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Am J Trop Med Hyg. Author manuscript; available in PMC 2008 May 5.

Published in final edited form as:
Am J Trop Med Hyg. 2008 March ; 78(3): 492–498.

Improved Molecular Technique for the Differentiation of Neotropical Anopheline Species

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Abstract

We evaluated a PCR-RFLP of the ribosomal internal transcribed spacer 2 region (ITS2) to distinguish species of *Anopheles* commonly reported in the Amazon and validated this method using reared F_1 offspring. The following species of *Anopheles* were used for molecular analysis: *An. (Nys.) benarrochi, An. (Nys.) darlingi, An. (Nys.) nuneztovari, An. (Nys.) konderi, An. (Nys.) rangeli,* and *An. (Nys.) triannulatus sensu lato* (s.l.). In addition, three species of the subgenus *Anopheles*, *An. (Ano.) forattini, An. (Ano.) mattogrossensis, and An. (Ano.) peryassui* were included for testing. Each of the nine species tested yielded diagnostic banding patterns. The PCR-RFLP method was successful in identifying all life stages including exuviae with small fractions of the sample. The assay is rapid and can be applied as an unbiased confirmatory method for identification of morphologic variants, disputed samples, imperfectly preserved specimens, and life stages from which taxonomic keys do not allow for definitive species determination.

INTRODUCTION

Since the early 1990s, the incidence of malaria has been increasing in Peru. Peru's largest department, Loreto, is in the Amazon basin and has an estimated population of 920,000. It is the major focus of the current malaria epidemic on the national level. In 2007, through epidemiologic week 19, Loreto has reported 13,645 cases of *P. vivax* malaria and 3,013 cases of *P. falciparum* malaria, which accounts for 70% of all malaria cases and 93% of *P. falciparum* cases in Peru over this time period (Peruvian Ministry of Health, unpublished).

Peru shares a number of its primary and potentially important malaria vectors with the other countries of the Amazon (Bolivia, Brazil, Colombia, Ecuador, French Guiana, Guyana, Suriname, and Venezuela). The distribution of these vectors across the Amazon basin is dynamic. Their accurate identification is the most basic requisite for understanding vector biology, malaria risk and epidemiology, and for designing and adequately measuring the impact of disease control interventions. Studies on mosquito susceptibility to plasmodium infection, behavior, and geographic distribution are all dependent on the capacity for the correct species designation of acquired samples.

The identification of anophelines in the Amazon is complicated by the biodiversity that characterizes the region. There are 43 species of *Anopheles* in Peru, 29 of which are present in the political department of Loreto.[1] Members of the subgenus *Nyssorhynchus* are both

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particularly problematic for morphologic identification and the major vectors of malaria in Loreto.

More important than the number of vectors is that intraspecies variability is common and poorly characterized, limiting the utility of taxonomic keys because key characteristics are often overlapping. This leads to erroneous identification; flawed vector implication has been the result on several occasions. The most significant overlap in key characteristics occurs between *An. nuneztovari, An. rangeli, An. benarrochi, An. evansae, An. oswaldoi*, and *An. konderi*.[2] *An. evansae* was implicated as a vector of importance in Colombia for 14 years[3–5] when in fact the species under study was *An. benarrochi,* as confirmed by sequence analysis of the ITS2 region and a segment of cytochrome oxidase subunit I.[6,7] A morphologic variant of *An. benarrochi*, the form most common in Peru, is easily confused with *An. oswaldoi*. The taxonomic keys of Faran,[8] Faran and Lithicum,[9] and Rubio-Palis[10] state that the basal dark spot of hindtarsomere 2 is less than 25% of the total length of that tarsomere in *An. oswaldoi*, whereas it was significantly longer in *An. benarrochi* (40–55%). Morphometric studies of variants of *An. benarrochi* have been shown to have mean values of 26% (range 17–33%),[2] which puts it at the limit of determination of the dichotomous key feature (25%) and leads to misclassification between *An. oswaldoi*, *An. benarrochi*, and to a lesser extent *An. rangeli*. A recent study that sequenced the ITS2 region of F_1 An. benar*rochi* and *An. oswaldoi* in Colombia revealed that GenBank entry AF055071 labeled *An. oswaldoi* and included in a published report describing sister species in the *An. oswaldoi* complex[11] was *An. benarrochi*.[12] A careful morphometric study in Colombia by Calle and others[2] clearly demonstrated that *An. nuneztovari*, *An. rangeli*, *An. benarrochi*, and *An. oswaldoi* had significant overlap in features used in the most commonly used taxonomic keys. *Anopheles* (*Nys.*) *konderi* should also be added to this list because it is only reliably distinguished from *An. oswaldoi* by the examination of male genitalia.[13,14] Unreliable species identification may play a role in the misinterpretation of a significant body of entomologic work done in the Amazon region and likely contributes to the inefficiency of disease control efforts.

A number of *Anopheles* species of the subgenus *Nyssorhynchus* have been described to be of importance in malaria transmission in the Amazon region of South America. *An. benarrochi* is an important vector in the western regions in Peru where high degrees of transmission occur in areas in the absence of *An. darlingi*,[15–17] and in the Putumayo region of Colombia.[6] *An. triannulatus* s.l. is considered to be an important vector in eastern Peru.[18] *An. nuneztovari* is an efficient vector in areas of the Amazon.[19,20] *An. oswaldoi* and *An. rangeli* have both been incriminated as vectors, most strongly in Brazil, Venezuela, and Colombia.[21–24] The inability to reliably distinguish *An. konderi* from *An. oswaldoi* in the absence of a genital examination of males has limited available data regarding its distribution and behavior but it is able to be infected with *Plasmodium vivax*.[24] The ability to efficiently and unequivocally identify these species is a priority to obtain a clear understanding of malaria transmission in the region.

PCR-RFLP is an optimal strategy for the testing of field isolates because of the ease of standardization and high reproducibility of the assay that will facilitate its uptake and application in reference laboratories in the endemic areas.[25,26] Studies of the second internal transcribed spacer region (ITS2) of rDNA have revealed interspecies differences among many Anophelines of the subgenus *Nyssorhynchus*[27] and the number of GenBank sequences available for Anophelines commonly reported in the Amazon region, which facilitates the analysis of experimental results. We structured a double enzyme RFLP digest to improve the accuracy of identification of a number of key vectors that display significant morphologic overlap within the *Nyssorhynchus* subgenus.

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MATERIALS

Anopheline collections and geographical origin

Human landing catches were performed to obtain adult females of nine local *Anopheles* species from which F_1 sibling generation progeny broods could be generated and used for molecular testing. The species presented are the following: *An. (Nys.) benarrochi, An. (Nys.) darlingi, An. (Nys.) nuneztovari, An. (Nys.) konderi, An. (Nys.) rangeli, An. (Nys.) triannulatus* s.l., *Anopheles*, *An. (Ano.) forattini*, *An. (Ano.) mattogrossensis*, and *An. (Ano.) peryassui.* These include all species for which progeny broods were available.

Specimens were collected over a broad geographical area within Peru, and in a number of different collection localities to ensure that the PCR-RFLP assay designed herein would account for intraspecific variation. These collections were performed in or around the following locations in the department of Loreto: Libertad, Mazan, Moralillo, Puerto Almendras, Quistococha, Zungarococha, and in the department of Ucayali: San Jose (see Table 1).

Human landing collections were performed from 1800–2300h, although on occasion were continued until 0600h the following morning. Captured adult females were blood fed on a live chicken immediately after capture. Blood-fed females found resting on foliage or stable structures within 10 meters of cattle ranches were collected via aspiration. Live mosquitoes were kept in humid chambers, transported to the laboratory, and fed with sugar water (25%) for 36 hours immediately after capture. Specimens were then stunned using ethyl acetate, one wing was removed, and females were placed in humidity vials for stressed oviposition. Eggs were collected and set in closed rearing trays, one tray per progeny brood, fed twice a day with fish food, and reared to adults for morphologic identification and molecular analysis. A subsample of larval and pupal exuviae, and fourth instar larvae from each progeny brood were stored in 70% alcohol for mounting and species confirmation.

Nine species of *Anopheles* were available after successful rearing of F_1 offspring: *An.* (*Nys.*) *benarrochi*, *An.* (*Nys.*) *darlingi*, *An.* (*Nys.*) *nuneztovari*, *An.* (*Nys.*) *konderi*, *An.* (*Nys.*) *rangeli*, *An.* (*Nys.*) *triannulatus* s.l., *An.* (*Ano.*) *forattini*, *An.* (*Ano.*) *mattogrossensis*, and *An.* (*Ano.*) *peryassui*.

Species identifications of mosquito specimens were made with a Meiji EMZ stereomicroscope (MEIJI Techno Co., Ltd) with a magnification of 10–45× and a 100 unit micrometer (1 ocular unit = 23.3 μM at 45×), using the keys of Faran.[8,9] Identification of each progeny brood was based on both morphologic characteristics and morphometrics of emerged adult females and a number of fourth instar larvae. Individual F_1 progeny broods were considered correctly identified based on three emerged adult females and fourth-stage larvae. In the case of *An. konderi*, species confirmation was also based on morphology of the male genitalia of emerged males using the methods and key of Causey.[13,14] Only progeny broods with consistent species identifications were used for molecular analysis. A number of wild-caught females from different localities collected both during this investigation and in prior years, whose species identification was re-confirmed in our laboratory using the aforementioned methods, were included for testing. These specimens served to confirm accurate species identifications based on morphology and the molecular assay. Mounted adults, fourth instar larva, and male genitalia have been saved as vouchers and are held at Laboratories of A.B. Prisma in Iquitos, Peru.

METHODS

Species used and generation of restriction fragments from ITS2

We designed a PCR-RFLP assay with the goal of distinguishing among species of Neotropical *Anopheles*, some of which are of medical importance (Table 2).[1,27] The enzymes *AluI*, *FspI*,

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and *BsrB1* were selected for double endonuclease restriction following *in silico* digests using NEBcutter (New England Biolabs) using previously published sequences for the amplified region of the ITS2 region.[27] The Genbank accession numbers and expected PCR-RFLP fragments (using *BsrB1* and *AluI*) for these species and others commonly found in South America are listed in Table 2.

DNA extraction

DNA was extracted from mosquito specimens as follows: Each individual mosquito, exuvia, or larva was homogenized in 200 μL lysis buffer (1 mL: 100 μL Tris 1M, 20 μL EDTA 0.5M, 200 μL SDS 10%, 10 μL Prot K, 670 μL H_2O). After incubating for 1 hr at 58°C, 14 μL of NaCl 5M and 6 μL of CTAB 10% were added and the sample was incubated for 10 min at 65° C. Extraction was performed with a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) (1×), and chloroform: isoamyl alcohol (24:1) (1×), followed by centrifugation (5 min, 13,000 g); 500 μL cold ethanol and 5 μL sodium acetate 3M were added to precipitate DNA. After centrifugation for 10 min at 13,000 rpm the pellet was washed with 500 μL ethanol 70%. Three 70% alcohol washes were alternated with centrifugation at 13,000 rpm for 5 min a cycle. The pellet was allowed to dry at room temperature and re-suspended in 80 μL of TE buffer (10 mL: 100 μL Tris 1M, 20 μL EDTA 0.5M).

PCR amplification and endonuclease digestion of ITS2 region

Amplification of the ITS2 region of the rDNA was performed using the CP16/CP17 primers and protocol as described previously by Marrelli,[27] with some modifications. In a final reaction tube of 25.5 μL, reagent concentrations are as follows (all reagents from Invitrogen, Carlsbad, CA unless otherwise specified): 1× reaction buffer; 2.5 mM $MgCl_2$; 250 μM dNTP's; 2.5 μM primers CP16 and CP17; 0.25 μg/μL BSA; and 1.0 U/μL recombinant Taq Polymerase; using 3 μL of extracted DNA as described above, and 2 μL and 5.9 μL of Cresol Red and H_2O respectively per reaction tube. The PCR reactions were performed for 25 cycles (94°C for 1 min, 50°C for 2 min, and 72°C for 2 min) and the products were visualized by ethidium bromide staining after electrophoresis in a 3% ultrapure agarose gel.

Double restriction digests were performed using *AluI* and *BsrB1* or alternatively *AluI* and *FspI* (New England Biolabs). In each sample: 10 μL of PCR products was double digested with 0.3 μL of each restriction enzyme, 1.5 μL of the corresponding buffer (New England Biolabs), and 6.2 μL of distilled water. The reaction tube was left at room temperature for 24 hrs and products visualized with ethidium bromide after electrophoresis in a 3% ultrapure agarose gel; band sizes were determined using Kodak Gel Logic 200 Imaging System and Kodak Molecular Imaging Software version 4.0.

RESULTS

Product sizes after amplification of nuclear rDNA using the specified primers ranged between 581 and 482 as described for each species in Table 3. Primary PCR product bands were clearly visible on the gels but differentiation between species based solely on PCR fragment size was not possible due to small size differences, often of less than 10 base pairs between species (Figure 1). Offspring of the species *An. benarrochi*, *An. konderi*, and *An. triannulatus* s.l. occasionally demonstrated the presence of an additional variable band(s) after initial amplification to the PCR product. In each case, this variable product caused a second minor RFLP pattern for that species. The patterns were restricted to one of these two types, the normal and the minor variant, which did not reveal overlap with other species tested in this assay. These variable PCR products and their resulting RFLP patterns (*BsrB1* & *AluI*) are listed below in the following format: *Species*, PCR product size in bp (variable bands); RFLP fragment sizes in bp (variable bands).

An. benarrochi, PCR product 518 (492); RFLP fragments 261 (232)

An. konderi, PCR product 520 (545); RFLP fragments 266, 248, 144, 134; (305, 173, 95)

An. triannulatus s.l., PCR product 574 (633, 590); RFLP fragments 212, 186, 175 (257, 245)

All other species yielded a single RFLP pattern. The PCR-RFLP assays used in this study were successful in generating distinct diagnostic banding patterns for all individuals of each of the nine species tested. The restriction digest using *AluI* & *BsrBI1* (Figure 2) or *AluI* & *FspI* each resulted in a different diagnostic pattern for each species specific to that set of enzymes (Table 3).

To determine whether the diagnostic patterns varied within a species, an average of three siblings from each progeny brood raised was tested (Figure 3). In addition, for each species, at least five wild-caught females and/or adults raised from larval collections in areas surrounding our laboratory in Iquitos were tested for pattern comparison. In each case, RFLP patterns from wild-caught specimens coincided with laboratory-raised specimens in their diagnostic patterns. Both male and female adults, larval and pupal exuviae, and heat-killed larva samples resulted in the same pattern when tested. At least five specimens from each species were re-tested, which confirmed these patterns. A total of 93 progeny broods were tested and yielded no significant intraspecific variability (beyond that described for *An. benarrochi, An. konderi*, and *An. triannulatus* s.l.) that could lead to the misidentification of species by the pattern of restriction digests. In all, over 220 individuals were tested using the assay and none of these tests lead to ambivalent or equivocal findings using the assay. Every specimen tested with the RFLP assay was easily identified to species using its respective banding pattern.

DISCUSSION

Due to the problematic nature of identifying species of the subgenus *Nyssorhynchus* of *Anopheles* mosquitoes based on existing morphologic keys, molecular methods of identification are likely to prove useful as tools for confirming species identities and in identifying ambiguous or damaged field samples. Accurate species identification will likely hasten our understanding of vector malaria transmission and assist disease control strategies.

To address this challenge, we used published sequences of the ITS2 region of *Anopheles* mosquitoes to design a simple PCR-RFLP assay to differentiate among nine *Anopheles* species, six belonging to the subgenus *Nyssorhynchus* and three to the subgenus *Anopheles*, all of which are commonly found in the study region of Loreto, Peru. The species evaluated included principal, occasional, or possible vectors of malaria in geographical regions in the political department of Loreto and Ucayali, Peru. The molecular assay proved to be a rapid and accurate method of discrimination between species, was successful with all life stages including exuviae samples, and required only minute portions of the original sample. The tests also reduce the need to rear samples for morphologic diagnosis, thereby ensuring all samples, rather than just survivors, can be identified.

Recently, Zapata and others have developed a similar PCR-RFLP (single digest with *AluI*) assay that successfully distinguishes *An. rangeli*, *An. triannulatus*, *An. nuneztovari*, *An. darlingi*, and *An. albitarsus* among others.[28] We opted instead for a double digest due to the fact that *An. benarrochi* and *An. nuneztovari* were found to be indistinguishable (352 bp, 92 bp, 76 bp versus 352 bp, 90 bp, 73 bp respectively) following *in silico* digests of published sequences with *AluI* (AF462384.127 and AF461749.1[29]). Ruiz[12] had also developed a single digest RFLP assay using *HaeIII* to distinguish *An. benarrochi* from *An. oswaldoi* sensu lato but *in silico* digests of published sequences with *HaeIII* reveal that *An. oswaldoi* (AF055069.1)

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and *An. rangeli* (AF462382.1) would have bands of 457 bp, 45 bp and 449 bp, and 45 bp respectively, which would be indistinguishable on agarose gels. *In silico* digests of *An. nuneztovari* (AF461749.1) and *An. benarrochi* (AF462384.1) for *HaeIII* revealed products of 320 bp, 150 bp, 45 bp and 320 bp, 153 bp and 47 bp, respectively, suggesting that this digest would also fail to successfully differentiate these species. Our testing involved nine sympatric species of medical importance and no two species resulted in the same banding pattern. The assay appears to differentiate the species that are both important secondary vectors and the members of the subgenus *Nyssorhynchus* that are most difficult to distinguish from one another on morphologic characteristics of females. The ease of PCR-RFLP as well as its ability to give patterns for all *Nyssorhynchus* species tested makes this assay an alternative to the PCR multiplex assay described to differentiate *An. rangeli*, *An. trinkae*, *An. strodei*, and *An. triannulatus*.[30]

It is important to note that a number of species complexes have been described or suggested that include the species tested in our assay. *An. oswaldoi* s.l. may encompass at least four cryptic species as suggested by ITS2 analysis[11] of which *An. oswaldoi* sensu stricto (s.s.) and *An. konderi* are both a part, and of which the other two groups may constitute lineages or species but have not been previously described morphologically or taxonomically. Mitochondrial coenzyme I analysis has also revealed four cryptic species[31] but it should be noted that the taxonomic status in this species complex remains to be clarified. These species have been described as existing sympatrically or as isolated populations in different regions.[32] Based on morphology of male genitalia, we have confirmed the individuals used in this study to belong to *An. konderi*. This finding is supported by the suggestion that only *An. konderi*, and not *An. oswaldoi* sensu stricto (s.s.) has been found in Peru.[14] In addition, *in silico* restriction digests were performed (NEBcutter 2.0, New England Biolabs) using published sequences for *An. oswaldoi* (AF055069 and AY684976) and *An. konderi* sequence U92348 to compare with the pattern generated in the RFLP digest. Published sequences for *An. konderi*, in all instances, lack a restriction site for *AluI* that is present in *An. oswaldoi* sequences 88 bp from the 5′ end of the amplified fragment. Digestion with *AluI* resulted exclusively in two fragments (368 bp, 137 bp) as predicted with *An. konderi* sequences, not three fragments (278 bp, 136 bp, 88 bp) that would be expected with *An. oswaldoi*, further confirming the species identification. The *An. konderi* used in this study were caught in two locations, showing distinct biting behavior in each (caught via human landing catch or blood fed resting near cattle), both in forested areas along the Iquitos-Nauta highway. All samples, however, showed the same diagnostic banding pattern when digested with enzymes in RFLP analysis. Because our assay has only been applied to these samples collected in a limited geographic area a logical next step will be to apply this assay to other geographically distant specimens of other members of the oswaldoi complex. Our findings of the use of ITS2 analysis for clarifying relationships in the identification of mosquitoes in the oswaldoi complex are consistent with that of previous studies in the region.[12]

The *An. benarrochi* used for testing came from two separate localities in the departments of Loreto and Ucayali, in northeastern Peru. Ongoing morphometric analysis of female *An. benarrochi* siblings in our laboratory indicate the presence of two morphologically different populations, based on the ratio of the dark spot on hind tarsomere 2 to the entire length of that tarsomere (unpublished data), one group whose characteristics are similar to those studied in southern Colombia (0.17–0.33),[2] which may constitute the highly anthropophilic species designated as *An. benarrochi* B by Ruiz,[12] and the other whose ratios are more consistent of those described in traditional keys (0.40–0.55, > 0.45).[9,10] Both were collected biting humans in Ucayali, whereas only one specimen, falling in the smaller of the two ratio ranges, was collected in Loreto. Whether these groups constitute a species complex remains unclear, but work by Ruiz and others[12] supports this premise. Following the application of the PCR-RFLP assay, there were clear distinctions between the RFLP fragments generated by each

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morphotype (this difference was also visible by gel electrophoresis of the original PCR product, as noted in the results section concerning variable bands) for each enzyme. This difference supports the idea that the morphotypes represent species or insipient species. However, as only three progeny broods of the standard morphotype described in keys were available for testing, further assays will be needed to determine if these apparent genetic differences are significant. Testing species from different areas of the Amazon is a priority given the morphologic variability of *An. benarrochi*.

This PCR-RFLP assay designed for identification of *Anopheles* proved quick, accurate, and highly affordable. The success of this study shows the feasibility of molecular identification tools to aid in situations where traditional taxonomic methods yield equivocal or conflicting findings. To be applicable on a national and international level within the Neotropics, further studies should include a larger number of species tested, a wider range of geographical distribution from which samples are collected, and sequence analysis for comparison with previously published results. Such work will give insight to important, yet problematic, species complexes and morphologic variants.

Acknowledgements

The authors thank Maribel Paredes Olortegui and ST Unit for logistical support and Graciela Meza Sanchez and Carmen Montalvan for critical review and encouragement.

Financial support: The work was supported by the Malaria Research Institute from the Department of Molecular Microbiology and Immunology at Johns Hopkins (PI: Margaret Kosek). Ryan Matson was supported by T37MD001580. Margaret Kosek is supported by the Fogarty International Center K01 TW05717 and the project was further supported by R01AI067727 and K24AI068903 to Joseph M. Vinetz.

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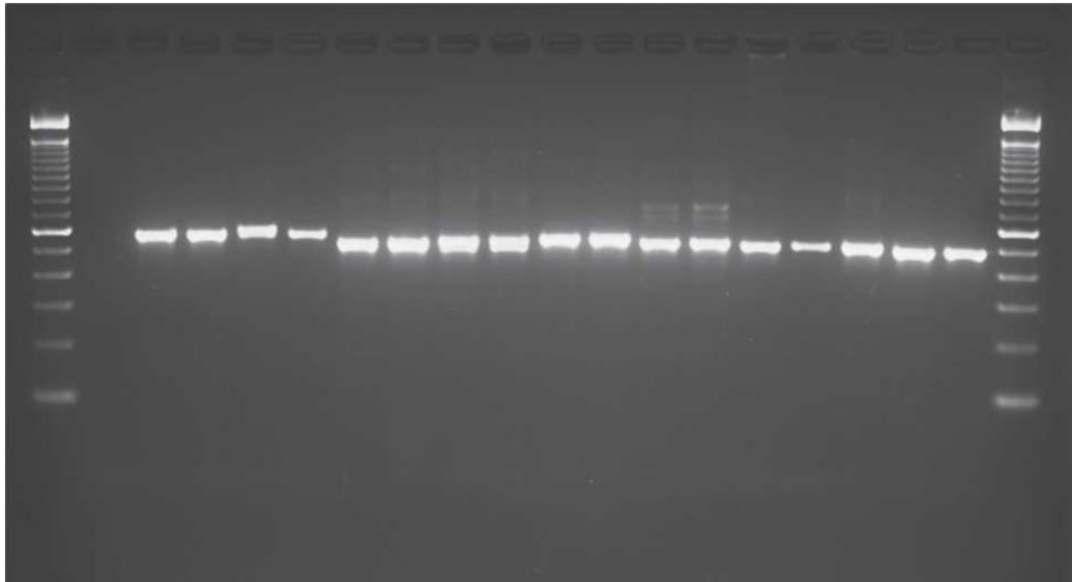

Figure 1.

PCR amplicons. Lane contents: 1&20 100 bp ladder; 2 negative control; 3&4 *A. darlingi*; 5&6 *An. triannulatus* s.l.; 7&8 *An. konderi*; 9&10 *An. benarrochi*; 11&12 *An. nuneztovari*; 13&14 *An. rangeli*; 15 *An. peryassui*; 16&17 *An. mattogrossensis*; 18&19 *An. Forattinii.*

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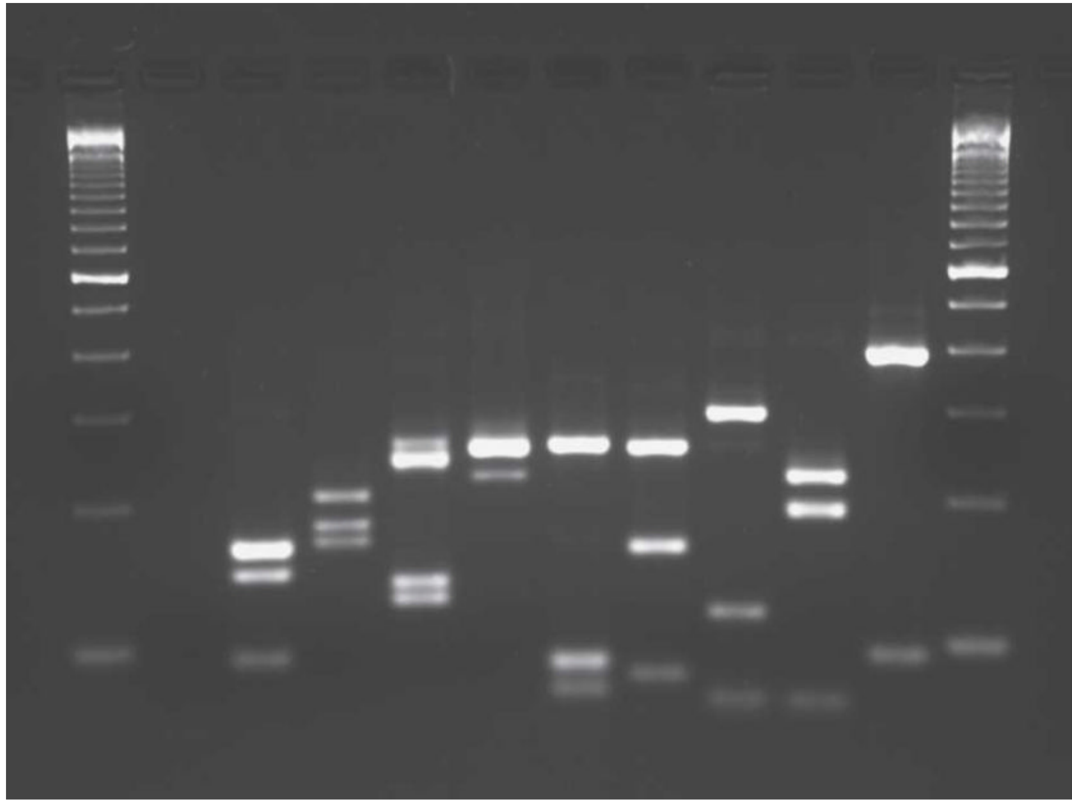

Figure 2.

RFLP diagnostic banding patterns using *BsrBI* & *AluI*. Lane contents: 1&12 100 bp ladder; 2 negative control; 3 *An. darlingi*; 4 *An. triannulatus* s.l.; 5 *An. konderi*; 6 *An. benarrochi*; 7 *An. nuneztovari*; 8 *An. rangeli*; 9 *An. peryassui*; 10 *An. mattogrossensis*; 11 *An. forattinii*. Band sizes in Table 3.

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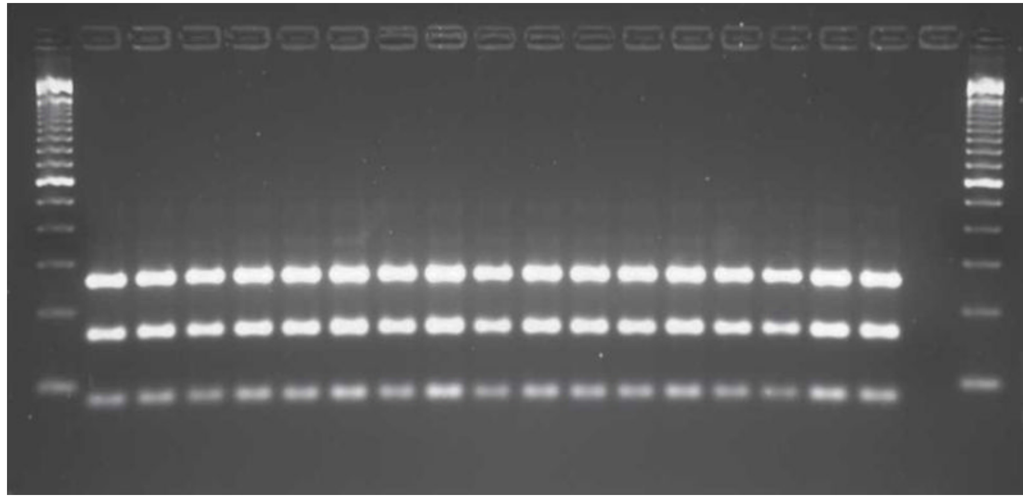

Figure 3.

RFLP of *An. rangeli* individuals using *BsrBI* & *AluI*. One individual was taken from each of 21 progeny broods (17 shown here) and tested to determine if variability existed between individuals or geographic distribution. All lanes show the same banding pattern with bands at 263 bp, 168 bp, and 83 bp. The collection location is listed in parentheses. Lane contents: 1&20 100 bp ladder; 2–15 *An. rangeli* (Quistococha); 16&17 *An. rangeli* (Moralillo); 18 *An. rangeli* (San Jose), 19 negative control.

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Table 1

Anopheline species analyzed, listed with geographic origin, capture date, and number of progeny broods reared

Genus (subgenus) species	Country	State	Town (collection area) in	Coordinates* Easting	Coordinates* Northing	Date	Progeny broods
Anopheles (*Nyssorhynchus*) benarrochi Gabaldon, Cova Garcia & Lopes	Peru	Ucayali	San Jose (town)	544059.940	907832.570	May 2007	20
	Peru	Loreto	Quistococha (cattle ranch)	685613.541	9577561.131	May 2007	1
Anopheles (*Nyssorhynchus*) *darlingi* Root	Peru	Loreto	Zungarococha (town)	683171.196	9576959.971	May 2007	4
Anopheles (*Nyssorhynchus*) *nuneztovari* Gabaldon	Peru	Loreto	Zungarococha (forest)	683376.154	9577109.143	April 2007	12
	Peru	Loreto	Quistococha (cattle ranch)	685613.541	9577561.131	May 5, 2007	1
Anopheles (*Nyssorhynchus*) *konderi* Galvão & Damasano)	Peru	Loreto	Moralillo (forest)	684720.851	9567751.324	April 2007	17
	Peru	Loreto	Moralillo (cattle ranch)	684141.266	9568458.198	May 2007	2
Anopheles (*Nyssorhynchus*) *rangeli* Gabaldon, Cova Garcia & Lopes	Peru	Loreto	Moralillo (cattle ranch)	684141.266	9568458.198	May 2007	2
	Peru	Loreto	Quistococha (cattle ranch)	685613.541	9577561.131	May 2007	17
	Peru	Ucayali	San Jose (town)	544059.940	907832.570	May 2007	2
Anopheles (*Nyssorhynchus*) *triannulatus* Neiva & Pinto	Peru	Loreto	Puerto Almendras (forest)	680548.746	9576497.479	March 2007	1
	Peru	Loreto	Zungarococha (forest)	683376.154	9577109.143	April 3 and May 2007	7
Anopheles (*Anopheles*) *forattinii* Wilkerson & Sallum	Peru	Loreto	Puerto Almendras (forest)	680548.746	9576497.479	March 2007	4
Anopheles (*Anopheles*) mattogrossensis Lutz & Neiva	Peru	Loreto	Moralillo (forest)	684720.851	9567751.324	March 2007	2
Anopheles (*Anopheles*) *peryassui* Dyar & Knab	Peru	Loreto	Quistococha (cattle ranch)	685613.541	9577561.131	May 2007	1

* Coordinate System: UTM. Zone: 18 South. Datum: WGS1984. Units: meters.

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Table 2

Predicted restriction fragments following *in silico* digestion with *BsrBI* and *AluI* based on GenBank sequences

Species	GenBank accession	PCR product	Expected restriction fragment sizes (*BsrBI* & *AluI*)				
			Fragment 1	Fragment 2	Fragment 3	Fragment 4	Fragment 5
An. albitarsis[*]	AF462385	508	264	159	79	6	
An. aquasalis	AF462376	500	255	134	69	37	5
An. benarrochi	AF462383	523	259	172	92		
An. braziliensis	AF461753	504	262	242			
An. darlingi	AF462389	562	164	162	144	92	
An. deaneorum[*]	AF461751	508	264	159	79	6	
An. dunhami	AF462378	494	233	136	125		
An. eiseni	AF462380	484	222	190	72		
An. Evansae[*†]	AF461750	517	258	93	92	74	
An. forattinii	n/a	n/a	n/a	n/a	n/a	n/a	n/a
An. konderi[†]	U92348[*]	505	243	137	125		
An. mattogrossensis	AF461754	482	220	190	72		
An. mediopunctatus	AF462379	476	476				
An. nuneztovari[*]	AF461749	515	258	94	90	73	
An. oswaldoi	AF055069	502	153	136	125	88	
An. peryassui	AF461755	483	294	117	72		
An. rangeli	AF462382	494	254	157	83		
An. triannulatus	AF462377	551	205	180	166		

[*] *An. albitarsis* and *An. deaneorum* may be indistinguishable using the PCR-RFLP digest described herein as predicted fragment sizes from *in silico* digests are identical. Similarly, *in silico* predicted fragments of *An. nuneztovari* and *An. evansae* would be difficult to distinguish due to only small differences in fragment sizes.

[†] *An. darlingi* sequence on flanking ends added to reach primers used (149 bp added).

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Table 3

Primary amplicon size and digestion products using two restriction enzyme combinations reveals species restriction of PCR-RFLP patterns for all Anopheline species evaluated. *An. benarrochi* var denotes a second pattern that correlated with morphometric differences in females when compared with *An. benarrochi*

Species	PCR primary fragment	RFLP *AluI/FspI*				RFLP *AluI/BsrBI*			
		Fragment 1	Fragment 2	Fragment 3	Fragment 4	Fragment 1	Fragment 2	Fragment 3	Fragment 4
An. benarrochi[*]	518	261				459	45		
An. benarrochi Var	520,492	263	232			446	432	42	
An. darlingi	581	169	151	96		317	172	101	
An. nuneztovari	517	261	94	76		303	93	77	42
An. konderi[*]	520	266	248	144	134	277	114	84	45
An. rangeli	506	263	168	83		291	169	43	
An. triannulatus[*]	574	212	186	175		217	190	120	47
An. forattinii	482	391	94			435	41		
An. mattogrossensis	485	225	194	64		231	159	44	10
An. peryassui	497	305	124	69		305	124	69	

[*] Denotes that a variable band is often present in addition to listed band.