Polyphyly in Urbanus and Astraptes (Hesperiidae: Eudaminae) Assessed Using Mitochondrial DNA Barcodes, with a Reinstated Status Proposed for Achalarus

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ABSTRACT. Phylogenetic analysis of mitochondrial DNA (cytochrome c oxidase subunit I; COI) barcode sequences is shown here to be an effective tool to aid in assessing polyphyly in selected genera of the subfamily Eudaminae (Hesperiidae). Results of phylogenetic analyses using both Bayesian inference and the neighbor-joining (NJ) algorithm were largely congruent and confirm and considerably expand on results from previous studies based on morphology and multilocus genetic data suggesting that the genera *Urbanus* and *Astraptes* consist of polyphyletic taxa as currently circumscribed. The genera *Narcosius* and *Ridens*, closely-related to *Urbanus* and *Astraptes*, emerged as monophyletic, although barcodes were available for only several species from each genus. Amino acid composition of the barcode segment in these and other closely allied genera was also useful in assessing polyphyly. Based on an analysis of barcodes, external morphology and the original description, a reinstated status is proposed for *Achalarus jalapus* (Plötz, 1881). We suggest that the phylogenetic barcode approach presented here to identify probable misplaced taxa can be used successfully in other taxa of the Eudaminae as well as in other subfamilies of Lepidoptera.

Additional key words: barcode amino acids; Bayesian inference; cytochrome c oxidase subunit I; neighbor-joining analysis; phylogenetic signal; *Thessia*

Since its inception more than a decade ago, the DNA barcode initiative has resulted in an extensive database of nucleotide sequence data with the expressed goal of providing a "reliable, cost-effective and accessible" means to identify known animal species and aid the discovery of new ones (Hebert et al. 2003, Hebert & Gregory 2005, Ratnasingham & Hebert 2007). The use of DNA barcodes, consisting of an approximately 650 base pair (bp) fragment beginning near the 5' end of the mitochondrial cytochrome c oxidase subunit I gene (COI or *cox1*), for species identification and discovery has also generated much controversy, with numerous authors pointing out its limitations (e.g., Brower 2006, Taylor & Harris 2012, Kwong et al. 2012, Kvist 2013). In particular, specific problems related to molecular taxonomy and phylogeography of the Lepidoptera that arise from using single-locus genetic data, including introgressive hybridization, incomplete lineage sorting, presence of symbiotic bacteria, and limited geographic sampling, have been discussed in detail (Forister et al. 2008, Zakharov et al. 2009, Kodandaramaiah et al. 2013). While caution clearly must be exercised when interpreting results based on COI barcodes, the barcode database is continuously growing (Ratnasingham & Hebert 2007, Zahiri et al. 2014) and thus represents a rich and unprecedented source of genetic information, much of which is publicly available.

Our goal in the present paper is to test whether barcodes could also be used to assess polyphyly in selected taxa of the Hesperiidae. Although higher level phylogenetic relationships are now typically assessed using large multilocus datasets (Lin and Danforth 2004, Regier et al. 2013, Wahlberg et al. 2013, McCormack et al. 2013), the single locus mitochondrial COI barcode segment shows substantial phylogenetic signal in Lepidoptera below the family level (Wilson 2011). Specifically, barcodes have proven their utility in interspecific phylogenetic studies in lasiocampid pine moths, *Dentrolimus* (Dai et al. 2012), noctuid moths, *Lasionycta* (Zahiri et al. 2014), nymphalid butterflies, *Junonia* (Pfeiler et al. 2012a, Gemmel and Marcus 2015) and *Hermeuptychia* (Seraphim et al. 2014), as well as in grasshoppers, Orthoptera (Huang et al. 2013) and caddisflies, Trichoptera (Boyle & Adamowicz 2015).

We focused our analysis on the hesperiid genera *Urbanus* and *Astraptes*, and their close relatives *Achalarus*, *Thessia*, *Ridens* and *Narcosius*, of the subfamily Eudaminae (previously a tribe [Eudamini] of the subfamily Pyrginae, recently elevated to a subfamily [Warren et al. 2009]). We chose this group of genera for two reasons. Firstly, detailed morphological analyses had previously suggested that *Urbanus* and *Astraptes* each consisted of a polyphyletic group of taxa in need of taxonomic revision (Steinhauser 1981, 1986, 1987). In particular, Steinhauser (1981) pointed out that some species of *Astraptes* appear to be more closely related to the so-called "*proteus* group" of *Urbanus* than some of the brown-bodied species assigned to *Urbanus*. The "*proteus* group" currently comprises about 18 species that show little variation in morphology and are characterized by green, blue or turquoise bodies and brown tails (Steinhauser 1981, Bertrand et al. 2014). Overall, the genus *Urbanus* consists of about 37 species as currently circumscribed (Mielke 2005, Bertrand et al. 2014). The genus *Astraptes* consists of about 38 species (Brower 2009), which includes the "ten species in one" proposed for *A. fulgerator* (Hebert et al. 2004, Brower 2010). Secondly, a comprehensive molecular phylogenetic study of the Hesperiidae based on maximum parsimony (MP) analysis of combined mitochondrial and nuclear genes showed that the two species of *Urbanus* analyzed, *U. dorantes* and *U. simplicius*, did not emerge as sister species (Warren et al. 2008), consistent with the earlier views of Steinhauser that the genus is polyphyletic. Most of the approximately 200 genera analyzed by Warren et al (2008), however, were represented by single species, including *Astraptes fulgerator*, *Narcosius colossus*, *Achalarus albociliatus*, *Autochton longipennis* and *Thorybes pylades* which were shown to be closely related to *U. dorantes* and *U. simplicius*. Thus, relationships among most species of *Urbanus* and *Astraptes*, and taxa in the other genera mentioned above, remain unclear. Complete (658 bp) COI barcode data are presently available in GenBank for a variety of nominal species of *Urbanus* (*n* = 16, nine of which are from the "*proteus* group") and *Astraptes* (*n* = 15). These sequences, together with those from closely allied genera, allowed us to assess in more detail whether barcodes could aid in assessing polyphyly in the Eudaminae and reveal taxa in need of taxonomic revision.

MATERIALS AND METHODS

Taxon sampling. Most of the barcode sequences examined here were taken from GenBank, primarily from the long-term biodiversity study of the butterfly fauna of Area de Conservación Guanacaste (ACG), Guanacaste Province, Costa Rica (Janzen et al. 2011). We also included new sequences for *Achalarus toxeus*, *A. albociliatus*, *Urbanus procne* and *U. dorantes* collected at coastal sites on the Gulf of California near Guaymas (27.961° N, 110.988° W) (Pfeiler et al. 2012b, 2016) and at Alamos (27.017° N, 108.947° W) in southern Sonora, Mexico. Links to additional information on collection data and adult photographs (where available) for voucher specimens deposited in the Barcode of Life Data Systems (BOLD) (Ratnasingham & Hebert 2007) and the ACG database (Janzen & Hallwachs 2009) can be found in GenBank under the accession numbers provided on Fig. 1. In most cases, we have omitted subspecies designations because of their inconsistent use in GenBank and BOLD.

DNA extraction and data analysis. Methods for extracting DNA from butterfly legs, and for amplifying and sequencing the 658 bp barcode segment are found in Pfeiler et al. (2012a). GenBank accession numbers for the new COI sequences obtained for taxa from Sonora, Mexico are KT290034-KT290038 and KU041804-KU041805.

Alignments of all sequences were easily conducted by eye. Base composition showed little variation among sequences, with CG content averaging 29.5% (*n* = 69). Aligned sequences were translated in MEGA version 5.0.5 (Tamura et al. 2011). No gaps or stop codons were found. Together these results suggest that the barcode sequences analyzed represent mitochondrial DNA (mtDNA), and are not nuclear mitochondrial pseudogenes (numts) which have been reported for the COI gene in insects (Song et al. 2008). Calculations of Kimura (1980) 2-parameter (K2P) genetic distances (*d*) and uncorrected *p*-distances were carried out in MEGA. Although the use of K2P distances in barcode studies has been criticized (Srivathsan & Meier 2012), we found that *p*-distances and K2P distances were similar, or identical, in most of the taxa examined, consistent with results from previous studies in other closely-related congeneric arthropod taxa reported by us (Pfeiler et al. 2009, 2010) and others (Hamilton et al. 2014).

Phylogenetic relationships among 45 ingroup taxa of the Eudaminae were assessed using Bayesian inference implemented in MrBayes version 3.1 (Huelsenbeck & Ronquist 2001). The model of nucleotide substitution that best fit the data set, determined with Modeltest 3.7 (Posada & Crandall 1998) using the Akaike Information Criterion, was GTR + G. Bayesian analyses were run under the parameters of this model for 1,000,000 generations, sampled every 250th generation (4,000 trees sampled), using the default random tree option to begin the analysis. Clade support for Bayesian trees was estimated utilizing a Markov chain Monte Carlo

(MCMC) algorithm and expressed as posterior probabilities. We also examined relationships using the neighbor-joining (NJ) algorithm of Saitou and Nei (1987) carried out in MEGA using a matrix of K2P distances. The NJ analysis provides a rapid assessment of relationships and is the clustering method used to identify taxa from barcode data deposited in BOLD (Ratnasingham & Hebert 2007). Statistical support for nodes was obtained by bootstrap analyses using 1000 pseudoreplicates (Felsenstein 1985). We chose *Polythrix asine*, a species of Eudaminae which was not closely related to the ingroup taxa of interest (*d* >12.0%; *p*-distance >11.0%), and *Pyrgus communis* (Pyrginae) as outgroups.

Results and Discussion

Phylogenetic analysis of barcodes in selected genera of the Eudaminae yielded five highly-supported clades (labeled clades 1, 2, 3*a*, 3*b*, and 4) in the Bayesian tree (Fig. 1). Clade 3*b* was further subdivided in two moderately supported subclades 3*b** and 3*b***. Of the five main clades, only two were monophyletic, clade 2 (*Narcosius*) and clade 4 (*Ridens*). The genera *Urbanus* and *Astraptes* were shown to be polyphyletic as predicted from morphological studies (Steinhauser 1981, 1986, 1987). *Urbanus* species resolved in three separate clades, 1*a*, 1*b* and 3*b**. *Astraptes* species clustered mainly in clades 3*a* and subclade 3*b***. With the exception of the inclusion of *Astraptes tucuti*, subclade 3*b** was comprised entirely of the "*proteus* group" of *Urbanus*. External morphology of *A. tucuti* is similar to its congeners resolving in clades 3*a* and 3*b*** and its clustering with the "*proteus* group" of *Urbanus* may reflect either limits on the phylogenetic resolution of barcodes, or possibly mitochondrial introgression from a species of *Urbanus*. Phylogenetic analyses, and amino acid composition of barcodes (Table 1), suggest that that subclades 3*b** and 3*b*** are very closely related (net *d* = 2.8%). It is also noteworthy that two additional species of *Astraptes* did not cluster with their congeners in clades 3*a* and 3*b***. *Astraptes phalaecus* resolved in clade 1*a* and *A. egregius* was sister to *U. dorantes* in clade 1*b* (discussed further below).

Clade 3*a*, comprised of *A. aulus, A. janeira* and *A. enotrus*, resolved basally to subclades 3*b** and 3*b*** and was highly supported in both Bayesian (posterior probability = 1.00; Fig. 1) and NJ (96% bootstrap support) phylogenetic trees. The interior branch labeled clade 3 on Fig.1, however, was poorly supported in both trees (<0.75 posterior probability and 50% bootstrap support, respectively). Barcode amino acid composition of the three species of *Astraptes* resolving in clade 3*a* also showed an apparent fixed substitution at position 71

compared with subclades 3*b** and 3*b*** (Table 1; discussed further below).

Brown-bodied *Urbanus* (*U. dorantes, U. procne, U. simplicius, U. teleus, U. tanna, U. doryssus* and *U. albimargo*) clustered in clade 1(1*a* and 1*b*) along with a number of species from other genera, including *Astraptes* (as mentioned above), *Thorybes*, *Achalarus*, *Thessia* and *Autochton*. The same resolution of the five clades found in the Bayesian tree, as well as the partitioning of taxa within clades, also was seen in the NJ tree (not shown).

Clustering of *U. simplicius, U. dorantes, Thorybes pylades, Achalarus albociliatus* and *Autochton longipennis* in clade 85 of the MP tree of Warren et al. (2008) was also recovered in clade 1 of our Bayesian tree and NJ trees based on barcodes alone, although *Autochton zarex* (GenBank JF753725) was substituted for *A. longipennis* because only one partial barcode (387 bp) was available in GenBank. Also consistent with the results of Warren et al. (2008) was that *U. simplicius* and *U. dorantes* were not sister species in our trees, and *Narcosius colossus* and *Astraptes fulgerator* resolved outside of clade 1. In Warren et al. (2008), *N. colossus* and *A. fulgerator* clustered as sister species in a separate clade (clade 81) basal to clade 85. Finding similar results in the two studies is noteworthy given that Warren et al. (2008) used genetic data from nuclear genes together with a segment of the mitochondrial COI gene that did not overlap the 658 bp barcode segment. Overall, our barcode analyses provide clear support for Steinhauser's (1981) view that some species of *Astraptes* appear more closely related to the "*proteus* group" of *Urbanus* than do some of the brown-bodied species of *Urbanus*.

Barcode amino acid composition, based on examination of more than 1880 sequences from 45 species (Table 1), provided additional characters that support the polyphyly of *Urbanus* and *Astraptes*. All nine species of the "*proteus* group" of *Urbanus* resolving in subclade 3*b** (including *A. tucuti*), in addition to the nine species of *Astraptes* in subclade 3*b***, the sister clade to the "*proteus* group", shared the same amino acid substitution at site 71 (methionine) in the 219 amino acid barcode segment, a substitution not seen in any of the other clades, including clade 3*a* which contained three additional species of *Astraptes* (Table 1). All species from the various genera resolving in clades 1, 2, 3*a* and 4, including five species of *Astraptes* and seven species of *Urbanus*, show a leucine at site 71. The five species of *Ridens* shared a threonine at site 118 whereas all other species analyzed possessed a serine at this site, with the only exception being an alanine found in *U. tanna* (Table 1). All four species of *Narcosius* (clade 2) showed an invariant amino acid profile at sites 67, 71, 118 and 169
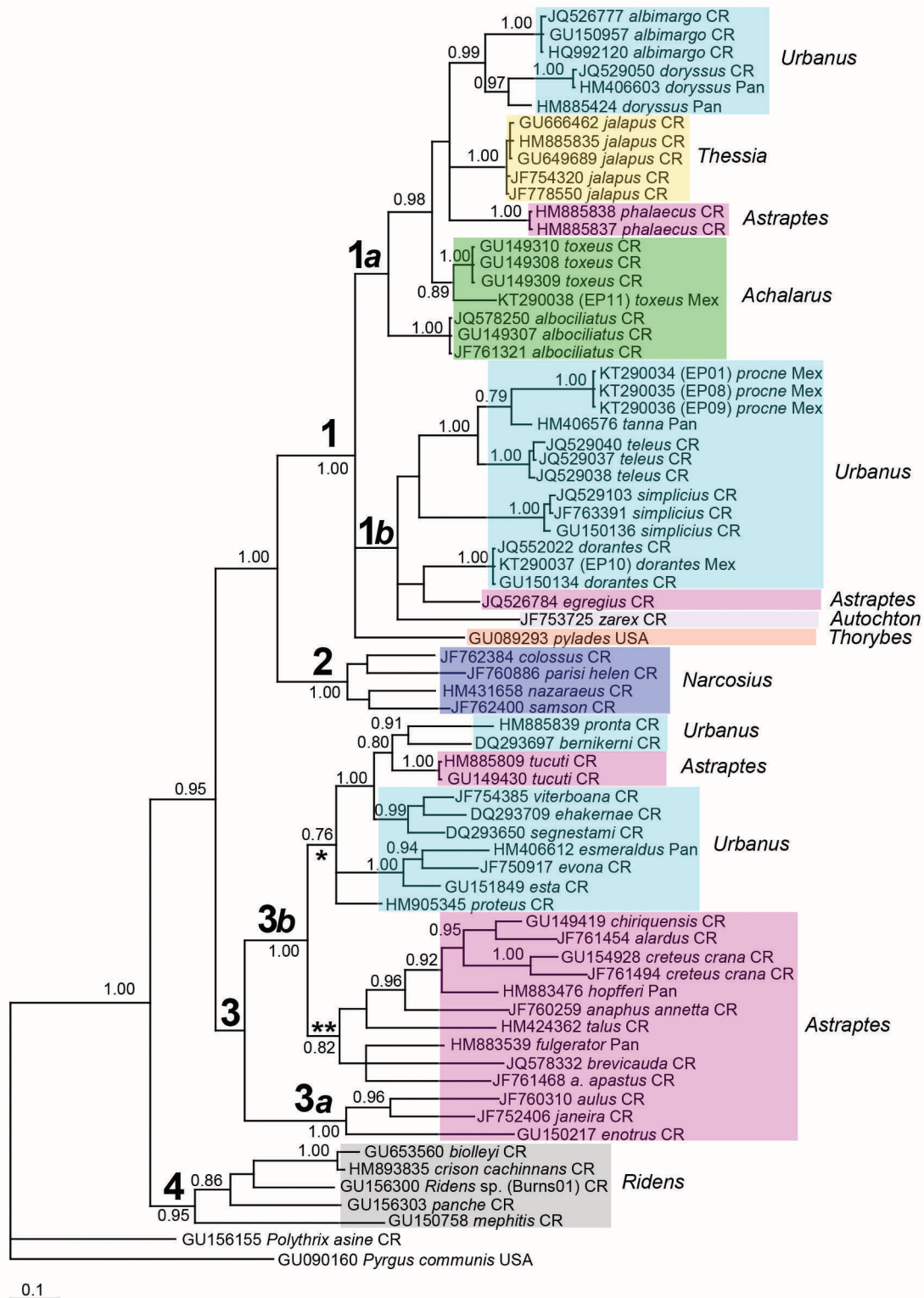
FIG. 1. Bayesian 50% majority rule consensus tree showing relationships among closely–related taxa of the Eudaminae based on COI barcode sequences. GenBank accession numbers are given for each sequence. Locality abbreviations: CR, Costa Rica; Pan, Panama; Mex, Mexico. Clade support values (posterior probabilities) are shown on branches; values <0.75 were omitted. Numbering of the main clades discussed in the text are shown in large bold type. The hesperiids *Pyrgus communis* (Pyrginae) and *Polythrix asine* (also in the Eudaminae but not closely related to the taxa of interest) were used as outgroups. The scale represents expected substitutions per site.

TABLE 1. Informative amino acid characters in the COI barcode protein segment (219 amino acids) in 45 species of the Eudaminae. Clades correspond to those in Fig. 1.

Species/Clade	N	67	71	118	169
Clade 1					
Thorybes pylades	1	I	L	S	I
1a					
Urbanus albimargo	7	I	L	S	I
Urbanus doryssus	81	V/I	L	S	I
Thessia jalapus	26	I	L	S	I
Astraptes phalaecus	3	I	L	S	I
Achalarus toxeus [a]	6	I	L	S	I
Achalarus albociliatus	10	V	L	S	I
1b					
Urbanus procne	3	V	L	S	I
Urbanus tanna	1	V	L	A	I
Urbanus teleus	13	I	L	S	I
Urbanus simplicius	18	V	L	S	I
Urbanus dorantes	20	V	L	S	I
Astraptes egregius	12	V	L	S	I
Autochton zarex	13	V	L	S	I
Clade 2					
Narcosius colossus	32	V	L	S	I
Narcosius parisi helen	8	V	L	S	I
Narcosius nazareus	1	V	L	S	I
Narcosius samson	29	V	L	S	I
Clade 3					
3a					
Astraptes aulus	5	V	L	S	I
Astraptes janeira	16	V	L	S	V
Astraptes enotrus	100	V	L	S	V

Species/Clade	N	67	71	118	169
3b*					
Urbanus pronta	21	V	M	S	V
Urbanus bernikerni [b]	68	V	M	S	V
Astraptes tucuti	23	V	M	S	V
Urbanus viterboana	16	V	M	S	V
Urbanus ehakernae [b]	31	V	M	S	V
Urbanus segnestami [b]	177	V	M	S	V
Urbanus esmeraldus	134	V	M	S	V
Urbanus evona	18	V	M	S	V
Urbanus esta	78	V	M	S	V
Urbanus proteus	150	V	M	S	V
3b**					
Astraptes chiriquensis	7	V	M	S	V
Astraptes alardus	30	V	M	S	V
Astraptes creteus crana DHJ01	14	V	M	S	V
Astraptes creteus crana DHJ02	82	V	M	S	I
Astraptes hopfferi	69	V	M	S	V
Astraptes anaphus annetta	122	V	M	S	V
Astraptes talus	25	V	M	S	V
Astraptes fulgerator	243	V	M	S	V
Astraptes brevicauda	22	V	M	S	V
Astraptes a. apastus	3	V	M	S	V
Clade 4					
Ridens biolleyi	11	V	L	T	V
Ridens crison cachinnans	6	V	L	T	V
Ridens panche	55	V	L	T	V
Ridens mephitis	49	V	L	T	I
Ridens sp. (Burns01)	13	V	L	T	I
Total	1872				

N, number of barcodes analyzed; *N* values for *Urbanus proteus* and *Astraptes fulgerator* are each subsamples of >400 available sequences. *Astraptes fulgerator* is now considered a complex of species-level taxa (Hebert et al. 2004; Brower 2010); all members of the complex were examined and included under *A. fulgerator*. Amino acid abbreviations: A, alanine; I, isoleucine; L, leucine; M, methionine; S, serine; T, threonine; V, valine. Partitioning of taxa of *Astraptes* and *Urbanus* among clades, and amino acid composition at sites 67, 71, 118 and 169 of all species, are highlighted.

[a] Includes *Achalarus* sp. cf. *toxeus* (EP11 and EP27);

[b] *Urbanus segnestami*, *U. bernikerni*, and *U. ehakernae*, previously *U. belli* DHJ01, –02, and –03 (Bertrand et al. 2014).

(VLSI) which was identical to that seen in *A. aulus* (clade 3*a*), most of the species in clade 1*b*, *Achalarus albociliatus*, and 30 of the 81 *U. doryssus* from clade 1*a* (labeled *U. doryssus* DHJ01 in Janzen et al. 2011).

It is important to point out that the amino acid compositions at the four sites shown in Table 1 were informative for evaluating relationships of the ingroup taxa analyzed here, but are not diagnostic when considering the entire Eudaminae. For example, we randomly chose several additional taxa outside of our ingroup and found that the amino acid pattern VLSI was also present in *Chioides zilpa* and *Proteides mercurius*. Likewise, VLTV, a pattern characteristic of three species of *Ridens* (clade 4) was also present in *Codatractus imalena, C. melon, C. carlos, C. alcaeus*.

**Narcosius and Ridens.** The genus *Narcosius* was erected by Steinhauser (1986) to include nine similar species of the Eudaminae, five of which had previously been placed in *Astraptes* (Evans 1952). Subsequently, Austin (1996) described three additional species from western Brazil. Of these 12 species, barcodes were available for four, *N. parisi helen, N. colossus, N. nazaraeus* and *N. samson*. Adults of the four species are essentially indistinguishable (see Fig. 4 in Janzen et al. 2011) but can be separated by genitalia, caterpillar facies, foodplant choice and/or DNA barcodes (Steinhauser 1986; Austin 1996; Janzen et al. 2011). Phylogenetic analyses of barcodes (Fig. 1) resolved these four species in a highly supported monophyletic clade (clade 2), supporting the taxonomic arrangement proposed by Steinhauser (1986) based mainly on genitalic morphology. Additionally, our analyses indicated that *N. samson* and *N. nazaraeus* resolved as sister species, also in agreement with Steinhauser (1986). Although *N. samson* and *N. nazaraeus* show the most similarities in genitalic morphology, most of the

remaining species of *Narcosius*, including *N. parisi helen* and *N. colossus* show substantial differences, especially in the male valvae (Steinhauser 1986; Austin 1996). Because we were limited in our analysis to only four of the 12 nominal species of *Narcosius*, and five of the approximately 20 nominal species of the genus *Ridens* (Evans 1952; Steinhauser 1983), additional molecular studies on the missing taxa, which includes the type species *Narcosius narcosius* and *Ridens ridens*, will be required to confirm the monophyly of both genera.

Clade 1 *Urbanus* and *Astraptes*. The observation that *Urbanus proteus*, the type species of the genus *Urbanus*, resolved in clade 3*b** along with its morphologically similar and turquoise-colored congeners, suggests that the name *Urbanus* be confined to this group and that the genetically distinct brown-bodied species of *Urbanus* clustering in clade 1 be transferred to a different genus (or genera). Otherwise, the genus *Urbanus* would remain polyphyletic. But it is clear that additional data, including life history information and molecular data from both mitochondrial and nuclear genes, will be required to sort out and stabilize the taxonomy of clade 1 *Urbanus*. Increased taxon sampling, especially in the genera *Thorybes* and *Autochton*, will also be required. For example, *Thorybes pylades* and *Urbanus dorantes* resolved as sister lineages in the study of Warren et al. (2008), and a close relationship among the two taxa was also seen in our study (d = 5.4%). The genetic relationship between *U. dorantes* and other species currently assigned to *Thorybes*, however, is unclear, as *T. pylades* currently is the only species with publicly available barcodes. Of particular interest was that *Astraptes egregius*, a species not included in the study of Warren et al. (2008), resolved as sister to *U. dorantes* (Fig. 1, clade 1*b*; also see Janzen et al. 2011), although the branch was poorly supported in both Bayesian and NJ (55% bootstrap value) trees. The close relationship between the two taxa (d = 4.4%) seems surprising given that the facies of the turquoise-colored *A. egregius* is typical of its congeners from clade 3, with the notable exception of *A. anaphus annetta*. More than a century ago, however, Godman & Salvin (1887-1901) commented that male genitalia of *Thymele* (=*Astraptes*) *egregius* were similar to *Eudamus* (=*Urbanus*) *simplicius*, again suggesting a closer relationship between *A. egregius* and the brown-bodied *Urbanus* species than superficial tail size and color differences would suggest. Barcode amino acid composition of *A. egregius* also is typical of most brown-bodied *Urbanus* and distinct from most other species of *Astraptes* (Table 1).

In the original description, it was noted that *Thymele* (=*Astraptes*) *phalaecus*, the Yellow-edged flasher, was superficially similar to *Telegonus* (=*Astraptes*) *anaphus anaphus*, the Yellow-tipped flasher, but that *Astraptes phalaecus* could be easily distinguished by the presence of three semihyaline subapical spots near the costa, as well as by the presence of a costal fold in males and differences in the shape of the valvae (Godman & Salvin 1887–1901). Phylogenetic analysis (Fig. 1), genetic distance (d >11.0%; p-distance >10.0%), and three differences in barcode amino acid composition (Table 1), also support the view that, although superficially similar, *A. phalaecus* and *A. anaphus annetta* (sensu Evans 1952) are not closely related and belong in different genera. Although barcode analysis suggests that *A. phalaecus*, as well as *Urbanus doryssus* and *U. albimargo*, are closely related to *Achalarus*, any taxonomic changes in these taxa and other apparently misplaced taxa of *Astraptes* will first require additional data, especially given that publicly available barcodes are currently lacking for *Achalarus lyciades* and *Astraptes aulestes*, the type species of their respective genera. Barcodes, however, are available for *Astraptes janeira* (see Table 1) which was listed by Steinhauser (1987) as a synonym of *Telegonus* (= *Astraptes*) *aulestes* (= *aulestis*) and which resolved in clade 3*a* (Fig. 1). Clearly, much additional work is needed to clear up the taxonomic questions in these genera and the Eudaminae in general. We suggest, however, that at this time there is enough evidence, detailed below, to propose reinstating *Thessia jalapus* to the genus *Achalarus*.

Genetic differentiation in *Achalarus*. In a recent biodiversity study on coastal butterflies of the state of Sonora in northwestern Mexico (Pfeiler et al. 2016), a specimen of what appeared to be *Achalarus toxeus* (Fig. 2a, b) was found, but because *A. toxeus* cannot be confidently distinguished from *Thessia jalapus* on external examination (Janzen et al. 2011) a barcode was obtained (voucher EP11) and queried in the BOLD database. The EP11 barcode showed 99.85% similarity (i.e. a single nucleotide difference) to three sequences of *A. toxeus* from the state of Jalisco, Mexico. The BOLD query of EP11, however, also showed about 3% sequence divergence (d) from *A. toxeus* from Costa Rica (Table 2; also see Figs. 1 and 3a, b) and the states of Sinaloa and Jalisco, Mexico. The EP11 sequence is labeled *A. toxeus* in Fig 1, but we suggest that an undescribed cryptic species, *Achalarus* sp. c.f. *toxeus*, may occur in northwestern Mexico together with nominate *A. toxeus* in Jalisco and possibly adjacent regions. Recently we obtained an additional specimen of *Achalarus* sp. c.f. *toxeus* from Alamos, Sonora (EP27;
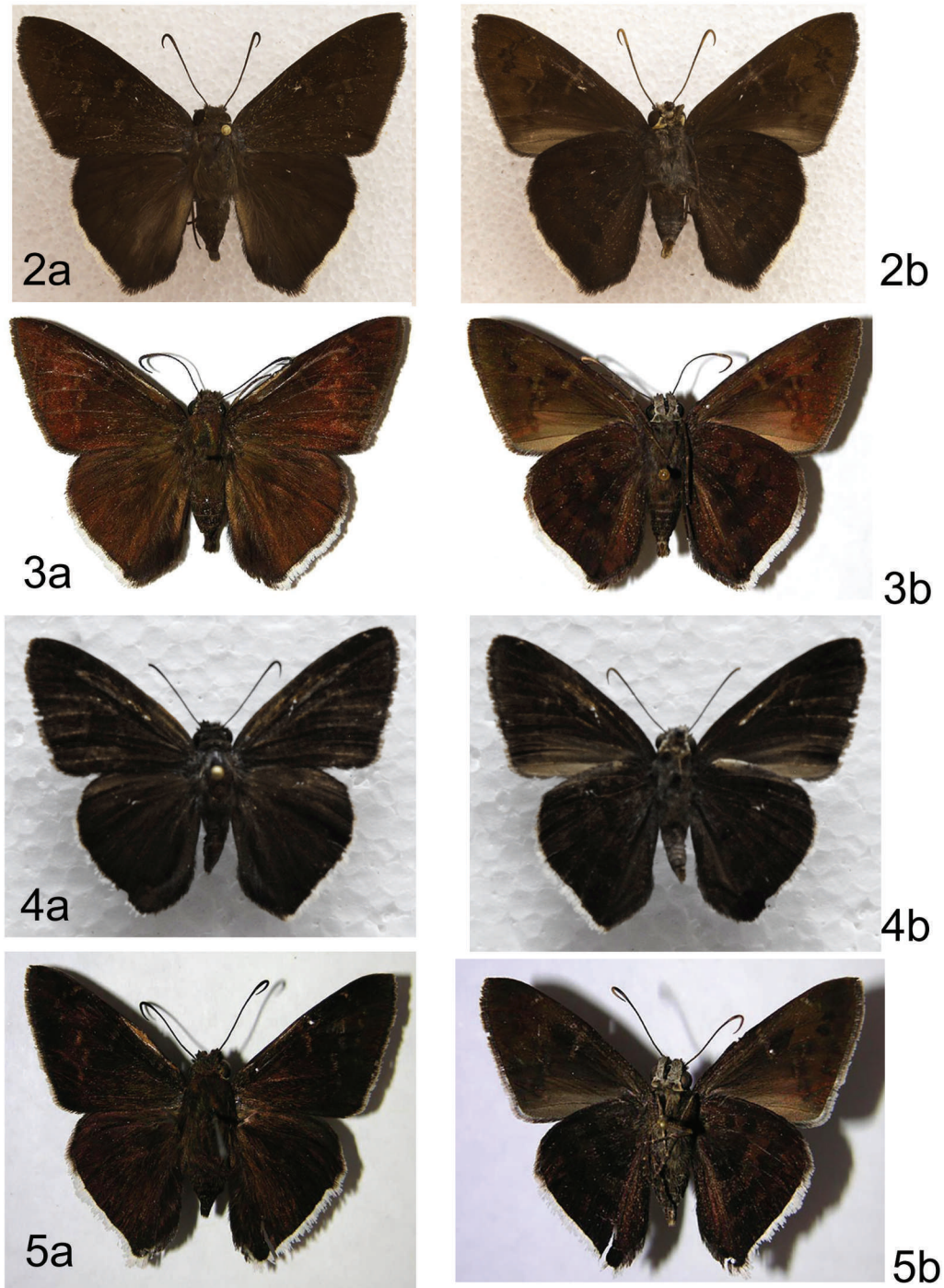
Figs. 2–5. Comparison of dorsal (**a**) and ventral (**b**) images of adult males of *Achalarus* species. Wingspans are given in mm; ACG, Area de Conservación Guanacaste, Guanacaste Province, Costa Rica. (**2**) *Achalarus* sp. cf. *toxeus*, 47 mm, San Carlos, Sonora, Mexico, 8 September 2014 (voucher EP11; GenBank KT290038); (**3**) *Achalarus toxeus*, 50 mm, ACG (voucher 02–SRNP–10179; GenBank GU149309); (**4**) *Achalarus albociliatus*, 43 mm, Alamos, Sonora, Mexico, 4 August 2015 (voucher EP28; GenBank KU041805); (**5**) *Achalarus jalapus* **reinstated status**, 44 mm, ACG (voucher 07–SRNP–21140; GenBank JF763249). Photograph credits: 2, Wain Evans; 3 and 5, Janzen & Hallwachs (2009); 4, E. Pfeiler.

GenBank KU041804) in which the barcode was identical to EP11.

The barcode from a specimen of *A. albociliatus* from Alamos (EP28; Fig. 4a, b) was also queried in BOLD and found to be identical to seven specimens of *A. albociliatus* from Costa Rica, apparently confirming its identity. BOLD results, however, also showed the Alamos sequence to be 100% identical to three sequences labeled *A. toxeus* from Mexico (Jalisco and Sinaloa), which we suggest is the result of misidentification of the Mexican specimens. Interestingly, however, the BOLD query also showed another genetically divergent clade of eight specimens labeled *A. albociliatus albociliatus* from southeastern Mexico (states of Campeche, Quintana Roo and Yucatán; no public records), resolving sister to the clade from Sonora and Costa Rica (d ~3.2%). This value of genetic divergence suggests that a cryptic species of *A. albociliatus* may also occur in southeastern Mexico. Thus, preliminary evidence for substantial genetic differentiation of *A. albociliatus* and *A. toxeus* in Mexico adds to the growing list of potential cryptic species of Lepidoptera revealed by barcode analyses (Dincă et al. 2015).

Thessia. Steinhauser (1989) erected the genus *Thessia* (type species: *Thessia athesis*) to include *Achalarus jalapus* (sensu Evans 1952) and *Urbanus athesis* (sensu Evans 1952), based mainly on similarities in genitalia characters among the two taxa compared with other related species of the Eudaminae. Although *T. jalapus* is generally recognized as a valid name (Opler & Warren 2005; Pelham 2008; ITIS 2015), its placement in *Thessia* rather than *Achalarus* has met with criticism (e.g. Cassie et al. 2001). Based on morphological and molecular evidence presented below we formally propose that *T. jalapus* should be returned to *Achalarus*.

TABLE 2. K2P genetic distances (*d*) (below the diagonal) and uncorrected *p*–distances (above the diagonal) among species of *Achalarus* based on COI barcodes. Values for within taxa genetic distances are shown in bold type along the diagonal. (**1**), *Achalarus* sp. c.f. *toxeus* (Sonora, Mexico; *n* = 2); (**2**), *A. toxeus* (Costa Rica; *n* = 3); (**3**), *A. albociliatus* (Sonora, Mexico and Costa Rica; *n* = 8); (**4**), *A. jalapus* **reinstated status** (Costa Rica; *n* = 5)

	1	2	3	4
1	**0.000**	0.029	0.062	0.050
2	0.030	**0.000**	0.046	0.036
3	0.066	0.047	**0.000**	0.051
4	0.052	0.037	0.054	**0.003**

Achalarus jalapus (Plötz, 1881), **reinstated status**
(Fig. 5a, b)
Eudamus jalapus Plötz 1881: 100
Achalarus jalapus: Evans 1952: 128
Thessia jalapus: Steinhauser 1989: 12-13
     =*Telegonus xerxes* Bell 1934: 90-92
     (synonym)

We suggest that morphology of male genitalia was a poor diagnostic character to be used by Steinhauser (1989) as the main criterion for erecting the genus *Thessia* and was not consistent with his use of genitalia characters in earlier comprehensive taxonomic work on the Eudaminae. Specifically, Steinhauser (1986) had previously shown that diagnostic differences in genitalia morphology, especially in male valvae, were critical for separating species of *Narcosius* in which adults are so similar that they cannot be confidently placed using adult external characters alone (see above section on *Narcosius* and *Ridens*). A comparison of the valvae of *T. jalapus* and *T. athesis* with those of *A. toxeus* and *A. albociliatus* reveals that the interspecific differences among the four taxa are no greater than those seen among the 12 nominal species of *Narcosius* (Steinhauser 1986; Austin 1996). Other than the absence of a costal fold in males of *A. albociliatus*, adults of *A. albociliatus*, *A. toxeus* and *T. jalapus* are so similar (Figs. 2–5) that confident identifications often require additional genetic and/or life history information (Janzen et al. 2011), as mentioned earlier. Mean pairwise genetic distance between *A. toxeus* and *T. jalapus* based on barcodes (d = 3.7%) is less than the value seen between *A. toxeus* and *A. albociliatus* (d = 4.7%) and is similar to that seen between the two genetically distinct populations of both *A. toxeus* (d = 3.0%; Table 2) and *A. albociliatus* (d ~3.2%) in Mexico, providing additional support for the view that *Thessia jalapus* should be returned to the genus *Achalarus*.

We also considered transferring *Thessia athesis* to *Achalarus* and formally suppressing the name *Thessia*. Unfortunately, no barcodes for *T. athesis* are currently available in BOLD or GenBank to assess the genetic relationship of *T. athesis* to *A. jalapus* and the other taxa of the Eudaminae. Because *T. athesis* is the type species of the genus (Steinhauser 1989), further genetic studies will be required before considering any change in its taxonomic status.

Concluding remarks. We have shown that phylogenetic analysis of COI barcodes is an effective tool in screening for polyphyly, revealing taxa that probably require taxonomic revision, in selected genera of the hesperiid subfamily Eudaminae. We suggest that this method could also be successfully used to help

untangle confused taxonomy in other closely related species of the Eudaminae, as well as in other subfamilies of Lepidoptera. We envision this tool as an initial screening to be used mainly in conjunction with multilocus genetic data (Forister et al. 2008, Rubinoff & Holland 2005) and traditional methods for species descriptions that also incorporate ecological, behavioral, and life history data into an integrative taxonomy framework (Dayrat 2005). But as we have also shown here, single locus barcodes, assessed together with external morphological characters and critical analysis of original species descriptions, can provide important information in support of taxonomic rearrangements.

Although an extensive database of barcodes is publicly available for use in phylogenetic studies of the type proposed here, several limitations warrant attention. Species coverage in GenBank for *Urbanus*, *Astraptes*, *Ridens* and *Narcosius* was about 40–50%, sufficient to confirm the polyphyly of *Urbanus* and *Astraptes*, and to hypothesize that *Ridens* and *Narcosius* are monophyletic as currently circumscribed. Additional species of both *Ridens* and *Narcosius* will need to be analyzed, however, to test the hypothesis that both genera are monophyletic. Also, data records in both GenBank and BOLD are known to contain taxonomic misidentifications and other errors that question their reliability (Wilson 2010, Shen et al. 2013). One suspect species identification of *Achalarus* was mentioned above. Possible introgression of mitochondrial DNA from other species also needs to be considered. But of particular concern is the question of the robustness of phylogenetic analyses based on the single locus mitochondrial barcode segment compared with that from large data sets obtained from next generation sequencing (McCormack et al. 2013). By limiting the phylogenetic barcode analyses to taxa at the subfamily level we suggest that potential problems with homoplasy and loss of phylogenetic signal of a single locus mitochondrial marker is probably minimal. The observation that our barcode results on closely related species of the Eudaminae are consistent with those obtained using multilocus genetic data (Warren et al. 2008) supports this suggestion.

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