CONTENTS

Preface
A. Austin (Editor-in-Chief) iii

TERRESTRIAL ARTHROPODS
DNA barcoding and morphological identification of Argentine species of Apanteles (Hymenoptera: Braconidae), parasitoids of cactus-feeding moths (Lepidoptera: Pyralidae: Phycitinae), with description of a new species
J. J. Martínez, C. Berta, L. Varone, G. Logarzo, P. Zamudio, A. Zaldívar-Riverón and R. G. Aguilar-Velasco 435

DNA barcodes and morphology reveal a hybrid hawkmoth in Tahiti (Lepidoptera: Sphingidae)
R. Rougerie, J. Haxaire, J. J. Kitching and P. D. N. Hebert 445

Molecular identification support most traditional morphological species of Ruspolia (Orthoptera: Conocephalinae)
Z. J. Zhou, R. L. Li, D. W. Huang and F. M. Shi 451

High species turnover of the ant genus Solenopsis (Hymenoptera: Formicidae) along an altitudinal gradient in the Ecuadorian Andes, indicated by a combined DNA sequencing and morphological approach

Continued on inside back cover
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Cover illustrations: Top left: Nautilites pompilius, enigmatic cephalopod and ancient relic, Coral Sea, Australia (photograph by Billy Sinclair); top right: Rupiaula dauta (Orthoptera: Conocephalidae), Mount Emei, Sichuan, China (photograph by Shi Fu Ming); bottom left: above, an F-2 hybrid female granddaughter of pure grandfather Eacles imperialisDHJ02 and hybrid mother Eacles imperialisDHJ02 (dry forest), both grandfather and father being Eacles imperialisDHJ02i (rain forest), naturally mating with (below) a fly-free wild and pure Eacles imperialisDHJ02 male. These two species of ACG ‘Eacles imperialis’ are 8% different in their barcodes, yet remain interfertile through all possible crosses at least through the 4th generation. Tropical dry forest, Área de Conservación Guanacaste (ACG), north-western Costa Rica (photograph by D. H. Janzen); bottom right: Hydroides elegans (Ammelida: Serpulidae), a dominant fouling species in tropical waters, extending the tentacle crown to capture food from the water column (photograph by Jian-Wen Qiu).

Invertebrate Systematics
CONTENTS
Continued from outside back cover
Challenging species delimitation in Callolobina: cryptic diversity among common springtails unveiled by DNA barcoding
What happens to the traditional taxonomy when a well-known tropical saturnid moth fauna is DNA barcoded?
INVASIVE INSECTS
DNA barcoding invasive insects: database roadblocks
L. M. Bocky, K. Armstrong, L. Kubatko and P. De Barro 506
FORENSICS
Comprehensive evaluation of DNA barcoding for the molecular species identification of forensically important Australian Sarcophagidae (Diptera)
K. A. Melkejøn, J. F. Walmann, S. L. Cameron and M. Dovton 515
TERRITORIAL ANTARCTIC INVERTEBRATES
Antarctic Tardigrada: a first step in understanding molecular operational taxonomic units (MOTUs) and biogeography of cryptic meiota
MARINE INVERTEBRATES
COI barcoding of Hydrodidae: a road from impossible to difficult
Y. Sun, E. K. Kuprijaneva and J. W. Qiu 539
DNA barcoding in Nautilus pompilius (Mollusca: Cephalopoda): evolutionary divergence of an ancient species in modern times
R. C. Williams, S. J. Newman and W. Sinclair 548
Molecular barcode and morphological analyses reveal the taxonomic and biogeographical status of the striped-legged hermit crab species Cibianus sclopetarius (Herbst, 1790) and Cibianus vitatus (Bliss, 1802) (Decapoda: Dactyridae)
M. Negri, L. G. Pileggi and F. L. Mantelatto 561
Investigating the taxonomy and systematics of marine wood borers (Bivalvia: Teredinidae): combining evidence from morphology, DNA barcodes and nuclear locus sequences
L. M. S. Borges, H. Sinkarya, A. M. Hoxius, J. R. Shipway, S. M. Cragg and F. O. Costa 572
SUBTERRANEAN INVERTEBRATES
Difficulties barcoding in the dark: the case of crustacean stygofauna from eastern Australia
M. G. Asmyhr and S. J. B. Cooper 583
MUSEUM SPECIMENS
An optimised protocol for barcoding museum collections of decapod crustaceans: a case-study for a 10–40-years-old collection
D. Zuccon, J. Brisset, L. Conbari, N. Pullandare, J. Utige and S. Samadi 592
What happens to the traditional taxonomy when a well-known tropical saturniid moth fauna is DNA barcoded?


Abstract. Biodiversity of tropical Saturniidae, as measured through traditionally described and catalogued species, strongly risks pooling cryptic species under one name. We examined the DNA barcodes, morphology, habitus and ecology of 32 ‘well known’ species of dry forest saturniid moths from Area de Conservacion Guanacaste (ACG) in north-western Costa Rica and found that they contain as many as 49 biological entities that are probably separate species. The most prominent splitting of traditional species – Eacles imperialis, Automeris zugana, Automeris tridens, Othorene verana, Hylesia dalina, Dirphia avia, Syssphinx molina, Syssphinx cola, and Syssphinx quadrilineata – is where one species was believed to breed in dry forest and rain forest, but is found to be two biological entities variously distinguishable by DNA barcodes and morphology, habitus, and/or microecological distribution. This implies that ‘standard’ biological information about each traditional species may be an unconscious mix of interspecific information, and begs renewed DNA barcoding, closer attention to so-called intraspecific variation, and increased museum collection and curation of specimens from more individual and ecologically characterised sites – as well as eventually more species descriptions. Simultaneously, this inclusion of sibling species as individual entities in biodiversity studies, rather than pooled under one traditional name, reduces the degree of ecological and evolutionary generalisation perceived by the observer.

Introduction

Neotropical Saturniidae are large, and mostly nocturnal, species-rich moths (e.g. Lemaire 1980, 1988, 2002; D’Abera 1998). In 1978, DHJ and WH set out to find all the species of saturniids living and breeding in the ~100-km² terrestrial portion of Parque Nacional Santa Rosa (PNSR), a dry forest small national park on the Pacific coastal plain of north-western Costa Rica. Today, this small national park is Sector Santa Rosa of the 12-times larger and
much more ecologically diverse terrestrial portion of Area de Conservacion Guanacaste (ACG) that surrounds it (Fig. 1) (Janzen 2002a; Janzen and Hallwachs 2011; Janzen et al. 2011). As roughly mapped in Fig. 1, the terrestrial portion of ACG is a mosaic of contiguous dry forest, rain forest, cloud forest, and a multitude of intergrades, all of which have suffered four centuries of anthropogenic rural disturbances (Janzen 1988, 2000).

Through seven years of intensive search for caterpillars and hundreds of nights of light-trapping, we had found by 1985 the caterpillars of all 32 of the traditional species of saturniids breeding in Sector Santa Rosa (Table 1, column A), as well as light-trapped single adult waifs of three other species (Arsenura sylla, Automeris hamata, Hylesia rosacea); we now know that these three species maintain ACG breeding populations in the ACG wetter cloud forests and rain forests to the east of Sector Santa Rosa. All but one of the 32 breeding species of Santa Rosa saturniids were found to be already described (Lemaire 1978, 1980, 1988), and were readily identified by their morphology (habitus and genitalia) by Lemaire himself (Janzen 2002b) from specimens that we sent to him in France in the 1980s; he also described what appeared to be the only undescribed and endemic Sector Santa Rosa species, Schausiella santarosensis (Lemaire 1988).

In short, for 26 years we felt we knew the Santa Rosa dry forest species-level saturniid moth fauna, and produced papers about their ecology (Janzen 1984a, 1984b, 1984c, 1986, 1987, 1993, 2003; Janzen and Waterman 1984; Bernays and Janzen 1988; Janzen and Gauld 1997; Costa et al. 2003; Janzen et al. 2005; Pinto-Tomas et al. 2007) as well as an illustrated field guide to the saturniids of Sector Santa Rosa (Janzen 1982). These species also became registered as the resident saturniids in the dry forest portion of the (still ongoing) overall inventory of the caterpillars (and adults) of ACG Lepidoptera (Janzen et al. 2009, 2011; Janzen and Hallwachs 2012), along with various extensions of these species into adjacent ACG rain forest and cloud forest. It was commonplace to view a species as breeding in both rain forest and dry forest.

However, in 2004, we began to routinely DNA barcode (Hebert et al. 2003) all of the species of Lepidoptera found by the overall ACG inventory (e.g. Hebert et al. 2004; Janzen et al. 2005, 2009; Hajibabaei et al. 2006). We quickly discovered, just as with other ACG higher insect taxa (e.g. tachinid flies (e.g. Smith et al. 2006, 2007), parasitic wasps (e.g. Smith et al. 2008), hesperiid and other butterflies (e.g. Hebert et al. 2004; Janzen et al. 2005, 2009, 2011; Burns et al. 2007, 2008, 2009, 2010), that the 32-species taxonomic portrait of the saturniids of Santa Rosa (and the many more traditional species in the remainder of ACG)

Fig. 1. Area de Conservacion Guanacaste, north-western Costa Rica. Four ecosystems: light blue – marine; yellow – dry forest; green – rain forest; dark blue – cloud forest. Sector Santa Rosa, the original Parque Nacional Santa Rosa (0–300 m elevation, 10 000 ha), is entirely dry forest.
Table 1. The 32 traditional species of Saturniidae breeding in the dry forest of Sector Santa Rosa, Area de Conservacion Guanacaste (ACG), arranged from left to right by the time period in which they are being considered.

Brown are barcode-defined dry forest entities, green are rain forest entities, and blue are entities that breed in both dry forest and rain forest. Scientific name changes from 2007 to 2008–2012 are based on published species descriptions.

<table>
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<th>Saturniinae</th>
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was not nearly as straightforward and simple as it had appeared to be when names and identifications were based on the various degrees of morphological scrutiny of habitus and genitalia that are normally bestowed on Lepidoptera found by field inventories and collecting trips (for non-saturniid examples of this with non-saturniid moths see the colour figures in Janzen et al., 2005, 2009, 2011).

Here, we dissect the taxonomic situation for just the 32 original species found breeding in Sector Santa Rosa dry forest, species by species, in order to begin to expose its taxonomic complexity, remaining puzzles, and uncertainties. We suspect that this unexpected (by us) degree of biological – and therefore taxonomic – complexity will be encountered in any tropical ecosystem that is rich in traditionally defined species of...
Saturniidae. It is especially likely where two major ecosystems
are parapatric, as is the ACG situation.

**Methods**

Collecting and rearing methods in the ongoing Lepidoptera
inventory of ACG since 1978 are standard (butterfly nets,
baiting, light traps, rearing wild-caught caterpillars) and
described elsewhere (e.g. Janzen et al. 2009; Janzen and
Hallwachs 2011, 2012). All individuals, caterpillars or adults,
are individually databased at the time of capture/preservation
and provided with a unique voucher code (yy-SRNP-xxxxxx).
In general, codes with six digits in the suffix (a consecutive
number within the year) are on adults that were caught by light
or net, while voucher codes with less than six digits in the suffix
are normally on wild-caught caterpillars (or pupae); however,
ocasionally, a less-than-6-digit suffix voucher code is placed on
a wild-caught adult if captured serendipitously by the caterpillar-
collecting team of ACG parataxonomists (Janzen et al. 2009;
Janzen and Hallwachs 2011). (We add parenthetically that
caterpillar parasitoids and hyperparasitoids are also databased
and vouchered this way (for singletons or lots), but in addition
to having the host yy-SRNP-xx.xx voucher code as collateral
information, the parasitoid specimen bears its own unique

All specimen records are public open-access through the
ACG inventory website (Janzen and Hallwachs 2012) and all
database vouchers (whether for DNA barcodes or otherwise) are
eventually deposited in public museums. The latter usually occurs
at the time that they are being taxonomically studied by members
of the taxasphere. All specimens of ACG saturniids referred to
in this account have been donated to, and deposited in, the
National Museum of Natural History (Smithsonian Institution)
in Washington, DC, except for some holotypes and paratypes
distributed to other public collections (INBio, Paris Museum, The
Natural History Museum).

The process of DNA barcoding the adult saturniid moths
analysed here is the same as that used throughout the ACG
Lepidoptera inventory (Janzen et al. 2005, 2009; Hajibabaei
et al. 2006). A single leg (or portion of a leg) is taken from an
oven-dried (in the field) pinned and spread voucher specimen,
placed in the well of a lysis plate (with a microdrop of 95% 
ethanol for glue), databased as having been de-legged for DNA
barcoding, and the lysis plate couriered to BIO (Biodiversity
Institute of Ontario) at the University of Guelph for extraction
and analysis (Hajibabaei et al. 2005; Ivanova et al. 2006).
DNA barcode records and Neighbour-Joining (NJ) trees
are then obtained through BOLD (www.boldsystems.org/),
and compared with the vouchers (to eliminate cases of
contamination or data-entry errors). Identifications that are
confirmed (scientific names or interim informal names) are
entered into the master database that is maintained at the
University of Pennsylvania (Janzen and Hallwachs 2012). The
content of this master database is periodically uploaded as an
update to BOLD, which is also then content-copied to GenBank
and to other members of the taxasphere who are working with
ACG inventory specimens, such as the national inventory
conducted by INBio (outskirts of San Jose, Costa Rica; see
also contain many duplicate ACG specimens of all the species
of Saturniidae mentioned here, as well as much larger series of
specimens from other parts of the country.

All genitalic dissections referenced here were done by DJH in
the NMNH/Smithsonian, and are deposited there, except that the
multigenitalia image in Fig. 11 was done by RR at BIO/University
of Guelp. All digital imaging of these genitalia is non-digitally
enhanced and was done by KD.

The taxonomic nomenclatorial convention used in this
account is as follows. When what has been described (and
identified) as a single Santa Rosa species by traditional
inspection is found by DNA barcoding wild-caught individuals
(adults and or caterpillars) to constitute two (or more) ‘distinctive’
groups of DNA barcodes in a standard NJ tree as produced by
the BOLD website (http://www.boldsystems.org/), the members
of the two groups are then informally interim tagged as, for
example, *Caio* championDHJ01 and *Caio* championDHJ02.
In this particular case, ‘distinctive’ encompassed a range of
1–3% divergence in the barcode region of COI; however,
as will become apparent below, we do not use any particular
percentage difference between barcode clusters as definitive
for species segregation among cryptic species complexes. This
informal interim nomenclature insures that as a ‘work-in-
progress’ there remains a cross-reference to the ‘classical’
name applied to that traditionally recognised entity while its
actual biological status is being explored. The initials cross-
reference to the person who baptised it.

The informal interim species epithet is explicitly formulated so
as to not be a valid scientific name (it contains numbers and capital
letters, and is neither ‘Latinised’ nor italicised). If it is found that
the two (or more) DNA barcode groups correlate with other
morphological (including colours and patterns), behavioural,
or ecological traits (such as microgeographic traits associated
with different ecosystems), then the informal interim species epithets
are retained in the inventory database (and on the website) until
baptised with a ‘true’ new and scientifically formal taxonomic
name or associated with an available scientific species epithet
(which may have previously been synonymised or not previously
understood to occur in ACG).

If no correlated traits are encountered, the informal interim tag
may still be retained provisionally while there is further study of
the situation. It is eventually discarded if (for any of a variety
of causes) it is concluded that the two (or more) clusters of DNA
barcodes do not, in fact, represent two (or more) species. It has
been our experience that the greater the percentage difference
between the two clusters of DNA barcodes, the more likely that
there will be encountered subtle correlates that cause us (and the
taxasphere) to eventually decide that indeed they are two
(or more) cryptic species (see especially examples in Smith
2011). However, as emphasised earlier, we do not use any
particular percentage difference between barcode clusters as
definitive for species segregation among cryptic species
complexes. Since the DNA barcode is effectively a kind of
‘only partly linear molecular clock not driven by direct
selection for the traits examined here’, it is self-evident that
the pace of evolution of species-level differences recognisable
by humans, or by the moths themselves, need not closely track
the pace of progression of emerging DNA barcode differences
between those same species. Species that are to us quite similar may have quite different barcodes (e.g. *Eacles imperialis*, see below), and quite different-appearing species may have quite similar barcodes.

For the purposes of this ACG inventory, a ‘species’ is what we infer or know to be a breeding population that does not freely exchange genetic information with other similar sympatric or parapatric species, though it may occasionally do so (just as dogs and coyotes are treated as two species yet produce quite viable hybrids). Given the very small area of ACG and the generally high mobility of lepidopteran adults over distances of a few kilometres, we consider every species breeding within ACG to be usually sympatric or parapatric with one or more of its cryptic (similar) species. In other words, the ACG rain forest population of *Othorene verana* is parapatric with the ACG dry forest population of *Othorene verana* (now described as *Othorene bernardespinozai* by Brechlin and Meister 2011b) where these two ecosystems interdigitate over an ecotone several kilometres wide. In this case, the two species are distinguished by their habitus, genitalia, and body size, as well as their DNA barcodes, which differ by only 6 base pairs (0.893%).

The longest distances between ACG collecting points are ~70 km (from the furthest Caribbean rain forest at 70–100 m elevation (Estacion Llanura, Estacion Botarrama) to the Pacific coast dry forest at Estacion Argelia, Sector Santa Rosa, and at the end of the Santa Elena Peninsula, Sector Santa Elena), but the more usual distance is 0.5–10 km. The ACG three terrestrial ecosystems (Fig. 1) are overlain with a fine-scale mosaic of restoring newly abandoned pastures and crop fields (and their trailsides and roadsides), through all ages of secondary forest succession and even patches of old-growth forest (Janzen 2000, 2002a; http://janzen.bio.upenn.edu/saveit.html). An individual moth can circulate among all kinds and degrees of (now being restored) disturbed forest within a night of normal flight, and while one presumed species may be separated from its similar congeners in some part of ACG by a few kilometres of species-free habitats, in some other portion of its range in ACG the congeners are parapatric or sympatric.

The data, specimens and their analysis described here are the product of a very large team of people and many laboratories, each offering different portions of the effort. This leads to a very long list of coauthors, which includes the current ACG parataxonomists (Janzen and Hallwachs 2011). As biodiversity inventory becomes a seamlessly integrated product from the wild newly caught (or newly observed) animal all the way through to a product such as this report or a species page on the web, such multiauthorship will be commonplace instead of the traditional finitely stuttered decades of ‘expedition-it, collect-it, store-it, sort-it, describe-it, analyse-it, publish-it, present-it’. For the present case, the authorship roughly divides into four major parts: (1) the collecting, rearing and field databasing, and preparing for barcoding, conducted by the ACG Costa Rican parataxonomists and a few international visitors, (2) the DNA barcoding and analysis done by the BIO (iBOL) BOLD team at the University of Guelph, Guelph, Canada, (3) the morphology-based taxonomic analysis done by members of the highly distributed taxasphere, and (4) the ecological-biodiversity overview offered by the on-site architects and overall managers of the ACG Lepidoptera inventory.

For the purposes of this interim analysis of ACG dry forest Saturniidae, their DNA barcodes (as well as habitus, and where possible, genitalia) were compared with the DNA barcodes of other similar species and species complexes in the BOLD database, with the permission of the DNA barcode information owners, in hopes of locating non-ACG described conspecifics. An example is the act of replacing the informal interim name *Automeris zugana* with the newly described (Brechlin and Meister 2011c) *Automeris zozimanaguana*, as reported below. However, we leave a broader and very species-rich geographic comparative analysis for other analyses both because far more specimens are needed for conclusions and because the BOLD DNA barcodes are at least partly non-public-access until their owners release their barcodes. In each case where the ACG saturniid inventory retains an informal interim name as used here, there was no definitive evidence that a name already exists for a conspecific occurring outside of ACG.

All sequence records together with trace files and images are available on BOLD as a single citable dataset (http://dx.doi.org/10.5883/DATASET-SATURN1) that is also cross-linked to GenBank.

As of 2011, the ACG inventory has reared and collected 48,000+ specimens of Saturniidae (among 540,000+ individual Lepidoptera records). Throughout this report, wherever it is important that males and females are correctly associated within their species, there are no doubts because wild-caught specimens are associated by their DNA barcodes as well as by other traits, and male–female sib groups have been reared for all traditional ACG species discussed here.

**Results**

**1978–2004**

During these 26 years, nothing in the examination of the habitus, genitalia, phenology, or caterpillar ecology of the 32 species of saturniids breeding in Sector Santa Rosa dry forest and in adjacent rain forest suggested anything other than that there are a straightforward 32 species. They were listed and portrayed that way in the inventory website and published that way (Janzen 1982, 2003; Lemaire 1980, 1988, 2002). However, in the discussions with taxonomists at the times of identification, there were the usual discussions of whether the specimens from Santa Rosa dry forest were ‘the same’ as what appeared to be their conspecifics from ACG rain forest, the USA, Mexico, El Salvador, other parts of Costa Rica, Panama, and countries to the south. In each of the 32 cases, Lemaire and other saturniid taxonomists, and comparisons by DHJ with museum specimens in the National Museum of Natural History (Smithsonian Institution), in the Natural History Museum (London), and in scattered other collections, suggested that there is ‘some geographic variation’ among members of ‘a species’, but none of it was considered to be sufficient to conclude that the Santa Rosa dry forest population was a distinctive species or even ‘subspecies’ (whatever that is meant to be). The situation for Santa Rosa dry forest, and as the saturniid inventory spread to include all of ACG and Costa Rica, was that the 32 species appeared to be taxonomically normal and unambiguous (though ‘variable’) at the species level, just as is the situation commonly
encountered elsewhere for some particular collecting area in the neotropics.

However, such a view, while traditionally used by neotropical ecologists and taxonomists in plying their trade (e.g. Janzen 1984a, 1984b, 1984c, 1986, 1987, 1993, 2003), now appears to gloss over and unknowingly aggregate taxonomic, and therefore biological, complexity.

2004–2006

In 2004 we applied DNA barcoding (Hebert et al. 2003) to what appeared to be a taxonomically and ecologically complex array of ACG skipper butterflies (Hesperiidae) (Hebert et al. 2004). We found that the ACG members of what had been regarded as a single ‘suspiciously variable’ species (Astraptes fulgerator) were indeed a complex of very similar sympatric and parapatric species that had quite different larval ecologies and colour patterns, slightly but consistently different DNA barcodes, and some traits of adult habitus that distinguished some of them (Hebert et al. 2004). While this wake-up call was followed up most intensively with hundreds of species of ACG Hesperidae (e.g. Janzen et al. 2011), we also began to DNA barcode all the voucher specimens that had been long accumulated by the ACG Lepidoptera inventory, as well as all newly reared and adult-captured specimens (Janzen et al. 2009).

The immediately visible impact of this broad ACG barcoding on the ‘unambiguous’ taxonomy of the Santa Rosa dry forest 32 species of breeding Saturniidae (Table 1) was to find that while all 32 were readily distinguished with 100% accuracy by their DNA barcodes (Fig. 2, Supplementary Fig. S1), four of them – Caio champions, Syssphinx colla, Syssphinx molina, Piloscola dargei – were potentially complexes of at least two cryptic species each; each pair of these four were captured in the same dry forest light trap (Luces, Sector Santa Rosa) at the same time, and the members of each pair were reared from wild-caught caterpillars from the same species of food plants in the same few hectares at the same time. These four ‘mine canaries’ are still today the subject of intense scrutiny and inventory to determine whether each is a set of highly sympatric cryptic species (see below), or whether each ‘pair’ simply reflects intraspecific barcode variation (i.e. intraspecific variation in the barcode region, unintentional amplification of pseudogenes, laboratory errors). Parenthetically, there is no indication that this within-species variation is Wolbachia-caused; all samples whose DNA barcodes are used in this report have been screened to eliminate Wolbachia sequences (see protocol in Smith et al. 2012). If the ACG inventory had nothing but these four cases of intraspecific DNA barcode variation to worry over, the data would probably have disappeared into the void of intraspecific taxonomic variation that appears in any inventory. However, such cases appeared everywhere in ACG and with hundreds of species of Lepidoptera, parasitic Hymenoptera, and parasitic Diptera (Janzen et al. 2009, 2011).

2006–2007

By 2006–2007 the ACG Lepidoptera inventory had expanded its rearing and light-trapping into all of its sectors (in addition to the initial dry forest Sector Santa Rosa), so that DNA barcoding was applied to saturniid specimens from all three major terrestrial ACG ecosystems (Fig. 1) and many habitats. This territorial expansion heavily impacted the seemingly straightforward identification status of the 32 dry forest species.

DNA barcoding now showed that the original 32 morphology-based species contained 9 additional species that also were potentially complexes of 2 or 3 species, for a total of 13 of the original 32 dissolving into possible complexes (Table 1). By luck, representatives of all of the ‘new’ entities living in adjacent ecosystems, as well as specimens from dry forest, had been sent over the years to Lemaire and identified by him as being the same species as the Santa Rosa specimens. However, it is unclear to what degree he examined genitalia as well as habitus of these specimens from other ACG ecosystems (see Discussion), and we did not ask for the return of these specimens; since he is deceased, we cannot ask about this detail. What we can know is that at the standard and careful level of morphology-based taxonomy traditionally applied to these large moths by a world-level authority (Lemaire 1978, 1980, 1988, 2002), they were comfortably identified as 32 species. Following DNA barcoding of many specimens from all ACG dry forest, cloud forest, and rain forest ecosystems (see Supplementary Fig. S1 for an NJ tree of all specimens), by 2007 it appeared that there are possibly at least 45 species of what were believed to be 32 species of ACG dry forest saturniids living in an area the size of London and its suburbs (and many more if the species of saturniids confined to cloud forest and rain forest are included, as will be discussed in another report). It is noteworthy, however, that 11 of the additional ‘new’ species occur only in ACG dry forest (but are parapatric with ACG rain forest sibling species) while 11 occur only in ACG rain forest (but are parapatric with ACG dry forest sibling species).

Of the 13 species (out of 32 traditional dry forest species) that are potential within-ACG species complexes, 10 of the species contain species pairs or triplets that are classically parapatric, with one or more members being a rain forest or rain–dry ecotone species and the other being a dry forest species. Parenthetically, such a species pair (but not here counted as such because it was not located by DNA barcoding) had already been previously recognised by saturniid taxonomists; the ACG dry forest Copaxa moinieri is parapatric with the within-ACG cloud forest and rain forest look-alike Copaxa rufinas, which was presumably discovered when the quite different genitalia of C. moinieri were examined: Lemaire 1978). Four of the species complexes (Othorene verana, Automeris zugana, Dirphia avia, Hylesia dalina) had already been noticed by the inventory as having ecosystem-specific variation in their habitus, but these were taxonomically ignored on the grounds of the commonplace feeling that ‘oh, the rain forest members of the species are generally larger and darker than are the dry forest members from a few kilometres away’. In these four cases, this is the case but the ‘larger and darker morph’ has turned out to be indeed another species than its dry forest counterpart. However, in seven of the species complexes that span ACG dry forest and rain forest, there is no obvious difference in habitus within each of the species pairs.

Incidentally, the caterpillar and adult collecting sites are chosen for the usual biologically serendipitous traits of light-trapping and searching for caterpillars, and therefore are usually not located at the microgeographic interfaces of later-revealed
Fig. 2. Neighbour-joining tree of the traditionally defined 32 species of Saturniidae breeding in Sector Santa Rosa, Area de Conservacion Guanacaste (ACG), and their apparent or possible segregates. Green names occur only in ACG rain forest, brown names occur only in ACG dry forest, and blue names occur in both. Specimens selected for inclusion have complete barcodes 655–658 bp in length.
intraspecies variation. Such collecting sites therefore do not usually accurately sample the interface between cryptic species pairs. This means, as has been the case, that discovery of intraspecific variation by DNA barcoding leads to yet more DNA barcoding of what appears to be the same species in attempts to explore and define the zones of moth parapatry or partial sympatry.

2007 to present

We comment briefly below, approximately from the more doubtful to the most certain cases of species complexes, on the current exploratory status for each of the 13 possible species complexes within ACG (Table 1) and a brief description of their current formal taxonomic status. In addition, in four cases in which there were no splits, barcoding combined with other traits resulted in upgrading of species names (e.g. Arsenura armida to Arsenura arianae, Othorene purpurascens to Othorene intermedia, Syssphinx mexicana to Syssphinx jennyphillipsiae, and Automeris metzli to Automeris digmariae: Table 1). In no case has the within-traditional-species DNA barcode variation resulted in identification confusion among the array of total ACG Saturniidae or even among all morphologically defined species of Costa Rican Saturniidae, to the degree they are known.

1. Caio championi (Druce, 1886) (Fig. 3)

The barcode of Caio championiDHJ01 differs from that of Caio championiDHJ02 by only 0.8% (5 bp) (Fig. 2, Supplementary Fig. S1) and no morphological or habitus traits have been found to distinguish them (two genitalic dissections) (Fig. 7). Adults of both distinct barcode clusters have been collected at the same dry forest light trap at the same time, and on one occasion, caterpillars of both were found in the same crown of an individual of the dry forest food tree (Pachira quinata, Malvaceae). However, such slight differences in barcode clusters have been found to correlate with morphologically different cryptic species of other ACG Lepidoptera (e.g. Hebert et al. 2004; Burns et al. 2007), and we consider that the shallow but conspicuously distinct split in C. championi barcodes (Supplementary Fig. S1) merits more intense examination of potential correlates from both ACG dry forest and rain forest. A further clue to distinctiveness is that all 10 Caio championiDHJ02 but one were light-trapped, while Caio championiDHJ01 are very common as both caterpillars and light-trapped victims.

Despite this being a common moth, the initial sample size barcoded was small because it was initially assumed to be a single species, its large size makes housing large numbers of specimens costly, it is highly variable in details of colour and pattern, and the vouchers were collected mostly in the early 1980s—with the consequence that these specimens are now too old to cheaply barcode. However, later rearing of entire sib groups of ACG Caio championi has found each to be monomorphic for the C. championiDHJ01 haplotype. Both DNA barcodes occur in ACG dry forest and rain forest, and the rain forest individuals of C. championi are generally larger and darker than are those from dry forest, though within a single sib group all extremes can be present.

All these potential clues taken together indicate the need for a closer look; while Lemaire (1980) records C. championi as occurring from Mexico to Colombia, the lectotype is from Volcan Chiriqui (1300 m) in northern Panama and may well be of the same population as that occurring in ACG. Parenthetically, when Lemaire first identified the ACG specimens of C. championi, he misidentified them as Caio undilinea (see Lemaire 1980, p. 85) but later (in litt.) reported that they are indeed C. championi. However, Lemaire also did not have the full range of extreme variation in pattern and colour intensity displayed by ACG specimens, nor their barcodes, to examine.

2. Citheronia bellavista Draudt, 1930 (Fig. 3)

Citheronia bellavista was a common moth in ACG dry forest light traps in the early 1980s when the inventory began, so common that few individuals were collected (and the advanced age of these specimens today renders them hard to barcode). However, by the late 1980s, C. bellavista appeared to be locally extinct in ACG dry forest in Sector Santa Rosa, presumably owing to the omnipresent warming and drying of this ecosystem from climate change since the mid 1980s. The common ACG rain forest C. bellavistaDHJ04 differs by 0.9% (6 bp) (Fig. 2, Supplementary Fig. S1) from the other two rain forest (rare) barcodes; notably, of the six genitalic dissections, each genitalia is conspicuously different from the other five, as in the pair in Fig. 7. If the three specimens from dry forest in 1982 have been accurately barcoded, C. bellavistaDHJ04 extended into Sector Santa Rosa dry forest at that time. The sample sizes of Citheronia bellavistaDHJ01 and Citheronia bellavistaDHJ03 are too small for any conclusion other than that these two barcode clusters, and the extreme intraspecific variation in genitalia morphology, merit a substantially larger sample of recently collected specimens to confirm or deny the existence of separate entities. Since Citheronia bellavista ranges from Nicaragua (presumably the same population as in northwestern Costa Rica) to Ecuador (holotype from Colombia: Lemaire 1988), there is ample opportunity for several species to be hidden within this single name.

3. Ptiloscola dargei Lemaire, 1971 (Fig. 3)

The barcode of Ptiloscola dargeiDHJ01 is 1.6% (10 bp) different from that of Ptiloscola dargeiDHJ02 (Fig. 2, Supplementary Fig. S1), but they do not have consistently different genitalia (10 genitalia dissections) (Fig. 7). While Fig. 7e and f appear to constitute one genitalic morph that is different from that of Fig. 7g and h, in fact Fig. 7e and g are P. dargeiDHJ01, and Fig. 7f and h are P. dargeiDHJ02.

While both barcodes occur throughout ACG dry forest, P. dargeiDHJ01 is almost entirely in Sector Santa Rosa (core dry forest, 0–300 m elevation) where it is common on its food plant and in light-traps, while P. dargeiDHJ02 is almost entirely in the more dry forest–rain forest ecotonal area of Sector Mundo Nuevo (400–700 m elevation) and at the same elevation ecotone on the western bases of the ACG northern volcanos (Sector Cacao, Sector Del Oro). However, P. dargeiDHJ01, the ‘most dry forest species’, has also been encountered four times on the cropland–forest rain forest margin on the Caribbean side of ACG (Fig. 8). This is likely to be a case of it following its dry forest weedy food plant – Senegal tenuifolia – into the fields and pastures cut out of the ACG rain forest. This phenomenon has
been encountered with many other ACG dry forest species of Lepidoptera that are following their dry forest food plants as they invade the anthropogenically dried rain forest agroscape.

Neither the genitalia nor DNA barcodes of ACG ‘Ptiloscola dargei’ match any of the new species of Ptiloscola recently described from South America (Brechlin and Meister 2008, 2011b). ACG P. dargei genitalia do appear to approximately match the diagram of those of the holotype from Pacific coastal Nicaragua (Lemaire 1988), a northern extension of the ACG dry forest ecosystem. We postulate that when the holotype or its population is DNA barcoded, it will be found that P. dargeiDHJ01 is the ‘true’ P. dargei. Whether P. dargeiDHJ02 is then a very poorly separated sibling species that occupies the somewhat more moist volcano slopes (Fig. 8) is yet to be determined.

4. Syssphinx colla Dyar, 1907 (Fig. 3)

The barcode of Syssphinx collaDHJ01 differs from that of Syssphinx collaDHJ02 by only 0.9% (6 bp) (Fig. 2, Supplementary Fig. S1) but they can be distinguished by the shape of the valve of the genitalia in lateral view (16 genitalic dissections) (Fig. 9). Numerous adults of both have been collected
at the same dry forest light trap at the same time (Luces, Sector Santa Rosa), and caterpillars of both have been found in the same crown of a *Samanea saman* (Fabaceae) food tree in Sector Santa Rosa.

The adults of both are extremely variable in colour pattern, variation that is exacerbated by the wings being lighter in colour if the moth ecloses about the date of the first rains in May, and darker in colour if eclosed in the weeks following the first rains (a seasonal colour polymorphism or ‘polymorphism’ (Shapiro 1984)) that is also present in ACG dry forest *Rothschildia lebeau* (Janzen 1984a) and *Adeloneivaia isara*). The caterpillars of *S. colla* are very variable in expression of the degree of dorsal spine development and silvery colour of the spines; however, this trait was noticed (but not recorded for each reared moth) long before the thought existed that there might be two cryptic species in *Syssphinx colla* in Sector Santa Rosa. Directed sampling is now needed to explore whether this larval variation correlates with the two barcodes and genitalia differences.

Lemaire (1988) synonymised *Syssphinx jaccu*, *Syssphinx pollens*, *Syssphinx talamanca*, and *Syssphinx vilderi* under the single name *Syssphinx colla* with a distribution from Mexico to Venezuela; he categorised it as being extremely variable. One or more of these five names may be found to be correctly applicable to the two ACG dry forest morphs of *Syssphinx colla*. It is worth noting, however, that the genitalia of *Syssphinx collaDHJ01* and *Syssphinx collaDHJ02* do not match the genitalia of Dyar’s holotype of *Syssphinx colla* in the NMNH (Smithsonian), a specimen from Mexico (Orizaba). The holotypes of *S. pollens*, *S. talamanca*, and *S. vilderi* are all from Costa Rican mid-elevation Caribbean rain forest (Sixola River). This ACG ecosystem has not produced any specimens of *S. colla* (other than one light-trapped singleton (06-SRNP-5783) that may be a stray from dry forest only a few kilometres away) in the ACG inventory, though there are specimens of *S. colla* from Costa Rican lowland and medium-elevation rainforest 100–200 km to the east of ACG.

5. *Syssphinx molina* (Cramer, 1780) (Fig. 4)

The barcode of *Syssphinx molinaDHJ01* differs from that of *Syssphinx molinaDHJ03* by 2.9% (19 bp) (Fig. 2, Supplementary Fig. S1); this pair cannot be distinguished by their genitalia (9 genitalic dissections) in lateral view (Fig. 9). *S. molinaDHJ01* is the (rare) ACG rain forest *S. molina* and *S. molinaDHJ03* is the (common) ACG dry forest *S. molina*, but *S. molinaDHJ02* occurs in both ecosystems at very low density in light traps. The two specimens of *Syssphinx molinaDHJ02* differ from the other two barcode clusters sufficiently in their barcodes and genitalia to suggest that this is a third ACG species in this complex, but this requires more sampling to determine. The rain forest *Syssphinx molinaDHJ01* has a somewhat larger and more intense black spot in the middle of the hind wing than does the dry forest *Syssphinx molinaDHJ03*, but is otherwise indistinguishable by habitus.

As is the case with *Syssphinx colla*, *S. molina* displays seasonal polymorphism in having darker and ‘muddier’ markings on its beige forewings when eclosed in the full rainy season as compared with eclosing at the time of the first rains in mid-May; this seasonal polyphenism (Shapiro 1984) renders searching for correlations of habitus with DNA barcodes more difficult. The lectotype of *S. molina* is from Suriname, and the six species names synonymised within it range from Mexico to Argentina (Lemaire 1988), though none of their holotypes are from Central America; it is possible that none will be found to actually match the ACG specimens of *S. molina*.

6. *Othorene verana* Schaus, 1900 (Fig. 4)

The barcode of *Othorene veranaDHJ01* (now newly described as *Othorene bernardoespinozai*) (Brechlin and Meister 2011b) is only 0.893% (6 bp) different from that of *Othorene veranaDHJ02* (Fig. 2, Supplementary Fig. S1). However, these two cryptic species are conspicuously different not only by their consistent barcode difference, and by the former being the ACG ‘dry forest *Othorene verana*’ and the latter being the ‘rain forest *Othorene verana*’, but also by their different genitalia (8 genitalic dissections) (Fig. 9). Adult rain forest *O. veranaDHJ02* are also noticeably larger and darker than adults of *O. veranaDHJ01* (and much more rarely taken in light traps). While only *O. bernardoespinozai* has a yellow morph, distinguishing the dark morphs of this pair by their habitus is made difficult because both species vary from light to dark brown (though *O. veranaDHJ02* is generally darker brown than *O. bernardoespinozai*). The frequency of the yellow versus brown colour morphs of *O. bernardoespinozai* does not appear to be associated with the detailed timing of the rainy season, in contrast to the *Syssphinx*, *Adeloneivaia* and *Rothschildia* seasonal polymorphisms in ACG (Janzen 1984a).

We have not yet been able to locate the rain forest food plant of *Othorene veranaDHJ02*, though we assume that it is likely to be a species of oak tree (*Quercus* spp.) because *Othorene veranaDHJ01* is host specific to *Quercus oleoides* in the ACG dry forest, and because there is one or more species of *Quercus* in all ACG forests where *O. veranaDHJ02* occurs as free-flying adults. We therefore cannot comment on how different are the caterpillars of these two species, if indeed they differ in appearance.

The holotype of *Othorene verana* (from Jalapa, Veracruz, Mexico) is in the NMNH and its dissected genitalia do not match either those of *O. bernardoespinozai* or *O. veranaDHJ02*. This is striking because the ecosystem of the holotype is very similar to the mid-elevation rain forest that is occupied by *O. veranaDHJ02* in ACG. As in the ACG situation, the ecosystem of the holotype is only a few tens of kilometres away. The barcode of *Othorene bernardoespinozai* was collected in the middle of the ACG population of what has been called *Othorene* veranaDHJ01, in Sector Santa Rosa, and its DNA barcode matches perfectly the many other previously baptised *O. veranaDHJ01* barcodes from Sector Santa Rosa. *Othorene veranaDHJ02* has never been encountered in the ACG dry forest and *Othorene bernardoespinozai* has never been encountered in the ACG rain forest. Both, however, have been caught as free-flying
adults a few hundred metres from the edge of the ACG dry forest–rain forest ecotone in Sector Del Oro of ACG.

Our intent with this report is not to explore outside of ACG. However, it is worth mentioning, in the spirit of complexity generated by barcoding, that examination of the habitus of the long national-level series of ‘Othorene verana’ in the INBio collections, with *O. bernardoespinozai* and *O. veranaDHJ02* in mind, strongly suggests that there is yet a third (yet larger and darker) species in this complex in the high elevations of Costa Rica (ICG, unpublished).

7. *Eacles imperialis* (Drury, 1773) (Fig. 4)

The barcode of *Eacles imperialisDHJ01* is 8% (52 bp) different from that of *Eacles imperialisDHJ02* in ACG (Fig. 2, Supplementary Fig. S1). These two cryptic parapatric species are conspicuously different not only by the large difference in their barcodes, but also by the former being the ‘rain forest *Eacles imperialis*’ and the latter being the ‘dry forest *Eacles imperialis*’ in ACG; this ecosystem fidelity is documented with hundreds of specimens (Fig. 10). This pair of species is currently under intense

![Fig. 4. All males. (a) Syssphinx molinaDHJ01, 06-SRNP-5772. (b) Syssphinx molinaDHJ03, 06-SRNP-15966. (c) Othorene bernardoespinozai, 05-SRNP-15274 (formerly Othorene veranaDHJ01). (d) Othorene veranaDHJ02, 07-SRNP-103770. (e) Eacles imperialisDHJ01, 09-SRNP-65584. (f) Eacles imperialisDHJ02, 10-SRNP-12102. (g) Automeris tridensDHJ06, 03-SRNP-283. (h) Automeris tridensDHJ07, 05-SRNP-70368.](image-url)
genetic and microecogeographic exploration in ACG (C. Bertrand, D. H. Janzen, W. Hallwachs and M. Hajibabaei, unpubl. data), but several salient traits are clear (DHJ and WH, unpublished):

1) in the dry forest–rain forest ecotone (and there only), males of both species have been captured in the same trap baited with a virgin female of either species (Santa Cecilia on the edge of Sector Pitilla), and wild caterpillars of both species have been found within a few hundred metres of each other (Gongora, Sector Cacao).

2) hybrid offspring of the two species are achieved reliably by allowing an ‘introduced’ tethered virgin female to call and mate with a free-flying male of the other species, in the male’s home ecosystem and many kilometres from the female’s home ecosystem; these offspring are fully viable out to at least the fourth generation of backcrosses with free-flying wild males of the female’s home ecosystem.

3) deep genetic exploration reveals that there is probably substantial gene flow between the two species in the dry forest–rain forest ecotone (C. Bertrand, D. H. Janzen, W. Hallwachs and M. Hajibabaei, unpubl. data), a gene flow that is probably generated by circulating males while the females remain faithful to their natal ecosystem.

4) individual specimens cannot be reliably distinguished by their habitats but a museum drawer of spread males of one species can be distinguished from a drawer of the other, in that the free-flying wild rain forest males are slightly larger with slightly more falcate forewing tips than are free-flying wild dry forest males, and

5) genitalia of the rain forest males are more robust (larger, heavier) than are the genitalia of the dry forest males (Fig. 11), though they do not differ in their superficial morphological profiles (11 genitalic dissections).

Since Eacles imperialis is an extreme generalist in its use of food plants (Janzen 2003; Janzen and Hallwachs 2012), and within-sib group caterpillars range from light green to orange to nearly black in both rain forest and dry forest (Janzen and Hallwachs 2012), the appearance of the caterpillars cannot be used to discriminate between the two species. While the barcodes of these two species are 8% different from each other, they are only ~5% different from the barcodes of Eacles imperialis in the eastern USA; the latter moth is, however, quite distinct in habitat from either species in ACG. ‘Eacles imperialis’ has a range from Canada to Argentina (Lemaire 1988), and has been the subject of substantial taxonomic subdivision into so-called ‘subspecies’ (Lemaire 1988; Brechlin and Meister 2011a) but all of this is clearly premature categorisation in view of the DNA barcoding results that are beginning to emerge from close examination of E. imperialis throughout its range (R. Rougerie and P.D. N. Hebert, unpubl. data).

8. Automeris tridens Herrich-Schaffer, 1855 (Fig. 4)

The barcode of Automeris tridensDHJ06 is 1.4% (9 bp) different from that of Automeris tridensDHJ07 (Fig. 2, Supplementary Fig. S1), and they do not have different genitalia (16 genitalia dissections) (Fig. 12). However, despite that, moths from the two barcode clusters cannot be distinguished by the habitus of males or females in ACG either (see Automeris zugana below for a different situation). their ecological segregation strongly suggests that these are two distinct species: A. tridensDHJ06 is an exclusively dry forest and ecotone moth (except for two individuals 07-SRNP-113884, 07-SRNP-113883 light-trapped in a rain forest pasture, perhaps invasive) while A. tridensDHJ07 inhabits the rain forest (but for two exceptions, see below) (Fig. 13).

There has been discussion as to whether A. tridens of Costa Rica is the ‘same’ as A. rubrescens of Mexico; Lemaire (2002) elected to apply the former name to the ACG specimens (unaware of the split discovered by barcoding) and to place rubrescens as a junior synonym of A. tridens. Very recently, Brechlin and Meister (2011c) revalidated the name A. rubrescens for the moths of this group flying in Mexico and Guatemala, but it now remains unclear as to whether these are conspecific with any of the two genetically and ecologically distinct groups found in ACG and that we consider to represent two distinct species. It is our opinion that the ACG A. tridens do not differ in genitalia from the neotype of A. tridens from Costa Rica. However, it is unknown as to which of the two ACG A. tridens barcodes match that neotype, though A. tridensDHJ07 is the most likely candidate. The males of both A. tridensDHJ06 and A. tridensDHJ07 are highly variable with respect to the degree of yellow and orange-pink shading of the forewings (e.g. Fig. 4g, h). A drawer of males of the dry forest moths seems to be slightly lighter in colour than a drawer of males of the rain forest moths. The shape and development of the hind wing false eyespots, and the yellow patterning of both forewings and hind wings, is highly variable within both groups of barcodes and within sib-groups, and it does not appear to be diagnostic for distinguishing the two species.

While Automeris tridensDHJ07 is essentially the only A. tridens encountered in ACG rain forest (Fig. 13), its caterpillar (04-SRNP-15216) has been found in one distinctive wet microhabitat in ACG dry forest. Near the outer end of Peninsula Santa Elena (Sector Santa Elena: Fig. 1) there is a pocket of evergreen freshwater swamp forest at the mouth of the seasonal Rio Potrero Grande. This wetter site contains some plant species commonly thought of as ‘rain forest’ species (e.g. Entada polystachya, Fabaceae) and some ‘rain forest’ Lepidoptera as well, such as a single record of an A. tridensDHJ07 caterpillar. A. tridensDHJ06 occurs throughout Sector Santa Elena dry forest, as well as in this same swamp forest. Dry forest ‘invasion’ is documented by a single male A. tridensDHJ07 light-trapped in central eastern Sector Santa Rosa (Luces, 06-SRNP-16975) dry forest (Fig. 13), but we interpret this as a wide-flying stray male from the dry forest–rain forest ecotone ~10 km to the north-east, since this light trap has also captured single males of three other species of ACG rain forest saturniids.

The group of NJ tree barcodes of Automeris tridensDHJ06 contains a distinctive subgroup of specimens from many parts of ACG dry forest. However, examination of the genitalia of these specimens showed no differences from other A. tridensDHJ06, and since they are not associated with any other trait, we regard them as representing intraspecific genetic variation.

9. Automeris zugana Druce, 1886 (Fig. 5)

ACG Automeris zugana broke unambiguously into three clusters when DNA barcoded (Fig. 2, Supplementary Fig. S1).
These clusters match the three ecologically distinct habitats and are considered here to represent three species: a dry forest (A. zuganaDHJ01) species, an upper-middle-elevation rain forest (700–1100 m; A. zuganaDHJ02) species, and a lower-elevation rain forest (360–700 m; A. zuganaDHJ03) species (Fig. 14). To date, only the very common A. zuganaDHJ01, newly described as *Automeris zozimanaguana* (Brechlin and Meister 2011c), has been encountered in the ACG dry forest and ecotones, with the other two species seemingly restricted to ‘pure rain forest’.

The barcode of *Automeris zuganaDHJ01* is 2.7% different from that of *Automeris zuganaDHJ02* and 3.4% different from that of *Automeris zuganaDHJ03*. The barcode of A. zuganaDHJ02 is 4.5% different from that of A. zuganaDHJ03. The genitalia of all three (15 dissections) (Fig. 15) are not individually distinguishable but a group of each of the three appear slightly different from a group of each of the other two, but not in a way sufficiently reliable to be diagnostic. While the caterpillars of all three appear to be identical, the females of all
three are readily distinguished by subtle differences in habitus, and the males as well, though less so, if the viewer is aware of the traits to be noticed (Fig. 5). When Lemaire identified (pers. comm.) all ACG males as being A. zugana in the 1980s, it was probably because the range of differences in male habitus fell within what he considered to be a single variable and widespread species (Lemaire 2002); we suspect that he did not examine the genitalia of the specimens sent to him. We did not have females associated unambiguously with conspecific males at that time, and did not send him females. We associate Automeris zuganaDHJ01 with the newly described Automeris zozimanaguana (Brechlin and Meister 2011c) by barcode and habitus similarity with specimens collected in Nicaraguan dry forest (Dept. Managua) at 865 m elevation, a site that appears to be an extension of the ACG dry forest ecosystem (though the elevation is greater than that generally occupied by A. zuganaDHJ01 in ACG). The barcodes of the other two ACG species within A. zugana do not match those of any of the many other Neotropical species of Automeris in the BOLD database and we suspect that they are undescribed, though perhaps quite local, species.

10. Syssphinx quadrilineata Grote & Robinson, 1867 (Fig. 6)

The barcode of Syssphinx quadrilineataDHJ01 is 1.3% (8 bp) different from that of Syssphinx quadrilineataDHJ02 (Fig. 2, Supplementary Fig. S1), but they display no difference in genitalia (10 genitalia dissections) (Fig. 15). Both barcodes occur in dry forest and rain forest (Fig. 16) and cannot be distinguished by their habitus in ACG. Free-flying males of both species have been captured in the same light trap in the Sector Mundo Nuevo dry forest–rain forest ecotone (Estacion La Perla, 400 m elevation). However, S. quadrilineataDHJ02 is known only from light-trapped individuals (n = 20), while S. quadrilineataDHJ01 is relatively common as both caterpillars and light-trap victims (n = 81). Since the ACG caterpillar inventory search process has been running for 34 years at 0–3 m above the ground, this implies that the caterpillar of S. quadrilineataDHJ02 may well be in the forest canopy.

The holotype of Syssphinx quadrilineata is from an unknown locality in Mexico (Lemaire 1988). In view of the wide distribution attributed to this species and its synonyms (Mexico to Venezuela, Lemaire 1988) there is a high chance that neither of the two ACG entities within ACG S. quadrilineata are conspecific with the population from which the Mexican holotype was collected. The finding that it may break into two within ACG suggests that it is a reasonable candidate for having a larger species complex contained within its single name elsewhere in the neotropics.

11. Dirphia avia (Stoll, 1780) (Fig. 6)

The barcode of Dirphia aviaDHJ01 is 4.4% (29 bp) different from that of Dirphia aviaDHJ02 (Fig. 2, Supplementary Fig. S1), and they have different genitalia (4 genitalia dissections) (Fig. 15). We consider them to be two distinct species, of which the one flying in the ACG dry forest, namely D. aviaDHJ01, is smaller and lighter than its rain forest counterpart. To date, all 51 specimens of D. aviaDHJ01 have been light-trapped or reared from the dry forest in ACG, whereas all six specimens of D. aviaDHJ02 have been light-trapped in rain forest (no females encountered to date). The lectotype of D. avia is from Suriname but traditional D. avia occurs from Nicaragua to Paraguay (Lemaire 2002); it has recently been divided into many species (Brechlin and Meister 2011d) and one reinstated name, Dirphia norita (Druce, 1897), described from Volcan de Chiriqui (Panama) matches the habitus and barcode of D. aviaDHJ02 convincingly and we elect to follow that nomenclature.

12. Hylesia dalina Schaus, 1911 (Fig. 6)

The barcode of Hylesia dalinaDHJ01 is only 0.9% (6 bp) different from that of Hylesia dalinaDHJ02 (Fig. 2, Supplementary Fig. S1), and moths from these two clusters of barcodes do not have distinguishable genitalia (12 genitalia dissections) (Fig. 15), caterpillars or habitus. Their genitalia also cannot be distinguished by morphology from the genitalia of the holotype of Hylesia dalina described from Costa Rican low-elevation Caribbean rain forest (Sixola close to the Caribbean coast). However, the genitalia of the rain forest H. dalinaDHJ01 seem to be more robust than are those of H. dalinaDHJ02 (thereby echoing the situation with Eacles imperialis), but this trait is not adequate for diagnosis.

H. dalinaDHJ02 has been reared and light-trapped only in ACG dry forest (n = 33), while H. dalinaDHJ01 has been reared and light-trapped only in ACG rain forest (n = 22). The latter is the most likely candidate to be the ‘true’ H. dalina, since the holotype is from Costa Rican rain forest (and since its genitalia seem slightly more robust than are those of H. dalinaDHJ02). While the two entities cannot be distinguished by their habitus in ACG, the dry forest specimens are slightly smaller than are the rain forest specimens. When Lemaire (pers. comm.) identified specimens from both ACG ecosystems as Hylesia dalina in the late 1980s, he commented that they were unlikely to have ‘good’ genitalic characteristics to distinguish them from other similar species of Hylesia, and repeats this comment later (Lemaire 2002). The strict ecological segregation between the two barcode clusters strongly suggests that they represent two distinct cryptic species that cannot presently be reliably separated specimen by specimen by their morphology.

13. Periphoba arcaeii (Druce, 1886) (Fig. 6)

The barcode of Periphoba arcaeiiDHJ01 is 1.7% (12 bp) different from that of Periphoba arcaeiiDHJ02 (Fig. 2, Supplementary Fig. S1). The only two ACG specimens of P. arcaeiiDHJ01 are reared females from middle-elevation rain forest (the common P. arcaeiiDHJ02 is found throughout ACG dry forest and rain forest) so male genitalia cannot be compared. Specimens of P. arcaeiiDHJ01 are not distinguishable from specimens of P. arcaeiiDHJ02 by their habitus or caterpillars (the extremely generalist caterpillars (Janzen 2003) appear to be identical in both ecosystems, though only two caterpillars of P. arcaeiiDHJ01 have been observed). It would be premature to conclude that P. arcaeiiDHJ01 is a different species (or the true Periphoba arcaeii) without inspecting a larger sample of them, but when contrasted with a wide geographic sample of ‘P. arcaeii’ in BOLD, its barcode matches the barcodes of specimens north
to Guatemala and Mexico, while *P. arcaeiDHJ02* matches ‘true’ *P. arcaei* from other parts of Costa Rica and to the south (the holotype is from Chiriqui in northern Panama). It is noteworthy that there are many distinct groups of *P. arcaei* DNA barcodes in BOLD, suggesting that this single name has been used for a speciose complex over its range from Mexico to South America. Within ACG, *P. arcaeiDHJ02* males are quite similar in habitus other than differing dramatically in their general ground colour from beige to grey to reddish; females are much more variable in wing pattern and intensity of ground colour differences. The two specimens of *P. arcaeiDHJ01* encompass the full range from reddish (Fig. 6g) to very dark grey.

**Discussion**

This barcoding, morphological, and ecological explorative dissection of the taxonomic underpinning of the 32 traditionally circumscribed species of saturniid moths breeding in ACG dry forest, as well as often breeding elsewhere in ACG, is revealing that while 19 of the traditional species are apparently
DNA barcoding of a tropical saturniid moth fauna

Invertebrate Systematics 493

Fig. 7. Male genitalia, ventral view: (a) *Caio* championiDHJ01, 05-SRNP-57001. (b) *Caio* championiDHJ02, 05-SRNP-56756. (c) *Citheronia* bellavistaDHJ03, 07-SRNP-103782. (d) *Citheronia* bellavistaDHJ04, 06-SRNP-5824. (e) *Ptiloscola* dargeiDHJ01, 05-SRNP-15985. (f) *Ptiloscola* dargeiDHJ02, 05-SRNP-57725. (g) *Ptiloscola* dargeiDHJ01, 10-SRNP-114232. (h) *Ptiloscola* dargeiDHJ02, 10-SRNP-114746.
just that, 13 (40%) of what were believed to be single species constitute potential or actual complexes of parapatric or sympatric cryptic species. The 9 names *Eacles imperialis*, *Automeris zugana*, *Automeris tridens*, *Othorene verana*, *Hylesia dalina*, *Dirphia avia*, *Syssphinx molina*, *Syssphinx colla*, and *Syssphinx quadrilineata* contain what appear to be 19 species that are each recognisable by their DNA barcodes, morphological traits (including habitus), and/or microgeographic distributions within ACG. *Caio championi*, *Citheronia bellavista*, and *Periphoba arcaei* each contain sufficient sympatric barcode variation, though not morphological or ecological correlated variation (within our small sample size), to be suspected of containing two cryptic species each, once more samples have been examined. *Ptiloscola dargei* has both two clusters of barcodes and a suspicious microgeographic separation of the two barcodes. And among the other 19 species there are also a few with suspicious shallow splits (Supplementary Fig. S1) that also merit a closer look (e.g. *Rothschildia lebeau*, *Othorene intermedia*, *Copaxa moinieri*, *Molippa nibasa*, and *Automeris zurobara*).

This result with 13 traditional species potentially increases the original 32 ACG dry forest species to being part of what could be as many as 49 species among the total ACG saturniid fauna. The 32 dry forest species were initially ~30% of the total of 88 traditionally recognised ACG species of Saturniidae. We now realise that this total is an unknown number that will eventually be found to be well over 100. This fine tuning and increase in species richness is congruent with what has been encountered by similarly dissecting the traditional taxonomic framework for other ACG Lepidoptera, parasitoid flies (Tachinidae), and parasitoid wasps (Braconidae, Ichneumonidae, Eulophidae, etc.). It is also congruent with similar dissections of other Lepidoptera faunas (e.g. in Bavaria: Hausmann et al. 2011; in Yucatan, Mexico: Prado et al. 2011).
Fig. 9. Male genitalia, lateral view: (a) Syssphinx collaDHJ01, 06-SRNP-15866. (b) Syssphinx collaDHJ02, 06-SRNP-15862. (c) Syssphinx collaDHJ01, 06-SRNP-15893. (d) Syssphinx collaDHJ02, 06-SRNP-15883. (e) Syssphinx molinaDHJ01, 06-SRNP-5772. (f) Syssphinx molinaDHJ02, 07-SRNP-103007. Male genitalia, ventral view: (g) Othorene bernardoespinozai, 06-SRNP-64438. (h) Othorene veranaDHJ02, 07-SRNP-103243. (i) Othorene bernardoespinozai, 96-SRNP-4701.2. (j) Othorene veranaDHJ02, 07-SRNP-103770.
With this interim analysis in hand, the entire set of ACG saturniids will be examined more carefully and more intensively over the coming years as the inventory continues for all ACG Lepidoptera. This will require substantially more and more focused specimen collection, more vouchering and specimen deposition, and more DNA barcoding, morphological examination, and hybridising experiments (such as those ongoing with the two parapatric species of *Eacles imperialis*).

Such activity had largely been discontinued during the past five years, because ‘we knew them all’. Our primary reason for rearing more wild-caught saturniid caterpillars was to explore further their parasitoid loads, and the primary reason for rearing sib groups was for caterpillar stock to place out as parasitoid traps. Collecting and rearing for redundant taxonomy was reduced because it is costly in time and funds, but we are very thankful that we were able to keep most of the voucher specimens reared over the years.

This exploration generates a perturbation to the orderly exploration of biodiversity. The scientific community has catalogued several centuries of taxonomic, and associated morphological and ecological, information under traditional species names, and continues to do so today. The implication of these ACG results is that such information for a given species may often be an unknowable and unrecoverable pooling of the information about a set of sibling species. The belief that, for example, a single species occurs from Mexico to Suriname (or in the case of *Eacles imperialis*, Canada to Paraguay), and that in any one small area what looks like one species is just that, has led to museum collections often deciding that just a very few specimens of ‘a species’ from an entire country is ‘enough’ for taxonomic purposes. However, such rarified collections are not adequate raw material with which to drill down into speciose complexes of species of sympatric or parapatric species, even with (often expensive) DNA barcoding of (often old) specimens and quite close morphological scrutiny. The six ACG *Citheronia bellavista* genitalia examined here offer the extreme example of all six being so different that each might well have been registered as a different morphology-based species; samples of several hundred...
Fig. 11. Male genitalia (all to same scale): (a), (c), (e) are the slightly more massive *Eacles imperialis* DHJ01 from rain forest. (b), (d), (f) are the slightly less massive *Eacles imperialis* DHJ02 from dry forest. (g1) 06-SRNP-5826, (g2) 06-SRNP-5828, (g3) 06-SRNP-5828, are the heavier genitalia from free-flying rain forest males. (g4) 05-SRNP-64411, (g5) 05-SRNP-64430, (g6) 05-SRNP-64412, are the slightly less massive genitalia from free-flying dry forest males.
Fig. 12. Male genitalia (ventral view) of Sector Santa Rosa, Area de Conservacion Guanacaste (ACG) dry forest *Automeris tridens*DHJ06 (left) and rain forest *Automeris tridens*DHJ07 (right). We conclude that these two species cannot be distinguished by the morphology of their genitalia. The specimens lacking a food plant name are light-trapped wild males, while the others were found as caterpillars naturally feeding on the food plants listed.
fresh ACG specimens may be required to sort out the situation with this common species that ‘everyone knows’.

Our extensive experience with identification of species during the decades of the ACG Lepidoptera inventory has encountered a second situation related to that described above. Owing to the convenience of the holotype concept (=one specimen is the gold standard for a species), there is a tendency to create a mental and/or written caricature of that species based on ‘diagnostic’ characters (including illustrations) of the holotype. When each new specimen is to be identified, there is a strong temptation to push that specimen, irrespective of its (often slight) variation, into that caricature. This in turn obscures variation that is correlated with other traits and can hide signals of the presence of another cryptic species. This ACG analysis, and others like it, show that the DNA barcode clusters are a convenient second look at an array of variable species, and the clusters may be found to correlate with yet other variables, especially using membership in a cluster to guide the analysis of ‘slight’ differences. In ACG, this is particularly notable in the many species pairs that are dry forest–rain forest in their ecologies.

Many of the so-called ACG dry forest 32 traditional species had ranges encompassing both dry forest and rain forest, rendering them conspicuous candidates for containing pairs of ecosystem-faithful sibling species. It might be thought that when the total set of 88 traditional ACG species of saturniids has been intensely barcoded (as well as examined closely for morphology and ecology), this set will not expand as strongly as did the dry forest set because many of the rain forest species seem to occur only in ACG cloud forest or rain forest. However, there is an ACG cloud forest-to-rain forest ecotone and ecosystem partitioning along that axis; we already know that it contains at least three cryptic species pairs (in Gamelia, Lonomia, and Automeris). Additionally, both Automeris zuganaDHJ02 and Automeris zuganaDHJ03, and Periphoba arcaei, demonstrate clearly that...
what appear to be single rain forest species can be cryptic complexes. While not discussed here, the traditional Automeris postalbida, common throughout ACG rain and cloud forest, comprises at least three parapatric/sympatric rain forest species in ACG. The traditional ACG species extend far from ACG into many other parts of (very small) Costa Rica (50,000 km²). It remains to be seen which of the newly recognised ACG species extend to where. It is likely that many of the ACG rain forest entities will be found throughout much of the country, since much of the country is (was) rain forest. The dry forest, more restricted to the Pacific coast, and more visibly fragmented by contour and local rainfall variation, may well not be as thoroughly occupied by the ACG dry forest morphs. On the other hand, it is much more difficult to see habitat and sub-ecosystem categories in the mostly evergreen rain forest than in the dry forest. A detailed analysis of these rain forest moths, where they have not yet been extinguished by the agroscape, may highlight habitat and sub-ecosystem categories previously overlooked, or differently defined by more human-perceived characteristics.

**Automeris tridens** is a conspicuous example of a pair of cryptic species that are generally faithful to their respective dry forest and rain forest ecosystems (Fig. 13), differ clearly in their non-overlapping DNA barcodes by 1–2% (Fig. 2, Supplementary Fig. S1), but do not display evident morphological differentiation in their genitalia (Fig. 12). It could be that their ‘barcode clock’ is running faster than is generally the case in Lepidoptera, and therefore they have split apart more recently than it would appear from their shallow DNA barcode differences. More likely, we suspect that there has not been selection favouring the evolution of genitalia differences because the moths differentiate among themselves by behaviour or pheromones, and/or the females of the two species are behaviourally faithful to their respective ecosystems. It should be emphasised that genitalia and habitus are subject to two quite different suites of selection, and are not expected to march forward in lock step in the evolution of sibling species.

It is easy to imagine the species pair within *Eacles imperialis* as being a similar situation. They also offer a reflection on taxonomic
practice. These two species can be distinguished through the rain forest species having more bulky genitalia (Fig. 11), but experimental matings of free-flying males with allospecific females experimentally placed in the males’ ecosystem shows that these genitalic differences do not block interspecific mating in nature (D. Janzen and W. Hallwachs unpubl. data). While it is

**Fig. 15.** Male genitalia, lateral view: (a) Syxphinx quadrilineataDHJ01, 00-SRNP-20581. (b) Syxphinx quadrilineataDHJ02, 07-SRNP-107355. Male genitalia, ventral view: (c) Automeris zuganaDHJ01, 04-SRNP-23090. (d) Automeris zuganaDHJ02, 04-SRNP-55730. (e) Automeris zuganaDHJ01, 91-SRNP-3128. (f) Automeris zuganaDHJ03, 08-SRNP-70716. (g) Dirphia aviaDHJ01, 07-22475. (h) Dirphia nora, 07-SRNP-108294. (i) Hylesia dalinaDHJ01, 05-SRNP-3961. (j) Hylesia dalinaDHJ02, 05-SRNP-16642.
generally assumed that visible differences in genitalia encountered between similar species of Lepidoptera correlate with mating incompatibility, there must be many cases where incompatibility is due to other traits and (relatively small) genitalic differences are of no or little consequence. Such differences probably originate in random non-detrimental changes, and in that sense, they are similar to DNA barcode differences (but of course need not emerge at the same rate).

The massive DNA barcode database for Neotropical Saturniidae that is rapidly accumulating in BOLD (www.boldsystems.org/), both privately and publically, allows three brief observations.

First, joining the intensive examination/analysis within just ACG with the light sprinkling of barcoded saturniid specimens from Mexico to Paraguay emphasises that yet much more collecting and analysis is required to make real sense of neotropical saturniid biodiversity at the species level, and that there are many tens, if not hundreds, more species to be discovered/defined, despite their several centuries of attention by collectors. We refrain here from beginning this taxonomic exploration outward from ACG, and recognise that it will require both extensive sampling, and some intense sampling at a few biologically diverse areas such as is occurring at ACG. All of this sampling needs to take into account that the males – the specimens most commonly captured with light traps – are strong fliers and can circulate over tens of square kilometres, areas quite large enough to encompass multiple ecosystems and habitats. This potential mixing of microdistributed breeding faunas at a single collecting site renders taxonomic analysis only from museum specimens all the more difficult.

Second, a real dissection and exposure of neotropical speciose saturniid biodiversity does require the addition of the new DNA barcoding tool to the long established repertoire of examining habitus and genitalia, with some support from ecosystem and microgeographic partitioning. DNA barcoding illuminates and underlines the relevance of subtle differences in all these traits,
differences that have tended to be traditionally swept into the bucket of ‘variation’. One of the outcomes of this ongoing analysis will undoubtedly be that while neotropical saturniid caterpillars will remain to be known as quite ‘generalist’ in their biologies (e.g. Janzen 2003), they also will be less so as it becomes recognised that the traits of a single traditional species are an unwittingly mixed array of traits of several species that are more specialist on at least some axes of their ecologies, much as has been found to be the case with ACG parasitic flies and wasps (e.g. Smith et al. 2006, 2007, 2008) and Hesperiidae (e.g. Burns et al. 2007, 2008; Janzen et al. 2011).

Third, traditional saturniid taxonomy tends to describe the genitalia and habitus in quite general terms, producing a variable caricature for a species, a caricature into which, as mentioned above, a variety of potentially species-level variants can be placed. This often results in the synonymy of multiple species described from different parts of the Neotropics. It also results in newly encountered similar species being summarily relegated to well established traditional names in the field and museum identification process. Syssphinx molina offers a clear example – if the moth has a crenulate/undulating wing margin (Fig. 4), it is S. molina and that is that (Lemaire 1988). This style of morphology-based taxonomy tends to hide species complexes inside a single name, leading to more generalised and ‘variable’ behaviours, colour patterns, food plants, microgeographies, etc. – i.e. it creates generalists. DNA barcoding offers the stimulus to suspect and dissect these potential species complexes.

Taxonomic concepts change over time. In general, they become more refined as more data emerge about a species, as taxonomist and other biologists have cause to examine traits more carefully, and they become more refined as we gradually slide away from our anthropocentric viewpoint – that of a large diurnal mammal – towards one that takes into account actual traits of the species rather than just what is easily perceived by us. All three of these refinements are on display here. DNA barcoding has allowed a closer look at traits not readily apparent, exposing both cryptic yet unambiguously different species, and arrays of specimens still catalogued under one scientific name that may be found to be different with yet closer examination of yet more cryptic traits.

It is a glaring trait of the 13 species examined closely because of their barcode splits that the members of each potential species pair in Figs 3–6 are extremely similar in habitus. Furthermore, for the three pairs (Othisorene verana, Dirphia avia, Automeris zugona) containing a diagnostically different habitus, each had already had one species in each pair or triplet recognised by non-barcoding, traditional means. Equally glaring is that while each pair of compared genitalia in Figs 7, 9, 12, 15 is grossly different from all of the other pairs of traditionally described species, the differences within a pair (except for the possible anomalous case of Citheronia bellavista) are absent or very subtle; all of them, except for C. bellavista, would traditionally be considered to be within normal intraspecific variation. Finally, by DNA barcode, each of the 32 barcode dry forest saturniids (Table 1) is unambiguously distinguishable from all others (Fig. 2, Supplementary Fig. S1), as is true for all traditionally described ACG Saturniidae (Janzen et al. 2005; Hajibabaei et al. 2006); the percentage differences within pairs of cryptic species, however, range from 8% to less than 1%. All of these traits emphasise that this taxonomic exploration of ACG dry forest saturniids is examining the saturniid fauna not only after many have long ago evolved into distinct and genetically strongly isolated lineages, but also added into the overall saturniid analysis some to many species that are much younger in their evolution and therefore potentially still allo-specifically exchanging genetic information (as is obvious in the case of the two ACG species of Eacles imperialis that produce viable hybrids up to the fourth generation: D. H. Janzen and W. Hallwachs, unpubl. data). While this leads to a more complex and muddy place-based taxonomic platform, the details of which are only discernable by both DNA barcoding and very detailed information about specimens, it also is closer to biological reality than is treating ecological and evolutionary processes as if they are constituted solely of species that are so grossly distinctive that a large diurnal vision-oriented mammal can easily distinguish them. Species are, on average, less ecologically generalist and less genetically isolated than we are inclined to think.

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References


Title : Search Result
Date : 3-May-2012
Data Type : Nucleotide
Distance Model : Kimura 2 Parameter
Marker : COI-5P
Codon Positions : 1st, 2nd, 3rd
Labels : SampleID, Sequence Length
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