

Taxonomic reassessment of species within the *chrysaoros* group of *Calisto* (Lepidoptera : Nymphalidae : Satyrinae)

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Abstract. The taxonomic status of the Hispaniolan species of *Calisto* Hübner, 1823 included in the *chrysaoros* group is reassessed. Morphological data and COI barcode sequences were analysed through different methods: Neighbour-Joining clustering, ABGD, Median-Joining Haplotype Network, Maximum Likelihood, and Bayesian Inference. Analyses yielded two deeply separated groups or putative species: *C. clydoniata* and *C. chrysaoros*. A shallower split was found, except in the Bayesian Inference approach, among populations of southern and northern Hispaniola palaeo-islands. In light of these findings we propose the recognition of two species: *C. clydoniata* and *C. chrysaoros*. Within the latter, the populations of the southern and northern Hispaniola palaeo-islands should be recognised as different subspecies: *C. chrysaoros chrysaoros* Bates and *C. chrysaoros galii* Schwartz, respectively. *Calisto clenchi*, syn. nov. is a synonym of *C. chrysaoros chrysaoros* whereas *C. galii galii*, syn. nov. and *C. galii choneupsilon*, syn. nov. are synonyms of *C. chrysaoros galii*.

Additional keywords: ABGD, barcoding, Bayesian Inference, COI, diagnostic sites, Greater Antilles, Haplotype Network, Hispaniola, Maximum Likelihood, monophyly, new synonym.

Received 14 April 2016, accepted 27 April 2017, published online 17 November 2017

Introduction

The genus *Calisto* Hübner, 1823 is restricted to the Greater Antilles, the Bahamas and Anegada (British Virgin Islands) and is the sole representative of the Satyrinae in the region (Smith *et al.* 1994; Lamas 2004). The genus comprises ~52 species and has received great attention due to its endemism and speciation on the islands (Sourakov and Zakharov 2011; Núñez Aguila *et al.* 2012, 2013; Matos-Maraví *et al.* 2014; Pérez-Asso *et al.* 2016).

The present work is focussed on several taxa formerly included within the *archebates* and *hysius* species groups created by Bates (1935). The *archebates* group was first established based on *Calisto archebates* (Ménétriés, 1832), *C. loxias* Bates, 1935, and *C. chrysaoros* Bates, 1935. Subsequently, additional taxa were added by Bates (1939), Schwartz (1983, 1985), and Schwartz and Gonzalez (1988): *C. arcas* Bates, 1939, *C. galii* Schwartz, 1983, *C. galii choneupsilon* Schwartz, 1985, and *C. wetherbeeii* Schwartz & Gonzalez, 1988 respectively. The *hysius* group includes a large number of species, including *C. clydoniata* Schwartz & Gali,

1984 and *C. clenchi* Schwartz & Gali, 1984, which were added by Schwartz and Gali (1984).

A recent molecular study by Matos-Maraví *et al.* (2014) based on six mitochondrial and nuclear markers showed that the *archebates* and *hysius* groups were not monophyletic. *C. archebates* was placed alone and distant from all other members of the genus with strong support. In the same analyses, the position of *C. arcas* was unresolved or weakly supported as sister of a strongly supported clade formed by *C. chrysaoros*, *C. galii*, *C. clydoniata* and *C. clenchi*, which has since been referred to as the *chrysaoros* group (Matos-Maraví *et al.* 2014). Here we also need to make a correction since the specimen identified as *C. clenchi* and coded as RN06-01 by Matos-Maraví *et al.* (2014) is actually another *C. galii* collected at the Cordillera Central in the Hispaniolan North palaeo-island. *C. clenchi* inhabits only the Sierra de Bahoruco in the South palaeo-island. In addition to the latter species, there are no published sequences of *C. loxias*, *C. galii choneupsilon*, and *C. wetherbeeii*. In the present work, we provide a reassessment of the taxonomic status of *C. chrysaoros*, *C. g. galii*, *C. galii*

choneupsilon, *C. clydoniata* and *C. clenchi* by using COI barcode sequences and morphological data.

Material and methods

Molecular analysis

Specimens were collected during expeditions from 2008 to 2014 in several areas of the Dominican Republic including the type localities of *C. galii choneupsilon*, *C. clydoniata* and *C. clenchi*. We employed the morphological characters traditionally used in the literature for *Calisto* (Bates 1935; Schwartz 1983, 1985, 1989; Schwartz and Gonzalez 1988).

Molecular data acquisition and analysis, DNA extraction, PCR amplification, and sequencing of the COI barcode region were performed at the Canadian Centre for DNA Barcoding and followed standard protocols.

A single pair of primers was used: LepF1 (ATTCAACCA ATCATAAAGATATTGG) and LepR1 (TAAACTTCTGGA TGTCCAAAAAATCA) (Hebert *et al.* 2004), which recovers a 658-bp region near the 5' end of COI including the 648-bp barcode region for the animal kingdom (Hebert *et al.* 2003). Sequence editing and alignment were done manually using BioEdit 7.0.9 (Hall 1999). DNA sequences have been submitted to GenBank (see Appendix 1 for accession numbers). DNA voucher specimens were deposited at the Victor González Research Collection, San Juan, Puerto Rico. Additional DNA sequences from *chrysaoros* group members were available at GenBank.

The classic DNA barcoding (Hebert *et al.* 2003) calculates a genetic distance between specimens using Kimura's 2-parameter (K2P) distance (Kimura 1980) to reveal a barcode gap or the break in the distribution among genetic distances of specimens belonging to the same species and those of different species. However, uncorrected genetic distances, p-distance, are used here instead as K2P could be inappropriate when employing it for closely related taxa (Srivathsan and Meier 2011). The genetic distances were calculated using MEGA5.1 (Tamura *et al.* 2011).

We conducted a character base approach of the COI sequences to identify the presence or absence of discrete nucleotide substitutions as character states. These substitutions potentially allow the identification of species or even populations (Rach *et al.* 2008; Tavares and Baker 2008; Brower 2010). After the Neighbour Joining implementation, we arranged the clustered sequences of the hypothetical species first and within each by localities in a single FASTA file. Then this file was visually inspected as a MAS alignment in MEGA looking for unique substitutions at each site within hypothetical species and in some cases within populations.

The Automatic Barcode Gap Discovery method (Puillandre *et al.* 2012a, 2012b) was used to sort the available 59 sequences into genetic clusters or hypothetical species. This algorithm automatically finds the inflection point in the frequency distribution of ranked pairwise genetic distances between aligned homologous sequences, and does so recursively to get the finest partition of the dataset into candidate species (Puillandre *et al.* 2012a, 2012b). We calculated a matrix of pairwise uncorrected p-distances in MEGA 5.1 excluding all ambiguous positions between each pair of sequences. We used

the ABGD web-interface available at <http://www.wabi.snv.jussieu.fr/public/abgd/> (accessed 10 October 2015) using the default values for all parameters. The analysis was performed employing the three implemented models (Jukes–Cantor, K2P, and Simple Distance) without differences in the species delimitation. Median Joining Haplotype Network was constructed using popart 1.7 (<http://popart.otago.ac.nz>, accessed 15 November 2015) from the available dataset but excluding sequences with ambiguities.

We reconstructed the phylogenetic relationships of the available species in the *chrysaoros* group by maximum likelihood and Bayesian inference. The main goal was to identify any phylogenetic structure within the complex by using COI sequences. In both cases *C. nubila* (Puerto Rico) was used as an outgroup as the ancestors of this species apparently diverged earlier than all extant species of the genus (Matos-Maraví *et al.* 2014). For the maximum likelihood reconstruction, we found the best-fitting model of molecular evolution as determined by the AICc in the MEGA 5.1 program (Tamura *et al.* 2011). We conducted a heuristic search using the Subtree Pruning Regrafting (Level 5). Nodal support was determined by 1000 bootstrap replicates. In the Bayesian approach, we inferred the best-fitting model of molecular evolution and partition scheme to apply using PartitionFinder 1.0.1 (Lanfear *et al.* 2012). The best-fitting partition/substitution model scheme, as determined by the AICc, was implemented in a Bayesian inference analysis with MrBayes 3.2 (Ronquist *et al.* 2012). Two independent MCMC analyses with four simultaneous chains (one cold and three heated) for each analysis were run for 20 million generations and the sampling of trees and parameters were set to every 1000 generations. Convergence of the two runs was determined by the stationary distribution plot of the log-likelihood values against number of generations and confirmed by the average standard deviation of split frequencies, which in all the cases were lower than 0.05. We discarded the first 5 million generations as burn-in and trees were summarised under the 50% majority rule method.

Abbreviations and acronyms used are as follows: ABGD, automatic Barcode Gap Discovery; BI, Bayesian Inference; FW, forewing; HW, hindwing; MCZ, Museum of Comparative Zoology, Harvard, United States; ML, Maximum Likelihood; NJ, Neighbour Joining; UN, underside; UNFW, underside of forewing; UNHW, underside of hindwing; UP, upperside; UPFW, upperside of forewing; UPHW, upperside of hindwing.

Systematics

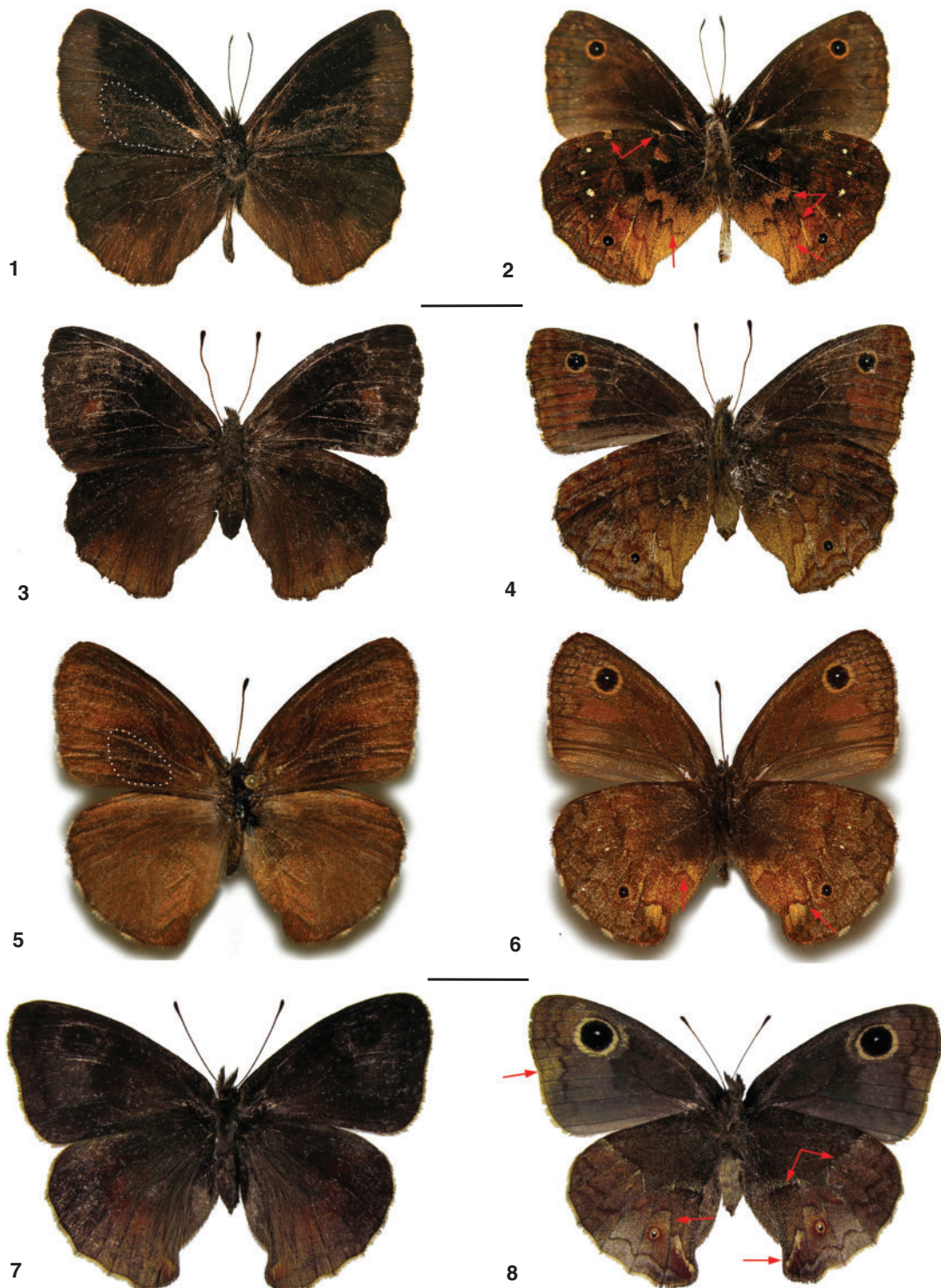
Here we propose the following systematic treatment for the species of the *chrysaoros* group of *Calisto*:

Calisto clydoniata Schwartz & Galii (Figs 1–4, 17, 18, 24, 27, 30–40)

Calisto clydoniata Schwartz & Gali, 1984: 10.– Schwartz, 1989: 439; Smith *et al.*, 1994: 54; Lamas, 2004: 207.

Material examined

Holotype (label data). HOLOTYPE *Calisto clydoniata* A. Schwartz – Frank Gali/890. Rep. Dom.: La Estrella: 1 km SW Puesto Pirámide 204, 6200 ft. 5 August 1982 F. Gali/MGCL/FLMNH Specimen no 47129/Allyn



Figs 1–8. Adults of the *chrysaoros* species group of *Calisto*. 1, UP *C. clydoniata* male, Pirámide 204 (type locality), Sierra de Neiba, white dotted line indicating androconial patch; 2, UN *C. clydoniata* male, same data; 3, UP *C. clydoniata* female, same data; 4, UN *C. clydoniata* female, same data; 5, UP *Calisto* sp. male, specimen formerly assigned to *C. clenchi*, 1.2 km S Scierie, Massif de la Selle, l’Ouest, Haiti, white dotted line indicating androconial patch; 6, UN *Calisto* sp. male, same data; 7, UP *C. chrysaoros chrysaoros* female, Los Arroyos (type locality of *C. clenchi* synonym of *C. chrysaoros chrysaoros*), Sierra de Bahoruco; 8, UN *C. chrysaoros chrysaoros* female, same data. Arrows indicate distinctive features. Scale bar 10 mm. All photographs were taken by Antonio Pérez-Asso, except 5 and 6 by Kim Davis, Mike Stangeland and Andrew Warren.

Museum Acc. 1983–37. [Examined through pictures available at Warren *et al.* (2016).]

Other material examined. Dominican Republic, Elias Piña, Puesto Pirámide 204, 16.vi.2011, A. Pérez-Asso & A. López (5♂, 2♀) (DNA vouchers: JAGWI-804, 805, 807, 808).

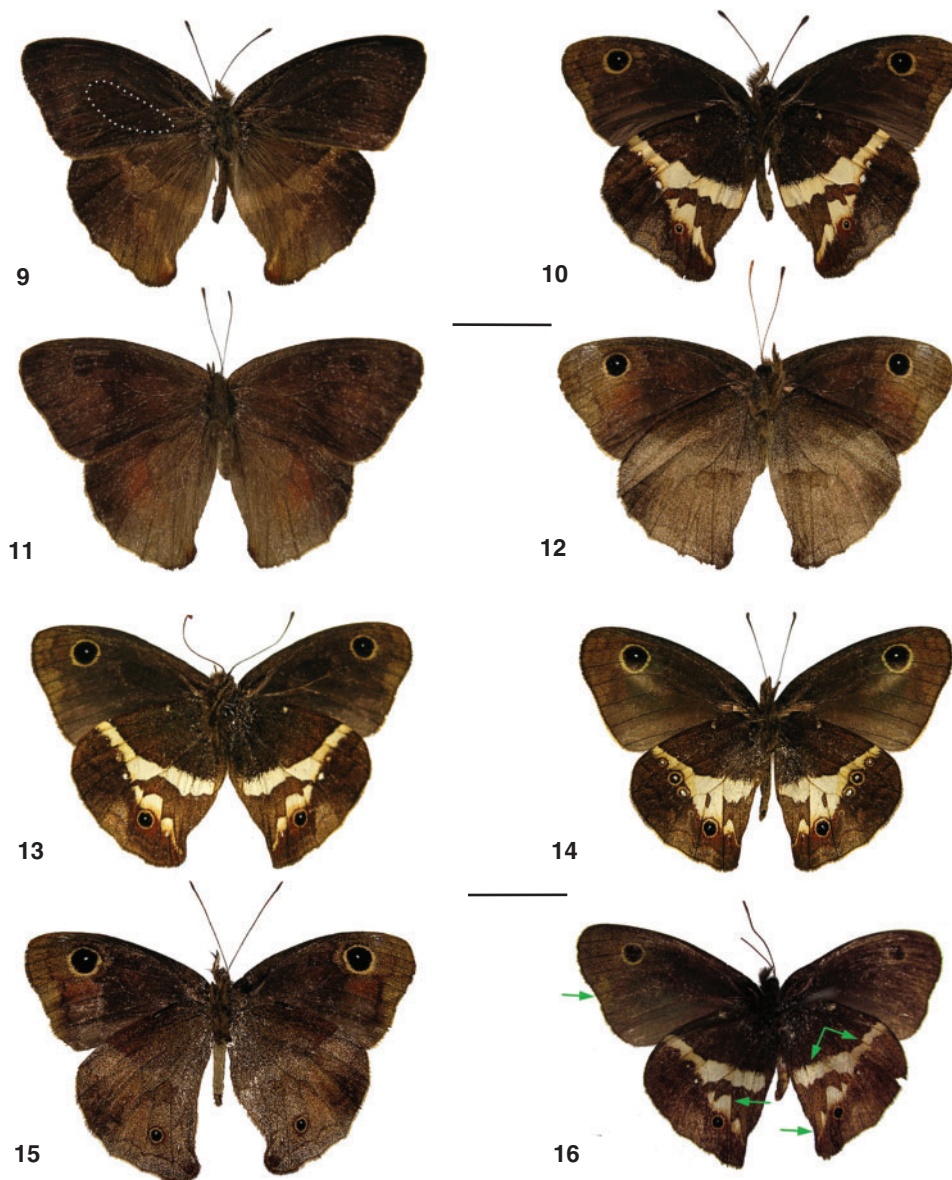
Diagnosis

Calisto clydoniata is easily separated from all other *Calisto* species by the orangish scales on the UNHW (Figs 2, 17, 18).

There is also a distinctive orange dot on the costal margin at the end of the postdiscal line. The indentation pattern of both discal and postdiscal lines is also unique (Figs 2, 4, 17, 18).

Distribution

Calisto clydoniata is restricted to the Dominican Republic portion of the Sierra de Neiba, separated from the northern Cordillera Central by the San Juan Valley and from the southern Sierra de Bahoruco by the Neiba Valley (Fig. 30).



Figs 9–16. Adults of the *chrysaoros* species group of *Calisto*. 9, UP *C. chrysaoros chrysaoros* male, Caseta 2, Sierra de Bahoruco, white dotted line indicating androconial patch, 10, UN *C. chrysaoros chrysaoros* male, same data; 11, UP *C. chrysaoros chrysaoros* female, same data; 12, UN *C. chrysaoros chrysaoros* female, same data; 13, UN *C. chrysaoros galii* male, La Nuez, Valle Nuevo, Cordillera Central; 14, UN *C. chrysaoros galii* male, same data; 15, UN *C. chrysaoros galii* female, same data; 16, UN *C. chrysaoros galii* male, Pirámide 204 (type locality), Sierra de Neiba, arrows indicating similarities with red arrows in Fig. 8. Scale bar 10 mm. Photographs by Antonio Pérez-Asso.

Biology

The few data known on this species appeared in the original description and have been repeated by subsequent authors. The species flies at an altitude of 1600–1900 m, inhabits deciduous and pine forest and mixed pine–broadleaf forest. Its life history is unknown.

Calisto chrysaoros Bates

Diagnosis

This species is easily separated from all known *Calisto* by the possession of a very pronounced anal lobe and a series of yellowish white spots arranged as an inverted ‘Y’ on the UNHW. There is a large amount of variation in these spots with some females, formerly described as *C. clenchi*, syn. nov., lacking them. Schwartz (1983) illustrated this variation using line drawings and we do the same here, showing distinctively patterned specimens (Figs 8, 10, 12–16).

The populations of the southern and northern Hispaniola mountains, geographically isolated by the Cul de Sac/Enriquillo depression, differ slightly and can be recognised on the basis of a few morphological characters and also by exclusive fixed positions of their COI barcode sequences and therefore we propose to treat them as two subspecies.

Calisto chrysaoros chrysaoros Bates

(Figs 8–12, 19–21, 25, 28, 30–40)

Calisto chrysaoros Bates, 1935: 235.– Schwartz, 1989: 389; Smith *et al.*, 1994: 50; Lamas, 2004: 207.

Calisto clenchi Schwartz & Gali, 1984: 10.– Schwartz, 1989: 441; Smith *et al.*, 1994: 54; Lamas, 2004: 207. Syn. nov.

Material examined

Holotype (label data). HOLOTYPE *Calisto clenchi* A. Schwartz–Frank Gali/8511. Rep. Dom.: Pedernales: 22 km SW El Aguacate, 5200 ft. 1 August 1982 A. Schwartz/Allyn Museum Acc. 1983–37. [Examined through pictures available at Warren *et al.* (2016).]

Holotype (label data). *Calisto chrysaoros* Bates Holotype/M.C.Z. Type 21986/Roche Croix La Hotte, Haiti, 5000', 13-X-'34, P.J. Darlington/antennae and LFW repaired 26 SEP 1980 R.E. Silberglied/Allyn Museum Photo No. 091980 11-12/Allyn Museum Photo No. 092280 A1-2. [Examined through pictures available online at Warren *et al.* (2016) and the MCZ Insect Type Database (<http://140.247.96.247/mcz/>, accessed 12 October 2015).]

Other material examined. *Calisto chrysaoros*: República Dominicana, caseta 2, Sierra de Bahoruco, viii.2010, A. López, A. Pérez-Asso & J.A. Genaro (3♂) (DNA vouchers: JAGWI-498–500). República Dominicana, Pedernales, caseta 2, Sierra de Bahoruco, 23.vi.2011, A. Pérez-Asso & A. López (3♂, 2♀) (DNA vouchers: JAGWI-801–803). Same data as preceding except 7.vi.2011 (2♂, 2♀) (DNA voucher: JAGWI-809). Same data as preceding except 22.xi.2011 (3♂, 4♀) (DNA vouchers: JAGWI-912–918). Type locality of *Calisto clenchi*: República Dominicana, Pedernales, Los Arroyos, 6.viii.2014, A. Pérez-Asso & A. López (4♀) (DNA vouchers: JAGWI-1036–1039).

Diagnosis

Size larger on average compared with *C. chrysaoros galii*, FWL: 18.2 mm in males ($n=11$), 19.6 mm in females ($n=12$). Other differences are the darker rusty brown background on the underside of the wings, better noted when comparing males

(females are too variable) (Figs 20, 22), the reduced size of male genitalia in relation to animal size, and the shorter ductus bursae of the female genitalia compared with the northern subspecies (Figs 28, 29). COI barcodes have cytosines in Positions 287 and 548, whereas in northern populations only a thymine was present.

Distribution

Calisto chrysaoros chrysaoros inhabits the southern mountains of Haiti and Dominican Republic: Massif de la Hotte, Massif de la Selle, and Sierra de Bahoruco (Fig. 30).

Biology

This subspecies is found from middle to high altitude between 1007 and 1910 m (Schwartz 1989). It can be found in several types of wooded habitats including pine, deciduous and mixed pine–broadleaf forest. The species seems to be associated with climbing grasses of the genera *Arthrostyidium* and *Chusquea*, the first of them forming dense curtains in the forest, a fact that has been noted by many authors (Schwartz 1989; Smith *et al.* 1994; Schwartz and Wetherbee 1996; Sourakov 1996).

Calisto chrysaoros galii Schwartz

(Figs 13–16, 22, 23, 26, 29–40)

Calisto galii Schwartz, 1983: 3.– Schwartz, 1985: 3; Schwartz, 1989: 391; Smith *et al.*, 1994: 50; Lamas, 2004: 207. Syn. nov.

Calisto galii choneupsilon Schwartz, 1985: 2.– Smith *et al.*, 1994: 50; Lamas, 2004: 207. Syn. nov.

Material examined

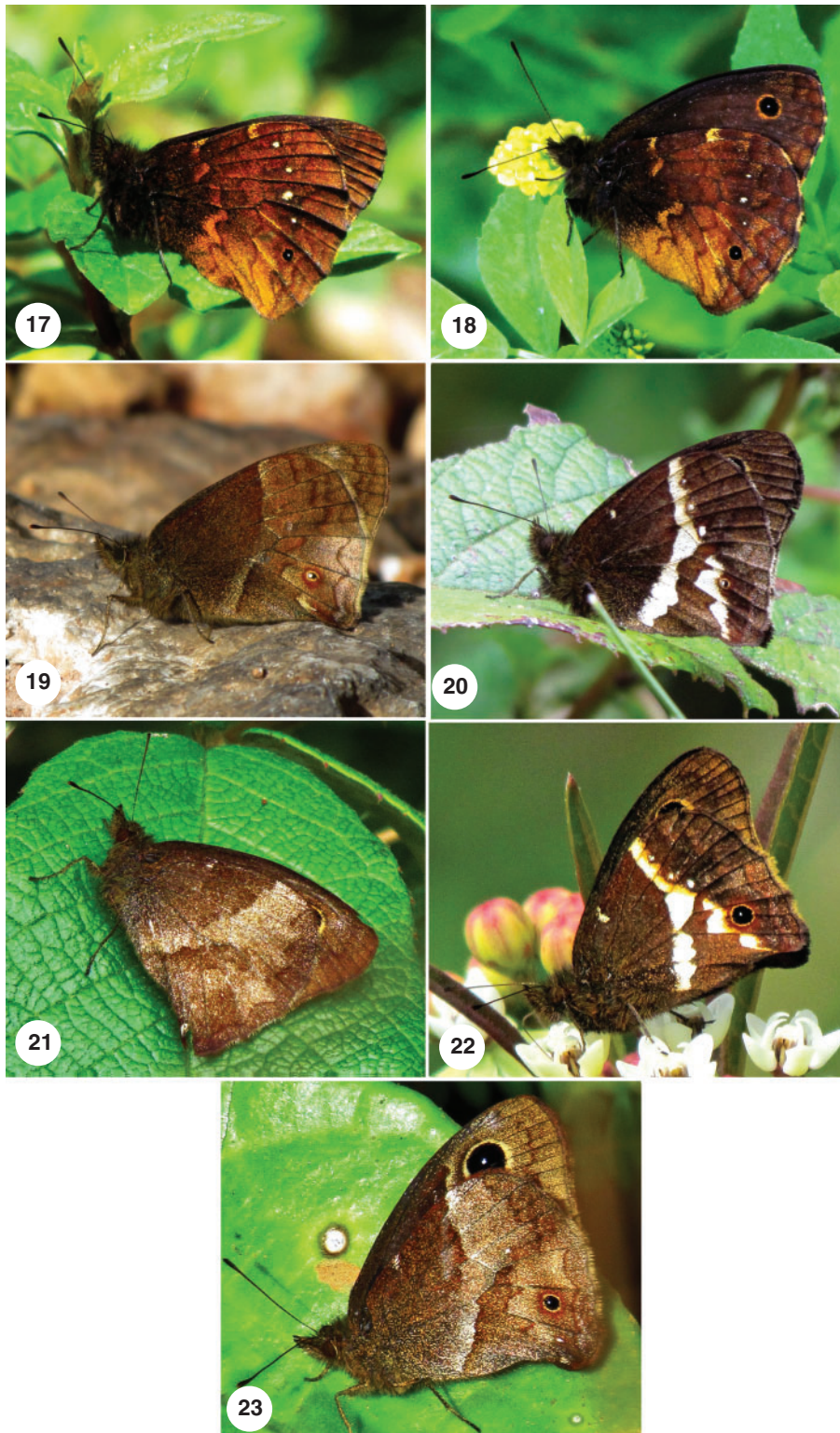
Holotype (label data). HOLOTYPE *Calisto galii* Albert Schwartz/3768. Rep. Dom.: La Vega: 18 km SE Constanza, 5200 ft. 5 July 1980 A. A. Gineika/Genitalia vial no. M-6341♂ Lee D. Miller. [Examined through pictures available at Warren *et al.* (2016).]

Holotype (label data). HOLOTYPE *Calisto galii choneupsilon* Albert Schwartz/912. Rep. Dom.: La Estrella: 2 km NE Puesto Pirámide 204, 5200 ft. 6 August 1982 F. Gali/Allyn Museum Acc. 1983–37. [Examined through pictures available at Warren *et al.* (2016).]

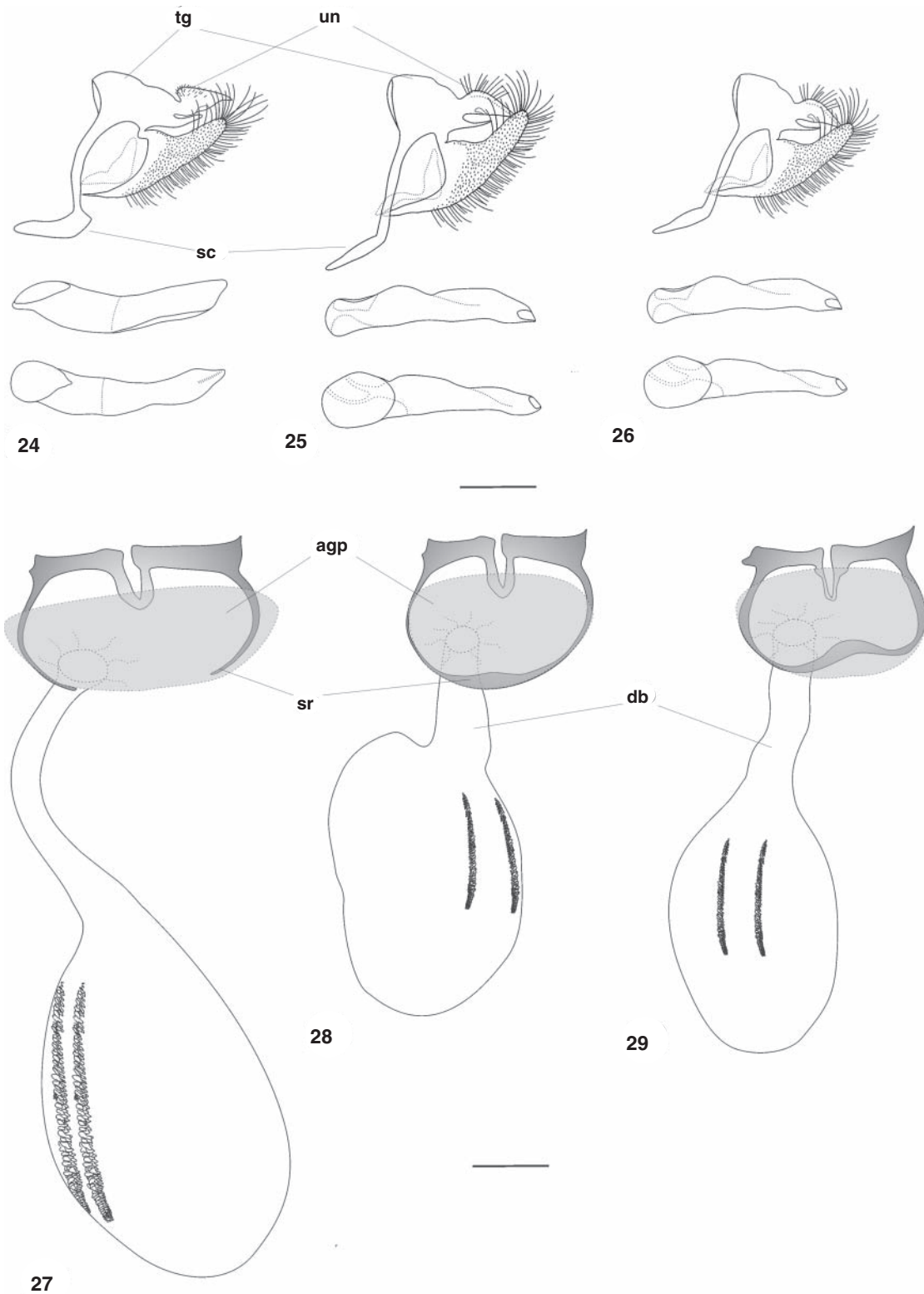
Other material examined. *Calisto galii*: República Dominicana, La Vega, Constanza, Valle Nuevo, xi.2008, J.A. Genaro & A. Pérez-Asso (1♂) (DNA voucher: JAGWI-546). República Dominicana, La Vega, Valle Nuevo, La Nuez, 19.vi.2011, A. Pérez-Asso & A. López (3♂) (DNA vouchers: JAGWI-796–797). Same data as preceding except La Pirámide, 20.vi.2011 (2♂, 2♀) (DNA voucher: JAGWI-798). República Dominicana, La Vega, El Cruce, Alto de la Cotorra, 29.xi.2011, A. Pérez-Asso & A. López (5♂, 8♀) (DNA vouchers: JAGWI-919–931). *Calisto galii choneupsilon*: Dominican Republic, Elias Piña, Puesto Pirámide 204, 16.vi.2011, A. Pérez-Asso & A. López (3♂, 1♀) (DNA vouchers: JAGWI-949, 950). Same data as preceding except 17.vi.2011 (2♂) (DNA vouchers: JAGWI-799, 800). Same data as preceding except 21.vii.2012 (2♂) (DNA vouchers: JAGWI-952, 953).

Diagnosis

Average FWL: 16.3 mm in males ($n=18$), 17.8 mm in females ($n=11$). In this subspecies the underwing background colour of males is a paler olive brown (Fig. 22), the male genitalia are proportionally larger, and the female genitalia have a larger ductus bursae compared with that of *C. chrysaoros chrysaoros* (Figs 28, 29). Sampled specimens show two exclusive fixed



Figs 17–23. Live adults of the *chrysaoros* species group of *Calisto*. 17, *C. clydoniata* male, Piramide 204 (type locality), Sierra de Neiba; 18, *C. clydoniata* female, Piramide 204, Sierra de Neiba; 19, *C. chrysaoros chrysaoros* female, Los Arroyos (type locality of *C. clenchi*, synonym of *C. chrysaoros chrysaoros*), Sierra de Bahoruco; 20, *C. chrysaoros chrysaoros* male, Los Arroyos, Sierra de Bahoruco; 21, *C. chrysaoros chrysaoros* female, Caseta 2, Sierra de Bahoruco; 22, *C. chrysaoros galii* male, Valle del Tetero, Cordillera Central; 23, *C. chrysaoros galii* female, Valle del Tetero, Cordillera Central. Photographs by Antonio Pérez-Asso.



Figs 24–29. Genitalia of the *chrysaoros* species group of *Calisto*. 24, male genitalia of *C. clydoniata* (top: main body in lateral view, middle aedeagus in lateral view, aedeagus in dorsal view); 25, male genitalia of *C. chrysaoros chrysaoros*; 26, male genitalia of *C. chrysaoros galii*; 27, female genitalia of *C. clydoniata* (ventral view); 28, female genitalia of *C. chrysaoros chrysaoros*; 29, female genitalia of *C. chrysaoros galii*. Scale bars 0.5 mm. tg, tegument; un, uncus; sc, saccus; agp, anterior genital plate; sr, sclerotised ring; db, ductus bursae.

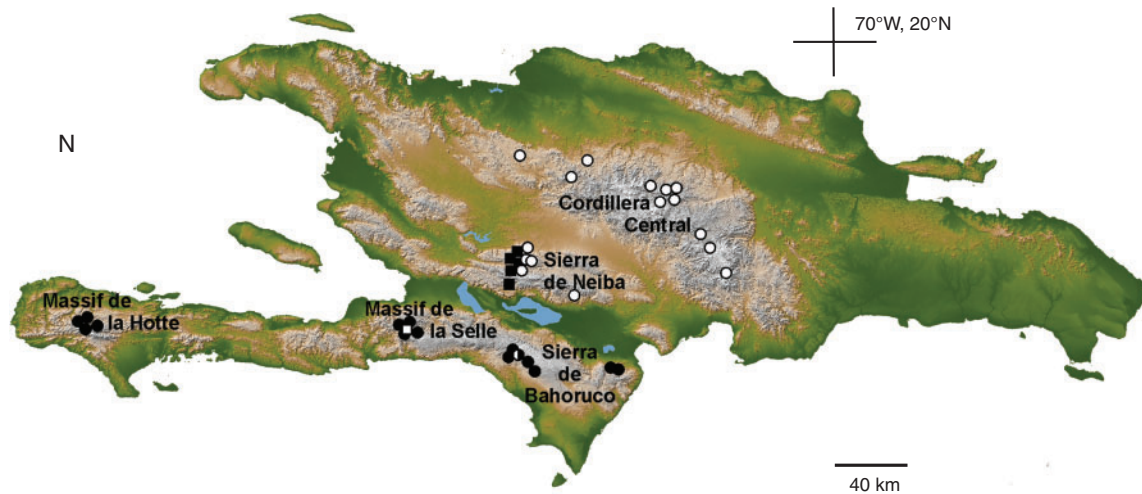


Fig. 30. Geographic distribution of the *chrysaoros* species group of *Calisto*. Black squares, *C. clydoniata*; black circles, *C. chrysaoros chrysaoros*; white circles, *C. chrysaoros galii*; black and white circle, type locality of *C. clenchi*, synonym of *C. chrysaoros chrysaoros*: Los Arroyos, Sierra de Bahoruco); white square, locality of the *Calisto* male formerly assigned to *C. clenchi* (Scierie, Massif de la Selle).

positions in their COI barcodes in positions 319 and 514, both cytosines, whereas in southern populations a thymine is present.

Distribution

Calisto chrysaoros galii inhabits the Dominican portion of the central Sierra de Neiba and the northern Cordillera Central (Fig. 30).

Biology

Like the southern subspecies *C. chrysaoros galii* flies at mid and high elevations, from 976 to 1891 m in the Sierra de Neiba and 549 to 2227 m in the Cordillera Central (Schwartz 1989). It is present in the same wooded habitats with climbing grasses (Schwartz 1989; Smith *et al.* 1994; Schwartz and Wetherbee 1996; Sourakov 1996). The egg and first-instar larva of the Cordillera Central population were described by Sourakov (1996).

Morphological comparisons

The comparison of available specimens showed marked differences between *C. clydoniata* and the other taxa. The only known male attributed to *C. clenchi* also showed features distinguishing it from all other *chrysaoros* group members including its 'conspecific' females. We also found similarities between specimens with a colour pattern formerly assigned to *C. clenchi* (all females) and *C. chrysaoros* specimens from both the southern and northern populations, the later previously included under the name *C. galii*.

Wing shape. Both sexes of *C. clydoniata* and the male specimen from Scierie, Haiti, attributed to the former *C. clenchi* have more rounded wings (Figs 1–6). They also lack the slight concavity in the median portion of the outer margin of the FW present in all specimens of *C. chrysaoros* across its range (Figs 7–16). In addition, the anal lobe of the HW is reduced in the Scierie male whereas it is more developed in both sexes of all populations of *C. chrysaoros* (Figs 5–16).

Androconial patch. Males of *C. clydoniata* possess a large androconial patch occupying the basal two-thirds of the UPFW below the cell (dotted white line in Fig. 1). In the male from Scierie the androconial patch is smaller, occupying only the mid portion of UPFW below the distal half of the cell (dotted line in Fig. 5). Males from all *C. chrysaoros* populations have an elongated diagonal patch along the posterior limit of the FW cell but distant from the posterior margin of the wing (dotted line in Fig. 9).

Wing pattern. UPFW colour pattern is very distinctive in *C. clydoniata* males due to the dark basal two-thirds of the wing, above the cell, hiding the androconial patch (Fig. 1). In all other taxa the background is homogenous.

Both sexes of *C. clydoniata* have a very distinctive UNHW pattern. The discal and postdiscal lines are heavily waved (red arrows in Fig. 2). An orangish suffusion, stronger in males, covers most of the UNHW surface and two yellowish costal spots by the discal and postdiscal lines are also distinctive. Males have four dots in the ocellar row but these are pale yellow instead white, as in most other *Calisto*. These dots are reduced in females, being only two or three in number with those at Rs–M1 and/or M1–M2 interspaces absent (Figs 4, 18). The male from Scierie possesses heavily waved discal and postdiscal lines but these differ from *C. clydoniata* in how and where these meet the posterior margin of the UNHW (red arrows in Fig. 6). The single known specimen has only two tiny whitish dots in the ocellar row. Both sexes of *C. chrysaoros* also have a heavily waved postdiscal line on the UNHW but indentations, similar in all specimens examined, are more irregular and differ from those on *C. clydoniata* and the male from Scierie (red arrows in Fig. 8, green arrows in Fig. 16). In the same manner, all specimens have a slightly curved and relatively smooth discal line. This line is bent inward from costal two-thirds to basal one-third of the anal margin (red arrows in Fig. 8, green arrows in Fig. 16). *C. chrysaoros* possesses a very distinctive UNHW pattern consisting of an inverted whitish 'Y'. The contour of the inverted Y is highly variable in the specimens examined from

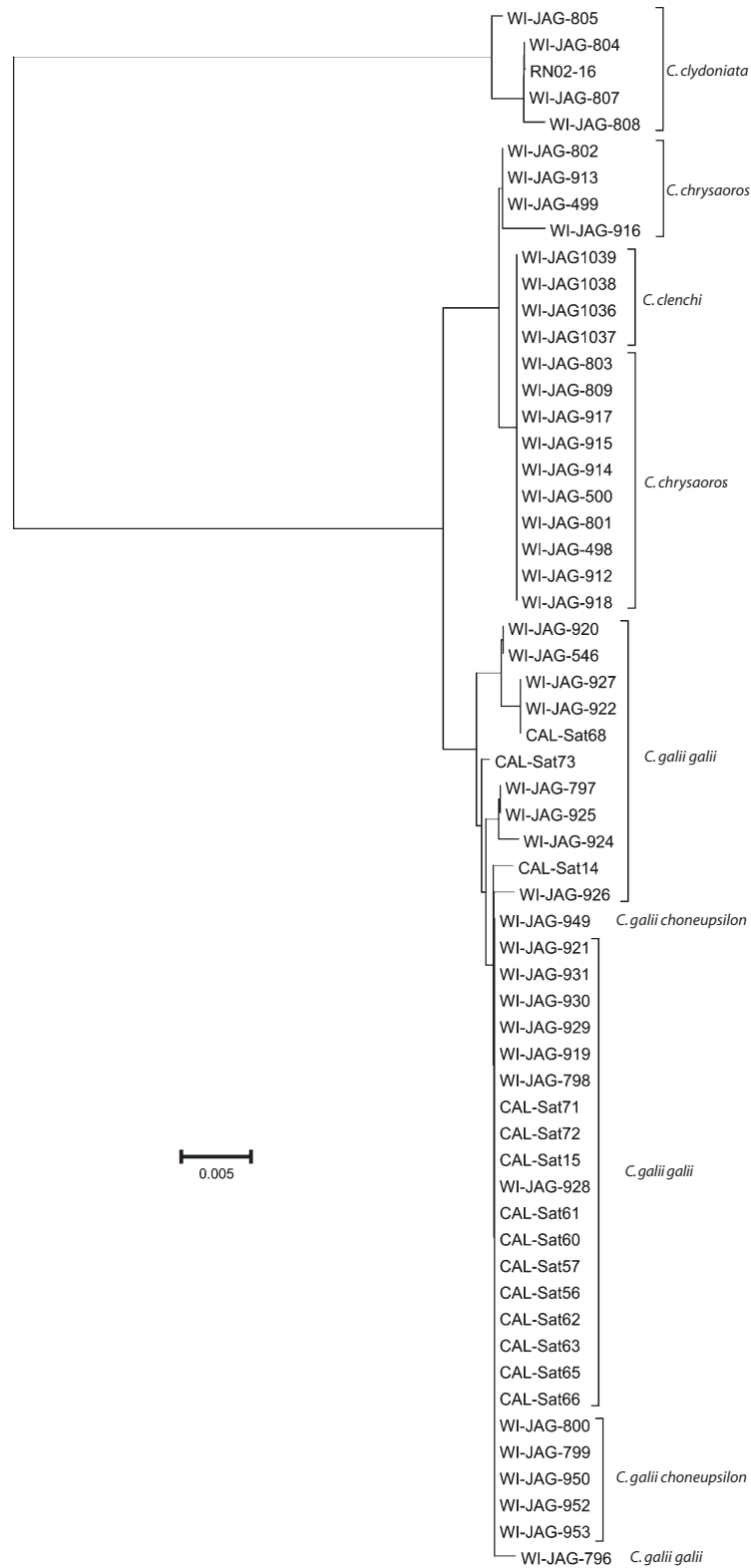
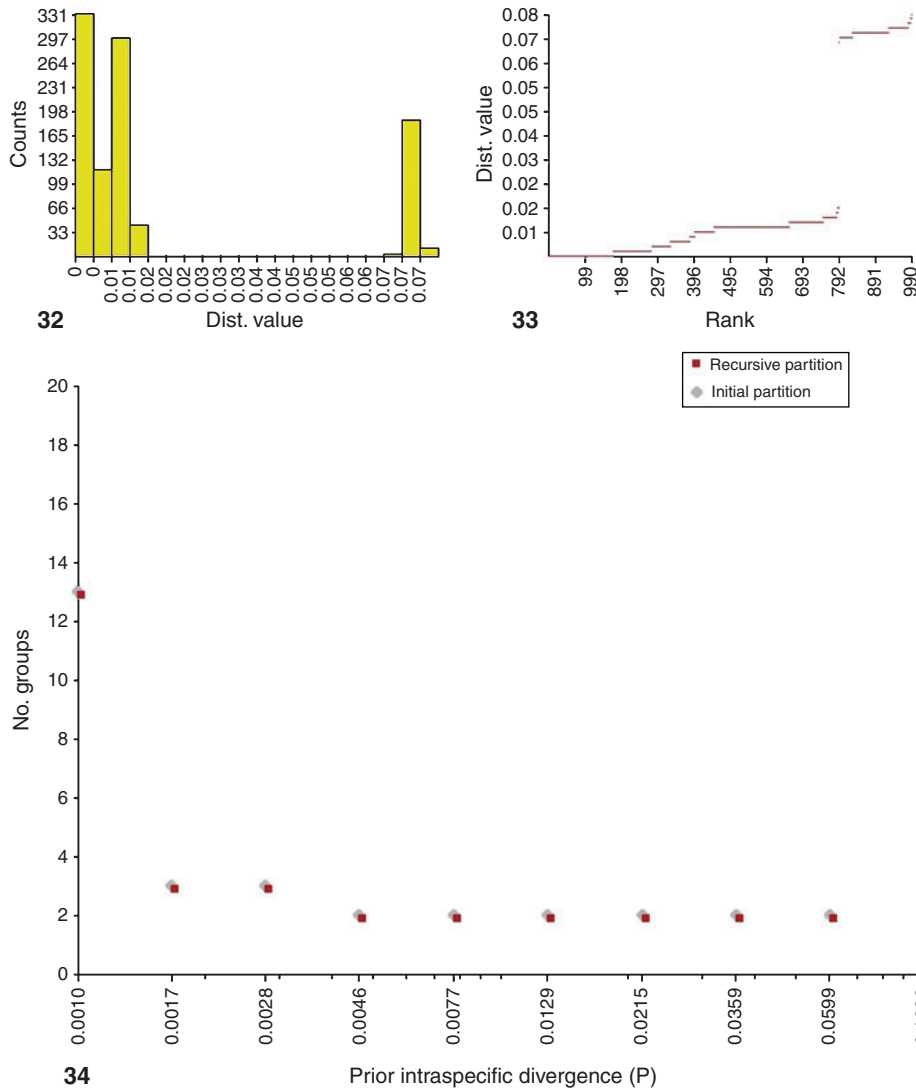


Fig. 31. Neighbour-joining tree of the COI barcode sequences of species belonging to the *chrysaoros* group of *Calisto*. Gray bars group sequences of both subspecies of *C. chrysaoros*. Black sections within the grey bars indicate the position of sequences from specimens formerly attributed to *C. clenchi* (type locality Los Arroyos, Sierra de Bahoruco), now within *C. chrysaoros chrysaoros*, and *C. galii choneupsilon* (type locality Sierra de Neiba), now within *C. chrysaoros galii*.



Figs 32–34. 32, Histogram of genetic distances; 33, ranked distances; 34, recursive and initial partitions obtained by using the ABGD web interface for the available COI sequences of *Calisto clydoniata*, *C. chrysaoros chrysaoros*, and *C. chrysaoros gali*.

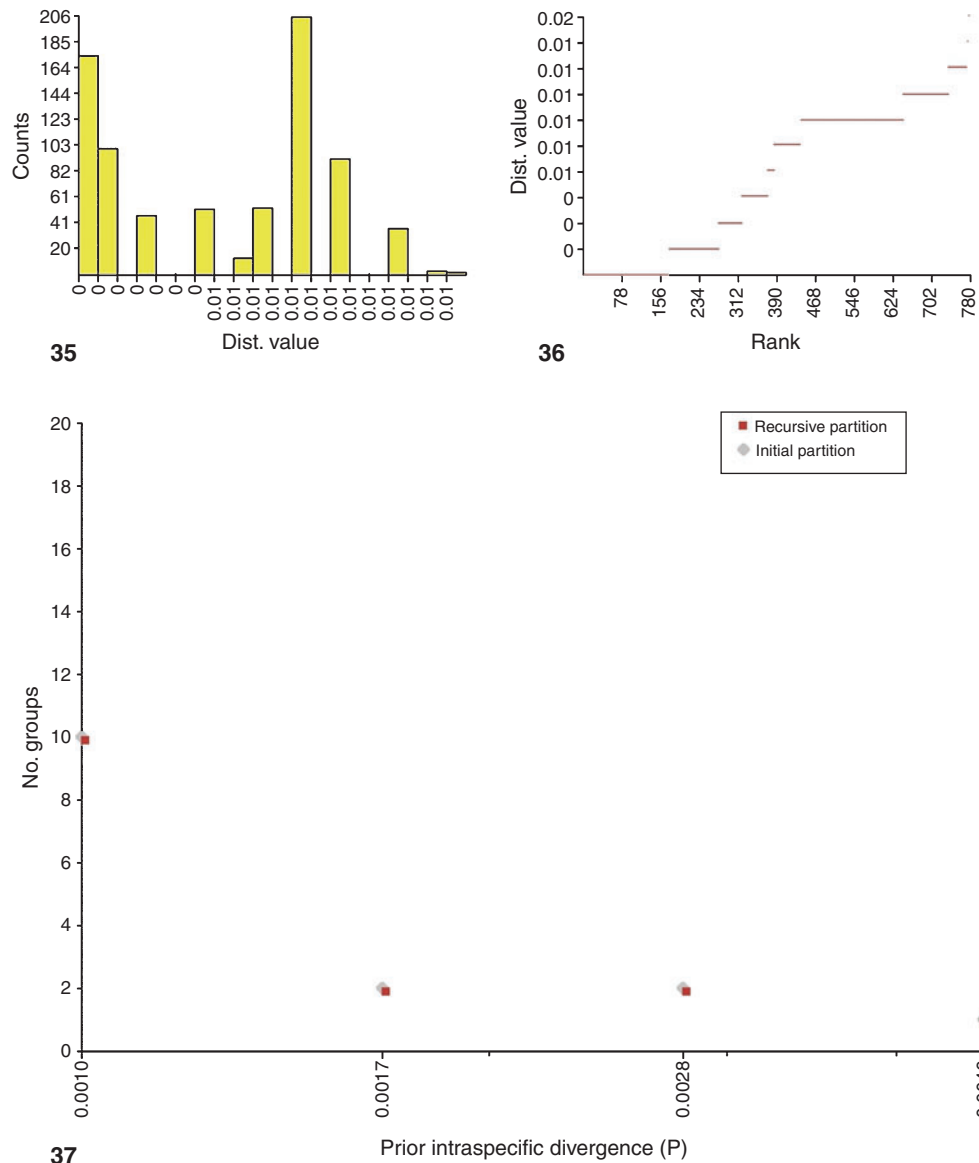
both palaeo-islands as well as the whitish dot on the costa near the wing base (Figs 10, 13, 14, 20, 22). Females from all three mountain ranges may have this pattern diffuse or almost entirely absent (Figs 8, 12, 15, 19, 21, 23), as occurs in females from Los Arroyos included in the type series of *C. clenchi*. Another feature used to distinguish *C. clenchi*, the reduction of the UNHW ocellus, is also present in specimens of *C. chrysaoros* from other localities (Figs 12, 20, 21).

Genitalia. Comparisons of male genitalia showed differences only between *C. clydoniata* and *C. chrysaoros* (Figs 33–35). The main differences are the shape of the tegumen, uncus (including hair length) and saccus, and in the shape of the aedeagus. There are no major differences in the male genitalic structure in *C. gali gali* and *C. gali choneupsilon* except it is slightly smaller in size. Comparisons of female genitalia found a longer and more slender ductus bursae in *C. clydoniata* that, together with its incomplete sclerotised ring

and elongated anterior genital plate (Fig. 27), also allows recognition. Female structures of *C. chrysaoros* from all populations showed almost no differences between pairs of each palaeo-island (Figs 28, 29). Differences were restricted to the length of the ductus bursae, being slightly shorter in southern populations (Figs 28, 29). The convexity in the middle portion of the sclerotised ring in the latter pair is illustrated to show the variation in this trait which occurs in both North and South palaeo-island populations (Figs 28, 29).

DNA barcoding, haplotypes network, and species delineation by ABGD

We obtained 45 COI sequences belonging to *C. clydoniata* (4) and *C. chrysaoros* (41). The latter included 17 sequences from Cordillera Central (north), six from Sierra de Neiba (central), and 20 from Sierra de Bahoruco (south). We attempted to obtain



Figs 35–37. 35, Histogram of genetic distances; 36, ranked distances; 37, recursive and initial partitions obtained by using the ABGD web interface for the available COI sequences of *C. chrysaoros chrysaoros* and *C. chrysaoros galii*.

the marker from a *C. wetherbeeii* specimen but were unsuccessful. We also downloaded 14 more sequences of *C. chrysaoros* and another of *C. clydoniata* from GenBank.

The Neighbour Joining algorithm performed resulted in two deeply separated clusters, one with the four *C. clydoniata* sequences and the other containing the *C. chrysaoros* ones (Fig. 31). The latter showed a shallower split in two groups, one containing all the sequences from the South palaeo-island, *C. chrysaoros chrysaoros*, and the other all the sequences from the Cordillera Central and Sierra de Neiba, both belonging to the North palaeo-island, *C. chrysaoros galii*.

Comparison of the p-distances showed high values, above 7.1%, for *C. clydoniata* sequences compared with any *C. chrysaoros* population and low or very low distances between sequences of the different populations of the latter (Table 1).

The average genetic distance among the sequences from southern specimens, *C. chrysaoros chrysaoros*, was 0.07% whereas divergence within the northern specimens, Cordillera Central and Sierra de Neiba, *C. chrysaoros galii*, was 0.11% (Table 1). Average distances among the subspecies or between any population from the south compared with any northern population ranged from 0.89 to 0.96% (Table 1).

The ABGD algorithm yielded the same results when applied in the three implemented models (JC, K2P, Simple Distance). The method identified a barcode gap centred approximately at 4% of divergence among all available COI sequences (Figs 32, 33). The analysis delineated the existence of three species in one recursive partition with a prior intraspecific genetic divergence value of 0.28% or two species in six recursive partitions with prior intraspecific genetic divergence values between 0.46%

Table 1. Uncorrected p-distance values among 658-bp COI sequences of *Calisto* taxa belonging to the *chrysaoros* group *n*, number of sequences (*n*), and the intra taxa average p-distance among specimens

	<i>n</i> /intra p-distance	p-distance	<i>C. c. chrysaoros</i> ^A	<i>C. c. chrysaoros</i>	<i>C. c. galii</i>
<i>C. clydoniata</i>	5/0.18				
<i>C. chrysaoros chrysaoros</i> ^A	4/0.00	7.15			
<i>C. chrysaoros chrysaoros</i>	14/0.11	7.22	0.07		
<i>C. chrysaoros galii</i>	30/0.19	7.15	0.96	0.95	
<i>C. chrysaoros galii</i> ^B	6/0.00	7.15	0.91	0.89	0.11

^ALos Arroyos, type locality of synonym *C. clenchi*.

^BSierra de Neiba, type locality of synonym *Calisto galii choneupsilon*.

and 5.9% (Fig. 34). These results we considered more likely than 15 species with intraspecific divergence values below 0.17% or a single species with intraspecific divergence values greater than 10% (Fig. 34, S1, available as supplementary material to this paper).

We performed a second analysis excluding the *C. clydoniata* sequences (Figs 35–37, and S2, available as supplementary material). The algorithm divided the sequences in 12 hypothetical species with prior intraspecific genetic divergence values below 0.17% or a species at values above 0.28% (Figs 35–37, and S2, available as supplementary material). We consider the last one to be the most likely hypothesis. In addition, the histogram of distances did not show the existence of a gap (Figs 35, 36).

Character base

By this approach the high differentiation between *C. clydoniata* and its relatives was evident. The available COI sequences of *C. clydoniata* have 18 positions with exclusive fixed positions (not mentioned here). On the other hand, sequences from specimens from Los Arroyos (type locality of *C. clenchi*) have no diagnostic features, being inseparable from the also southern *C. chrysaoros chrysaoros*. Together, they can be diagnosed by having a cytosine at both 287 and 548 positions. In the same way, northern *C. chrysaoros* from the Cordillera Central and Sierra de Neiba can only be distinguished together; they also have two exclusive fixed positions, also cytosines, at 319 and 514 positions.

Median-joining haplotype network

The median-joining haplotypes network produced a clustering similar to that of NJ, ML and BI phylogenies (Figs 38, 39). The results show the *C. clydoniata* haplotypes separated by 42 mutations from *C. chrysaoros* from Cordillera Central. The latter and specimens from Sierra de Neiba formed a group separated by four mutations from the southern group of *C. chrysaoros* (Fig. 38). Geographically, the northern Cordillera Central harbored the greater number of haplotypes (10) whereas one of these was the only haplotype present among six samples from Sierra de Neiba (Figs 38, 39). This haplotype was present at four localities of the Cordillera Central, being the most widespread (Figs 38, 39). At the southern Sierra de Bahoruco, only three haplotypes were present among the *C. chrysaoros* samples, one of which was the only one present in the samples from Los Arroyos, the type locality of *C. clenchi* (Figs 38, 39).

Phylogeny

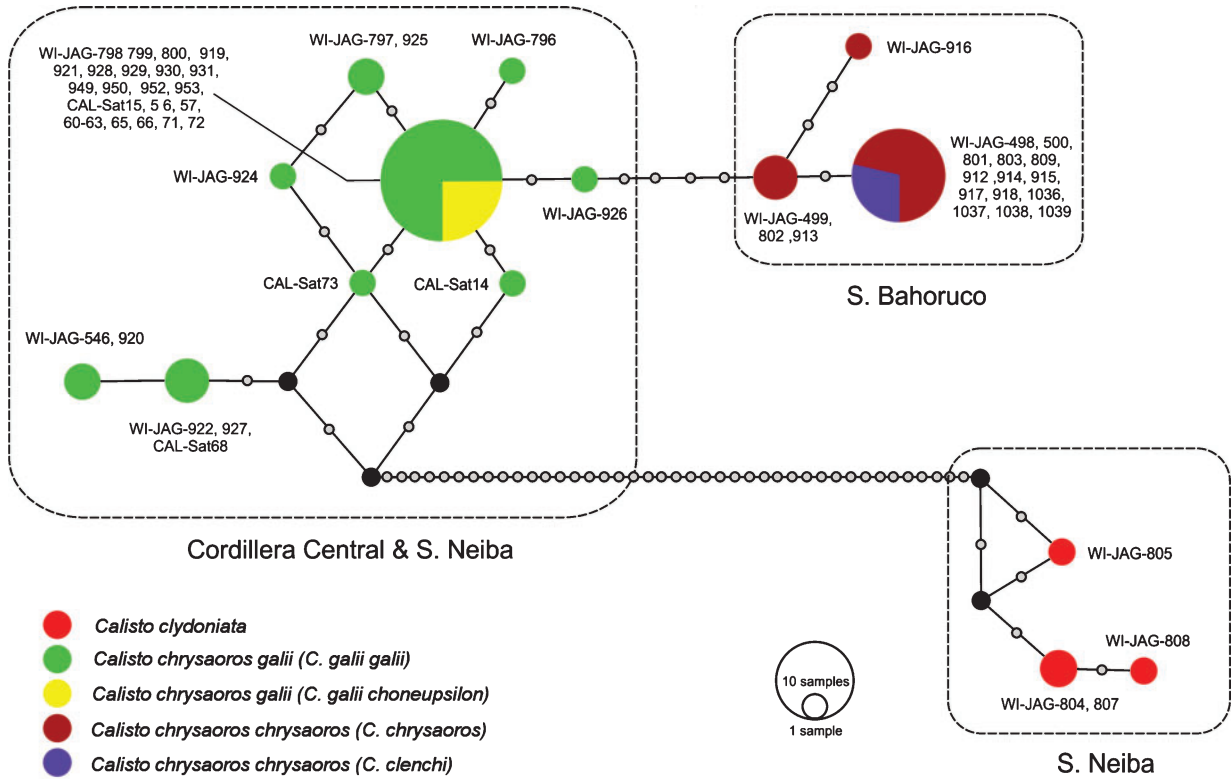
The ML topology matches the NJ tree and the Haplotype Network with *C. clydoniata* as sister of a clade containing all the sequences of *C. chrysaoros*. The latter clade was split in a pair of monophyletic clusters, South and North, although support was weak for the latter (Fig. 40). The BI topology was similar in obtaining two monophyletic clusters: one including all *C. clydoniata* sequences and the other grouping the remainder (Fig. 40). However, the structure inside the latter clade was different, with four *C. chrysaoros* sequences from the South positioned as sister of the northern ones, all subclades being weakly supported (not shown).

Discussion

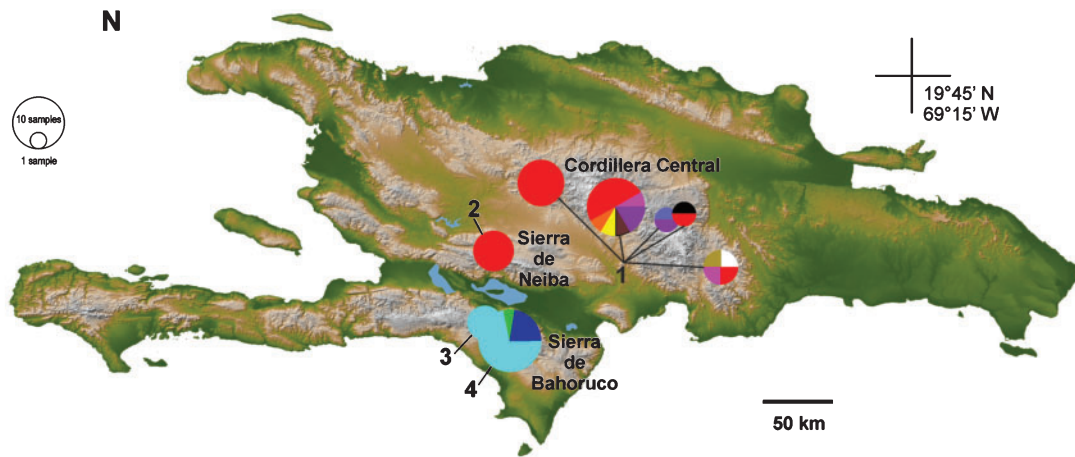
The convergence of the different methods employed for sequence analysis strongly supports the recognition of two species within the taxa analysed, *C. clydoniata* and *C. chrysaoros*. The latter comprises all the populations previously included under the names *C. clenchi*, *C. galii galii* and *C. galii choneupsilon*. Morphologically this hypothesis stands since wing pattern variability occurs in the northern and southern Hispaniolan palaeo-islands, and genitalic features also overlap except for a few minor differences. Females formerly assigned to *C. clenchi* match the highly diffuse pattern mentioned by Bates (1935) when describing *C. chrysaoros* females and by Schwartz (1983) for some *C. galii* females.

We propose the subspecies rank for the *C. chrysaoros* allopatric populations inhabiting both Hispaniolan palaeo-islands. The subspecies category has been controversial for a long time; however, recent points of view (Braby *et al.* 2012; Wilson *et al.* 2013; Patten 2015; Vinarski 2015) have shown its utility towards the recognition of populations with a level of differentiation under the species level, highlighting them as important units for evolution but also for conservation. Our decision to assign a subspecies to northern and southern populations of *C. chrysaoros* matches some of the requisites proposed by Braby *et al.* (2012) for subspecies recognition: they are allopatric and also genetically distinct and reciprocally monophyletic, according to their COI barcode sequences. Morphologically they are very similar but still exhibit a few slight but constant differences in average FWL, length of ductus bursae, and background colour underside, these being features also noted by past workers (Johnson *et al.* 1987; Schwartz 1989).

The split among southern and northern populations may have occurred in recent times, probably at the end of the multiple marine flooding of the Cul de Sac/Enriquillo depression, that



38



39

Figs 38–39. 38, Haplotype network of COI sequences belonging to *Calisto clydoniata*, *C. chrysaoros chrysaoros* and *C. chrysaoros galii* constructed in popart 1.7 using the median-joining algorithm (each circle represents a unique haplotype; colours represent the taxa recognised before the present study; circle size is proportional to number of samples; black dots denote missing haplotypes; grey dots denote mutational steps); 39, geographic distribution of the different COI haplotypes belonging to *Calisto clydoniata*, *C. chrysaoros chrysaoros* and *C. chrysaoros galii* (circle size is proportional to number of samples; each colour represents a unique haplotype; numbers: localities for *C. chrysaoros galii* at 1. Cordillera Central, 2. Sierra de Neiba (Puesto Pirámide 204, type locality of synonym *C. galii choneupsilon*), localities for *C. chrysaoros chrysaoros* at Sierra de Bahoruco, 3. Los Arroyos (type locality of synonym *C. clenchi*), 4. Caseta 2).

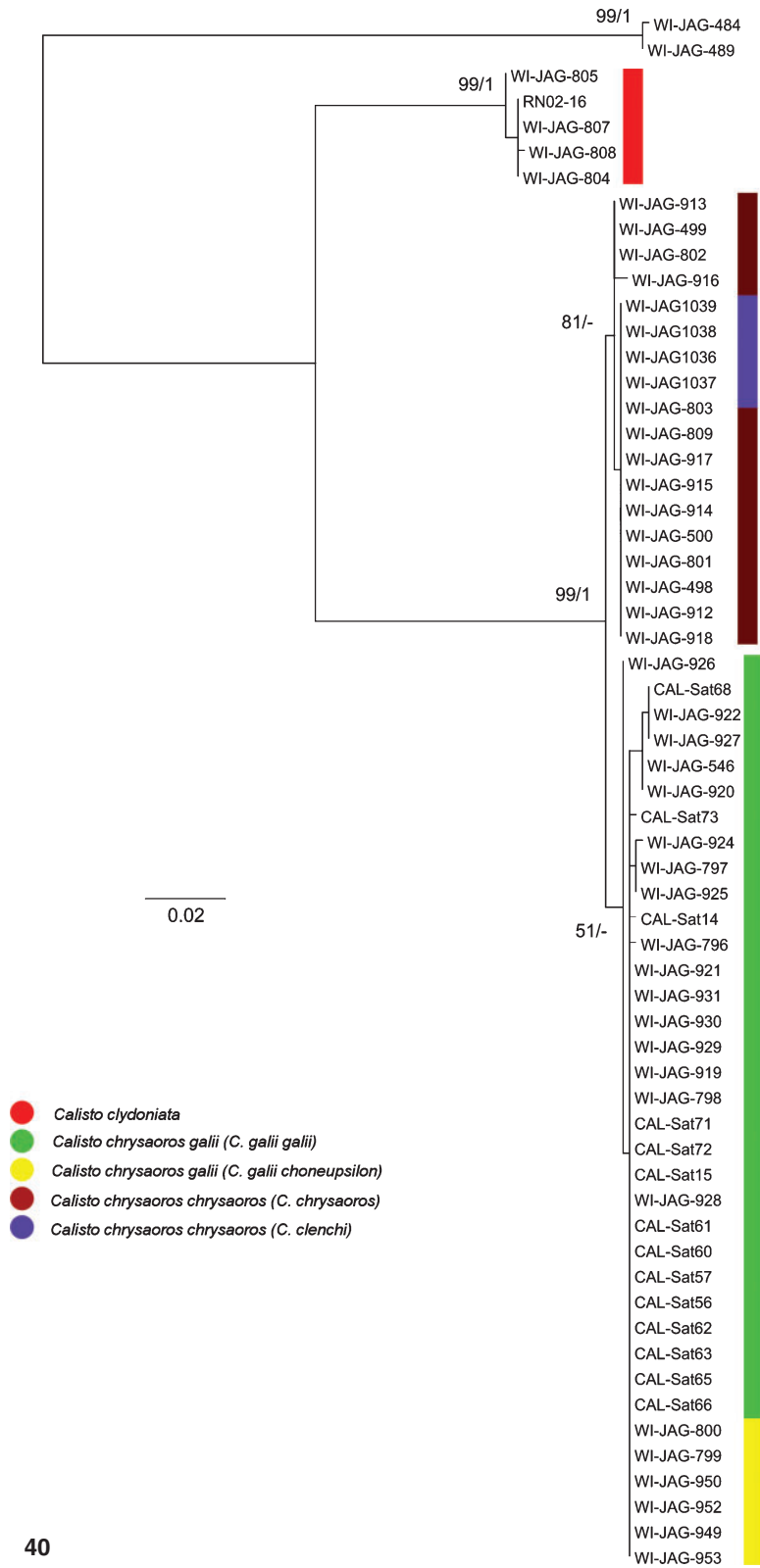


Fig. 40. Phylogenetic relationships among *Calisto clydoniata*, *C. chrysaoros chrysaoros* and *C. chrysaoros galii* reconstructed by Bayesian Inference and Maximum Likelihood. Node values are bootstrap support and posterior probabilities. Colour bars at the right side represent the taxa recognised before the present study.

divides the palaeo-islands, that occurred during the Plio–Pleistocene (Maurrasse *et al.* 1980; Graham 2003).

The only male formerly assigned to *C. clenchi* (Schwartz 1989; Smith *et al.* 1994) is not conspecific with *C. chrysaoros*. This is indicated by the morphological differences mentioned above and by the fact that all other known specimens previously assigned to *C. clenchi* (= *C. chrysaoros*) were females. Schwartz (1989) mentioned that Frank Gali collected two specimens at Scierie but no further comments on the second specimen have been found in the literature. The above specimen was available to us only through pictures at the *Butterflies of America* website (Warren *et al.* 2016). Even when wing pattern is distinctive, we can only come to further conclusions on its identity when its genitalia have been examined and when more material becomes available. Our comparison was only intended to demonstrate that the specimen was not conspecific with *C. clenchi* as an additional way of showing that specimens included under the latter name, now a synonym, are only *C. chrysaoros* bearing an extreme pattern.

The phylogenetic placement of *C. clydoniata* and *C. chrysaoros* needs to be reassessed in the future. A previous attempt by Matos-Maraví *et al.* (2014) failed to elucidate the relationships of the members of *chrysaoros* group with the extant congeners despite the use of six mitochondrial and nuclear genes. Further studies should include the inclusion of potential relatives such as *C. wetherbeeii* and the use of additional molecular markers or a phylogenomic approach.

Acknowledgements

We are grateful to Carlos Suriel and Celeste Mir (National Museum of Natural History, Santo Domingo, Dominican Republic), Yvonne Arias and Héctor Andujar (Grupo Jaragua Inc.) and Ruth Bastardo (Autonomous University of Santo Domingo, Dominican Republic) for their support during fieldwork and study of collections at Dominican Republic. We are thankful to Kim Davis, Mike Stangeland and Andrew Warren for their permission to use their picture of the male from Scierie, Massif de la Selle, l'Ouest, Haiti. We also thank Tim Norriss for his review of the English. We are extremely grateful to Victor González (Puerto Rico) for his financial support for travel to Dominican Republic and DNA sequencing at Guelph University, Ontario. This research was also partially funded by a grant from the Systematics Research Foundation (SRF). We are grateful to Andrei Sourakov and other anonymous referees for reviewing the manuscript during the editorial process.

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Handling editor: Lars Krogmann

Appendix 1. List of specimens with COI barcode sequences analysed and their corresponding collection data and GenBank accession numbers
Voucher codes are unique identifiers referring to specimens at repositories and to individual records in the Barcode of Life Datasystems (BOLD, www.boldsystems.org, accessed 27 May 2017)

Species	Voucher code	Locality	GenBank accession number
<i>Calisto nubila</i>	JAG-WI-484	Puerto Rico, Coto Laurel	KX536775
<i>Calisto nubila</i>	JAG-WI-489	Puerto Rico, Quebradillas	KX536711
<i>Calisto chrysaoros</i>	JAG-WI-498	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	JN299975
<i>Calisto chrysaoros</i>	JAG-WI-499	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	JN299976
<i>Calisto chrysaoros</i>	JAG-WI-500	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	JN299977
<i>Calisto chrysaoros</i>	JAG-WI-801	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172250
<i>Calisto chrysaoros</i>	JAG-WI-802	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172239
<i>Calisto chrysaoros</i>	JAG-WI-803	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172216
<i>Calisto chrysaoros</i>	JAG-WI-809	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172249
<i>Calisto chrysaoros</i>	JAG-WI-912	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172243
<i>Calisto chrysaoros</i>	JAG-WI-913	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172233
<i>Calisto chrysaoros</i>	JAG-WI-914	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172217
<i>Calisto chrysaoros</i>	JAG-WI-915	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172222
<i>Calisto chrysaoros</i>	JAG-WI-916	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172225
<i>Calisto chrysaoros</i>	JAG-WI-917	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172240
<i>Calisto chrysaoros</i>	JAG-WI-918	Dominican Republic, Independencia, Sierra de Bahoruco, Caseta 2	MF172236
<i>Calisto clenchi</i>	JAG-WI-1036	Dominican Republic, Pedernales, Los Arroyos	MF172232
<i>Calisto clenchi</i>	JAG-WI-1037	Dominican Republic, Pedernales, Los Arroyos	MF172221
<i>Calisto clenchi</i>	JAG-WI-1038	Dominican Republic, Pedernales, Los Arroyos	MF172242
<i>Calisto clenchi</i>	JAG-WI-1039	Dominican Republic, Pedernales, Los Arroyos	MF172215
<i>Calisto clydoniata</i>	JAG-WI-804	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172212
<i>Calisto clydoniata</i>	JAG-WI-805	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172248
<i>Calisto clydoniata</i>	JAG-WI-807	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172229
<i>Calisto clydoniata</i>	JAG-WI-808	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172227
<i>Calisto clydoniata</i>	RN02-16	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	KF054341
<i>Calisto galii choneupsilon</i>	JAG-WI-949	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172241
<i>Calisto galii choneupsilon</i>	JAG-WI-950	Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172226
<i>Calisto galii choneupsilon</i>	JAG-WI-952	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172228
<i>Calisto galii choneupsilon</i>	JAG-WI-953	Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172211
<i>Calisto galii choneupsilon</i>	JAG-WI-799	Dominican Republic, Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172220
<i>Calisto galii choneupsilon</i>	JAG-WI-800	Elías Piña, Sierra de Neiba, Puesto Pirámide 204	MF172235
<i>Calisto galii galii</i>	JAG-WI-546	Dominican Republic, Constanza, Valle Nuevo	JN300022
<i>Calisto galii galii</i>	JAG-WI-919	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172230
<i>Calisto galii galii</i>	JAG-WI-920	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172218
<i>Calisto galii galii</i>	JAG-WI-921	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172223
<i>Calisto galii galii</i>	JAG-WI-922	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172231
<i>Calisto galii galii</i>	JAG-WI-923	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172251
<i>Calisto galii galii</i>	JAG-WI-924	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172219
<i>Calisto galii galii</i>	JAG-WI-925	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172244
<i>Calisto galii galii</i>	JAG-WI-926	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172245
<i>Calisto galii galii</i>	JAG-WI-927	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172214
<i>Calisto galii galii</i>	JAG-WI-928	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172234
<i>Calisto galii galii</i>	JAG-WI-929	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172247
<i>Calisto galii galii</i>	JAG-WI-930	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172246
<i>Calisto galii galii</i>	JAG-WI-931	Dominican Republic, La Vega, El Cruce, Alto de la Cotorra	MF172238
<i>Calisto galii galii</i>	JAG-WI-796	Dominican Republic, La Vega, Valle Nuevo, La Nuez	MF172237
<i>Calisto galii galii</i>	JAG-WI-797	Dominican Republic, La Vega, Valle Nuevo, La Nuez	MF172213
<i>Calisto galii galii</i>	JAG-WI-798	Dominican Republic, La Vega, Valle Nuevo, La Pirámide	MF172224
<i>Calisto galii galii</i>	McGuire09-CAL-Sat14	Dominican Republic, Santiago	JN197326
<i>Calisto galii galii</i>	McGuire09-CAL-Sat15	Dominican Republic, Santiago	JN197325
<i>Calisto galii galii</i>	McGuire09-CAL-Sat56	Dominican Republic, Elías Piña	JN197323
<i>Calisto galii galii</i>	McGuire09-CAL-Sat57	Dominican Republic, Elías Piña	JN197322
<i>Calisto galii galii</i>	McGuire09-CAL-Sat60	Dominican Republic, Elías Piña	JN197321
<i>Calisto galii galii</i>	McGuire09-CAL-Sat61	Dominican Republic, Elías Piña	JN197320
<i>Calisto galii galii</i>	McGuire09-CAL-Sat62	Dominican Republic, Elías Piña	JN197319

(continued next page)

Appendix 1. (continued)

Species	Voucher code	Locality	GenBank accession number
<i>Calisto galii galii</i>	McGuire09-CAL-Sat63	Dominican Republic, Elias Piña	JN197318
<i>Calisto galii galii</i>	McGuire09-CAL-Sat65	Dominican Republic, Elias Piña	JN197317
<i>Calisto galii galii</i>	McGuire09-CAL-Sat66	Dominican Republic, Elias Piña	JN197316
<i>Calisto galii galii</i>	McGuire09-CAL-Sat68	Dominican Republic, Elias Piña	JN197315
<i>Calisto galii galii</i>	McGuire09-CAL-Sat71	Dominican Republic, La Vega	JN197314
<i>Calisto galii galii</i>	McGuire09-CAL-Sat72	Dominican Republic, La Vega	JN197313
<i>Calisto galii galii</i>	McGuire09-CAL-Sat73	Dominican Republic, La Vega	JN197312