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Novel genetic diversity within *Anopheles punctimacula* s.l.: Phylogenetic discrepancy between the Barcode cytochrome *c* oxidase I (*COI*) gene and the rDNA second internal transcribed spacer (ITS2)

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ABSTRACT

Anopheles punctimacula s.l. is a regional malaria vector in parts of Central America, but its role in transmission is controversial due to its unresolved taxonomic status. Two cryptic species, *An. malefactor* and *An. calderoni*, have been previously confused with this taxon, and evidence for further genetic differentiation has been proposed. In the present study we collected and morphologically identified adult female mosquitoes of *An. punctimacula* s.l. from 10 localities across Panama and one in Costa Rica. DNA sequences from three molecular regions, the three prime end of the mitochondrial cytochrome *c* oxidase I gene (3′ *COI*), the Barcode region in the five prime end of the *COI* (5′ *COI*), and the rDNA second internal transcribed spacer (ITS2) were used to test the hypothesis of new molecular lineages within *An. punctimacula* s.l. Phylogenetic analyses using the 3′ *COI* depicted six highly supported molecular lineages (A–F), none of which was *An. malefactor*. In contrast, phylogenetic inference with the 5′ *COI* demonstrated paraphyly. Tree topologies based on the combined *COI* regions and ITS2 sequence data supported the same six lineages as the 3′ *COI* alone. As a whole this evidence suggests that *An. punctimacula* s.l. comprises two geographically isolated lineages, but it is not clear whether these are true species. The phylogenetic structure of the *An. punctimacula* cluster as well as that of other unknown lineages (C type I vs C type II; D vs E) appears to be driven by geographic partition, because members of these assemblages did not overlap spatially. We report *An. malefactor* for the first time in Costa Rica, but our data do not support the presence of *An. calderoni* in Panama.

1. Introduction

Incorrect species identification is a serious issue when dealing with malaria vectors (Diptera: *Anopheles*), because choosing proper mosquito control methods depends on accurate knowledge of the species' taxonomic status, ecology and behavior. Traditionally, female adult morphology has been used to distinguish *Anopheles* species, and this approach is critically important for some groups (Harbach, 2004). Nevertheless, morphological characters have been shown to overlook key genetic divergence, which may be related to differences in vectorial capacity or insecticide resistance (Zarowiecki et al., 2011). Furthermore, the increasing number of cryptic species complexes in *Anopheles* mosquitoes indicates that reliance on morphology alone could compromise vector control strategies (Marrelli et al., 2006; Li and Wilkerson, 2007; Paredes-Esquivel et al., 2009; Bourke et al., 2010; Cienfuegos et al., 2011), and this may be the case for most non-Amazonian countries of Latin America where *Anopheles* taxonomy studies are rather incomplete (Loaiza et al., 2012; Arevalo-Herrera et al., 2012).

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Table 1
Reported distributions of the twenty-four species comprising the Arribalzagia Series (Root, 1922) of the subgenus *Anopheles*. *Indicates species reported from Panama; species highlighted in bold have the potential to also be present in Panama; species names underlined share the morphological character of presence of upper mesepimeral scales in adult female stage. The country of the type locality for each species is capitalized.

Arribalzagia Series	Authors	Geographic Records
An. anchietai	Corrêa & Ramalho	BRAZIL
*An. apicimacula**	Dyar & Knab	Belize, Bolivia, Colombia, Costa Rica, Ecuador, El Salvador, GUATEMALA, Guyana, Honduras, Mexico, Panama, Suriname, Venezuela
An. bustamentei	Galvão	BRAZIL
An. calderoni	Wilkerson	Colombia, PERU, Venezuela
An. costai	Fonseca & Ramos	BRAZIL, Suriname
An. evandroi	da Costa Lima	Argentina, BRAZIL
An. fluminensis	Root	Argentina, Bolivia, BRAZIL, Peru
An. forattini	Wilkerson & Sallum	BRAZIL, Colombia, French Guiana, Peru
An. gabaldoni	Vargas	Guatemala, MEXICO
An. guarao	Anduze & Capdevielle	VENEZUELA
An. intermedius	(Peryassu)	Belize, BRAZIL, French Guiana, Guatemala, Guyana, Mexico, Peru, Suriname, Trinidad and Tobago
An. maculipes	(Theobald)	Argentina, BRAZIL, French Guiana, Trinidad and Tobago, Uruguay
*An. malefactor**	Dyar & Knab	PANAMA
An. mattogrossensis	Lutz & Neiva	Bolivia, BRAZIL, Colombia, Peru, Venezuela
*An. mediopunctatus**	(Lutz)	Argentina, BOLIVIA, Brazil, Colombia, Ecuador, French Guiana, Guyana, Panama, Peru, Suriname, Trinidad and Tobago, Venezuela
An. minor	da Costa Lima	Argentina, BRAZIL, French Guiana, Paraguay, Suriname
*An. neomaculipalpus**	Curry	Argentina, Belize, Brazil, Colombia, Costa Rica, El Salvador, Mexico, PANAMA, Paraguay, Peru, Trinidad and Tobago, Venezuela
An. peryassui	Dyar & Knab	Bolivia, BRAZIL, Colombia, Peru, French Guiana, Guyana, Suriname, Venezuela
An. pseudomaculipes	(Peryassu)	BRAZIL
*An. punctimacula**	Dyar & Knab	Argentina, Belize, Bolivia, Brazil, Colombia, Costa Rica, Ecuador, Mexico, PANAMA, Peru, Uruguay, Suriname, Trinidad and Tobago, Venezuela
An. rachoui	Galvão	BRAZIL
An. shannoni	Davis	Bolivia, BRAZIL, Guyana, Peru, Suriname
An. veruslanei	Vargas	MEXICO
*An. vestitipennis**	Dyar & Knab	Belize, Colombia, Costa Rica, Cuba, Dominican Republic, El Salvador, GUATEMALA, Haiti, Honduras, Jamaica, Mexico, Nicaragua, Panama, Puerto Rico

Two DNA regions have been commonly used to assist with species identification in the genus *Anopheles*: the Folmer or "Barcode" region at the five prime end of the mitochondrial cytochrome *c* oxidase I gene (5′ *COI*) and the rDNA second internal transcribed spacer (ITS2). Both molecular markers have been employed to test hypotheses of molecular taxonomy and systematics in malaria vectors using phylogenetic analysis (Wilkerson et al., 2005; Marrelli et al., 2006; Li and Wilkerson, 2007; Paredes-Esquivel et al., 2009; Bourke et al., 2010; Ruiz-Lopez et al., 2012), to investigate patterns of population structure, and to validate species distribution records (González et al., 2010; Loaiza et al., 2010b, 2012; Laboudi et al., 2011). The ITS2 is recognized as the gold standard for species identification in most *Anopheles* sibling complexes; length differences and fixed substitutional changes between ITS2 sequences are taken as proof of lineage splitting especially if the lineages are geographically co-distributed (Walton et al., 2007a,b). Similarly, fixed mutations and more than three percent divergence among Barcode sequences may indicate cessation of gene flow and speciation (Crywinska et al., 2006; Kumar et al., 2007; Ruiz-Lopez et al., 2012), although Foley et al. (2006) suggested that the threshold level to define *Anopheles* species with the Folmer region could be set lower than 3% to minimize false negatives. Despite the fact that agreement between the Barcode *COI* and the ITS2 should provide a robust demonstration of species' taxonomic status, discrepancies may correspond to evidence of different evolutionary processes acting at different genetic levels (Paredes-Esquivel et al., 2009; Bourke et al., 2010). Conflicting results between mitochondrial genes and the ITS2 are relatively common in the literature, but no studies have addressed whether different portions of the *COI* gene harbor similar phylogenetic signals. Molecular discrepancies among different fragments of the *COI* gene and between the *COI* and ITS2 could be caused by unequal mutation rates across the former, which may disrupt the molecular signal(s) or may be due to significant geographic variation in the latter, respectively (Dusfour et al., 2007; Bower et al., 2008; Bora et al., 2009). Different mutation rates, effective population sizes and selective forces acting within and between these markers could also explain these discrepancies (Loaiza et al., 2012).

The Arribalzagia Series (Root, 1922) of the subgenus *Anopheles* comprises 24 recognized species (Table 1). Of these, *Anopheles punctimacula* s.l. Dyar and Knab, *An. malefactor* Dyar & Knab and *An. neomaculipalpus* Curry were described from Panama, with three other species reported from the country: *An. apicimacula* Dyar and Knab, *An. vestitipennis* Dyar and Knab and *An. mediopunctatus* Lutz. However, the latter species was confirmed afterwards to be present only in Sao Paulo State, Brazil (Sallum et al., 1999). In addition, six other species within this Series may be found in Panama due to their reported geographical distributions (Table 1). *Anopheles punctimacula* s.l. ranges from Mexico through Argentina and into the Caribbean Islands (Forattini, 1962; Knight and Stone, 1977). This species was found infected with *Plasmodium* parasites in Panama and corroborated afterwards as a malaria vector in laboratory experiments (Simmons, 1936a,b, 1937). However, the incrimination of *An. punctimacula* s.l. as a malaria vector occurred prior to the recognition of *An. malefactor* and *An. calderoni* (Wilkerson, 1990, 1991), therefore its involvement in transmission is questionable. *Anopheles punctimacula* s.l. and *An. malefactor* co-occur in the same larval breeding sites in Panama and have also been collected biting humans, but *An. calderoni* has not been reported from the country (Loaiza et al., 2008, 2009). To date, considerable variability in egg, larval and adult morphology has been reported from specimens identified as *An. punctimacula* s.l., and several authors

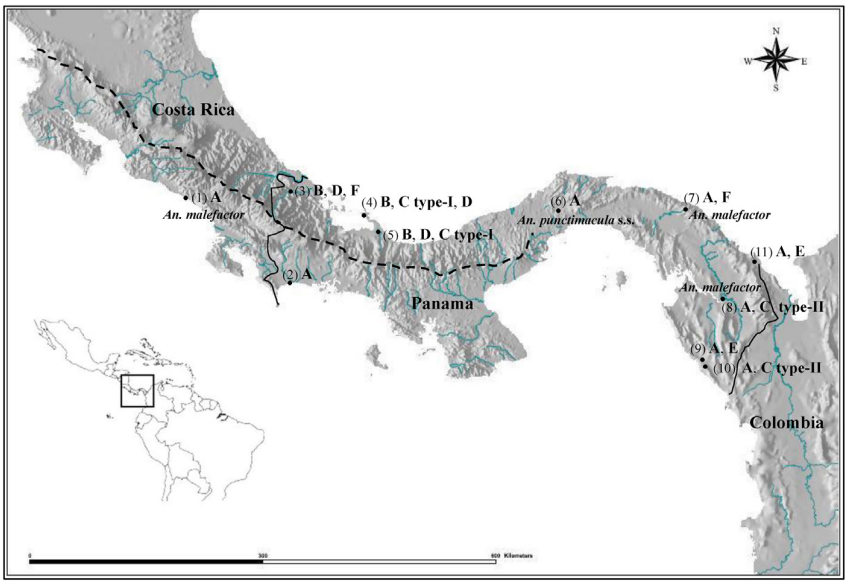

Fig. 1. Geographic distribution of the *An. punctimacula* cluster (Lineage A = *An. punctimacula* s.s. and lineage B), C type I, C type II, D, E, F = *An. neomaculipalpus* and *An. malefactor*, across southern Central America. The numbers in parenthesis correspond to localities in Table 2, positioned on the map according to the longitude and latitude of the site. The dashed line divides the Atlantic coast of Costa Rica and western Panama (localities 3–5) from the Pacific coast of Costa Rica, western and central-eastern Panama (localities 1, 2, 6–11). Inset map depicts the geographic position of the study area.

have revealed bio-ecological evidence supporting the existence of a species complex (Wilkerson, 1990, 1991; Achee et al., 2006; Ulloa et al., 2006; Loaiza et al., 2008; Cienfuegos et al., 2011). Hence, it is possible that *An. punctimacula* s.l. (in the past confused with *An. malefactor* and *An. calderoni*) consists of more than three cryptic species. Moreover, unlike other Neotropical malaria vectors, some of which are straightforward to identify with adult identification keys, *An. punctimacula* s.l. can be also confused with other species within the Arribalzagia Series (Simmons, 1937; Loaiza et al., 2008; Cienfuegos et al., 2011). Clearly, all these facts could have significant implications for vector control and malaria transmission across the species range, especially in Panama, where *An. punctimacula* s.l. is believed to be an important vector of *Plasmodium vivax* and *P. falciparum* (Simmons, 1936a,b, 1937; Wilkerson, 1990; Loaiza et al., 2008, 2009).

Our goal in this study was to determine the taxonomic status of adult female *An. punctimacula* s.l. collected from various localities in southern Central America. We sought to identify new molecular lineages within *An. punctimacula* s.l. by testing for topological congruencies within and between two independent DNA markers, the *COI* gene and the ITS2. First, we constructed phylogenetic trees using a more variable region in the three prime end of the *COI* gene, and then, employed molecular information from more conventional taxonomic regions such as the 5′ *COI* or "Barcode region" and the ITS2 marker. Finally, we investigated the presence of *An. calderoni* in Panama, which may have been misidentified as *An. punctimacula* s.l.

2. Materials and methods

Female *Anopheles* mosquitoes were collected and processed as previously described in Loaiza et al. (2010a) from 10 localities across Panama and one in Costa Rica (Fig. 1 and Table 2). They were identified morphologically following the key of Wilkerson and Strickman (1990). Genomic DNA was extracted from mosquito abdomens using the DNeasy® Blood & Tissue extraction kit (QIAgen®, Hilden, Germany), following manufacturers' recommendations and stored at −80 °C until needed. Two hundred and eighty five mosquitoes, roughly 30 per site, except from Guayabo and Gamboa (Table 2), were selected for PCR amplification and sequencing of the three prime end of the *COI* gene (hereafter 3′ *COI*). The 3′ *COI* region was amplified with the UEA3F and UEA10R primers (Lunt et al., 1996) following the protocols and conditions reported in Mirabello and Conn (2006). After initial phylogenetic analysis using the 3′ *COI* sequences, a subset of individuals representing the most likely molecular lineages were also sequenced for the 5′ *COI* and the ITS2 regions (Table 2). These samples were selected from the 3′ *COI* topology (i.e., most common haplotypes in well supported phylogenetic lineages) initially defined by various phylogenetic analyses (see details below). The amplification of 658 bp of the 5′ *COI* region was carried out using the primer pair LCO 1490F and HCO 2198R (Folmer et al., 1994) and the following thermocycler parameters: 95 °C for 5 min, then 34 cycles of 95 °C for 30 s, 48 °C for 30 min and 72 °C for 45 s, followed by 72 °C for 5 min and a 10 °C hold. The amplification of the ITS2 region was achieved with the primer pair 5.8SF and 28SR (Collins and Paskewitz, 1996) and the protocols and conditions reported in Linton et al. (2001). All PCR products were purified with Exo-SAP-IT (Affymetrix, Inc., Santa Clara, CA) prior to sequencing. The Applied Genomics Technology Core at Wadsworth Center carried out the sequencing of the 3′ *COI* using either an ABI 3730 DNA Analyzer or 3130 Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA). The 5′ *COI* and the ITS2 sequences were generated in the Sequencing Facility of the Natural History Museum, London, using a Big Dye Terminator kit (PE Applied BioSystems, Warrington, England). Forward and reverse sequences were edited using Sequencher™ version 4.8 (Genes Codes Corporation, Ann Arbor, MI). Complete consensus sequences for both the 3′ *COI* and the 5′ *COI* were aligned using the ClustalW application in BioEdit v 7.0 (Swofford, 2004). The alignment of the ITS2 sequences was done manually in MEGA v 4.0 (Kumar et al., 2004). Maximum parsimony (MP), Bayesian and Neighbor-joining (NJ) analyses were conducted initially using only unique sequences (i.e., haplotypes) of the 3′ *COI* region. A MP tree was generated after one hundred replicates of a heuristic search with an initial random stepwise addition of sequences and TBR branch swapping. Bootstrap values were calculated with 1000 replicates using PAUP v

Table 2
Locality details, including numbers, codes and geographic coordinates (see also Figs. 1 and 2 and Additional files 1–3) plus sample size for *Anopheles punctimacula* s.l. and other taxa from the Arribalzagia Series of the subgenus *Anopheles* collected in Panama and Costa Rica.

Number	Locality	Code	Coordinates	Species	3′COI	5′COI	ITS2
1	Parrita (Costa Rica)	PAR	09°51′ N, −84°33′ W	*An. punctimacula* s.l.	34	–	–
				An. malefactor	3	3	3
2	Guayabo	GYO	08°24′ N, −82°52′ W	*An. punctimacula* s.l.	4	–	–
				An. punctimacula s.l.	33	12	12
3	White Bridge	WB	09°27′ N, −82°37′ W	*An. vestitipennis*	2	2	2
				An. neomaculipalpus	1	1	1
				An. punctimacula s.l.	30	10	10
4	Hilo Creek	HC	09°09′ N, −81°53′ W	*An. apicimacula*	1	1	1
5	Diablo River	DR	09°10′ N, −81°54′ W	*An. punctimacula* s.l.	30	10	10
6	Gamboa	GAM	09°14′ N, −79°32′ W	*An. punctimacula* s.l.	8	–	–
				An. punctimacula s.l.	26	10	10
7	Playon Chico	PC	09°18′ N, −78°18′ W	*An. malefactor*	1	1	1
				An. punctimacula s.l.	30	13	13
8	Yaviza	YAV	08°07′ N, −77°39′ W	*An. malefactor*	2	1	1
9	Jaque	JAQ	07°30′ N, −78°08′ W	*An. punctimacula* s.l.	30	10	10
				An. punctimacula s.l.	30	10	10
10	Biroquera	BI/BR	07°59′ N, −78°07′ W	*An. neomaculipalpus*	1	1	1
11	Puerto Obaldia	PO	08°63′ N, −77°43′ W	*An. punctimacula* s.l.	30	10	10
				Total	296	95	95

4.0b10 (Swofford, 2000). Bayesian inference (BI) was performed in MrBayes v 3.1.1. (Ronquist and Huelsenbeck, 2003), partitioned by codon position using the model of nucleotide substitution calculated with jModelTest (Posada, 2008). The settings were two simultaneous runs of the Markov Chain Monte Carlo (MCMC) for 20 million generations, sampling every 2000 and discarding the first 25% as burn-in. The NJ tree was unrooted with all characters equally weighted, treated as unordered and calculated in Geneious v 5.4 (http://www.geneious.com/) (Drummond et al., 2010, 2011). We also constructed MP, BI and NJ trees for a subset of 5′ *COI* and ITS2 sequences, as well as for the combined *COI* regions, 5′ *COI* plus 3′ *COI* (hereafter the combined *COI*). *Anopheles malefactor* was used as outgroup in all the phylogenetic analyses due to close systematic relationship and overlapping distribution with *An. punctimacula* s.l. in Panama (Fig. 1 and Table 2).

To determine whether our specimens of *An. punctimacula* s.l. were mistakenly identified as *An. calderoni* or confused with other species from the Arribalzagia Series, we confirmed species identity of samples belonging to well supported *COI* and ITS2 phylogenetic lineages using three molecular strategies: (1) Sequence comparisons using standard nucleotide BLAST searches (http://blast.ncbi.nlm.nih.gov/) to the *COI* and ITS2 sequences in GenBank and also to the 5′ *COI* sequences of members of the Arribalzagia Series from the public records of the Mosquito Barcoding Initiative (MBI) section of Barcode of Life Data Systems v. 2.5 (BOLD; www.boldssystems.org); (2) PCR-ITS2-RFLP assays to compare ITS2-RFLP profiles of representatives from our phylogenetic lineages with those of several morphologically assigned species from Colombia including *An. punctimacula* s.l., *An. apicimacula* and *An. neomaculipalpus* as well as other species from the Peruvian Amazon such as *An. mattogrossensis*, *An. peryassui* and *An. forattinii*, respectively (Zapata et al., 2007; Matson et al., 2008; Cienfuegos et al., 2011); (3) Phylogenetic analysis including the 5′ *COI* and the ITS2 regions of other members of the Arribalzagia Series that coexist with *An. punctimacula* s.l. in Panama, but do not have sequences deposited in GenBank or in the MBI data sets (i.e., *An. apicimacula*, *An. neomaculipalpus* and *An. vestitipennis*) (Table 2).

3. Results

In total, 73 and 20 haplotypes were obtained from 285 (3′ *COI*) and 85 (5′ *COI*) individuals of *An. punctimacula* s.l. sequenced, respectively. Both the 3′ *COI* and 5′ *COI* sequences were unambiguously aligned and no insertions or deletions were found. The absence of pseudogenes was established by the lack of stop codons, low pairwise divergence and clear electropherograms. Individual length for the 3′ *COI* and 5′ *COI* sequences ranged from 723 bp to 995 bp, and from 550 bp to 701 bp, with a final alignment length of 1084 bp and 610 bp, respectively. In addition, seventeen ITS2 variants were detected from the same 85 individuals sequenced for the 5′ *COI* region (Fig. 2; Table 2); individual lengths among ITS2 sequences ranged from 394 to 494 bp (Fig. 4). Unique sequences generated during this research were submitted to GenBank (accession numbers: JX212783–JX212823) using new features of BankIT (http://www.ncbi.nlm.nih.gov/WebSub/).

The MP, BI and NJ trees using only the 21 most frequent 3′ *COI* haplotypes depicted the same topology and therefore we only present the NJ tree. Six distinct and well-supported phylogenetic lineages (A–F) were clearly differentiated, none of which were *An. malefactor* (Fig. 2). These lineages have no shared haplotypes and most of them appear to occur in different geographic areas (Figs. 1 and 2). In contrast, the 5′ *COI* region did not support the 3′ *COI* topology and grouped the sequences in five different lineages. These lineages were also well supported, but two of them included mixed sequences that belonged to lineages A, B, C and D from the 3′ *COI* tree, thus depicting paraphyly (Additional file 1). These conflicting results could be due to unequal mutation rates across the *COI* gene, and thus, insufficient phylogenetic signal in the Barcode region, perhaps as a result of lower rate of sequence evolution (Hebert et al., 2003; Crywinska et al., 2006; Kumar et al., 2007; Ruiz-Lopez et al., 2012). Sequence divergence among the 5′ *COI* lineages was not calculated because of the uncertain molecular signal of this region. In addition, MP, BI and NJ trees constructed with unique ITS2 variants and the combined *COI* data set supported the same six lineages detected in the 3′ *COI* analysis (Fig. 2; Additional

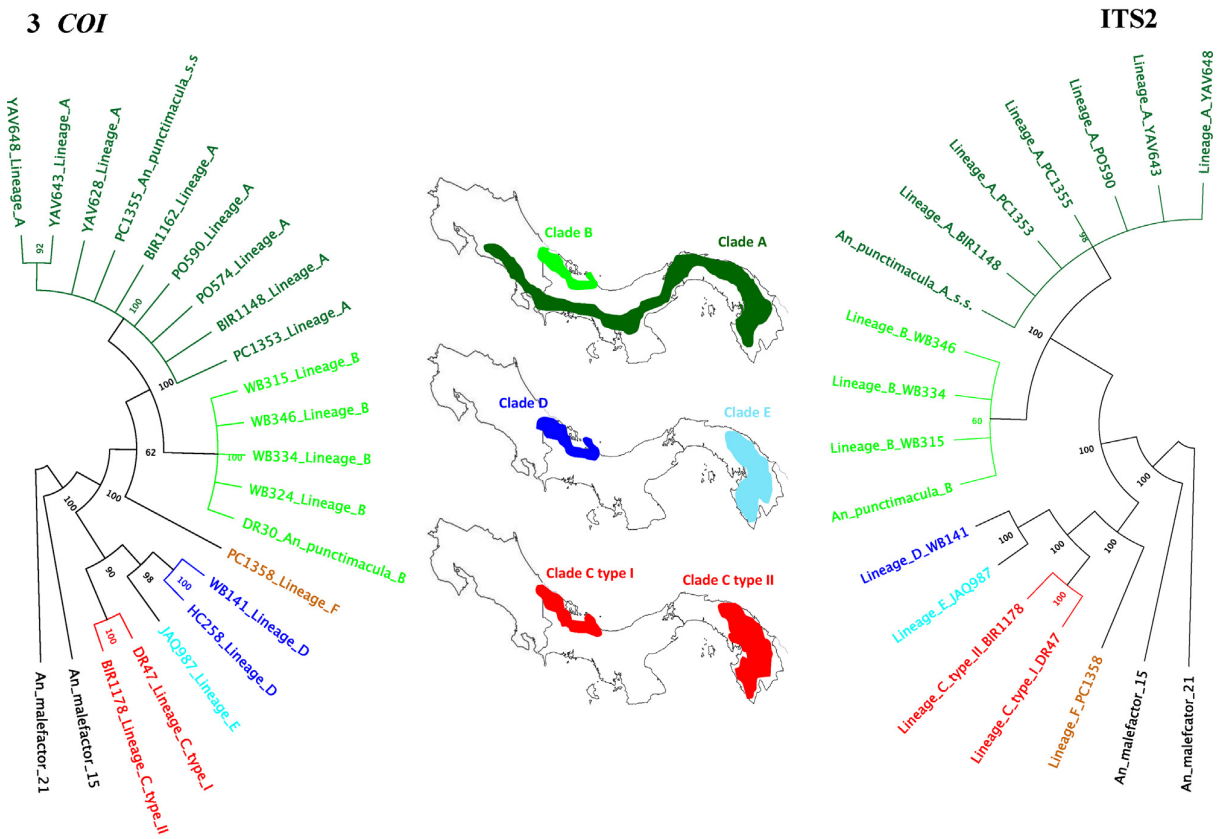

Fig. 2. Right side: NJ tree using unique variants of the rDNA-ITS2 region showing six distinct lineages belonging to samples identified as *An. punctimacula* s.l. (A–F) and *An. malefactor*. Center: Geographic distribution of lineages A, B, C type I, C type II, D and E. Left side: NJ tree using the 3′*COI* haplotypes showing six distinct lineages belonging to *An. punctimacula* s.l. (A–F) and *An. malefactor*. Lineage characterization as follows: Lineage A: corresponds to *An. punctimacula* s.s. Lineage B: has different ITS2 sequence and ITS2-RFLP profile than *An. malefactor*. Lineage C type I: the same ITS2 sequence and ITS2-RFLP profile as *An. apicimacula* from western Panama. Lineage C type II: the same ITS2-RFLP profile as *An. apicimacula* from Colombia, but different ITS2 sequence and ITS2-RFLP profile than C type I. Lineage D: the same as the barcode sequence of *An. apicimacula* from the MBI dataset. Lineage E: the same ITS2-RFLP profile as lineage D, but different ITS2 sequence and ITS2-RFLP profile than C type I, C type II, and *An. apicimacula* from Panama and Colombia. Lineage F: the same ITS2 sequence and ITS2-RFLP profile as *An. neomaculipalpus* from Panama and Colombia. Sample codes correspond to the names of sampling localities in Table 2 (e.g., YAV = Yaviza). Samples YAV628, PC1355, WB324 and DR30 were included in the ITS2 analysis, but merged with other sequences with identical rDNA ITS2 haplotyes.

files 2, 3). All six ITS2 lineages (A–F) had either length differences or fixed substitutions among their sequences as well as distinct ITS2-RFLP banding profiles, except lineages A and B. Moreover, lineage C depicted two different ITS2-RFLP types (I and II) (Figs. 2–4; Additional file 2).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2013.06.012.

Two hundred and twelve (74%) of the samples initially identified as *An. punctimacula* s.l. based on morphological characters and corresponding to lineages A and B in the 3′ *COI*, combined *COI* and ITS2 trees, matched with 99% homology the *COI* sequence of *An. punctimacula* s.l. from Nicaragua (accession number: AF417719) (Sallum et al., 2002). These sequences also matched with 99% homology the ITS2 of *An. punctimacula* s.l. from Nechi, Colombia (BOLD; www.boldssystems.org) and depicted a similar ITS2-RFLP banding profile (Fig. 3; Additional file 2) as other samples identified as *An. punctimacula* s.l. from Colombia (Zapata et al., 2007; Cienfuegos et al., 2011). We refer to these sequences hereafter as the *An. punctimacula* cluster. In contrast, 73 samples (25%) corresponding to lineages C, D, E and F in the 3′ *COI*, combined *COI* and ITS2 trees, had low values of sequence homology (i.e., <94%) with the Nicaraguan *COI* sequence (AF417719) and dissimilar ITS2-RFLP banding profiles and fragment sizes compared with Colombian *An. punctimacula* s.l. (Figs. 2–4; Additional file 2). As suggested in the

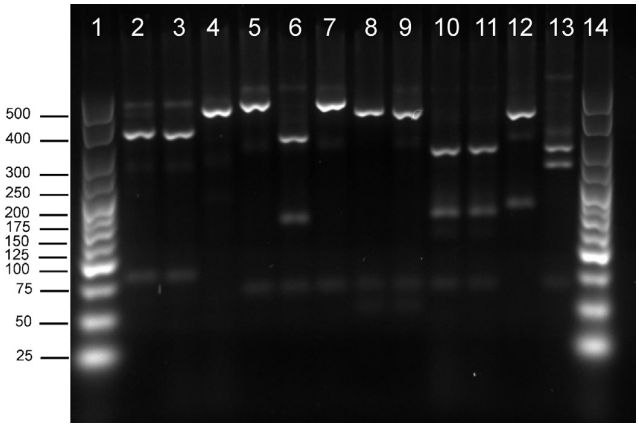

Fig. 3. PCR-ITS2-RFLP *AluI* digest banding patterns following Zapata et al. (2007), Matson et al. (2008) and Cienfuegos et al. (2011) – 2.5% agarose gel. Lanes 1 and 14, 25–500 bp molecular ladder; Lane 2 = Lineage A = *An. punctimacula* s.s.; Lane 3 = Lineage B; Lane 4 = *An. malefactor*; Lane 5 = Lineage C type I; Lane 6 = Lineage C type II; Lane 7 = *An. apicimacula* collected in western Panama; Lane 8 = Lineage D; Lane 9 = Lineage E; Lane 10 = Lineage F; Lane 11 = *An. neomaculipalpus* collected in Panama; Lanes 12 and 13 = *An. albimanus* and *An. vestitipennis* collected from Panama, respectively.

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[                          1 1111111112 2222222223 3333333334 4444444445 5555555556 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        tgtgaactgc aggacacatg aacatcgata agttgaacgc atattgcgcg tcgggcgaca
Lineage(F)        .......... .......... .......... .......... .......... ..........
Lineage(D)        .......... .......... .......... .......... .......... ..........
Lineage(E)        .......... .......... .......... .......... .......... ..........
An_punctimacula(A) .......... .......... .......... .......... .........a ...a.a.tt.
An_punctimacula(B) .......... .......... .......... .......... .........a ...a.a.tt.
An_malefactor(15)  .......... .......... .......... .......... .........a ...a.a.tt.

[                                                    1 1111111111 1111111111 ]
[                 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        cagctcgacg cacatatcat tgagagtcca tatttgttag tcggagacac ttacga-gcg
Lineage(F)        .......... .......... .......... .......... ....g..... ......-...
Lineage(D)        .......... .......... .......... ......ga.. ..c.t....- ......ta..
Lineage(E)        .......... .......... .......... ......ga.. ..c.t....- ......ta..
An_punctimacula(A) at......t. ........t. .......... ..g...cat- ------...- ...aaca...
An_punctimacula(B) ag......t. ........t. .......... ..g...cat- ------...- ...aaca...
An_malefactor(15)  at......t. ........t. .......... ..g...cat- ------...- ...aacta..

[                 1111111111 1111111111 1111111111 1111111111 1111111111 1111111111 ]
[                 2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        tccgtgccac ccgttgggcc ccgtgcccgg gg-tggaccc acgcggtcac tcgtttcctg
Lineage(F)        .......... .......... .......... ..-....... .......... ..........
Lineage(D)        ........g. t.cc--..t. ....a..g.. ..a..... ..........a ..........
Lineage(E)        ........g. t.cc--..t. ....a..g.. ..a..... ..........a ..........
An_punctimacula(A) .g..------ ---------- -.t...---- --------g. ...t.-c..g .t.------.
An_punctimacula(B) .g..------ ---------- -.t...---- --------g. ...t.-c.gg .t.------.
An_malefactor(15)  .g..------ ---------- -.t...tt.a ---..ag.a. ...t.-c.gg .t.------.

[                 1111111111 1111111112 2222222222 2222222222 2222222222 2222222222 ]
[                 8888888889 9999999990 0000000001 1111111112 2222222223 3333333334 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        gggtagcgcc tcgcagagag cggtgccctt aagacagtgg agcgcgttcc ctcgtccg-t
Lineage(F)        .......... .......... .......... .......... .......... ........-.
Lineage(D)        ........g. ..--.agagc .......... .......... .......... ........-.
Lineage(E)        ........g. ..--.a.agc .......... .......... .......... ........-.
An_punctimacula(A) ..tgg...t. g.---a..gc g.c.a..... ..a.tct... ..a.t..c.a .cgt....c.
An_punctimacula(B) ..tgg...t. g.---a..gc g.c.a..... ..a.tct... ..a.t....a .cgt....c.
An_malefactor(15)  ..tgga..t. g.---a..gc g.t.at...g ..a..ct.c. ..a.t....a .cgt...ta.

[                 2222222222 2222222222 2222222222 2222222222 2222222222 2222222223 ]
[                 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        tagcgaccgt cacaagcgcc cggcgttgga ccaacacctc acctcaccac catagagtgt
Lineage(F)        .......... .......... .......... .......... .......... ...g......
Lineage(D)        -......... .......... .......... .......... .....ct... a.--...c..
Lineage(E)        -......... .......... .......... .......... .....ct... a.--...c..
An_punctimacula(A) gg.ttct... t..c..t.gg .a...g...- ..gg..... .....tttc. a..-...c..
An_punctimacula(B) gg.ttct... t..c..t.gg .a...g...- ..gg..... .....tttc. a..-...c..
An_malefactor(15)  gg.tact... t..c..t.gg .a...g...- ...g..... .....tttc. a..-...c..

[                 3333333333 3333333333 3333333333 3333333333 3333333333 3333333333 ]
[                 0000000001 1111111112 2222222223 3333333334 4444444445 5555555556 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        cgatgcgtgt tcggacccca gtcgtcggga -gcgacggta ccaggtcggg atcgttcgtg
Lineage(F)        .......... .......... .......... -......... .......... ..........
Lineage(D)        t......... g......... ac...tt... t......... .a...g.... .g..ag...t
Lineage(E)        t......... g......... ac...tt... t......... .a...g.... .g..ag...c
An_punctimacula(A) t..gaa.... ....-t.g.. tg..ct-.c. ca......gt --.ct.t... .cg.cgt.gc
An_punctimacula(B) t..gaa.... ....-t.g.. tg..ct-.c. ca......gt --.ct.t... .cg.cgt.gc
An_malefactor(15)  a..gaa...c ....-t.g.. tg..ct-.ct ca......g- --.ccct..a .cg.agatcc

[                 3333333333 3333333333 3333333333 3333333334 4444444444 4444444444 ]
[                 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112 ]
[                 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)        tcacccactg a---gtcggg tgtacccca cagg-gctac ggccggg--- -ggtacagac
Lineage(F)        .......... .---...... .......... ....-..... .......--- -.........
Lineage(D)        agct.t..cc gcat.c...a gca.tt.tgg ....a.g... ac..a.accc a...c.g.gg
Lineage(E)        .gct.t..cc gcat.c...a gca.tt.tgg ....a.g... ac..a.accc a...c.g.gg
An_punctimacula(A) c.tt.t---- ---------- ---------- ---------- --t.aaa--- -.c.tt..tt
An_punctimacula(B) c.tt.t---- ---------- ---------- ---------- --t.aaa--- -.c.tt..tt
An_malefactor(15)  c.tt.t---- ---------- ---------- ---------- --t..aa--- -ct.ctt.ct

[                 4444444444 4444444444 4444444444 4444444444 4444444444 4444444444 ]
```

Fig. 4. Variable base alignment of unique ITS2 variants for six phylogenetic lineages (A–F) obtained from 85 *Anopheles punctimacula s.l.*, and outgroup taxa: *Anopheles malefactor*.

```
[                   2222222223 3333333334 4444444445 5555555556 6666666667 7777777778 ]
[                   1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 ]
Lineage(C)          atgagagaga gcactccttc agtggagcta tgtggacctc aattgatgtg tgactacccc
Lineage(F)          .......... .......... .......... .......... .......... ..........
Lineage(D)          g..t-.cg.. .......... ........a. -......... .......... ..........
Lineage(E)          g..t-.c... .......... ........a. -......... .......... ..........
An_punctimacula(A)  tc..-----. ..g...t... g......-.. ca........ ..a....... ..........
An_punctimacula(B)  tc..-----. ..g....... g......-.. ca........ ..a....... ..........
An_malefactor(15)   tct.-----. ..g....... g......-.. ca........ ..a....... ..........

[                   4444444444 4444]
[                   8888888889 9999]
[                   1234567890 1234]
Lineage(C)          ctaaatttaa gcat
Lineage(F)          .......... ....
Lineage(D)          .......... ....
Lineage(E)          .......... ....
An_punctimacula(A)  .......... ....
An_punctimacula(B)  .......... ....
An_malefactor(15)   .......... ....
```

Fig. 4. (*Continued*).

introduction, it is possible that a portion of our samples were morphologically misidentified as *An. punctimacula* s.l., but may instead represent other species within the Arribalzagia Series.

Comparison between fractions of the unknown sequences corresponding to lineage C (12/73 = 16%), D (57/73 = 78%), and E (2/73 <3%) (Fig. 2; Additional files 1, 2) with others available in GenBank, the MBI data sets, and some generated in this study revealed contradictory results. For example, all samples belonging to lineage D matched with >98% sequence homology the 5′ *COI* sequence of *An. apicimacula* s.l. from the MBI data sets (BOLD; www.boldssystems.org). However, the ITS2 fragments of the same individuals in lineage D differed significantly in length from another sample of *An. apicimacula* s.l. identified from Hilo Creek in western Panama (Table 2). The latter clustered instead with ITS2 sequences in lineage C (Additional file 2). Likewise, the ITS2-RFLP profiles of all sequences in lineage D differed from the Panamanian sample of *An. apicimacula* s.l., which in turn depicted a similar ITS2-RFLP profile to lineage C type I (Fig. 3). In addition, lineage E harbored very different 3′ *COI* and ITS2 sequences compared with lineages C type I, C type II and D; yet it depicted a similar ITS2-RFLP banding profile to that of lineage D (Figs. 2 and 3; Additional files 2, 3). Moreover, lineage C type II (Fig. 3) depicted a similar ITS2-RFLP banding profile to the recently reported Colombian sample of *An. apicimacula* s.l. (Cienfuegos et al., 2011), but sequences in lineages C type I, D or E did not. Although these outcomes may point to a mistaken identification of our Panamanian sample of *An. apicimacula* s.l., they could also be due to low resolution of the 5′ *COI* region to discriminate among different members of the Arribalzagia Series in Panama (Additional file 1). In this study, the uncertain taxonomic value of the 5′ *COI* region makes it hard to draw firm conclusions about species identity. Consequently, given the phylogenetic congruency between the 3′ *COI*, the combined *COI* and the ITS2 regions, we rely more on these regions to distinguish among species of the Arribalzagia Series.

Samples belonging to lineages C type I, C type II, D, E and F did not match any of the following sequences: the *COI* of *An. intermedius* from Brazil [AF417718], the ITS2 of *An. fluminensis* from Bolivia [DQ328638], the ITS2 of *An. mattogrossensis* from Brazil [AF461754], the ITS2 of *An. mediopunctatus* from Brazil [AF462379], the ITS2 of *An. peryassui* from Brazil [AF461755], and the ITS2 sequences from *An. vestitipennis* collected from western Panama (Table 2; Additional file 3). Moreover, none of them depicted similar ITS2-RFLP banding profiles as the ones reported for *An. mattogrossensis*, *An. peryassui* and *An. forattinii* in Matson et al. (2008). It is noteworthy that two unidentified ITS2 sequences belonging to lineage F (2/73 <3%) had the same length and matched with high sequence homology (99%) the ITS2 sequences of two samples identified as *An. neomaculipalpus* from western and eastern Panama, respectively (Fig. 1; Table 2; Additional file 2). Also, these sequences shared a similar ITS2-RFLP banding profile (Fig. 3) with *An. neomaculipalpus* identified from Colombia (Cienfuegos et al., 2011), and therefore, we conclude that they have been mistakenly assigned to *An. punctimacula* s.l.

4. Discussion

Anopheles punctimacula s.s., *An. malefactor* and *An. calderoni* were considered a single species formerly, and consequently, their roles as vectors of human Plasmodia are still uncertain (Simmons, 1936a,b, 1937; Wilkerson, 1990, 1991). The first two species occur in Panama, and now we report *An. malefactor* for the first time in Costa Rica. However, based on the molecular evidence presented here, it seems unlikely that *An. calderoni* occurs in Panama (Additional files 1, 2). Although our findings do not support the presence of *An. calderoni* in Panama they suggest that a significant number of our samples were misidentified as *An. punctimacula* s.l., and might instead represent other species within the Arribalzagia Series. This appears to be the case for two samples confirmed as *An. neomaculipalpus*, yet most of the unknown DNA sequences belong to lineages C, D and E, which molecular taxonomic statuses remain controversial based on the present data set. A possible explanation for this outcome is that *An. apicimacula* s.l. is a species complex encompassing sequences from C type I, C type II, D and E. This view agrees at least partially with the conflicting results between the 5′ *COI* and ITS2 sequences plus the puzzling outcomes of the PCR-ITS2-RFLP assay using samples of *An. apicimacula* s.l. from different countries and data sources. Reared specimens of *An. apicimacula* s.l. from the type locality are needed to clarify the identity of all these lineages (Table 1).

We did not have problems distinguishing *An. punctimacula* s.l. from *An. malefactor* because none of the specimens were mistakenly assigned to this taxon. The fact that *An. punctimacula* s.s. and *An. malefactor* are more similar morphologically to each other than to any other species of Arribalzagia supports the likelihood that lineages C, D, and E are different species of Arribalzagia rather than novel lineages within *An. punctimacula* s.l. It also underscores the occurrence of underreported species in Panama, which may be undescribed. Due to the uncertain taxonomic status of some lineages in the present study, we focused primarily on discussing the findings concerning the *An. punctimacula* cluster. This grouping comprises two different molecular lineages (A and B), both of which were clearly distinct from *An. malefactor*, but were closer genetically to this taxon than to other species (Additional file 2). We name the members of lineage A, *An. punctimacula* s.s. hereafter,

because haplotypes from this lineage were recovered from specimens collected in Central Panama, near the type locality (Fig. 1). These two lineages received moderate to high levels of support for the 3′ *COI*, the combined *COI* and the ITS2 trees (Fig. 2; Additional files 2, 3). Sequence analysis of the ITS2 region from individuals of *An. punctimacula* s.s. and lineage B showed no differences in length (both 395 bp long), but uncovered four fixed substitutional changes in positions 62, 131, 181, and 330, respectively (Fig. 4). This finding agrees with their similar ITS2-RFLP banding profiles, and could suggest a very recent history of diversification or high levels of contemporary gene flow between them (Fig. 3).

The phylogenetic structure in the *An. punctimacula* cluster appears to be driven by geographic separation as haplotypes from *An. punctimacula* s.s. were recovered from across the entire sampling area except localities on the Atlantic coast of western Panama. In contrast, individuals belonging to lineage B were only recovered from this region (Figs. 1 and 2). This inter-lineage divergence is more consistent with recent allopatric fragmentation rather than with old species diversification; future studies will be needed to confirm sympatry without heterozygotes between *An. punctimacula* s.s. and lineage B (Walton et al., 2000, 2007a,b). It is of interest that all of the other lineages appear to have the same discrete geographic distributions as the *An. punctimacula* cluster. Lineage D was found exclusively in the Atlantic coast of western Panama, where it co-occurs with lineages B and C type I. Likewise, lineages C type II and E were only recovered from eastern Panama where they co-occur with *An. punctimacula* s.s (Figs. 1 and 2). This pattern of geographic partition is also supported by regional differences in the ITS2-RFLP banding profiles between Colombian and Panamanian samples of *An. apicimacula* s.l. For instance, lineage C type I, collected only from Hilo Creek and Diablo River (Fig. 1; Table 2), showed similar ITS2-RFLP banding profiles to *An. apicimacula* s.l. collected on the Atlantic coast of western Panama, whereas lineage C type II occurred only in eastern Panama, closer to *An. apicimacula* s.l. from Colombia, with which it shares a similar ITS2-RFLP banding profile. This may partially explain errors in the identification and might suggest that significant intraspecific variability in *An. apicimacula* s.l. could be similar to the local morphological variation of *An. punctimacula* s.l. or vice versa. The issue of misidentification seems more problematic in the Atlantic coast of western Panama, where roughly 90% of the unknown specimens (corresponding to lineage C type I, 10/73 = 13% and lineage D, 57/73 = 78%) were mistakenly identified as *An. punctimacula* s.l. As pointed out previously, *An. apicimacula* s.l. might be a species complex of at least two distinct taxa, one encompassing lineages C type I and C type II and another comprising lineages D and E, both further subdivided geographically in the same fashion as *An. punctimacula* s.s. and lineage B (Figs. 1 and 2).

Previous studies uncovered considerable genetic structuring in *Anopheles albimanus* Wiedemann across southern Central America; four divergent and non-randomly distributed *COI* haplogroups were found between populations from Costa Rica and western Panama and those from central-eastern Panama (Loaiza et al., 2010a,b). The genetic structure of *An. albimanus* was hypothesized to be the result of Pleistocene bottleneck, geographic fragmentation and subsequent secondary contact via demographic expansion. Our present findings suggest that the pattern of genetic structure in *An. albimanus* and the *An. punctimacula* cluster, as well as the one from lineages C type I, C type II, D and E, was driven by the same historical demographic process. The fact that a similar geographic pattern was found across molecular lineages and species using different DNA regions reinforces this view (Loaiza et al., 2010b, 2012). However, at this point it is uncertain whether this is due to past unstable demography (i.e., temporal fluctuation in the effective population size), currently restricted gene flow across the study area (i.e., the Central American Cordillera could act as a physical barrier to genetic exchange) or to a combination of both (Figs. 1 and 2). A population study that includes additional collecting sites from central Panama, a larger sample size and more taxa, is in progress to distinguish between these hypotheses.

Several studies have confirmed that traditional *Anopheles* identification based only on morphological characters frequently fails to represent the true intra-inter specific extent of genetic variability (Linton et al., 2001; Crywinska et al., 2006; Foley et al., 2006; Kumar et al., 2007; Paredes-Esquivel et al., 2009; Bourke et al., 2010; González et al., 2010; Cienfuegos et al., 2011; Ruiz-Lopez et al., 2012). The problem of incorrect *Anopheles* species designation may be even more prominent for those taxa with slightly less medical relevance, such as those in the Arribalzagia Series. Our results strongly support this view and indicate that more research is needed to comprehensively assess the systematic relationship between *An. punctimacula* s.l. and *An. apicimacula* s.l. across Central America. Furthermore, the lack of phylogenetic resolution within *An. punctimacula* s.l. using the 5′ *COI* region in the present study suggests that caution should be exercised when using this fragment alone to discriminate among poorly studied species. Our results support the use of the combined *COI* regions plus the ITS2 as the most robust evidence of cladogenesis in *An. punctimacula* s.l. and other members of the Arribalzagia Series of the Subgenus *Anopheles*. Studies on *Plasmodium* infection rates, longevity and host-feeding preferences should be conducted across Panama to investigate the potential transmission role of *An. punctimacula* s.s. and lineage B.

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