker 1.04 and sized in Adobe Photoshop 2021 v. 22.5.1 (San Jose, CA).

DNA sequence data and phylogenetic analyses

DNA was extracted from a metathoracic leg from 67 frozen specimens encompassing 9 species of *Paranthrene* and 5 color forms along with two outgroup species (Table 5) using a Qiagen DNeasy blood and tissue kit (Hilden, Germany) following the manufacturer's protocol. The remaining bodies were vouchered in the A. J. Cook Arthropod Research Collection. The purified DNA was used in PCR for mitochondrial cytochrome oxidase I, elongation factor 1a, and wingless. All three genes have been used for previous phylogenetic studies of sesiids (Taft and Cognato 2017, Cognato et al 2023). EXO-SAP-IT (USB Corp., Cleveland, OH, USA) was used to ready the PCR products for sequencing at the Michigan State University Research Technology Support Facility using Big-Dye Terminator v 1.1 (Applied Biosystems, Foster City, CA, USA) and an ABI 3730 Genetic Analyzer (Applied Biosystems). Sense and antisense strands were compiled using Sequencher (Ann Arbor, MI) to trim sequences of primer sequences, align the sequences and to create consensus sequences. Final sequences were deposited in Genbank (Table 5) and assembled in a Nexus file for a total of 1421 nucleotides (639 from COI, 365 from ef1a, and 417 from Wingless) which included 254 parsimony-informative characters.

Phylogenetic parsimony analysis of the aligned sequences consisted of a branch and bound search using default options in PAUP v4.0a (build 168; Swofford 2002). Gaps were treated as missing data. Bootstrap values were determined with 500 pseudo-replicates each conducted by heuristic search with simple stepwise addition. Percent pairwise DNA difference was calculated as p-distance in PAUP*. In addition, Bayesian analysis under a likelihood optimality criterion was used to assess phylogenetic relationships using this dataset. Using Mr. Bayes 3.2.6 (Ronquist et al. 2012) two simultaneous analyses were conducted in which each gene was partitioned by codon position and a model of general time reversal + gamma + proportion of invariable sites was applied to each partition (unlinked parameters). Four Metropolis-coupled Markov chain Monte Carlo searches (one cold, three heated) were analyzed for 5 million generations. Each analysis was sampled every 100th iteration and all parameters reached stability. Bayesian posterior probabilities of clades were based on 75,002 trees—the total of both runs after a 25% burn-in.

Results

The PAUP* analysis found 600 most parsimonious trees that were mostly resolved in the strict consensus of those trees (Fig. 16). Intraspecific and interspecific relationships of all *P. simulans* forms, *P. pellucida* and *P. hilairemontis* were unresolved (Fig. 16) resulting in a single clade with a 100% bootstrap value. All other clades of species except *P. robiniae* and *P. dollii* had 100% bootstrap values and values for internal nodes varied (Fig. 16). The topology of the Bayesian tree was generally similar to the parsimony tree (Fig 17). Posterior probabilities (PP=1) were high for all species clades except *P. pellucida*, *P. hilairemontis*, *P. robiniae* and, *P. dollii*. *Paranthrene pellucida* and *P. hilairemontis* were intermixed with *P. simulans.*

Within the *P. simulans* clade the placement of *P. pellucida* in both parsimony and Bayesian trees and *P. hilairemomtis* in the Bayesian tree rendered *P. simulans* as paraphyletic. *Paranthrene simulans* had an average intraspecific COI divergence of 1.56% and interspecific values of 1.17% and 1.27% compared to *P. pellucida* and *P. hilairemontis* respectively (Table 6). The divergence for nuclear genes between these species was <1% and <1.35% for Wg. These distances are similar to other intraspecific and interspecific distances of sister species within *Paranthrene* (Tables 9 and 12). Given the COI divergence was < 2%, as compared to the sister species, *P. fenestrata* (Table 6), the paraphyly of *P. simulans*, and a lack of genitalic differences among *P. simulans, P. hilairemontis* and *P. pellucida*, we synonymize the latter species with *P. simulans.* The luggeri form was the only monophyletic clade among *P. simulans* color forms and it rendered *P. simulans* paraphyletic. This clade is weakly supported in the parsimony (64% bootstrap value) and Bayesian trees (PP = 0.83) (Fig 16 and 17) and had a larger sequence divergence than any other color forms (Table 6).

Neither the typical black or yellow forms of *P. fenstrata* were monophyletic in either tree. They resolved polyphyletic within the clade representing *P. fenstrata* (Fig. 16 and 17). There was no DNA divergence or morphological evidence to support the recognition of these forms as species (Table 6).

Paranthrene robiniae and *P. dollii* resulted in a single weakly supported clade with specimens collected at different localities monophyletic with a 100% bootstrap value (Fig. 16). In the Bayesian tree, individual geographic clades of *P. robiniae* and *P. dollii* were less supported

with a posterior probability ranging from 0.68-1 (Fig. 17) but had similar placement to the parsimony tree. The placement of P*. dollii* rendered *P. robiniae* paraphyletic in both trees. The palescens form *P. robiniae* was polyphyletic, occurring in three separate clades (Fig. 16 and 17). The interspecific COI divergence among the three *P. robiniae* populations and *P. dollii* ranged between 6% and 9% (Table 7). The intraspecific COI divergence for each group was < 1%, except the Nevada population which was 7%. The great divergence observed in the Nevada population was caused by a single individual and removal of this single specimen decreased the average divergence to 1%. This specimen is an anomaly and requires investigation in future studies. External morphologies between the three populations showed striking similarities with little to negligible difference between the Nevada and New Mexico populations. The monophyletic Arizona and New Mexico populations had diagnostic differences of the male genitalia consistent with currently recognized species (Fig. 19 and 20). The two species are described below.

Taxonomy

Paranthrene **Hubner**

Males of *Paranthrene* have an apical spine on the aedeagus (Eichlin & Duckworth 1988)

***Paranthrene oasis* Smith, Taft and, Cognato, new species**

Type material

Holotype, male, United States: Arizona., Base and Meridian Wildlife Area, 280m ele., 33°22'37"N 112°18'24"W. 21vi2023. W, Smith Col (MSUC), second label, "DNA voucher BT195". One paratype as previous except male genitalia dissected and with second label, "DNA voucher BT 194" (MSUC).

Diagnosis

Paranthrene oasis is most similar to the palescens form of *P. robiniae*. The two species are remarkably similar in gross appearance but can be differentiated by color pattern. In *P. oasis* the prothoracic collar and first abdominal segment are yellow in their entirety and in *P. robiniae* the collar is bicolored, brown-orange proximal to the head and yellow distally, and the first abdominal segment is pigmented orange to brown (Fig. 18c-d and 21). The tarsal spines of *P. oasis* are orange spines while spines of *P. robiniae* are black (Fig. 18a-b). The saccus of the male genitalia, of *P. oasis* is slender and terminates in a point, while the saccus of *P. robiniae* is spatulate. (Fig. 19 a-c). The aedeagus spine in *P. oasis* is pointed and thorn-like with few teeth, while the spine on *P. robiniae* is broad and ridge-like with many teeth (Fig 20).

Description

Male (Fig. 14c). Head: Vertex covered in long yellow to light orange scales; Frons light yellow; Labial palps roughened, yellow with burnt orange scales along ventral side; Haustellum coiled and longer than labial palps; Antenna burnt orange. Thorax: Prothorax covered in a solid collar of yellow scales (Fig. 18c). Scutum brown and burnt orange; Scutellum brown and burnt orange bordered posteriorly with light yellow; Tegulas brown and burnt orange anteriorly transitioning to yellow posteriorly; Pluerons with broad flat burnt orange and yellow scales; Metanotum yellow, lateral anterior margins with long dense burnt orange setae; Legs primarily yellow with some burnt orange ventrally on all segments; Coxae yellow and burnt orange; Prothoracic femur concaved posteriorly and line with burnt orange setae; Mid-tibia with a

single pair of ventrally apical spurs; Hind tibia with two pairs of yellow to burnt orange ventral spurs; All tarsi with ventral burnt orange spines (Fig. a). Forewing: Mostly burnt orange to brown scales. Orange scales brightest along the base and basal portion of the inner margin; A faint orange discal spot; R, M, and C veins with dark brown scales; Brown fringe along marginal edge. Hindwing: Hyaline with dark brown fringe. Small patch of basal orange scales; Dark brown scales along all veins except R-M and M veins closing the discal cell. Abdomen (Fig 21d): Predominantly yellow with a thin anterior dorsal band of burnt orange on segment two and thin dorsal posterior bands on segments five and six. Segment three yellow and burnt orange circumferentially with a dorsally posterior band of dark brown scales; Anal tufts short and golden yellow. Male Genitalia: Saccus is shaped in a narrow triangular prism and terminates in a point. The subscaphium squares off bluntly behind the transtilla. The spine on the aedeagus is pointed and thorn like (Fig. 20c).

Female

Unknown.

Host

Unknown

Distribution

Known only from two specimens collected in the Base and Meridian Wildlife Area south of Phoenix Arizona near Estrella Mountain.

Etymology

"Oasis" used as a noun in apposition. This species is named after the environment it was found, that is, in the narrow vegetated area along the Gila River in the deserts of Arizona.

Remarks

Paranthrene oasis was collected in a trap hanging from a Fremont cottonwood *Poplus fremontii* baited with Scentry Western Poplar Clearwing Moth Lures © from Great Lakes IPM © (Table 4). There was also an abundance of Gooding's Willows *Salix goodingii* in the habitat.

***Paranthrene gilaensis* Smith, Taft, and Cognato, new species**

Type material

Holotype, male, United States: New Mexico., Lake Roberts, Gila National Forest, 1846m ele., 33°01'44"N 108°09'04"W. 18vii2017. W, Taft Col.(MSUC), second label, "DNA voucher BT149". Paratypes with same locality label. One with male genitalia dissected and second label "DNA voucher BT 148" and one with second label "DNA voucher BT 150" (2 MSUC).

Diagnosis

Paranthrene gilaensis most closely resembles *P. robiniae*. Diagnosis is difficult due to the color polymorphism in *P. robiniae*. *Paranthrene gilaensis* has darker forewings and the hindwing fringe is slightly darker and thicker. On the second abdominal segment of *P. gilaensis* there are three different colored bands, anterior band is black, followed by a thin red band, and a thicker yellow posterior band (Fig. 21c). In *P. robiniae* this middle red band is missing, although some red scales may occur but not enough to form a continuous band around the abdomen. On abdominal segment three, *P. gilaensis* is black dorsally and transitions laterally to red and ventrally to orange. In *P. robiniae* this band is black, or black and yellow for the entire circumference of the segment. Male genitalia provides unambiguous diagnostic an feature. The subscaphium in *P. gilaensis* is bifurcated basally while in *P. robiniae* ends in a single point. The presence of dark scales on the hindwing differentiate *P. dollii* from *P. gilaensis* in which the dark scales are absence.

Description

Male (Fig. 14d). Head: Vertex covered by long yellow to burnt orange scales; Frons covered in orange scales with occipital margins pale yellow, all covering a layer of smooth black scales underneath. These black scales might not be seen unless the upper layer of orange scales is rubbed off; Labial palps roughened, almost entirely light yellow and light orange except for the base which is burnt orange red and a scattering of long slender black scales; Antenna orange to orange-brown with black scales on the dorsal portion at the apical end. Thorax: Prothorax with a bicolored collar of burnt orange on the anterior margin and yellow on the posterior. The anterior orange scales cover a layer of smooth black scales similar to the frons; Scutum black with some long hair-like red scales: Scutellum black bordered posteriorly with

yellow, this yellow border may be speckled with occasional red scales; Tegulas black anteriorly with many long red hairlike scales, then yellow posteriorly giving the appearance of red and yellow "shoulders" (Fig 22b); Pluerons black; Legs, Coxae black with dorsal tufts of orange scales, Femurs orange on the anterior side and black on the posterior, Mid-tibia with a single pair of ventrally apical spurs; Hind tibia with two pairs of yellow to burnt orange ventral spurs, all Tibias orange and yellow, all Tarsi orange and yellow with black spines; Forewing: Densely covered with brown and dark orange scales, Ata the base of the wing dense black scales along the anterior margin and dense red scale posteriorly. Hindwing: Hayline with a thick dark brown fringe, bright orange scales along basal edge, all veins with brown scales except brown and burnt orange scales covering the R-M and M veins closing the discal cell. Abdomen: Predominantly yellow except for the first three segments, Segment one black, segment two with an anterior black band, then a thin red band followed by a posterior yellow band, abdominal segment three is black dorsally and transitions red laterally to yellow or orange ventrally, there is some variation with the lateral red scales forming a dorsal band between black bands, and the black scales forming a very thin circumferential anterior band; Male Genitalia: Saccus broadly spatulate and narrowed slightly at the base (Fig. 19d). The subscaphium long extending behind the transtilla and terminates in a bifurcated structure.

Female

Unknown.

Host

Unknown

Distribution

Known only from Lake Roberts New Mexico, within the Gila National Forest.

Etymology

This species is named after the type locality in the Gila National Forest.

Remarks

Paranthrene gilaensis was collected in traps baited with Western Poplar Borer (EZ 3,13 OH/ZZ 3,13 OH) 50:50 pheromone blend.

***Paranthrene simulans* Grote 1881**

Trichilium simulans Grote, 1881, *Bull. Brooklyn Ent. Soc., 3:78.*

Fatua palmii Hy. Edwards, 1887, *Can. Ent.,* 19:145.

Trochilium luggeri Hy. Edwards, 1891, *Psyche,* 6:108.

Paranthrene pellucida Greenfield and Karandinos, 1979, *Proc. Ent. Soc Washington,* 81:499. **New Synonymy**

Paranthrene hilairemontis Handfield and Handfield, 2021, *Journ. Lep. Soc.* 75(4):252-258. **New Synonymy**

Diagnosis

Paranthrene simulans can be separated from other North American *Paranthrene* by a combination of hyaline hindwings, occipital fringe black dorsally.

Redescription

Male (Fig. 12). Head: Vertex black, except in palmii where it is black and yellow-orange; Occipital fringe black; Frons black with occipital margins white-yellow to orange-yellow; Labial palps white-yellow to orange-yellow, black at the base and various amounts of black ventrally; Antenna black with dark orange tips; Thorax: Prothorax with bicolored collar, anterior margin black and posterior band ranges from pale white-yellow, yellow to golden orange; Scutum and scutellum black with fringes ranging from pale white-yellow, yellow to golden orange: Tegulas black with a patch of "shoulder" scales ranging from pale white-yellow, yellow, to golden yellow; Legs, bicolored, black basally ending from the trochanter to the apical end of the tibia, second color ranges from pale white-yellow, yellow to golden orange-yellow; Forewings: except in the pellucida form, covered in brown, orange-brown, to black scales and hyaline in the tornus and outer margin, scaling much more dense in the palmii form, forewings of the pellucida form is hyaline with a brown discal spot and brown to black scales only along veins, brown to black fringe; Hindwings: Hyaline with brown to black scales along veins and brown to black fringe, base of inner margin either black, yellow or orange; Abdomen: Highly variable, Each segment may contain black scales ranging from small patches on the posterior margin (palmii), narrow bands (luggeri), to many whole segment black (hilairemontis), non-black portions of the abdomen range from pale white-yellow, yellow to golden orange-yellow; Male Genitalia: Valves

rounded, may have a slight point apically and many dense setae, Saccus spatulate, thickness of apical end may vary.

Remarks

Paranthrene simulans, *P. pellucida*, and *P. hilairemontis* are synonymized given the inclusion of the latter in a well-supported monophyletic group of widely distributed *P. simulans* individuals (Figs. 16 and 17) and the <2% interspecific DNA divergence of both mitochondrial and nuclear genes (Tables 6, 9, and 12). Genitalic morphology is undistinguishable between *P. simulans, P. pellucida* and *P. hilairemontis* and the extent of color polymorphism is consistent with the variability observed with other *P. simulans* color forms.

Discussion

Traditional taxonomy studies synonymized many color forms (Engelhardt 1946, Eichlin and, Duckworth 1988). Several of these findings are upheld in this study such as the synonymies of the *P. simulans* and *P. robiniae* forms. *Paranthrene pellucida* and *P. hilairemontis,* along with palmii and luggeri, should be recognized as unique color forms of *P. simulans*, as well as, palescens as a form of *P. robiniae*. Conversely, this study and previous Sesiidae molecular phylogenetic studies have supported the recognition of color forms as species (Taft et al 2016, Smith et al 2024). However, of the nine color forms we examined only two are monophyletic, have comparable DNA sequence divergence, and associated with species diagnostic morphology. Thus, color pattern is not a predictable indicator for species boundaries.

Maintenance of color polymorphism among *Paranthrene* species is likely a complexity of many factors including natural selection and genetic drift (Huxley 1955, Gray and McKinnon 2006). Color polymorphism of aposematic Lepidoptera can be maintained through selection against hybrids in narrow hybrid zones (Mattel and Barton 1989). A zone of overlap occurs for the simulans and palmii forms (Solomon, 1995, Eichlin and Duckworth 1988 and, Englehardt 1946), but this is not the case for the majority *Paranthrene* forms whose ranges are entirely sympatric. For example, the geographic ranges of the luggeri, pellucida, and hilairemontis forms are completely sympatric with the range of the simulans form (Handfield and Handfield 2021, Eichlin 1989, Eichlin and Duckworth 1988). The range of the *P. robiniae* palescens color form also completely overlaps with the robiniae color form (Englehardt 1946). Within these sympatric ranges, local abundance of particular models may provide selection at small geographic scales and/or for a limited time thus creating a patchwork of color forms without mating barriers (Mattel and Barton 1989). Little is known concerning the distribution of *Paranthrene* color forms and model hymnopteran species or predation intensity against poor mimics of local models.

Alternatively, sexual selection may drive the diversity of color forms (refs). However, sesiids use pheromones to locate mates (Eichlin and Duckworth 1988). All color forms of *P. simulans* were collected with the same two semiochemical blends (Taft et al 1991). These include the semiochemicals used in the collection of the hilairimontis and pellucida forms

(Greenfield and Karandinos 1979, Taft et al 1991, Handfield and Handfield 2021). Also, all three *P. robiniae* populations and *P. dollii* were collected with the same semiochemical blend (Table 4). It is unlikely that color form and pheromone type are genetic linked. Thus, sexual selection likely does not contribute to maintenance of *Paranthrene* color forms although it is unknown if color pattern is used in mate recognition.

We hypothesis genetic drift is an important factor in the fixation of color forms and it is coincidental with allopatric speciation. In this study and others, species were described or validated from mountainous sky islands and oasises (Taft and Cognato 2017, Cognato et al. 2023). The sky islands and deserts of southwestern United States are notable for their role in speciation due to geographic isolation (Masta 2000, Mitchell and Ober 2013, Edwards et al 2016). Given that much of sesiid diversity occurs in this region, it is likely that additional cryptic or pseudocryptic species exists among color polymorphic species given the apparent recent species radiation of several sesiid genera (Cognato et al. 2023). The abberant *P. robiniae* palescens form specimen (BT193) may represent a cryptic species where color form has not coincided with DNA divergence.

Color pattern is a poor indicator of species boundaries in *Paranthrene*. A combination of monophyly, morphology, and DNA divergence reliably delimit *Paranthrene* species boundaries. These species may include populations of different color forms. Geographic barriers appear to play an important role in limiting gene flow, especially in the American Southwest. A more comprehensive survey of geographically separated populations of *Paranthrene* and of their hymenopteran models is needed to better understand the maintenance of color forms.

Figures

Figure 11. *Paranthrene spp* (right)beside potential hymenoperan models (left).

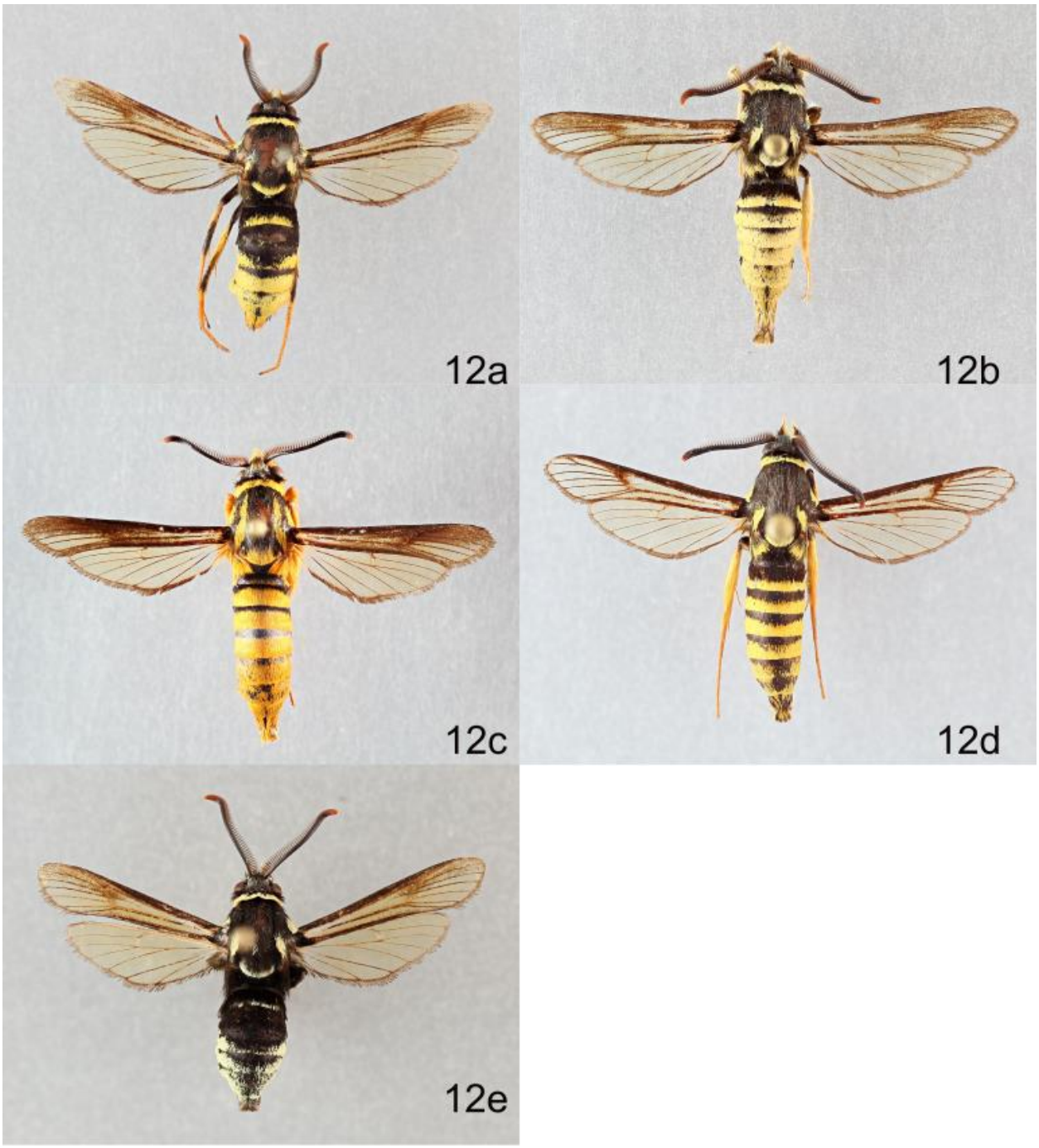

Figure 12. a) *Paranthrene simulans* "simulans" **b)** *Paranthrene simulans* "luggeri" **c)** *Paranthrene simulans* "palmii" **d)** *Paranthrene pellucida* **e)** *Paranthrene hilairemontis.*

Figure 13. *Paranthrene fenestrata* **a)** yellow **b)** black.

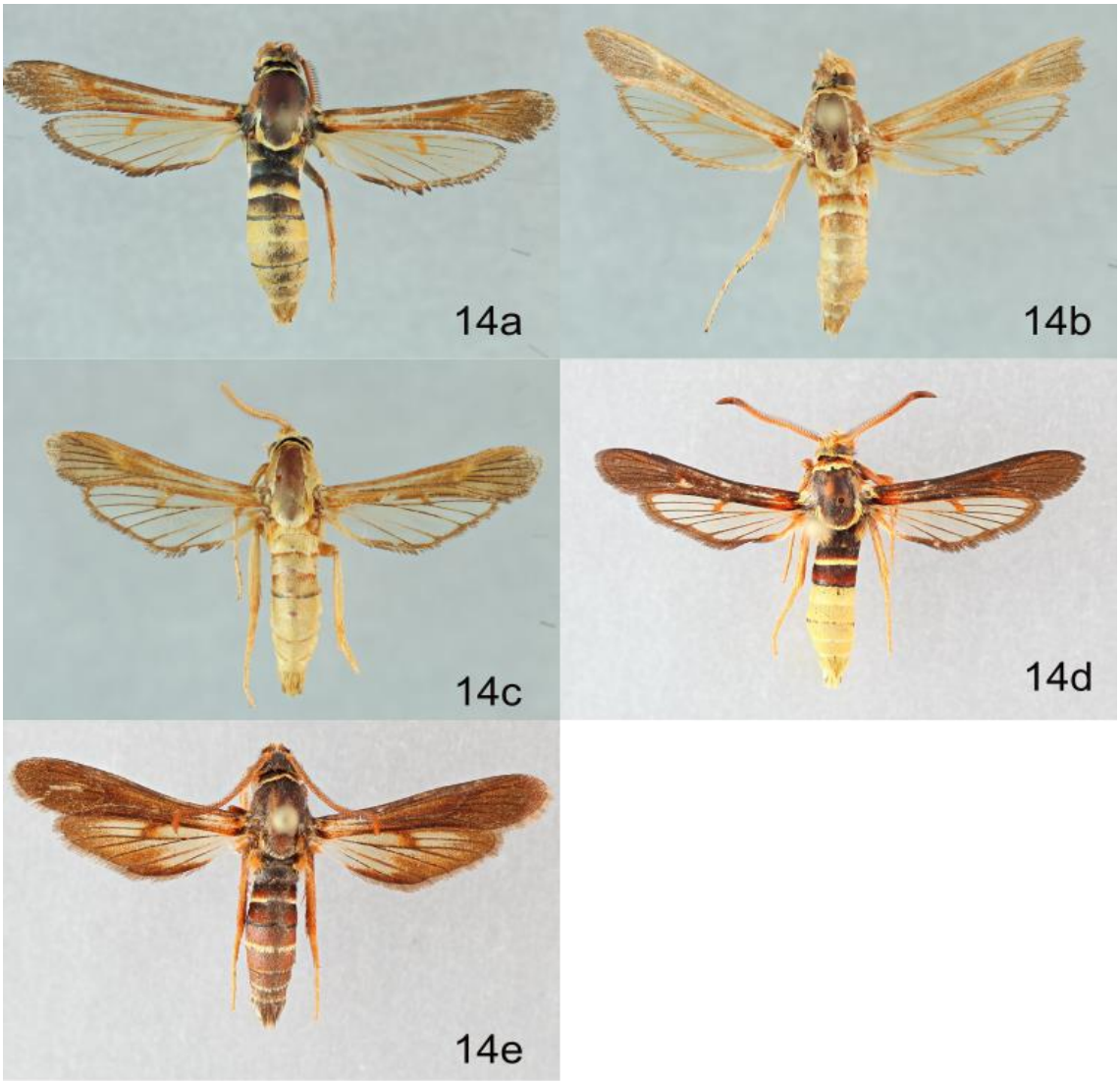

Figure 14. a)*Paranthrene robiniae* "robiniae" **b)** *Paranthrene robiniae* "palescens" **c)** *Paranthrene oasis* **d)** *Paranthrene gilaensis* **e)** *Paranthrene dollii.*

Figure 15. a) *Paranthrene tabaniformis* **b).** *Paranthrene tabaniformis* "oslari" **c)** *Paranthrene sogaardi* **d)** *Paranthrene asilipennis.*

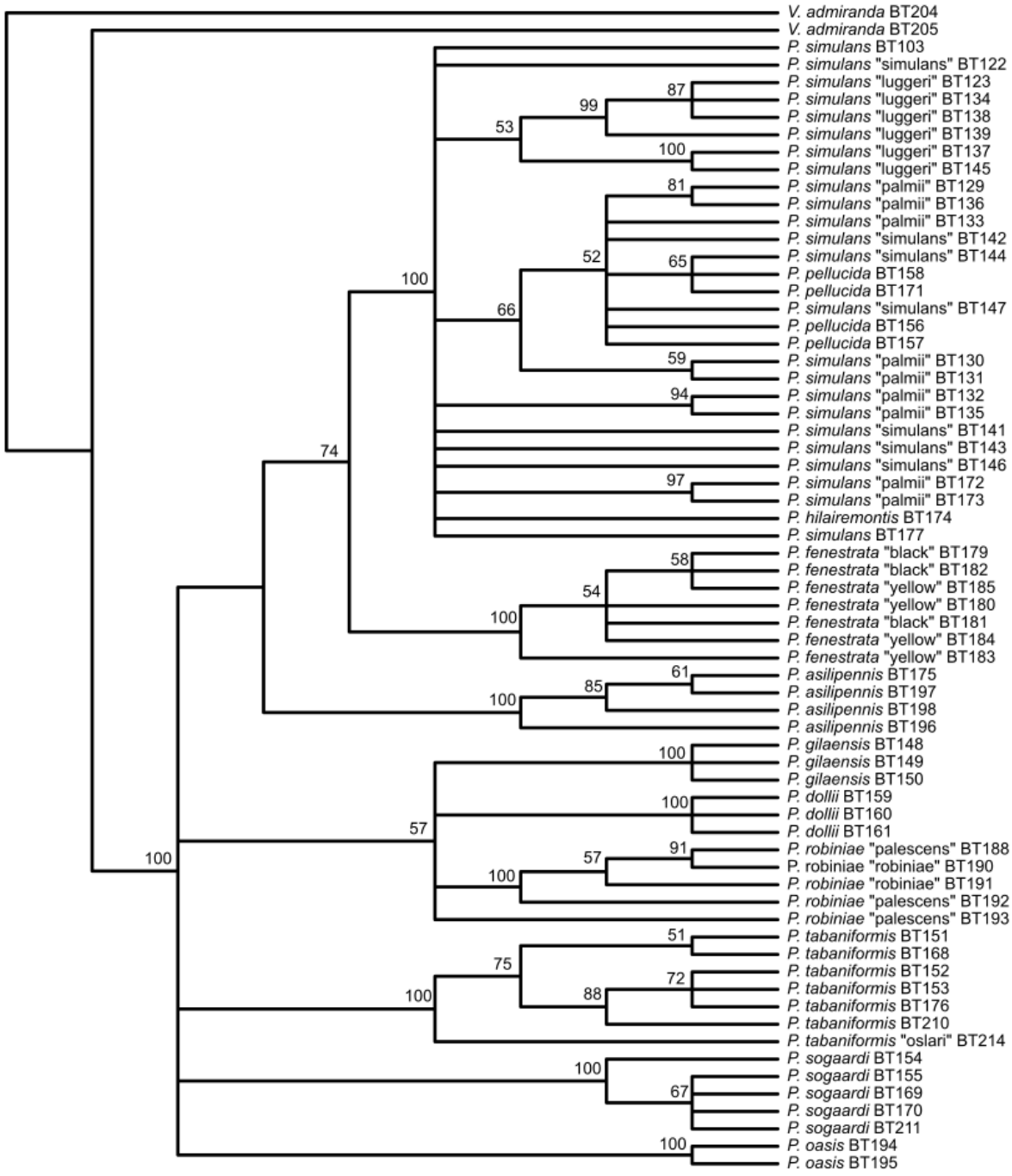

Figure 16. Strict consensus of 600 most parsimonious trees. Numbers above branches are bootstrap values.

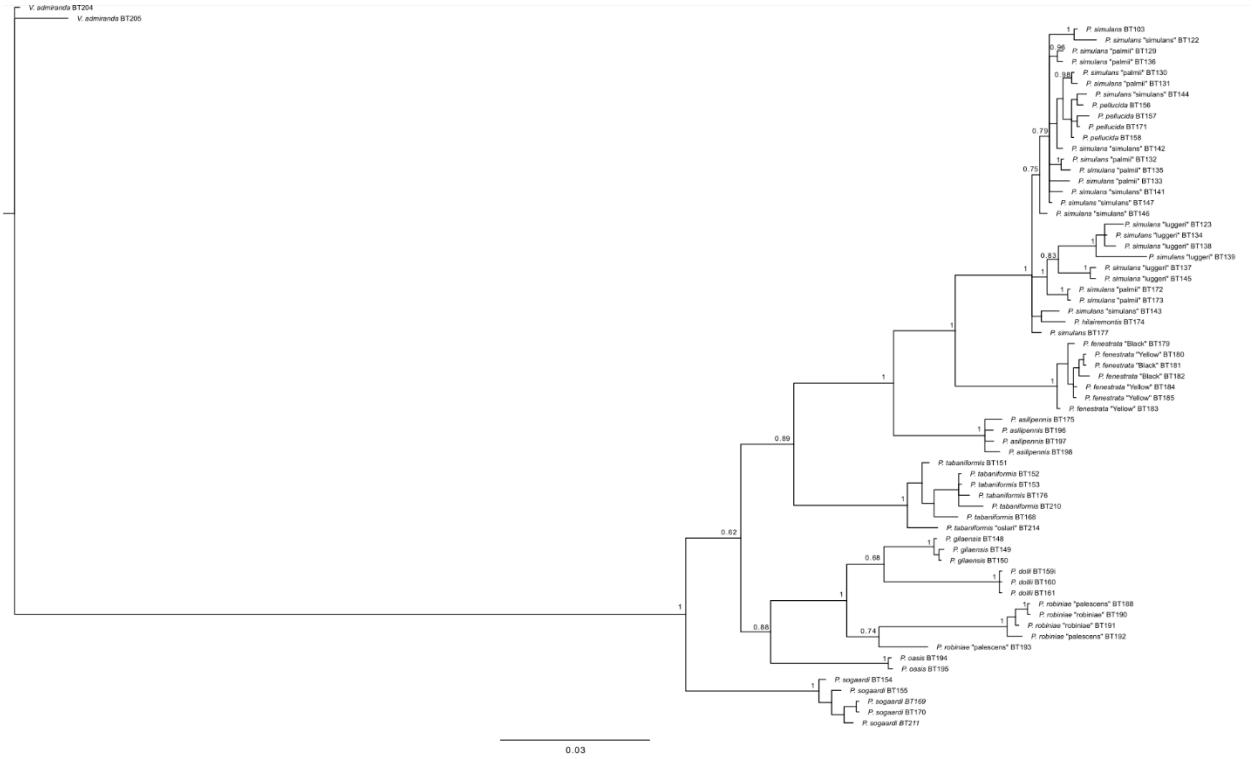

Figure 17. Bayesian analysis. Numbers above nodes are posterior probabilities based on 75,002 trees.

Figure 18. Paranthrene oasis and P. robiniae comparison **a)** *Paranthrene oasis* tarsal spines **b)** *Paranthrene robiniae* tarsal spines **c)** *Paranthrene oasis* prothoracic collar **d)** *P. robiniae* prothoracic collar.

Figure 19. Genitalia **a)** *Paranthrene robiniae* **b)** *Paranthrene dollii* **c)** *Paranthrene oasis* **d)** *Paranthrene gilaensis.*

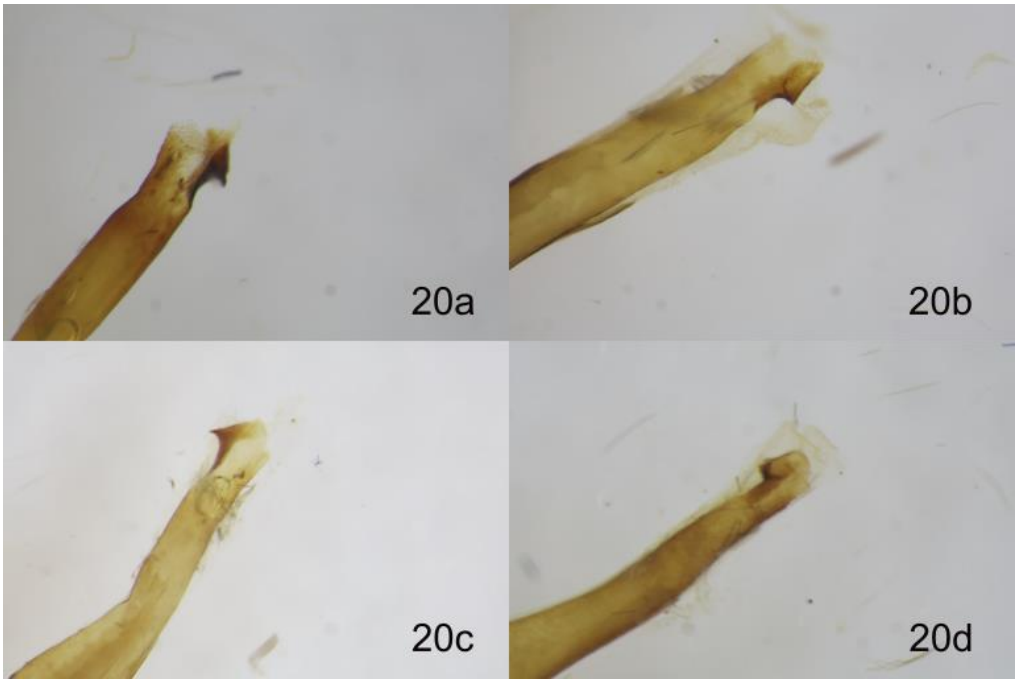

Figure 20. Adeagus spines **a)** *Paranthrene robiniae* **b)** *Paranthrene dollii* **c)** *Paranthrene oasis* **d)** *Paranthrene gilaensis.*

Figure 21. Abdominal color patterns **a)** *Paranthrene robiniae* "robiniae" **b)** *Paranthrene robiniae* "palescens" **c)** *Paranthrene gilaensis* **d)** *Paranthrene oasis.*

Figure 22. Tegula "shoulder" scales **a)** *Paranthrene robiniae* **b)** *Paranthrene gilaensis.*

Tables

Table 4. Pheromones used for species collection.

Blend	Retailer	Target Species
ZZ 3,13 OH/EZ 3,13 OH (50:50)	Great Lakes IPM	*P. robiniae* *P. dollii* *P. oasis* *P. gilaensis* *P. tabaniformis* *P. sogaardi*
ZZ 3,13 A/EZ 2,13 A/Z 13 A (80: 15: 5)	Alpha Scent	*P. fenestrata* *P. simulans*
EZ 3,13 A/ZZ 3,13 A (4:1)	Alpha Scents	P. pellucida
ZZ 3,13 A/ZZ 2,13 OH (75:25)	Great Lakes IPM	*P. simulans* *P. pellucida*
EZ 2,13 A	Anglian Lepidopterist Supplies	*P. hilairemontis*
EZ2,13 A/Z 13 A (96:4)	Alpha Scents	*P. asilipennis*

Table 5. Species, specimens, localities and Genbank numbers.

Species	Color Form	DNA voucher #	Location	Genbank # COI	Genbank # Wg	Genbank # Ef1a
V. admiranda		BT 204	Medicine Park, OK	PP333558	PP338691	PP661425
V. admiranda		BT 205	Medicine Park, OK	PP333559	PP338692	PP661426
P. simulans	Unkown	BT103	Frankin, NC	PP654332	PP661526	PP661427
P. simulans	Unkown	BT 177	North Lima, OH	PP654361	PP661563	PP661471
P. simulans	simulans	BT 122	Minneapolis, MN	PP654333	PP661523	PP661428
P. simulans	simulans	BT 141	Manistee County, MI	PP654345	PP661536	PP661441
P. simulans	simulans	BT142	Manistee County, MI	PP333561	PP338694	PP661442
P. simulans	simulans	BT 143	Manistee County, MI	PP654346	PP661538	PP661443
P. simulans	simulans	BT 144	Clinton County, MI	PP654347	PP661539	PP661444
P. simulans	simulans	BT 146	Clinton County, MI	PP654349	PP661541	PP661446
P. simulans	simulans	BT 147	Clinton County, MI	PP654350	PP661542	PP661447
P. simulans	luggeri	BT 123	Minneapolis, MN	PP654334	PP661524	PP661429
P. simulans	luggeri	BT 134	Princeton, MN	PP654339	PP661530	PP661435
P. simulans	luggeri	BT 137	Princeton, MN	PP654342	PP661533	PP661438
P. simulans	luggeri	BT 138	Princeton, MN	PP654343	PP661534	PP661439
P. simulans	luggeri	BT 139	Fairfax, VA	PP654344	PP661535	PP661440
P. simulans	luggeri	BT 145	Clinton County, MI	PP654348	PP661540	PP661445
P. simulans	palmii	BT 129	Jacksonville, NC	PP333560	PP338693	PP661430
P. simulans	palmii	BT 130	Godsden County, FL	PP654335	PP661526	PP661431
P. simulans	palmii	BT 131	Palatka, FL	PP654336	PP661527	PP661432
P. simulans	palmii	BT 132	Jacksonville, NC	PP654337	PP661528	PP661433
P. simulans	palmii	BT 133	Jacksonville, NC	PP654338	PP661529	PP661434
P. simulans	palmii	BT 135	Jacksonville, NC	PP654340	PP661531	PP661436
P. simulans	palmii	BT 136	Jacksonville, NC	PP654341	PP661532	PP661437
P. simulans	palmii	BT 172	Gainsville, FL	PP654357	PP661558	PP661466
P. simulans	palmii	BT 173	Gainsville, FL	PP654358	PP661559	PP661467
P. pellucida		BT 156	Clinton County, MI	PP654352	PP661551	PP661456
P. pellucida		BT 157	Gratiot County, Mi	PP654353	PP661552	PP661457
P. pellucida		BT 158	Gratiot County, Mi	PP654354	PP661553	PP661458
P. pellucida		BT 171	Clinton County, MI	PP654356	PP661557	PP661465
P. Hilairemontis		BT 174	Quebec, Canada	PP654359	PP661560	PP661468
P. fenestrata	typical	BT 179	Emory Pass, NM	PP333575	PP338706	PP661472
P. fenestrata	typical	BT 181	Pinos Altos, NM	PP333577	PP338708	PP661474
P. fenestrata	typical	BT 182	Pinos Altos, NM	PP654362	PP661567	PP661475
P. fenestrata	yellow	BT 180	Emory Pass, NM	PP333576	PP338707	PP661473
P. fenestrata	yellow	BT 183	Emory Pass, NM	PP333578	PP338709	PP661476
P. fenestrata	yellow	BT 184	Pinos Altos, NM	PP654363	PP661569	PP661477
P. fenestrata	yellow	BT 185	Emory Pass, NM	PP654364	PP661570	PP661478
P. gilaensis	typical	BT 148	Grant County, NM	PP333562	PP661543	PP661448
P. gilaensis	typical	BT 149	Grant County, NM	PP333563	PP661544	PP661449

Table 5 (cont'd)

P. gilaensis	typical	BT 150	Grant County, NM	PP654351	PP661545	PP661450
P. robiniae	typical	BT 190	Henderson, NV	PP654366	PP661572	PP661480
P. robiniae	typical	BT 191	Henderson, NV	PP654367	PP661573	PP661481
P. robiniae	palescens	BT 188	Henderson, NV	PP654365	PP661571	PP661479
P. robiniae	palescens	BT 192	Henderson, NV	PP654368	PP661574	PP661482
P. robiniae	palescens	BT 193	Henderson, NV	PP654369	PP661575	PP661483
P. oasis		BT 194	Pheonix, AZ	PP654370	PP661576	PP661484
P. oasis		BT 195	Pheonix, AZ	PP654371	PP661577	PP661485
P. tabaniformis	typical	BT 151	McClellanville, SC	PP333564	PP338697	PP661451
P. tabaniformis	typical	BT 152	Bath Township, MI	PP333565	PP338698	PP661452
P. tabaniformis	typical	BT 153	Bath Township, MI	PP333566	PP338699	PP661453
P. tabaniformis	typical	BT 168	Ravalli County, MT	PP333571	PP338702	PP661462
P. tabaniformis	typical	BT 176	North Lima, OH	PP654360	PP661562	PP661470
P. tabaniformis	typical	BT 210	Princeton, MN	PP333580	PP338711	PP661489
P. tabaniformis	oslari	BT 214	Comanche County, OK	PP654374	PP661583	PP661491
P. dollii		BT 159	Clinton County, MI	PP654355	NA	PP661459
P. dollii		BT 160	Clinton County, MI	PP333569	NA	PP661460
P. dollii		BT 161	Clinton County, MI	PP333570	NA	PP661461
P. sogaardi		BT 154	Bath Township, MI	PP333567	PP338700	PP661454
P. sogaardi		BT 155	Bath Township, MI	PP333568	PP338701	PP661455
P. sogaardi		BT 169	Clinton County, MI	PP333572	PP338703	PP661463
P. sogaardi		BT 170	Clinton County, MI	PP333573	PP338704	PP661464
P. sogaardi		BT 211	Princeton, MN	PP333581	PP338712	PP661490
P. asilipennis		BT 175	North Lima, OH	PP333574	PP338705	PP661469
P. asilipennis		BT 197	Swanton, OH	PP654372	PP661579	PP661487
P. asilipennis		BT198	Swanton, OH	PP654373	PP661580	PP661488
P. asilipennis		BT196	Swanton, OH	PP333579	PP338710	PP661486

Table 6. Average percent COI "p" - distance between *Paranthrene simulans, Paranthrene pellucida, Paranthrene hilairemontis* and*, Paranthrene fenestrata* species and color forms.

	P. simulans simulans	*P. simulans* luggeri	*P. simulans* palmii	*P. hilairemontis*	*P. pellucida*	*P. fenestrata* "black"	*P. fenestrata* "yellow"
P. simulans simulans	0.65%						
P. simulans luggerii	2.40%	2.03%					
P. simulans palmii	0.94%	2.63%	1.15%				
P. hilairemontis	0.83%	2.27%	1.11%	NA			
P. pellucida	0.55%	2.66%	0.90%	1.10%	0.26%		
P. fenestrata "black"	6.95%	7.51%	7.04%	6.36%	7.17%	0.31%	
P. fenestrata "yellow"	6.76%	7.36%	6.84%	6.26%	6.97%	0.23%	0.16%

Table 7. Average percent COI "p" - distance between *Paranthrene robiniae, Paranthrene dollii, Paranthrene oasis* **n. sp**. and*, Paranthrene gilaensis* **n.sp.** species and color forms.

	P. oasis n.sp	*P. gilaensis* n.sp	*P. robiniae* typical	*P. robiniae* palescens	*P. dollii*
P. oasis n.sp	0.16%				
P. gilaensis n.sp	7.43%	0.00%			
P. robiniae typical	9.00%	7.98%	0.63%		
P. robiniae palescens	8.01%	7.25%	2.74%	5.12%	
P. dollii	7.43%	6.26%	8.29%	7.88%	0.00%

Table 8. Average percent COI "p" - distance between *Paranthrene* species.

	P. asillipennis	*P. dollii*	*P. fenestrata*	*P. hilairemontis*	*P. pellucida*	*P. oasis* n.sp	*P. gilaensis* n.sp	*P. robiniae*	*P. simulans*	*P. sogaardi*	*P. tabaniformis*	*V. admiranda*
P. asillipennis	0.65%											
P. dollii	8.57%	0.00%										
P. fenestrata	7.28%	10.49%	0.22%									
P. hilairemontis	6.30%	8.76%	6.31%	NA								
P. pellucida	7.00%	9.31%	7.06%	1.10%	0.26%							
P. oasis n.sp	7.39%	7.43%	8.11%	7.90%	8.45%	0.16%						
P. gilaensis n.sp	8.26%	6.26%	10.06%	9.08%	9.15%	7.43%	0.00%					
P. robiniae	8.21%	7.38%	10.20%	9.08%	9.43%	8.04%	7.62%	5.20%				
P. simulans	7.17%	9.41%	7.00%	1.27%	1.17%	8.55%	9.66%	9.55%	1.56%			
P. sogaardi	7.32%	7.67%	9.77%	8.29%	9.09%	7.56%	8.70%	8.40%	8.93%	0.47%		
P. tabaniformis	7.60%	8.03%	9.98%	7.38%	7.96%	7.49%	8.87%	8.65%	7.86%	7.46%	1.56 %	
V. admiranda	13.62%	14.87%	13.90%	13.22%	13.46%	12.83%	14.95%	14.28 %	13.51%	13.26 %	14.14 %	0.47%

Table 9. Average percent EF1a "p" - distance between *Paranthrene simulans, Paranthrene pellucida, Paranthrene hilairemontis* and*, Paranthrene fenestrata* species and color forms.

	P. simulans simulans	*P. simulans* luggeri	*P. simulans* palmii	*P. hilairemontis*	*P. pellucida*	*P. fenestrata* "black"	*P. fenestrata* "yellow"
P. simulans simulans	0.48%						
P. simulans luggerii	0.64%	0.45%					
P. simulans palmii	0.47%	0.50%	0.36%				
P. hilairemontis	0.64%	0.98%	0.91%	N/A			
P. pellucida	0.34%	0.35%	0.26%	0.70%	0.00%		
P. fenestrata "black"	0.69%	0.66%	0.59%	0.67%	0.39%	0.38%	
P. fenestrata "yellow"	0.56%	0.60%	0.53%	0.57%	0.29%	0.29%	0.29%

Table 10. Average percent EF1a "p" - distance between *Paranthrene robiniae, Paranthrene dollii, Paranthrene oasis* **n. sp**. and*, Paranthrene gilaensis* **n.sp.** species and color forms.

	P. oasis n.sp	*P. gilaensis* n.sp	*P. robiniae* typical	*P. robiniae* palescens	*P. dollii*
P. oasis n.sp	0.00%				
P. gilaensis n.sp	0.29%	0.19%			
P. robiniae typical	0.29%	0.67%	0.00%		
P. robiniae palescens	0.19%	0.49%	0.19%	0.29%	
P. dollii	0.58%	0.47%	0.86%	0.78%	0.00%

Table 11. Average percent EF1a "p" - distance between *Paranthrene* species.

	P. asillipennis	*P. dollii*	*P. fenestrata*	*P. hilairemontis*	*P. pellucida*	*P. oasis* n.sp	*P. gilaensis* n.sp	*P. robiniae*	*P. simulans*	*P. sogaardi*	*P. tabaniformis*	*V. admiranda*
P. asillipennis	0.15%											
P. dollii	2.07%	0.00%										
P. fenestrata	1.27%	2.64%	0.32%									
P. hilairemontis	1.35%	3.07%	0.62%	NA								
P. pellucida	0.93%	2.26%	0.34%	0.70%	0.00%							
P. oasis n.sp	1.51%	0.58%	2.06%	1.99%	1.72%	0.00%						
P. gilaensis n.sp	1.83%	0.47%	2.35%	2.61%	2.12%	0.29%	0.19%					
P. robiniae	1.74%	0.81%	2.29%	2.33%	1.95%	0.23%	0.56%	0.22%				
P. simulans	1.28%	2.52%	0.60%	0.84%	0.32%	1.94%	2.34%	2.20%	0.48%			
P. sogaardi	2.90%	2.60%	3.18%	3.44%	2.83%	2.04%	2.40%	2.28%	3.17%	0.40%		
P. tabaniformis	2.06%	2.01%	2.55%	2.76%	2.29%	1.41%	1.80%	1.60%	2.40%	2.36%	0.42 %	
V. admiranda	5.95%	5.55%	5.69%	5.31%	5.53%	5.03%	5.17%	5.11%	5.62%	5.40%	4.48 %	3.44 %

Table 12. Average percent Wg "p" - distance between *Paranthrene simulans, Paranthrene pellucida, Paranthrene hilairemontis* and*, Paranthrene fenestrata* species and color forms.

	P. simulans simulans	*P. simulans* luggeri	*P. simulans* palmii	*P. hilairemontis*	*P. pellucida*	*P. fenestrata* "black"	*P. fenestrata* "yellow"
P. simulans simulans	0.43%						
P. simulans luggeri	0.89%	0.00%					
P. simulans palmii	0.52%	0.97%	0.70%				
P. hilairemontis	0.85%	0.78%	1.10%	NA			
P. pellucida	0.74%	1.04%	0.95%	1.35%	0.28%		
P. fenestrata "black"	0.75%	1.14%	1.06%	0.94%	1.18%	0.16%	
P. fenestrata "yellow"	0.77%	1.16%	1.08%	0.96%	1.20%	0.10%	0.12%

Table 13. Average percent Wg "p" - distance between *Paranthrene robiniae, Paranthrene dollii, Paranthrene oasis* **n. sp**. and*, Paranthrene gilaensis* **n.sp.** species and color forms.

	P. oasis n.sp	*P. gilaensis* n.sp	*P. robiniae* typical	*P. robiniae* palescens	*P. dollii*
P. oasis n.sp	0.00%				
P. gilaensis n.sp	2.72%	0.16%			
P. robiniae typical	2.62%	0.08%	0.00%		
P. robiniae palescens	2.62%	0.08%	0.00%	0.00%	
P. dollii	0.00%	0.00%	0.00%	0.00%	0.00%

Table 14. Average percent Wg "p" - distance between *Paranthrene* species.

	P. asillipennis	*P. dollii*	*P. fenestrata*	*P. hilairemontis*	*P. pellucida*	*P. oasis* n.sp	*P. gilaensis* n.sp	*P. robiniae*	*P. simulans*	*P. sogaardi*	*P. tabaniformis*	*V. admiranda*
P. asillipennis	0.36%											
P. dollii	0.00%	0.00%										
P. fenestrata	0.83%	0.00%	0.11%									
P. hilairemontis	1.23%	0.00%	0.95%	NA								
P. pellucida	1.69%	0.00%	1.19%	1.35%	0.28%							
P. oasis n.sp	3.98%	0.00%	3.50%	3.89%	3.93%	0.00%						
P. gilaensis n.sp	3.37%	0.00%	2.82%	3.64%	3.69%	2.72%	0.16%					
P. robiniae	3.24%	0.00%	2.79%	3.60%	3.68%	2.62%	0.08%	0.00%				
P. simulans	1.47%	0.00%	0.99%	0.95%	0.90%	3.76%	3.53%	3.51%	0.68%			
P. sogaardi	3.58%	0.00%	3.14%	4.02%		2.47%	2.31%	2.23%	3.85%	0.29%		
P. tabaniformis	2.32%	0.00%	1.85%	2.28%	2.70%	3.60%	2.89%	2.79%	2.51%	3.24%	0.20 %	
V. admiranda	7.51%	0.00%	7.08%	7.92%	7.34%	7.15%	6.58%	6.43%	7.71%	6.45%	6.85 %	0.48 %

CONCLUSION

Our phylogeny of North American *Paranthrene* does not demonstrate an association between color morphology and species boundaries. We hypothesize that other factors may be playing a role in speciation within the genus. *Paranthrene* demonstrates unique allopatric color polymorphism making them a potential model organism for the study of polymorphic color maintenance in Batesian mimicry.

Future work

This study was unable to evaluate all color morphologies for all North American species. More specifically the intraspecific relationships between all *P. dolli* and *P. robiniae* forms along with allopatric populations need further investigation. The phylogeny in Handfield and Handfield (2021) showed *P. dolli* rendering the perlucida and robiniae forms of *P. robiniae* as paraphyletic along with the single Robiniae anomaly recognized in chapter two demonstrate unresolved relationships within the species. In addition, observations of several *P. robiniae* specimens in the AJ Cook Arthropod Research Collection found unique, unnamed color morphologies of *P. robiniae* from the mountains of southern Arizona. A more comprehensive phylogeny of *P. dolli* forms and *P. robiniae* populations is still needed.

Polymorphic color maintenance in *Paranthrene* remains under explored. Our phylogeny did not demonstrate monophyletic clades of allopatric color forms. Selective pressure for these morphologies still need investigation. We recommend a comprehensive survey of the abundance of potential hymenopteran models and their forms along corresponding *Paranthrene* ranges.

Finally, phylogenetic analysis of Sesiidae centers on COI and a select few nuclear genes (Cognato et al, 2023; Lait and Herbert, 2018; McKern et al., 2008; Taft et al., 2016; Taft and Cognato, 2017; Handfield and Handfield,2021). These nuclear genes were relatively uninformative. In the multi-gene phylogenies of both chapters there was little support for some of the internal nodes. Exploration into other potentially informative genes is needed to help elucidate these interspecific relationships.

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