

# Color pattern divergence in *Napeocles jucunda* Hübner, 1808 (Nymphalidae) is accompanied by shifts in host plant and habitat use

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**Abstract:** *Napeocles jucunda* (Nymphalidae: Victorinini) has blue wings more reminiscent of canopy-flying Charaxinae (Nymphalidae) than its phylogenetic relatives *Siproeta* and *Anartia*, which live primarily in forest edges and disturbed areas. Here, we present photos and descriptions of its immature stages and host plant (*Ruellia* sp. nov. (Acanthaceae)) from northern Peru, together with remarks on adult behavior. Taken in conjunction with previously published data from French Guiana, our observations suggest that color pattern divergence in *N. jucunda* has been accompanied by specialisation on scandent host plants, and flying in the mid-story and canopy of forests. We discuss hypotheses for the adaptive significance of blue wing patterns in such environments.

**Keywords:** *Napeocles jucunda*, *Ruellia*, habitat shift, host plant shift, immature morphology, motion dazzle.

## INTRODUCTION

*Napeocles jucunda* Hübner, 1808 (Nymphalidae: Victorinini) is a poorly studied Neotropical butterfly and the sole representative of its genus. It is distributed throughout much of the Amazon basin and Guianas (Fig. 1), although its geographic range is somewhat unclear as the species is not well represented in museum collections. Adults exhibit striking wing color patterns that more closely resemble canopy-flying members of the Charaxinae than their phylogenetic relatives in the Victorinini, which typically live in forest edges and disturbed areas. For example, while species in *Siproeta* (the sister genus to *Napeocles* (Wahlberg *et al.*, 2009)) are characterized by bold green, orange or white wing patterns, *N. jucunda* evokes *Memphis* or *Archaeoprepona*, with a blue and black dorsal side, and a cryptic ventral side resembling a dead leaf. *Napeocles jucunda* is sexually dimorphic, with the dorsal wing surfaces of males characterized by a brilliant, almost electric blue, and females exhibiting a more greyish blue, with some off-white patches. Brévignon (1990) previously published observations on *N. jucunda* in French Guiana, which included a host plant record along with descriptions of the immature stages, photographs of 4<sup>th</sup> and 5<sup>th</sup> instars, and a figure of the pupae. Here, we present photographs and descriptions of the eggs, 2<sup>nd</sup> to 5<sup>th</sup> instars, pupae and a new host plant from the eastern edge of the Andes in northern Peru, together with anecdotal observations on adult behavior. In light of the data from French Guiana, we then discuss the evidence that wing color pattern divergence has been accompanied by shifts in other ecologically relevant traits.

## METHODS

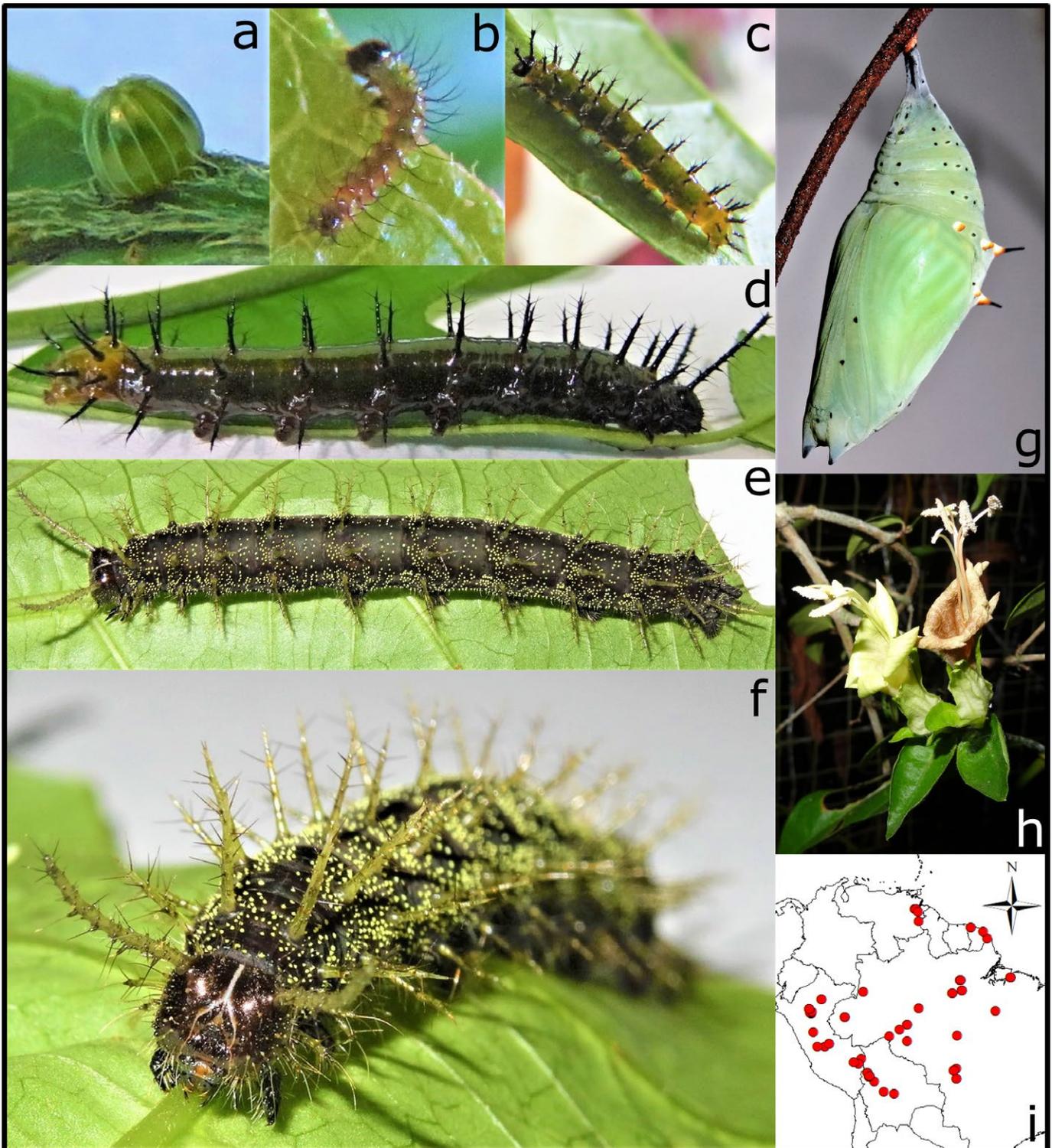
Observations of butterflies and their host plants were made by the authors in the vicinity of Tarapoto, Peru. To confirm the phylogenetic placement of the host plant, *Ruellia* sp. nov., we sequenced 431 bases of the whole internal transcribed

spacer (ITS) region of nuclear ribosomal DNA, using standard DNA extraction and PCR protocols. Approximately 1 cm<sup>2</sup> of leaf tissue was crushed using a TissueLyser and then DNA extracted using the QIAGEN DNeasy Plant Kit following the standard protocol. ITS-u1/ITS-u4 universal primers were used to amplify the whole ITS region by PCR (Cheng *et al.*, 2016). The PCR was carried out in a 10 µl volume using 5× reaction buffer (Promega), 0.5 µM each of forward and reverse primers, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs and 0.25 U GoTaq DNA polymerase (Promega). PCR cycling conditions were as follows: initial denaturing at 95°C for 2 min; followed by 35 cycles of 95°C for 45 sec, 57°C for 45 sec, 72°C for 45 sec; with a final elongation at 72°C for 5 min. The PCR product was purified with AMPure XP beads (Beckman Coulter), cycle sequenced from both ends using the Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems), and analysed on an AB3730xl capillary sequencer (Applied Biosystems). Sequence traces were then inspected and assembled manually.

A BLAST search with the *Ruellia* sp. nov. sequence revealed 98% sequence similarity with *R. exserta* Washh. & J. R. I. Wood, *R. siraensis* Washh. and *R. steyermarkii* Washh. We then downloaded the available sequences for these and other related species (Tripp, 2007; Tripp & McDade, 2013) from GenBank, aligned all the sequences using ClustalW, and trimmed the downloaded sequences to same region successfully sequenced for *Ruellia* sp. nov. We used MEGA7 (Kumar *et al.*, 2016) to estimate the maximum likelihood (ML) nucleotide substitution model and phylogeny (Fig. 2).

## RESULTS AND DISCUSSION

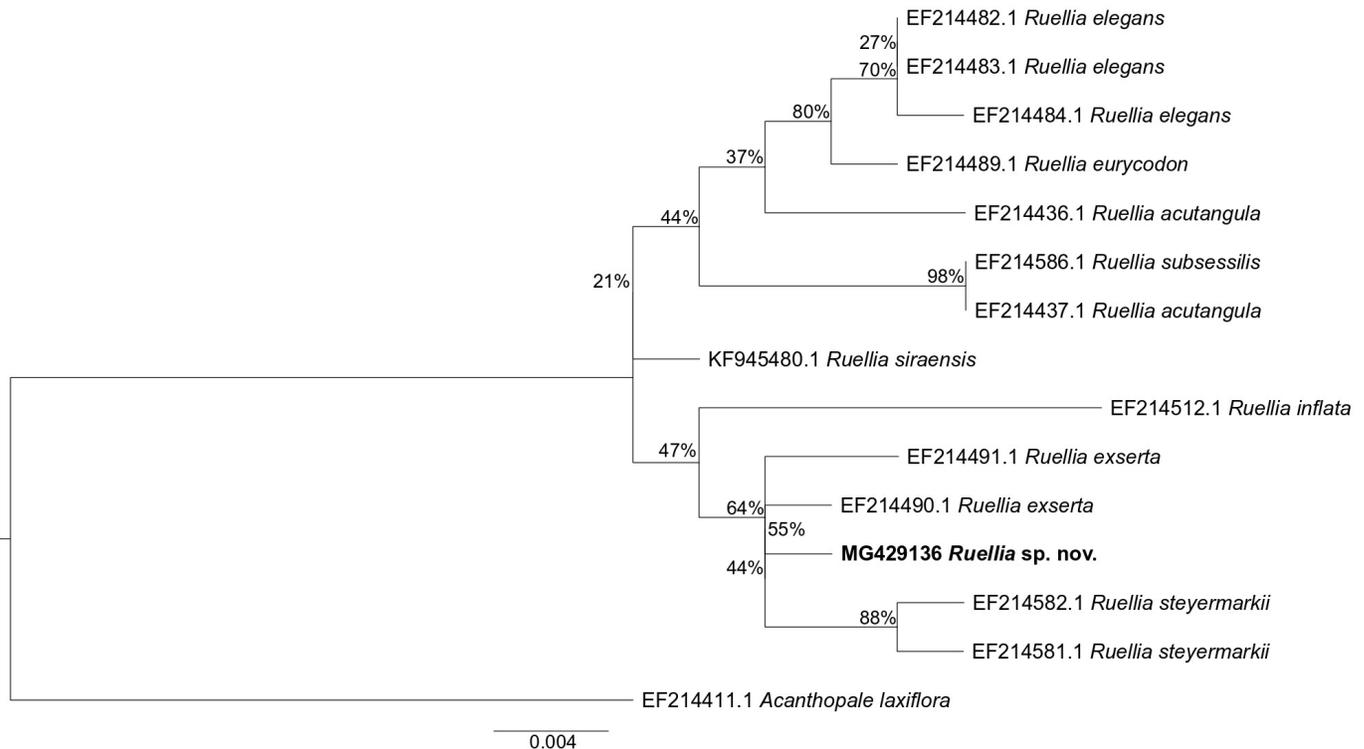
The most frequently collected individuals around Tarapoto were males, which were most often encountered when puddling at the edges of water bodies or on wet unpaved jungle roads. However, they were never found far from forest, through which they could be observed flying erratically at high speed in, or even above, the canopy, while occasionally pausing to rest



**Figure 1.** The immature stages of *N. jucunda*, its host plant in Peru, and its geographic distribution. a) egg, b) 2<sup>nd</sup> instar, c) 3<sup>rd</sup> instar, d) 4<sup>th</sup> instar, e-f) 5<sup>th</sup> instar, g) pupa, h) *Ruellia* sp. nov., i) *N. jucunda* distribution map (see Table 1 for locality data).

very briefly on vegetation. In their behavior, the males closely resembled and often co-occurred with *Historius odius* Fabricius, 1775, although we did not note them performing sorties from a perch, as does *H. odius*. At one particular site on the Rio Cumbaza near San Antonio, males could be observed puddling and visiting *Casearia* sp. (Salicaceae) flowers on the riverbanks, or flying in the adjacent forest, on almost 100% of visits on sunny days after 10:00. Males were also regularly encountered

at Pucayaquillo near Shapaja, where we saw them flying in the canopy and puddling in a ford on the road. In addition, we saw males puddling on dirt roads at San Roque de Cumbaza (n=1), and Pongo-Barranquita km 7 (n=1), and on the banks of the Rio Shilcayo (n=1). Females were more rarely seen than males, and we encountered them only 11 times in the wild. As with males, they appeared only on sunny days in the hours around midday, but flew more sedately, frequently pausing to rest on



**Figure 2.** ML phylogenetic tree for ITS sequences of *Ruellia inflata* and allies assuming the Tamura 3-parameter nucleotide substitution model (Tamura, 1992) as selected by Bayesian Inference Criterion, and rooted using *Acanthopale laxiflora*. GenBank accession numbers are provided for each sequence. Branch lengths are measured as the number of substitutions per site. Node values indicate bootstrap support from 1000 replicates. Although the clade comprising the scendent *Ruellia* (*R. steyermarkii*, *R. exserta* and *R. inflata*) did not have high bootstrap support here, it was very strongly supported in a previous comprehensive phylogenetic analysis of the *Ruellia* (Tripp, 2007).

vegetation. At San Antonio, we observed a female flying inside the forest about 5 m above the ground, and another resting on a rock by the river. At Pongo-Barranquita km 7 we observed a female flying about 6 m above the ground, which frequently paused to rest on plants and appeared to be in search of its host. On the 22<sup>nd</sup> of October 2013, we observed a female ovipositing on a scendent *Ruellia* sp. (Acanthaceae) in the understory of primary forest lining a ravine at Pucayaquillo, near Shapaja. Oviposition occurred on new growth about 1.5 m above the ground, but the plant was around 5 m high, with the upper parts reaching the canopy due to the steepness of the ravine. Eggs were collected from the same plant or another individual nearby on most subsequent visits to the site, and reared to adulthood on several occasions. We observed females here on three more occasions, flying in the understory and midstory up to about 5 m in height, where they flitted from plant to plant, presumably searching for their host. Finally, we observed *N. jucunda* females on four occasions at Sangamayoc in the Amazon lowlands. One female was seen inside the forest, another on the path, and two in cleared land right at the edge of the forest. Each was close to the ground and in the immediate vicinity of a more pubescent but otherwise identical species of *Ruellia* (only the buds of new growth were pubescent in the Shapaja plants, whereas both the young leaves and stem were pubescent in the Sangamayoc plants). We did not confirm whether *N. jucunda* was using these plants in the wild. However, some were placed in a large insectary in Tarapoto with a female *N. jucunda*, which oviposited numerous eggs on them. The female survived fairly well in captivity, and was fed with rotting bananas.

Females laid eggs singly, deep in the axils of new growth. The eggs are qualitatively identical to those of *Siproeta stelenes* (Linnaeus, 1758): bright green and round, with light green vertical striations (Fig. 1a). The larval stages comprise five instars, with instars 1-4 also very similar to those of *S. stelenes* (Fig. 1b-d). However, the final instar is quite different, being characterized by a dark maroon basal color, with the thorax and abdomen flecked by numerous yellow specks (Fig. 1e-f). In our Peruvian specimens, this yellow speckling is at a lower density than in the specimen figured in Brévignon (1990), which may indicate geographic variation in this character. The head and thoracic legs are of a darker color than the thoracic and abdominal segments. The head capsule is setaceous, particularly laterally and ventrally, and has yellow specks laterally. It has a pair of semi-translucent yellow cephalic scoli. A ring of semi-translucent yellow scoli also protrude from the midpoint of each thoracic and abdominal segment, and the ventral side of the thorax and abdomen are setaceous, particularly around the thoracic legs and prolegs. The pupae are larger but virtually identical to that of *S. stelenes*, with a bright green basal color and the cremaster attached by red tissue (Fig. 1g). Dorsally and laterally the pupa is characterized by black or orange spots and protuberances, which on the abdominal segments A2, A3 and A4 protrude to form orange conical structures with white bases. The two pairs in the centre of dorsal side of segments A3 and A4 extend to such an extent that they form black tipped spines. The spiracles are tinged with black. On the ventral side there is some black spotting, and a pair of black protuberances at the anterior end of the anal groove. An adult butterfly enclosed

29 days after it was first found in the wild as an egg, with the final instar lasting six days, and pupation lasting eight days. The larva measured about 6 cm long halfway through the final instar, with the maximum dimensions of the head capsule about 0.45 cm x 0.45 cm. The pupa measured 2.85 cm in length and 1.1 cm at the widest point.

The host plant from Shapaja was taken into cultivation in Tarapoto, and flowered in late July, 2015. The yellow-green flowers opened at night and blossomed for a matter of hours, leading us to presume that the species is bat-pollinated (Fig. 1h). Photos were sent to Erin Tripp (University of Colorado), who identified the plant as being closely allied to *Ruellia exserta* and *R. beckii* Wassh. & J. R. I. Wood from Brazil and Bolivia, respectively. However, it was noted to be clearly different from both due to the highly distinctive calyx with acuminate apices, and is probably an undescribed species (E. Tripp, pers. comm). We therefore refer to this species as *Ruellia* sp. nov. Although the largest plant we have seen was about 5 m high, it is possible that *Ruellia* sp. nov. grows larger, as *R. beckii* is said to grow up to 10 m in height (Wasshausen & Wood, 2004). Our phylogenetic analysis of the ITS sequences for the plant and close relatives confirm the plant's close phylogenetic relationship with members of the *R. inflata* Rich. clade, *sensu* Tripp (2007).

Butterflies are known to fly in the same microhabitats as their host plant, and this includes the vertical as well as the horizontal dimensions of the environment (Beccaloni, 1997). Brévignon (1990) recorded the host plant of *N. jucunda* in French Guiana as *R. cordifolia* Lindau, now considered a junior synonym of *Ruellia inflata* (Funk *et al.*, 2007). Together, the data from Peru and French Guiana thus suggest that *N. jucunda* specializes on species in the *R. inflata* clade. Interestingly, all are scrambling vines or lianas, which is an unusual trait in *Ruellia* (Tripp, 2007). We have also observed *S. stelenes* using *Ruellia* sp. nov. at Sangamayoc. However, *S. stelenes* is a generalist that typically occurs at forest edges and in disturbed areas, and correspondingly its primary hosts around Tarapoto are shrubs and herbs found in these environments, such as *R. vel.* aff. *tarapotana* Lindau, *R. chartacea* (T. Anderson) Wassh., and the introduced *R. simplex* C. Wright. Finding that *N. jucunda* has shifted away from the shrubs and herbs used by its relatives to specialize on lianas thus adds strong support to our anecdotal observations that the butterfly lives primarily in the mid- or upper strata of forests, as might be expected by its wing color pattern.

The adaptive significance of the blue color pattern for life in the forest is rather unclear. It has previously been hypothesized that some strong-flying butterflies participate in "escape mimicry", in which wing color patterns signal strong flying ability and consequent unprofitability to predators (van Someren & Jackson, 1959). For example, some *Archaeoprepona* and *Prepona* have bright blue bands that strongly resemble those of certain *Doxocopa*, and all three genera are very strong fliers that inhabit the canopy (Pinheiro & Freitas, 2014). When flying, *N. jucunda* bears some resemblance to *Archaeoprepona* species, through both its behavior and coloration, and it could conceivably be included in a blue-banded escape mimicry ring. However, the weak sexual dimorphism observed in *N. jucunda*

is not easily explained by this hypothesis, which predicts convergence between sexes occupying the same mimicry ring. It is also difficult to explain through female preference for a showy male trait, which typically predicts more marked differences between the sexes (Darwin, 1871; Kemp, 2007). Furthermore, *Napeocles* males cannot be attracted with a blue cloth (unlike some *Morpho* species), suggesting that at least the males are not using blue for species recognition. An alternative possibility is that *N. jucunda*'s coloration may have evolved as an effective means to interfere with predator's ability to judge speed and direction when the butterfly is flying, perhaps through interaction with dappled light in the canopy (Stevens *et al.*, 2011). This "motion dazzle" camouflage would protect the butterfly until it lands, at which point it conceals itself through its leaf-like ventral wing surfaces. Weak sexual dimorphism might then be more easily explained through the sexes' different flight behaviors and microhabitats. Despite the divergence in wing color pattern, host plant and habitat use, *N. jucunda* still retains some affinities to related genera. With the exception of the final instar, immature morphology is highly conserved, with eggs, instars 1-4 and pupae all essentially identical to those of *Siproeta*. This suggests either that these traits are less labile, or that the selective pressures shaping them are similar across habitats.

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#### LITERATURE CITED

- Beccaloni, G. W. 1997. Vertical stratification of ithomiine butterfly (Nymphalidae: Ithomiinae) mimicry complexes: the relationship between adult flight height and larval host-plant height. *Biological Journal of the Linnean Society* 62: 313–341.
- Brévignon, C. 1990. Quelques élevages guyanais: *Napeocles jucunda* Hübner (Lep. Nymphalidae), *Papilio torquatus* Cramer, *Papilio anchisiades* Esper, *Eurytides ariarathes* Esper (Lep. Papilionidae). *Bulletin de la Société de Sciences Naturelles* 68: 19–21.
- Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., Zhou, S. 2016. Barcoding the kingdom Plantae: new PCR primers for ITS regions of plants with improved universality and specificity. *Molecular Ecology Resources* 16: 138–149.
- Darwin, C. 1871. *The Descent of Man, and Selection in Relation to Sex*. London, John Murray. ix + 688 pp.
- Funk, V., Hollowell, T., Berry, P., Kelloff, C., Alexander, S. N. 2007. Checklist of the plants of the Guiana Shield (Venezuela: Amazonas, Bolívar, Delta Amacuro; Guyana, Surinam, French Guiana). *Contributions from the United States National Herbarium* 55: 1–584.
- Kemp, D. J. 2007. Female butterflies prefer males bearing bright iridescent ornamentation. *Proceedings of the Royal Society of London B: Biological Sciences* 274: 1043–1047.

- Kumar, S., Stecher, G., Tamura, K.** 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution* 33: 1870–1874.
- Neild, A. F. E.** 2008. *The Butterflies of Venezuela Part 2: Nymphalidae II (Acraeinae, Libytheinae, Nymphalinae, Ithomiinae, Morphinae)*. London, Meridian Publications.
- Pinheiro, C. E. G.** 1996. Palatability and escaping ability in Neotropical butterflies: tests with wild kingbirds (*Tyrannus melancholicus*, Tyrannidae). *Biological Journal of the Linnean Society* 59: 351–365.
- Pinheiro, C. E. G., Freitas, A. V. L.** 2014. Some possible cases of escape mimicry in Neotropical butterflies. *Neotropical Entomology* 43: 393–398.
- Van Someren, V., Jackson, T.** 1959. Some comments on protective resemblance amongst African Lepidoptera (Rhopalocera). *Journal of Lepidopterists' Society* 13: 121–150.
- Stevens, M., Searle, W. T. L., Seymour, J. E., Marshall, K. L., Ruxton, G. D.** 2011. Motion dazzle and camouflage as distinct anti-predator defenses. *BMC Biology* 9: 81.
- Tamura, K.** 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Molecular Biology and Evolution* 9: 678–687.
- Tripp, E., McDade, L.** 2013. Time-calibrated phylogenies of hummingbirds and hummingbird-pollinated plants reject a hypothesis of diffuse co-evolution. *Aliso: A Journal of Systematic and Evolutionary Botany* 31: 89–103.
- Tripp, E. A.** 2007. Evolutionary relationships within the species-rich genus *Ruellia* (Acanthaceae). *Systematic Botany* 32: 628–649.
- Wahlberg, N., Leneveu, J., Kodandaramaiah, U., Peña, C., Nylin, S., Freitas, A. V. L., Brower, A. V. Z.** 2009. Nymphalid butterflies diversify following near demise at the Cretaceous/Tertiary boundary. *Proceedings of the Royal Society B: Biological Sciences* 276: 4295–4302.
- Wasshausen, D. C., Wood, J. R. I.** 2004. Acanthaceae of Bolivia. *Contributions from the United States National Herbarium* 49: 1–152.

**Table 1.** Localities for *N. jucunda* obtained from published literature, on-line databases, our own records, communications from other researchers, and specimens held in the Natural History Museum, London.

Locality	Latitude	Longitude	Source or Collection
Camp. Rio Grande, Bolívar, Venezuela	8.05	-61.63	Neild (2008)
Col. Frco. Villa, Rio Grande, Bolívar, Venezuela	8.05	-61.63	Neild (2008)
Vía Puchima, ca. 15 km north of El Palmar, Bolívar, Venezuela	8.02	-61.88	Neild (2008)
El Hormiguero, Meseta de Nuria, Bolívar, Venezuela	7.67	-61.33	Neild (2008)
El Dorado to Santa Elena km 67, Bolívar, Venezuela	6.29	-61.32	Neild (2008)
St. Laurent du Maroni, French Guiana	5.50	-54.03	NHM (London)
St. Laurent du Maroni, French Guiana	5.48	-54.03	C. Brevignon, pers. comm.
Cayenne, French Guiana	4.93	-52.33	NHM (London)
Saint-Georges de l'Oyapock, French Guiana	3.89	-51.8	Brévignon (1990)
Pará, Brazil	-1.45	-48.48	NHM (London)
Obidos, Brazil	-1.91	-55.52	GBIF - YPM
Itaituba to Obidos, Brazil	-3.27	-55.33	NHM (London)
Sao Paulo, Brazil	-3.47	-68.95	NHM (London)
Juhuty, Amazonas, Brazil	-3.68	-56.57	NHM (London)
Chambireyacu, près Yurimaguas, Peru	-4.53	-74.77	NHM (London)
Lr. Rio Madeira, Brazil	-5.75	-61.25	NHM (London)
Rio Cachiaco, Iquitos, Peru	-5.83	-76.55	NHM (London)
Carajas mountains, Brazil	-6.14	-50.65	Pinheiro (1996)
Sangamayoc, Peru	-6.24	-76.13	Mori Pezo and Rosser
Pongo - Barranquitas Km 7, San Martin, Peru	-6.29	-76.23	Mori Pezo and Rosser
San Antonio de Cumbaza, Peru	-6.38	-76.4	Mori Pezo and Rosser
San Roque, Peru	-6.39	-76.44	Mori Pezo and Rosser
Rio Shilcayo, Peru	-6.45	-76.35	Mori Pezo and Rosser
Pucayaquillo, Shapaja, Peru	-6.58	-76.22	Mori Pezo and Rosser
Haut Amazons, Rio Jurua, Brazil	-7.02	-71.55	NHM (London)
Calama, Rio Madeira, Brazil	-8.03	-62.87	NHM (London)
Porto Velho, Brazil	-8.76	-63.90	NHM (London)
Tingo María, Peru	-9.13	-75.9	GBIF - HYO
Cristalino Jungle Lodge, Brazil	-9.6	-55.93	GBIF - naturgucker
Abuna, Lr. Madeira, Brazil	-9.70	-65.35	NHM (London)
Rancho Grande, Brazil	-10.3	-62.88	GBIF - FSCA
Atalaya, Peru	-10.73	-73.77	A. Hoskins, pers. comm.
Shima, Peru	-10.77	-73.73	A. Hoskins, pers. comm.
San Ramón, Peru	-11.13	-75.35	NHM (London)
Puerto Ocopa, Peru	-11.15	-74.32	A. Hoskins, pers. comm.
Explorers Inn, Peru	-12.84	-69.3	GBIF - Cons. Int.
Yahuarmayo, Peru	-13.30	-70.30	NHM (London)
La Unión to S. Domingo, Carabaya, Peru	-13.53	-69.63	NHM (London)
Lower R. Arinos, Brazil	-14.17	-56.07	NHM (London)
Bush of R. Paraguay, n. Diamantina, Brazil	-14.41	-56.45	NHM (London)
Santa Cruz, Diamantina, Rio Paraguay, Brazil	-14.41	-56.45	NHM (London)
Apolobamba, Bolivia	-14.72	-68.42	NHM (London)
20 miles above Mapiri, Bolivia	-15.25	-68.17	NHM (London)
San Augustin, Mapiri, Bolivia	-15.32	-68.20	NHM (London)
Cuyabá, Mato Grosso, Brazil	-15.58	-56.08	NHM (London)
Taipiplaya, Bolivia	-15.94	-67.48	GBIF - naturgucker
Cuyaba-Corumba River System, Mato Grosso, Brazil	-17.36	-56.71	NHM (London)
Cochabamba, Bolivia	-17.43	-66.17	GBIF - HYO
Cochabamba, Yungas del Espirito Santo, Bolivia	-17.43	-66.17	NHM (London)
San Mateo, Bolivia	-17.70	-64.70	NHM (London)
R. Coriahuira, Bolivia.	unknown	unknown	NHM (London)
Juguai, Bolivia	unknown	unknown	NHM (London)