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Genomic DNA sequencing reveals two new North American species of *Staphylus* (Hesperiidae: Pyrginae: Carcharodini)

Jing Zhang^{1,2}, Qian Cong^{1,2}, Jinhui Shen², Leina Song², and Nick V. Grishin^{2,3,4*}

¹Eugene McDermott Center For Human Growth & Development, Departments of ²Biophysics and ³Biochemistry, and

⁴Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9050, USA; *Corresponding author: grishin@chop.swmed.edu

ABSTRACT. Two new skipper butterfly (Hesperiidae) species are described from the United States: *Staphylus floridus* Grishin, **sp. n.** (type locality in Florida, Volusia Co.) and *Staphylus ecos* Grishin, **sp. n.** (type locality in Texas, Brewster Co.). They are cryptic and hence escaped recognition. They differ from their sister species by the relative size and morphology of genitalia and by genotype—including and beyond the COI barcode—thus suggesting genetic isolation that argues for their species-level status. A **lectotype** is designated for *Helias ascalaphus* Staudinger, 1876. *Staphylus opites* (Godman & Salvin, 1896), **stat. rest.** is a species-level taxon and not a synonym of *Staphylus vincula* (Plötz, 1886), while *Pholisora iguala* Williams & Bell, 1940, **syn. n.** is a junior subjective synonym of *S. vincula*.

Additional key words: biodiversity, cryptic species, suture zones, genomics, phylogeny.

ZooBank registration: <http://zoobank.org/CCF018F4-0188-451A-BE75-006F64938595>

INTRODUCTION

New methods bring new insights and reveal aspects not previously apparent. For centuries, zoology relied on phenotype-based research and classification. New species were traditionally discovered via phenotypic differences. Phenotypes are encoded by genotypes, and direct investigation of genotypes by the analysis of DNA sequences may be a more direct and powerful approach to classifying nature. Even short segments of DNA, such as COI barcodes, have been insightful in revealing species diversity not readily evident from phenotypes (Hebert et al. 2004).

Genome-scale phylogenetic approaches show promise in revolutionizing our understanding of butterfly evolution and refining their taxonomy (Toussaint et al. 2018; Allio et al. 2019; Li et al. 2019; Zhang et al. 2019a; Zhang et al. 2019b; Toussaint et al. 2021; Robbins et al. 2022; Toussaint et al. 2022). Accurate phylogenetic trees constructed from millions of base pairs give better confidence in the results and frequently reveal inconsistencies with the current classification (Cong et al. 2019b; Zhang et al. 2021; Toussaint et al. 2022; Zhang et al. 2022). While this approach is instrumental for higher classification and taxonomy above species level, its application to species discovery and description has been limited (Janzen et al. 2017; Cong et al. 2020). Even for COI barcodes, a major obstacle is the DNA analysis of extant primary type specimens which is required to properly place discovered new species in the context of previously named taxa (Pfeiler 2018).

Next-generation sequencing technology allows us to obtain whole genome shotgun sequences of some of the oldest type specimens, thus resolving this impediment (Cong et al. 2021). Sequencing of primary types is essential to confidently address nomenclatural questions. Imperative for cryptic species

complexes, it could also be helpful in seemingly less difficult cases. Oftentimes type localities were incompletely documented or type specimens may have been mislabeled. Genomic comparison across a species' range places type specimens among them and clarifies type localities (Cong et al. 2021). Sequencing of primary types on a large scale (Zhang et al. 2022) offers unprecedented opportunities for species-level taxonomy, both in correcting previous mistakes and new species discovery.

Our research group carries out large-scale butterfly genomic sequencing across taxonomic groups and continents. As a result, specimens currently assigned to one species sometimes partition into distinct and strongly supported clades in phylogenetic trees. We construct three maximum likelihood trees from all protein coding genes encoded by: nuclear genome partitioned into (1) autosomes and (2) the Z chromosome, and (2) mitochondrial genome. The Z chromosome holds a special place in the evolution of Lepidoptera (Sahara et al. 2012; Mongue and Walters 2018; Cong et al. 2019a; Mongue et al. 2022) and its phylogeny frequently agrees best with how species boundaries are defined based on previous work. Well-supported clades observed in all three trees within "species" prompts a more detailed investigation.

Here, we analyze two such cases dealing with North American species of *Staphylus* Godman & Salvin, 1896 (type species *Helias ascalaphus* Staudinger, 1876). Genomic trees that include primary type specimens of the relevant names reveal a split into two clades in each of the two current species of *Staphylus*. Following up on these results with morphological analyses, two new species are proposed.

MATERIALS AND METHODS

The specimens were inspected and photographed/sampled for DNA in the following collections: American Museum of Natural History, New York, NY, USA (AMNH), Natural History Museum, London, UK (BMNH), Carnegie Museum of Natural History, Pittsburgh, PA, USA (CMNH), Museum für Naturkunde, Berlin, Germany (MFNB), Texas A&M University Insect Collection, College Station, TX, USA (TAMU), National Museum of Natural History, Smithsonian Institution, Washington, DC, USA (USNM), Zoologische Staatssammlung München, Germany (ZSMC), and the personal collection of John V. Calhoun, Palm Harbor, FL, USA. Historical documents, such as unpublished drawings, were inspected in the Natural History Museum, London, UK and National Museum of Natural History, Smithsonian Institution, Washington, DC, USA. Photographs of specimens and illustrations were taken with Nikon D800 camera through 105 mm macro lens; dissected genitalia were photographed in glycerol with the Nikon D200 camera without a lens and through a microscope at 4.5x magnification. Images were assembled and edited in Photoshop CS5. Genitalia photographs were taken in several focus slices and stacked in Photoshop to increase depth of field. Genomic DNA was extracted from a leg, libraries were prepared, and sequenced as previously reported (Li et al. 2019). The details of phylogenetic tree construction were given in Zhang et al. (2022). In brief, the maximum-likelihood tree was constructed from protein-coding regions in the Z chromosome using IQ-tree v1.6.12 under GTR+GAMMA model (Nguyen et al. 2015). To estimate the confidence of each node, we generated 100 replicates of 10,000 codons randomly sampled from the total set of codons in the Z chromosome and constructed maximum-likelihood trees for each replicate. The support values of each node were summarized from these replicate trees using sumtrees routine in dendropy package (Sukumaran and Holder 2010). The method to find diagnostic DNA characters is described in Cong et al. (Cong et al. 2019b). Sequence datasets obtained in this work are deposited in the NCBI database <<https://www.ncbi.nlm.nih.gov/>> as BioProject PRJNA831579 (BioSample entries of the project contain the locality and collection data of the sequenced specimens shown in the trees), and COI barcodes have GenBank accessions ON351018–ON351022. Exon sequences with diagnostic characters highlighted are also available from <<https://osf.io/tygxc/>>. DNA characters are given as abbreviations, e.g., aly728.44.1:G672C means position 672 in exon 1 of gene 44 from scaffold 728 of *Cecropterus lyciades* (formerly in *Achalarus* Scudder, 1872, thus aly) reference genome (Shen et al. 2017) is C, changed from G in the ancestor. Similar notations are used for the COI barcode characters, e.g., A78G means position 78 is G, changed from A in the ancestor, or T264T(not C) means position 264 is T, unchanged from the ancestor, and not C as in sister taxon.

RESULTS

All three phylogenetic trees—based on nuclear genome: (1) autosomes and (2) Z chromosome, and (3) mitochondrial genome—consistently reveal a well-supported split into two clades in each of the two North American species of *Staphylus*: *S. hayhurstii* (W. H. Edwards, 1870) and *S. ceos* (W. H. Edwards, 1882). These two names have no known synonyms (Pelham 2008; Pelham 2022), and therefore one of the clades in each species is likely to represent a new taxon. A combination of genomic and morphological analyses suggests that these unnamed taxa are new species, which are herein described. In the first case, nuclear genome diverged stronger than the mitogenome. In the second case, the opposite occurred. Furthermore, genomic comparison of old primary type specimens placed among more recently collected specimens suggests a number of taxonomic changes that are detailed below.

Staphylus floridus Grishin, new species

<http://zoobank.org/B7CE2D54-7E0F-4FBD-96B6-4A96BCE02C6D>

(Figs. 1 part, 2, 3a, b, e)

Definition and diagnosis. Genomic analysis of specimens identified as *Staphylus hayhurstii* (W. H. Edwards, 1870) (type locality USA: Missouri, possibly in Pettis Co.) from across its range, including the lectotype (NVG-15096H05), reveals their split into two prominent clades with 100% statistical support in all three trees (Fig. 1 red and blue clades). The red clade consists of specimens from Florida, and the blue clade includes the rest, where the lectotype of *S. hayhurstii* belongs. Genetic differentiation between the two clades is more pronounced in the nuclear genome than in the mitogenome: *Fst*/*Gmin* computed on the Z chromosome are 0.49/0.02, suggesting that the two clades are two distinct species (Cong et al. 2019a); but mitogenomes of the two species diverged less, e.g., their COI barcodes differ by 1.1% (7 bp), a value by itself insufficient to substantiate species-level distinction. Comparison of male and female genitalia offers additional support for the species-level status of the new taxon, and we conclude that the red clade (Fig. 1) represents a new species. This species (Fig. 2) is superficially similar to its sister *S. hayhurstii*, and we were not able to find wing pattern characters that

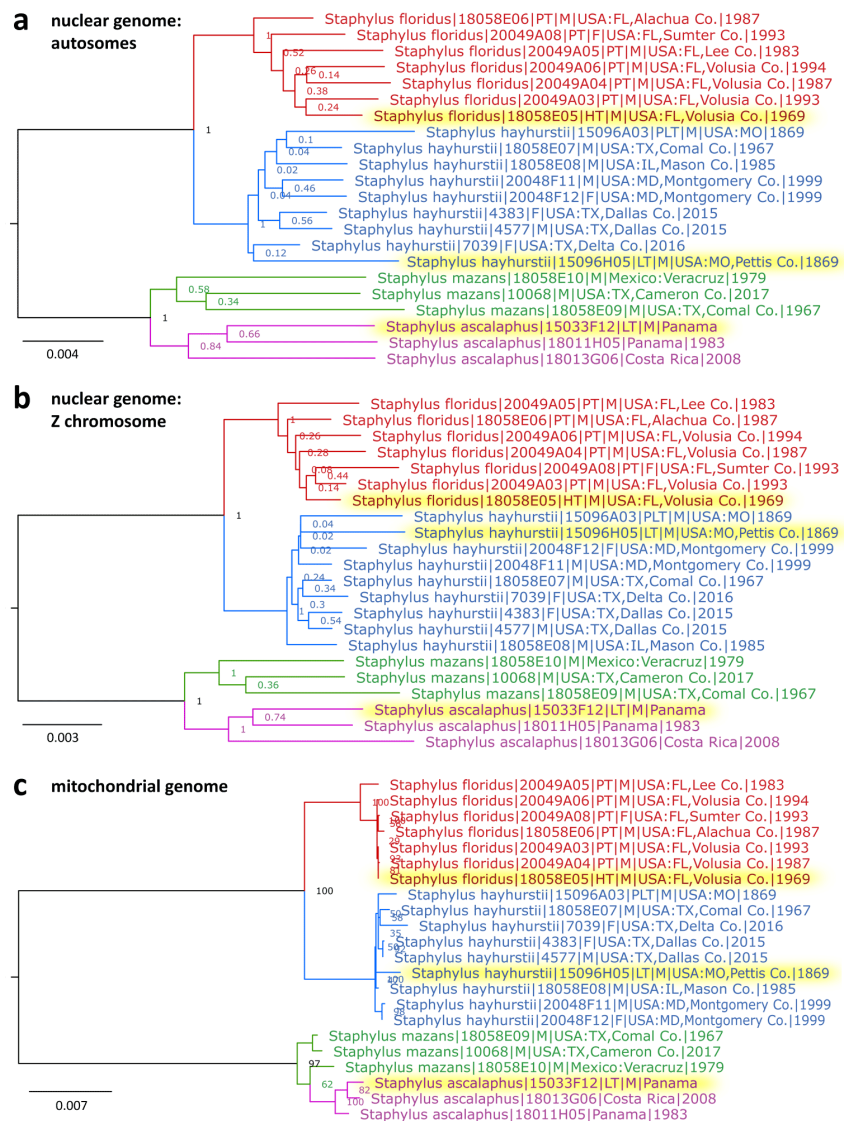


Fig. 1. Phylogenetic analysis of *Staphylus floridus* sp. n. and *S. hayhurstii*. The trees are constructed from protein-coding regions in: a. autosomes, b. Z chromosome, c. mitochondrial genome. Different species are shown in different colors. Names of primary type specimens are highlighted in yellow.

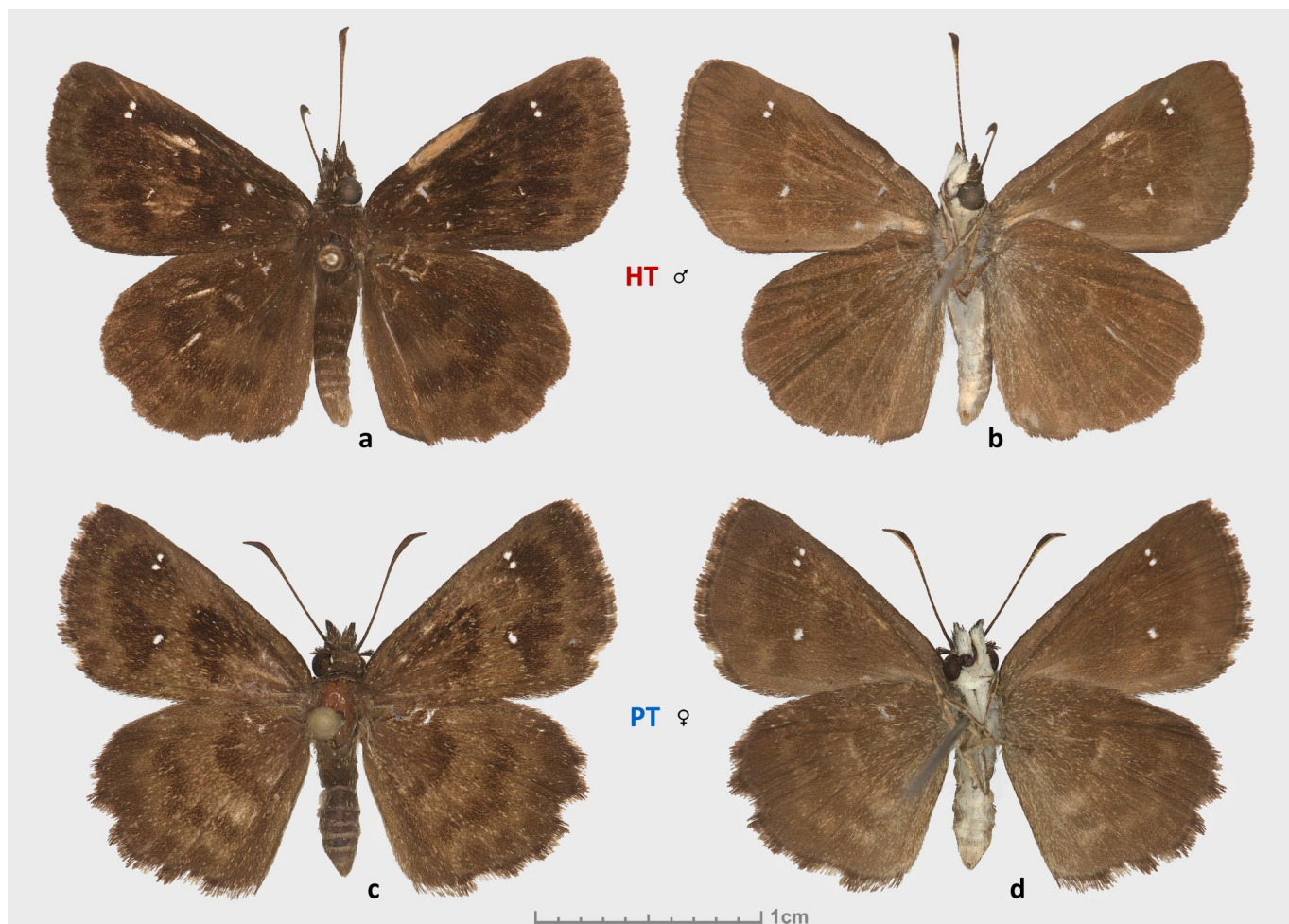


Fig. 2. *Staphylus floridus* sp. n. holotype, male, NVG-18058E05 (a, b), USA: Florida, Volusia Co. and a female paratype NVG-20049A08 (c, d), USA: Florida, Sumter Co., in dorsal (a, c) and ventral (b, d) views. Other data are in text.

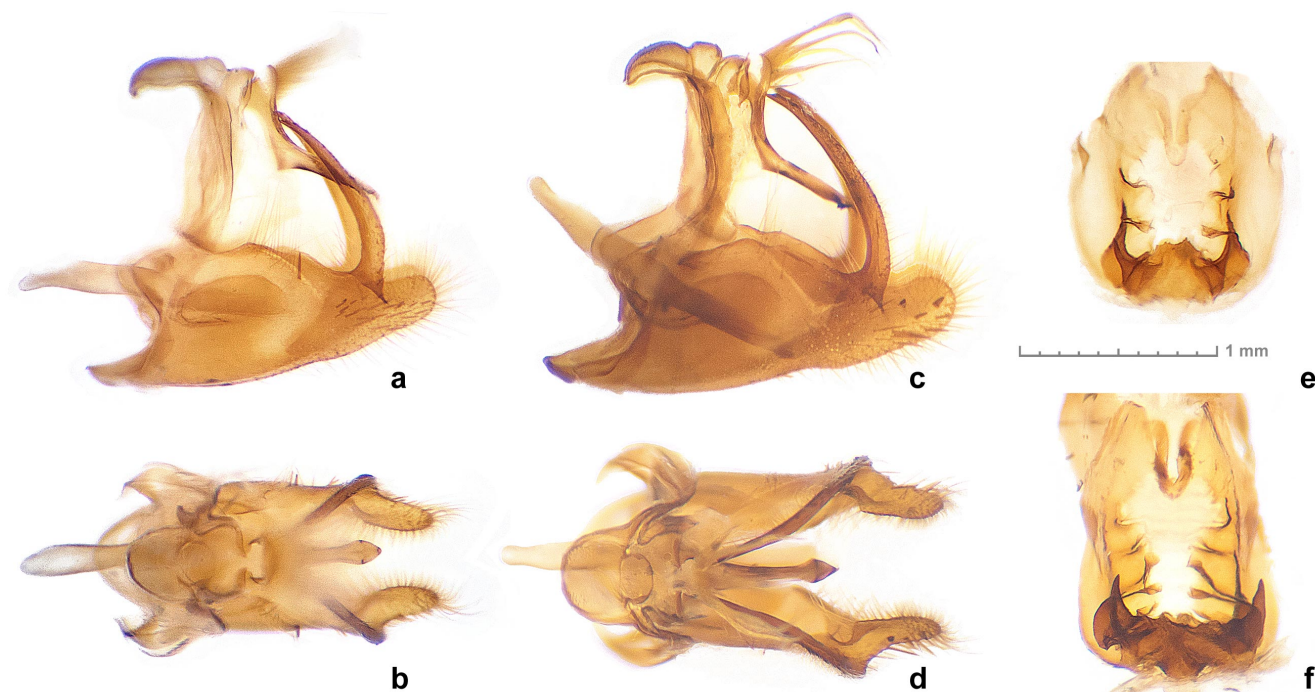


Fig. 3. Genitalia of *Staphylus floridus* sp. n. paratypes (a, b. NVG-20049A03 and e. NVG-20049A07, data in text) and *S. hayhurstii* from USA: Texas (c, d. NVG-12597, Dallas Co. and f. NVG-7042, Delta Co.), males (a–d) and females (e, f), in left lateral (a, c), dorsal (b, d), and ventral (e, f) views. Whole genitalic capsule is shown for males and sterigma for females.

distinguish them. This is hardly surprising for *Staphylus*, a genus that includes dozens of species nearly impossible to identify by facies even for more distant relatives, such as *Staphylus mazans* (Reakirt, [1867]) (type locality in Mexico: Veracruz) (Fig. 1 green), which is sympatric with *S. hayhurstii* in central Texas (e.g., in Comal Co., Fig. 1).

The new species differs from *S. hayhurstii* by less robust and relatively smaller genitalia, in both males (Fig. 3a–d) and females (Fig. 3e, f): illustrated genitalia are of specimens with similar forewing length. In male genitalia, tegumen longer and narrower, vs. more compact but broader tegumen in *S. hayhurstii*; harpe narrower (in lateral view), straighter, and bent less from the body of valva at its junction (best seen in dorsal view). In female genitalia, lamella postvaginalis less sculptured, typically with fewer and shorter ridges; lamella antevaginalis with comparatively smaller tooth on each side and more expanded middle section protruding caudad, with a central notch, vs. nearly crescent-shaped (tooth to tooth) lamella with irregular middle margin in *S. hayhurstii*. These genitalic characters are rather subtle, and due to individual variation the most confident identification can be achieved using DNA sequences. Combinations of the following DNA characters are diagnostic: in COI barcode: C271T, T322A, C401T, and T613C, and in the nuclear genome: aly1849.21.2:G3621A, aly1849.21.2:G3078A, aly1113.5.4: T1282A, aly1849.21.2:A702G, aly1656.10.4:T414G. Each of the barcode characters taken individually distinguishes the new species from *S. hayhurstii* in a sample of specimens we sequenced.

Barcode sequence of the holotype: Sample NVG-18058E05, GenBank ON351018, 658 base pairs:

```
AACTTTATATTTTATTTTGGTATTTGATCAGGAATAGTAGGAACCTCTTAAAGTATTTCTTATTCGTTCTGAATTAGGAACACCTGGATCTTTAATTGGAGATGATCAAATTTATAATACT
ATTGTAACAGCTCATGCTTTTATATAATTTTATATAGTTATACCTATTATAATTGGAGGATTCGGAATTGACTTGTTCTCTTATATTAGGAGCCCTGATAGCTTTTCCTCGAA
TAAATAATATAAGATTTTGATTATTACCTCCATCTTAACTTTTAAATTTCAAGAAGAATTGTAGAAAATGGAGCAGGAACCTGGATGAACAGTTTACCCCCCTTCAGCCAATATTGC
TCATCAAGGTCTCTCTAGATTAGCTATTTTCTTACATTTAGCAGGTATTTCTTCTATTTTAGGAGCAATTAATTTTATTACAACATATTATTAATATACGAATTAATAATTATCA
TTTGATCAAATACCTTTATTTGTTTGAGCTGTTGGAATTACAGCATTACTTTTATCTTTTACCAGTATTAGCAGGTGCTATTACTATACTTTTAACAGATCGAAATCTTAATACAT
CATTCTTCGATCTGCTGGTGGAGGAGATCCTATTTTATATCAACATCTATTC
```

Type material. Holotype: ♂ (Fig. 2a, b), deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA (USNM), bears the following three rectangular white printed (date partly handprinted) labels: [FLORIDA | Volusia Co. | New Smyrna Beach | 13 June 1969 | G. W. Rawson leg.], [DNA sample ID: | NVG-18058E05 | c/o Nick V. Grishin], [USNMMENT | {QR Code} | 01466715] and one red printed [HOLOTYPE ♂ | *Staphylus* | floridus Grishin]. **Paratypes:** 5♂♂ and 2♀♀, all from USA: Florida: 1♂ NVG-18058E06, Alachua Co., Alachua, leg. Scott W. Gross, 29-Aug-1987; others leg. John V. Calhoun: 3♂♂ Volusia Co.: NVG-20049A04, New Smyrna Beach, 2495 N Dixie Fwy, 5-Mar-1987; NVG-20049A03, W side of US1, 1 mi N of Brevard Co. line, 7-Mar-1993; and NVG-20049A06, Reed Grove Rd. N of Stacey Grove Rd., SW of Oak Hill, 20-Mar-1994; 1♂ NVG-20049A05 & 1♀ NVG-20049A07, Lee Co., W of IH75, off Carrell Rd. nr. Bonita Springs, 25-Mar-1983; 1♀ NVG-20049A08, Sumter Co., E side SR301, N side of Shady Brook, 14-May-1993 (coll. J. V. Calhoun, Palm Harbor, FL, USA).

Type locality. USA: Florida, Volusia County, New Smyrna Beach.

Distribution. Currently known only from USA: Florida with specimens sequenced from Alachua, Sumter, Volusia, and Lee Counties. This species may be allopatric with *S. hayhurstii*, and geographically separated from it by the lack of records in northern Florida and nearby parts of other states. This apparent gap in the distribution of *Staphylus* would be interesting to investigate further.

Etymology. Being a reference to Florida, the state from which the type series is known, a Latin word translated as flowery or blooming was chosen as the name for this not so garish species from Florida that nevertheless flaunts rather elaborate pattern of browns, nearly as flamboyant as gothic styles. The name is a masculine adjective.

Suggested English name. Florida Sootywing.

Lectotype designation for *Helias ascalaphus* Staudinger, 1876

Helias ascalaphus Staudinger, 1876 (type locality in Panama), the type species of *Staphylus*, was described from a series of specimens (Staudinger 1876), and several syntypes are in the MFNB collection.

We sequenced a male syntype already bearing a red "Lectotypus" label (designation unpublished), and the results agreed with the current application of this name (Fig. 1). To stabilize nomenclature, N.V.G. hereby designates this sequenced syntype, in the Museum für Naturkunde, Berlin, Germany bearing the following 8 rectangular labels [*Lectotypus*], [Origin.], [Panama | Ribbe], [*ascalaphus*], [*Ascalaphus* | *Stdgr.*], [GEN.PREP., | MIELKE | 1996], [{QR Code} <http://coll.mfn-berlin.de/u/908619>], and [DNA sample ID: | NVG-15033F12 | c/o Nick V. Grishin] as the **lectotype** of *Helias ascalaphus* Staudinger, 1876. The COI barcode sequence of the lectotype (GenBank ON351019) is:

AACTTTATATTTTATCTTTGGTATTTGATCTGGAATAGTAGGAACCTCTTTAAGTATTTCTATTCTGTTCTGAATTAGGAACCTCTGGATCTTTAATTGGAGATGATCAAAATTTATAATACT
ATTGTAACAGCTCATGCTTTTATTATAATTTTATAGTTATACCTATTATAATTGGAGGTTTGGAAATTGACTTGTTCTCTTATATTAGGAGCCCTGATATAGCTTTTCCCGAA
TAAATAATAAAGATTTGATTATTACCCCATCTTTAATACCTTTAATTTCAAGTAGAATCGTAGAAATGGAGCAGGTACTGGATGAACAGTTTATCCCCCTTTTCAGCTAACATTGC
CCATCAAGGTTCTTCTGTAGATTAGCTATTTTCTTTACATTAGCAGGTTTCTTCTATTTTGGAGGCAATTAATTTTATTACAATATTATCAATATACGAATTAATTAATTTATCC
TTTGATCAAATACCCTTATTGTTTGGAGCAGTTGGAATTACAGCATTATTATTACTTTTATCTTTACCAGTATTAGCAGGTGCTATTACTATACTTTTACAGATCGAAATCTTAATACAT
CATTTTTTGATCTGCTGGTGGAGGAGATCTATTATTTATATCAACATTATTC

Staphylus ecos Grishin, new species

<http://zoobank.org/33FF40E5-1E83-4E3E-8A0F-C85A81A0521A>

(Figs. 4 part, 5, 6a, b)

Definition and diagnosis. Genomic analysis of specimens identified as *Staphylus ceos* (W. H. Edwards, 1882) (type locality in USA: Arizona, Graham Co.) from across its range, including the lectotype (NVG-15096H06), reveals their split into two prominent clades with 100% statistical support in the mitochondrial genome tree (Fig. 4c red and blue clades), substantiated by a shallow but consistent split in both nuclear genome trees (Fig. 4a, b). The red clade consists of specimens from the eastern part of the range and the blue clade includes the rest, where the lectotype of *S. ceos* belongs. Genetic differentiation between the two clades is limited in the nuclear genome, but is substantial in the mitogenome. *Fst*/*Gmin* computed on the Z chromosome are 0.08/0.15, well within the range of one species (Cong et al. 2019a). Mitogenomes of the two groups of specimens (red and blue) diverged strongly, e.g., their COI barcodes differ by 2.7% (18 bp), a value typically sufficient to support species-level distinction (Hebert et al. 2003), but only in the presence of phenotypic differences. Comparison of male genitalia reveals the differences that are typical of closely

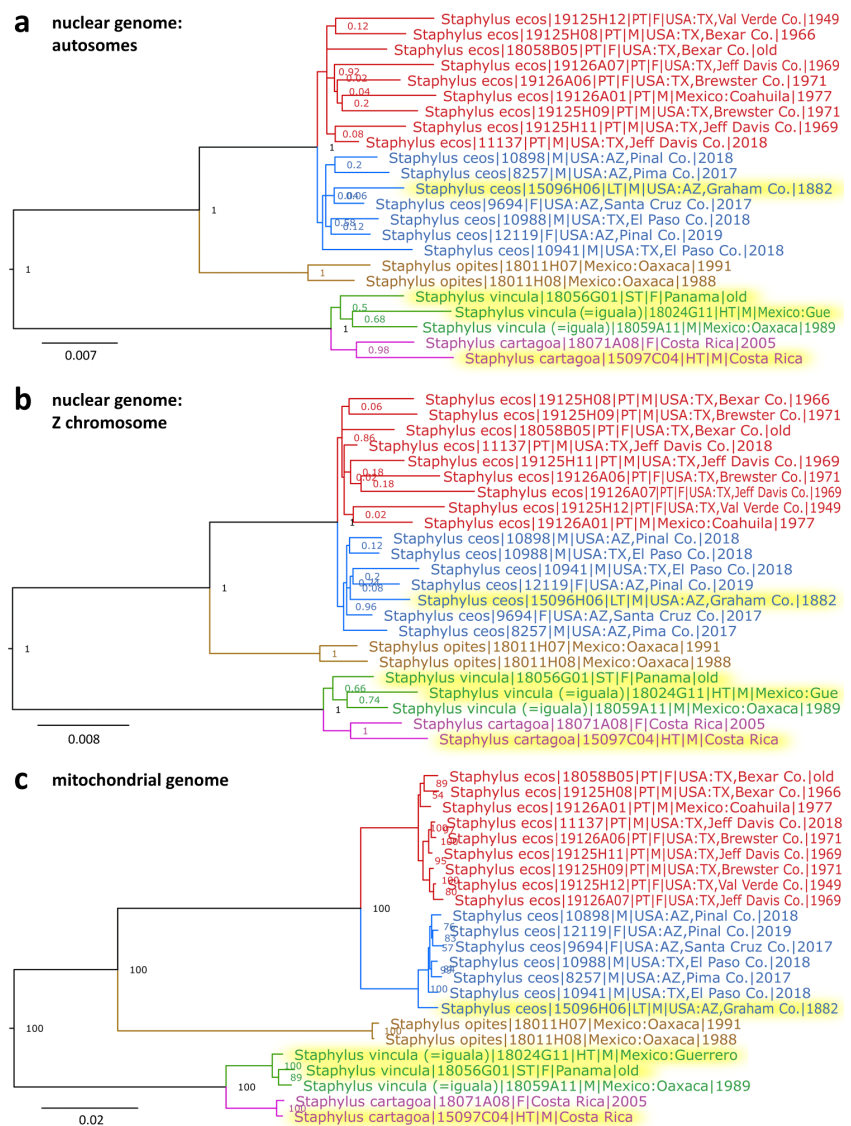


Fig. 4. Phylogenetic analysis of *Staphylus ecos* sp. n. and *S. ceos*. The trees are constructed from protein-coding regions in: **a.** autosomes, **b.** Z chromosome, **c.** mitochondrial genome. Different species are shown in different colors. Names of primary type specimens are highlighted in yellow.

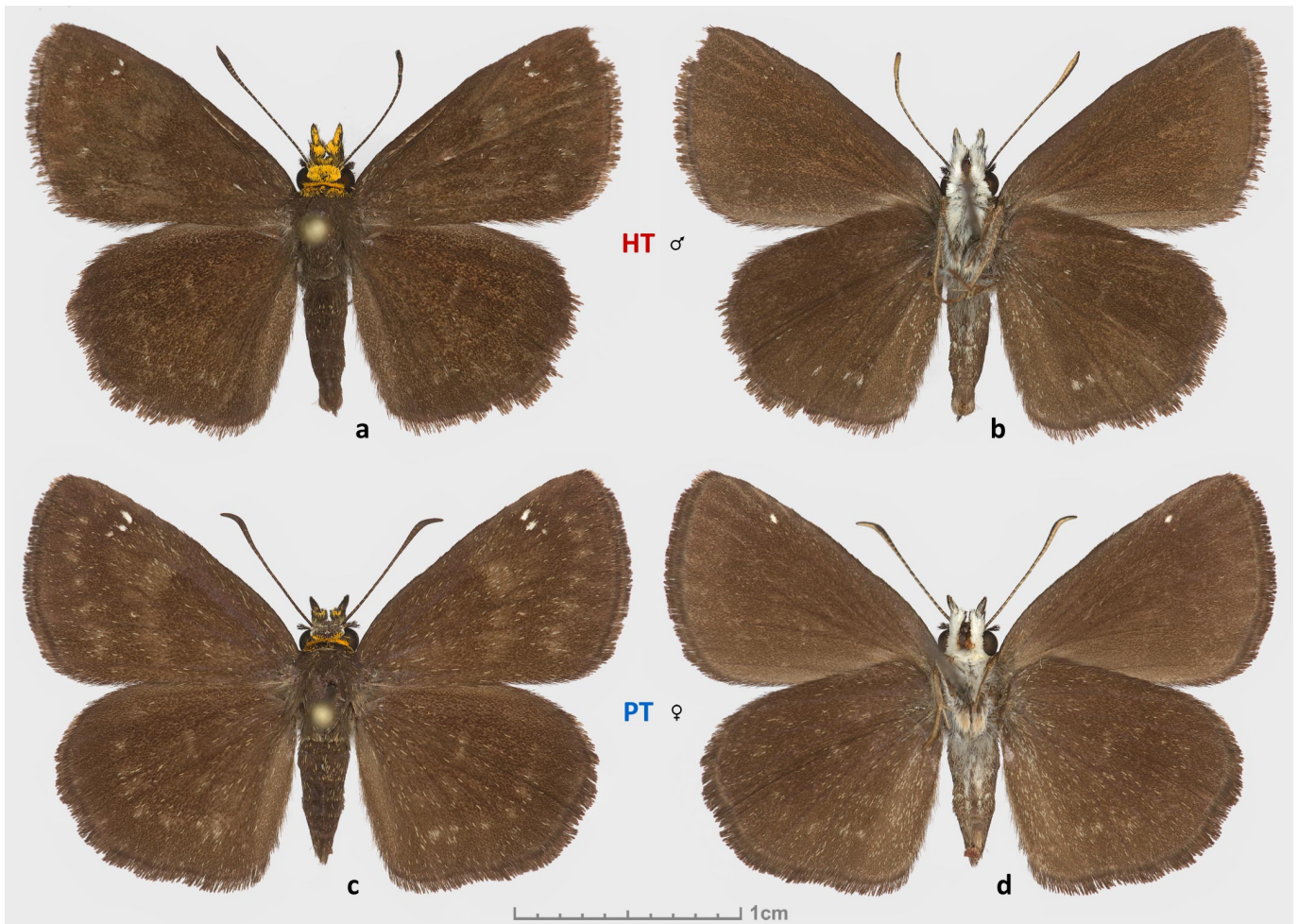


Fig. 5. *Staphylus ecos* sp. n. holotype, male, NVG-21113E02 (a, b) and a female paratype NVG-21113E03 (c, d), from USA: Texas, Brewster Co., Big Bend National Park, Chisos Mountains, in dorsal (a, c) and ventral (b, d) views. Other data are in text.

related but distinct species, and therefore we conclude that the red clade (Fig. 4) represents a new species. This species (Fig. 5) is superficially similar to its sister *S. ceos*, and we were not able to find meaningful wing pattern characters to differentiate them. This is typical for *Staphylus*, a genus that includes a number of species with similar appearance, even for more distant relatives.

The new species differs from *S. ceos* by more robust and relatively larger male genitalia (Fig. 6): illustrated genitalia are of specimens with similar forewing length. Ampulla of valva with nearly straight distal margin (in lateral view), not concave as in typical *S.*

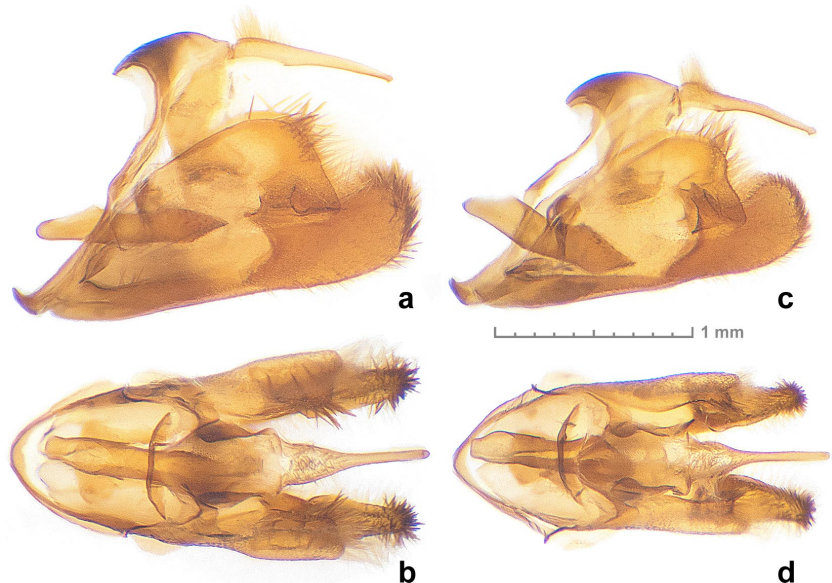


Fig. 6. Male genitalia of *Staphylus ecos* sp. n. (a, b), paratype NVG-11137, USA: Texas, Jeff Davis Co. and *S. ceos* (c, d), NVG-10941, USA: Texas, El Paso Co. in left lateral (a, c) and dorsal (b, d) views, whole genitalic capsule.

ceos; harpe longer relatively to its height (in lateral view), less curved dorsad, with longer dorsal ridge that projects farther anteriad (towards vinculum) connecting to valva at several points that look like "teeth" in lateral view, and with thicker short spines at the distal end, vs. *S. ceos*, which has a shorter, more square-shaped harpe that is terminally more curved dorsad, the dorsal ridge that connects to valva closer to the ampulla and well before the anterior portion of valva along ventral margin, the "teeth" formed by connecting points of the ridge with valva that appear smaller, and distal spines that are more minute. These genitalic characters are rather subtle, and due to individual variation, the most confident identification can be achieved using DNA sequences. Combinations of the following DNA characters are diagnostic: in COI barcode: T67C, T205C, T337A, T478C, and T514C, and in the nuclear genome: aly1113.5.4:A1684G, aly1849.21.2:A8067G, aly1849.21.2:A8055G, aly1113.5.4:A1338C. Each of the barcode characters taken individually distinguishes the new species from *S. ceos* in a sample of specimens we sequenced.

Barcode sequence of a paratype: Sample NVG-19126A06, GenBank ON351020, 658 base pairs:

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AACCTTTATATTTTATTTTGGTATTTGATCTGGAATAGTAGGAACCTCTTTAAGTATTTCTTATTCGCTCAGAATTAGGAACCCAGGATCTTTAATTGGAGATGATCAAAATTTATAATACT
ATTGTAACAGCTCATGCTTTTATTATAATTTTATATAGTAATACCTATTATAATCGGAGGTTTGGAAATTGATTAGTACCCCTAATATTAGGAGCCCCAGATATAGCTTTCCCTCGAA
TAAATAATATAAGTTTCTGATTATACCCCTCTCTTATACCTTTAATTTCAAGTAGTATTGTAGAAAATGGAGCAGGTACAGGATGAAGTATACCCCTCTTTTACGCTAATATTGC
TCATCAAGGATCATCTGTAGATTAGCTATTTTCCCTTCATTTAGCCGGAATTTCTTCAATTTTAGGGGCAATTAATTTTATTACAATATTATTAATATACGAATTAATAACTTATCA
TTTGATCAAATACCTTTATTTGTTGAGCCGTAGGTATTACAGCATTAATTTTACTTTTATCTCTCCAGTATTAGCTGGTGTCTATTACTATACTTTTACAGATCGAAATCTTAATACAT
CATTTTGTGACCTGCAGGAGGTGGAGATCCTATTTTATACCAATTTTATTT
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Type material. Holotype: ♂ (Fig. 5a, b), deposited in the National Museum of Natural History, Smithsonian Institution, Washington, DC, USA (USNM), bears the following two rectangular white printed labels: [Green Gulch | Big Bend Nat. Park | Brewster Co. TX | 12-Jun-2004 USA | leg. Grishin N. V.], [DNA sample ID: | NVG-21113E02 | c/o Nick V. Grishin], and one red printed [HOLOTYPE ♂ | *Staphylus* | *ecos* Grishin]. **Paratypes:** 8♂♂ and 5♀♀, most collected by Roy O. Kendall & C. A. Kendall (ROK) and Nick V. Grishin (NVG), from USA: Texas: 1♂ NVG-19125H10, Comal Co., New Braunfels, Panther Canyon, leg. W. W. McGuire, 6-May-1972; Bexar Co.: 1♂ NVG-19125H08, USH281 north at Salado Creek, ROK, 4-Jun-1966; 1♀ NVG-18058B05, O. C. Poling, no date, estimated around 1900; 1♀ NVG-19125H12, Val Verde Co., Del Rio, leg. Hugh Avery Freeman, 12-Jul-1949; Brewster Co., Big Bend National Park: 1♂ NVG-19125H09, 22-Sep-1971 & 1♀ NVG-19126A06, 14-Sep-1971, K-Bar Research Station, ROK; 1♀ NVG-21113E03, Chisos Basin Campground, NVG, 29-May-2004; Jeff Davis Co.: 1♂ NVG-19125H11, 10-Jul-1969 & 1♀ NVG-19126A07, 7-Jul-1969, Musquiz Canyon, SH118, ROK; 1♂ NVG-11137, Jeff Davis Co., Fort Davis city limits, NVG, 18-May-2018; 2♂♂ NVG-21113E04 & 5, Jeff Davis Co., Davis Mts. State Park, nr. Indian Lodge, NVG, 25-Mar-2005; and 1♂ NVG-19126A01, Mexico: Coahuila, Hwy 57 ca. 18 mi S of Monclova, ROK, 14-Sep-1977.

Type locality. USA: Texas, Brewster County, Big Bend National Park, Green Gulch, elevation 1960 m, GPS 29.2769, -103.2836.

Distribution. Confirmed from USA: Texas, in the following counties: Jeff Davis, Brewster, Val Verde, Bexar, and Comal; and from Mexico: Coahuila, ca. 18 mi S of Monclova. Specimens we sequenced from El Paso County Texas were *S. ceos* (Fig. 4). The two sister species are currently allopatric, and the boundary between them in the USA lies somewhere between the Davis Mountains and the Franklin Mountains in Texas with no known intervening populations. Further studies of these species may be productive in Chihuahua, Mexico, where they may closely approach one another.

Etymology. The species that ec[h]o[e]s *ceos* with distortion and the name is its anagram. For a simple mnemonic, think of "e" for "east" for this more eastern species. The name is a noun in apposition.

Suggested English name. Texas Sootywing.

***Staphylus opites* (Godman & Salvin, 1896), reinstated status**

Scantilla opites Godman & Salvin, 1896 (type locality in Guatemala) was placed as a junior subjective synonym of *Staphylus vincula* (Plötz, 1886) (type locality in Panama) by Evans (1953). Genomic sequencing of *Tagiades vincula* Plötz, 1886 syntype in ZSMC (NVG-18056G01, Fig. 7a–c) reveals that it

is in a different clade from the two specimens of *S. opites* from Mexico: Oaxaca (Fig. 4). This syntype, a female (Fig. 7a–c), agrees with the original description of *T. vincula*. It is close in appearance to the specimen from Costa Rica in BMNH that Godman considered to be similar to the original Plötz illustration (Godman 1907), and is curated as a type in ZSMC. Therefore, we accept it as a true syntype, and due to significant genetic differentiation between the two taxa, reinstate species-level status for *Staphylus opites* (Godman & Salvin, 1896), **stat. rest.** The COI barcode sequence of the *T. vincula* syntype (GenBank ON351021) is:

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AACTTTATATTTTATTTTGGTATTTGATCCGGTATAGTAGGAACCTCTTTAAGTATTCTTATTCGTTTCCAGAACTAGGAACCTCTGGATCTTTAATTGGAGATGATCAAAATTTACAATACT
ATTGTAACAGCTCATGCTTTTATTATAATTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGATTAGTACCTTAATATTAGGAGCTCCTGATATAGCTTTCCCTCGAA
TAAATAATATAAGTTTGTATTATACCCCTCTCTTATACCTTTAATTTCAAGTAGTATTGTAGAAATGGAGCAGGTACAGGATGAACCTTTTACCACCTCTTTCAGCAATATTGC
TCATCAAGGAGCATCTGTAGATTAGCTATTTTCCCTTCATTAGCAGGAATTTCTCAATTTTAGGAGCAATTAATTTTATTACAATATTATTAATATACGAATTAATTAATTATCA
TTTGATCAAAATACCTTTATTCGTTTGAAGCTGTAGGAATTACAGCTTTACTTTTACTTTTACCAGTATTAGCAGGTGCTATTACTATACTTTTAACTGATCGAAATCTTAATACAT
CATTTTTTGATCCAGCAGGAGGAGGAGATCCAATTTTATATCAACATTTTATT
```

Additionally, we found an illustration of *T. vincula* (Fig. 7d) pinned in a *Staphylus* drawer in USNM. This color illustration depicts the left dorsal surface and is rather similar to a Costa Rican specimen from the Godman & Salvin collection in BMNH, which lacks the abdomen and is spread in a similar manner. Furthermore, the illustration shows three subapical hyaline spots, as the BMNH specimen, but the syntype has two, as stated in the original description. Hence, this illustration is possibly of the BMNH specimen, rather than a copy of the original Plötz's drawing.

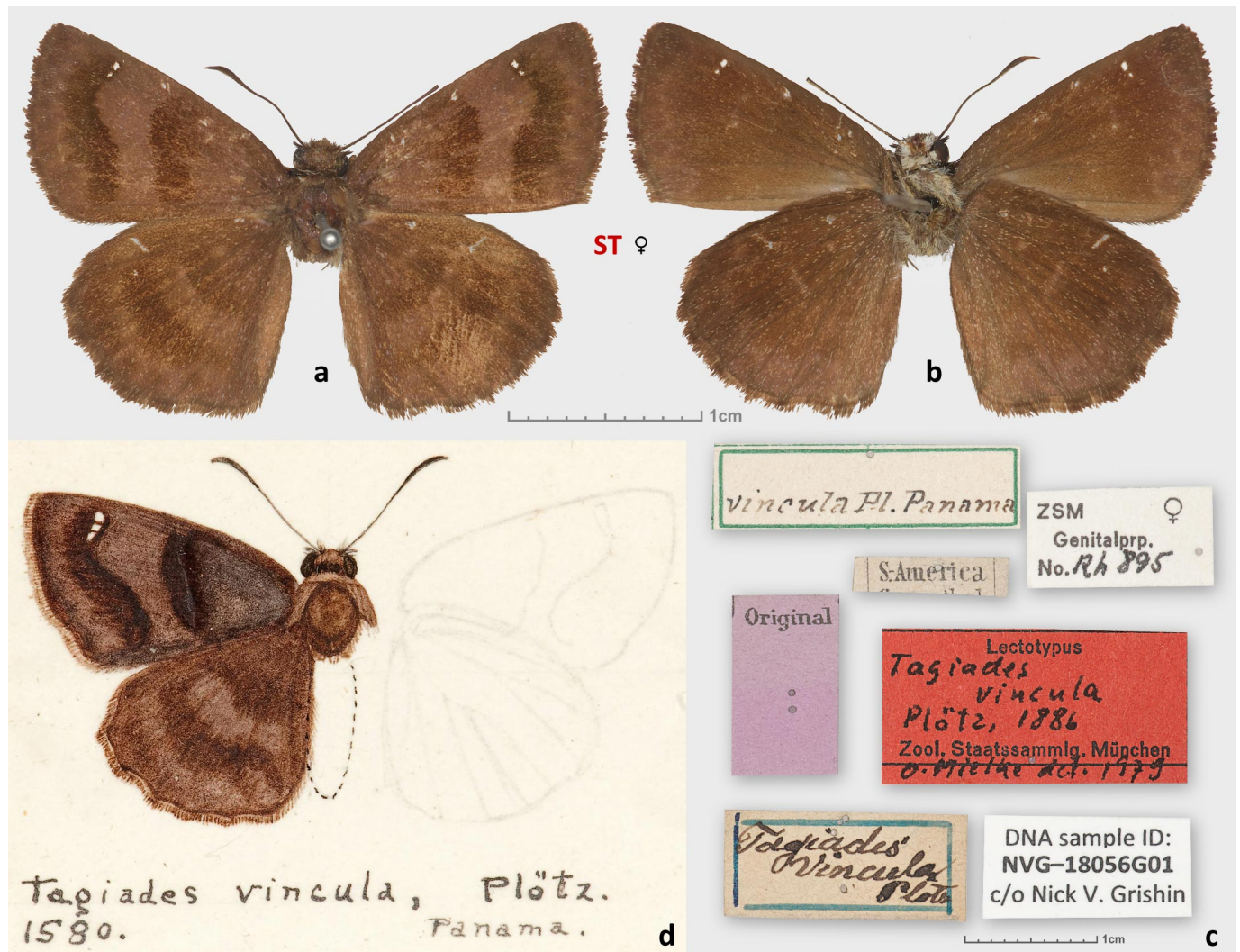


Fig. 7. *Staphylus vincula* syntype, female, NVG-18056G01 in ZSMC: **a.** dorsal and **b.** ventral views, **c.** labels (lectotype designation was not published and the specimen remains a syntype); **d.** an illustration pinned in a *Staphylus* drawer in USNM, possibly of a specimen from Costa Rica in BMNH, which Godman (1907) considered to be similar to the original illustration of *Tagiades vincula* by Plötz. Larger scale bar refers to the specimen and illustration and smaller scale bar refers to labels (reduced compared to the specimen).

***Pholisora iguala* Williams & Bell, 1940 is a junior subjective synonym of *Staphylus vincula* (Plötz, 1886)**

As we have shown above, *Staphylus opites* (Godman & Salvin, 1896), stat. rest. (type locality in Guatemala) and *Staphylus vincula* (Plötz, 1886) (type locality in Panama) are distinct, non-sister species (Fig. 4). While *S. vincula* is sister to *Staphylus cartagoa* (Williams & Bell, 1940) (type locality in Costa Rica), *S. opites* is sister to the clade consisting of *Staphylus ceos* (W. H. Edwards, 1882) (type locality in USA: Arizona, Graham Co.) and *Staphylus ecos* sp. n. (type locality in USA: Texas, Brewster Co.). Genomic sequencing of the holotype of *Pholisora iguala* Williams & Bell, 1940 (type locality in Mexico: Guerrero) in the AMNH (NVG-18024G11), together with a more recently collected specimen identified as *S. iguala* (NVG-18059A11), reveals that they are genetically close to the syntype of *S. vincula*, in both nuclear and mitochondrial genomes. Moreover, their COI barcodes differ by only 0.3% (2 bp). Therefore, we propose that *Pholisora iguala* Williams & Bell, 1940 **syn. n.** is a junior subjective synonym of *Staphylus vincula* (Plötz, 1886). As a result of this change, current synonyms of *P. iguala* become junior subjective synonyms of *S. vincula*. This genetic similarity with Mexican specimens casts certain doubt on the Panamanian origin of the only known *S. vincula* syntype, and this question should be investigated further by sequencing of additional specimens. Furthermore, the syntype bears two conflicting locality labels: "Panama" and "S[outh] America" (Fig. 7d), but the original description gives Panama as the locality, where it was supposedly collected by Ribbe. Ribbe is not mentioned on the labels of the syntype. Genomic sequencing suggests that its South American (and maybe even Panamanian) origin is unlikely, and therefore the specimen was possibly mislabeled. For these reasons, we refrain from designating it a lectotype (it already bears a lectotype label, but this designation remains unpublished) waiting for further research. However, this is the only credible syntype we located thus far, and we are using it to define the identity of *S. vincula*. The COI barcode sequence of the *P. iguala* holotype (GenBank ON351022) is:

```
AACTTTATATTTATTTTGGTATTTGATCCGGTATAGTAGGAACCTCTTAAGTATTTCTATTCGTTTCAGAACTAGGAACCTCTGGATCTTTAATTGGAGATGATCAAATTTACAATACT  
ATTGTAACAGCTCATGCTTTTATTATAATTTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTTGATTAGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCCTCGAA  
TAAATAATATAAGTTTTTGATTATTACCTCCTTCTTATACCTTTTAATTTCAAGTAGTATTGTAGAAAATGGAGCAGGTACAGGATGAACCTTTACCCACCCCTTCAGCCAATATTGC  
TCATCAAGGAGCATCTGTAGATTAGCTATTTTCCCTTCATTTAGCAGGAATTTCTTCAATTTTAGGAGCAATTAATTTTATTACAACCTATTATTAATATACGAATTAATAATTATCA  
TTTGATCAAATACCTTTATTCGTTTGAGCTGTAGGAATTACAGCTTTTACTTTTATCTTTTACCAGTATTAGCAGGTGCTATTACTATACTTTTAACTGATCGAAATCTTAATACAT  
CATTTTTTGATCCAGCAGGAGGAGGAGATCCAATTTTATATCAACATTTATTT
```

DISCUSSION

Screening of butterfly populations across their distribution ranges by genomic sequencing to probe genetic diversification is productive in revealing new taxa. Addition of primary type specimens to these datasets puts this genetic approach on a solid nomenclatural footing. Traditionally done using only COI barcodes, such screens have been insightful (Hebert et al. 2004; Burns and Janzen 2005; Burns et al. 2007; Burns et al. 2008), but total genomic coverage alleviates the problems with introgression and other irregularities of COI barcodes. Here, we applied the genomic approach that led to the discovery of two new, partly cryptic butterfly species in the United States. Genetic differences are reflected in genitalic differences, although these are rather subtle and therefore difficult to evaluate without genetic differentiation.

Geographically, the two allopatric species in each *Staphylus* pair are separated by well-documented suture zones (Remington 1968; Rising 1983), which form known boundaries for other butterfly taxa. For instance, the North Florida suture zone separates *Erynnis somnus* (Lintner, 1881) from *Erynnis brizo* (Boisduval & Le Conte, [1837]) (Burns 2020), as well as *Staphylus floridus* sp. n. from *S. hayhurstii*, among many subspecies-level taxa (Warren et al. 2016). The Central New Mexico suture zone separates a number of species pairs, such as *Megathymus violae* D. Stallings & Turner, 1956 from *Megathymus ursus* Poling, 1902 (Zhang et al. 2020), *Poladryas minuta* (W. H. Edwards, 1861) from *Poladryas arachne* (W. H. Edwards, 1869), *Microtia elada* (Hewitson, 1868) from *Microtia perse* (W. H. Edwards, 1882), and *Junonia coenia* Hübner, [1822] from *Junonia grisea* Austin & J. Emmel, 1998 (Lalonde and Marcus 2019), among others, together with *Staphylus ecos* sp. n. from *S. ceos*. Notably, in

this case, the exact geographic position of the boundary between the two species in every pair varies somewhat: it could be to the west (as for *Megathymus*) or to the east (as for *Staphylus*) of El Paso.

Textbooks can be written about species delimitation and its practice. For practical applications, we reason that genetic differentiation is by itself sufficient to substantiate species-level status of a taxon under certain conditions. This differentiation should be: 1) strongly supported by statistics, e.g., bootstrap-like values near 100%; 2) consistent throughout the genomes, both nuclear and mitochondrial, e.g., Fig. 1 red and blue clades; and 3) of a magnitude documented for well-studied species (Cong et al. 2019a). This is even more meaningful when coupled with differences in genitalia, although these differences would necessarily be subtle for closely related species. The second case (Fig. 4 red and blue clades) provides more food for thought. Although mitochondrial genome differentiation is prominent (Fig. 4c), and agrees with that of closely related but distinct species (2.7% COI barcode difference), nuclear genomes did not diverge much, and this divergence does not pass our conservative criteria for speciation. However, nuclear genomic differences are consistent between autosomes and the Z chromosome (Fig. 4a, b) and therefore are a reflection of distinct evolutionary paths of these clades. Due to the small divergence, we considered proposing *S. ecos* sp. n. as a subspecies of *S. ceos*. However, this opinion changed after genitalic comparison. The differences in male genitalia are consistent in magnitude with those used to support new species even in the absence of DNA evidence. Further studies of *S. ceos* species complex in Mexico are likely to enrich our understanding of speciation.

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