Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*

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***Astraptes fulgerator*, first described in 1775, is a common and widely distributed neotropical skipper butterfly (Lepidoptera: Hesperiidae). We combine 25 years of natural history observations in northwestern Costa Rica with morphological study and DNA barcoding of museum specimens to show that *A. fulgerator* is a complex of at least 10 species in this region. Largely sympatric, these taxa have mostly different caterpillar food plants, mostly distinctive caterpillars, and somewhat different ecosystem preferences but only subtly differing adults with no genitalic divergence. Our results add to the evidence that cryptic species are prevalent in tropical regions, a critical issue in efforts to document global species richness. They also illustrate the value of DNA barcoding, especially when coupled with traditional taxonomic tools, in disclosing hidden diversity.**

We are driven to find and describe our planet's unrecognized biodiversity because it is disappearing before our eyes. Yet some of this uncharacterized biodiversity has been staring us in the face, almost from the taxonomic start. Consider the neotropical skipper butterfly *Astraptes fulgerator* (Hesperiidae) (Fig. 1) described in 1775 (1). This butterfly has long been regarded as a single species that is common, variable, and wide-ranging: from the far southern United States to northern Argentina, from the near desert to deep rain forest, from lowlands to middle elevations, and from urban gardens to pristine habitats. However, this view blocks perception of its real complexity.

The rearing of >2,500 wild-caught caterpillars of *A. fulgerator* during 25 years of biodiversity inventory in the dry forest, rain forest, and cloud forest of the Area de Conservación Guanacaste (ACG) in northwestern Costa Rica (http://janzen.sas.upenn.edu; refs. 2–5) revealed a range of dicotyledonous food plants far too broad for one species of pyrgine hesperiid (as demonstrated by some 31,000 other ACG pyrgine rearing records (http://janzen.sas.upenn.edu). Moreover, divergent color patterns of the caterpillars (Fig. 2) segregated in accord with food plants. Although dissections of 67 male and female genitalia disclosed none of the morphological differentiation that often distinguishes cryptic species of skippers (see, for example, refs. 6–9), close study of adults, sorted by their caterpillar food plant, showed subtle differences in color, pattern, size, and wing shape. Synthesis of information on food plant use, caterpillar color pattern, and adult external phenotypes indicated that *A. fulgerator* from the ACG was a complex of at least six or seven species. However, it seemed that several more years of linking caterpillar and adult characteristics with food plants would be needed to fully delimit species.

While this query was proceeding, it became apparent that DNA sequencing of a standard gene region or "DNA barcoding" (10) might speed a solution. DNA barcoding can be helpful in species diagnosis because sequence divergences are ordinarily much lower among individuals of a species than between closely related species (11–13). For example, congeneric species of moths show an average sequence divergence of 6.5% in the mitochondrial gene cytochrome *c* oxidase I (COI), whereas divergences among conspecific individuals average only 0.25% (11). Similar values were obtained in birds, with intraspecific divergences at COI averaging 0.27%, whereas congener divergences averaged 7.93% (14).

In this study, the addition of DNA barcodes to data on food plants, ecological distributions, caterpillar color patterns, and adult facies indicates that *A. fulgerator* consists of 10 largely sympatric species in the ACG. This result raises the prospect that, over its huge neotropical range, *A. fulgerator* may comprise many more hidden species. Imagine the biodiversity implications of this result for other wide-ranging, common, and "somewhat variable" species of neotropical animals and plants.

Materials and Methods

Field Biology. The ACG is a 110,000-hectare mosaic of many ages of succession and old growth tropical dry forest, rain forest, and cloud forest, as well as their various intergrades under conservation in northwestern Costa Rica (www.acguanacaste.ac.cr and http://janzen.sas.upenn.edu). Since 1978, tens of thousands of caterpillars have been reared annually from thousands of species of plants (e.g., refs. 2–5 and 15). Through 2003, these rearings included 2,592 caterpillars of *A. fulgerator.* Each caterpillar was reared individually, and its rearing data were collated under a unique voucher code (e.g., 93-SRNP-3774), which is accessible on the project web site (http://janzen.sas.upenn.edu).

Approximately half of these *A. fulgerator* caterpillars produced adults; 968 were frozen on the day of eclosion, thawed within 2 months, pinned, spread, oven-dried, and stored at ambient temperatures. These specimens were collected under multiple research and export permits issued to D.H.J. by the Ministerio del Ambiente y Energía of Costa Rica, and they have been deposited in the National Museum of Natural History, where they remain stored at ambient temperatures.

We attempted to assign DNA barcodes to 484 of these adults. Where possible, those chosen included at least 20 individuals reared from each species of food plant, extremes and intermediates of adult and caterpillar color variation, and representatives from the three major ACG terrestrial ecosystems (dry forest, cloud forest, and rain forest) and their intergrades. All 30 available individuals from wild-caught pupae were barcoded even though their food plants are unknown (caterpillars of *A. fulgerator* often pupate off their food plant). One leg was plucked from each individual, placed in a dry Eppendorf tube, and sent to the University of Guelph for DNA analysis. Sampled adults

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Abbreviations: ACG, Area de Conservación Guanacaste; COI, cytochrome *c* oxidase I; NJ, neighbor-joining.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY666597–AY667060, AY7224411, and AY7224412).

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Fig. 1. Newly eclosed female *A. fulgerator* (species LOHAMP, voucher code 02-SRNP-9770) from the ACG.

received yellow labels stating "Legs away/for DNA." Digital photographs of the upper and lower side of each adult, together with its collection details, are available on the Barcodes of Life (BoLD) web site (www.barcodinglife.com) and on the inventory web site, which also has images of hundreds of the adults and their caterpillars.

Genetic Analysis. Total DNA was extracted from each of the 484 dry legs by using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma Genosys) according to the manufacturer's specifications, and the resultant DNA was eluted in 30 μl of double-distilled H_20. Analysis ordinarily examined sequence diversity in a specific 648-bp fragment of the mitochondrial COI gene (the COI 5′ region). This sequence was amplified by using the following primer pair designed for Lepidoptera: LEP-F1, 5′-ATTCAACCAATCATAAAGATAT-3′; and LEP-R1, 5′-TAAACTTCTGGATGTCCAAAAA-3′. When PCR amplification with these primers failed to generate a product, the LEP-F1 primer was combined with another reverse primer (5′-CTTATATTATTTATTCGTGGGAAAGC-3′) to generate a 350-bp product. This combination was necessary in ≈5% of the extractions; it was necessary more frequently with specimens 10–20 years old than with newer material.

All PCR mixes had a total volume of 50 μl and contained 2.5 mM $MgCl_2$, 5 pmol of each primer, 20 μM dNTPs, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 10–50 ng (1–5 μl) of genomic DNA, and 1 unit of *Taq*DNA polymerase. The thermocycling profile consisted of one cycle of 1 min at 94°C, six cycles of 1 min at 94°C, 1 min and 30 sec at 45°C, and 1 min and 15 sec at 72°C, followed by 36 cycles of 1 min at 94°C, 1 min and 30 sec at 51°C, and 1 min and 15 sec at 72°C, with a final step of 5 min at 72°C. PCR products were electrophoresed in 1.0% TBE agarose gels, stained with ethidium bromide, and visualized under UV light. Two microliters (20–50 ng) of the PCR products from these reactions were cycle sequenced without further cleanup by using the LEP-F1 primer, the ABI Prism *Taq*FS dye terminator kit (Applied Biosystems), and BIG DYE (version 3.1). Sequencing reactions had a total volume of 10 μl and included 10 pmol of each primer. The sequencing amplification protocol consisted of one cycle of 1 min at 96°C, followed by 30 cycles of 10 sec at 96°C, 5 sec at 55°C, and 4 min at 60°C. Sequences were analyzed on an ABI 377 sequencer (Applied Biosystems) and were aligned subsequently by eye in BIOEDIT (16). Sequence divergences among individuals were quantified by using the Kimura-2-Parameter distance model (17) and graphically displayed in a neighbor-joining (NJ) tree (18). All sequences obtained in this study and the original chromatograms are available in a completed project file (*Astraptes fulgerator* Complex) on the BoLD web site. The sequences have also been deposited in GenBank (accession nos. AY666597–AY667060, AY7224411, and AY7224412).

Results

COI Divergences. A full-length PCR product was recovered from 465 of the 484 individuals (96%), and a 350-bp product was recovered from 14 of the 19 remaining specimens. The COI sequences were easily aligned, as no insertions or deletions were detected. However, 13 sequences showed heterozygosity (as evidenced by dual peaks of similar height in the electropherograms) at 16–28 nucleotide sites, suggesting either heteroplasmy or coamplification of a nuclear pseudogene with its mitochondrial counterpart. A second DNA extraction from these 13 individuals, followed by sequence analysis, confirmed their heterozygosity. We discuss these individuals below, but we excluded them from our initial analyses. The 137 different COI sequences among the remaining individuals displayed considerable divergence (Appendix 1, which is published as supporting information on the PNAS web site), with Kimura-2-Parameter distances among individuals averaging 2.76% (range, 0.0–7.95%).

The 10 Taxa. Mapping caterpillar/adult morphology and food plants onto the NJ tree of COI divergences reveals 10 haplotype clusters that covary with morphological and ecological traits (Fig. 3), suggesting the presence of 10 species. Sequence divergences for the 45 pairwise NJ comparisons among these 10 taxa average 2.97% and range from 0.32% to 6.58% (Appendix 2, which is published as supporting information on the PNAS web site). To aid discussion, we code each species by key biological attributes: 7 of the 10 taxa are coded according to their primary food plants (TRIGO, CELT, LONCHO, LOHAMP, HIHAMP, BYTTNER, and INGCUP), and the other three taxa are coded by their main food plants plus a color character of the adult (SENNOV, YESENN, and FABOV). Two small COI groups of three (MYST) and four (NUMT) individuals are treated separately for reasons that are justified later. In the remainder of this section, we briefly describe key features (ecological, ethological, and morphological) for each of the 10 presumptive taxa.

The yellow-ringed caterpillars of TRIGO eat the two species of *Trigonia* (Trigoniaceae) in the ACG, whereas those of CELT eat only *Celtis iguanaea* (Celtidaceae/Ulmaceae). These food plants are ignored by the remainder of the *A. fulgerator* complex (and by other ACG hesperiids, as well). Conversely, TRIGO and CELT do not use the food plants of the other eight members of the complex. The lone record of TRIGO eating *Licania arborea* (Chrysobalanaceae) is real but exceptional (the other ≥750 caterpillar records from this species of plant are of other species of Lepidoptera). CELT and TRIGO are sympatric in the ACG lowland rain forest (up to ≈400 m), but only TRIGO extends into the dry forest.

LOHAMP and LONCHO, which have similar yellow-disk-marked caterpillars, are likewise faithful to their food plants, although with some instructional exceptions. LOHAMP normally eats *Hampea appendiculata* (Malvaceae), but 1 of 47 barcoded individuals ate *Lonchocarpus oliganthus* (Fabaceae), and 2 individual ate *Styrax argenteus* (Styracaceae), a plant that was not otherwise fed on by any member of the *A. fulgerator* complex. LONCHO regularly eats *L. oliganthus* or *L. costaricensis*, but 9 of 41 barcoded individuals used *Senna* (Fabaceae), and 2 used *H. appendiculata*. Hence, LOHAMP and LONCHO can survive on each other's principal food plant, whereas LONCHO also rarely eats the primary food plants (*Senna*) of three other *A. fulgerator* species. LONCHO is sympatric with five species in the complex at the lower

ECOLOGY

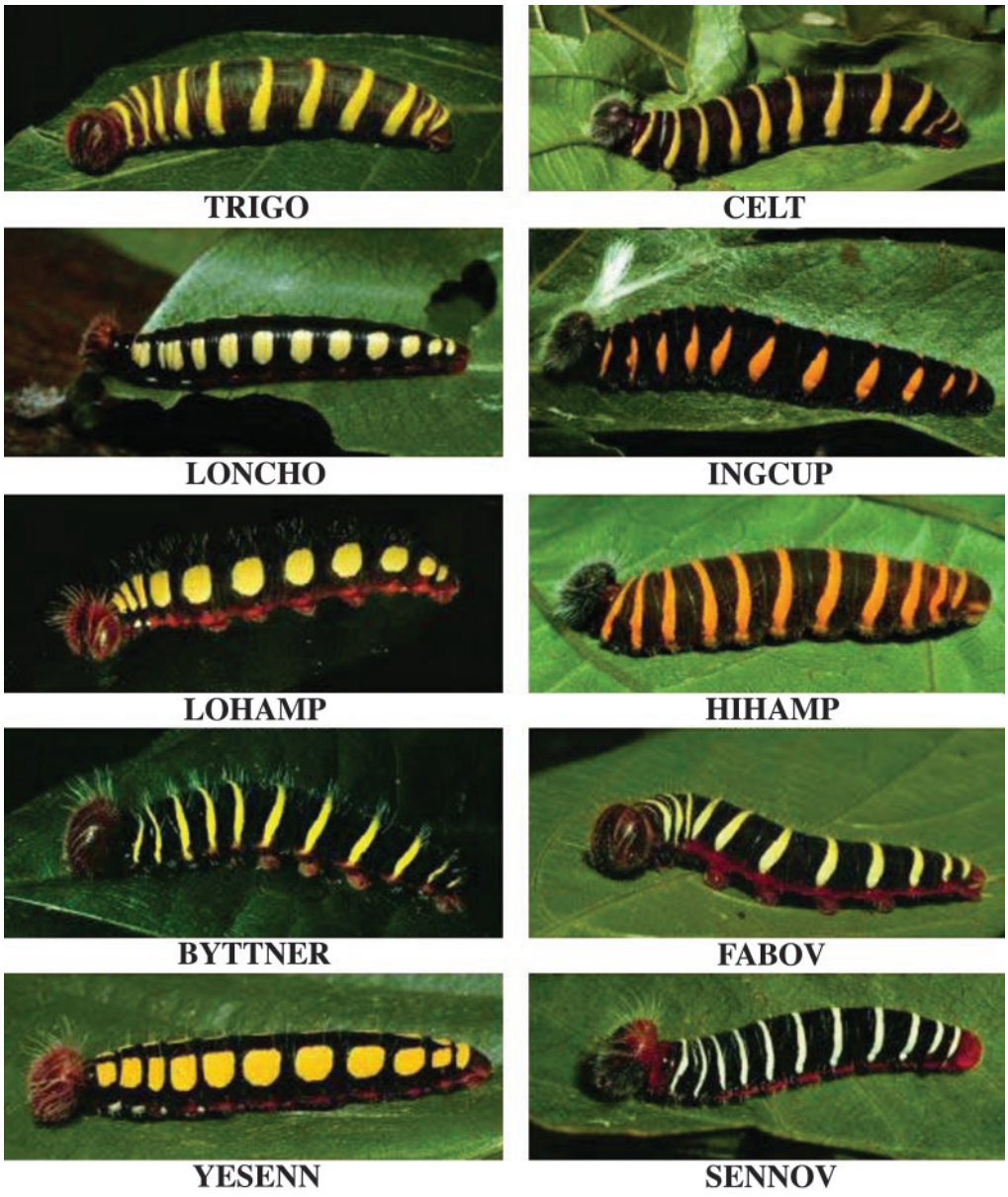


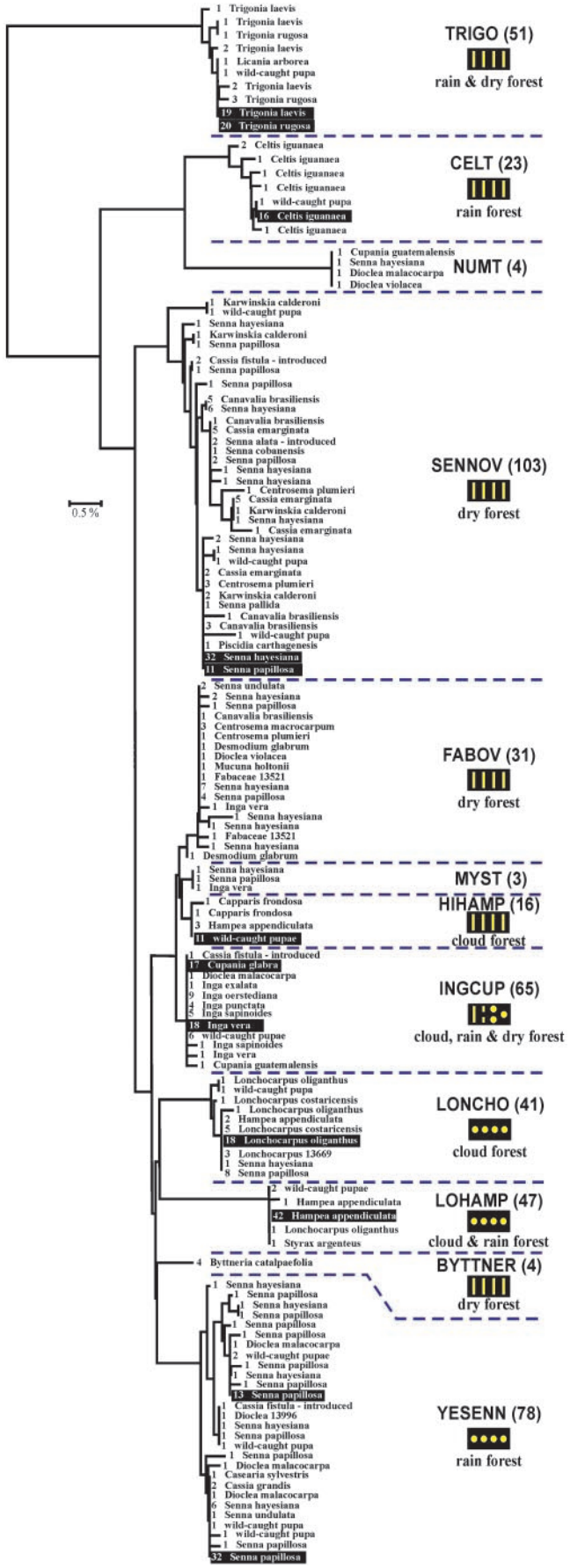
Fig. 2. Last-instar caterpillars of 10 species in the *A. fulgerator* complex from the ACG. Interim names reflect the primary larval food plant and, in some cases, a color character of the adult.

margin of the ACG cloud forest but does not follow the many species of *Lonchocarpus* into the rain forest or dry forest lowlands. By contrast, LOHAMP follows *H. appendiculata* throughout its highland to lowland range and so coexists with eight other members of the *A. fulgerator* complex. The blue on the upperside of the wings of fresh reared adults is perceptibly deeper and darker in LONCHO than it is in LOHAMP.

DNA barcoding revealed HIHAMP in an unexpected manner. The original HIHAMP group included just three adults reared from caterpillars but 11 adults from wild-caught pupae. Each of these pupae was found 1–2 m above the ground, under a different tall adult *H. appendiculata*. One caterpillar was in mature foliage of an adult *Hampea* crown, whereas both records "from" *Capparis frondosa* (Capparidaceae) were likely prepupal caterpillars descended from the *Hampea* overhead. A directed search in 2004 located three more HIHAMP caterpillars in the crowns of adult *Hampea*, two of which survived to produce adults. Both had sequences identical with those of the other HIHAMP. The caterpillars of HIHAMP are ringed (whereas those of LOHAMP bear yellow discs), and they feed on mature foliage in the *Hampea* crown, whereas those of LOHAMP feed on low, young foliage (usually on saplings). HIHAMP appears to be a middle-elevation cloud-forest species that coexists with five other members of the *A. fulgerator* complex, whereas the partly sympatric LOHAMP ranges down into the rain forest lowlands. However, HIHAMP is ecologically and microgeographically parapatric with FABOV, which is closest to it in the NJ tree, and feeds on a very different plant family than FABOV.

The ringed caterpillars of BYTTNER resemble those of several other *A. fulgerator* species. Their apparent monophagy on *Byttneria catalpaefolia* (Sterculiaceae) is still tentative because just four adults of this dry forest species were available for analysis. Despite their rarity, these specimens were obtained over a 15-year period, providing evidence for a persistent linkage between this food plant and a particular COI lineage.

INGCUP is striking because it eats multiple species in two



genera, *Inga* (Fabaceae) and *Cupania* (Sapindaceae), in different plant orders, in all three major ACG ecosystems. Only 3 of 66 barcoded INGCUP used other *A. fulgerator* food plants. Although their color patterns on both food plant genera vary from complete rings to dorsally broken rings to lateral patches that range from intense yellow to deep orange, the barcode group is extremely cohesive, with 95% having identical sequences.

Early on, reared adults whose caterpillars were mainly found on the same two species of *Senna* (Fabaceae) were sorted by J.M.B. into the following two groups by ventral body color: SENNOV with an orange venter and YESENN with a yellow one. It was later recognized that their caterpillars are, respectively, fully ringed and laterally disked. Moreover, SENNOV is a dry forest species using chiefly *Senna hayesiana*, whereas YESENN is a rain forest species using chiefly *Senna papillosa*. Both species also occur in cloud forest clearings and in the intergrade between dry and rain forests where, again, their two main food plants are common, and an individual plant may have both species on it. Although both eat a smattering of other legumes, SENNOV appears to be the more polyphagous because it occasionally eats *Karwinskia calderoni* (Rhamnaceae). Like adult ventral coloration and caterpillar patterns, barcodes clearly separate these two species.

The COI sequences also point to yet another species (FABOV), whose adults were initially grouped by J.M.B. with SENNOV because of their orange venters and their caterpillar food plants. However, when FABOV was revealed, it was seen that, on average, its ventral orange is slightly paler than that of SENNOV. Although predominantly *Senna* eaters (21 of 33 cases), the ringed caterpillars of FABOV feed on at least seven species in six other genera of Fabaceae. This taxon occurs in the lowland ACG, mostly in dry forest and in the intergrade between dry forest and rain forest (once recorded in deep rain forest), but it is not common anywhere.

Individuals with "Heterozygous" COI Sequences. Adults with two different COI sequences came from caterpillars collected on *Celtis* (1), *Cupania* (1), *Hampea* (1), *Inga* (3), *Lonchocarpus* (1), and *Senna* (6). Because of the strong associations between caterpillar food plants and COI sequences in other individuals, it was possible to ascertain the likely genotypic characteristics of these "heterozygotes." By assuming that one of the COI sequences in each individual matched the typical sequence for other *A. fulgerator* found feeding on its food plant, the second sequence could be determined by subtraction. For example, the three heterozygous individuals collected on *Inga* were assumed to possess the standard sequence found in individuals of INGCUP and a second sequence that differed from it at all heterozygous positions. This analytical approach revealed that all 13 COI heterozygotes possessed a second sequence with high similarity. This congruence, despite recovery from varied members of the *A. fulgerator* complex, supports a pseudogene origin because nuclear pseudogenes of mitochondrial origin (also known as Numts; see ref. 19) regularly show sequence conservation among lineages with divergence in the corresponding mitochondrial sequence (20). These presumptive pseudogene sequences are closely similar or identical to the NUMT sequence, suggesting that the four individuals in this group represent cases in which only this pseudogene amplified.

Fig. 3. NJ tree based on Kimura-2-Parameter distances for COI DNA sequences from 466 individuals of the *A. fulgerator* complex from the ACG. Numbers in parentheses indicate the total sample size for each interim taxon, rectangles caricature caterpillar color patterns, and black backgrounds indicate groups of ≥10 conspecifics with identical sequences.

Discussion

Despite the infancy of DNA barcoding protocols, our study demonstrates that dry museum specimens up to 23 years old can be sequenced with considerable success (≈98%; but often only 350-bp sequences were obtained from older specimens). Although preservation methods can damage DNA (21, 22), earlier studies have recovered PCR products from insect specimens over 1 century old (23, 24). Moreover, augmented PCR protocols with an initial DNA repair step promise advances in DNA recovery, suggesting that a comprehensive barcode library could be assembled through the sequencing of museum specimens.

Numts pose a potential interpretational hazard for any PCR-based survey of mitochondrial DNA diversity (25), and 2.8% of our COI sequences showed probable coamplification of a Numt with its mitochondrial counterpart. However, the taxonomic impact of these coamplifications was small; all such individuals were identified as belonging to the *A. fulgerator* complex, and most individuals could be assigned to one of its 10-component taxa when the pseudogene sequence was determined. We emphasize, as well, that when sequencing is done with fresh specimens, the use of RT-PCR provides strong protection against Numt amplification (26), suggesting the use of this approach in taxa with COI pseudogenes.

Our sequencing results support the prior conclusion that *A. fulgerator* is a species complex. Its levels of COI diversity are much higher than those typical of single-species populations. More importantly, there is clear covariation of the COI sequences with morphological, ethological, and ecological traits. Although studies of classical characters indicated six or seven species, the addition of COI data raised the count to 10. This increase reflects the way in which the COI data added meaning to isolated records on unusual food plants (e.g., *Byttneria*), to lineages with behavioral and caterpillar color pattern divergence (e.g., LOHAMP vs. HIHAMP), and to taxa with nearly indistinguishable adult facies (e.g., SENNOV vs. FABOV). Whereas our study reveals the power of DNA barcoding in helping to resolve complex taxonomic situations, it also indicates the imperative of large sample sizes and supplemental morphology and natural history. We emphasize that barcodes differ from the standard traits used for species discrimination in the following important way: they can be obtained in a mechanized manner. Hence, they can be used without much background knowledge, both for routine identifications and for the detection of hidden species (13, 27).

Despite the variation in caterpillar color pattern and food plants, adults in the *A. fulgerator* complex show little phenotypic diversity. Their similarity probably reflects not only recent common ancestry but also stabilizing selection arising from membership in a massive mimicry ring. At least 35 species in three subfamilies of hesperiids from the ACG range from general to exact mimics of *A. fulgerator* (see images of adults at http://janzen.sas.upenn.edu). In the neotropics as a whole, many more skipper species swell this mimetic assemblage. As expected from their morphological similarity, members of the *A. fulgerator* complex show less sequence divergence than most other congeneric species pairs (10, 13). Only two taxa (CELT and TRIGO) have minimum COI divergences (3.4% and 5.4%) from all other members of the complex that exceed the sequence threshold (3%) typically encountered between congeneric species pairs recognized by morphological approaches (14). Divergences among the remaining taxa are lower, but all exceed 1.1%, which is well above usual intraspecific values, except among members of the triad FABOV, HIHAMP, and INGCUP, which show <0.5% divergence. These three species possess distinct COI sequence arrays (Fig. 3), feed on very different food plants, and show subtle differences in both caterpillar and adult facies. The MYST lineage apparently represents an exception to this pattern of mitochondrial divergence as it includes one individual from *Inga* and two from *Senna*, suggesting that it is shared by INGCUP and FABOV. As such, this likely represents a case in which lineage sorting is incomplete, a result that might have been expected given the low genetic divergence (and presumed recent origin) of these groups.

Past work has provided conflicting perspectives on the likely efficacy of mtDNA markers in delineating species boundaries. Some studies, including extensive analyses of GenBank data, have indicated that even closely related species ordinarily show marked mitochondrial divergence (10, 12). However, others suggest that mtDNA markers will often encounter problems in species resolution (28–30). For example, a review of case studies (31) concluded that nearly one-fourth of all animal species fail the test of mitochondrial monophyly. The *A. fulgerator* complex represents a case in which mitochondrial markers might have been expected to fail because its component species are both extremely similar and sympatric, providing opportunities for hybridization. However, our detection of reciprocal monophyly for COI variants among its members means that shared ancestral polymorphisms have been lost, either as a consequence of stochastic lineage pruning or selective sweeps. Moreover, the lack of shared haplotypes indicates either strict reproductive isolation or ongoing selection against mitochondrial exchange between members of the complex. Female Lepidoptera are both the heterogametic sex and the primary agents of food plant selection, which are factors that can also act to ensure rapid divergence in mitochondrial markers (32, 33).

Should the 10 species of *A. fulgerator* identified in this study be formally described despite their morphological similarity? Yes. Although their recognition was facilitated by DNA barcoding, the combination of their genetic distinctiveness and their covarying caterpillar color patterns, food plant usage, and adult morphology demonstrates that they are reproductively isolated populations. The fact that these populations are largely sympatric argues even more strongly for traditional binomials.

Diversification in the *A. fulgerator* complex is clearly linked to food plants, suggesting the importance of a detailed analysis of shifting food plant use. The complex likely derives from a species that fed on Fabaceae because most other species of *Astraptes* and many of those in 19 allied genera (34) feed largely on plants in this family (2). Based on standard calibrations for rates of mitochondrial evolution (e.g., refs. 35 and 36), TRIGO separated from the basal *fulgerator* clade ≈4 million years ago, probably onto *Trigonia*, whereas CELT separated ≈2 million years ago, likely onto *Celtis*. Subsequent speciation has involved less radical food plant shifts, but some of these events probably occurred within the last half-million years (e.g., FABOV, HIHAMP, and INGCUP). This diversification may be linked to regional variation in the species composition of plant communities and to the inclusion of novel species in the food plant repertoire of some taxa. For example, only SENNOV eats *K. calderoni*, a species that is chemically and morphologically divergent from its standard food plants. If isolated in a setting lacking its usual food plants, SENNOV might rapidly evolve into a *Karwinskia* specialist.

Although members of the *A. fulgerator* complex are young in evolutionary terms, their ranges have surely expanded well beyond their areas of origin. As a result, we doubt that any of the lineages revealed in this study are endemic to the ACG or to Costa Rica. Rather, we expect that most of their distributions span many degrees of latitude, extending wherever food plants and ecological conditions permit (37). Given the high diversity of the South American hesperiid fauna, and the fact that *A. fulgerator* ranges from the southern United States to northern Argentina, a comprehensive survey might uncover many more species.

This study has altered our view of a "species" that has been known to science for more than 2 centuries. Its transformation from a single, common, variable, and wide-ranging taxon to a complex of ≥10 food plant specialists with differing ecological attributes reveals a layer of biological complexity that needs exploration. How often are widespread "species," such as *A. fulgerator,* really an amalgam of specialized, reproductively isolated lineages? The answer to this question is crucial to refining estimates of species diversity in the animal kingdom because the level of food plant specialization in tropical arthropods is a critical modulator of these values (e.g., refs. 38 and 39). Although few DNA-based studies have examined this issue, our results contribute to an emerging pattern. The number of species in a neotropical cerambycid beetle genus doubled after an analysis of only 48 individuals from a small geographic region (40). Similar evidence for cryptic species was found in varied tropical pseudoscorpion lineages (41). Collectively, these results may motivate a broad-ranging evaluation of the incidence of cryptic species in the tropics, an effort that could be expedited by use of an efficient screening procedure like DNA barcoding.

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