Mosquitoes of eastern Amazonian Ecuador: biodiversity, bionomics and barcodes

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Two snapshot surveys to establish the diversity and ecological preferences of mosquitoes (Diptera: Culicidae) in the terra firme *primary rain forest surrounding the Tiputini Biodiversity Station in the UNESCO Yasuní Biosphere Reserve of eastern Amazonian Ecuador were carried out in November 1998 and May 1999. The mosquito fauna of this region is poorly known; the focus of this study was to obtain high quality link-reared specimens that could be used to unequivocally confirm species level diversity through integrated systematic study of all life stages and DNA sequences. A total of 2,284 specimens were preserved; 1,671 specimens were link-reared with associated immature exuviae, all but 108 of which are slide mounted. This study identified 68 unique taxa belonging to 17 genera and 27 subgenera. Of these, 12 are new to science and 37 comprise new country records. DNA barcodes [658-bp of the mtDNA cytochrome c oxidase (*COI*) I gene] are presented for 58 individuals representing 20 species and nine genera. DNA barcoding proved useful in uncovering and confirming new species and we advocate an integrated systematics approach to biodiversity studies in future. Associated bionomics of all species collected are discussed. An updated systematic checklist of the mosquitoes of Ecuador (n = 179) is presented for the first time in 60 years.*

Key words: Ecuador - Amazon - Culicidae - DNA barcodes - species list - habitat

The Yasuní Biosphere Reserve and National Park cover an area of 9,823 km² between the Napo and Curaray rivers in the Napo, Pastaza and Orellano provinces of northeastern Ecuador (00º10' 01º45'S, 75º20' 77º00'W). The reserve comprises the largest remaining pristine tropical forest in eastern Ecuador and is characterised by low plains alternating with the foothills of the Andes Mountain Range. The area has long been recognised internationally as a biodiversity hotspot and was designated UNESCO Biosphere status in 1989 at the request of the Ecuadorian government. Plant diversity is extremely high and it is one of only nine places worldwide known to support over 4,000 vascular plant species per 10,000 km². A single hectare is reported to support more than 100,000 species of insect. More than 204 species of mammals, including 13 primates, 550 birds and 382 fish (including 43 endemic vertebrates) have been reported from the Park (Bass et al. 2010) (wcsecuador.org).

Not only is Yasuní National Park arguably one of the World's most significant biodiversity hotspots, it is also home to a wealth of human cultural diversity, including the Tagaeri and Taromenane peoples, as well as some of the last remaining uncontacted tribes. Unfortunately, as well as richness in human, plant and animal biodiversity, Yasuní is under serious threat due to its plentiful oil reserves and the accompanying impacts that oil extraction, human disturbance and associated activities bring (e.g. deforestation, illegal logging and hunting, reduction in large mammal numbers) (Zapata-Ríos et al. 2006).

In 1995, the Tiputini Biological Station (TBS) was established by the Universidad San Francisco de Quito in collaboration with Boston University, to facilitate research and understanding of forest ecosystem management within the Yasuní Biosphere Reserve and National Park. TBS is situated in a 650-hectare (1,500-acre) tract of primary lowland rainforest to the south of the Park and encompasses a variety of habitats, ranging from upland forests, seasonally flooded lowland forest, palm swamps, small oxbow lakes and streams and other wetlands. The main forest type surrounding the station is *terra firme* (Oxford et al. 2012).

The mosquito faunal list for Ecuador was last revised by Leví-Castillo (1953) and there is no updated species list, nor current regional taxonomic keys available, making species verifications laborious. Very little information is known on the species diversity or ecological parameters of the Culicidae of the Ecuadorian Amazon (Leví-Castillo 1953, Heinemann & Belkin 1979). This study focuses on documenting the sylvan mosquito diversity and ecological parameters for species collected in the forest environs surrounding the TBS, through collections of immature stages and adult mosquitoes in November 1998 and May-June 1999.

Mosquitoes were collected in forest environments, mainly as immatures. Specimens were link-reared fol-

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lowing the procedures advocated by Belkin et al. (1965) providing high quality associated males and females with larval and/or pupal skins for rigorous taxonomic verification and integrated systematics study. DNA barcodes, 658-bp fragments of the mtDNA cytochrome c oxidase (*COI*) gene, were generated for some specimens to aid identification. Exact distribution coordinates and details on bionomics for all species collected are given. Species recorded herein were combined with other published documents and reports and an updated checklist for the 179 Culicid species known from Ecuador is presented for the first time in 60 years.

MATERIALS AND METHODS

Specimen collection - Mosquito collections were carried out in a range of natural and artificial habitats in the forest environment within a 10 km circumference of the TBS, Yasuní Biosphere Reserve and National Park, Orellana province, Ecuador (Fig. S1).

Collections (n = 121) of immature mosquitoes were carried out in aquatic habitats within the forest, including permanent standing water bodies (e.g., stream margins, lakes), temporary water bodies (e.g., ground pools, tree holes) and container habitats (e.g., rainwater collections in bromeliad and *Heliconia* axils as well as artificial containers and oviposition traps). Each discreet habitat was registered as a separate collection and detailed notes on habitat and locality information noted on a standardised collection form, which can be downloaded from the Walter Reed Biosystematics Unit (WRBU) website (wrbu.si.edu/Techniques.html). Where gaps in the forest canopy permitted, GPS readings were taken. Otherwise, detailed notes on each locality were documented in relation to positions along the TBS trails, allowing retrospective georeferencing using Google Earth. All coordinates were converted to decimal degrees (Foley et al. 2009) and distributions records are freely available in MosquitoMap (mosquitomap.org).

Depending on habitat, immature mosquitoes were collected using either a standard larval dipper (BioQuip, CA, USA) or a turkey baster and carefully transferred to a WhirlPak® plastic bag, using wide-mouth glass pipettes for transport to the laboratory. Specimens were individually link-reared to adults, following the modified procedures of Belkin et al. (1965) summarised in Gaffigan and Pecor (1997), ensuring a good series of high-quality morphological voucher adult specimens (of both sexes) with associated larval and/or pupal skins upon which to undertake the morphological assessment. Adults were mounted on paper points attached to stainless steel insect pins. The associated immature exuviae were preserved in 80% ethanol and subsequently mounted on microscope slides. A subset of male genitalia were clipped and mounted on microscope slides for further taxonomic verification. In addition to morphological vouchers, some whole larvae and reared adult specimens (particularly males) of perceived morphotaxa were preserved in 100% ethanol for later DNA analysis.

Human landing catches were not carried out in this study, but a few individuals found landing on the collectors whilst up the tower in the canopy were collected using manual aspirators and later pinned (EC126, EC168). In addition, three Shannon Traps (EC124, EC139, EC259), one indoor resting (EC219) and one outdoor resting (EC127) collection yielded adults (Fig. S1, Supplementary data 1). In some cases, identification was impossible due to poor quality (rubbed) specimens and lack of differentiating characters in the adult female life stage. Only those adults identified with certainty are reported to species herein; all others are identified to subgenera.

Morphological identification - There is no single morphological key to facilitate the identification of all known mosquitoes of Ecuador. The multi-entry web-based keys developed by the WRBU for South America (wrbu.si.edu/southcom_MQkeys.html) were used to determine generic level identifications and species level identification for some better-known taxa. These multi-entry keys facilitate identification of damaged specimens by the ability to skip questions directed at missing or damaged body parts. In addition, available dichotomous keys and original species descriptions of all life stages and the male genitalia were compiled through literature freely available on the WRBU website (wrbu.org).

Important references used for species identifications by taxonomic groups included: *Tribe Aedini* (Berlin 1969, Schick 1970, Zavortink 1972, Arnell 1973), *Anopheles* (Cova-Garcia 1961, Zavortink 1973, Faran 1980, Faran & Linthicum 1981, Wilkerson & Sallum 1999, González & Carrejo 2007), *Chagasia* (Harbach & Howard 2009), *Culex* (Rozeboom & Komp 1950, Bram 1967, Valencia 1973, Berlin & Belkin 1980, Pecor et al. 1992, Sallum & Forattini 1996), *Haemagogus* (Zavortink 1972, Arnell 1973), *Onirion* (Harbach & Peyton 2000), *Psorophora* (Guedes & Souza 1964), *Sabethes* (Harbach 1991, Hall et al. 1999), *Trichoprosopon* (Stone 1944, Zavortink 1979a, b) and *Wyeomyia* (Judd 1998). Lane (1953) was used as a general reference. Authors associated with species names, along with standardised generic and subgeneric abbreviations, are given in the final Ecuador species list (Supplementary data 2).

Molecular identification - DNA from single legs plucked from pinned adults or whole ethanol-preserved larva or adults were extracted using the QIAgen® DNeasy Blood & Tissue Kit on the BioSprint 96 automated DNA extraction platform (QIAgen®, Germantown, MD, USA). A 710-bp amplicon (658-bp less primers) corresponding to the universal "barcoding" region of the mitochondrial *COI* I gene was amplified using the LCO1490 & HCO2198 primers of Folmer et al. (1994), using the standard protocol of the Mosquito Barcoding Initiative (headed by Y-ML and RCW). Each PCR contained 1 µL of DNA template, 1 x NH_4 buffer, 0.5 mM of each deoxynucleotide triphosphate, 2 mM MgCl, 0.3 µM each primer, 0.2 U of Taq polymerase (BioLine, Taunton, MA, USA), made up to a total volume of 10 µL using ddH_20. The polymerase chain reaction (PCR) cycle was: 95ºC for 5 min, 35 cycles of 95ºC for 30 s, 48ºC for 30 min and 72ºC for 30 s, 72ºC for 5 min and a 10ºC hold.

Two microlitres of the PCR product was removed and mixed with 2 µL of 5 x loading dye before loading into wells in a 1.5% agarose gel containing 0.5 mg/mL

of ethidium bromide. Gels were run at 100 V for 8 min, prior to ultraviolet visualisation. PCR products were cleaned by adding 2 µL of ExoSAP-IT® (USB Co, Cleveland, OH, USA), diluted 1:4 with ddH_20, to the remaining 8 µL of positive PCR products. Samples were put back into the thermocycler and run at 37ºC for 30 min, followed by 80ºC for 20 min.

Bi-directional sequencing was carried out on an ABI 3730 automated sequencer (PE Applied BioSystems™, Warrington, England) using the original PCR primers and the Big Dye Terminator Kit® (PE Applied BioSystems™). Sequences were edited in Sequencher™ v.4.8 (Genes Codes Co, Ann Arbor, MI, USA). Similarities with publicly available sequences were assessed using Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi). Comparisons with unpublished barcode records were checked through the Identification System of the Barcode of Life Database (BOLD) (barcodinglife.org/index.php/IDS_OpenIdEngine). *COI* sequences generated contribute to the larger dataset of the Mosquito Barcoding Initiative. Intra and interspecific variation was calculated using Kimura two-parameter distance algorithm (Kimura 1980) in MEGA v.5.2.2 (Tamura et al. 2011). Distance calculations involved all 658 bp of the barcode fragment. Sixty variable nucleotide sequences were detected in the 21 species dataset and all nucleotides were included in pairwise comparisons.

Voucher specimens - Details of voucher specimens stored in the Culicid and frozen collections of the Smithsonian Institution, National Museum of Natural History (NMNH), including associated exuviae, genitalia preps and DNA, are explicitly listed by species in Supplementary data 3. Full collection site details, including georeferences and species determined by collection site are listed in Supplementary data 1. Collection numbers run sequentially, but EC101-102, EC109, EC119, EC129, EC182, EC185, EC190-192, EC211, EC260-261 and EC265 yielded no specimens and are omitted. Collections EC220-EC253 are also excluded as they occurred outside the study area.

Fully digitised specimen level data records and the original collection sheets are held in the WRBU accession ACC1682 and are incorporated into the MosquitoMap section of VectorMap (vectormap.org) (headed by DHF). A schematic map of the trails surrounding TBS is available from Y-ML on request. Details of specimens used for DNA, along with *COI* barcode sequences and the original chromatograms can be accessed through the BOLD (boldsystems.org) under the project Mosquitoes of the Ecuadorian Amazon. DNA sequences are available in GenBank (ncbi.nlm.nih.gov/genbank) under accessions KF670990-KF671024 and KF671027-KF671044. The DNA barcode sequences of some *Anopheles (Ker.) lepidotus* from this collection were published previously (Harrison et al. 2012) (JQ041282-JQ041286).

RESULTS AND DISCUSSION

Mosquito collections were carried out in the forest environs around the TBS between 29 October-13 November 1998 (WRBU ACC1682; collections EC100-EC219) and 25 May-2 June 1999 (WRBU ACC1682; collections EC254-EC270). These focussed primarily on collections of immatures for link-rearing, but also included a few adult collections (Shannon trap, incidental human landing, indoor resting and outdoor resting). A total of 2,284 voucher specimens are preserved and these are housed in the mosquito collections of the Smithsonian Institution, NMNH. Of these, 1,671 specimens have associated immature exuviae, all but 108 of which are slide mounted. To further verify species identifications, the male genitalia of 121 individuals were softened overnight, then clipped, cleared and permanently mounted on slides. Slide-mounted exuviae and male genitalia preps are labelled with the same unique specimen numbers as their associated adults. Full details of associated life stages (including GenBank accessions for sequenced specimens) are listed in Supplementary data 3.

Biodiversity survey (morphology and DNA barcodes) - This study identified 68 unique taxa belonging to 17 genera and 27 subgenera as follow: *Anopheles* (*An.*) [subgenera *Anopheles* (*Ano.*), *Kerteszia* (*Ker.*), *Lophopodomyia* (*Lph.*), *Nyssorhynchus* (*Nys.*), *Stethomyia* (*Ste.*)], *Chagasia* (*Ch.*), *Culex* (*Cx.*) [subgenera *Anoedioporpa* (*And.*), *Carrollia* (*Car.*), *Culex* (*Cux.*), *Melanoconion* (*Mel.*), *Microculex* (*Mcx.*)], *Haemagogus* (*Hg.*) [subgenus *Haemagogus* (*Hag.*)], *Limatus* (*Li.*), *Lutzia* (*Lt.*) [subgenus *Lutzia* (*Lut.*)], *Ochlerotatus* (*Oc.*) [subgenera *Chrysoconops* (*Chs.*), *Protoculex* (*Pcx.*), *Protomacleaya* (*Pro.*)], *Onirion* (*On.*), *Orthopodomyia* (*Or.*), *Psorophora* (*Ps.*) [subgenera *Grabhamia* (*Gra.*), *Janthinosoma* (*Jan.*)], *Runchomyia* (*Ru.*) [subgenus *Ctenogoeldia* (*Cte.*)], *Sabethes* (*Sa.*) [subgenera *Peytonulus* (*Pey.*), *Sabethes* (*Sab.*)], *Toxorhynchites* (*Tx.*) [subgenus *Lynchiella* (*Lyn.*)], *Trichoprosopon* (*Tr.*), *Uranotaenia* (*Ur.*) [*subgenus Uranotaenia* (*Ura.*)] and *Wyeomyi*a (*Wy.*) [subgenera *Decamyia* (*Dec.*), *Dodecamyia* (*Dod.*), *Hystatomyia* (*Hys.*), *Miamyia* (*Mia.*), *Wyeomyia* (*Wyo.*)]. A quantitative list of all confirmed species are given (Fig. S1, Supplementary data 3) along with information on bionomics and collection localities (Fig. S1, Supplementary data 1).

In conjunction with morphological analysis of available life stages, DNA barcodes were generated for 58 questionable specimens and used to determine their specific identity. *COI* sequences were compared with topotypic and/or expertly identified specimens in the databank of the Mosquito Barcoding Initiative and those available in GenBank. Comparison of these 58 sequences allowed the verification of 20 species in nine genera, including five of the new species discovered: *An.* (*Ano.*) sp. nr. *fluminensis*, *An.* (*Nys.*) sp. nr. *konderi*, *Ch.* sp. nr. *fajardoi*, *Cx.* (*And.*) sp. nr. *conservator*, *Wy.* (*Dod.*) sp. nr. *aphobema*, as well as the confirmation of *An.* (*Ste.*) sp. undet. (Figure, Supplementary data 3).

Somewhat incredibly, 37 of the 68 species confirmed here from Yasuní National Park comprise new country records for Ecuador. These include 12 species new to science and one further undetermined species in *Anopheles* (*Stethomyia*), a subgenus not previously reported from Ecuador (Supplementary data 2). More than half of all specimens collected (53.3%) comprised the following five species: *Wy. (Dec.) ulocoma* (n = 462), *Tr. digitatum* (n = 279), *Cx. (Cux.) mollis* (n = 181), *An.* (*Nys.*) sp. nr.

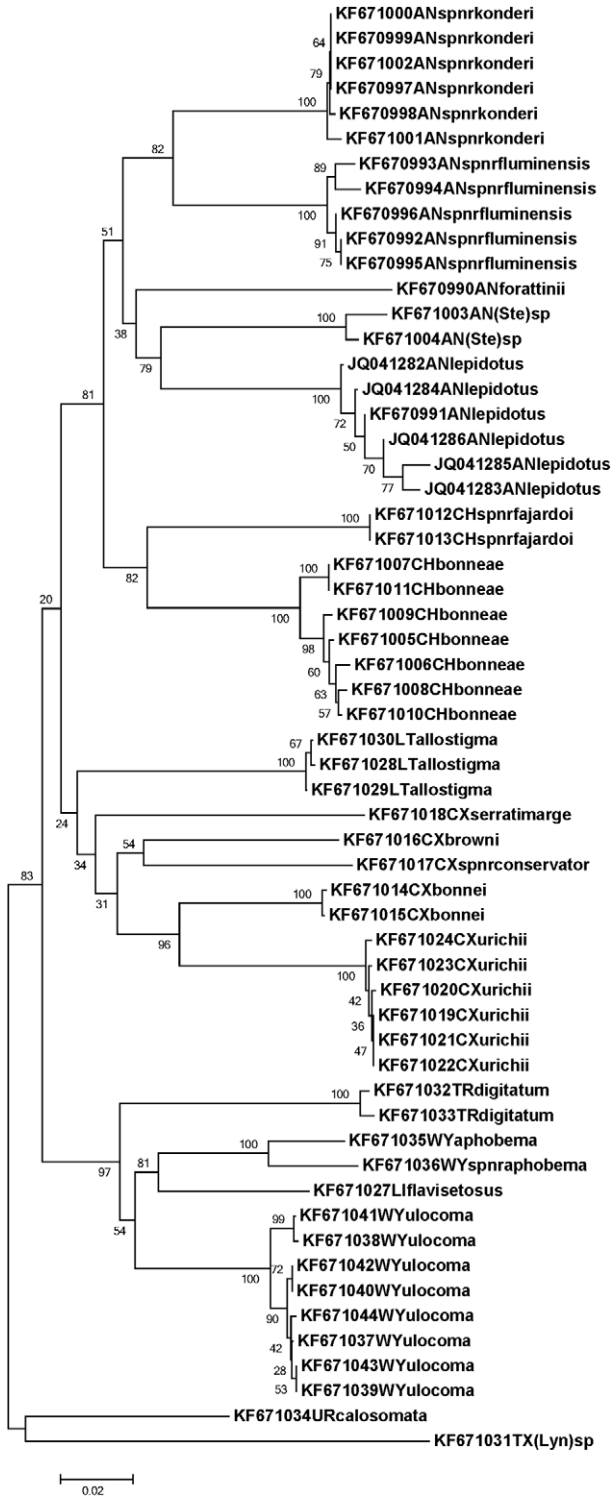


Figure: bootstrap consensus tree of cytochrome c oxidase sequences (n = 58) inferred using the neighbour-joining method of Saitou and Nei (1987). The bootstrap consensus tree was inferred from 5,000 replicates (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% of replicates are collapsed. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. Evolutionary distances were calculated using Kimura two-parameter distance algorithm (Kimura 1980). Scale bar represents sequence (%) divergence between samples. The final dataset comprised 658 nucleotides, including all codon positions. Analysis was carried out in MEGA5 (Tamura et al. 2011).

konderi (n = 154) and *Cx. (Car.) urichii* (n = 141) (Fig. S1, Supplementary data 3). Species identified are listed by taxonomic groupings below.

Subfamily Anophelinae - Anopheles: Eight species of *Anopheles* were collected, representing five subgenera: *Anopheles*, *Kerteszia*, *Lophopodomyia*, *Nyssorhynchus* and *Stethomyia*. Integrated morphological and molecular study identified the following taxa: *An.* (*Ano.*) *forattinii*, *An. (Ano.) mattogrossensis*, *An.* (*Ano.*) sp. nr. *fluminensis*, *An. (Ker.) bambusicolus*, *An. (Ker.) lepidotus*, *An. (Lop.) squamifemur*, *An.* (*Nys.*) sp. nr. *konderi* and three specimens of an undetermined species of *Anopheles* subgenus *Stethomyia* (Fig. S1, Supplementary data 3). The specific identification of three specimens as belonging to *Anopheles* subgenus *Stethomyia*, a new subgeneric record for Ecuador, was hampered as only two females remain. The only male exemplar, stored in ethanol, was wholly destroyed in an earlier DNA study and therefore its genitalia could not be examined. The two *COI* sequences obtained (Figure) (as *Anopheles* sp. TIP1) were unique with regard to others in BOLD or GenBank, thus it remains unclear whether the specimens collected represent a new species or corresponds to one of the five already described species within the subgenus *Stethomyia* (*An. acanthotorynus* Komp, *An. canorii* Floch & Abonnenc, *An. kompi* Edwards, *An. nimbus* (Theobald) or *An. thomasi* Shannon). While it was not possible to positively confirm the identity of the species detected here, *COI* sequence comparison with the three available *Stethomyia* species sequences have excluded *An. acanthotorynus*, *An. kompi* and *An. nimbus*. Comparative DNA sequences are not available for *An. canorii* or *An. thomasi*, however the former has only been reported from French Guiana so its presence in Ecuador seems unlikely. Mostly probably, these DNA sequences represent *An. thomasi* or perhaps a new species within the subgenera.

Anopheles (Ano.) fluminensis was originally described from Itaperuna, state of Rio de Janeiro, Brazil and has never been reported from Ecuador. Comparison of sequences from five specimens identified as "*An. fluminensis*?" by morphology showed 2.75-3.77% sequence differences from two unpublished Brazilian specimens of *An. fluminensis* in the Mosquito Barcoding Initiative (MBI) dataset in BOLD (MBII823-09 and MBII825-09) from Pariquera Acú, state of São Paulo, Brazil. Retrospective comparisons with Brazilian specimens revealed morphological differences and the species, designated herein as *An.* sp. nr. *fluminensis* awaits formal description.

Six *COI* sequences were generated for specimens morphologically identified as "*An. oswaldoi*?". These clustered together, varying by only 0.2-0.61% over the 658-bp fragment, indicating these comprised the same species. Sequences were 99.01-100% identical to a new species determined from Colombia (Caquetá), Ecuador (Orellana) and Peru (Madre de Dios, Loreto) in Ruiz-Lopez et al. (2013) and 98.32% identical to GenBank entry JF437965 (AC18-16 of Sallum & Laporta from Acre Brazil, listed as *An. konderi*). The true identity of *An. konderi* remains unresolved. Efforts to resolve the molecular identity of *An. konderi* and *An. oswaldoi* (Ruiz-Lopez et al. 2013) inadvertently uncovered the presence of more than one species in the specimens listed in the neotype description (Flores-Mendoza et al. 2004). Our specimens here correspond to one of the species barcoded from the neotype series, but it remains unclear whether this is "true" *An. konderi*. Efforts are underway to sequence the neotype, but in the meantime we designate this species *An.* sp. nr. *konderi* [= that of same name in Ruiz-Lopez et al. (2013)].

DNA barcodes are presented here for *An. (Ano.) forattinii* (n = 1) for the first time. This is most similar to the published sequence of *An. (Ano.) costai* (95.17-96.18% identity, JX205127 and HM022403 from Colombia). *Anopheles* (*Ano.*) sp. nr. *fluminensis*, *An.* (*Nys.*) sp. nr. *konderi*, *An. (Ano.) forattinii*, *An. (Ano.) mattogrossensis*, *An. (Lph.) squamifemur* and the undetermined *Anopheles* (*Ste.*) species comprise six new country records for *Anopheles* in Ecuador.

Chagasia - Ch. bonneae was the only recognised species in the collection by morphology. DNA extractions and *COI* sequencing was carried out on nine purported *Ch. bonneae* specimens. Of these, two specimens were found to be highly genetically distinct (minimum 10.84% sequence divergence). Retrospective detailed morphological study confirmed morphological differences and showed this novel taxa was most similar to *Ch. fajardoi*, thus we informally designated this species *Ch.* sp. nr. *fajardoi* (n = 12). Mean intra-specific sequence variation ranged from 0% in *Ch.* sp. nr. *fajardoi* (n = 2) to 1.3% in *Ch. bonneae* (n = 7). These are the first available DNA barcodes for these two species.

Subfamily Culicinae - Tribe Aedini - Eight species of the Tribe Aedini were collected in this study, representing three genera and six subgenera, as follow: *Hg. (Hag.) janthinomys*; *Oc. (Chs.) fulvus*, *Oc. (Pcx.) serratus*, *Oc. (Pro.) argyrothorax*; *Ps. (Gra.) cingulata*, *Ps. (Jan.) albigenu*, *Ps. (Jan.) albipes* and *Ps. (Jan.) lanei* (Fig. S1, Supplementary data 3). *Oc. (Pro.) argyrothorax*, *Ps. (Jan.) albigenu* and *Ps. (Jan.) lanei* are new country records for Ecuador.

Tribe Culicini - Twenty-six species of Tribe Culicini were collected in this study (Fig. S1, Supplementary data 3). *Culex*: twenty-five species of *Culex* were collected, representing five subgenera: *Cx. (And.) bamborum*, *Cx. (And.) browni*, *Cx.* (*And.*) sp. nr. *conservator*, *Cx. (Car.) bonnei*, *Cx. (Car.) iridescens*, *Cx. (Car.) secundus*, *Cx. (Car.) urichii*, *Cx. (Car.) wilsoni*, *Cx. (Cux.) bonneae*, *Cx. (Cux.) camposi*, *Cx. (Cux.) coronator s.l.*, *Cx. (Cux.) mollis*, *Cx. (Cux.) usquatus*, *Cx. (Mcx.) chryselatus*, *Cx. (Mcx.) pleuristriatus*, *Cx. (Mcx.) stonei*, *Cx. (Mel.) eastor*, *Cx. (Mel.) elevator*, *Cx. (Mel.) evansae*, *Cx. (Mel.) iolambdis*, *Cx. (Mel.) pilosus*, *Cx. (Mel.) serratimarge*, *Cx. (Mel.) symbletos*, *Cx. (Mel.) theobaldi* and *Cx. (Mel.) vomerifer*.

The most commonly encountered *Culex* species were *Cx. (Cux.) mollis* (n = 181) and *Cx. (Car.) urichii* (n = 141). Ten species of *Culex* are reported from Ecuador for the first time: *Cx. (And.) bamborum*, *Cx. (Car.) iridescens*, *Cx. (Car.) wilsoni*, *Cx. (Cux.) bonneae*, *Cx. (Cux.) us-*

quatus, *Cx. (Mel.) serratimarge*, *Cx. (Mel.) symbletos*, *Cx. (Mcx.) pleuristriatus* and *Cx. (Mcx.) stonei*, including the newly discovered taxa *Cx.* (*And.*) sp. nr. *conservator* (Supplementary data 2).

DNA barcodes were generated for five *Culex* species: *Cx. (And.) browni* (n = 1), *Cx.* (*And.*) sp. nr. *conservator* (n = 1), *Cx. (Car.) bonnei* (n = 2; intra-specific distance, 0.15%), *Cx. (Car.) urichii* [n = 6; mean distance, 0.18% (range 0-0.46%) and *Cx. (Mel.) serratimarge* (n = 1)]. Mean intra-specific distances within *Culex* species ranged from 0-0.62% (mean 0.21%) whereas inter-specific differences ranged from 9.0-15.69% (mean 13%). These sequences represent the first published DNA barcodes for all these species.

Culex (*And.*) sp. nr. *conservator* was discovered through DNA analysis. The sequence of one specimen, morphologically identified as *Cx. (And.) conservator*, was compared against unpublished sequences in the MBI dataset generated from near topotypic specimens from Trinidad. These sequences differed by 9.46%, confirming the novel taxa in Ecuador. The *Cx. (And.) browni COI* sequence shared 96.77-96.93% sequence similarity with the unpublished MBI *Cx. (And.) conservator* sequences from Trinidad. *Culex (Car.) bonnei COI* sequences were most similar to *Cx. (Car.) urichii* (91.13-91.44%, herein) and *Cx. (Mel.) serratimarge* to *Cx. (Mel.) iolambdis* (92.3-92.66%) (MBI unpublished).

Fifty specimens were identified as belonging to the *Cx. (Cux.) coronator group.* Of the six recognised species in this complex (*Cx. covagarcia* Forattini, *Cx. camposi* Dyar, *Cx. coronator* Dyar & Knab, *Cx. ousqua* Dyar, *Cx. usquatus* Dyar and *Cx. usquatissimus* Dyar) (Bram 1967), the presence of only *Cx. camposi* (n = 2) and *Cx. usquatus* (n = 3) were verified through examination of slide-mounted male genitalia. The remaining specimens from Yasuní National Park and the group as a whole need further examination.

Lutzia - The 14 specimens of *Lt.* (*Lut.*) *allostigma* collected herein represent the first report of this species in Ecuador (Fig. S1, Supplementary data 3). Heinemann and Belkin (1979) previously reported an undetermined species of the genus (as *Culex* subgenus *Lutzia*), but it is unclear whether these were *Lt. allostigma*. All specimens collected here were found in water collected in Palm bracts on the ground or in Bamboo stumps filled with boiled leaf water (Fig. S1, Supplementary data 1). DNA barcodes were generated for three specimens of *Lt.* (*Lut.*) *allostigma* (n = 3). Intra-specific variation ranged from 0.15-0.31% and was closest to the published sequence of *Cx. (Cux.) hutchinsoni* (DQ149239) at 93.45% similarity.

Tribe Orthopodomyiini - Orthopodomyia: Three specimens of *Or. fascipes* were collected as immatures in a small tree hole (n = 2, EC175) and one in the internode of fallen, living bamboo (EC197) (Fig. S1, Supplementary data 1, 3).

Tribe Sabethini - Limatus: Two species of the genus *Limatus* were recorded: *Li. durhamii* and *Li. flavisetosus*; the latter is a new country record. Here we present the *COI* barcode data for *Li. flavisetosus* (n = 1) for the first time. This is the first public barcode available for this species and while it 100% matched six undetermined specimens in BOLD, the next most similar unpublished sequence was that of *Li. durhamii* at 91.59-92.2% similarity. *Onirion*: five specimens of *On. imparis* were reared from bamboo internodes (Supplementary data 1). *Runchomyia*: one female *Ru. (Cte.) magna* was identified, reared from a pupa collected in ground seepage (EC130). *Sabethes*: a new species, *Sa.* (*Pey.*) sp. nr. *luxodens*, was discovered as immatures in a bamboo internode (EC132, n = 4), along with an adult female, identifiable only to subgenus *Sabethes* (EC219). *Trichoprosopon*: *Tr. digitatum* (n = 279) was the second most numerous species collected in this study. DNA barcodes were generated for two specimens of *Tr. digitatum* (0.62% difference); these comprise the first published *Trichoprosopon COI* sequences. Two specimens of a new species, *Tr.* sp. nr. *lampropus* were collected from water collected in a Palm bract, lying on the ground (Supplementary data 1, 3). *Wyeomyia*: due to lack of updated keys, species of the genus *Wyeomyia* are notoriously difficult to identify. Many *Wyeomyia* specimens have been identified only to subgenus and these specimens form part of a larger revisionary study of the genus by CHP. Of the 12 *Wyeomyia* species identified herein, six were new to science: *Wy.* (*Dec.*) sp. CP2013, *Wy.* (*Dod.*) sp. nr. *aphobema* (identified by DNA), *Wy.* (*Hys.*) sp. nr. *lamellata*, *Wy.* (*Mia.*) sp. nr. *codiocampa*, *Wy.* (*Wyo.*) sp. nr. *albosquamata* and *Wy.* (*Wyo.*) sp. nr. *medioalbipes*. Of the remaining six species [*Wy. (Dec.) pseudopecten*, *Wy. (Dec.) ulocoma*, *Wy. (Dod.) aphobema*, *Wy. (Hys.) autocratica*, *Wy. (Mia.) codiocampa*, *Wy. (Mia.) oblita*], all but *Wy. (Dod.) aphobema* are new country records for Ecuador (Fig. S1, Supplementary data 3).

DNA barcodes were generated for three species of *Wyeomyia*, namely: *Wy. (Dec.) ulocoma* (n = 8; mean distance 0.7, range 0-1.55%), *Wy. (Dod.) aphobema* (n = 1) and *Wy.* (*Dod.*) sp. nr. *aphobema* (n = 1), with the latter discovered by DNA. Sequence variation between the three species sequenced ranged from 4.6-10.66% (mean 9.94%). These sequences comprise the first published *COI* barcodes for *Wyeomyia* species.

Tribe Toxorhynchitini - Toxorhynchites: Two species of the *Toxorhynchites* subgenus *Lynchiella* were identified: *Tx. (Lyn.) bambusicola* and *Tx. (Lyn.) haemorrhoidalis*. The three link-reared specimens of *Tx. (Lyn.) bambusicola* reported here were collected in three separate collections (EC197, EC203, EC254) and these comprise a new country record for Ecuador (Fig. S1, Supplementary data 1). Only one DNA barcode was determined for an undetermined species of *Toxorhynchites* (as *Toxorhynchites* sp. TIP1 in Figure). This sequence was most similar (87.7%) to the published sequence of *Tx. (Tox.) splendens* (HQ398877).

Tribe Uranotaeniini - Uranotaenia: The two male specimens of *Ur. (Ura.) calosomata* collected as pupae in the large lake (EC112) form another new country record (Fig. S1, Supplementary data 3). The DNA barcode sequenced from one of these specimens is closest to *Ur. (Ura.) lateralis* (HQ398881) at 89.14% sequence similarity.

An integrated morphological and molecular approach such as advocated here facilitates robust identification for the species found in Amazonian Ecuador. DNA barcodes were useful in the discovery of several new species, which were at first overlooked by morphological assessment alone. Efforts will continue to obtain species-diagnostic DNA barcode signatures for all other species documented in this collection and elsewhere in Ecuador. A reference library of DNA sequences, generated from well-vouchered morphological specimens, will invariably facilitate the correct identification of adult specimens, the stage most commonly captured in vector surveillance studies and currently most difficult to identify using morphology alone.

Bionomics - Immature habitats - Mosquito species are often highly specific in their choice of oviposition site. In this study, larval habitats could be grouped into temporary and permanent groundwater habitats, artificial containers and natural (plant) containers (Fig. S1). Excepting *An. (Ker.) bambusicola* (exclusive to bamboo internodes) and *An. (Ker.) lepidotus* (exclusive to bromeliads), immatures of all other species of *Anopheles* and *Chagasia* were collected with ground water habitats: *An.* sp. nr. *fluminensis* was also found in a ground pool. *Culex* (*Mel.*) immatures were all recovered from ground water habitats, including ground pools, seepage and lake, swamp and stream margins (Fig. S1).

Permanent water bodies (lakes, swamps, stream margins) in the forest environs of the TBS yielded a high diversity of taxa. Species collected at the edge of the four-hectare lake (EC112, EC199, EC263) included *An. (Ano.) mattogrossensis*, *An.* (*Nys.*) sp. nr. *konderi*, *Cx. (Mel.) pilosus*, *Cx. (Mel.) serratimarge*, *Cx. (Mel.) theobaldi*, *Cx. (Mel.) eastor*, *Cx. (Mel.) symbletos*, *Ur. (Ura.) calosomata* and further undetermined specimens of *An.* (*Ano.*) and *Cx.* (*Mel.*) (Fig. S1, Supplementary data 1). Eighteen collections carried out at stream margins (EC100, EC125, EC127, EC128, EC145, EC146, EC152, EC153, EC163, EC169, EC170, EC172, EC198, EC216, EC257, EC258, EC260, EC266) yielded immatures of the following taxa: *An.* (*Ano.*) sp. nr. *fluminensis*, *An. (Lph.) squamifemur*, *An.* (*Nys.*) sp. nr. *konderi*, *Cx. (Mel.) elevator*, *Cx. (Mel.) evansae*, *Cx. (Mel.) iolambdis*, *Cx. (Mel.) theobaldi*, *Ch. bonneae*, *Ch.* sp. nr. *fajardoi* and undetermined specimens of *An.* (*Ano.*) and *An.* (*Ste.*). Larvae of *An.* sp. nr. *fluminensis*, *An.* (*Nys.*) sp. nr. *konderi*, *Cx. (Mel.) theobaldi* and undetermined specimens of *Cx.* (*Mel.*), *An.* (*Ano.*) and *Chagasia* were found at the edges of a large swamp (EC255, EC269). Ground pools (EC137, EC148, EC200) were occupied by *An.* (*Ano.*) sp. nr. *fluminensis*, *Cx. (Cux.) coronator s.l.*, *Cx. (Mel.) evansae*, *Oc. (Pcx.) serratus*, *Ps. (Jan.) albigenu* and undetermined specimens of *Cx.* (*Mel.*). *Culex (Cux.) camposi*, *Cx. (Cux.) coronator s.l.*, *Cx. (Cux.) usquatus*, *Cx. (Mel.) pilosus* and *Ru. (Cte.) magna* were collected from ground seepage in open sunlight (EC130, EC134) (Fig. S1, Supplementary data 1).

Many species were found occupying a variety of natural and artificial habitats at ground level. *Culex* (*And.*) sp. nr. *conservator*, *Cx. (Cux.) mollis*, *Cx. (Car.) urichii*, *Lt. (Lut.) allostigma*, *Li. durhamii*, *Li. flavisetosus*, *Tr. digitatum* and *Tr.* sp. nr. *lampropus* were collected in small rainwater pools in palm bracts on the ground (EC103, EC110, EC113-7, EC120-1, EC140-1, EC143, EC171, EC175, EC186-7, EC194). *Culex (Cux.) mollis*, *Tr. digitatum* and *Wy.* (*Dod.*) sp. nr. *aphobema* were collected from rainwater pockets in a folded tarpaulin on the forest floor (EC157). Larvae of *Cx. (And.) browni*, *Cx.* (*And.*) sp. nr. *conservator*, *Cx. (Cux.) mollis*, *Li. flavisetosus* and *Tr. digitatum* were collected in a tree hole in a fallen tree (EC118). Larvae of *Li. durhamii* were collected in a discarded 1.5 L plastic bottle lying on the forest floor (EC188) in which rainwater had collected.

Dark plastic oviposition traps were placed at different heights up a tower in the forest: at ground level (EC209, EC212), at 5m (EC210), at 8 m (EC164, EC167), at 25 m (EC213) and at the top of the tower (34 m, EC208). *Tr. digitatum* was collected in the oviposition cups (ovi-cup) placed at every height (EC167, EC208, EC209, EC210, EC213). An undetermined species of *Toxorhynchites* was reared from an ovi-cup (EC212) at ground level, *Cx. (Mcx.) pleuristriatus* at 8 m (EC164) and *Cx. (Mcx.) chryselatus* and *Tr. digitatum* were collected together in an ovi-cup placed 34 m up at the top of the tower (EC208).

Bamboo offers many discrete microhabitats for immature stages of forest mosquitoes; these microhabitats appear to be closely associated with certain mosquito species. *Culex (Car.) bonnei* and *Cx. (Cux.) mollis* were collected from inside bamboo into which a hole had been artificially cut (EC154) and from cut, dry bamboo lying on the ground (EC136), along with *Li. durhamii*, *Li.* sp. undet. and *Li. flavisetosus*. Split aged bamboo sections were filled with boiled leaf water and left on the forest floor to promote oviposition (EC177-181, EC183-184, EC214-215, EC217). *Culex (Cux.) mollis*, *Lt. (Lut.) allostigma*, *Tr. digitatum* and *Wy. (Dec.) ulocoma* were reared from these containers. *Culex (Car.) wilsoni*, *Cx. (Car.) iridescens*, *On. imparis*, *Tx. (Lyn.) bambusicola* and *Tx. (Lyn.) haemorrhoidalis were* collected from internodes in dead bamboo (EC202-203, EC206, EC270) whereas *An. (Ker.) bambusicolus*, *Cx. (And.) bamborum*, *Cx. (And.) browni*, *Cx. (Mcx.) pleuristriatus*, *Cx. (Car.) secundus*, *Cx. (Car.) urichii*, *Cx. (Car.) wilsoni*, *On. imparis*, *Sa.* (*Pey.*) sp. nr. *luxodens*, *Tx.* (Lyn.) *theobaldi*, *Wy. (Mia.) codiocampa* and *Wy. (Mia) oblita* were collected from internodes in living bamboo (EC131-133, EC196, EC204). *Tx. (Lyn.) bambusicola* and *Or. fascipes* were collected from a bamboo internode in fallen, living bamboo (EC197) and *Cx. wilsoni* from a bamboo internode in fallen, cut bamboo (EC205). *Li. durhamii*, *Li. flavisetosus* and *Tr. digitatum* were collected from bamboo stumps (EC155, EC174, EC201) (Fig. S1, Supplementary data 1).

Anopheles mosquitoes of the subgenus *Kertezsia* breed almost exclusively in plant containers and serve as highly effective malaria vectors in forested areas (Zavortink 1973). Whereas *An. (Ker.) bambusicolus* (EC131, EC254) was collected from internodes in standing bamboo, we found that immatures of *An. (Ker.) lepidotus* were restricted to bromeliad axils (EC104, EC151, EC166, EC256). No details were noted for EC165, but as *An.* (Ker.) *lepidotus* was collected along with *Tr. digi-*

tatum, it seems likely that this collection was also a bromeliad axil collected from the tower.

Water collections from bromeliad axils yielded a high diversity of mosquito species including: *An. (Ker.) lepidotus*, *Cx. (Car.) urichii*, *Cx. (Car.) bonneae*, *Cx. (Mcx.) chryselatus*, *Cx. (Mcx.) stonei*, *Cx.* (*Mcx.*) sp. undet., *Tx. (Lyn.) bambusicola*, *Wy. (Dod.) aphobema*, *Wy. (Hys.) autocratica*, *Wy.* (*Hys.*) sp. nr. *lamellata, Wy. (Dec.) ulocoma*, *Wy.* (*Wyo.*) sp. nr. *albosquamata*, *Wy.* (*Wyo.*) sp. nr. *medioalbipes*, *Wy.* (*Dec.*) sp. undet., *Wy.* (*Hys.*) sp. undet. and *Wy.* (*Wyo.*) sp. undet. (EC104-105, EC108, EC135, EC138, EC147, EC151, EC156, EC161, EC166, EC207, EC256, EC268). *An. (Ker.) lepidotus*, *Cx. (Car.) bonneae*, *Cx. (Mcx.) chryselatus*, *Cx. (Mcx.) stonei*, *Cx.* (*Mcx.*) sp. undet. and *Tx. (Lyn.) bambusicola* were all collected from bromeliad axils in high (> 30 m) in the forest canopy (EC166, EC207, EC256), accessed from the tower and canopy walkway. Bromeliad axils that fall to the ground (EC122-123) seem to loose their exclusivity and are occupied instead by non-bromeliad-specific species such as *Culex (Car.) bonnei*, *Cx. (Cux.) mollis*, *Cx. (Car.) urichii* and *Lt. (Lut.) allostigma*, which appear to occupy almost all ground habitats. Excluding the fallen bromeliads, nine species were collected in this unique habitat, including three of the new *Wyeomyia* species.

Species found in the axils of *Heliconia* plants (EC106, EC107, EC144, EC158-160, EC173, EC189, EC193, EC195, EC218, EC264) were limited to *Tr. digitatum* and *Wyeomyia* (*Dec.*) species namely *Wy. (Dec.) pseudopecten*, *Wy. (Dec.) ulocoma*, *Wy.* (*Dec.*) sp. CP2013 and *Wy.* (*Dec.*) sp. undet. Immatures of *Tx.* (*Lyn.*) *haemorrhoidalis* were found in the axil of a palm frond (EC267). Immatures of *Cx. (Cux.) mollis*, *Hg. (Hag.) janthinomys*, *Oc. (Pro.) argyrothorax*, *Or. fascipes* and *Wy. (Dec.) ulocoma* were collected in holes in living trees (EC111, EC142, EC162, EC173).

In addition, *Cx. (Car.) bonneae* and *Cx. (Cux.) mollis* females opportunistically laid egg rafts in rearing pans in the laboratory (EC149-150) (Supplementary data 1).

Adult collections - Due to the paucity of diagnostic female characteristics, little effort was placed on collection of adults so only 142 adults were collected in seven discrete collections. Three collections were carried out using Shannon traps (EC124, EC139, EC259), two human landing collections (EC126, EC168), one indoor resting (EC219) and one outdoor resting collection (EC127) (Supplementary data 1). Only those indisputable specific identifications are listed, with most adult females being identified to subgeneric level only (Supplementary data 3).

An un-baited Shannon trap set some 1.5 m above the ground (EC124) yielded only one *Lt. allostigma*, whereas setting the trap near a gas lantern resulted in the collection of many more specimens as well as a wider variety taxa (EC259, n = 84): *An.* (*Nys.*) sp. nr. *konderi*, *Cx.* (*Cux.*) sp. undet., *Cx. (Mel.) pilosus*, *Cx. (Mel.) theobaldi*, *Cx. (Mel.) vomerifer*, *Cx.* (*Mel.*) sp. undet., *Hg.* (*Hag.*) sp. undet., *Oc. (Chs.) fulvus*, *Oc.* sp. undet., *Ps. (Jan.) albipes* and *Tr. digitatum*. Another Shannon trap (EC139) set high in the forest canopy (c. 34 m at the top of the tower) collected *Oc. (Chs.) fulvus*, *Tr. digitatum*, *Ps. (Jan.) albigenu* and *Cx.* (*Cux.*) sp. undet., as well as the only specimens of *Ps. (Gra.) cingulata* (n = 1) and *Ps. (Jan.) lanei* (n = 1) detected in this study (Supplementary data 1, 3).

Tr. digitatum was collected outdoor resting near the edge of a stream (EC127) and indoor resting within the research station, along with undetermined species of *Cx. (Cux.)* and *Ps. (Jan.)* and the only *Sa. (Sab.)* specimen collected in this study (EC219). *An. (Ker.) lepidotus* was collected on two separate occasions trying to bite the collectors some 34 m up in the canopy on the tower (EC126, EC168) (Supplementary data 1). Despite the unfocussed collecting effort, five of the 68 species reported (7.3%) were collected only as adults, showing that adult collections are a valuable addition to biodiversity surveys in this region.

Current list of Ecuadorian Culicidae - Given the relatively narrow spatial and temporal range of this study, the biodiversity of mosquitoes collected was quite remarkable. That more 16% of all taxa identified from the Yasuní National Park in these snapshot collections were new to science and 54% are new country records clearly indicates that taxonomic study of Ecuadorian fauna has been woefully neglected, at least in the eastern Amazonian region. This prompted the authors to review the available literature and to determine a current faunal list for the mosquitoes of Ecuador (Supplementary data 2).

By far the most productive mosquito taxonomist in Ecuadorian history was Roberto Levi-Castillo, who published several species lists (Leví-Castillo 1945, 1949, 1953) and many new species in the 40s and 50s, 30 of which remain valid species today (Supplementary data 2). Levi-Castillo published several reports (e.g., Leví-Castillo 1956) in which he listed species collected in Ecuador, but these were composite lists, without associated collection data or specimens. While there is no doubting his contribution to the understanding of the mosquito fauna of Ecuador, his disregard for preserving voucher specimens has previously been highlighted (e.g., Zavortink 1979a) (p. 13). In their revisionary work, "A Catalog of the Mosquitoes of the World", Knight and Stone (1977) included the distributions of only verifiable records.

To create this updated species list for Ecuador, we used Knight and Stone (1977) as a basis and included all species reported in verified peer reviewed publications since, reflecting revisions in taxonomy (Knight 1978, Faran 1979, Heinemann & Belkin 1979, Zavortink 1979a, b, Berlin & Belkin 1980, Sirivanakarn 1982, Ward 1984, Forattini & Sallum 1989, Hall et al. 1999, Sallum & Hutchings 2003, Reinert et al. 2004, 2005, 2006, 2008, 2009, Rueda et al. 2004, Harbach & Howard 2007, González et al. 2010, Harrison et al. 2012, Ruiz-Lopez et al. 2013). Our findings show that 179 mosquito species have been recorded in Ecuador to date, representing 26 genera and 39 subgenera (Supplementary data 2). This is the first published list of Ecuadorian Culicidae since Leví-Castillo (1953) and we hope this serves as a useful systematics platform for future work on the mosquitoes of Ecuador.

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