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Evaluation of a PCR-RFLP- ITS2 assay for discrimination of *Anopheles* species in northern and western Colombia

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Abstract

Anopheles mosquitoes are routinely identified using morphological characters of the female that often lead to misidentification due to interspecies similarity and intraspecies variability. The aim of this work was to evaluate the applicability of a previously developed PCR-RFLP-ITS2 assay for accurate discrimination of anophelines in twelve localities spanning three Colombian malaria epidemiological regions: Atlantic Coast, Pacific Coast, and Uraba-Bajo Cauca-Alto Sinu Region. The evaluation of the stability of the PCR-RFLP patterns is required since variability of the ITS2 has been documented and may produce discrepancies in the patterns previously reported. The assay was used to evaluate species assignment of 939 mosquitoes identified by morphology. Strong agreement between the morphological and molecular identification was found for species *An. albimanus*, *An. aquasalis*, *An. darlingi* and *An. triannulatus* s.l. ($p \geq 0.05$, kappa=1). However, disagreement was found for species *An. nuneztovari* s.l., *An. neomaculipalpus*, *An. apicimacula* and *An. punctimacula* ($p \leq 0.05$; kappa ranging from 0.33–0.80). The ITS2-PCR-RFLP assay proved valuable for discriminating anopheline species of northern and western Colombia, especially those with overlapping morphology in the Oswaldoi Group.

Keywords

Anopheles; PCR-RFLP; ITS2; species identification; malaria

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1. INTRODUCTION

Colombia is among the 31 countries with the highest malaria burden in the world, and it has the second highest number of malaria cases in Latin America, with an average of 145,000 cases per year, although, it is estimated that more than 400,000 cases occur annually in the country (WHO, 2008, 2009). The social and economical problems caused by malaria have turned the control and elimination of this parasitic disease into one of the priorities of public health programs in several endemic countries (WHO, 1993, 2005a). The Global Malaria Program has claimed that vector control is one of the most efficient strategies for reducing disease burden (WHO, 1993, 2005b, 2005c). Successful malaria control, however, is critically dependent on the accurate identification of *Anopheles* species involved in parasite transmission, since targeted vector control strategies should be designed according to species specific aspects, such as ecology and behavior. For example, in Binh Thuan province of Vietnam, *Anopheles varuna* Iyengar was mistargeted as the malaria vector after being erroneously identified as *An. minimus* Theobald, another species recognized as a primary vector in southeastern Asia (Van Bortel et al., 2001). Using a PCR-RFLP assay, *An. dirus* s.l. (Peyton & Ramalingam, 1988) was correctly identified as the primary vector species in Binh Thuan province and subsequently targeted for control (Van Bortel et al., 2001; Van Bortel et al., 2000). Similar reports of morphological misidentification of potential vectors are found for Colombian and Peruvian anopheline species. Quiñones et al. (2001) and Fernandez et al. (2004) found that specimens of *An. benarrochi* s.l. Gabaldon were erroneously identified as *An. evansae* (Brèthes) in the Department of Putumayo in southern Colombia and in the region of Ucayali in Peru. *Anopheles benarrochi* s.l. is considered a zoophilic species in Brazil (Faran, 1980), but recent studies have shown it could be implicated in parasite transmission in other areas (Fernandez et al., 2004; Flores-Mendoza et al., 2004). Specimens of *An. benarrochi* s.l. collected in malaria endemic areas in eastern Peru were found infected with *Plasmodium falciparum* and *Plasmodium vivax* VK210 and VK247, suggesting that this species played a role in transmitting and maintaining *Plasmodium* species at least locally and that misidentification could have adverse public health impacts (Flores-Mendoza et al., 2004).

Species of the Oswaldoi Group of *Anopheles* subgenus *Nyssorhynchus*, such as *An. benarrochi* s.l., *An. evansae*, *An. nuneztovari* s.l. Gabaldón, *An. rangeli* Gabaldón, Cova Garcia & Lopez, *An. oswaldoi* (Peryassú) and *An. aquasalis* Curry (Harbach, 2004), are difficult to distinguish because of the well-known intraspecific variability and the substantial morphological overlap in some characters used for their discrimination (Calle et al., 2002; Fajardo et al., 2008; Faran and Linthicum, 1981). The morphologically similar species of the Oswaldoi Group differ in their contribution to parasite transmission in several regions of South America. *Anopheles nuneztovari* s.l. is a primary malaria vector in Colombia (Brochero et al., 2006; Gutierrez et al., 2008; Gutierrez et al., 2009; Olano et al., 2001), where it was recently found naturally infected with *Plasmodium vivax* (Gutierrez et al., 2009); but it has little importance in parasite transmission in the Brazilian Amazon where it has zoophilic behavior and other vectors have a primary role in parasite transmission (da Rocha et al., 2008; Pova et al., 2006; Tadei and Dutary Thatcher, 2000). *Anopheles aquasalis* is a vector in Venezuela, Trinidad and Tobago, Brazil and Guyana (Berti et al., 1993; Chadee and Kitron, 1999; Laubach et al., 2001; Pova et al., 2003), but until now its involvement in parasite transmission in Colombia has not been demonstrated, except for an epidemiological implication in a malaria focus in the Department of Guajira in 2000 (INS, 2001). Other species such as *An. rangeli* and *An. oswaldoi* s.l. have only recently been implicated in transmission in southern Colombia (Quiñones et al., 2006). Such differences in the vectorial roles of morphologically similar species make it critical to develop alternative identification strategies to complement the traditional use of morphological keys (Collins et al., 2000).

In Colombia, several studies have contributed to the design and standardization of molecular tools for *Anopheles* species discrimination. An assay was developed for the molecular identification of *An. oswaldoi* s.l. and *An. benarrochi* s.l. specimens from Putumayo, southern Colombia (Ruiz et al., 2005). Later, a PCR-RFLP for discrimination of seven anopheline species collected in Antioquia, northwestern Colombia was designed by Zapata et al. (2007). In this study, spacers ITS1 and ITS2 of the ITS region were evaluated, however only the ITS2 region was used in the assay since it proved to be more reproducible and reliable as a marker for species differentiation. The ITS2 PCR-RFLP patterns were better defined and more clearly distinguishable among species than those for the ITS1. Also, various studies have found greater levels of sequence and length variation in the ITS1 compared with the ITS2 (Fairley et al., 2005; Bower et al., 2008; 2009). The Zapata et al. (2007) assay was subsequently optimized to include other species of the Oswaldoi Group reported in the country (Cienfuegos et al., 2008). Considering the importance of correct species discrimination in malaria endemic regions of Colombia, the aim of the present work was to evaluate a PCR-RFLP-ITS2 strategy (Cienfuegos et al., 2008; Zapata et al., 2007) in 12 localities of northern and western Colombia, spanning three malaria epidemiological regions: the Atlantic and Pacific Coasts, and the Uraba-Bajo Cauca-Alto Sinu region. The assessment of the ITS2 stability in specimens of localities from different areas is required since variability of this region has been documented and is due to the presence of insertions, deletions, transitions and transversions which could result in changes of the patterns previously reported. However, the PCR-RFLP-ITS2 band patterns for the specimens evaluated herein were in agreement with the band patterns reported for specimens from San Pedro de Uraba, Antioquia, and with the *An. aquasalis* pattern confirmed by Cienfuegos et al. (2008), suggesting that the assay can be applied to identify anopheline mosquitoes collected in the three malaria epidemiological regions. The implementation of this assay in mosquito surveillance would provide a vital tool for malaria control programs.

2. MATERIALS AND METHODS

2.1. Collection sites

Specimens were collected in 12 localities spanning three Colombian epidemiological regions: Atlantic Coast (annual parasite index, API: 0.4), Pacific Coast (API: 10.8) and Uraba- Bajo Cauca-Alto Sinu (API: 25.5) (INS, 2006). The latter two regions present the highest risk of parasite transmission in Colombia (API ≥ 10 : high risk) (PAHO, 2001). The localities sampled are listed in Table 1 and depicted in Figure 1. The closest distances among collections sites were 12 km between VAL and TAL, followed by MLI and PLT (27 km); the rest have distances > 50 km. Adult mosquitoes were collected by human landing catches under an informed consent agreement and procedures approved by the Bioethics Committee for Human Research at University Research Center (SIU) of the University of Antioquia. In addition, isofemale lines and reared larvae with associated exuviae were obtained to support the identification of collected *Anopheles* species. Details on collection procedures in this study are found in Gutierrez et al. (2008).

2.2. Morphological identification of anopheline mosquitoes

Identification of wild-caught females and larvae collected in the field and reared in the laboratory was performed using available morphological keys (Faran, 1980; Faran and Linthicum, 1981; González and Carrejo, 2007; Rubio-Palis, 2000; Wilkerson and Strickman, 1993). Mosquitoes were dissected, and the abdomen, legs and wings of each mosquito were placed in vials containing 95% ethanol. In addition, one wing and one hindleg were mounted on a glass slide with Euparal and conserved as part of the *Anopheles* species collection of the Molecular Microbiology Group, University of Antioquia.

2.3. PCR-RFLP-ITS2 analysis

Mosquito legs or wings were used as the source of DNA for PCR reactions. When amplification from this material was not accomplished, dissected abdomens were used for DNA extraction (Birungi and Munstermann, 2002). The ITS2 primers used were from Beebe and Saul (1995). PCR conditions were as previously described (Zapata et al., 2007).

Previous bioinformatic analyses (Cienfuegos et al., 2008) indicated that a PCR-RFLP-ITS2 assay performed with *AluI*, *FspI* or *DraIII* is useful for identification of species belonging to the Oswaldoi Group. As such, amplified ITS2 products (50 to 100 ng/μl) were selected for the enzyme restriction analysis and digested according to manufacturer's instructions (Promega, Madison, WI and New England Biolabs, Ipswich, MA, USA). Each restriction digest contained 1X buffer, 2 μg acetylated BSA, 1 unit *AluI* (Promega, Madison, WI, USA), 1 μg ITS2 amplicon and sterile deionized water in a 20 μl final volume. In addition to single restriction digests, double restriction digests with *AluI/FspI* were used to discriminate *An. nuneztovari* s.l. from *An. benarrochi* s.l. (Cienfuegos et al., 2008; Matson et al., 2008). These double digests were performed in a 20 μl reaction containing 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 1 unit *FspI* and 1 unit *AluI* (New England Biolabs, Ipswich, MA, USA), and incubated at 37°C for 4 hours or overnight. Digested products were visualized as previously described (Zapata et al., 2007). Positive controls corresponding to the ITS2 digest of each species being analyzed were included in every gel. These controls were prepared from sequenced clones containing complete ITS1 and ITS2 regions from individuals of isofemale lines or immature stages. *Anopheles aquasalis* controls were clones from voucher specimens collected in Suriname. In order to avoid bias during the PCR-RFLP pattern analyses, the morphological identification and the molecular confirmations were carried out by two different persons and species assignments were confirmed only at the end of the PCR-RFLP process; in addition, the patterns were interpreted by two observers, one of them not involved in the processing of the samples.

2.4. Statistical analysis

The percentage of concordance between the morphological and molecular identifications was calculated by comparison of the number of specimens given the same or different morphological and molecular identification for each species. To test the null hypothesis of no real disagreement between molecular and morphological identification, a χ^2 was calculated using a McNemar test and the strength of agreement was evaluated using a kappa index.

3. RESULTS

Nine hundred and thirty-nine anopheline specimens were analyzed and their morphological identifications were evaluated by PCR-RFLP-ITS2 (Table 2, Figure 2). A 100% concordance was found between morphological and molecular identification for species *An. albimanus*, *An. aquasalis*, *An. darlingi*, *An. nuneztovari* s.l., *An. triannulatus* s.l., *An. neomaculipalpus* and *An. apicimacula*, only 30% for *An. punctimacula*, and no concordance was found for *An. rangeli*, *An. oswaldoi* s.l., *An. evansae* and *An. strodei* s.l. (Table 3). Results of the McNemar test for species *An. albimanus*, *An. aquasalis*, *An. darlingi* and *An. triannulatus* s.l., did not show disagreement between the identification methods ($p \geq 0.05$), and the kappa statistic showed a strong agreement ($\kappa=1$), between both methods. Disagreement between molecular and morphological identification was found for specimens *An. nuneztovari* s.l., *An. neomaculipalpus*, *An. apicimacula* and *An. punctimacula* ($p \leq 0.05$). The strength of agreement was higher in *An. neomaculipalpus* ($\kappa=0.8$), but lower in *An. nuneztovari* ($\kappa=0.36$), *An. apicimacula* ($\kappa=0.33$) and *An. punctimacula*.

($\kappa=0.45$). The McNemar and kappa tests could not be calculated for *An. rangeli*, *An. oswaldoi* s.l., *An. evansae*, *An. strodei* s.l. since none of the specimens analyzed were identified as such by the PCR-RFLP method (Table 3).

The PCR-RFLP-ITS2 allowed the identification of *An. aquasalis* ($n=4$), a species that was not included in the previous study (Zapata et al., 2007). The restriction pattern for *An. aquasalis* agreed with the one predicted *in silico* using *An. aquasalis* ITS2 sequences retrieved from GenBank (Cienfuegos et al., 2008) and with that from voucher material (Figure 3). In *An. albimanus* specimens processed in this study, a slight variation of 15 bp was observed in the banding pattern in specimens of all localities (Figure 4). Sequence analyses confirmed that this banding alteration likely resulted from a single point mutation in the recognition site of the *AluI* enzyme.

Only specimens of *An. punctimacula* from ACH ($n=6$) and PLT ($n=3$) showed a PCR-RFLP pattern similar to the one described in San Pedro de Uraba (317 and 77 bp) (Zapata et al., 2007). However, specimens from SRL ($n=3$) and MON ($n=14$) that were morphologically consistent with *An. punctimacula* yielded RFLP results consistent with specimens identified as *An. neomaculipalpus* (95, 155 and 264 bp) and specimens from NUQ ($n=4$) yielded RFLP results consistent with specimens identified as *An. apicimacula* (372, 61 and 47 bp, deduced by bioinformatic analysis of sequences of individuals identified by morphology as such). Specimens from SRL and MON identified morphologically as *An. neomaculipalpus* were confirmed as such by their RFLP pattern (Table 2). One specimen from NUQ was identified by both morphology and RFLP pattern as *An. apicimacula*.

Specimens of the subgenus *Nyssorhynchus* of the Oswaldoi Group that were identified morphologically as *An. evansae*, *An. rangeli*, *An. benarrochi* s.l., and *An. oswaldoi* s.l. were confirmed by PCR-RFLP of ITS2 as *An. nuneztovari* s.l. (Table 2). Amplimers from specimens identified morphologically as *An. nuneztovari* s.l. ($n=94$) were double digested with *AluI*/*FspI* for discrimination between *An. nuneztovari* s.l. and *An. benarrochi* s.l. (Cienfuegos et al., 2008; Matson et al., 2008). All specimens [MLT ($n=21$), VAL ($n=5$), PLT ($n=67$)] were confirmed as *An. nuneztovari* s.l. according to band pattern expected for this species (296, 83, 57, 45 and 16 bp), which differs from that for *An. benarrochi* s.l. (341, 83, 60 and 16 bp) (Figure 5).

4. DISCUSSION

Numerous investigators have reported conflicting morphological identification of female mosquitoes of species of the Oswaldoi Group (Calle et al., 2002; Fajardo et al., 2008; Quiñones et al., 2001; Zapata et al., 2007). In particular, *An. nuneztovari* s.l. can be confused with *An. rangeli*, with *An. benarrochi* s.l. or *An. aquasalis*, and to some extent, with *An. trinkae* Faran, *An. strodei* s.l. Root or *An. evansae* (Fajardo et al., 2008). Although alternative approaches have been proposed for the accurate discrimination of these problematic *Anopheles* species, e.g., traditional and geometric morphometric analyses (Calle et al., 2008; Calle et al., 2002), these strategies are not entirely effective, nor especially practical, and therefore molecular identification assays should be evaluated from across the known geographic distribution of each species. In response to this issue, our PCR-RFLP-ITS2 strategy showed that species-specific band patterns were conserved throughout malaria-endemic localities in Uraba-Bajo Cauca-Alto Sinu and the Atlantic and Pacific coasts, and agreed with those obtained in specimens from San Pedro de Uraba (Zapata et al., 2007).

Among the species in the Oswaldoi Group, previous bioinformatic analysis showed that the *AluI* restriction pattern obtained *in silico* for *An. benarrochi* s.l. was similar to the one for

An. nuneztovari s.l. (Cienfuegos et al., 2008; Matson et al., 2008). This occurs because a deletion of a G at position 349 of the *An. benarrochi* s.l. sequence **AF462384** of a specimen from Brazil creates a new *AluI* recognition site (AGGCT→AGCT). Of relevance is that differences in behavior, morphology of male genitalia and ITS2 sequences suggest the existence of different species within *An. benarrochi* s.l. in Brazil, Venezuela, Colombia and Peru (Flores-Mendoza, 2004; Quiñones, 2006; Deane et al. 1948, Klein et al. 1991; Sallum et al. 2008). The *An. benarrochi* B from southern Colombia (Ruiz et al., 2005) and Peru are anthropophilic, and in some areas of Peru have been found naturally infected by *Plasmodium* spp. (Flores-Mendoza, 2004; Quiñones, 2006). Conversely, *An. benarrochi* s.l. from Brazil showed zoophilic behavior and was not susceptible to infection by *Plasmodium* (Deane et al., 1948; Klein et al., 1991). In addition, preliminary descriptions of two morphological Forms (I and II) based on differences in male genitalia of specimens from Acrelandia, Brazil, showed identical ITS2 sequences (Sallum et al., 2008). Both forms are similar in ITS2 sequences (97%) to *An. benarrochi* B from Colombia (GenBank accession nos. **AY684984-AY684976**), while ITS2 sequences from Rondonia, Brazil only showed 86% similarity (GenBank accession nos. **AF462383** and **AF462384**), to sequences of *An. benarrochi* B (Sallum et al., 2008). Specimens identified morphologically in the present study as *An. nuneztovari* s.l. and double digested with *AluI*/*FspI* for their discrimination from *An. benarrochi* s.l. were confirmed as *An. nuneztovari* s.l. In addition, bioinformatic predictions of the ITS2 digest of *An. benarrochi* B from Colombia reveal an *AluI* PCR-RFLP pattern that is distinct from *An. benarrochi* s.l. from Brazil and from *An. nuneztovari* s.l., suggesting that in Colombia these species should not be confused when a digest with *AluI* is performed.

The small variation in the band pattern observed for *An. albimanus* was likely the result of a single transversion (G→C) in the recognition site of the *AluI* enzyme (AGCT, mutation: ACCT), rendering this site unavailable for restriction. Accordingly, two *An. albimanus* PCR-RFLP patterns can be defined: a wild type or “A” pattern of three bands (381, 128, and 15 bp) similar to that identified by Zapata et al. (2007) and a transversion or “B” pattern of two bands (381 and 143 bp). These patterns were consistent with those predicted *in silico* for the wild type and transversion sequences (GenBank accession nos. **GU477267- GU477274**). In spite of this variation, patterns A and B were suitable for the discrimination of *An. albimanus* (Figure 4). Further analysis of the wild type and transversion sequences will be necessary to test the extent of concerted evolution in *An. albimanus* populations.

Within the Arribalzagia Series of subgenus *Anopheles*, *An. apicimacula*, *An. punctimacula*, *An. neomaculipalpus*, and *An. calderoni* have presented additional challenges. In particular, *An. apicimacula*, *An. punctimacula*, *An. neomaculipalpus* share a subset of adult female morphological characters (González and Carrejo, 2007; Wilkerson and Strickman, 1993). However, our analyses revealed that specimens of these species were differentiated by their PCR-RFLP-ITS2 patterns. The species specificity of these patterns will be confirmed with PCR-RFLP-ITS2 analyses of progeny-reared samples. Among the other species in this series, *An. calderoni* can be confused with *An. punctimacula* (Rubio-Palis and Moreno, 2003; Wilkerson, 1991). *Anopheles calderoni* was recently reported from two localities in Antioquia State, which is located in the Uraba-Bajo Cauca-Alto Sinu region (González and Carrejo, 2009). The fact that *An. punctimacula* was collected in the present study in this same region (PLT, SLU) affirms the importance of evaluating mosquitoes at different life stages to definitively identify *An. calderoni*.

Based on our data, we suggest that the PCR-RFLP-ITS2 assay evaluated in this study is, at this time, the most accurate method for discrimination of anopheline species of Colombia. A similar assay that discriminates *An. benarrochi* s.l. and *An. oswaldoi* s.l. in southern Colombia was proposed previously (Ruiz et al., 2005). However, bioinformatic predictions

using ITS2 sequences retrieved from GenBank, selected based on the correctness of species assignation according to analyses conducted by Marrelli et al. (2006), showed that each of the patterns identified by Ruiz et al. (2005) can be confused with other species in the Oswaldoi Group. For example, *An. benarrochi* s.l. (GenBank accession nos. **AF462383** and **AF462384** from field-collected specimens in Brazil) and *An. nuneztovari* s.l. (GenBank accession nos. **AY028081-AY028126**) would yield the same pattern under the conditions proposed by Ruiz et al. (2005). Likewise, *An. oswaldoi* s.l. (GenBank accession nos. **AF055068-AF055070**), *An. aquasalis* (GenBank accession nos. **DQ020123-DQ020137**) and *An. rangeli* (GenBank accession nos. **AF462381, AF462382, Y09239**) would yield similar patterns (Cienfuegos et al., 2008).

In conclusion, the results suggest that the *AluI* PCR-RFLP assay previously developed in San Pedro de Uraba, Colombia (Zapata et al., 2007), has applicability in different localities of Colombian malaria endemic regions. The assay was useful for discriminating species of the Oswaldoi Group and species within the Arribalzagia Series. In general, the results suggest less species diversity in the localities than previously reported, which among others, may be attributable to misidentification of the adults of species of the Oswaldoi Group that are frequently confused with *An. nuneztovari* s.l. This molecular tool is easy to perform, affordable and highly accurate for discriminating anopheline species, and therefore it is recommended that this or a similar molecular methodology be implemented for high throughput analysis and correct identification of *Anopheles* species in malaria endemic regions of Colombia. In addition, the fact that the ITS2 region is generally conserved and the patterns are reproducible in specimens collected from different geographical regions, suggest that it has the potential to be used as a nuclear barcoding marker for *Anopheles* species identification.

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Figure 1. Localities sampled for *Anopheles* mosquitoes (1 to 12). San Pedro de Urabá (SP) was the source of specimens for standardizing the *AluI* PCR-RFLP-ITS2 assay of Zapata et al., 2007.

Figure 2.

PCR-RFLP-ITS2 analysis of anopheline mosquitoes of northern and western Colombia. 2.5% agarose gel. Lanes: M: Molecular weight marker; 1 and 2: *An. albimanus*; 3 and 4: *An. nuneztovari* s.l.; 5 and 6: *An. aquasalis*; 7 and 8: *An. triannulatus* s.l.; 9 and 10: *An. punctimacula*; 11 and 12: *An. apicimacula*; 13 and 14: *An. neomaculipalpus*; 15: negative control.

Figure 3. PCR-RFLP of ITS2 region following *AluI* restriction of amplicons from *An. aquasalis* on 2.5% agarose gel. Lanes: M: Molecular weight marker; 1–3: *An. aquasalis* individuals collected in Los Achiotas; 4: positive control, clone of *An. aquasalis* ITS2 region.

Figure 4. PCR-RFLP-ITS2 analysis of *An. albimanus*. 2.5% agarose gel. Lanes: M: Molecular weight marker; 1: positive control, clone of *An. albimanus* ITS2 region; 2–9: *An. albimanus* specimens collected in Los Achiotos.

Figure 5. Double digest (*AluI/FspI*) of ITS2 region amplified from individuals identified by the PCR-RFLP with *AluI* as *An. nuneztovari* s.l. Lanes: M: Molecular weight marker; 1: positive control, clone of *An. nuneztovari* s.l. ITS2 sequence; 2–12: specimens collected in Puerto Libertador.

Table 1

Collection data for specimens analyzed in this study.

Region	Department / Locality	Abbreviations	Coordinate Latitude, Longitude *	No. specimens analyzed	Collection date
Atlantic	<i>Magdalena</i>				
	Los Achiotos	ACH	11.2578–73.6144	28	2005–2006
	<i>Bolívar</i>				
	Santa Rosa de Lima	SRL	10.435–73.3558	78	2005–2006
Uraba-Bajo Cauca-Alto Sinú	<i>Córdoba</i>				
	Moñitos	MOÑ	9.2561–16.1092	128	2005–2007
	Puerto Libertador	PLT	7.9064–75.6733	200	2007
	Tierra Alta	TAL	8.1942–76.0786	49	2007
	Montelibano	MLT	7.9853–75.4214	223	2007
	Valencia	VAL	8.2617–76.1528	17	2007
	<i>Antioquia</i>				
	Zaragoza	ZAR	7.4944–74.8678	50	2008
	<i>Chocó</i>				
	Pizarro	PIZ	2.0511–78.6247	44	2005
Pacific	Nuquí	NUQ	5.7111–77.2639	39	2005–2006
	<i>Valle del Cauca</i>				
	Buenaventura	BUE	3.4292–77.0531	51	2005–2006
	<i>Nariño</i>				
	Tumaco	TUM	1.8067–78.7717	41	2005–2006

* Coordinates are expressed in decimal degrees

Table 2
Morphological and molecular identification of *Anopheles* mosquitoes from localities of three Colombian malaria endemic regions.

Region	Locality	No. individuals collected per species	Morphological Identification	No. specimens analyzed by PCR-RFLP	Molecular Identification
Atlantic Coast	ACH	66	<i>An. albimanus</i>	17	<i>An. albimanus</i>
		6	<i>An. punctimacula</i>	6	<i>An. punctimacula</i>
		4	<i>An. aquasalis</i>	4	<i>An. aquasalis</i>
		1	<i>An. darlingi</i>	1	<i>An. darlingi</i>
	SRL	1583	<i>An. triannulatus</i> s.l.	35	<i>An. triannulatus</i> s.l.
		945	<i>An. albimanus</i>	37	<i>An. albimanus</i>
		3	<i>An. punctimacula</i>	3	<i>An. neomaculipalpus</i>
		4	<i>An. neomaculipalpus</i>	3	<i>An. neomaculipalpus</i>
		2099	<i>An. albimanus</i>	50	<i>An. albimanus</i>
		113	<i>An. triannulatus</i> s.l.	30	<i>An. triannulatus</i> s.l.
Urabá-Bajo Cauca-Alto Sinu	MON	40	<i>An. neomaculipalpus</i>	34	<i>An. neomaculipalpus</i>
		15	<i>An. punctimacula</i>	14	<i>An. neomaculipalpus</i>
		144	<i>An. nuneztovari</i> s.l.	117	<i>An. nuneztovari</i> s.l.
		52	<i>An. rangeli</i>	43	<i>An. nuneztovari</i> s.l.
		31	<i>An. darlingi</i>	14	<i>An. darlingi</i>
		17	<i>An. triannulatus</i> s.l.	10	<i>An. triannulatus</i> s.l.
	PLT	6	<i>An. oswaldoi</i> s.l.	6	<i>An. nuneztovari</i> s.l.
		5	<i>An. evansae</i>	5	<i>An. nuneztovari</i> s.l.
		4	<i>An. punctimacula</i>	3	<i>An. punctimacula</i>
		2	<i>An. strodei</i> s.l.	2	<i>An. nuneztovari</i> s.l.
		145	<i>An. nuneztovari</i> s.l.	15	<i>An. nuneztovari</i> s.l.
		134	<i>An. oswaldoi</i> s.l.	119	<i>An. nuneztovari</i> s.l.
	MLI	78	<i>An. rangeli</i>	70	<i>An. nuneztovari</i> s.l.
		8	<i>An. evansae</i>	8	<i>An. nuneztovari</i> s.l.
		8	<i>An. strodei</i> s.l.	8	<i>An. nuneztovari</i> s.l.
		5	<i>An. darlingi</i>	3	<i>An. darlingi</i>
		127	<i>An. nuneztovari</i> s.l.	11	<i>An. nuneztovari</i> s.l.
		22	<i>An. rangeli</i>	20	<i>An. nuneztovari</i> s.l.
TAL	TAL	127	<i>An. nuneztovari</i> s.l.	11	<i>An. nuneztovari</i> s.l.
		22	<i>An. rangeli</i>	20	<i>An. nuneztovari</i> s.l.

Region	Locality	No. individuals collected per species	Morphological Identification	No. specimens analyzed by PCR-RFLP	Molecular Identification
Pacific Coast	VAL	8	<i>An. oswaldoi</i> s.l.	8	<i>An. nuneztovari</i> s.l.
		1	<i>An. triannulatus</i> s.l.	1	<i>An. triannulatus</i> s.l.
		37	<i>An. nuneztovari</i> s.l.	10	<i>An. nuneztovari</i> s.l.
	ZAR	11	<i>An. oswaldoi</i> s.l.	4	<i>An. nuneztovari</i> s.l.
		3	<i>An. darlingi</i>	3	<i>An. darlingi</i>
		58	<i>An. triannulatus</i> s.l.	33	<i>An. triannulatus</i> s.l.
	NUQ	32	<i>An. nuneztovari</i> s.l.	10	<i>An. nuneztovari</i> s.l.
		9	<i>An. darlingi</i>	3	<i>An. darlingi</i>
		3	<i>An. nuneztovari</i> s.l./ <i>rangeli</i>	3	<i>An. nuneztovari</i> s.l.
	BUE	1	<i>An. nuneztovari</i> s.l./ <i>aquasalis</i>	1	<i>An. nuneztovari</i> s.l.
		3099	<i>An. albimanus</i>	34	<i>An. albimanus</i>
		5	<i>An. punctimacula</i>	4	<i>An. apicimacula</i>
	PIZ	1	<i>An. apicimacula</i>	1	<i>An. apicimacula</i>
		1630	<i>An. albimanus</i>	45	<i>An. albimanus</i>
		6	<i>An. rangeli</i>	6	<i>An. nuneztovari</i> s.l.
TOTAL	TUM	47	<i>An. albimanus</i>	44	<i>An. albimanus</i>
		1687	<i>An. albimanus</i>	41	<i>An. albimanus</i>
				939	

In bold: specimens without concordance between morphological and molecular identification

Table 3

Concordance between morphological vs. molecular identification.

Species Identified by Morphology	No. Specimens	No. Specimens confirmed as each species by PCR-RFLP	Molecular Identification	% Concordance	p ^b	Kappa
<i>An. albimanus</i>	268	268	<i>An. albimanus</i>	100	1.0	1.0
<i>An. aquasalis</i>	4	4	<i>An. aquasalis</i>	100	1.0	1.0
<i>An. darlingi</i>	24	24	<i>An. darlingi</i>	100	1.0	1.0
<i>An. nuneztovari</i> s.l.	167	167	<i>An. nuneztovari</i> s.l.	100	0.0	0.36
<i>An. triannulatus</i> s.l.	109	109	<i>An. triannulatus</i> s.l.	100	1.0	1.0
<i>An. neomaculipalpus</i> ^a	37	37	<i>An. neomaculipalpus</i> ^a	100	0.0	0.80
<i>An. apicimacula</i> ^a	1	1	<i>An. apicimacula</i> ^a	100	0.12	0.33
<i>An. punctimacula</i>	30	9	<i>An. punctimacula</i> (n=9)	30	0.0	0.45
			<i>An. neomaculipalpus</i> ^a (n=17)			
			<i>An. apicimacula</i> ^a (n=4)			
<i>An. rangeli</i>	139	0	<i>An. nuneztovari</i> s.l.	0	NC	NC
<i>An. oswaldoi</i> s.l.	137	0	<i>An. nuneztovari</i> s.l.	0	NC	NC
<i>An. evansae</i>	13	0	<i>An. nuneztovari</i> s.l.	0	NC	NC
<i>An. strodei</i> s.l.	10	0	<i>An. nuneztovari</i> s.l.	0	NC	NC

^a A different PCR-RFLP pattern was found for specimens identified by morphology as *An. neomaculipalpus* and *An. apicimacula*. The species specificity of these patterns should be confirmed by PCR-RFLP-ITS2 analyses of progeny-reared samples

^b p: significance level; the null hypothesis of agreement between the morphological and molecular identification methods is rejected when $p \leq 0.05$. The statistics were calculated comparing the morphological and molecular identifications of each species

NC: Value not calculated since molecular identification for these species was not obtained