Confirmation of *Anopheles (Anopheles) calderoni* Wilkerson, 1991 (Diptera: Culicidae) in Colombia and Ecuador through molecular and morphological correlation with topotypic material

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The morphologically similar taxa Anopheles calderoni, Anopheles punctimacula, Anopheles malefactor *and* Anopheles guarao *are commonly misidentified. Isofamilies collected in Valle de Cauca, Colombia, showed morphological characters most similar to* An. calderoni, *a species which has never previously been reported in Colombia. Although discontinuity of the postsubcostal pale spots on the costa (C) and first radial (R_1) wing veins is purportedly diagnostic for* An. calderoni, *the degree of overlap of the distal postsubcostal spot on C and R_1 were variable in Colombian specimens (0.003-0.024). In addition, in 98.2% of larvae, seta 1-X was located off the saddle and seta 3-C had 4-7 branches in 86.7% of specimens examined. Correlation of DNA sequences of the second internal transcribed spacer and mtDNA cytochrome c oxidase subunit I gene (COI) barcodes (658 bp of the* COI *gene) generated from Colombian progeny material and wild-caught mosquitoes from Ecuador with those from the Peruvian type series of* An. calderoni *confirmed new country records. DNA barcodes generated for the closely related taxa,* An. malefactor *and* An. punctimacula *are also presented for the first time. Examination of museum specimens at the University of the Valle, Colombia, revealed the presence of* An. calderoni *in inland localities across Colombia and at elevations up to 1113 m.*

Key words: *Anopheles calderoni* - morphology - DNA barcodes - COI - ITS2 - Colombia - Ecuador - new country records

The Central and Southern American mosquito species *Anopheles (Anopheles) calderoni* Wilkerson, 1991, *Anopheles (Anopheles) guarao* Anduze and Capdevielle (1949), *Anopheles (Anopheles) malefactor* Dyar & Knab 1907 and *Anopheles (Anopheles) punctimacula* Dyar & Knab 1906 (Arribalzagia Series, Laticorn Section) (Harbach 2004) are highly isomorphic and closely related (Wilkerson 1990, 1991). Following reassessment of specimens from both the type series and type locality [Panama, Colon (Canal Zone)] of *An. punctimacula*, Wilkerson (1990) detected exemplars of *An. malefactor* in the syntype series and resurrected the nominal species from synonymy with the former. According to Wilkerson (1990, 1991), *An. punctimacula* is reported from Mexico to northern Colombia and Venezuela, whereas *An. malefactor* appears to be limited to Panama and northeastern Colombia. So far, the distribution of *An. guarao* is thought to be limited to its type locality of Caripito, Estado Monagas, Venezuela (Anduze & Capdevielle 1949).

Further assessment of *An. punctimacula* s.l. specimens from Piura, Salitral, Peru, revealed yet another cryptic species, which Wilkerson (1991) formally named *An. calderoni*. The description suggested that prior records of *An. punctimacula* in the low-lying areas (< 250 m) of the Pacific Coast of Colombia, Ecuador and Peru (Levi-Castillo 1949, Russell et al. 1963, Calderón et al. 1974) could, at least in part, refer to *An. calderoni*. Based on characters of the adult females, Rubio-Palis and Moreno (2003) confirmed *An. calderoni* from the states of Táchira and Barinas, in Venezuela. In light of this geographical discordance, its presence in Colombia and Ecuador seems highly likely. That *An. calderoni* has been found positive for *Plasmodium vivax* in the Peruvian regions of Lima, Salitral, Querecotillo, Lourdes Paz and Palo Blanco (Kroeger & Alarcón 1993, Calderón et al. 1995), makes this taxonomic query also one of biomedical importance.

Literature records and museum holdings indicate only the presence of *An. punctimacula* and *An. malefactor* in Colombia (González & Carrejo 2007), and the morphological keys most commonly used to identify Colombian *Anopheles* (Suárez et al. 1988) predate the description of *An. calderoni*. Following the detection of atypical *An. punctimacula* s.l. specimens from various localities in the valley of the Rio Cauca, progeny broods were obtained to facilitate a thoroughly integrated systematic assessment of these Colombian variants. Identifications of archive specimens of *An. punctimacula* s.l. in the Entomological Museum, Universidad del Valle, Colombia (MUSENUV) were revised, alongside wild-caught material from Ecuador.

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MATERIALS AND METHODS

Specimens - Wild-caught adult females of *An. punctimacula* s.l. were collected in August 2007, between 18 pm-21 pm, either resting or in human landing catches, at Corporacion Autonoma Regional del Valle del Cauca in Laguna de Sonso, Buga, Valle del Cauca, Colombia (03º52'32''N, 76º20'53''W) at an elevation of 960 m. Females were induced to oviposit according to the standard protocols of Belkin et al. (1965) and once eggs were laid, females were pinned and stored in the MUSENUV collections and labelled with the same code as the corresponding progeny. Larvae were fed on a mix of brewer's yeast and rabbit food. Some 4th instar larvae were separated, killed by boiling water and stored in acetic acid, glycerol and alcohol fixative (10 parts 60% ethyl alcohol with 1 part glacial acetic acid and 1 part glycerol) for future morphological examination. Larval and pupal exuviae of link-reared specimens were stored in 75% ethanol prior to slide-mounting following the protocols in González and Carrejo (2007). Emergent adults were allowed to feed on 10% sucrose solution and were later killed by freezing. Adults were pinned and labelled with the same code as the mother for further integrated studies.

Morphological assessment - Morphological identifications were carried out using the diagnostic characters for *An. punctimacula* s.l. (Wilkerson 1990) and the original species description of *An. calderoni* (Wilkerson 1991). Wing character nomenclature used herein follows that of Wilkerson and Peyton (1990) (Fig. 1, Tables I-III). Measurements were taken using a micrometer mounted in a Nikon SMZ-645 Stereo Zoom Microscope. For each female (n = 79), variation in the overlap of the postsubcostal dark spot with the preapical dark spot (Table I), the respective lengths of the costal wing spots (Table II) and the ratio of the length of the costal wing spot relative to the total wing length (measured from the humeral cross-vein) (Table III) were calculated. The presence of key diagnostic characters of *An. calderoni* larvae - numbers of branches on seta 3-C and the positioning of seta 1-X in relation to the saddle (Wilkerson 1991) - were assessed in all mounted larvae (n = 144) (Table IV). Verification of specimens held in the archive collections of the MUSENUV and which had been identified as *An. punctimacula* were also reassessed (Supplementary data).

Molecular analysis - Full locality details of specimens used in the molecular part of this study are given in Table V. DNA extraction, polymerase chain reaction (PCR) amplification and product clean-up took place on board The ScholarShip (M/V Oceanic II) as part of the activities of the Mosquito Barcoding Initiative (MBI). DNA was extracted from the abdomens of pinned specimens using the QIAGEN® BioSprint robotic DNA extraction machine and the BioSprint 96 DNA Tissue Kit (QIAGEN®, Crawley, UK), following the manufacturers instructions. The universal LCO and HCO barcoding primers of Folmer et al. (1994) were used to amplify the cytochrome c oxidase subunit I gene (COI) region (658 bp, less primers). The PCR comprised 1 µL template DNA, 1 µL 10 x NH_4 buffer, 0.5 µL dNTPs at 2.5 mM, 0.3 µL each primer at 10 µM, 0.4 µL $MgCl_2$ at 50 mM and 0.2 µL of Taq polymerase (BioLine, London, England), made up to 10 µL with ddH_20. The PCR reaction comprised initial denaturing at 95ºC for 5 min, then 34 cycles of 95ºC for 30 s, 48ºC for 30 min and 72ºC for 45 s, followed by a 5-min extension at 72ºC and a 10ºC hold. Two microliter of each PCR product was visualized on 1% agarose gels, containing 0.5 mg/mL of ethidium bromide. Amplification of the nuclear ITS2 rDNA region was achieved using the 5.8SF and 28SR primers of Collins and Paskewitz (1996), following the protocol listed in Linton et al. (2001).

PCR products were purified using ExoSAP-IT® (USB, Cleveland, Ohio), following the manufacturers instructions. Bi-directional DNA sequences were generated either in the Sequencing Facility of the Natural History Museum, London, or at the Museum Support Center of the Smithsonian Institution, Maryland, using the Big Dye® Terminator Kit (PE Applied Biosystems, Warrington, England) and run on ABI 3730 automated sequencers (PE Applied Biosystems). Sequences were edited using

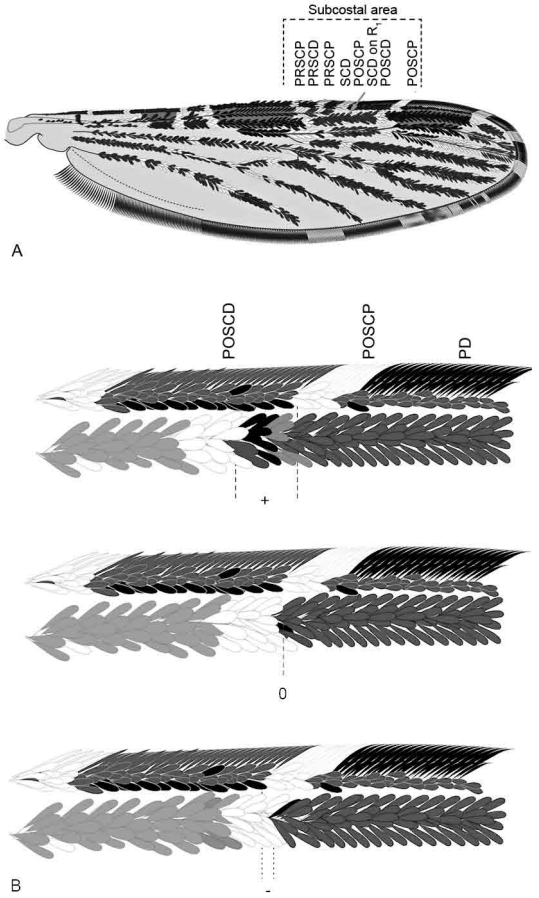

Fig. 1: schematic diagram of the wing of *Anopheles calderoni*. A: the position of postsubcostal pale spot (POSCP) with relation to its position on the first radial vein (R_1); B: variation in the position of the POSCP with regard to its reportedly standard position on R_1. PD: preapical dark; POSCD: postsubcostal dark. PRSCD: presubcostal dark; PRSCP: presubcostal pale; SCD: subcostal dark.

TABLE I

Variation in the extent of overlap (in mm) of the distal part of postsubcostal dark spot with the proximal part of the preapical dark spot on vein first radial vein from 79 female progeny of iso-female lines of *Anopheles calderoni* generated from females collected in Valle de Cauca, Colombia

	Isofamily number									
Isofamily	1	2	3	4	5	6	7	8	9	10
1[a]	0.020	-0.010	0.080	0.010	0.060	0.040	0.050	-0.040	0.100	0.020
2	0.040	-0.040	0.110	0.026	0.090	0.080	-0.060	0.000	0.040	0.060
3	0.030	0.000	0.050	0.054	0.060	0.000	0.000	0.010	0.036	0.004
4[a]	0.026	-0.026	0.074	0.020	0.060	0.070	0.070	0.000	0.050	0.000
5[a]	0.000	-0.020	0.040	0.040	0.090	0.020	0.110	0.000	0.006	0.070
6	-	-0.020	0.070	0.060	0.054	0.030	0.000	-0.040	0.026	0.050
7	-	-0.034	0.080	-	0.130	0.000	0.020	-0.030	-0.010	0.020
8	-	-0.040	0.050	-	-	0.020	0.000	-	-	0.020
9	-	-0.030	0.026	-	-	-	-0.040	-	-	0.000
10	-	-0.050	0.050	-	-	-	-0.030	-	-	-
Mean	0.023	-0.024	0.063	0.035	0.078	0.033	0.012	-0.014	0.035	0.027
SD	± 0.015	± 0.015	± 0.024	± 0.020	± 0.028	± 0.030	± 0.052	± 0.021	± 0.035	± 0.026

a: DNA-typed isofamily; SD: standard deviation. Negative or zero values signify lack of overlap.

Sequencher™ version 4.8 (Genes Codes Corporation, Ann Arbor, Michigan) and aligned in CLUSTALX (Jeanmougin et al. 1998) (Fig. 2). Similarity of the sequences generated in this study was compared with those available in GenBank using Blast (blast.ncbi.nlm.nih.gov/) and sequences were analyzed according to standard barcoding methodology (Hebert et al. 2003). Sequence statistics, calculation of pairwise distance parameters using Kimura 2-parameter algorithm (Kimura 1980) and generation of the bootstrapped neighbor-joining tree (10,000 replicates) were carried out in MEGA v.4.0 (Tamura et al. 2007).

Details of isofamily specimens of *An. calderoni* sequenced from Colombia (n = 7) and wild-caught specimens from Ecuador (n = 5) in this study are given in Table V. In addition, one specimen each of the closely related taxa *An. punctimacula* [Gamboa, Panama (09º06'N, 79º41'W); colls.: J Loaiza, Y Linton, F Ruiz and students of The ScholarShip LLC] and *An. malefactor* [Yaviza, Panama (08º11'N, 77º41'W); coll.: J Loaiza] were included in the analysis, but no specimens of *An. guarao* were available for inclusion. The COI electropherograms and specimen details (including exact localities details with georeferences, details of voucher type and housing, GenBank accessions) are available in the public records of the MBI section of the Barcode of Life Data Systems v. 2.5 (boldsystems.org) (Ratnasingham & Hebert 2007) and appear in GenBank as barcode red flag data, indicating their high quality and voucher standards under the following accessions: *An. calderoni* (Colombia HQ642964-70, Ecuador HQ642971-74), *An. punctimacula* (Panama HQ622626) and *An. malefactor* (Panama HQ622625). The ITS2 sequences of *An. calderoni* generated in this study are available in GenBank under accessions numbers HQ622618-HQ622622.

RESULTS

Morphological assessment of Colombian *An. calderoni*

Adults - Nearly all the female progeny from families reared for this study exhibited characters concordant with those given in the original description of *An. calderoni* as follow: upper mesanepimeron with pale scales, wings with pale yellow scales mixed with white scales, distal postsubcostal pale spot on the costa and the postsubcostal pale spot on the first radial (R_1) not contiguous, frequently separated by dark scales at their respective ends, i.e. overlap of scales of the distal part of the postsubcostal dark spot with the proximal part of the preapical dark on vein R_1 (Fig. 1A, B); scales on vein R_1 between the proximal part of the presubcostal pale and the distal postsubcostal pale generally dark (Fig. 1B), i.e. R_1 in the subcostal area is usually all dark except for pale spots at the ends.

Measures of the overlap of scales of the distal part of the postsubcostal dark spot with the proximal part of the preapical dark spot of R_1 were found to be heterogeneous

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                      136 217 271 316 319 349 373 469 496 502 557 601 628
Haplotype1(COL=6) tgcggcaaaacta
Haplotype2(COL=1) ....a........
Haplotype3(EC=2)  c...at..g..cg
Haplotype4(EC=1)  c...atg.g..cg
Haplotype5(EC=1)  ca.aa.gg..ttg
Haplotype6(EC=1)  cataa.g..gctg
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Fig. 2: alignment of unique cytochrome c oxidase subunit I gene haplotypes of 13 *Anopheles calderoni* from Colombia (COL) (n = 7) and Ecuador (EC) (n = 6). Numbers above bases indicate position of variable bases along the 658 bp fragment (709 bp with primers). Points indicate bases identical to haplotype 1.

TABLE II

Measurements of the costal wing spots normalized for wing length (humeral cross vein to apex), from F_1 progeny of 10 iso-female lines of *Anopheles calderoni* generated from females collected in Valle de Cauca, Colombia

		Subcostal area		Presubcostal proximal pale		Presubcostal dark		Presubcostal distal pale		Subcostal dark		Postsubcostal proximal pale		Postsubcostal dark		Postsubcostal distal pale	
Isofamily	n	Mean	SD (±)	Mean	SD (±)	Mean	SD (±)	Mean	SD (±)	Mean	SD (±)	Mean	SD (±)	Mean	SD (±)	Mean	SD (±)
1[a]	5	0.252	0.009	0.039	0.006	0.015	0.004	0.020	0.003	0.029	0.004	0.018	0.004	0.107	0.013	0.024	0.005
2	10	0.243	0.014	0.015	0.004	0.030	0.010	0.021	0.006	0.036	0.006	0.014	0.003	0.106	0.005	0.022	0.004
3	10	0.261	0.013	0.014	0.004	0.047	0.008	0.014	0.005	0.032	0.011	0.016	0.005	0.112	0.014	0.025	0.005
4[a]	6	0.246	0.014	0.015	0.003	0.040	0.008	0.021	0.003	0.025	0.007	0.016	0.003	0.103	0.013	0.025	0.005
5[a]	7	0.219	0.016	0.013	0.003	0.030	0.007	0.017	0.004	0.036	0.004	0.014	0.006	0.088	0.014	0.022	0.005
6	8	0.242	0.015	0.016	0.002	0.040	0.007	0.020	0.004	0.027	0.008	0.015	0.003	0.099	0.007	0.025	0.007
7	10	0.240	0.028	0.013	0.005	0.043	0.012	0.016	0.004	0.024	0.007	0.015	0.005	0.112	0.020	0.026	0.005
8	7	0.232	0.008	0.017	0.004	0.043	0.013	0.018	0.006	0.037	0.008	0.020	0.004	0.075	0.006	0.028	0.005
9	7	0.243	0.016	0.016	0.003	0.034	0.011	0.019	0.005	0.035	0.008	0.015	0.004	0.094	0.014	0.030	0.006
10	9	0.232	0.017	0.012	0.006	0.036	0.009	0.019	0.004	0.034	0.004	0.016	0.002	0.093	0.012	0.022	0.006
Total	79	0.241	0.017	0.016	0.006	0.037	0.009	0.018	0.005	0.032	0.007	0.016	0.004	0.100	0.013	0.025	0.005
p		0.0004		0.0038		< 0.0001		0.1273		0.0002		0.2166		< 0.0001		0.1310	

a: DNA-typed isofamily; SD: standard deviation.

TABLE III

Measurements of the costal wing spots normalized for wing length (humeral cross vein to apex) from 79 females from 10 isofamilies of *Anopheles calderoni* generated from females collected in Valle de Cauca, Colombia, compared to those given in the original description of *An. calderoni* (Wilkerson 1991)

	Isofamilies (Colombia)			Wilkerson (1991)		
Wing spot	Mean	SD (±)	Range	Mean	SD (±)	Range
Humeral pale	0.01	0.010	0.01-0.03	0.02	0.010	0.01-0.03
Humeral dark	0.07	0.010	0.04-0.10	0.09	0.020	0.03-0.12
Presector pale	0.01	0.000	0.01-0.02	0.02	0.010	0.01-0.03
Presector dark	0.11	0.020	0.08-0.15	0.09	0.020	0.05-0.14
Sector pale	0.07	0.020	0.03-0.10	0.10	0.020	0.06-0.14
Sector pale accessory	0.04	0.010	0.02-0.07	0.06	0.020	0.04-0.10
Sector dark	0.18	0.020	0.02-0.23	0.15	0.020	0.11-0.18
Area subcostal	0.24	0.020	0.20-0.29	0.28	0.020	0.24-0.31
Presubcostal dark	0.04	0.010	0.01-0.06	0.04	0.010	0.02-0.06
Presubcostal pale (proximal)	0.02	0.010	0.01-0.05	0.02	0.010	0.01-0.06
Presubcostal pale (distal)	0.02	0.000	0.01-0.04	0.02	0.010	0.01-0.03
Postsubcostal dark	0.10	0.020	0.06-0.14	0.10	0.010	0.07-0.12
Postsubcostal pale (proximal)	0.02	0.000	0.01-0.03	0.02	0.010	0.01-0.04
Postsubcostal pale (distal)	0.02	0.010	0.02-0.04	0.04	0.010	0.02-0.06
Subcostal dark	0.03	0.010	0.01-0.05	0.02	0.001	0.01-0.04
Preapical dark	0.15	0.020	0.12-0.20	0.13	0.016	0.10-0.18
Preapical pale	0.10	0.010	0.07-0.12	0.11	0.010	0.09-0.12
Preapical dark accessory	0.05	0.010	0.01-0.07	0.05	0.010	0.04-0.07
Apical dark	0.03	0.010	0.01-0.04	0.02	0.010	0.01-0.03

SD: standard deviation.

TABLE IV

Variation in numbers of branches of seta 3-C and the positioning of seta 1-X with respect to the saddle in 144 specimens from 10 isofamilies of *Anopheles calderoni* generated from females collected in Valle de Cauca, Colombia

		Number of branches on seta 3-C				Position of seta 1-X		
	Individuals						Saddle	Border
Isofamily	n	Mean	Min	Max	Mode	n	%	%
1[a]	6	5.5	4	7	4-7	3	100	-
2	6	6.2	4	7	4-8	3	100	-
3	26	5.9	3	9	4	13	69.2	23.1
4[a]	26	4.5	3	7	4	12	91.7	8.3
5[a]	13	4.5	2	6	4-6	6	100	-
6	5	6.6	5	7	5	3	100	-
7	19	5.4	2	7	6	10	70	30
8	12	5.8	4	8	6	5	60	40
10	15	4.7	1	6	6	7	85.7	14.3
11	5	7.4	1	11	-	2	50	50
12	4	5.5	5	6	5-6	2	100	-
Total	137	5.4	1	11	6	66	81.8	16.7

a: DNA-typed isofamilies.

among families ($p < 0.0001$) and variable even among female siblings (Table I). This value is positive when there is overlap in the spots, but can also have a value of zero when the ends of the spot are immediately adjacent and a negative value when the spots are clearly separated (Fig. 1B). Overall, eight of the 10 isofamilies examined exhibited overlapping spots (0.003-0.024 mm), however many of the individuals in isofamily 2 and some from isofamilies 7-9 showed either no overlap but immediately adjacent spots or clear separation of the spots (Table I). An even alignment of the postsubcostal spot on C and R_1, reportedly typical of all other Arribalzagia Series species (Suárez et al. 1988, Wilkerson 1990, 1991, González & Carrejo 2007), was not observed in the *An. calderoni* specimens examined here. Considering the above observations, it is necessary to add a clarification of the original description of this character as follows: "distal postsubcostal pale spot on vein C generally not contiguous with the postsubcostal pale on vein R_1, separated (generally) or not by dark scales of the distal part of the postsubcostal dark and the proximal part of preapical dark on R_1". For those specimens that agree with the remaining characters yet where the aforementioned overlap does not occur, it is possible complement species identification by examination of the scales between the proximal presubcostal pale and the distal postsubcostal pale spots located on R_1. These are usually all dark in *An. calderoni*, whereas they are predominantly pale in *An. punctimacula*, *An. malefactor* and *An. guarao* (Wilkerson 1991).

Analysis of costal spots within the isofamilies revealed that some specimens did not comply with a normal distribution, even after data transformation using wing length; hence a non-parametric Kruskal-Wallis test was used to compare the averages of these measurements among isofamilies.

Statistically significant differences were found when comparing lengths of the subcostal area (Fig. 3) as a whole among the isofamilies; yet, the relative size comparisons of individual spots within this area, i.e. the size of the most distal presubcostal pale spot, and the proximal and distal postsubcostal pale spots, did not statistically differ amongst isofamilies (Table II). Furthermore, the average values among isofamilies (Table III) were very similar to those presented in the original description (Wilkerson 1991).

The band or spot on hindtarsomere 5 was generally found not to be as pale as in the original description (Wilkerson 1991). Instead it was observed as a darker band, sometimes grayish or pale brown and was not well defined in 91.1% of the progeny specimens examined. This darker band was also noted in 94.6% of specimens collected in human landing catches from the same locality (Valle de Cauca, n = 37).

The original description of *An. calderoni* makes no reference to variation in the pale and dark spots of the 4th hindtarsomere 4, which are otherwise widely employed as diagnostic characters in several morphological keys for species of the Arribalzagia Series (Gorham et al. 1973, Wilkerson et al. 1990, González & Carrejo 2007). There are generally five spots of varying length on hindtarsomere 4: two dark and three pale (basal, apical and central). Spots reportedly differ in length, with the two pale spots (basal, apical) being smaller than the dark spots. In 91.6% of Colombian *An. calderoni* examined (n = 79), the central pale spot separated the two dark spots; whereas in the remaining specimens the central pale spot was not clear, allowing the two dark spots to merge and appear more like a band. The central pale spot was found to be of variable width, and was mostly light grey in color. This character was even found to be variable between legs of the same individual therefore we discourage the further use of this character for species diagnosis in the Arribalzagia Series.

Larvae - The number of branches in seta 3-C and the position of seta 1-X were assessed in all 144 specimens from 10 isofamilies (Table IV). That seta 1-X is not present on the saddle is a shared characteristic of both *An. punctimacula* and *An. calderoni*. In 98.2% of progeny examined, seta 1-X was not found on the saddle itself, with 81.5% attached outside this structure altogether and 16.7% placed on its border (Table IV). Most commonly, *An. calderoni* larvae exhibited 4-7 branches on seta 3-C (range 1-11; mean 5.4; mode 6). The mean number of branches is slightly higher than the 4.2 reported in the original description (Wilkerson 1991), most probably due to increased sample size. *An. punctimacula* differs significantly in having an average of 11.5 branches hence the combination of these two characters allows morphological differentiation of larval *An. calderoni* and *An. punctimacula*.

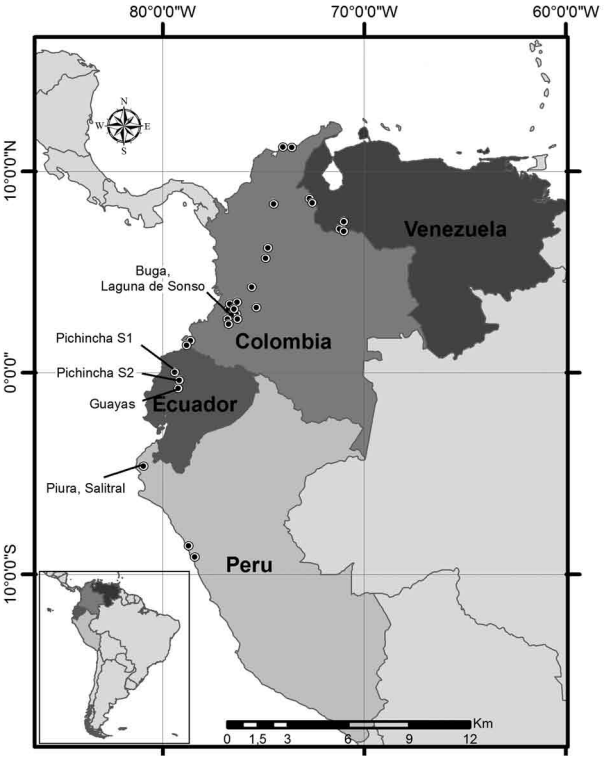

Fig. 3: map indicating the known distribution of *Anopheles calderoni* in Colombia, Ecuador, Peru and Venezuela, based on morphological and molecular verification, following this study and those of Wilkerson (1991) and Rubio-Palis and Moreno (2003).

DISCUSSION

Revision of MUSENUV archive specimens - Retrospective identification of archive specimens of *An. punctimacula* and *An. malefactor* held in the MUSENUV revealed a significant proportion of specimens were infact *An. calderoni*. Specimens of *An. calderoni* were detected in collections from 1948-2009, in 35 localities in 20 municipalities, representing 12 departments of Colombia (Supplementary data). Although the distribution of *An. calderoni* in Colombia appears predominantly coastal (both on the Pacific and Atlantic coasts) (Fig. 3), some of the departments (e.g. Tolima) are far inland. Additionally, several collection sites in Valle de Cauca and Quindio were over 1000 m in altitude (Supplementary data), considerably higher than the 250 m limit previously reported for early reports of *An. calderoni* in Peru (Wilkerson 1991) and 200-400 m reported in Venezuela (Rubio-Palis & Moreno 2003). Rather our data supports the findings of Calderón et al. (1995) who reported *An. calderoni* from sea level to 1080 m in Peru, suggesting that the distribution of *An. calderoni* could be considerably more extensive than at first thought.

Molecular characterization of An. calderoni - ITS2 rDNA sequences generated from two specimens [PE400(2)-29, PE400(2)-31] belonging to the type series of *An. calderoni* from Salitral (Wilkerson 1991) were identical to those from three specimens from Ecuador (Table V), thus verifying the new country record of *An. calderoni* in Ecuador. Correlation of the 658 bp COI DNA barcode of one of the verified Ecuadorian *An. calderoni* specimens (EC229-1) allowed the confirmation the conspecific identity of additional wild-caught adults from two other sites in Ecuador (Guayas, n = 4, Pichincha, n = 1) and progeny broods collected in Valle de Cauca, Colombia (n = 7 from isofamilies 1, 4 and 5) (Table V), used in the morphological assessment (Table V).

In total, DNA barcodes were generated for 12 specimens (Colombia, n = 7, Ecuador, n = 5), which varied in AT richness from 66-66.5%. Six unique haplotypes were determined, as follows: haplotypes 1 (n = 6) and 2 (n = 1) (both Valle de Cauca, Colombia), haplotype 3 [Guayas (n = 1) and Pichincha (n = 1), Ecuador] and haplotypes 4-6 (n = 1, each one from Guayas, Ecuador) (Figs 2, 3). Within Colombia, haplotypes 1 and 2 varied by only one base (A/G at base 319) (Fig. 2). Overall, 13 (1.97%) variable bases were noted, 10 of which were parsimony informative. Mean pairwise (K2P) sequence diversity within *An. calderoni* was 0.70%, well within values of intraspecific variation reported for the barcoding region in other mosquito species (Cywinska et al. 2006, Kumar et al. 2007, Azari-Hamidian et al. 2010). Haplotypes were unique by country, with mean intra-country pairwise distances of 0.04% and 0.77% within Colombian and Ecuadorian specimens, respectively, and an overall mean inter-country sequence divergence of 1.03%. Amino acid translation of the COI sequences revealed that all nucleotide changes were synonymous, suggesting that these represent population level differences only.

Differentiation of An. calderoni, An. punctimacula, An. malefactor and An. guarao - Given that *An. calderoni* has often been misidentified as *An. punctimacula* in Colombia (herein), we set out to assess the utility of DNA barcodes for species differentiation between these species and other closely related taxa - *An. malefactor* and *An. guarao.* No specimens of *An. guarao* were available for molecular comparison, but the morphological distinctiveness of this species makes it unlikely to be confused with *An. calderoni.* The robust boot strap values illustrated on the neighbor-joining tree generated using the COI barcode data of *An. calderoni*, *An. punctimacula* and *An. malefactor* confirmed the separate species status of these taxa (Fig. 4). In comparison to the 0.70% interspecific variation noted within *An. calderoni*, interspecific comparisons with those of Panamanian *An. punctimacula* and *An. malefactor* generated in this study, revealed 5.8% and 10.9% variation, respectively (Fig. 4). COI barcodes of *An. malefactor* (67.7% AT) were most similar to those of *An. punctimacula* (67.1% AT), differing by 9.6%.

Neither *An. calderoni* nor *An. malefactor* are currently represented in GenBank and thus these sequences also comprise the first DNA sequences reported for these species. Although the GenBank entry AF417719 of Sallum et al. (2002) partially overlapped our *An. punctimacula* sequence by 93 bases, the sequences generated herein represent the first published DNA barcodes for that species. This study shows that DNA barcodes will aid correct identification of *An. calderoni*, *An. malefactor* and *An. punctimacula* in future studies and will allow the vector status of *An. calderoni* and *An. malefactor* to be more accurately assessed.

DNA verification of Ecuadorian and Colombian specimens as *An. calderoni* along with the morphological reassessment of archived MUSENUV specimens significantly adds to the knowledge of the distribution of *An. calderoni* in Latin America (Fig. 3). Given that the known distribution of *An. calderoni* now includes Ecuador and Colombia, as well as Peru and Venezuela, and its prior taxonomic confusion with *An. punctimacula* and *An. malefactor*, it is now prudent to carry out further collections to determine the true distributions and specific roles in malaria transmission of these three species across the Neotropical Region.

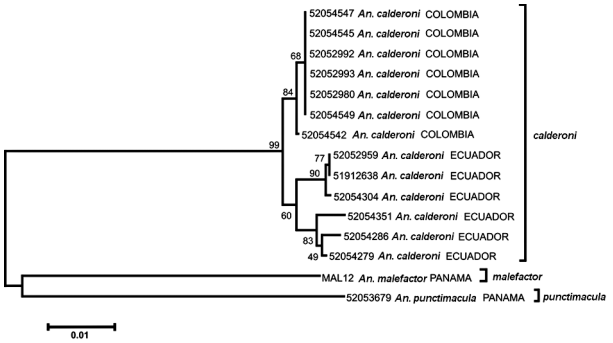

Fig. 4: neighbor-joining tree of *Anopheles calderoni*, *Anopheles punctimacula* and *Anopheles malefactor* based on 658 bp of the mtDNA cytochrome c oxidase subunit I gene gene. The tree was constructed using 10,000 replicates of pairwise genetic distances generated using the Kimura 2-parameter distance algorithm. Bootstrap confidence limits are shown on tree labels.

TABLE V
Exact localities, field codes, Mosquito Barcoding Initiative (MBI) number, ITS2, cytochrome c oxidase subunit I gene (COI) and haplotypes (HAP) collection information, GenBank accession and identifier for all specimens of *Anopheles calderoni* sequenced in this study

Country	Exact locality	Sample code	MBI	COI HAP	ITS2 HAP	GenBank accession	Identifier
Colombia (n = 7)	Valle del Cauca, Buga, Laguna de Sonso (03º52.032'N, 76º20.053'W)	MUSENUV	52052980	1	-	HQ642969	R González
		F08-P04	52054545	1	-	HQ642970	R González
		F18-P05	52054549	1	-	HQ642965	R González
		F20-P04	52052992	1	-	HQ642966	R González
		MUSENUV	52052993	1	-	HQ642967	R González
		F15-P01	52054547	1	-	HQ642964	R González
		MUSENUV	52054542	2	-	HQ642968	R González
Ecuador (n = 6)	Guayas, Yaguachi, Hacienda Eulalia (02º07.707'S, 79º41.886'W)	EC-YG-A1	52052959	3	-	HQ642971	J Alarcon
		EC-YG-A1	52054304	4	-	HQ642974	J Alarcon
		EC-YG-B1	52054351	5	-	HQ642972	J Alarcon
		EC-YG-H1	52054279	6	-	HQ642973	J Alarcon
	Pichincha, near Tinalandia S1 (00°17.783'S, 79°03.484'W)	EC229-1	51912638	3	1	HQ622618(ITS2)	R Wilkerson
		EC229-2	-	-	1	HQ622619	R Wilkerson
	Pichincha, near Tinalandia S2 (00°05.283'S, 79°16.567'W)	EC232-14	-	-	1	HQ622620	R Wilkerson
Peru (n = 2)	Piura, Salitral (04°49.999'S, 80°42.000'W)	PE400(2)-29[a]	-	-	1	HQ622621	R Wilkerson
		PE400(2)-31[a]	-	-	1	HQ622622	R Wilkerson

a: specimens from the type series of *An. calderoni*; MUSENUV: Entomological Museum, Universidad del Valle, Colombia.

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Collection details of specimens of *Anopheles calderoni* retrospectively identified in the collections of the Entomology Museum of the Universidad del Valle, Colombia

Department	Municipality	Locality	Elevation (m)	Collection date	Collector
Antioquia	Gómez Plata	El Brasil	244	10 May1984	SEM
	Puerto Nare	El Pescado	270	4 Oct 1983	SEM
	Santafe de Antioquia	Paso Real	666	17 Mar 1984	SEM
Bolívar	Achí	Platanal	23	26 Jun 1984	SEM
Caldas	La Dorada	Guarinocito	200	unknown	SEM
	Puerto Tejada	Perico Negro	990	unknown	SEM
Cauca	Villarica	Água Azul	990	18 Feb 2005 1 Mar 2006	Curso de E. Médica
Chocó	Riosucio	Caño Pavas	4	5 Jun 1981	SEM
Guajira	Riohacha	Las Flores	9	22 Jun 1986	H Ramirez (SEM)
Magdalena	Santa Marta	Guachaca	18	2 May 1983	Derrueco (SEM)
Nariño	Tumaco	Brazo Rio Mira	15	7 Apr 1986	SEM
		Bucheli	10	9 Jun 1987	Cortés (SEM)
		Chajal	9	7 Jun 1987	Cortés (SEM)
		Chilví-Línea	15	17 Jun 1986 22 Jul 1986 21 Jul 1986 23 Jul 1986	Cortés (SEM)
		El Descalzadero	15	28 Feb 1984	SEM
		Ing. del Carmen	15	23 Jun 1987 3 Jul 1987 10 Jul 1987 16 Jul 1987	Cortés (SEM)
		Ing. del Guadual	15	18 Jun 1987	Cortés (SEM)
		Nueva Union 2	15	1 Apr 1986	Castillo (SEM)
Norte de Santander	Tibú	Puerto Barco	56	17 Jun 1986 26 Jun 1987	Pabón (SEM)
		Isla La Ceiba	35	24 Sep 1986	SEM
		Caño Castillo	60	Sep 1983	SEM
Quindio	Quimbaya	La Montaña del Ocaso	1100	13 Nov 2003	P Lopez & I Valderrama
Tolima	Ataco	Bocas de S. Pedro	613	30 Nov 1981	D Tique (SEM)

↳

Department	Municipality	Locality	Elevation (m)	Collection date	Collector
Valle del Cauca	Buga	Laguna de Sonso	960	7 Jun 2007 9 Jul 2007 May 2009	R González, N Carrejo, DM Lucumí, CX Restrepo, DM Montoya & C Salas
		El Vinculo	970	2008	LM Martínez
		Chambimbal, El Cedral	928	May 2009	D M Montoya
	Bugalagrande	El Pital (Humedal)	926	29 Apr 2009	DM Montoya
	Candelaria	El Cabuyal	1005	May 2006 7 May 2007	MH Cuellar & LM Martínez
		El Otoño	1005	May 2008	DM Cundumí & CX Restrepo
	Florida	Chocosito	1045	15 May 2007	R González
		Florida (Cab)	1030	May 1948 20 Aug 1948	A Patiño (SEM)
	Jamundí	Urb. Terranova	980	28 Apr 2009	V Cerón
	Palmira	Ciat	962	Oct 2007	LM Martínez
		San Isidro-El Bolo	980	15 May 2005 24 Nov 2007	D Gómez & LM Martínez
	Tulúa	Mate Guadua	1113	28 Apr 2006	H Cadena, NJ Mina & C Robledo

SEM: Servicio de Erradicación de la Malaria, Colombia.