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Discrimination of Seven *Anopheles* Species from San Pedro de Urabá, Antioquia, Colombia, by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Analysis of Its Sequences

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Abstract. Accurate identification of anopheline species is essential for vector incrimination and implementation of appropriate control strategies. Several anopheline species are considered important malaria vectors in Colombia; however, species determination is complicated by cryptic morphology and intra-individual variation. We describe polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacer 2 (ITS2) sequences for differentiation of seven *Anopheles* species collected in a locality in Antioquia, Colombia, with high levels of malaria transmission. Each of these seven species can be identified by unique *Alu*I PCR-RFLP restriction patterns. Comparisons of morphologic identification with molecular identification of voucher specimens confirmed species designation for 886 wild-caught anophelines. This new method can be used as a diagnostic tool for discrimination of anopheline species of medical importance in this region, some of which have overlapping morphologic characters and for conducting complementary studies where rapid and accurate identification of large numbers of specimens is needed.

INTRODUCTION

Colombia is among the countries with the highest incidence of malaria in Latin America.[1] This disease constitutes an important health problem, especially in rural areas located at an elevation below 1,600 meters, where appropriate conditions for disease transmission and the presence of malaria vectors exist.[2] Based on epidemiologic and entomologic data, of 43 species of *Anopheles* reported in Colombia, only three in the subgenus *Nyssorhynchus* are recognized as primary malaria vectors: *Anopheles albimanus*, *An. darlingi*, and *An. nuneztovari*.[3] Although these are the traditionally accepted major vectors, there are reports of malaria in regions of Colombia where these species are absent and other species could prove to be important in malaria transmission.[4] Moreover, many of the species are difficult to distinguish using morphologic keys because of cryptic morphology and intraspecies variation.[5–7]

Recently, DNA based methods of identification have been used as tools for unequivocal species determination. Among them, molecular analysis of the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) has proved of great value in species differentiation (reviewed by Krzywinski and Besansky[8]). In *Anopheles*, ITS1 variation is readily detected among individuals of a single species,[9,10] and this variation has been used to identify populations or strains within a species.[9] In contrast, ITS2 variation is low or not detectable within a species and shows greater levels of sequence variation among species. Consequently, ITS2 variation has proved of great value in species differentiation. The array of molecular methodologies for anopheline discrimination using the ITS region include sequencing,[7,11,12] polymerase chain reaction (PCR) assays with species specific primers[5] and in a multiplex format,[13–16] and restriction fragment length polymorphism of PCR products (PCR–RFLP).[17–21]

In Colombia, a number of studies combining morphologic and molecular data have resolved the taxonomic status of various species. Sierra and others[22] demonstrated that *An. nuneztovari* comprises a single genetic species on both sides of the Andes mountains. Ruiz and others[4] used a PCR-RFLP assay to differentiate two species with overlapping morphology from southern Colombia, an *An. benarrochi* variant, and *An. oswaldoi*. Considering that accurate identification of anopheline species is essential for vector incrimination, the purpose of our study was to develop a high throughput assay based on PCR-RFLP of ITS sequences for reliable identification of the species present in a locality in Antioquia, Colombia, with high levels of malaria transmission. Our overall aim was to develop a molecular test to be used in the rapid and accurate identification of large numbers of specimens.

MATERIALS AND METHODS

Mosquito collection and processing. *Anopheles* mosquitoes were collected in San Pedro de Urabá, Antioquia state of Colombia. This locality was selected because it has one of the highest levels of malaria cases in the Urabá region; the average number of reported cases of malaria per year between 2000 and 2005 was 3,113.[23] Adult specimens were collected by aspiration from the walls and the roofs of dwellings where adults normally rest after feeding. Human landing catches of adults were conducted under a fully executed informed consent agreement using a protocol and collection procedures that were reviewed and approved by the Bioethics Committee for Human Research at University of Antioquia.

Mosquitoes analyzed were from three sources: 1) human landing catches, 2) individually reared progeny of wild-caught females or isofemale lines, and 3) adults reared from field collected larvae (series), both with associated exuviae that allowed positive morphologic identification. Identification was based on morphologic features of wild-caught adults,

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voucher specimens, and immature stage–associated exuviae, using existing keys.[24–27] After identification, specimens were stored in vials containing 95% ethanol until molecular assays were performed. For archival preservation of identified specimens, a wing and posterior leg from a series of adults from each identified species and a series of pupal exuviae from the same were mounted on glass slides with euparal. These voucher specimens have been stored at the Molecular Microbiology Laboratory at our institution.

DNA extraction and amplification. Template DNA was obtained from either a single leg or wing added to a PCR mixture or mosquito genomic DNA obtained with the phenol-chloroform extraction method described by Ashburner.[28] The ITS2 sequences from *Anopheles* mosquitoes were amplified using primers for conserved 5.8S and 28S flanking sequences and cycling conditions as reported by Beebe and Saul.[17] Primers for conserved *Anopheles* 18S and 5.8S flanking sequences used to amplify the ITS1 region and cycling conditions were as described by Beebe and others.[9] The PCR was carried out in a 25-μL reaction volume using an iCycler (Bio-Rad Laboratories, Hercules, CA). The final PCR mixture contained 2.5 μL of 10× buffer, 200 μM of each dNTP, 0.25 μM of each primer, 1.5 mM $MgCl_2$, 0.65 units of *Taq* DNA polymerase, and 5 μL of template DNA. When using a single leg or wing, the template DNA volume (5 μL) in the aforementioned reaction was replaced with water.

Digestion of PCR products. *Enzyme selection criteria.* The initial selection of enzymes to be used for digestion of ITS PCR products was based on previous reports on the use of *Msp*I[17] and *Alu*I (Ruiz F, unpublished data). To predict which of these enzymes would produce banding patterns that would be well-defined and species-specific, we conducted preliminary bioinformatic analyses with the program DNA Refinder (kindly provided by V. A. Giraldo and D. Zuleta, Universidad de Antioquia, Medellín, Colombia) for various ITS2 DNA sequences obtained from GenBank for *An. albimanus*, *An. nuneztovari*, *An. darlingi*, and *An. rangeli*. Based on these analyses, *Alu*I was selected and evaluated against individuals from series, isofemale line specimens, and adult mosquitoes collected in the field.

PCR-RFLP of ITS1 and ITS2 sequences. Restriction digests with *Alu*I (Promega, Madison, WI) were performed according to the manufacturer's instructions. The mixture was incubated for 3 hours at 37°C. Digested PCR products were fractionated by electrophoresis on 2% agarose gels (Agarose; Amresco, Solon, OH) and stained with ethidium bromide. Gels were photographed by using the Gel Doc 2000™ Gel Documentation System (Bio-Rad Laboratories), and band sizes were calculated by using the Quantity One® 1-D Image Analysis Software (Bio-Rad Laboratories). Morphologic identification results were compared with molecular profiles of confirmed voucher specimens to evaluate the degree of congruence of the two techniques.

Once the PCR-RFLP patterns for the different species were obtained and as another means of corroborating our results, sequences obtained in our laboratory for *An. albimanus*, *An. nuneztovari*, *An. darlingi*, *An. triannulatus*, and *An. punctimacula* and sequences for *An. rangeli* and *An. albitarsis* species complex obtained from GenBank were subjected to *in silico* restriction enzyme analysis with NEBcutter software.[29] Software predictions and PCR-RFLP patterns were then compared directly as further validation of our assay.

RESULTS

Mosquito identification. A total of 886 mosquitoes collected by human landing catches in San Pedro de Urabá, Antioquia, were identified by morphology and their species assignment was confirmed by ITS2 PCR-RFLP (Table 1). The species collected at the highest frequency (64.8%) was *An. nuneztovari*, which is generally considered a primary vector in Colombia. The next most abundant species was *An. rangeli*, comprising 18.7% of total catches. The other two species that are considered to be primary vectors, *An. darlingi* and *An. albimanus*, comprised 4.6% and 3.4%, respectively, of total catches. In addition, *An. punctimacula* and *An. pseudopunctipennis*, which are recognized as secondary malaria vectors, were found at this locality. Of the mosquitoes collected by human landing catches, *An. triannulatus* was one of the least abundant (4.2%). In contrast, this species was the second most abundant in larval collections, which suggested, as reported,[25] that adults of this species feed infrequently on humans. Eighty-two adults were reared from field-collected larvae (series) and 36 isofemale lines were obtained (Table 1). All species were verified morphologically based on immature stages from series. Establishment of isofemale lines of *An. darlingi*, *An. albitarsis* s.l., *An. triannulatus*, and *An. pseudopunctipennis* was not possible, in part because of the low number of individuals collected for these species.

PCR of ITS sequences. The ITS2 amplicon lengths ranged from 485 to 584 basepairs for the complete ITS2 with partial 5.8S and 28S rDNA regions, including primers target sites (Table 2). Individual ITS1 amplicon lengths ranged from 445

TABLE 1
Species and numbers of *Anopheles* mosquitos collected in San Pedro de Urabá, Antioquia, Colombia, identified by morphology*

Species	No. of mosquitoes (%)	No. of mosquitoes from series (%)	No. of isofemale lines (%)
Anopheles (Nyssorrynchus) nuneztovari	574 (64.8)	29 (35.4)	28 (77.8)
An. (Nyssorrynchus) rangeli	166 (18.7)	15 (18.3)	2 (5.6)
An. (Nyssorrynchus) darlingi	41 (4.6)	6 (7.3)	–
An. (Nyssorrynchus) albitarsis s.l.	4 (0.5)	1 (1.2)	–
An. (Nyssorrynchus) albimanus	30 (3.4)	2 (2.4)	5 (13.9)
An. (Nyssorrynchus) triannulatus	37 (4.2)	26 (31.7)	–
An. (Anopheles) punctimacula	23 (2.6)	2 (2.4)	1 (2.8)
An. (Anopheles) pseudopunctipennis	11 (1.2)	1 (1.2)	–
Total	886 (100)	82 (100)	36 (100)

* Species assignment was confirmed by internal spacer 2 polymerase chain reaction–restriction fragment length polymorphism patterns.

TABLE 2
Comparison of fragment sizes resulting from *Alu*I digestion of ITS2 PCR products with fragment sizes predicted by bioinformatic analysis*

Species	Agarose gel fragments (bp)		Bioinformatic prediction of fragments (bp)		Sequence identification
	PCR products	PCR–RFLP	PCR product	PCR–RFLP	
Anopheles nuneztovari	485	358, 96, 76	493	342, 73, 78	Our laboratory (unpublished)
An. albitarsis s.l.	524	280, 170, 95	478	*An. deanoerum*: 260, 135, 85	AF461752.1
				An. albitarsis: 259, 135, 84	AF462387.1
				An. marajoara: 258, 133, 85	U92334.1
An. punctimacula	397	334, 81	394	317, 77	Our laboratory (unpublished)
An. rangeli	516	340, 165	470	335, 135	AF462382.1
An. albimanus	566	377, 127	509	366, 128, 15	Our laboratory (unpublished)
An. triannulatus	584	240, 190, 168	563	226, 180, 157	Our laboratory (unpublished)
An. darlingi	575	321, 158, 78	540	303, 155, 82	Our laboratory (unpublished)
An. pseudopunctipennis	566	No fragments	510	No predicted fragments	U49735

* ITS2 = internal transcribed spacer 2; PCR = polymerase chain reaction; bp = basepairs; RFLP = restriction fragment length polymorphisms.

to 636 basepairs for complete ITS1 with partial 5.8S and 18S rDNA regions. Discrimination of most species based on ITS1 or ITS2 amplicon size was not possible. Therefore, we performed PCR-RFLP of ITS sequences.

PCR-RFLP. Preliminary bionformatic analyses of ITS2 DNA sequences obtained from GenBank predicted that *Alu*I digestion would produce banding patterns that would be well-defined and specific for each species. In contrast, the predicted patterns for *Msp*I digestion were not specific and included very small predicted fragments in some of the sequences that would be difficult to visualize by agarose electrophoresis. In addition, bioinformatic analyses predicted that *Msp*I recognition sites were not present in available *An. rangeli* ITS2 sequences. Given these results, we selected the enzyme *Alu*I for subsequent digestions of ITS1 and ITS2 PCR products.

PCR-RFLP of ITS2 sequences. The ITS2 amplicons digested with *Alu*I yielded banding patterns unique to each species (Figures 1 and 2). The discrimination of restriction fragments depended to a great extent on the agarose concentration used; 0.8% and 1.5% agarose gels did not show easy discrimination of bands that were smaller and similar in size. For example, electrophoresis with 2.0% or 2.5% agarose yielded good fragment separation for *An. nuneztovari* (Figure 3). Although the smallest fragments appeared diffuse (Figure 1, lanes 3 and 4 and lanes 12 and 13), all PCR-RFLP patterns showed species discrimination. Discrimination was improved by electrophoresis of larger volumes of PCR-RFLP reaction products, which sharpened banding patterns (Figure 2, lanes 5 and 6), as did amplification of some ITS2 sequences from plasmid clones prior to *Alu*I digestion. Fragment sizes produced from each species are shown in Table 2. Comparison of predicted fragment sizes with the PCR-RFLP data showed congruency in the results (Table 2). Small differences between predicted and actual fragment sizes are likely the result of user subjectivity of band capture, which is integral to analyses with Quantity One® 1-D Image Analysis Software.

PCR-RFLP of ITS1 sequences. The ITS1 amplicons digested with *Alu*I resulted in banding patterns that were distinctive for each species. *Anopheles darlingi*, *An. nuneztovari*, and, *An. punctimacula* showed RFLP patterns with multiple

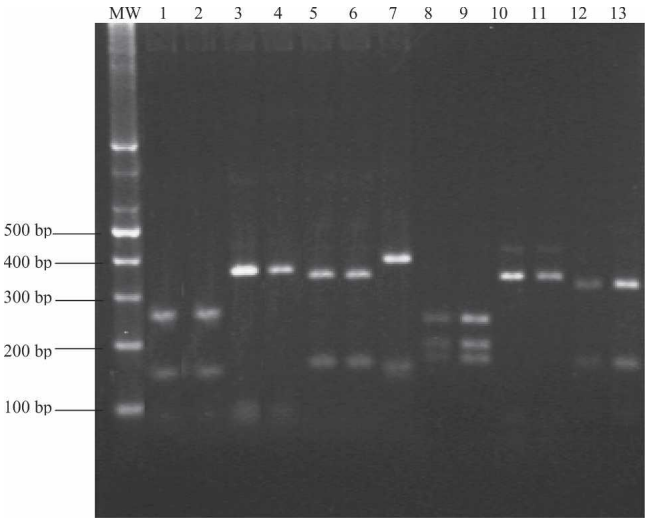

FIGURE 1. Internal transcribed spacer 2 polymerase chain reaction–restriction fragment length polymorphism (*Alu*I digestion) patterns of *Anopheles* species from San Pedro de Urabá, Antioquia, Colombia on a 2% agarose gel. Lane MW, molecular mass markers; lanes 1 and 2, *An. albitarsis* s.l.; lanes 3 and 4, *An. nuneztovari*; lanes 5 and 6, *An. rangeli*; lane 7, *An. albimanus*; lanes 8 and 9, *An. triannulatus*; lanes 10 and 11, *An. punctimacula*; lanes 12 and 13, *An. darlingi*. bp = basepairs.

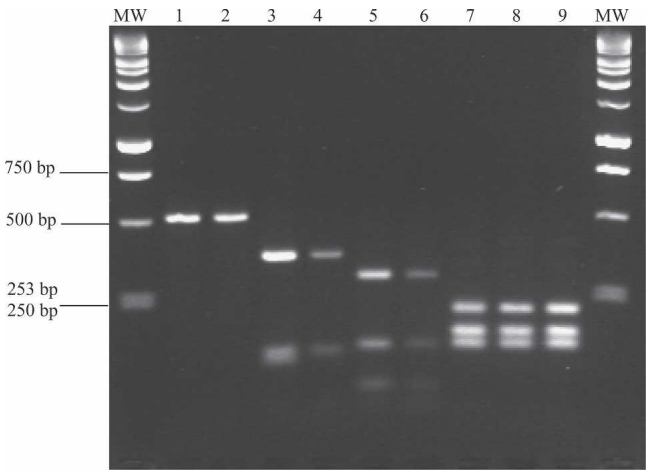

FIGURE 2. Internal transcribed spacer 2 polymerase chain reaction–restriction fragment length polymorphism (*Alu*I digestion) patterns of *Anopheles* species from San Pedro de Urabá, Antioquia, Colombia, on a 2% agarose gel. Lanes MW, molecular mass markers, lanes 1 and 2, *An. pseudopunctipennis*; lanes 3 and 4, *An. albimanus*; lanes 5 and 6, *An. darlingi*; lanes 7–9, *An. triannulatus*. bp = basepairs.

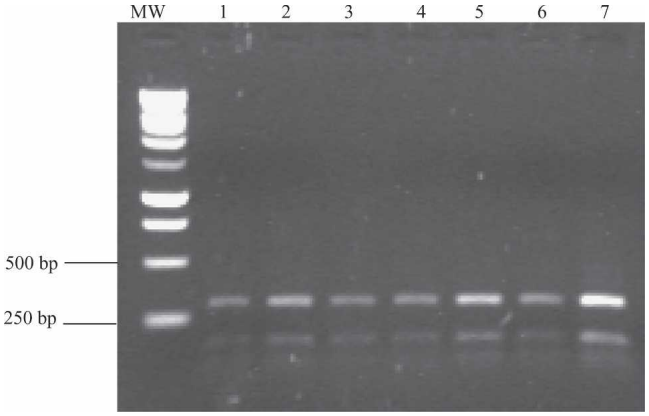

FIGURE 3. Internal transcribed spacer 2 polymerase chain reaction–restriction fragment length polymorphism (*Alu*I digestion) patterns of individual *An. nuneztovari* mosquitoes from San Pedro de Urabá, Antioquia, Colombia, on a 2.5% agarose gel. Lane MW, molecular mass markers; lanes 1–6, field-collected *An. nuneztovari*; lanes 7, *An. nuneztovari* derived from an isofemale line as a control. bp = basepairs.

bands of different sizes. Amplicons from *An. pseudopunctipennis*, *An. albimanus*, and *An. triannulatus* yielded two fragments, one that was slightly smaller than the ITS1 amplicon and one or more very small fragments that were difficult to discern on agarose gels; the larger bands derived from amplicons from *An. pseudopunctipennis* and *An. albimanus* could be differentiated by their size. The ITS1 PCR-RFLP pattern of the *An. triannulatus* amplicon was a double or wide band. Despite these differences among species, ITS1 PCR products were frequently unstable, becoming degraded after a short period of freezer storage, making results difficult to reproduce.

DISCUSSION

In Colombia, there is a general knowledge of the distribution of the primary and secondary vector species of malaria parasites.[3] However, some reports indicate that these purported vectors are absent in some malaria-endemic regions of the country, suggesting that other species are responsible for transmission in these areas.[4] To contribute to the assessment of species distribution and understanding of the entomologic aspects of malaria transmission, we developed a molecular assay for reliable identification of regional *Anopheles* species and evaluated this assay using specimens collected in the state of Antioquia, Colombia, at San Pedro de Urabá, a locality with high levels of malaria transmission. San Pedro de Urabá is located in northwestern Colombia at an altitude of 200 meters. The average temperature is 27°C. From 2000 to 2005, San Pedro de Urabá accounted for 15% of the reported cases in the Urabá region and approximately 80% of these cases were caused by *Plasmodium vivax*.[23]

In San Pedro de Urabá, we identified three species, *An. albimanus*, *An. darlingi*, and *An. nuneztovari*, that have been traditionally considered major malaria vectors in Colombia. Although *An. nuneztovari* accounted for nearly 65% of total landing counts, the other two major vector species combined accounted for only 8% of total catches. Interestingly, *An. rangeli* accounted for nearly 19% of total catches; this species was recently implicated in transmission of *P. vivax* in southern Colombia,[30] which suggested that careful assessment of this species is also warranted in northwestern Colombia. Furthermore, numerous studies have demonstrated that species abundance does not necessarily correlate with vector importance.[30–33] As such, future studies will be focused on vector assessment of all species identified in Antioquia.

The complexity involved in the identification of adult anophelines makes it difficult to understand the role of those species in particular malaria-endemic areas. In this study, identification of immature stages from isofemale lines and series helped to overcome difficulties arising during taxonomic determination of adults and provided reference samples to standardize the PCR-RFLP assay. Studies of immature stages, however, cannot be used routinely because they are laborious and time-consuming and require specially trained personnel. Therefore, larval studies are not appropriate for extensive malaria survey and control programs, especially in malaria-endemic countries where budgets for prevention and control are very limited. The availability of a molecular test such as the one described here will permit reliable and rapid identification of the species present at specific malaria-endemic areas.

In the standardization of our assay, we first attempted to use ITS amplification product sizes. However, we found that ITS2 amplicons did not allow the discrimination of species based only on band sizes. Other investigators have reported some degree of variation in ITS2 sequences and similarity in the sizes of the spacers among different neotropical anopheline species, ranging from 323 to 410 basepairs[7] and from 351 to 406 basepairs.[14] These observations show why ITS2 amplicon size cannot be used to discriminate neotropical anophelines. Analyses of ITS1 amplicons indicated that these were not reliable for species discrimination and, as such, were not pursued further.

Because ITS2 amplicon sizes were not diagnostic, we found that it was necessary to develop a PCR-RFLP assay for species discrimination. Our data clearly showed that PCR-RFLP of ITS2 amplicons yielded unique patterns for each of the seven species and, thus, allowed unambiguous identification. The PCR-RFLP patterns of type specimens confirmed species assignments. For example, some mosquitoes were identified from morphological characteristics as "possibly" *An. oswaldoi*, *An. rangeli*, and *An. strodei*, but ITS2 RFLP patterns from type specimens confirmed that all were *An. nuneztovari*. These results were not unexpected because Faran[24] reported that *An. rangeli* and *An. nuneztovari*, which are sibling species, have high interspecific similarity. Furthermore, studies carried out by Delgado and Rubio-Palis[34] in Venezuela showed that *An. oswaldoi, An. strodei*, and *An. nuneztovari* can be confused using the key of Faran[24] because of overlapping morphologic characters. This also applies to other species in the subgenus *Nyssorhynchus*.[5,7,35] In a comprehensive analysis of available ITS2 sequences of Latin American anophelines, Marelli and others[35] found that the ITS2 sequences for some species were distributed in more than one group of a neighbor-joining tree. Among the possibilities for this distribution pattern is the misidentification of field-collected females based only on morphologic characteristics. A significant benefit of our study is that the ITS2 PCR-RFLP assay was developed on the basis of the verification of mo-

lecular results with the morphologic identification of immature stages.

The patterns obtained with ITS1 PCR-RFLP were different among the species analyzed, which suggested that ITS1 analyses could be used for species diagnostics. However, ITS2 PCR-RFLP patterns were better defined and more clearly distinguishable among species. In addition, ITS1 sequences have been found to display high levels of sequence variation even among individuals.[10] As such, it will be necessary to determine the extent of ITS1 sequence variation within individuals of representative Colombian species before this sequence region can be used as a diagnostic marker. The level of variation of ITS1 sequences enabled the identification of populations within the species *An. farauti* s.s. using PCR-RFLP.[9] In our study, we did not detect ITS1 variation among *An. nuneztovari* specimens. This observation could result from a lack of utility of ITS1 sequence variation to identify population structuring in *An. nuneztovari* or that no population level differences exist at our study site. A third possibility is that intraspecies variability cannot be detected by this technique. Farley and others[10] investigated the level of intragenomic heterogeneity of the ITS array (ITS1, 5.8S, and ITS2) of *An. aquasalis* mosquitoes and found greater levels of sequence and length variation in the ITS1 than in the ITS2; point mutations were detected in both spacers but microsatellites were detected only in ITS1. Recently, Li and Wilkerson[36] studied the ITS2 sequences from four species belonging to the *An. albitarsis* complex and found that even these sequences were very similar ($\leq$ 1.17% divergence) and length and sequence variation were due to two variable microsatellite regions, a number of insertions/deletions (indels), and base substitutions. Preliminary data of ITS1 and ITS2 sequences from *An. albimanus*, *An. darlingi*, *An. nuneztovari*, and *An. triannulatus* show that point mutations were common to both spacers but microsatellites affecting the length of the sequence were only found in the ITS1 of *An. triannulatus* (Correa M and others, unpublished data).

In conclusion, PCR-RFLP of ITS2 reported in this study can be used as a diagnostic tool for discrimination of some Colombian *Anopheles* species with overlapping morphology. The correct species assignment is of great relevance because multiple species with geographic ranges that span Central and South America have been implicated in malaria transmission. The knowledge of species that are of regional importance will contribute to the design and implementation of effective vector control measures for malaria prevention. The ITS2 PCR-RFLP assay can allow for rapid and accurate identification of large numbers of mosquitoes, which enables the development of complementary studies in which other factors of vector biology can be determined, including extensive epidemiologic and vector incrimination studies.

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