

Article

DNA Barcoding and Taxonomic Challenges in Describing New Putative Species: Examples from Sootywing and Cloudywing Butterflies (Lepidoptera: Hesperidae)

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Abstract: DNA barcoding has resulted in the ‘discovery’ of a vast number of new species and subspecies. Assigning formal scientific names to these taxa remains a major challenge. Names sometimes are newly designated. Alternatively, available valid names can be resurrected from synonymy, based on barcode analyses together with classical taxonomic characters. For the most part, however, new putative species revealed by barcoding studies go undescribed. This situation is most often attributed to insufficient taxonomic expertise with the authors conducting the study, together with a critical lack of formally trained taxonomists. However, even with formal training, and additional supportive data from morphological, ecological or life history characters, other factors can arise that impede new species descriptions. In the present paper, several specific taxonomic challenges that have arisen from barcode analyses in two groups of skipper butterflies (Lepidoptera: Hesperidae), the Sootywings (*Pholisora catullus* and *P. mejicanus*) and the Coyote Cloudywing (*Achalarus toxeus*) are highlighted and discussed. Both *P. catullus* and *A. toxeus* show relatively large intraspecific genetic divergences of barcodes (2–3%) which suggests the possibility of previously unrecognized cryptic speciation within each group. Some of the challenges to providing formal names and clarifying taxonomic status of these cryptic taxa could be largely overcome by (1) barcoding type specimens, (2) clarifying imprecise and often vague or suspect type localities, and (3) by conducting in-depth comparative studies on genitalic morphology.

Keywords: *Achalarus*; cryptic species; cytochrome *c* oxidase subunit I; haplotype network; *Pholisora*; species delimitation

1. Introduction

Formal taxonomic descriptions of newly discovered cryptic species are critical for understanding the extent of regional biodiversity and for informing management decisions for conservation [1–3]. Although not often the case, assigning a scientific name to a cryptic lineage revealed by DNA barcoding can be straightforward and relatively rapid. Examples from our own studies include using barcodes, together with previously overlooked differences in morphology, ecology or life history, to reinstate available names that had been synonymized with an earlier described species or subspecies [4–6]. In general, however, taxonomic studies resulting in new species descriptions lag far behind the vast number of cryptic lineages now being ‘discovered’ by barcoding. The most obvious reason for this backlog is a shortage of funding and scarcity of formally trained taxonomists to keep up with the workload generated by molecular studies, resulting in what has been called the ‘taxonomic impediment’ [2,7,8]. However, even with formal training, additional challenges in describing new species can sometimes arise. These include a lack of designated types and type localities, or a vague

description of the type locality, in the original description of an already recognized species in a given taxon, as well as a lack of DNA barcode data for type specimens. This information is sometimes critical for making decisions on whether a new name is necessary for a newly discovered taxon, or whether a previously applied name is available. For example, many early descriptions of New World butterflies were made from the eighteenth to the early twentieth century, and it was not uncommon to find that a type specimen and type locality were not designated, or if they were designated, that the specimen was subsequently lost or the location of the type locality was suspect [9]. A further obstacle is that extremely broad geographic regions were often named as type localities (e.g., ‘America’, ‘Mexico’, etc.). Also, designated syntypes were sometimes chosen from individuals collected over wide geographic areas which are now known to include more than a single taxon of the species in question.

In the present study, genetic diversity in a 658 base pair (bp) segment beginning near the 5' end of the mitochondrial cytochrome *c* oxidase subunit I gene (COI or *cox1*) [10] was analyzed in two groups of skipper butterflies (Lepidoptera: HesperIIDae), the Sootywings (Pyrginae: *Pholisora*) and Cloudywings (Eudaminae: *Achalarus*) based on both newly sequenced samples from southern Sonora, Mexico and sequences available in GenBank and the Barcode of Life Data System (BOLD) [11]. The limitations of using a single locus mitochondrial DNA marker in studies on molecular taxonomy and phylogeography have been discussed extensively [12,13], and possible introgressive hybridization, nuclear mitochondrial pseudogenes (numts) and contamination by symbiotic bacteria represent potential sources of error [14–20]. Barcodes alone, although not always successful in identifying species, have shown impressive success rates (>90%) in Lepidoptera [21–23], and have been particularly useful in providing important diagnostic characters to support the delimitation of new cryptic butterfly species [24–27]. Barcode analyses reported here reveal previously unreported cryptic diversity in both Sootywings and Cloudywings. Several challenges that arise when attempting to determine species boundaries and assign formal scientific names to the putative new taxa in these two groups of butterflies are discussed.

2. Materials and Methods

2.1. Samples

Most of the barcode sequences for the HesperIIDae analyzed here were taken from GenBank and BOLD databases (Table 1). New barcode sequences for *Pholisora catullus* (Fabricius) and *Achalarus toxeus* (Plötz) were obtained from specimens collected in southern Sonora at Guaymas/San Carlos and La Aduana near Alamos (Table 1). These specimens have been deposited in the insect collection at the Centro de Investigación en Alimentación y Desarrollo (CIAD), Guaymas, Sonora. Owing to occasional species misidentifications in both GenBank and BOLD [28], downloaded records were carefully checked, and if a particular species was clustered in a clade containing many individuals of a different species, it was omitted. Specimen photographs are often shown in BOLD to help verify identifications. Also, the original electropherograms are often included in BOLD, and can be compared with sequence files to correct for any suspect nucleotide calls [26,29].

Table 1. GenBank and BOLD COI barcode data for *Pholisora catullus*, *P. mejicanus* and *Achalarus* spp. analyzed in this study.

Species/Locality	GenBank	BOLD	Specimen ID	Date Collected	Hap	bp
<i>Pholisora catullus</i>						
MEX; Sonora; San Carlos	MH310828		CIAD 14–EP12	02 Oct. 2014	1a	658
MEX; Sonora; San Carlos	MH310832		CIAD 14–EP14	02 Oct. 2014	1i	658
MEX; Sonora; San Carlos	MH310833		CIAD 14–EP15	24 Sep. 2014	3	658
MEX; Sonora; San Carlos	MH310830		CIAD 14–EP16	24 Sep. 2014	1c	658
MEX; Sonora; San Carlos	MH310831		CIAD 14–EP17	06 Jul. 2014	1e	658
MEX; Sonora; San Carlos	MH310829		CIAD 14–EP18	10 Jul. 2014	1d	658
MEX; Sonora; La Aduana	MH310827		CIAD 16–EP69	02 Aug. 2016	1a	658
USA; AZ; Pima Co.		BBLOC352-11	BIOUG01455-F12	11 Aug. 2011	1a	658

Table 1. Cont.

Species/Locality	GenBank	BOLD	Specimen ID	Date Collected	Hap	bp
USA; AZ; Pima Co.		BBLOC561-11	BIOUG01457-H07	11 Aug. 2011	1b	658
USA; AZ; Pima Co.		BBLOC563-11	BIOUG01457-H09	11 Aug. 2011	1f	658
USA; AZ; Pima Co.		BBLOC562-11	BIOUG01457-H08	11 Aug. 2011	'1'	621 ^a
USA; AZ; Pima Co.		BBLOC564-11	BIOUG01457-H10	11 Aug. 2011	3	658
USA; AZ; Pima Co.		BBLOC816-11	BIOUG01466-D02	11 Aug. 2011	3	658
USA; AZ; Pima Co.		BBLOE1210-12	BIOUG01985-A02	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1211-12	BIOUG01985-A03	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1212-12	BIOUG01985-A04	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1215-12	BIOUG01985-A07	15 Aug. 2011	1h	658
USA; AZ; Pima Co.		BBLOE1217-12	BIOUG01985-A09	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1221-12	BIOUG01985-B01	15 Aug. 2011	1g	658
USA; AZ; Pima Co.		BBLOE1223-12	BIOUG01985-B03	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1225-12	BIOUG01985-B05	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1226-12	BIOUG01985-B06	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1227-12	BIOUG01985-B07	15 Aug. 2011	1j	658
USA; AZ; Pima Co.		BBLOE1228-12	BIOUG01985-B08	15 Aug. 2011	1a	658
USA; AZ; Pima Co.		BBLOE1760-12	BIOUG01990-G05	15 Aug. 2011	1k	658
USA; CA; San Diego Co. ^b	KP895741			11 Apr. 2008	2a	657
USA; CA; San Diego Co. ^b	KP895742			11 Apr. 2008	2a	657
USA; CA; San Diego Co. ^b	KP895743	GBMIN81625-17		11 Apr. 2008	2b	657
USA; CA; San Diego Co. ^b	KP895744	GBMIN81626-17		11 Apr. 2008	2a	657
USA; CA; San Diego Co.		LOCBF4342-15	BIOUG23748-F06	07 Apr. 2013	'2'	588 ^a
USA; CA; San Diego Co.		LOCBF1343-13	BIOUG06805-H01	07 Apr. 2013	'2'	624 ^a
USA; CA; San Diego Co.		LOCBF1344-13	BIOUG06805-H02	07 Apr. 2013	'2'	625 ^a
USA; CA; San Diego Co.		LOCBF1345-13	BIOUG06805-H03	07 Apr. 2013	'2'	625 ^a
USA; TX; Randall Co.		BBLOD820-11	BIOUG01568-D06	09 July 2011	3	658
<i>Pholisora mejicanus</i>						
USA; NM; Colfax Co.		LNAUU1796-15	USNM_ENT 01201719	07 Aug. 1989	4a	658
USA; NM; Colfax Co.		LNAUU1795-15	USNM_ENT 01201718	07 Aug. 1989	4b	658
USA; NM; Torrance Co.		NGSFT3796-16	USNM_ENT 01201720	10 Aug. 1946	'4'	658 ^a
USA; CO; Chaffee Co.		NGSFT3797-16	USNM_ENT 01201721	No date	'4'	208 ^a
<i>Achalarus toxeus</i>						
MEX; Sonora; San Carlos	KT290038		CIAD 14-EP11	08 Sep. 2014	A	658
MEX; Sonora; Alamos	KU041804		CIAD 15-EP27	03 Aug. 2015	A	658
MEX; Sonora; San Carlos	MH807206		CIAD 16-EP62	28 Mar. 2016	B	658
MEX; Sonora; San Carlos	MH807207		CIAD 16-EP63	04 Apr. 2016	B	658
MEX; Sonora; San Carlos	MH807208		CIAD 16-EP64	10 Apr. 2016	B	658
MEX; Sonora; San Carlos	MH807209		CIAD 16-EP65	17 Apr. 2016	B	658
MEX; Sonora; San Carlos	MH807210		CIAD 17-EP72	06 Apr. 2017	B	658
MEX; Sonora; San Carlos	MH807211		CIAD 17-EP73	08 Apr. 2017	B	658
MEX; Sonora; San Carlos	MH807212		CIAD 17-EP75	14 Apr. 2017	B	658
Costa Rica; ACG	GU149310		03-SRNP-237	20 Jan. 2003 ^c	CR	658
Costa Rica; ACG	GU149309		02-SRNP-10179	31 Mar. 2002	CR	658
Costa Rica; ACG	GU149308		02-SRNP-10180	19 Apr. 2002	CR	658
Costa Rica; ACG	DQ291763		96-SRNP-8690	09 Oct. 1996	CR	568
Costa Rica; ACG	DQ291762		93-SRNP-7539	14 Dec. 1993	CR	393
<i>Achalarus albociliatus</i>						
MEX; Sonora; Alamos	KU041805		CIAD 15-EP28	04 Aug. 2015		658
Costa Rica; ACG	JF761321		07-SRNP-12984	08 Jun. 2007		658
Costa Rica; ACG	GU149307		02-SRNP-4786	15 Apr. 2002		658
Costa Rica; ACG	JQ578250		06-SRNP-101470	07 Apr. 2006		658
<i>Achalarus jalapus</i>						
Costa Rica; ACG	GU666462		08-SRNP-22900	26 Sep. 2008		658
Costa Rica; ACG	GU649689		08-SRNP-23350	05 Nov. 2008		658
Costa Rica; ACG	HM885835		09-SRNP-20140	02 Mar. 2009		658
Costa Rica; ACG	JF754320		08-SRNP-30979	20 May 2008		658
Costa Rica; ACG	JF778550		08-SRNP-22673	22 Sep. 2008		658

^a Barcodes of *P. catullus* and *P. mejicanus* shorter than 657 bp that were not used for the haplotype network but were included in phylogenetic analyses; for these shorter sequences, numbers in quotation marks in the haplotype column (Hap) indicate the main haplotype group in which the sequence resolved on the Bayesian tree (see Figure 3b). Sequence NGSFT3796-16 (*P. mejicanus*) is listed at 658 bp in BOLD but has 94 missing sites (see Results and Discussion). Output files for BOLD sequences LOCBF1343-13, 44-13 and 45-13 were edited after reviewing the high-quality electropherograms. Two haplotypes (A and B) were found in *Achalarus* sp. cf. *toxews* (see text) from Mexico, with only a single highly divergent haplotype (CR) seen in three complete barcodes (658 bp); the CR haplotype was also inferred from the two shorter sequences of *A. toxews* from Costa Rica although they were both missing several diagnostic sites (see text). ^b from Pratt et al. [30]. ^c collection date of reared larva; other dates in Costa Rica are adult eclosion dates from reared larvae.

2.1.1. Sootywings

The Common Sootywing, *Pholisora catullus*, is widely distributed in the USA, southern Canada and throughout much of Mexico [31]. Larvae feed on a variety of weedy and garden plants in the family Amaranthaceae, including *Amaranthus*, *Chenopodium*, and various other related genera, and the butterfly is often found flying in disturbed habitats [32]. *Pholisora catullus* occurs throughout much of the state of Sonora, Mexico, especially along the coastal plain of the Gulf, where it is commonly encountered [33,34] (see Figure 1a–h). Potential host plants, *Amaranthus*, *Chenopodium* and *Chenopodiastrum*, are common near Guaymas [35], but specific host(s) remain to be determined.

A closely related species, the Mexican Sootywing *Pholisora mejicanus* (Reakirt), is also widely distributed in Mexico (except for the Baja California Peninsula and Sonora), but shows a more restricted distribution in the USA (Colorado, New Mexico, Arizona and Kansas) [31,34,36]. As with its congener, *P. mejicanus* can be found in open and disturbed areas, and larvae are known to feed on *Amaranthus* and *Chenopodium* [37]. *Pholisora mejicanus* is very similar to *P. catullus*, but can be distinguished by differences in morphology of male genitalia [38] and by the jet-black veins on the underside of the hindwings [31] (see Figure 1i).

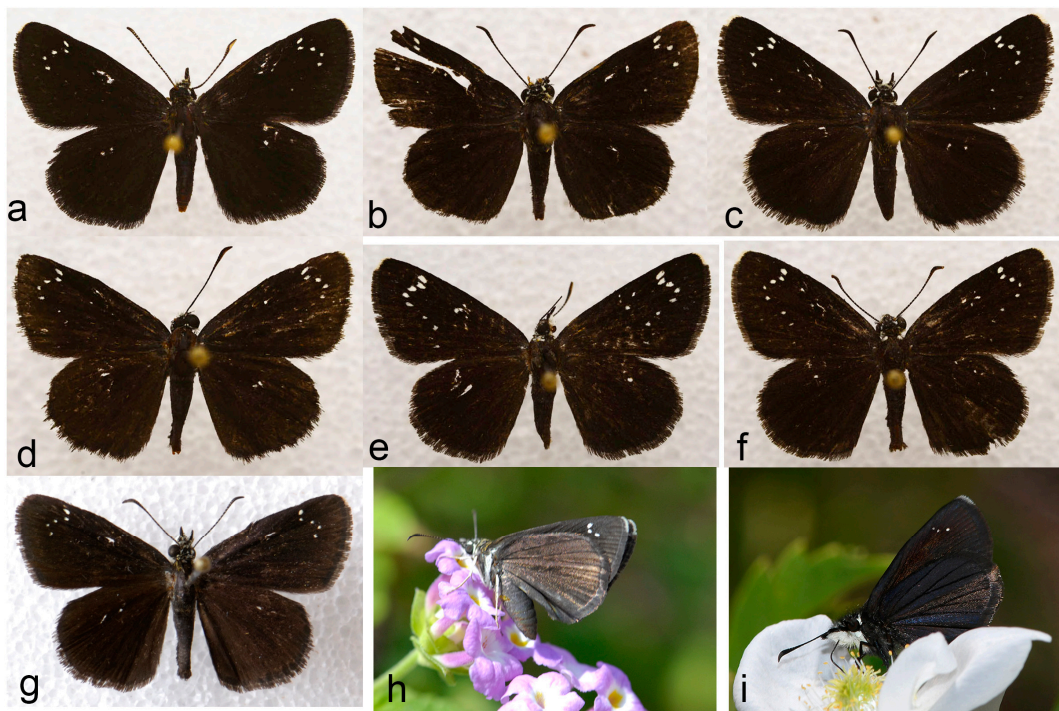


Figure 1. Photographs of *Pholisora catullus* samples from Sonora, Mexico used for barcoding (dorsal surface). Wingspans are given in mm. (a) CIAD 14–EP12, ♂, 27 mm; (b) CIAD 14–EP14, ♂, 24 mm; (c) CIAD 14–EP15, ♀, 27 mm; (d) CIAD 14–EP16, ♂, 23 mm; (e) CIAD 14–EP17, ♀, 26 mm; (f) CIAD 14–EP18, ♂, 24 mm; (g) CIAD 16–EP69, ♂, 27 mm. See Table 1 for collection data. All specimens are from COI haplotype group 1, except (c) CIAD 14–EP15 (haplotype group 3). The ventral surface of live adults (not barcoded) of *P. catullus* from Sonora, Mexico (h) and *P. mejicanus* from Torrance Co., New Mexico, USA (i); photo courtesy of Ken Kertell, shown to illustrate the diagnostic black veins on the hindwings of *P. mejicanus*.

2.1.2. Cloudywings

The Coyote Cloudywing, *Achalarus toxeus*, is broadly distributed in North America, found from west Mexico and southern Texas south to Panama [31]. *Achalarus toxeus* is morphologically very similar and extremely difficult to distinguish from two of its congeners that also occur in this region, *A. albociliatus* (Mabille) and *A. jalapus* (Plötz) [31,39–41]. The three species, however, can easily be

distinguished by their DNA barcodes and by genitalic examination of males [40]. In addition, males of *A. albociliatus* lack the costal fold present in the other two species [42]. Preliminary barcode studies indicated substantial genetic divergence (~3%) between populations of *A. toxeus* from Costa Rica and northwestern Mexico, suggesting that at least two cryptic species are presently assigned to this taxon [41]. The northwestern Mexico populations were provisionally referred to as *Achalarus* sp. cf. *toxus*. Barcode analysis from additional specimens recently obtained from southern Sonora, Mexico are analyzed here (Figure 2; Table 1) and provide additional insight into the findings obtained from the earlier study [41].

Host plants for larvae of *A. toxus* from Costa Rica are *Calliandra tergemina* and *Sphinga platylobo* (Fabaceae) [43]. Texas ebony (*Ebenopsis ebano* Fabaceae) is the larval foodplant in southern Texas [39]. The larval host(s) for *Achalarus* sp. cf. *toxus* in Sonora is unknown, but there are several species of fairy dusters (*Calliandra* spp.) in northwestern Mexico, in addition to various other species of Fabaceae, that might be used [44].

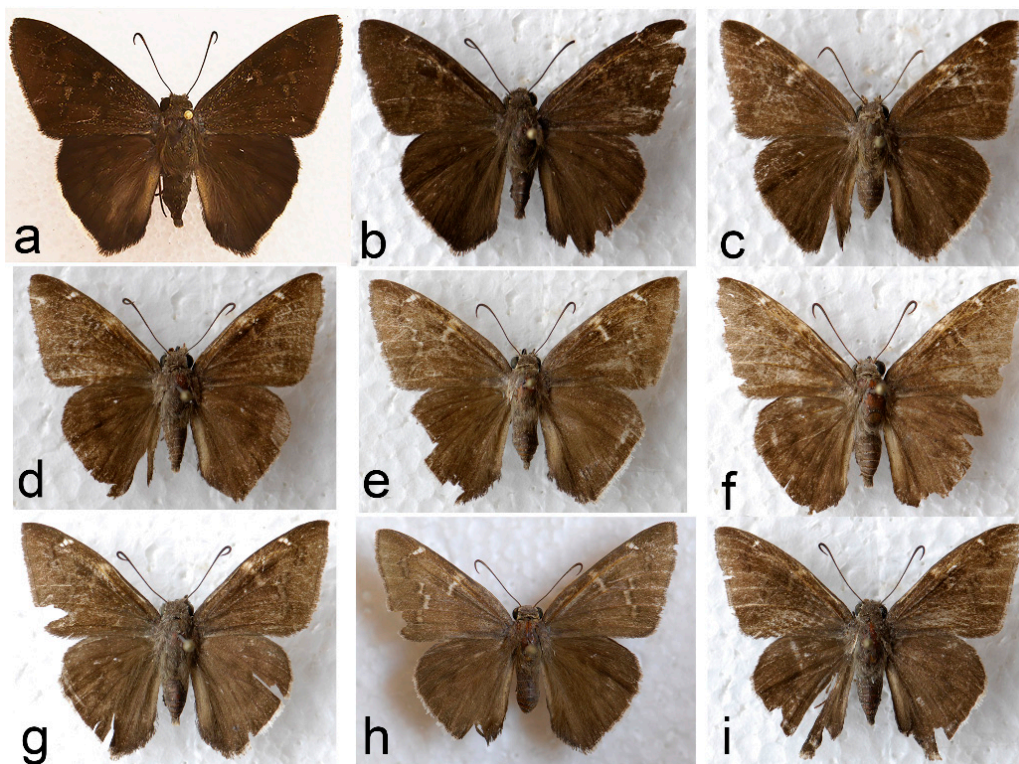


Figure 2. Photographs of *Achalarus* sp. cf. *toxus* samples from Sonora, Mexico (dorsal surface). Wingspans are given in mm. (a) CIAD 14–EP11, ♂, 47 mm; (b) CIAD 15–EP27, ♂, 44 mm; (c) CIAD 16–EP62, ♀, 41 mm; (d) CIAD 16–EP63, ♂, 39 mm; (e) CIAD 16–EP64, ♀, 46 mm; (f) CIAD 16–EP65, ♂, 40 mm; (g) CIAD 17–EP72, ♂, 41 mm; (h) CIAD 17–EP73, ♀, 47 mm; (i) CIAD 17–EP75, ♂, 44 mm. See Table 1 for collection data. Specimens (a,b) from a previous study [41] possess COI haplotype A; the remaining specimens show haplotype B (Table 1).

2.2. DNA Extraction and Sequence Analysis

Total genomic DNA was extracted from two legs of each butterfly using the DNeasy™ (QIAGEN Inc., Valencia, CA, USA) protocol. The polymerase chain reaction (PCR) was used to amplify the COI barcode segment [11] with primers LCO1490f and HCO2198r using standard PCR conditions [45]. Sequencing reactions were performed on an Applied Biosystems (Foster City, CA, USA) ABI 3730XL DNA sequencer at the Laboratorio Nacional de Genómica para la Biodiversidad (LANGEBIO) core DNA sequencing facility in Irapuato, Guanajuato, Mexico using the PCR primers. Translation of the gene segment in MEGA version 5.0.5 [46] revealed no frameshifts or stop codons. The content

of combined nucleotides CG ranged from 28.7% to 30.3%. These results suggest that the sequences for both species represent mtDNA, and are not numts, which have been reported for the COI gene in insects [15]. GenBank accession numbers for the new COI sequences for *P. catullus* and *A. toxeus* from Sonora are found in Table 1. The 658 bp COI segment corresponds to nucleotide positions 1516 to 2173 in the complete mitochondrial genome of the monarch butterfly, *Danaus plexippus* (GenBank KC836923) [47].

Haplotype networks of COI sequences in *Pholisora* were constructed using statistical parsimony implemented in TCS version 1.21 [48]. The first nucleotide was deleted in all 658 bp barcodes to match the four 657 bp sequences of *P. catullus* from California available in BOLD (see Table 1). The connection limit among haplotypes was set to a default value of 95%. Relationships among *Pholisora* sequences were also assessed with Bayesian inference implemented in MrBayes version 3.1 [49]. Bayesian analysis was conducted as described previously [41] after determining the most appropriate nucleotide substitution model. Outgroups were the hesperiids *Hesperopsis libya* (GenBank KP895740), *Staphylus ceos* (KY019902) and *Pyrgus communis* (AF170857). Clade support was estimated utilizing a Markov chain Monte Carlo (MCMC) algorithm and expressed as posterior probabilities. For barcodes from the divergent populations of *A. toxeus* from Sonora and Costa Rica, a median-joining haplotype network was constructed in POPART ver. 1.7 [50], and a Bayesian tree was constructed as described above using *P. communis* and *Urbanus proteus* (HM905345) as outgroups.

3. Results and Discussion

3.1. Sootywings

The TCS haplotype network of *Pholisora* barcodes (Figure 3a), which included new sequences of *P. catullus* from southern Sonora, Mexico ($n = 7$; Figure 1), together with barcodes for *P. catullus* and *P. mejicanus* sourced from GenBank and BOLD (total $n = 31$; Table 1), revealed a single network and four distinct haplotype groups (groups 1 to 4). Most ($n = 17$) of the sequences for *P. catullus* were obtained from a single locality (Catalina State Park near Tucson) in Pima County, Arizona. Figure 3a shows that all but two of the Arizona sequences, and all but one of the sequences from Sonora, resolved in haplotype group 1. The common haplotype of group 1 (haplotype 1a) was shared among Sonora and Arizona populations. The three outlier sequences, along with one from Texas, were identical (haplotype group 3) and were separated by ten mutational steps from haplotype group 1. Four GenBank sequences of *P. catullus* from southern California clustered in haplotype group 2, separated by a minimum of seven mutational steps from haplotype group 1. Two complete barcodes for *P. mejicanus* from BOLD resolved in haplotype group 4 and were separated from haplotype group 1 by a minimum of six mutational steps. Uncorrected mean genetic distance (p -distance) among the four haplotype groups ranged from 1.6 to 2.0%; minimum p -distance among groups ranged from 0.9 to 1.8% (inset in Figure 3a). Maximum p -distances within groups were 1.4% (group 1), 0.5% (group 2), 0.0% (group 3) and 0.2% (group 4). An additional 658 bp barcode for *P. mejicanus* was available in BOLD (NGSFT3796-16), but was not trimmed to 657 bp and used for the haplotype network because it was missing data at 94 of the 658 nucleotide sites (Table 1).

The Bayesian tree based on *Pholisora* barcodes (Figure 3b) generally showed the same resolution of the four haplotype groups as seen in the haplotype network. In addition, the sequence from *P. mejicanus* that was missing data (see above) clustered with the other two complete sequences from this species in haplotype group 4 (not shown). Statistical support for nodes was high for haplotype groups 2, 3 and 4, but low for group 1. The same resolution of clades and similar support values were found in a Maximum Parsimony tree constructed in MEGA using a matrix of uncorrected p -distances (not shown). Although only a single haplotype was found in the four individuals in group 3 with complete barcodes from Sonora, Arizona and Texas (Figure 3a), it is labeled as a haplotype 'group' based on the results of a query of a Sonora sequence (CIAD 14-EP15) in BOLD, which yielded a tree-based identification showing 19 additional sequences not publicly available, with a variety of presumed

haplotypes, from specimens collected over a wide geographic area (western Canada [British Columbia], western USA [Texas, Arizona, Colorado, Wyoming] and northeastern Mexico [Tamaulipas]), clustering in a single clade that included haplotype 3. Haplotype groups 1 and 3 of *P. catullus* were found sympatrically, and were flying on the same dates, both in southern Arizona (11 August 2011) and Sonora, Mexico (24 September 2014) (Table 1). Haplotype group 1 predominated in samples from both localities (16 of 18 individuals in Arizona and 6 of 7 individuals in Sonora) and to date has not been found elsewhere (Table 1).

The four haplotype groups of *Pholisora* also showed apparent fixed differences in single nucleotide polymorphisms (SNPs) (Table 2). *Pholisora mejicanus* (group 4) differs from the three groups of *P. catullus* at a single site (no. 43). A single SNP is diagnostic for group 1, and two diagnostic SNPs are seen in groups 2 and 3. Larger sample sizes of groups 2, 3 and 4, however, will be required to confirm whether these differences are robust.

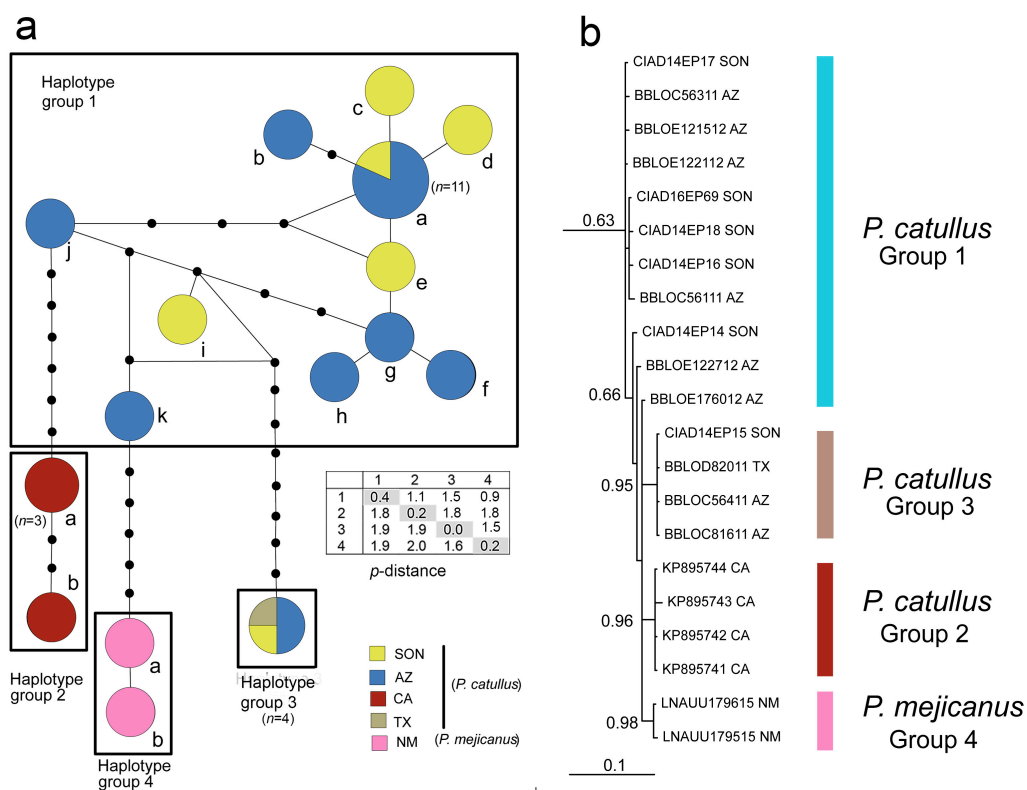


Figure 3. (a) TCS haplotype network of COI barcodes in *Pholisora* showing clustering of haplotypes into four groups. Different haplotypes in each group are identified with letters corresponding to those shown in Table 1. Numbers next to circles represent the number of individuals with that haplotype, if greater than one. Each line segment between haplotypes represents a single mutation. Inferred intermediate haplotypes that were not sampled are shown as black dots. Size of the circles is scaled approximately to haplotype frequency. State abbreviations: SON, Sonora (Mexico); AZ, Arizona; CA, California; TX, Texas; NM, New Mexico. Table inset shows mean pairwise *p*-distances (%) among groups 1 to 4 below the diagonal; shaded values shown along the diagonal are mean within-group *p*-distances; minimum pairwise *p*-distances among groups are shown above the diagonal. (b) Bayesian 50% majority rule consensus tree of barcode sequences showing clustering of the four COI haplotype groups (outgroups not shown). Clade support values (posterior probabilities) for the major clades are shown adjacent to branches. The scale represents expected substitutions per site.

Table 2. Variable nucleotide sites in the 658 bp COI barcode segment showing apparent fixed differences among samples of *Pholisora catullus* and *P. mejicanus*. For *P. mejicanus* at site 448, $n = 2$; for *P. catullus* at site 634, $n = 21$ for group 1 and $n = 4$ for group 2. Nucleotides shown in red are diagnostic for the haplotype group/species indicated. All substitutions are third codon position transitions, except at site 542 (first codon position).

Haplotype Group/Species	Nucleotide Site No.								
	43	58	271	355	367	418	448	542	634
1 <i>P. catullus</i> ($n = 22$)	A	T	C	T	C	T	T	C	C
2 <i>P. catullus</i> ($n = 8$)	A	C	C	C	C	C	T	C	T
3 <i>P. catullus</i> ($n = 4$)	A	C	T	T	T	C	C	T	C
4 <i>P. mejicanus</i> ($n = 3$)	G	C	T	T	T	T	T	C	C

Barcode analysis suggests that the taxonomy of *Pholisora* may not be as clear-cut as previously thought. *Pholisora catullus* and *P. mejicanus*, both currently recognized as valid taxa [9], show relatively small genetic divergences in COI barcodes (p -distance $\sim 2\%$; Figure 3a) and are connected in a single TCS haplotype network. In addition, *P. catullus* consists of at least three populations showing intraspecific divergences similar to the interspecific differences between *P. catullus* and *P. mejicanus*. Possible specimen misidentification of *P. mejicanus* available in BOLD is unlikely. The two complete (658 bp) barcodes of this species were high-quality electropherograms obtained from specimens collected by J.M. Burns, a taxonomic specialist on the HesperIIDae. The very short sequence (208 bp) of *P. mejicanus*, although not used for the haplotype network or the phylogenetic tree, clustered in haplotype group 4 in preliminary trees (not shown), and the specimen photograph in BOLD clearly showed the characteristic black veins on the ventral hindwing that distinguish this species.

The similarity in genetic divergences of COI barcodes among the four haplotype groups comprising two recognized species raises the possibility of three cryptic lineages of *Pholisora* in southwestern USA and northwestern Mexico that are currently placed in a single taxon, *P. catullus*. The taxonomic uncertainty that arises is whether to recognize the three cryptic lineages as distinct species or, alternatively, whether they should be named as subspecies of *P. catullus*. The latter option raises the possibility that *mejicanus* should also be placed as a subspecies of *P. catullus*. *Pholisora catullus* and *P. mejicanus*, however, have been collected flying together in central Colorado [51]. The observation that the two species are sympatric, together with differences in ventral hindwing vein color, genitalic differences, and barcode differences, suggest that they are indeed distinct species. To date, only a single subspecies of *P. catullus* has been formally proposed, *P. catullus crestar* J. Scott & Davenport, for localized populations from the southern Sierra Nevada of California [52]. However, in the revised online edition of 'A Catalogue of the Butterflies of the United States and Canada' [9] (see References for URL), the name *crestar* is placed as a junior subjective synonym of *P. catullus*. It should be added that barcodes were not reported for '*crestar*' [52] and thus it is unclear whether this population should be placed with haplotype 2 of *P. catullus* from San Diego County, California (Figure 3). The only reported diagnostic character of '*crestar*' was a submarginal line of small white spots on the dorsal hindwing, but this character is of limited taxonomic usefulness, being found at low frequency in other populations throughout the USA [52].

Confirmation of additional species-level (or subspecies-level) taxa currently assigned to *P. catullus*, and any change to the taxonomic status of *P. mejicanus*, will first require further molecular, morphological and ecological studies with larger sample sizes. Especially important will be thorough comparative studies of male genitalia in the different haplotype groups, given the importance of this character in separating *P. mejicanus* and *P. catullus*. In addition, barcodes from the type specimen of *P. catullus* could potentially provide important baseline data for future taxonomic studies [53,54]. However, the type(s) of *P. catullus* collected from a locality named 'Indiis' are probably lost [9]. Some have suggested that the type locality is 'probably Georgia' [32,55]. If a type locality can be fixed with reasonable certainty, neotypes could be selected, genitalia examined and barcodes determined and

compared with those presented here. Unfortunately, there are no published barcodes for specimens of *P. catullus* from the eastern USA, including Georgia, currently deposited in GenBank or BOLD.

3.2. Cloudywings

As mentioned earlier, a COI genetic divergence between populations of *Achalarus* sp. cf. *toxews* from Sonora (haplotype A, only) and *A. toxews* from Costa Rica of ~3% (p -distance = 2.9%; K2P distance = 3.0%) was found in a previous barcoding study [41]. A query of Sonora haplotype A in BOLD showed that it clustered with barcodes (not publicly available) found in three specimens from the state of Jalisco in western Mexico, with 99.85% sequence identity, and that an additional specimen from Jalisco and Sinaloa clustered with the Costa Rica haplotype CR, suggesting two distinct but sympatric cryptic species of *Achalarus toxews* are found in these two Mexican states [41]. Barcodes from the seven new specimens from San Carlos, Sonora possessed a unique haplotype (haplotype B) which differed at six nucleotide sites from Sonora haplotype A, as can be seen on a median-joining haplotype network (Figure 4a). The mean (and minimum) genetic divergence (p -distance) between haplotypes A and B was 0.9%. These results, as well as subtle color differences (Figure 2), suggest incipient speciation may be occurring among Sonora populations, but additional molecular, morphological and ecological studies on larger sample sizes of both haplotypes will be needed for confirmation. Here the combined Sonora sample is treated as a single undescribed species, *Achalarus* sp. cf. *toxews*. The mean (and minimum) p -distance between haplotype B and the Costa Rica population (haplotype CR) was 2.9%, the same result as between haplotype A and Costa Rica. Table 3 shows the 16 diagnostic barcode nucleotides that distinguish the Sonora (both A and B haplotypes) from Costa Rica samples of *A. toxews*. These diagnostic nucleotides represent important characters [24,27] for future taxonomic studies on this group.

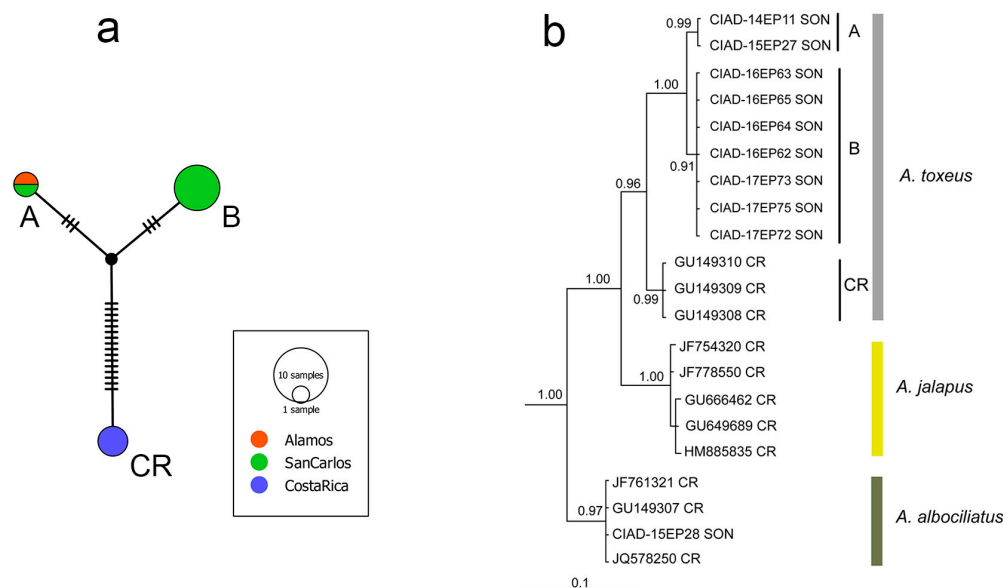


Figure 4. (a) Median-joining haplotype network of the 658 bp COI barcode segment in *Achalarus toxews* showing relationships among haplotypes A ($n = 2$) and B ($n = 7$) from Alamos and San Carlos, Sonora, and haplotype CR ($n = 3$) from the Area de Conservación Guanacaste (ACG), Costa Rica. Sizes of the circles are proportional to sample size. Each hatch mark on lines connecting haplotypes represents a single nucleotide substitution. The small black circle represents an inferred intermediate haplotype. (b) Bayesian 50% majority rule consensus tree of barcode sequences of the three haplotypes of *A. toxews* (A, B and CR), and the partitioning of clades of two other cryptic species in this complex, *A. jalapus* and *A. albociliatus* (see text). Clade support values (posterior probabilities) for the major clades are shown on branches. The scale represents expected substitutions per site. Abbreviations: SON, Sonora (Mexico); CR, Costa Rica.

Table 3. Diagnostic nucleotide differences in COI barcodes between Sonora (Son; haplotypes A and B; shaded) and Costa Rica (CR) specimens of *Achalarus toxeus*.

Haplotype	Nucleotide Site No.															
	46	79	205	212	217	277	283	313	407	415	421	472	490	508	574	628
Son A (<i>n</i> = 2)	C	A	T	C	A	C	T	C	C	G	C	C	C	T	C	G
Son B (<i>n</i> = 7)	C	A	T	C	A	C	T	C	C	G	C	C	C	T	C	G
CR (<i>n</i> = 3)	T	T	C	T	G	T	C	T	T	A	T	T	T	C	T	A
CR/DQ291763 *	T	T	C	T	G	T	C	T	T	A	T	T	T	C	–	–
CR/DQ291762 *	–	–	–	–	–	T	C	T	T	A	T	T	T	C	T	A

Nucleotide sites 212 and 407 are 1st codon position substitutions; the remainder are 3rd position. * Single barcodes < 658 bp from Costa Rica/GenBank No. (see Table 1).

The Bayesian tree based on barcodes from *A. toxeus* from Sonora and Costa Rica (Figure 4b) showed the same resolution of the three haplotypes (A, B and CR) as seen in the haplotype network. In addition, the partitioning of distinct clades of two other cryptic species of *Achalarus* mentioned earlier, *A. jalapus* and *A. albociliatus*, are included for comparison. The same resolution of *Achalarus* clades, and high clade-support values, were also found in a Maximum Parsimony tree (not shown).

Determining a formal scientific name to apply to *Achalarus* sp. cf. *toxeus* is especially challenging given the imprecise type locality information and brief species diagnoses in the original descriptions of *Aethilla* (= *Achalarus*) *toxeus* [56] and *Murgaria* (= *Achalarus*) *albociliata* var. *nigrociliata* [57], the latter now placed as a junior synonym of *A. toxeus* [9]. The type locality of *A. toxeus* was listed only as ‘Mexico’, without further locality details [56]. The type locality of *A. nigrociliata* was also not specific, listed only as ‘Mexique’ (Mexico) [57]. A specific type locality, however, was given in the description of *Eudamus* (= *Achalarus*) *coyote* (TL: ‘Southern Texas’) [58], a name also currently placed as a synonym of *A. toxeus* [9]. Later, the type locality of *coyote* was further restricted to Aaron, near Corpus Christi, Texas [59]. Thus, of the two potentially available names for newly discovered species in the *Achalarus toxeus* complex, only *A. coyote* is accompanied by a specific type-locality. A large data set of genitalic dissections from *A. toxeus* collected by other workers from throughout Mexico is now available [60], and these data, together with planned barcode analyses of these additional specimens, will hopefully be able to clarify which, if any, of the available names should be applied to new species of the *A. toxeus* complex from North America. It is also worth noting that the type specimen of *A. toxeus*, deposited in the Berlin Natural History Museum in Germany, is a female [42]. There is, however, no superficial character to reliably separate females of *A. toxeus* from *A. albociliatus* (or from *A. jalapus*) [42]. Because a COI barcode is not available for the type specimen, it is possible that Plötz [56] may have examined any one of the several cryptic species of *Achalarus* found in Mexico in his description of *A. toxeus* in 1882. This uncertainty both highlights the utility of determining barcodes for historical type material [53,54] and the challenges encountered in formally naming new cryptic species revealed by DNA barcoding.

4. Concluding Remarks

Although the precise number of cryptic species across animal taxa is unknown [61], there are probably thousands of species that have been delimited by molecular data alone, but that have not been formally described [62]. In butterflies, an early and now classic, but controversial, example of the underestimation of biodiversity by barcoding is the highly cited (presently 2685 citations returned by Google Scholar) ‘ten species in one’ paper on the hesperiid *Astraptes fulgerator* (Eudaminae) [63]. Because the ten species of *A. fulgerator* were inferred from their COI genetic divergences, but not formally described, that paper also highlights several of the taxonomic challenges in cryptic taxa that I have presented here. Several years after ‘ten species in one’ appeared, Brower [24] provided formal names for each species based on COI diagnoses alone. Although not considered ideal [24], at least each putative taxon now had a scientific name associated with it. However, as with *Achalarus toxeus*, several names had been previously proposed for members of the *Astraptes fulgerator* group, and it was uncertain if any of the newly proposed names would eventually end up as synonyms. I have chosen a

more conservative approach here by not providing a formal scientific name to *Achalarus* sp. cf. *toxus*, but with the disadvantage that this name is also less than ideal. Clearly, much work remains to be done before taxonomic impediments to providing valid scientific names to the ever-increasing number of cryptic species can be overcome.

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