CompCytogen 5(3): 191–210 (2011)
doi: 10.3897/CompCytogen.v5i3.1730
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

"Darwin's butterflies"? DNA barcoding and the radiation of the endemic Caribbean butterfly genus *Calisto* (Lepidoptera, Nymphalidae, Satyrinae)

Andrei Sourakov[1,†], Evgeny V. Zakharov[2,‡]

1 *McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA* **2** *Biodiversity Institute of Ontario, University of Guelph, Guelph, ON, Canada N1G 2W1*

Corresponding author: *Andrei Sourakov* (asourakov@flmnh.ufl.edu)

Academic editor: *V Lukhtanov* | Received 20 June 2010 | Accepted 4 July 2010 | Published 24 August 2011

Citation: Sourakov A, Zakharov EV (2011) "Darwin's butterflies"? DNA barcoding and the radiation of the endemic Caribbean butterfly genus *Calisto* (Lepidoptera, Nymphalidae, Satyrinae). Comparative Cytogenetics 5(3): 191–210. doi: 10.3897/CompCytogen.v5i3.1730

Abstract

The genus *Calisto* Hübner, 1823 is the only member of the diverse, global subfamily Satyrinae found in the West Indies, and by far the richest endemic Caribbean butterfly radiation. *Calisto* species occupy an extremely diverse array of habitats, suggestive of adaptive radiation on the scale of other classic examples such as the Galápagos or Darwin's finches. However, a reliable species classification is a key requisite before further evolutionary or ecological research. An analysis of 111 DNA 'barcodes' (655 bp of the mitochondrial gene COI) from 29 putative *Calisto* species represented by 31 putative taxa was therefore conducted to elucidate taxonomic relationships among these often highly cryptic and confusing taxa. The sympatric, morphologically and ecologically similar taxa *C. confusa* Lathy, 1899 and *C. confusa debarriera* Clench, 1943 proved to be extremely divergent, and we therefore recognize *Calisto debarriera* **stat. n.** as a distinct species, with *Calisto neiba* Schwartz et Gali, 1984 as a junior synonym **syn. n.** Species status of certain allopatric, morphologically similar sister species has been confirmed: *Calisto hysius* (Godart, 1824) (including its subspecies *C. hysius aleucosticha* Correa et Schwartz, 1986, **stat. n.**), and its former subspecies *C. batesi* Michener, 1943 showed a high degree of divergence (above 6%) and should be considered separate species. *Calisto lyceius* Bates, 1935/*C. crypta* Gali, 1985/*C. franciscoi* Gali, 1985 complex, also showed a high degree of divergence (above 6%), confirming the species status of these taxa. In contrast, our data suggest that the *Calisto grannus* Bates, 1939 species complex (including *Calisto*

grannus dilemma González, 1987, *C. grannus amazona* González, 1987, **stat. n.**, *C. grannus micrommata* Schwartz et Gali, 1984, **stat. n.**, *C. grannus dystacta* González, 1987, **stat. n.**, *C. grannus phoinix* González, 1987, **stat. n.**, *C. grannus sommeri* Schwartz et Gali, 1984, **stat. n.**, and *C. grannus micheneri* Clench, 1944, **stat. n.**) should be treated as a single polytypic species, as genetic divergence among sampled populations representing these taxa is low (and stable morphological apomorphies are absent). A widely-distributed pest of sugar cane, *Calisto pulchella* Lathy, 1899 showed higher diversification among isolated populations (3.5%) than expected, hence supporting former separation of this species into two taxa (*pulchella* and *darlingtoni* Clench, 1943), of which the latter might prove to be a separate species rather than subspecies. The taxonomic revisions presented here result in *Calisto* now containing 34 species and 17 subspecies. Three species endemic to islands other than Hispaniola appear to be derived lineages of various Hispaniolan clades, indicating ancient dispersal events from Hispaniola to Puerto Rico, Cuba, and Jamaica. Overall, the degree of intrageneric and intraspecific divergence within *Calisto* suggests a long and continuous diversification period of 4–8 Myr. The maximum divergence within the genus (ca. 13.3%) is almost equivalent to the maximum divergence of *Calisto* from the distant pronophiline relative *Auca* Hayward, 1953 from the southern Andes (14.1%) and from the presumed closest relative *Eretris* Thieme, 1905 (14.4%), suggesting that the genus began to diversify soon after its split from its continental sister taxon. In general, this 'barcode' divergence corresponds to the high degree of morphological and ecological variation found among major lineages within the genus.

Keywords

COI, biogeography, DNA barcoding, islands, intraspecific variation, Lepidoptera, Nymphalidae, Satyrinae, speciation, taxonomy

Introduction

The genus *Calisto* Hübner, 1823 is endemic to the West Indies, and, until the present revision, comprised 54 named taxa (Lamas et al. 2004) of small to medium sized butterflies in the subfamily Satyrinae, a diverse global radiation including ca. 2,200 described species. *Calisto* is considered a member of the neotropical subtribe Pronophilina, but while many *Calisto* are lowland dwellers, occurring as low as sea level, almost all other pronophilines are exclusively montane and/or temperate. Most of the extant described species of *Calisto* are found on the island of Hispaniola, with a single species on Jamaica, one on Puerto Rico, two species on the Bahama Islands, one on Anegada, and two on Cuba (Smith et al.1994).

Though *Calisto* are neither visually spectacular nor economically important (with the exception of *C. pulchella* Lathy, 1899 , which is a pest of sugar cane), a significant amount of information is available on the distribution of the more common species on Hispaniola from the general survey of the island's butterflies by Schwartz (1989). However, phylogenetic relationships of the genus are unclear and affinities to both South American and African taxa have been proposed based on adult morphology (Riley 1975, Miller and Miller 1989), although the most recent taxonomic treatments of the tribe (e. g., Viloria 1998; Lamas et al. 2004) kept *Calisto* in Pronophilina. The

montane neotropical genus *Eretris* Thieme, 1905 has been considered one of the closest relatives of *Calisto* by some (Miller 1968, De Vries 1987, Peña et al. 2011).

The morphology of immature stages has been utilized extensively in phylogenetic studies of butterflies (e. g., Kitching 1985, Murray 2001, Penz and DeVries 2001, Freitas and Brown 2004, Willmott and Freitas 2006), however this has been mostly at higher taxonomic levels. *Calisto* is one of the few satyrine genera for which the immature stages have been studied in detail at least for most major species groups, providing insights into a high degree of morphological diversification in the egg and larvae, atypical for other satyrine genera (Sourakov 1996, 2000). Structures that normally show little variation in the Satyrinae intragenerically, such as male and female genitalia, are also remarkably diverse in *Calisto* (Sourakov 1997). Until now, however, it has been unclear whether this spectacular morphological variation results from an ancient history of divergence, or from strong disruptive selection on traits potentially involved with fitness and reproductive isolation.

Many species of *Calisto* were described only recently, towards the end of the 20th century (e.g., Schwartz and Gali 1984, Gali 1985, Johnson and Hedges 1998) and are still known only from the type series. Small numbers of specimens, in conjunction with reliance on wing pattern elements alone, which often seem to be variable in better known taxa, makes the status of many of these recent names difficult to determine. For example, additional eyespots were used to define the species *C. neiba* Schwartz et Gali, 1984 and *C. amazona* González, 1987. Many of these names might thus prove to be synonyms, or, conversely, represent a formerly unexplored array of cryptic species that are only just being recognized. The taxonomic confusion is evident in Smith et al.'s (1994) comprehensive treatment of Caribbean butterflies; they listed all the described taxa, but for many species avoided illustrating them and provided inconclusive comments on the validity of many taxa. For instance, they did not illustrate *Calisto montana* Clench, 1943 for which only the male holotype is known, and of which even the precise collecting locality is uncertain. For *Calisto neiba*, Smith et al. (1994) stated that it has additional wing ocelli (which are, however, a variable character in many Satyrinae (e. g., Sourakov 1995, Kooi et al. 1996), repeatedly appearing within all species of *Calisto*, usually as an occasional aberration), and concluded that "the final estimate of the affinities of *C. neiba* cannot yet be made." Another un-illustrated species, *C. aleucosticha* Correa et Schwartz, 1986, described from a couple of individuals that could represent aberrant *C. batesi* Michener, 1943 females, was assessed as "very close to *C. hysius* (Godart, 1824), and discovery of the male may well clarify its status." The illustration of *Calisto micheneri* Clench, 1944 represents a taxon similar to our concept of *C. grannus dilemma* González, 1987, a taxon not illustrated by Smith et al. (1994) and said to be known "from a single specimen only. It is readily confused with other common species, and may well be more frequent than the rather sparse records would suggest." Also not illustrated were *Calisto phoinix* González, 1987, of which Smith et al. (1994) said that "there seems little doubt that this species is not conspecific with *C. grannus*, but their relationships remains to be established," and *C. dystacta* González, 1987, which "occurs at lower altitude than *C. phoinix*. The two are very similar and

may be conspecific." We examined type specimens and the original descriptions of Schwartz and Gali (1984) and González (1987) and could only conclude that these names most likely represent variants of *Calisto grannus* Bates, 1939 found at unusual elevations and slopes, and hence exhibiting slightly different phenotypes from typical specimens of the latter taxon.

A different issue is presented by the taxa that are clearly allopatric (and probably remained in isolation for a long time), but which are so morphologically similar that one must question the extent of diversification between them. For instance, Smith et al. (1994) treat *Calisto batesi* as a separate species, following treatment by Schwartz (1989), yet state that "this insect has generally been considered a subspecies of *C. hysius*." Originally, *batesi* was described as a subspecies of *hysius* and Smith et al. (1994) chose to illustrate *C. batesi*, but did not illustrate *C. hysius*, because, we presume, the main difference between these taxa aside from their distribution is their size (*batesi* 13–15 mm; *hysius* 16.5–17.5mm), while the wing patterns are identical.

We find allopatric similar taxa within other major species groups, such as *C. chrysaoros* Bates, 1935 (names include *Calisto galii* Schwartz, 1983 and *galii choneupsilon* Schwartz, 1985) and *C. lyceius* Bates, 1935 (names include *Calisto crypta* Gali, 1985 and *Calisto franciscoi* Gali, 1985). In the *Calisto confusa* Lathy, 1899 complex, the name *C. confusa debarriera* Clench, 1943 has been attributed to a form with reduced white discal and extradiscal bands on the underside, which is found throughout the geographic range of *C. confusa confusa* and is occasionally sympatric, though frequently replaces typical *C. confusa* phenotypes at higher elevations. *Calisto montana* Clench, 1943 was described from the same group based on a single very worn specimen which had an unusual double-pupiled eye-spot on the underside of its forewing (Fig. 7) – a character found occasionally throughout *Calisto*. Other taxa within *Calisto confusa* species complex have also been described, such as *C. gonzalezi* Schwartz, 1988 for which Smith et al. (1994) state that "the exact relationship between this species and *C. confusa* remains to be clarified should new populations of *C. gonzalezi* be discovered."

The above confusion over the recently described taxa is perhaps partly due to sole reliance of the authors on wing characteristics combined with distribution data in their approach to delineating new species, partly due to limited series and quality of specimens, and partly due to the exercising of the typological approach in its extreme form, with a disregard for interspecific variation. A possible solution to the problem is to use a new set of characters such as molecular sequence data. The technique of 'DNA barcoding' is based on the analysis of short, standardized gene regions; in the case of animals, this is a 655-bp segment of mitochondrial cytochrome oxidase subunit I (COI). DNA barcoding potentially provides an efficient method for species identification as well as for solving species-level taxonomical problems. Although the DNA barcode region can vary intraspecifically on a geographic scale as well as within populations (e. g., Lukhtanov et al. 2009, DeWalt 2011), and has shown varying degrees of success in species delimitation (e.g., Wiemers and Fiedler 2007), it has overall proved to be an excellent tool for species identification as illustrated in several large Holarctic

Lepidoptera groups (Hebert et al. 2010, Lukhtanov et al. 2009). In the present study, therefore, we explore the potential for DNA barcode data to attempt to answer long-standing questions concerning interspecific and intraspecific relationships within the genus by studying 21 species of *Calisto* (representing almost all of the major species groups). We examine a number of questionable taxa, such as representatives of *C. grannus* and *C. confusa*, and the *C. lyceius* species complex. Furthermore, this study allows us to examine the utility of the DNA barcoding method for species delimitation using a group, which, unlike the Holarctic fauna, probably underwent continuous diversification for a prolonged period without the major climatic stresses of glaciations. The results of this study should also add to our understanding of the extent to which DNA-barcode divergence correlates with morphological and ecological divergence. Prior to further phylogenetic work based on morphological, molecular or combined characters, it is key to establish species boundaries and the alpha taxonomy of the genus. In this study, we therefore use DNA barcodes to test the current species classification based on traditional characters, and to try to resolve the taxonomic status of a number of problematic phenotypes and populations.

Methods

A total of 110 *Calisto* specimens representing 31 putative taxa were sampled (Table 1). All specimens were collected in 1994–1999 by the first author. None of the specimens were subjected to any chemical treatment before desiccation. The climate of the regions ensured quick drying of specimens, which were stored at a room temperature (18–25°C) for over 10 years. DNA was extracted from a single leg removed from each specimen. Specimens were mostly unprepared (papered), with the exception of several individuals.

We amplified a 655-bp segment of mitochondrial cytochrome oxidase subunit I, from the *COI* barcode region. All polymerase chain reactions and DNA sequencing were carried out following standard DNA barcoding procedures for Lepidoptera as described previously (Hajibabaei et al. 2006, deWaard et al. 2008). Photographs of all specimens used in the analysis as well as specimen collection data and sequences are available in the Barcode of Life Data System (BOLD) at http://www.barcodinglife.org/ as well as in GenBank (accession numbers JN197297--JN197406). All voucher specimens are deposited at the McGuire Center for Lepidoptera and Biodiversity (Florida Museum of Natural History, University of Florida).

We chose two genera as outgroups: *Eretris*, which Miller (1968) thought to be *Calisto*'s closest relative on the mainland, based on wing shape and relative proximity to the Caribbean, and the southern Andean genus *Auca* Hayward, 1953 (Satyrinae: Pronophilina), which we have observed to be morphologically and behaviorally similar to *Calisto* (e. g., *Auca*'s association with bunch grass in arid lowland habitats is very similar to species in the *Calisto lyceius* complex) (Sourakov pers. obs.). Though geographically distant from *Calisto*, the inclusion of such a Pronophilina member from the southern Andes could provide insight into the origin of *Calisto* should the genus prove to be

non-monophyletic and also provides an additional point of comparison for the pairwise divergence analysis. Hence, we obtained five additional sequences from GenBank (table 1), including two species of *Auca*, *A. coctei* (GenBank number DQ338833) and *A. barrosi* (GenBank number DQ338832) (Peña et al. 2011), and two species of *Eretris*, *Eretris* sp. (GenBank number GQ357229) and *Eretris* sp.2 (GenBank number GQ864764) (Peña et al. 2006). We also obtained one additional sequence of *Calisto pulchella* (GenBank number GQ357225) (Peña et al. 2011).

Sequences were aligned using BioEdit software (Hall 1999) and manually edited. Sequence information was entered into the Barcode of Life Data System (http://www.barcodinglife.org) along with an image and collateral information for each voucher specimen. Detailed specimen records and sequence information, including trace files, are available in the LOWA project file in the BOLD website. All sequences are also available through GenBank.

Sequence data were analyzed using Bayesian inference (BI), as implemented in Mr Bayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). A GTR substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites was specified before running the program for 5,000,000 generations with default settings. The first 2500 trees (out of 10000) were discarded prior to computing a consensus phylogeny and posterior probabilities.

Maximum parsimony (MP) analysis was performed using a heuristic search as implemented in MEGA4 (Tamura et al. 2007). We used the close-neighbor-interchange algorithm with search level 3 (Nei and Kumar 2000) in which the initial trees were obtained by random addition of sequences (100 replicates). We used nonparametric bootstrap values (Felsenstein 1985) to estimate branch support on the recovered tree, with the bootstrap consensus tree inferred from 1000 replicates (MP tree is provided as Supplementary file). The Kimura 2-parameter model of base substitution was used to calculate genetic distances in MEGA4 software (Tamura et al. 2007). Dendroscope (Huson et al. 2007) was used to edit trees for publication.

Results

Fig. 1 shows the results of the Bayesian Inference analysis (BI). The maximum parsimony analysis revealed a similar topology, but deeper nodes were not strongly supported (bootstrap value < 0.5). Bootstrap values higher than 0.5 are shown on the MP tree (see Supplementary file). For the further analysis and discussion of results we refer to the BI tree. The BI analysis of the tree topology and the Kimura 2-parameter model estimation of genetic distances showed the following results:

1. The sympatric, superficially similar widespread species *Calisto confusa*, *C. obscura* and *C. batesi*, which frequently share the same habitat, proved to be extremely divergent. *Calisto confusa* appear to be related to the morphologically highly derived *C. arcas* Bates, 1939 Fig. 3 (Clades A, B). *Calisto obscura*, which is found throughout the lowlands and mid-elevations proved to be related to the *C. grannus* species group

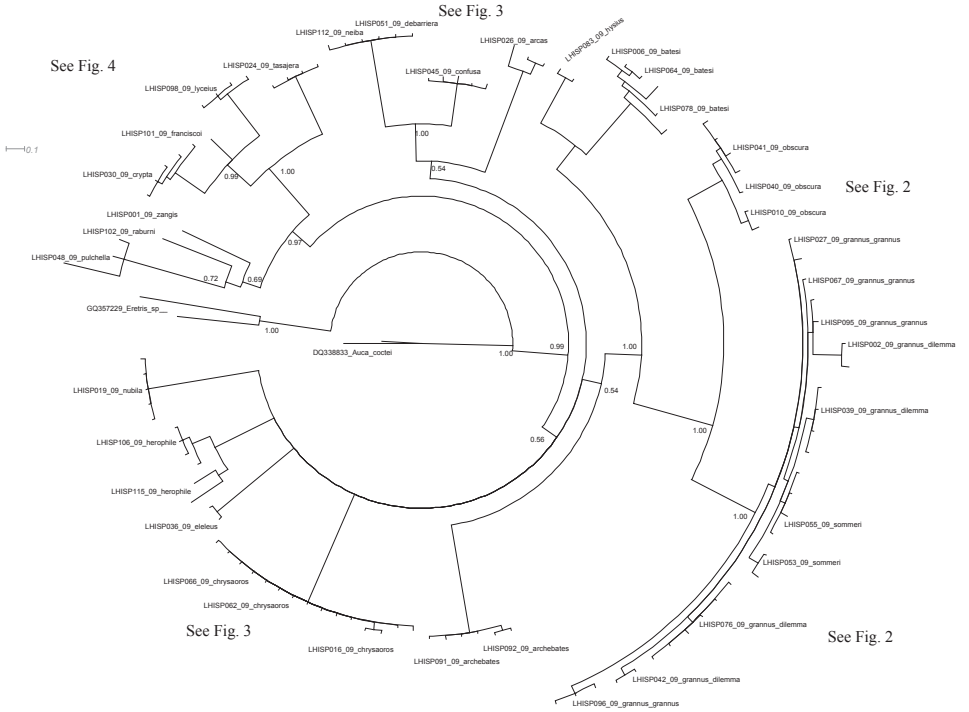

Figure 1. Bayesian inference phylogeny based on 655bp of COI for 111 specimens of the genus *Calisto* (representing ca. 20 species belonging to 26 named taxa), with outgroups of *Eretris* and *Auca* (Nymphalidae: Satyrinae: Prinophilini). The numbers at the nodes indicate posterior probability.

which is found locally throughout the island at higher elevations (Fig. 2, Clade B). Though the latter clade has *C. batesi*/*C. hysius* species complex as its sister clade (Fig. 2, Clade A), the divergence between *C. obscura* and *C. batesi* is substantial at approximately 9%.

2. The allopatric morphologically similar sister species *Calisto batesi*/*C. hysius* (Fig. 2, Clade A), whose species status was questionable based on adult morphology, and whose immature stages are also quite similar (Sourakov 1996), showed a high degree of divergence of ca. 6%, which is twice the rate seen in some sister species in Palearctic Satyrinae (Lukhtanov et al. 2009). For comparison, the divergence within *C. batesi* among well isolated populations throughout Cordillera Central, though still significant, is equal to or less than 1%.

3. *Calisto confusa* and *C. debarriera* appeared as two well-separated clusters (Fig. 3, Clade A). *Calisto debarriera* was originally treated as subspecies of *C. confusa* (Munroe 1951), and later regarded as color variant of *C. confusa* because of its frequent sympatry with the latter (Sourakov per. obs.), and because rearing did not indicate additional morphological characters (Sourakov 1996, 1997). Individuals of both taxa used in our analysis came from the same localities throughout the island, and while they showed interspecific divergence of over 6%, showed divergence of less than ca. 0.2% intraspe-

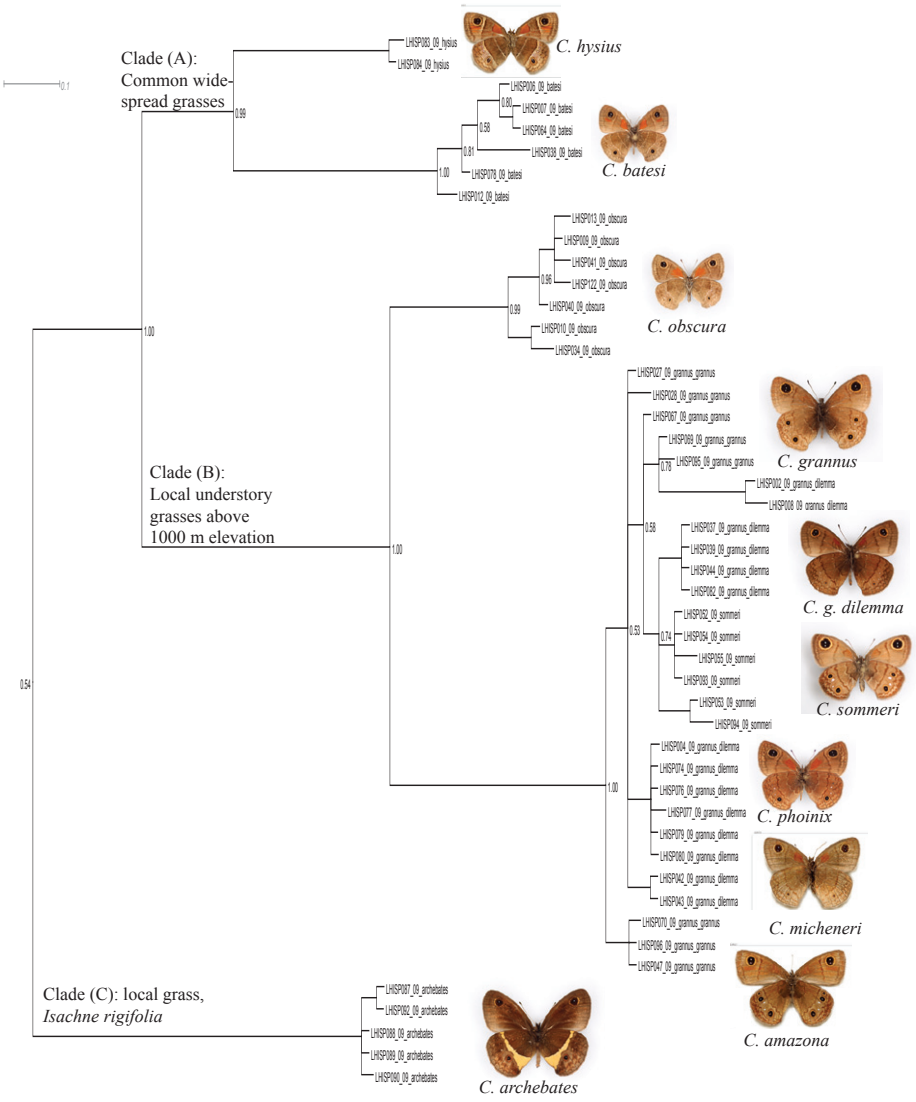

Figure 2. Fragment of the BI tree in Figure 1 with additional information about clades **Clade A:** *Calisto hysius* and *C. batesi* are found allopatrically on two Hispaniolan paleoislands **Clade B:** *Calisto obscura* is a widespread Hispaniolan species. The *Calisto grannus* complex is represented by a number of named populations, mostly but not exclusively found in Cordillera Central, the status of which are revised to subspecies in the present study **Clade C:** *C. archebates* is a local endemic of the southern paleoisland's Sierra de Bahoruco.

cifically. A single specimen with the phenotype of *Calisto neiba* (from Sierra de Neiba) was not divergent from the rest of *C. debarriera*, suggesting that the former is a synonym of the latter.

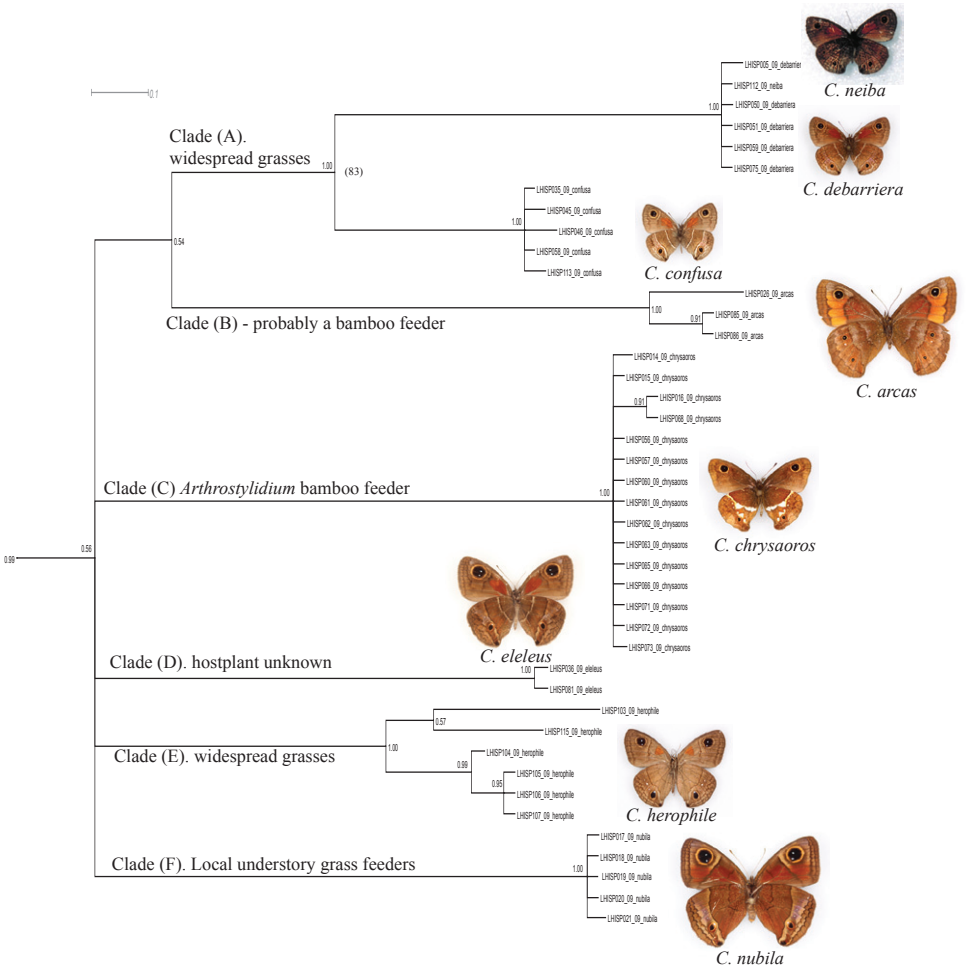

Figure 3. Fragment of the BI tree in Figure 1 with additional information about clades. **Clade A:** *Calisto confusa* and *C. debarriera*/*C. neiba* are morphologically similar and sometimes sympatric, though seemingly occupy different elevations **Clade B:** *Calisto arcas* is an endemic of Cordillera Central's Valle Nuevo area **Clade C:** *Calisto chrysaoros* is found at high elevations on both southern and northern paleoislands in the refugias associated with climbing bamboo grass *Arthrostylidium* **Clade D:** *Calisto eleleus* is now found extremely locally in the Cordillera Central **Clade E:** *Calisto herophile* is distributed on Cuba and Bahamas islands **Clade F:** *C. nubila* is a Puerto Rican endemic.

4. Within the *Calisto grannus* species complex (Fig. 2, clade B), we included at least nine isolated populations from different elevations, which we initially assigned to three taxa: *C. grannus grannus* of high elevations in the Cordillera Central (including a specimen representing the *C. amazona* phenotype), *C. grannus dilemma* (*grannus* individuals with red discal spot on the underside forewing, which includes such taxa as *dilemma*, *micrommata*, *dystacta*, *phoinix*, and *micheneri*) and *C. sommeri*, an isolate from Sierra de Bahoruco. The 28 individuals from these nine populations that are identi-

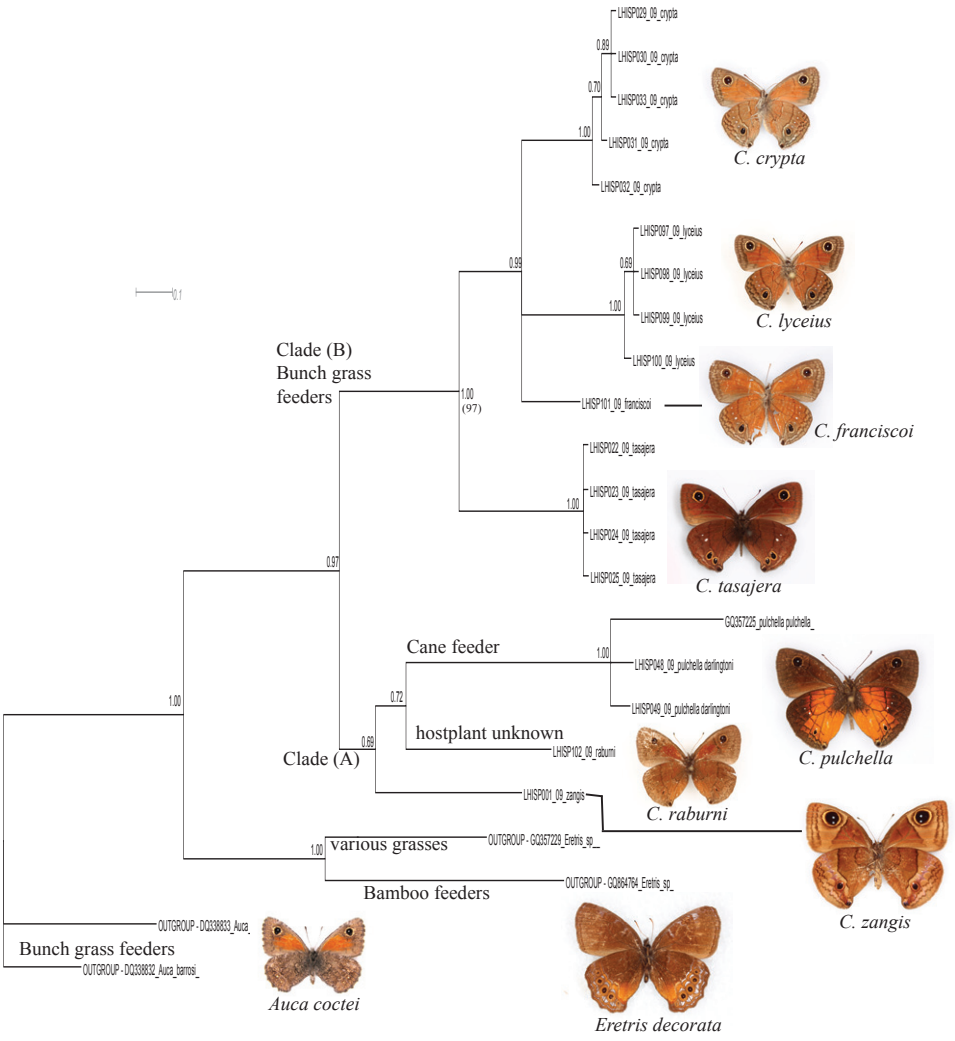

Figure 4. Fragment of the BI tree in Figure 1 with additional information about clades. The outgroups (*Auca* - bunch grass feeder from the southern Andes; *Eretris* - a bamboo-feeding group from Central and South America) and two basal *Calisto* clades **Clade A:** *C. zangis* of Jamaica which is aligned with the Hispaniolan *C. raburni* (a rare highly divergent species with an unknown life history) and *C. pulchella*, a well-known sugar cane pest (the native host plant is unknown) **Clade B:** *C. tasajera* (from the highlands of Cordillera Central) which feeds on *Danthonia domingenisis* bunch grass and *Calisto* of the *lyceius* group feeding on *Uniola virgata* bunch grass in the Hispaniolan lowlands.

fied on the barcode tree as *C. grannus grannus*, *C. grannus dilemma* and *C. sommeri* show geographic, rather than taxonomic, structure. In other words, individuals cluster within populations, separated from other such clusters by 0.5–1.5%, regardless of the taxonomic name applied. For instance, *C. sommeri* of Sierra de Bahoruco appears as

a sister clade to *C. grannus dilemma* from the extreme western portion of Dominican Cordillera Central. The lowland and very common widespread *C. obscura* appears to be a sister taxon to the *C. grannus* species complex, with a divergence of 5–7%.

5. Within the *Calisto lyceius* species complex (Fig. 4, Clade B), lowland desert isolates such as *C. crypta*, *C. franciscoi*, and *C. lyceius*, despite their superficial morphological similarities, proved to be divergent in their barcodes (ca. 4.5%). *Calisto tasajera* González, Schwartz et Wetherbee, 1991 proved to be their immediate relative, found at the high elevations.

6. A widely-distributed pest of sugar cane, *Calisto pulchella* (Fig. 4, Clade A) showed a high degree of divergence (3.5%) between its two described subspecies (*C. pulchella pulchella* from the lowlands and *C. p. darlingtoni* from the Cordillera Central).

7. Three species endemic to islands other than Hispaniola (*Calisto nubila* Lathy,1899, *C. zangis* (Fab., 1775) and *C. herophile* Hübner, 1823) appear to be derived lineages of various Hispaniolan taxa (Fig. 3, Clade D and E; Fig. 4, Clade A). Divergence of these island isolates, though high, does not exceed divergence found within the island of Hispaniola.

8. The maximum divergence within the genus (13.3% between *C. nubila* and *C. grannus*) is almost equivalent to the maximum divergence of *Calisto* from its distant pronophiline relative *Auca* from the southern Andes (14.1%), or from its presumed closest relative *Eretris* (14.4%) (Fig. 4). The average interspecific divergence in *Calisto* was found to be 10%.

Discussion

As a result of the present "DNA barcode" analysis, it is possible to draw a number of taxonomic conclusions (proposed taxonomic changes are summarized in Table 1). *Calisto grannus* represents a recent and incomplete diversification through allopatric isolation, and for now is best considered as a single species, with *C. g. dilemma*, *C. g amazona* stat. n., *C. g micrommata* stat. n., *C. g. dystacta* stat. n., *C. g. phoinix* stat. n., *C. g. sommeri* stat. n., and *C. g. micheneri* stat. n. representing subspecies. Within the *Calisto lyceius* complex, lowland desert isolates such as *C. crypta*, *C. franciscoi*, and *C. lyceius*, despite their superficial morphological similarities, proved to be sufficiently divergent in their barcodes to confirm their species status previously postulated based on male genitalia (Sourakov 2000). The observed divergence within *Calisto pulchella*, which is not only one of the most morphologically divergent species (Sourakov 1996, 1997), but also a widespread and economically important pest of sugar cane (Smyth 1920, Holloway 1933), calls for more research. Interestingly, these results correspond to earlier views (Munroe 1951, Wisor and Schwartz 1985) that there are at least two taxa in *pulchella*, one in the lowlands and another (*C. pulchella darlingtoni*) in the Cordillera Central at 3000–4000 ft elevation. Columbus introduced sugar cane to the island around 500 years ago (Deer 1949), so the current distribution of the species is likely different from its historical distribution. Perhaps, pre-Columbus *C. pulchella* ex-

Table 1. *Calisto* species examined in the present study and resulting nomenclatural changes.

Smith et al. 1994 name	Describer(s)	Status change	Proposed new status
aleucosticha	Correa & Schwartz, 1986	stat. n.	*hysius aleucosticha*
amazona	González, 1987	stat. n.	*grannus amazona*
arcas	M. Bates, 1939		
archebates	(Ménétriés, 1832) (Satyrus)		
batesi	Michener, 1943		
chrysaoros	M. Bates, 1935		
confusa	Lathy, 1899		
confusa debarriera	Clench, 1943	stat. n.	*debarriera*
crypta	Gali, 1985		
dystacta	González, 1987	stat. n.	*grannus dystacta*
eleleus	M. Bates, 1935		
franciscoi	Gali, 1985		
gonzalezi	Schwartz, 1988	syn. n.	*debarriera*
grannus	M. Bates, 1939		
grannus dilemma	González, 1987		
herophile	Hübner, [1823]		
hysius	(Godart, [1824]) (Satyrus)		
lyceius	M. Bates, 1935		
micheneri	Clench, 1944, repl. name	stat. n.	*grannus micheneri*
micrommata	Schwartz & Gali, 1984	stat. n.	*grannus micrommata*
montana	Clench, 1943	syn. n.	*debarriera*
neiba	Schwartz & Gali, 1984	syn. n.	*debarriera*
nubila	Lathy, 1899		
obscura	Michener, 1943		
phoinix	González, 1987	stat. n.	*grannus phoinix*
pulchella	Lathy, 1899		
pulchella darlingtoni	Clench, 1943		
raburni	Gali, 1985		
sommeri	Schwartz & Gali, 1984	stat. n.	*grannus sommeri*
tasajera	González, Schwartz & Wetherbee, 1991		
zangis	(Fabricius, 1775) (Papilio)		

isted as two non-interbreeding allopatric entities, which continued to maintain no or limited gene exchange following sugar cane introduction, but both were able to adapt a new hostplant. We suggest preserving subspecies status for these two entities until further research can be done, which should include multiple specimens from a number of populations, including studying this butterfly in its wild habitat in association with the native hostplant.

Munroe's view that *Calisto confusa* and *C. debarriera* stat. n. are good species is now supported by our DNA data. Munroe found differences only in aedeagus width/length ratio and immediately cast doubt on his finding: "No fresh material was examined, and such a difference might conceivably be the result of distortion of the preparations."

Munroe examined only four *debarriera* specimens, but stated that "in support of this evidence it may be noted that the material of *debarriera* comes from a limited altitude range, which is entirely contained in both the altitudinal and geographic range of the widely distributed *confusa*." In other words, Munroe, though only having available a few old collection specimens, already supposed that he was dealing with two sympatric taxa. Future workers reduced *debarriera* to subspecies (e. g., Smith et al. 1984) and even considered it a synonym of *confusa* after their peripatric/sympatric distribution became more and more evident. However, at the same time, additional representatives of *debarriera* were being described as separate species, such as *C. neiba* syn. n. and *C. gonzalezi* syn. n., based on aberrant isolated populations. Our DNA barcode analysis suggests that *confusa* and *debarriera* are indeed two reproductively isolated species, whose ranges overlap, perhaps as a result of secondary contact following initial speciation through niche partitioning, since *debarriera* is largely a highland species and *confusa* largely a lowland species. A similar confusing situation that existed within the *Calisto hysius* complex, which included *C. hysius*, *C. batesi* (often listed as *C. hysius batesi* (e. g., Munroe 1951)), and *C. aleucosticha* stat. n. is now resolved. *Calisto hysius* mostly occurs on the southern paleoisland (Fig. 5) and shows significant divergence from the mostly northern *C. batesi*, suggesting that these two are distinct species (Fig. 2). *Calisto aleucosticha*, which was described from a few aberrant females of *C. hysius* found on the northern paleoisland by Correa and Schwartz (1986), should be considered a subspecies of *C. hysius*.

Non-Hispaniolan island endemics (*Calisto nubila*, *C. zangis* and *C. herophile*) appear to be derived lineages of various Hispaniolan taxa, indicating several ancient dispersal events from Hispaniola to Puerto Rico, Cuba, and Jamaica. For instance, *Calisto herophile*, which occurs in Cuba and the Bahamas, appears to be a product of dispersal from Hispaniola of the widespread polyphagous *C. confusa* or its immediate ancestor. *Calisto nubila*, endemic to Puerto Rico, which bears morphological similarity to the rare and localized Hispaniolan *C. eleleus* Bates, 1935 (Fig. 3, Clade D), also most likely have originated by dispersal to Puerto Rico from the Hispaniolan clade. Divergence of these island isolates, though great, does not exceed divergence found within the island of Hispaniola, which suggests that they dispersed from Hispaniola when the genus was already undergoing diversification. The low diversity of species on non-Hispaniolan islands as well as the time-frame of *Calisto* evolution, suggests that such taxa arrived there by accidental dispersal, rather than by land bridges or vicariance as hypothesized previously by Miller and Miller (1989).

Calisto zangis, along with *C. pulchella* and *C. raburni* Gali, 1985, are the most morphologically divergent members of the genus in general wing pattern, male and female genitalic structures, and in the immature stages (at least for *pulchella*, for which life history has been studied) (Sourakov 1996, 1997). DNA barcodes also indicate that these three species are strongly separated, suggesting that the origin of Jamaican *C. zangis* is likely an ancient event. The fact that the *C. lyceius*/*C.tasajera* group of bunchgrass-feeding *Calisto* has a close affinity to cane-feeding *C. pulchella* and to the Jamaican *C. zangis*, together forming a clade sister to all other *Calisto*, is of great interest.

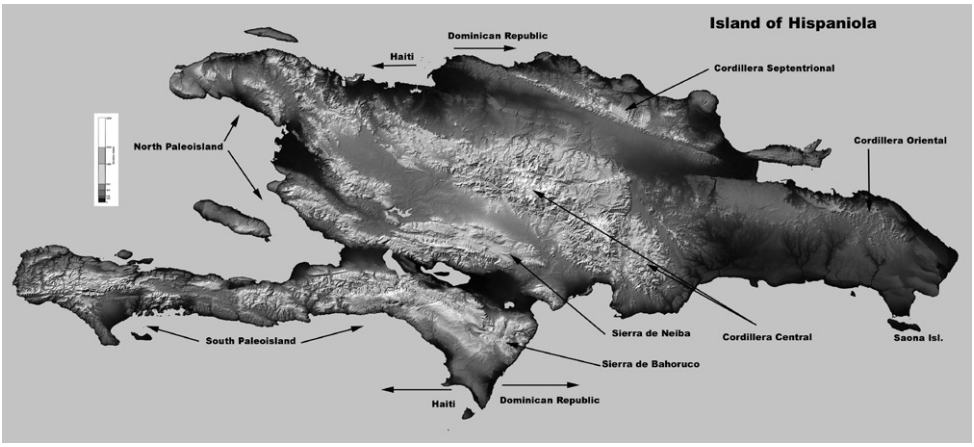

Figure 5. The island of Hispaniola, with some key geological features.

Although the bamboo-feeding *Eretris* were historically regarded as the closest relative to *Calisto* (Miller 1968), our results suggest that the south Andean genus *Auca* may be at least as closely related to *Calisto*, and we suspect that we need to search among lowland bunch-grass feeding satyrines for the closest mainland *Calisto* relative. Feeding on bunch-grasses in low elevation arid habitats may instead be the ancestral state in *Calisto* (e.g., the *C. lyceius* complex) that has been retained in other satyrine genera in Central and South America.

Our results highlight the usefulness of DNA-barcode analysis for routine species-level taxonomic work. DNA-barcoding allowed us to confirm previously observed morphological synapomorphies and test theories based on morphology and ecology alone. For example, the fact that the phenotypically divergent species *C. archebates* (Ménétriés, 1832), which has a yellow stripe traversing the hindwing underside, appeared as sister species to the *C. grannus*/*C. confusa*/*C. batesi* complex was already hypothesized based on immature stage morphology (Sourakov 1996). Further molecular research involving more genes is necessary to establish a robust phylogeny of *Calisto*.

The evolution of Satyrinae has been linked to the evolution and diversity of grasses (Peña and Wahlberg 2008). The DNA barcode divergence found in this study is associated with apparent ecological niche partitioning by species that inhabit a wide variety of habitats and utilize various host plants. We observe evolution of clades that is associated with shifts to new hostplant groups such as bunch grasses, bamboos, canes, etc. These clade-hostplant associations found today are shown in Fig. 2–4. For example, *Calisto arcas* and *C. chrysaoros* are two species whose adults are morphologically highly distinctive but whose life histories are poorly known. Sourakov (1996) described the eggs and first instar larvae of these two species and found that while the life history of *C. arcas* is surprisingly similar to many other *Calisto*, *C. chrysaoros*, which is strongly associated with bamboo, has egg and first instar larva that are highly divergent from the most common *Calisto* phenotype. In the present study, *C. arcas* formed a single clade in the middle of BI tree together with *C. confusa*/*C. neiba* complex (Fig. 3), which sup-

ports previously observed morphological synapomorphies. Yet, the average divergence of *C. arcas* from other *Calisto* (10–12%) is greater than that of *C. chrysaoros* (9–10%).

The butterfly fauna of Hispaniola has evidently been evolving for many millions of years. For instance, an extinct species of an extant neotropical genus of Riodinidae is known from Dominican fossil amber, dating from 15–25 Myr (Hall et al. 2004). Peña et al. (2011) suggested that *Calisto* might be a remnant of the initial colonization of South America by North American Satyrinae, in which case, *Calisto* might be a very old group. Several authors (e. g., Miller and Miller 1989) have suggested that much of the biological diversification found in *Calisto* may be associated with geological events. Indeed, the geological history of the Greater Antilles, the center of distribution for the genus, is complex. The archipelago originated more than 50 million years ago, and since then the component islands have undergone extensive metamorphosis, with Cuba and Hispaniola separating 20–25 million years ago (Pindell 1994). Though it is tempting to assume some role of geological events in speciation of *Calisto*, it has been shown repeatedly that adaptive radiation process is the main driving force behind evolution of species richness in the Caribbean (e.g., Losos et al. 2006). In our opinion, the genus shows a remarkable degree of diversification in comparison with other Caribbean clades, presumably because of low dispersal ability of these butterflies that interacts with topographic isolation within an island of Hispaniola and with exploitation of different habitats with varying rainfall patterns. Inter-island isolation, of course, also contributed to the overall diversity of the genus. However, it is the incredible diversity of habitats, ranging from the hot, dry deserts of the Hispaniolan lowlands to montane forests and grasslands at over 3000 m in elevation, that is responsible for the todays diversity of *Calisto*. These habitats are so variable due primarily to the high central mountain range, which creates strong gradients of temperature and rainfall. In harsher habitats (e. g., deserts, high mountain tops, peripheral localities (Fig. 6 shows two examples)) where numerous unique adaptations are necessary for survival, species may be very local, not spreading to neighboring areas despite the availability of unlimited resources and seeming absence of interspecific competition.

Butterflies, especially grass-feeding butterflies in such a hurricane-prone area, have thus had many chances to colonize every possible habitat and island through dispersal. Even though the genus appears more divergent than most other satyrine genera, it does not seem to be old enough to be influenced too much by geological events related to continental movement. Though recognizing the limited ability of a short DNA strand to give precise time estimates for observed divergence, most models assume that 1.5–3.5% divergence roughly equates to one million years of isolation (e. g., Brower 1994, Kandul et al. 2004, Papadopoulou et al. 2010, Vila et al. 2010). Hence, we can hypothesize based on available data that the genus *Calisto* underwent continuous diversification for some 4–8 Myrs, and thus ancient geological events of continental movement are unlikely to be a factor. Instead, it seems most likely that the diversification of *Calisto* into these numerous different habitats represents traditional Darwinian adaptive radiation, as suspected for other groups of Caribbean insects and vertebrates (e.g., Losos and Schluter 2000; Woods 1989; Liebher 1988).

Figure 6. Examples of habitat diversity on the island of Hispaniola. **A** Valle de Bao (1920 m elevation) at the foothill of Pico Duarte (3098 m elevation), covered with bunch grass, *Danthonia domingenis* - a hostplant of *Calisto tasajera* (top right) **B** Arid south eastern coastal habitat in Boca de Yuma, Altagracia provides an environment for sea oats, *Uniola virgata*, and associated *Calisto lyceius* (top right).

Figure 7. *Calisto montana* holotype (Museum of Comparative Zoology, Harvard, Massachusetts, USA).

Acknowledgements

We thank Keith Willmott for reviewing the manuscript and Alexandra Sourakov for proofreading the early versions. The National Geographic Society funded some of the first author's fieldwork through Committee for Research and Exploration grant #5717-96. Companions on the field trips, especially Thomas C. Emmel, were instrumental in getting the project to this stage. DNA sequence analysis was carried out at the Canadian Centre for DNA Barcoding through the International Barcode of Life project funded by NSERC, the Ontario Ministry of Research and Innovation and Genome Canada through the Ontario Genomics Institute.

References

Brower AVZ (1994) Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proceedings of the National Academy of Sciences 91: 6491–6495. doi: 10.1073/pnas.91.14.6491

Correa JC, Schwartz A (1986) The status of *Calisto hysius batesi* (Lepidoptera, Satyridae) with the description of a new species of *Calisto* from Hispaniola. Biological Sciences 49: 12–17.

De Vries PJ (1987) The Butterflies of Costa Rica and their Natural History. Volume I. Princeton University Press, Princeton, 327 pp.

Deer N (1949) The history of sugar. Vol. 1. Chapman and Hall Ltd., London, 258 pp.

DeWalt R E (2011) DNA barcoding: a taxonomic point of view. Journal of the North American Benthological Society 30(1): 174–181. doi: 10.1899/10-021.1

deWaard JR, Ivanova NV, Hajibabaei M, Hebert PDN (2008) Assembling DNA barcodes: analytical protocols. In: Martin C (Ed) Methods in molecular biology: environmental genetics. Humana Press, Totowa, NJ,\ 275–293.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791. doi: 10.2307/2408678

Freitas AVL, Brown KS (2004) Phylogeny of the Nymphalidae (Lepidoptera). Systematic Biology 53: 363–383. doi: 10.1080/10635150490445670

Gali F (1985) Five new species of *Calisto* (Lepidoptera: Satyridae) from Hispaniola. Contributions in biology and geology, Milwaukee Public Museum 63: 1–16.

González FL (1987) Three new species and one new subspecies of the *grannus* complex of Hispaniolan *Calisto* (Lepidoptera: Satyridae). Bulletin of the Allyn Museum 108: 1–17.

Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN (2006) DNA barcodes distinguish species of tropical Lepidoptera. Proceedings of the National Academy of Sciences 103: 968–971. doi: 10.1073/pnas.0510466103

Hall JPW, Robbins RK, Harvey DJ (2004) Extinction and biogeography in the Caribbean: new evidence from a fossil riodinid butterfly in Dominican amber. Proceedings of the Royal Society of London B, 271(1541): 797–801. doi: 10.1098/rspb.2004.2691

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analyses program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Hebert PDN, deWaard JR, Landry JF (2010) DNA barcodes for 1/1000 of the animal kingdom. Biology Letters 6(3): 359–362. doi: 10.1098/rsbl.2009.0848

Holloway TE (1933) Insect pests of sugar cane. The leaf eating insects. Army worms, cutworms, leaf miners, grasshoppers and others. Facts About Sugar, New York, 28(5): 216–219.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755. doi: 10.1093/bioinformatics/17.8.754

Huson DH, Richter DC, Rausch C, Dezulian T, Franz M, Rupp R (2007) BMC Bioinformatics 22, 8(1): 460.

Johnson K, Hedges SB (1998) Three new species of *Calisto* from southwestern Haiti (Lepidoptera: Nymphalidae: Satyrinae). Tropical Lepidoptera 9(2): 45–53.

Johnson K, Quinter EL, Matusik D (1987) A new species of *Calisto* from Hispaniola with a review of the female genitalia of Hispaniolan congeners (Satyridae). Journal of Research on the Lepidoptera 25(2): 73–82.

Kandul NP, Lukhtanov VA, Dantchenko AV., Coleman JWS, Sekercioglu CH, Haig D, Pierce NE (2004) Phylogeny of *Agrodiaetus* Hübner 1822 (Lepidoptera: Lycaenidae) inferred from mtDNA sof COI and COII and nuclear sequences of EF1-α: karyotype diversification and species radiation. Systematic Biology 53(2): 278–298. doi: 10.1080/10635150490423692

Kitching IJ (1985) Early stages of the milkweed butterflies (Lepidoptera: Danainae). Zoological Journal of Linnaean Society 85: 1–97. doi: 10.1111/j.1096-3642.1985.tb01516.x

Kooi RE, Brakefield PM, Rosie WEM-Th (1996) Effects of food plant on phenotypic plasticity in the tropical butterfly *B. anynana*. Entomologeia Experimentalis et Applicata 80: 149–151. doi: 10.1111/j.1570-7458.1996.tb00906.x

Lamas G, Viloria AL, Pyrcz TW (2004). Nymphalidae, Satyrinae, Satyrini, Pronophilina. Checklist: Part 4A. Hesperioidea – Papilionoidea. In: Heppner JB (Ed) Atlas of Neotropical Lepidoptera. Vol. 5A. Association for Tropical Lepidoptera, Scientific Publishers, Gainesville, 206–215.

Liebherr KJ (Ed) (1988) Zoogeography of Caribbean Insects. Cornell University Press, Ithaca, 285pp.

Losos JB, Schluter D (2000) Analysis of an evolutionary species–area relationship. Nature 408: 847–850. doi: 10.1038/35048558

Losos JB, Glor RE, Kolbe JJ, Nicholson K (2006) Adaptation, speciation, and convergence: a hierarchical analysis of adaptive radiation in Caribbean *Anolis* lizards. Annals of the Missouri Botanical Garden 93(1): 24–33. doi: 10.3417/0026-6493(2006)93[24:ASACAH]2.0.CO;2

Lukhtanov VA, Sourakov A, Zakharov EV, Hebert PDN (2009) DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. Molecular Ecology Resources: 9: 1302–1310. doi: 10.1111/j.1755-0998.2009.02577.x

Miller LD (1968) The higher classification, phylogeny and zoogeography of the Satyridae (Lepidoptera). Memoirs of American Entomological Society 24: 1–174.

Miller LD, Miller JY (1989) The biogeography of West Indian butterflies (Lepidoptera: Papilionidea, Hesperioidea): a vicariance model. In: Woods CA (Ed) Biogeography of the West Indies: Past, Present, and Future. Gainesville: Sandhill Crane Press, 29–262.

Munroe EG (1951) The systematics of *Calisto* (Lepidoptera: Satyridae) with remarks on the evolutionary and zoogeographic significance of the genus. Journal of the New York Entomological Society 58: 211–240.

Murray DL (2001) Systematic of euptychiine butterflies (Nymphalidae: Satyrinae: Euptychiina) based on larval morphology and DNA sequence data and the evolution of life history traits. Ph.D. dissertation, Louisiana State University, Baton Rouge, LA.

Nei M, Kumar S (2000) Molecular Evolution and Phylogenetics. Oxford University Press, New York, 333 pp.

Papadopoulou A, Anastasiou I, Vogler AP (2010) Revisiting the Insect Mitochondrial Molecular Clock: The Mid-Aegean Trench Calibration. Molecular Biology and Evolution 27(7): 1659–1672. doi: 10.1093/molbev/msq051

Peña C, Sören N, Wahlberg N (2011) The radiation of Satyrini butterflies (Nymphalidae: Satyrinae): a challenge for phylogenetic methods. Zoological Journal of the Linnaean Society 161: 64–87. doi: 10.1111/j.1096-3642.2009.00627.x

Peña C, Wahlberg N (2008) Prehistorical climate change increased diversification of a group of butterflies. Biology Letters 4: 274–278. doi: 10.1098/rsbl.2008.0062

Peña C, Wahlberg N, Weingartner E, Kodandaramaiah U, Nylin S, Freitas AVL, Brower AVZ (2006) Higher level phylogeny of Satyrinae butterflies (Lepidoptera: Nymphalidae) based on DNA sequence data. Molecular Phylogenetics and Evolution 40: 29–49. doi: 10.1016/j.ympev.2006.02.007

Penz CM, DeVries PJ (2001) A phylogenetic reassessment of *Thisbe* and *Uraneis* butterflies (Riodinidae, Nymphidiini). Contributions in Science Los Angeles 485: 1–27.

Pindell JL (1994) Evolution of the Gulf of Mexico and the Caribbean. In: Donovan SK, Jackson TA (Eds) Caribbean Geology, An Introduction. The University of the West Indies Publishers' Association, Kingston, 13–39.

Riley NR (1975) A Field Guide to the Butterflies of the West Indies. Detmer Press, 224 pp.

Ronquist F, Huelsenbeck JP (2003) MRBAYES3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. doi: 10.1093/bioinformatics/btg180

Smith DS, Miller LD, Miller JY (1994) The Butterflies of the West Indies and South Florida. Oxford University Press, Oxford, 264 pp.

Smyth HEG (1920) To keep out cane butterfly. Journal of Economic Entomology 13: 149.

Schwartz A (1989) Butterflies of Hispaniola. University of Florida Press, Gainesville, 580 pp.

Schwartz A, Gali F (1984) Five new species of *Calisto* (Satyridae) from Hispaniola. Bulletin of the Allyn Museum 85: 1–18.

Sourakov A (1995) Systematics, Evolutionary Biology and Population Genetics of the *Cercyonis pegala* Group (Nymphalidae: Satyrinae). Holarctic Lepidoptera 2(1): 1–21.

Sourakov A (1996) Notes on the genus *Calisto*, with descriptions of the immature stages (Part I) (Lepidoptera: Nymphalidae: Satyrinae). Tropical Lepidoptera 7(1: Part 2): 91–112.

Sourakov A (1997*)* Morphology of immature stages in satyrinae (Lepidoptera:Nymphalidae) and its application to systematics. Ph. D. Dissertation, University of Florida.

Sourakov A (2000) Notes on the genus *Calisto*, with descriptions of the immature stages (Part II) (Lepidoptera: Nymphalidae: Satyrinae). Tropical Lepidoptera 10(2): 73–79.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596–1599. doi: 10.1093/molbev/msm092

Vila R, Lukhtanov VA, Talavera G, Gil-TF, Pierce NE (2010) How common are dot-like distribution ranges? Taxonomical oversplitting in Western European *Agrodiaetus* (Lepidoptera, Lycaenidae) revealed by chromosomal and molecular markers. Biological Journal of the Linnean Society 101: 130–154. doi: 10.1111/j.1095-8312.2010.01481.x

Viloria AL (1998) Studies on the Systematics and Biogeography of Some Montane Satyrid butterflies (Lepidoptera). PhD Thesis, University of London, London.

Wiemers M, Fiedler K (2007) Does the DNA barcoding gap exist?- a case study in blue butterflies (Lepidoptera: Lycaenidae). Frontiers in Zoology 4: 8. doi: 10.1186/1742-9994-4-8

Willmott KR, Freitas ALV (2006) Higher-level phylogeny of the Ithomiinae (Lepidoptera: Nymphalidae): classification, patterns of larval hostplant colonization and diversification. Cladistics 22: 297–368. doi: 10.1111/j.1096-0031.2006.00108.x

Wisor RW, Schwartz A (1985) Status of *Calisto pulchella darlingtoni* Clench (Lepidoptera: Satyridae). Florida Scientist 48(1): 7–13.

Woods CA (Ed) (1989) Biogeography of the West Indies: Past, Present, and Future. Sandhill Crane Press, Gainesville, 878 pp.