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Molecular identification of Anopheles spp. (Diptera: Culicidae) in the Chapare/Carrasco Valleys, Cochabamba, Bolivia

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Molecular Identification of *Anopheles* spp. (Diptera: Culicidae) in the Chapare/Carrasco Valleys, Cochabamba, Bolivia.

By

Corey Brelsfoard

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

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2005

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Molecular Identification of *Anopheles* spp. (Diptera: Culicidae) in the Chapare/Carrasco Valleys, Cochabamba, Bolivia

By

Corey Brelsfoard

Eastern Illinois University

ABSTRACT

Studies on malaria in our "backyard," the Neotropics, have been hampered by the lack of an accurate means of species identification for mosquito vectors of the human malaria parasite (i.e. *Plasmodium* spp). Mosquitoes in the genus *Anopheles* are the principle malaria vectors in the Neotropics and are often composed of closely related morphologically similar species complexes. This thesis describes three polymerase chain reaction (PCR) protocols to aid in identifying four anopheline mosquitoes for any life stage, of which three have been incriminated as vectors of human malaria in several locations throughout S. America and might be potential vectors of human malaria in the Chapare and Carrasco valleys of Cochabamba, Bolivia. The first chapter describes a protocol that involves a multiplex PCR with two species specific primers that anneal within the internal transcribed spacer 2 (ITS2) to identify two members of the unresolved Oswaldoi species complex in the subgenus *Nyssorhynchus.* The second chapter describes a PCR protocol that produces a single amplicon of a section of the ITS2 specific for members of the Albitarsis species complex in the subgenus *Nyssorhynchus*; digestion of the amplicon with the restriction endonuclease Bfa-I produces two fragments specific to *An. marajoara* in Bolivia. In addition, I report a new species record for *An. marajoara* in Bolivia. The third chapter describes a PCR protocol that produces a single amplicon of a section of the ITS2 specific for *An. fluminensis,* a member of the Arribalzagia Series in the subgenus *Anopheles*. Examination of ITS2 sequences showed the existence of three short repeat units (aprox. 125 bp) within the ITS2 of *An. fluminensis*. This study also records the presence of *An. fluminensis* for the first time in Cochabamba, Bolivia. The PCR protocols described in this thesis, then, will simplify the task of identifying four

anopheline species and facilitate studies that aim to elucidate their basic biology, ecology, and behavior.

DEDICATION

To

My Parents

ACKNOWLEDGEMENTS

I would like to thank Dr. G. Fritz for providing me with this opportunity to be involved in such an exigent and enlightening project. In addition, I would like to thank him for is advice and guidance throughout the project, which was vital to my success. I would also like to thank the Bolivian Ministerio de Salud, the Cochabamba Ministerio de Salud, Dr. Rene Mollinedo, Dr. E. Vallejo, C. Lopez, Ing. M. Ledezma, Ing. G. Joffre and R. de Michel. I would also like to acknowledge R. Rodriguez for his immense help in collecting specimens and for his hospitality in my stay in Bolivia. Moreover, I would like to thank C. Li and R. Wilkerson for providing additional required specimens and assistance in identifying unknown specimens.

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CHAPTER 1

Introduction

1.1 Literature Review

The mosquitoes of the genus *Anopheles* (subfamily Anophelinae) are vectors of human malaria, an important tropical disease that threatens >40% of the world's population in >90 countries and territories (Sallum et al. 2000). It is estimated to kill 1-3 million people each year (World Health Organization 1996). This figure includes ~1,000,000 child deaths per year, which can be attributed to acute malaria alone or in association with other diseases (Sallum et al. 2000).

A major breakthrough in understanding malaria was made a hundred years ago by Sir Ronald Ross, who demonstrated that the malarial parasites, *Plasmodium* species, are transmitted by mosquitoes (Mitchell, 1901; Walton et Al 1999). Since Ross's discovery, one of the most successful methods of malaria prevention and eradication has been through control of the mosquito vector (Walton et al. 1999). The four *Plasmodium* spp. that cause human malaria are transmitted by mosquitoes in the genus of *Anopheles*; therefore, anopheline mosquitoes have been studied throughout the world. The number of *Anopheles* species recognized currently stands at almost 500, many of which occur as complexes of closely related, morphologically similar species distributed throughout the globe (Harbach 1994; Gwadz & Collins 1996).

Mosquitoes of a species complex are genetically distinct from each other yet appear morphologically indistinguishable; hence the term 'cryptic species' is often applied to them (Walton et al. 1999). The existence of cryptic species of *Anopheles* was first realized in the 1930s from the work of Grassi, Roubaud, and others in Europe

(Hackett 1937). Before that time, it was a mystery why malaria was more prevalent in some areas of Europe than others, though the same species of mosquito was present. Due to the discovery of cryptic species, it became clear that some species in such a complex had little, if any, capacity to transmit malaria (Walton et al. 1999).

Biological differences that exist between species of a complex, such as host preference, habitat preference, longevity, and refractoriness to *Plasmodium* spp., are key factors in determining whether a particular species is a vector of malaria (Walton et al. 1999). However, one of the major obstacles in studies attempting to determine the basic biology, behavior, and ecology of vectors of human *Plasmodium* spp. in the neotropics has been the difficulty of species identification. The identification of cryptic mosquito species is, therefore, an essential tool in epidemiological studies of transmission (Walton et al. 1999).

The vectors of malaria are well known in Africa and some regions of Asia, but significantly less is known about the transmission of this disease in our own "backyard," the Neotropics. Unlike Africa, where two or three species are responsible for most transmission in the continent (Gillies and Coetzee 1987), the epidemiology of malaria in the Neotropics is more complex due, in part, to the diversity of potential vectors (Arruda et al. 1986, Hayes et al. 1987, Haworth 1988, Branquinho et al. 1993, Lounibos and Conn 2000).

In the life cycle of anopheline mosquitoes there are four main developmental stages, i.e. eggs, larvae, pupae, and adult. The immature forms (eggs, larvae and pupae) are aquatic and may inhabit simple puddles or marshes, or large hydric collections such as rivers, lakes, lagoons etc. Physicochemical characteristics such as temperature, sun

incidence, vegetation, and microfauna, are some of the important factors of aquatic systems that may affect development of different anopheline species; these factors, and others, contribute to the diversity and distribution of species in the neotropics where the ecology is complex (Forattini 1962).

Historically, most studies on the epidemiology of malaria have examined adults, particularly females (since this is the only sex that feeds on blood and is attracted to traps) and have used morphological keys (e.g., Faran 1980) to identify specimens. According to Conn (1990) morphological keys for female mosquitoes are unreliable for many species due to intraspecific variation in characters. Both the investigation of genetic variability and the discovery and identification of cryptic species have relied primarily on cytotaxonomy, crossing experiments, and the use of molecular tools such as enzyme electrophoresis or DNA probes (Kitzmiller 1976; Hahon et al. 1976; Narang et al. 1989; Booth et al. 1991; Fritz et. al. 1994). Recent advances in DNA analysis, such as the polymerase chain reaction (PCR) and improved cloning and sequencing techniques, have facilitated the application of molecular biology to taxonomic problems (Porter and Collins 1991). New methods that are most widely used to detect and differentiate such cryptic species include mating incompatibility, morphometric analysis, polytene chromosome analysis, isoenzymes electrophoresis (Coluzzi et al. 1979, Collins et al. 1988, Rosa-Freitas et al. 1990, Coetzee et al. 1993, Foley and Bryan 1993), and DNA sequence analysis (Conn et al. 1993, Fritz 1997).

In a previous study done by Porter and Collins (1990) species-specific differences in restriction site patterns in the rDNA were reported between the cryptic species *An. freeborni* and *An. hermsi.* A recognition site for the restriction endonuclease *Pst*I that is

absent from *An. hermsi* was detected in the internal transcribed spacer 2 (ITS2) of *An. freeborni*. In a further study by Porter and Collins (1991) the extent of interspecies differences over the entire ITS2 sequence of these two species was reported. Their method of species diagnosis took advantage of the fact that the internal spacers are flanked by highly conserved ribosomal RNA coding regions, permitting amplification by PCR with primers derived from published sequences of even distantly related organisms (Porter and Collins 1991). The sequence information generated from the resulting PCR products is useful not only for generating diagnostic PCR primers, but can also clarify systematic relationships. Because of the relatively rapid rate at which new mutants are fixed in the rDNA spacers, these regions may distinguish closely related species that otherwise show little genetic divergence (Brown et al. 1972; Furlong and Maden, 1983; Tautz et al. 1987; Porter and Collins 1991). Also, rDNA is present in hundreds of tandem copies per cell nucleus in most multicellular organisms, thus providing greater sensitivity in PCR. Multiple copies of the rDNA units also undergo homogenization and concerted evolution. Therefore, PCR-based species diagnostics using rDNA spacer sequences offer a good general solution to the problem of 'cryptic species' identification (Porter and Collins 1991).

There are five species groups of anophelines that are responsible for most transmission of human *Plasmodium* spp. in the Neotropics; four of these groups are in the subgenus *Nyssorhynchus*. These four groups are the Albimanus, Albitarsis, Argyritarsis, and Oswaldoi groups. Within these four groups there are at least 27 species (Faran 1981, Peyton et al. 1992, Harback 1994), many of which are difficult to identify reliably using

morphological keys in any life stage, justifying the need for a quick and accurate molecular means of identification.

To date, there are no species within the subgenus *Nyssorhynchus* that have been shown to have identical ITS2 sequences, though the size of the ITS2 has generally not diverged substantially in size. Therefore, it is not feasible to distinguish species solely on the size of the complete ITS2 amplified by primers found in the conserved regions flanking the ITS2. Rather, primers elaborated from species-specific differences within the ITS2 are required that generate amplicons of discernable size difference on standard agarose gels. Fritz et al. (2004), for example, used such an approach to develop the first diagnostic multiplex PCR reaction to identify species in the subgenus *Nyssorhynchus*:*An. triannulatus, An. trinkae, An. rangeli,* and *An. Strodei.* Subsequently, Li and Wilkerson (personal communication) developed a series of PCR reactions to identify four *Nyssorhynchus* species in the Albitarsis complex, which include *An. marjoara* Galvão and Damasceno, *An. albitarsis* Lynch-Arribálzaga (Linthicum 1988), *An. albitarsis* B (Wilkerson 1995 a,b), and *An. deaneorum* Rosa-Freitas. There are, otherwise, no published DNA-based methods for identifying *Nyssorhynchus* spp.

1.2 Study Site

This study is part of a larger project examining the ecology of anopheline larvae in the Chapare and Carrasco Provinces, in the department of Cochabamba, Bolivia. Bolivia is one of South America's most isolated and poorest countries. In 1995 27,475 cases of malaria were reported in Bolivia (Velasco and Soriano 1998). Politically, Bolivia is divided into 9 departments, Cochabamba, Pando, Beni, La Paz, Oruro, Santa Cruz, Potosi, Chuquisaca, and Tarija (Fig. 1.1). Seven of the nine departments are

malarious; only residents of the most mountainous Andean departments, Oruro and Potosi, are free from the risk of malaria (Fig. 1.1). Most cases of malaria in Bolivia occur in the departments of Beni and Cochabamba. Cochabamba is divided into 16 provinces: Arque, Cercado, Campero, Ayopaya, Esteban Arce, Capinota, German Jordan, Quillacollo, Tapacari, Chapare, Carrasco Tropical, Carrasco Valley, Mizque, Punatai, Bolivar, and Tiraque. Of these provinces, the Chapare and Carrasco Valley (contiguous provinces) have the highest malaria incidence rates (between 8.1 to 19 recorded infections per 1000 individuals between 1996-97) (Velasco and Soriano 1998) (Fig. 1.2). Furthermore, a number of species in the subgenus *Nyssorhynchus* that have been incriminated/suspected as vectors of malaria elsewhere in South America have been collected in the Chapare/Carrasco provinces including: *An. evansae* Brethes (Fonseca an Uniti 1943), *An. nuneztovari* Galbaldon (Elliot 1972), *An. albitarsis* Lynch-Arribálzaga (Linthicum 1988), *An. oswaldoi* Peryassu (Branquinho et al. 1993, Klein 1991), *An. benarrochi* Gabadon, Covia Garcia and Lopez (Need et al. 1993), *An. trinkae* Faran (Hayes et al. 1987), *An. rangeli* Gabadon, Covia Garcia and Lopez (Hayes et al. 1987), *An. konderi* Galvao and Damasceno (Klein et al. 1992), *An. strodei* Root (Correa 1938), *An. marajoara* Galvão and Damasceno (Conn et al. 2002, Rubio-Palis et al. 1992), and *An. triannulatus* (Neiva and Pinto) (Arruda et al. 1986). Species in two other subgenera, *Kertezia* and *Anopheles*, are also found in the Chapare/Carrasco provinces and include vectors of human *Plasmodium*, but their overall impact is small relative to the species within the subgenus *Nyssorhynchus* (Lounibos and Conn 2000).

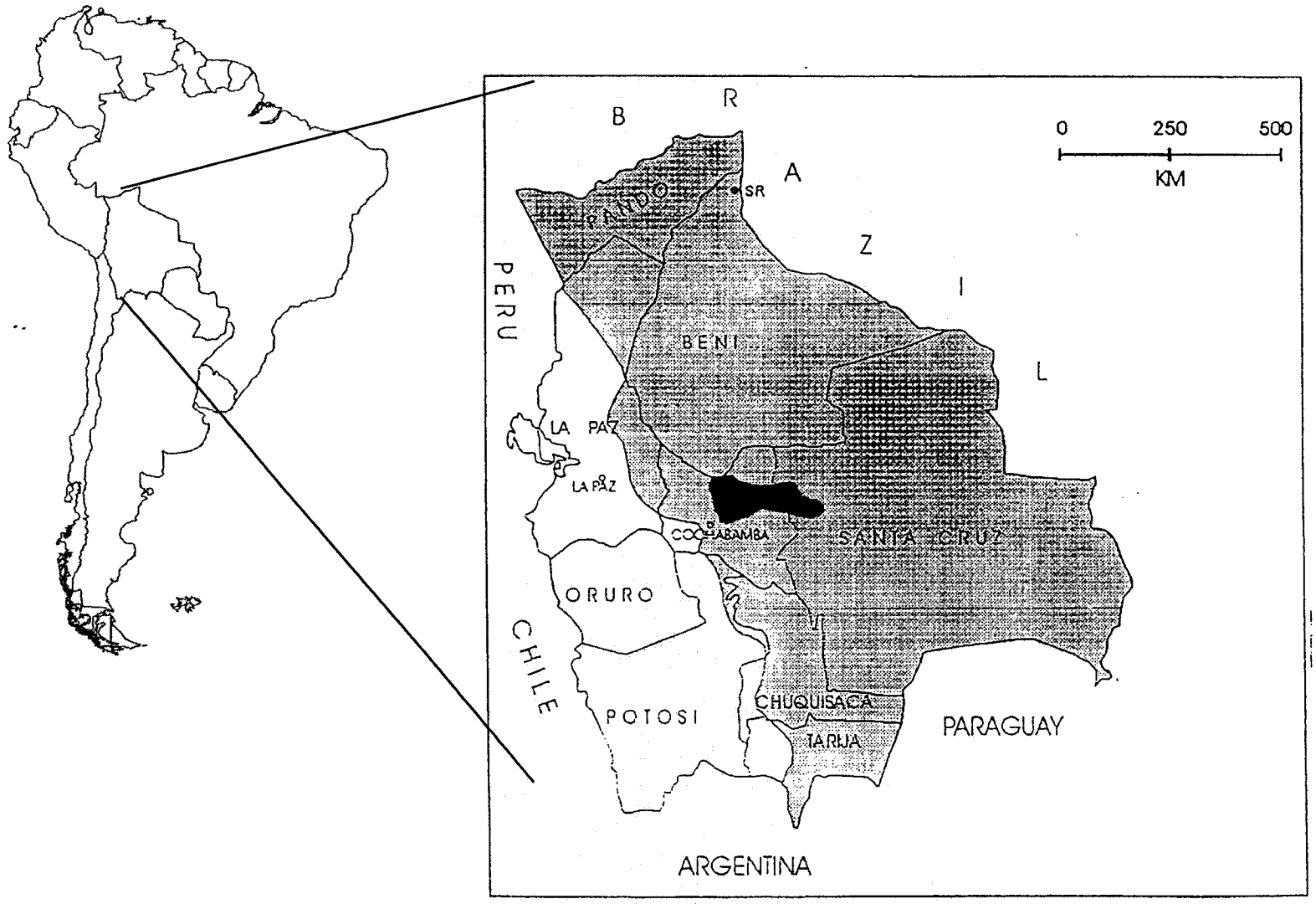

Fig. 1.1. Map of Bolivia showing the nine departments and the distribution of malaria (shaded region) based on government statistics (Ministry of Health 1998). The darkly shaded area of the Department of Cochabamba encompasses the river valley region of the provinces of Chapare Carrasco.

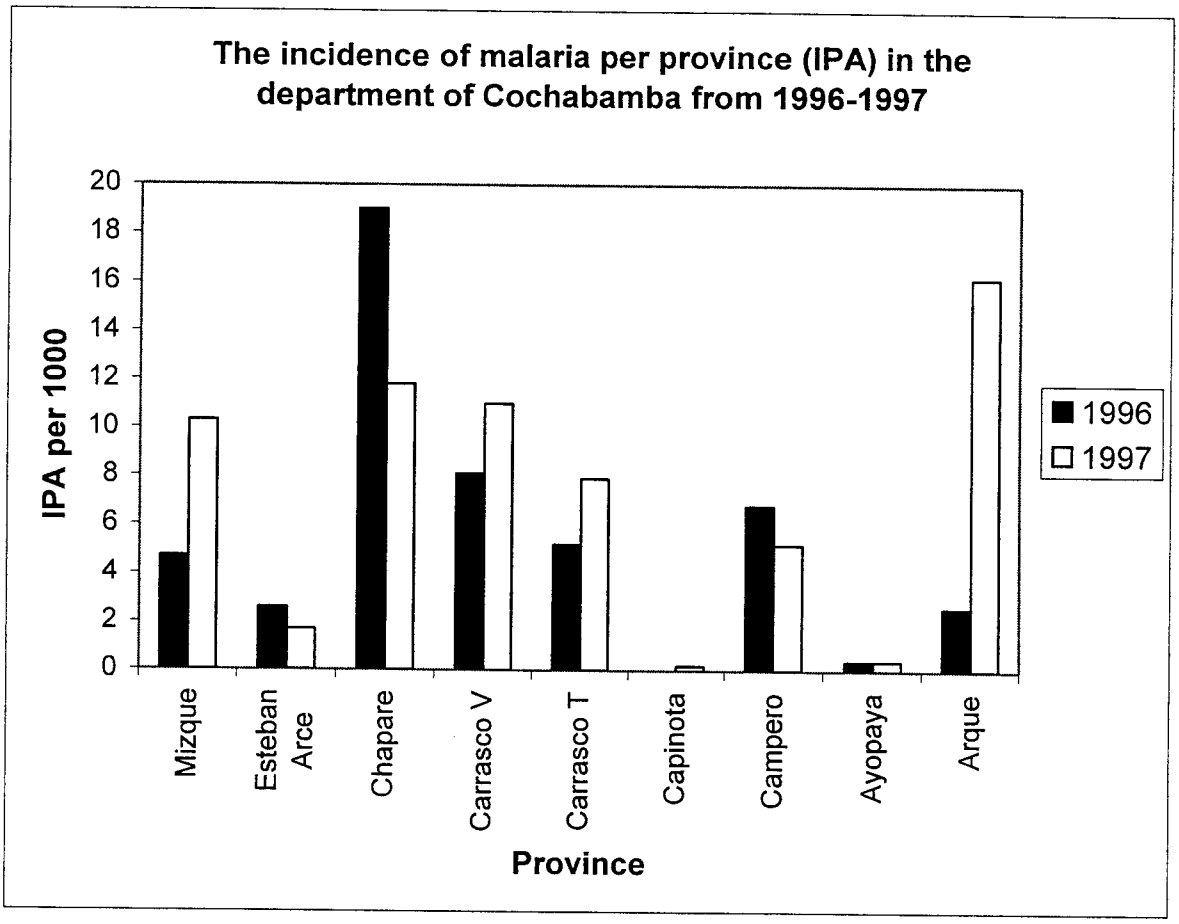

Fig.1.2 The incidence of malaria per province in the department of Cochabamba from 1996-1997.

The Chapare/Carrasco valley region with its relatively high incidence of malaria in the Chapare/Carrasco valleys, high diversity of anopheline mosquitoes, and varied ecology (piedmont to savannah grasslands), make this region of Bolivia a good model locality for examining the potential complexities of the epidemiology of this disease. Furthermore, the Chapare and Carrasco provinces are of particular interest to malariologists because they illustrate regional trends along the eastern slopes of the Andes Mountains that affect the epidemiology of malaria: intense logging of rainforest with concomitant conversion to secondary forest, livestock grazing, and monoculture, sustained by a seasonal migrant labor force. In this altered landscape, anopheline mosquito densities can increase many-fold (Tadei et al. 1998), and migrant laborers increase the possibility of chronic pathogen introduction (Conn et al. 2000). During the last ten years, the uncoordinated settlements and the consequent deforestation, especially in large urban centers, have been the main cause for the emergence or re-emergence of malaria in South America (Povoa et al. 2000).

The Chapare Valley begins in a gorge within the foothills of the Andes Mountains and is considered a sub-andean rain forest region intersected by large river systems and with relatively high temperatures (mean annual temperatures ranging from 20 to 25 °C) and some of the higest mean precipitations in the World (as high as 560 cm per year in Villa Tunari in the Chapare valley). The Chapare can be divided into three major portions: the upper portion, composed of piedmont and low hills, dense subtropical forest on steep slopes rising above numerous small valleys and streams. The middle portion, a stabilized alluvial plain of approximately the same size as the upper portion, containing high terraces with good drainage and is a continuation of the piedmont. Finally, the

lower plain, which covers almost three-fourths of the area, has mainly a sedimentary geology from erosion of the eastern slopes of the Andes. The soils are fertile, but annual floods undercut agricultural potential as the lower terraces are continually inundated. The topography of the valley is complex insofar as the elevation varies from around 600 m to 200 m above sea level. All of the aforementioned attributes illustrate the complex ecological habitat in the Chapare/Carrosco system (Velasoc and Soriano 1998). Therefore, the Chapare Valley provides a good model system for examining and understanding vector dynamics a region of the Neotropics where there is a great diversity of potential vector species and ecological habitats, concomitant with the effects of human activity on the ecology of the rainforest.

1.3 Objectives/Specific Aims

The aims of the project in Bolivia are to: 1) to determine species composition in the Chapare/Carrasco Valley system, 2) to determine species distributions, 3) correlate environmental variables in breeding habitats to species distributions, 4) develop DNA-based diagnostics for identifying species. My role in this project was the latter and specifically included:

- Development of species-specific primers for PCR-based identifications of some anopheline mosquitoes found in the Chapare/Carrasco Valley, Bolivia.
- Combine species-specific primers into multiplex reactions that can identify two or more specimens.

This thesis is organized into three subsequent sections that represent three manuscripts to be submitted to the Journal of Medical Entomology and the American

Journal of Tropical Medicine and Hygiene. Each section, therefore, includes portions of a manuscript including references.

1.4 References Cited

Booth, D. R., R. J. Mahon, and K. S. Sriprakash. 1991. DNA probes identify members of the Anopheles farauti complex. Med. Vet. Entomol. 5: 447-454.

Branquinho, M. S., C. B. T. Lagos, R. M. Rocha, D. Natal, J. M. S. Barata, A. H. Cochrane, E. Nardin, R. S. Nussenzweig, and J. K. Kloetzel. 1993. Anophelines in the state of Acre Brzil infected with *Plasmodium faciparum, P. vivax,* the variant *P. Vivax* VK247 and *P. malariae.* Trans. Soc. Med. Hyg. 87: 391-394.

Brown, D. D., Wensink, P. C. and Jordan, E. 1972. A comparison of the ribosomal DNA's of *Xenopus laevis* and *Xenopus mulleri*: the evolution of tandem genes. J. Mol. Biology. 63: 57-73.

Coetzee, M., R. H. Hunt and L. E. Braak. 1993. Enzyme variation at aspratate aminotransferase locus in members of the *Anopheles gambiae* complex (Diptera: Culicidae). J. Med. Entomol. 39: 303-308.

Collins, F. H., V. Finnerty and V. Petrarca. 1988. Ribosomal DNA probes differentiate five cryptic species in the *Anopheles Gambiae* complex. Passitologia 30: 231-240.

Collins F. H., C. H. Porter, S. E. Cope. 1990. Comparison of rDNA and mtDNA in the sibling species *A. freeborni* and *A. hermsi.* Am. J. Trop. Med. Hyg. 42: 417-423.

Colluzzi, M., A. Sabatini, V. Petrarca and M. A. Di Deco. 1979. Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex. Trans. R. Soc. Trop. Med. Hyg. 73: 483-497.

Conn, J. E., R. C. Wilkerson, M. N. O. Segura, R. T. L. De Souza, C. D. Schlichting, R. A. Wirtz, and M. M. Póvoa. 2002 Emergence of a new Neotropical malaria

vector facilitated by human migration and changes in land use. Am. J. Trop. Med. Hyg. 66(1): 18-22.

Conn, J. 1990. A genetic study of the malaria vector *Anopheles nuneztovari* from *western Venezuela.* J. of the American Mosquito Control Association 6: 400-405.

Conn, J., A. F. Cockburn and S. E. Mitchell. 1993. Population differentiation of the malaria vector *Anopheles aquasalis* using mitochondrial DNA. J. Hered. 84: 248-253.

Corrêa, R. R. 1938. O *Anopheles* (N.) *strodei* Root, 1926 como provável vector de malária. Rev. Biol. Hyg. 9:104-109.

de Arruda, M., M. B. Carvalho, R. S. Nussenzweig, M. Mracic, A. W. Ferreira, And A. H. Cockrane. 1986. Potential vectors of malaria and their different susceptibility to *Plasmodium faciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. Am. J. Trop. Med. Hyg. 35: 873-881.

Elliot, R. 1972. The influence of vector behavior on malaria transmission. Am. J. Trop. Med. Hyg. 21: 755-763.

Faran, M. E. and K. J. Linthicum. 1981. A handbook of the Amazonian species of *Anopheles (Nyssorhynchus)* (Diptera: Culicidae). Mosq. Syst. 13: 1-81.

Foley, D. H. and J. H. Bryan. 1993. Eletrophoretic keys to identify members of the *Anopheles punctulatus* complex of vector mosquitoes in Papua New Guinea. Med. Vet. Entomol. 7: 49-53.

Fonseca, J. A. B., O. Unti. 1943. Infeccão experimental de anofelinos de regiões indenes à malaria. Folia Clin. Biol. 15: 43-52.

Forattini, O. P. 1962. Família Culicidae, p 173-301. In Entomologia médica. O.P.

Forattini ed, EDUSP, Sâo Paulo.

Fritz, G. N., J. Conn, A. Cockburn, and J. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). Mol. Biol. Evol. 11(3): 406-416.

Fritz, G. N. 1997. Sequence Analysis of the rDNA internal transcribed spacer 2 of five species of South American human malaria mosquitoes. J. Sequencing and Mapping 84: 215-221.

Fritz, G. N., Engman, S. Rodriguez, R., and Wilkerson R. C. 2004. Identification of four vectors of human *Plasmodium spp.* by multiplex PCR: *Anopheles rangeli, A. strodei, A. triannulatus,* and *A. trinkae* (Diptera: Culicidae: *Nyssorhynchus*) J. Med. Ent. 41(6): 1111-1115.

Furlong J. C. and B. E. H. Madden. 1983. Patterns of major divergence between internal transcribed spacers of ribosomal DNA in *Xenopus borealis* and *Xenopus laevis*, and of minimal divergence within ribosomal coding regions. EMBO J. 2: 443-448.

Gillies, M.T., M. Coetzee. 1987. A supplement to the Anophelinae of Africa south of the Sahara (Afrotropical region). S. Afr. Inst. Med. Res. 55: 143.

Gwadz R. and F. H. Collins. 1996. Anopheline mosquitoes and the agents they transmit. In: Beaty B.J. Marquardt W. C., eds. The biology of disease vectors. Niwot: University Press of Colorado, 73-84.

Hackett L. W. 1937. Malaria in Europe Oxford: Oxford University Press.

Harbach R. E. 1994. Review of the internal classification of the genus *Anopheles* (Diptera: Culicidae): the foundation for comparative systematic and phylogenetic

research. Bulletin of Entomological Research 84: 331-342.

Haworth, J. 1988. The global distribution of malaria and the present control effort, pp. 1379-1420. *In* W. H. Wernsdorfer and I. McGregor (eds.), Malaria: principles and practices of marlariology, vol. II Churchill Livingston, New York.

Hayes, J., G. Calderon, R. Falcon, and V. Zambrano. 1987. Newly incriminated anopheline vectors of human malaria parasites in Junin Department, Peru. J. Am. Mosq. Contr. Assoc. 3:418-422.

Kitzmiller, J. B. 1976. Genetics, cytogenetics and evolution of mosquitoes. Ad. Genet. 18: 316-433.

Klein, T. A., B. P. Lima, A. Toda Tang. 1992. Vector incrimination and effects of antimalarial drugs on malaria transmission and control in the Amazon basin of Brazil. Mem. Inst. Osw. Cruz 87: 393-397.

Klein, T. A., J. B. P. Lima, M. S. Tada. 1991. Comparative susceptibility of anopheline mosquitoes in Rondonia, Brazil to infection by *Plasmodium vivax.* Am. J. Trop. Med. Hyg. 45: 463-470.

Linthicum, K. J. 1988. A revision of the Argyritarsis Section of the subgenus *Nyssorhynchus* of *Anopheles* (Diptera: Culicidae) Mosquito Systematics 20: 98-271

Lounibos, L. P., R. C. Wilkerson, J. E. Conn, L. J. Hribar, G. N. Fritz and J. A. Danoff-Burg. 1998. Morphological, molecular, and chromosomal discrimination of cryptic *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae) from South America. J. Med. Ent. 35: 830-838.

Lounibos, L. P. and J. Conn. 2000. Malaria vector heterogeneity in South America.

Amer. Ent. 46: 238-249.

Mitchell E. G. 1907. Mosquito Life. New York and London: GP Putman's Sons, The Knickerbocker Press.

Narang, S. K., S. R. Toniolo, J. A. Seawright, and P. E. Kaiser. 1989. Genetic differentiation among sibling species A, B, and C of the *Anopheles quadrimaculatus* complex (Diptera: Culcidae). Ann. Entomol. Soc. Am. 82: 508-515.

Need, J. T., R. A. Wirtz, E. D. Franke, R. Fernandez, F. Carbajal, R. Falcon, E. San Roman. 1993. *Plasmodium vivax* VK247 and VK210 circumsporozite proteins in Anopheles mosquitoes from Andoas, Peru. J. Med. Ent. 30(3): 597-600.

Peyton, E. L., R. C. Wilkerson and R. E. Harbach. 1992. Comparative analysis of the subgenera *Kertesia* and *Nyssorhynchus* of *Anopheles* (Diptera:Culicidae). Mosq. Syst. 24: 51-69.

Porter C. H., F. H. Collins. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles Hermsi* (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 45(2): 271-279.

Porter, C. H., F. H. Collins. 1990. Susceptibility of *Anopheles hermsi* to *Plasmodium vivax*. Am. J. Trop. Med. Hyg. 42: 414-416.

Povoa, M. M., Ana de N. M. da Silva, Carla C.B. dos Santos, Maria de Nazaré de O. Segura, R. L. D. Machado. 2000. Malaria transmission. Ciéncia e cultura J. of Braz. Assoc. for Adv. of Sci. 52: 208-212.

Rosa-Freitas, M. G., L. M. Deane and H. Momen. 1990. A morphological, isoenzymatic and behavioral study on ten populations on *Anopheles albitarsis* Lynch-Arribialzaga,

1978 (Diptera, Culcidae) including from the type-locality - Barader, Argentina. Mem. Inst. Oswaldoi Cruz 85: 275-289.

Rubio-Palis, Wirtz R.A., and Curtis C. F. 1992. Malaria entomological inoculation rates in western Venezuela, Acta Trop 52: 167-174.

Sallum M. A. M, T. R. Schultz, and R. C. Wilkerson. 2000. Phylogeny of Anophelinae (Diptera Culcidae) based on morphological characters. Systematics 93: 2000.

Tadei, W. P., B. D. Thatcher, J. M. M. Santos, V. M. Scarpassa, I. B. Rodriguez, and M. S. Rafeal. 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. Am. J. Trop. Hyg. 59: 325-335.

Tautz, D., C. Tautz, D. Webb, and G. A. Dover. 1987. Evolutionary divergence of promoters and spacers in the rDNA family of four *Drosphila* species. Implications for molecular coevolution in multigene families. J. Mol. Biol. 195: 525-542.

Velasco, M. J., J. C. S. Soriano. 1998. Analasis descriptivo de la situacion epidemiological de la malaria en el departamento de Cochabamba. Revista Medica 2(14): 5-10.

Walton C., R. G. Sharpe., S. J. Pritchard, N. J., Thelwell and R. K. Butlin. 1999. Molecular identification of mosquito species. Bio. J. of the Linnean Soc. 68: 241-256.

Wilkerson, R. C., T. V. Gaffigan, J. B. Lima. 1995a. Identification of species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). Mem. Inst. Oswaldoi Cruz 90: 721-732.

Wilkerson, R. C., T. J. Parsons, T. A. Klein, T. V. Gaffigan, E. Bergo, J. Consoli. 1995b. Diagnosis by random amplified polymorphic DNA polymerase chain reaction of four cryptic species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* (Diptera: Culicidae) from Paraguay, Argentina and Brazil. J. Med. Ent. 32: 697-704.

World Health Organization. 1996. World Malaria Situation. WHO Weekly Epidemiological Record 3: 17-22.

CHAPTER 2

Multiplex PCR for Identifying Two Species of the Oswaldoi Complex in Bolivia (Diptera: Culicidae: *Anopheles*)

2.1 Abstract

Anopheles Nyssorhynchus oswaldoi sensu lato has been shown to comprise an unresolved complex of cryptic species and has been incriminated as a vector of malaria in parts of Brazil, Venezuela, and Peru. We describe a multiplex PCR to identify two members of the Oswaldoi species complex found in the Chapare and Carrasco Valleys in Cochabamba, Bolivia, using species specific primers developed from internal transcribed spacer 2 (ITS2) sequences. One species specific primer anneals to a region of the ITS2, that is identical for three other taxa within the Oswaldoi complex, producing an amplicon of 335 bp (identifying the sample). The other primer anneals to a specific region of the ITS2 producing an amplicon of 298 bp that is unique to only one suspected member of the Oswaldoi complex.

2.2 Introduction

The entomological aspect of the epidemiology of malaria in South America is complex since anopheline mosquito species diversity is high, species compositions vary regionally and can include numerous primary and secondary vector species, and the ecology of the Neotropics is complex. Anopheline mosquitoes in the subgenus *Nyssorhynchus* are responsible for the majority of human malaria cases in South America. This subgenus includes at least 29 species (Faran 1980, Peyton et al. 1992, Harback 1994, Rubio-Palis and Zimmerman 1997, Lounibos et al. 1998) of which over 50% have been implicated or suspected as primary or secondary vectors of human *Plasmodium* spp. (e.g., Arruda et al. 1986, Goriup and Pull 1988, Hayes et al. 1987, Haworth 1988, Branquinho et al. 1993, Lounibos and Conn 2000); furthermore, a number of these taxa are thought to comprise cryptic species complexes (Rosa-Freitas et al. 1998), some of which are beginning to be resolved (e.g., Wilkerson et al. 1993, Marelli et al. 1999, Silva do Nascimento and Lourenço-de-Oliveira 2002).

A major impediment for studies on the basic biology, ecology, and behavior of *Nyssorhynchus spp.* has been the difficulty of identifying specimens with ease and accuracy, particularly immature stages and members of cryptic species complexes (e.g., Wilkerson 1995, Cornel et al. 1996, Lounibos et al. 1998, Marrelli, et al. 1999). The relatively recent advent of DNA-based techniques for discriminating species, such as the polymerase chain reaction, now provide methodologies offering greater resolution and accuracy when identifying all stages of development. Over a decade ago, Oaks et al. (1991) opined that the single most important laboratory technique that should be developed and transferred to field studies on malaria was simple and inexpensive

methods to differentiate anopheline mosquitoes. Although molecular identification techniques are now available for a number of Asian and African species, few have been developed for the multitude of species found in South America. At present, there are only two PCR-based methods for identifying *Nysorrhynchus* spp: primers that distinguish the four members of the *albitarsis* complex (Li and Wilkerson, unpublished data), and a multiplex PCR for discriminating *An. triannulatus* (Neiva and Pinto), *An. trinkae* Faran, *An. rangeli* Gabadon, Covia Garcia and Lopez and *An. Strodei* Root (Fritz et al. 2004).

As part of a study on the ecology of anopheline larvae in the Chapare and Carrasco Provinces, Department of Cochabamba, Bolivia, we have been developing molecular species-diagnostics allowing for the identification of all stages of mosquito development. Most cases of malaria in Bolivia occur in the departments of Beni and Cochabamba. Our study has focused on anophelines in the Chapare and Carrasco provinces of Cochabamba, where most cases of malaria are reported for this department (Velasco and Soriano 1998). Furthermore, the Chapare and Carrasco provinces are of particular interest to malariologists because they illustrate regional trends along the eastern slopes of the Andes Mountains that affect the epidemiology of malaria: intense logging of rainforest with concomitant conversion to secondary forest, livestock grazing, and monoculture, sustained by a seasonal migrant labor force. In this altered landscape, anopheline mosquito densities can increase many-fold (Deane 1988, Tadei et al. 1998), and migrant laborers increase the possibility of chronic pathogen introduction (Conn et al. 2000).

Here we describe a multiplex PCR for identifying two *Nyssorhynchus* species found in the Chapare and Carrasco valleys. Both species resolve as *An. oswaldoi* using

standard morphological keys for larvae, pupae and adult *Nyssorhynchus* spp. (Faran 1980, Faran and Linthicum 1981, Linthicum 1988) and, therefore, are members of the oswaldoi species complex. At present, the taxonomic status of *An. oswaldoi* is indeterminate since recent DNA sequence data suggests a species complex with at least four species including *An. konderi* Galvão & Damasceno (Marelli et al. 1999, R. Wilkerson and Y. Linton pers com.). *An. oswaldoi sensu lato* has been found to harbor human *Plasmodium* spp. in areas of Brazil, Venezuela and Peru, where it may serve as a secondary or primary vector (Arruda et al. 1986, Hayes et al. 1987, Rubio-Palis et al. 1992, Branquinho et al. 1993, 1996, Tadei et al. 1998, Póvoa et al. 2001).

For purposes of this study, we chose to call one of our species *An. oswaldoi* A and the other species *An. oswaldoi* B. Diagnostic primers were developed from sequence differences in the internal transcribed spacer 2 (ITS2). Ribosomal DNA is useful for the development of species specific primers and probes because rDNA units occur in tandem repeat arrays including conserved genes (18s, 28s, and 5.8s) that are separated by rapidly evolving spacers (Beckingham 1982, Marelli et al. 1999). Because of homogenization and concerted evolution, spacers may differentiate even closely related species that otherwise show little genetic divergence (Fritz 1994, Crabtree et al. 1995, Charlwood and Edoh 1996, Miller 1996, Fritz 2004). We also chose the ITS2 for the elaboration of species specific primers because the sequence of this spacer is available from genetic databases (e.g., GenBank) for most species within the subgenus *Nyssorhynchus* (22 of the 27 species) including *An. konderi* and other "species" in the Oswaldoi complex (Marelli et al. 1999).

2.3 Materials and Methods

Collection and Identification of Specimens

Mosquito larvae, pupae and adults used in this study were obtained from a subset of 56 collection sites in the Chapare and Carrasco Valleys in Cochabamba, Bolivia in May, 2003, sampled as part of a study on the larval ecology of *Nyssorhynchus* spp. Larvae and pupae were stored in 90% ETOH until used for morphological identifications, sequencing, and PCR-based identifications. Up to ten individuals from each site were also link-reared to obtain larval and pupal exuviae, and pinned adults as voucher specimens. Specific identities of link-reared specimens were determined by morphological keys of Faran and Linthicum (1981) and Linthicum (1988).

Mosquitoes identified initially as *An. oswaldoi* were subsequently shown to include two species with different ITS2 sequences (see Figure 2.1) and generally non-overlapping distributions (Fritz unpublished data). Thus, one of these two species was referred to as *An. oswaldoi* A, whereas the other species was referred to as species B.

Sequencing and Primer Design

DNA was isolated using DNeasy kits (Qiagen Inc., Valencia, CA) following the instructions for the isolation of DNA from animal tissues. The ITS2 was amplified by PCR using primers that annealed to flanking conserved regions of the 5.8s gene (5'-TGTGAACTGCAGGACACATG - 3') and the 28s gene (5'-ATGCTTAAATTTAGGGGGTAGTC - 3') (Porter and Collins 1991) using a Hybaid PCR Express Thermocycler (Thermo Electron Corp.) with the following temperature profile: 1 cycle of 1 min at 94°C, 30 sec. at 65°C, and 30 sec. at 72°C, 28 cycles of 30

sec. at 94°C, 30 sec. at 65°C, and 30 sec at 72°C, and 1 cycle of 30 sec. at 94°C, 30 sec. at 65°C, and 5 min. at 72°C. PCR reactants included: 2 µl of target DNA (from DNeasy kits isolation), ½ µl of TaKaRa Ex Taq, 5 µl 10X Ex Taq Buffer, 4 µl of dNTP Mixture (2.5 mM each) all obtained from Takara Bio Inc. (Shiga, Japan), 33.5 µl H_2O, and 2.5 µl of each of the two primers at 40 ng/µl (5.8s and 28s). Amplicons were subsequently purified using the Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA) according to manufacturer's instructions for a microcentrifuge.

Forward and reverse sequencing reactions were done with a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Inc., Fullerton, CA) following the manufacturer's recommendations for sequencing from a double stranded template. Sequencing was completed using a CEQ 2000 dye terminator sequencer (Beckman Coulter, Inc., Fullerton, CA). Forward and reverse sequences were then aligned using Sequencher™ 3.0 (Gene Codes Corp., Ann Arbor, MI) and manually checked for optimal alignment. Four specimens of *An. oswaldoi sensu lato* (species A) were sequenced from a locality in the Chapare Valley (Table 2.1) where we had identified this species based on morphological characteristics of larvae, pupae and adults. Similarly, nine specimens of *An. oswaldoi* species B were sequenced from five locations (Table 2.1).

Species specific primers were designed using the CLUSTALW alignment program (Thompson et al. 1994) to align the ITS2 sequences of both target species together with those of other species in the subgenus *Nyssorhynchus* available from the GenBank database: *An. dunhami, An. strodei, An. trinkae, An. triannulatus, An. rangeli, An. galvaoi, An. darlingi, An. marajoara, An. albimanus, An. nuneztovari, An. evansae, An. konderi, An. argyritarsis, An. braziliensis, An. deanorum, An. aquasalis, An.*

albitarsis, An. albitarsis B, *An. dunhami* and *An. rondoni* in addition to three putative species of the Oswaldoi complex as determined by Marelli et al.(1999). Primers were optimized using standard protocols and chosen to give easily resolved amplicons on 2% gels.

Each species specific primer, paired with a primer that annealed to the 5.8s conserved region, was first tested on its target species and subsequently tested for non-specific amplification with the DNA from 19 other species found in the subgenus *Nyssorhynchus* (Table 2.2): *An. trinkae, An. triannulatus, An rangeli, An. strodei, An. aquasalis, An albimanus, An. darlingi, An evansae, An. oswaldoi, An. marajoara, An. albitarsis, An. nuneztovari, An. galveoi, An. deanorum, An. bennarochi, An. konderi, An. braziliensis, An. argrytarsis,* and *An. oswaldoi* species A or B. Finally, both species specific primers were combined in a multiplex PCR with the 5.8s primer and re-tested on the DNA from the species listed above.

2.4 Results

Aligments of species A and B ITS2 sequences with those available for 22 other *Nyssorhynchus* species (GenBank) ITS2 identified unique sequence sites. Figure 2.1 shows the sequence alignment of species A and species B with those with which they shared most homology, two members of the Oswaldoi complex (GenBank and Marelli et al. 1999). The ITS2 sequence of species A was most similar to that of a clone of *An. oswaldoi sensu lato* collected from Costa Marques, Rondônia, Brazil (GenBank accession number AF055069); species B was most similar to a clone of *An. oswaldoi sensu lato* from specimens collected at Espirito Santo, Brazil (Genbank accession number

AF055072) (Fig. 2.1). Two regions of the ITS2 were chosen for the development of primers that would provide diagnostic amplicons (Table 2.3, Fig. 2.1). One sequence region is unique to species B and the primer that anneals there does not amplify any other species of *Nyssorhynchus* spp. tested to date (Fig. 2.1). The second primer (amplifies species A) anneals to a region of the ITS2 that is identical for three other taxa within the Oswaldoi complex (Fig. 2.1). The conserved 5.8s primer used in combination with species A and B primers either yields a 335 bp fragment (species A) or a 298 bp fragment (Species B) (Fig. 2.2).

Amplicons suitable for sequencing by multiplex PCR were generated in 50 µl reactions and included: 5 µl of 10X buffer, 8 µl of DNTP mix at 1.25 Mm, ½ µl of taq. polymerase at 5 U/µl, 21.0 µl of H_2O, 6 µl of MgCl at 25 mM, 2.5 µl of each of the three primers at 40 ng/µl (for species A, B, and the 5.8s), and 2 µl of target DNA . Thermal cycling parameters were tested, especially the annealing temperature, to optimize amplifications. A Hybaid PCR Express Thermocycler (Thermo Electron Corp.) was used for all PCR reactions. The following temperature profile was found to provide the most amplicon product with the least amount of non-specific amplification: 30 cycles of 30 sec at 94° C, 30 sec. at 59.5° C, and 30 sec. at 72° C; the last cycle had an extension time of 5 min. Amplification products were electrophoresed on 1.5% gels and visualized with ethidium bromide stain.

The multiplex PCR produced no unexpected amplicons when tested on DNA samples of other species in the subgenus *Nyssorhynchus* (data not shown), some from multiple locations throughout South America (Table 2.2). However, the species A species primer also produced an amplicon for *An. konderi*, as expected; since *An. konderi*

shares an almost identical ITS2 sequence with *An. oswaldoi* and until recently has been regarded as a junior synonym (Marrelli et al. 1999). The multiplex primer was tested on additional samples of larvae from locations in the Chapare and Carrasco valleys where adult specimens indicated the presence of *An. oswaldoi sensu lato*. Species A or B were found in 26 out of 56 sampling locations and had, overall, mutually exclusive distributions (Fritz et al. unpublished data). All samples produced a single amplicon of the expected two sizes and no hybrids were observed.

2.5 Discussion

An. oswaldoi sensu lato has a very broad distribution in South America and it is not surprising, as suggested (Klein et al. 1991, Marelli et al. 1999), that this taxon is a cryptic species complex. As mentioned previously, the taxonomic status of species in the Oswaldoi complex is presently unresolved. Marelli et al (1999) suggested four species based on sequence analysis of cloned ITS2. It is thus difficult to say whether or not species A or B in our study correspond to any of the species examined by Marelli et al. (1999) and others. Although the two taxa we examined have unique sequences, it is certainly possible they are geographic variants of species present in other regions and whose sequences are included in GenBank.

The two primers developed in this study were tested against most other species in the subgenus *Nyssorhynchus* and some from different geographic locations. Neither primer produced non-target amplicons, which implies these primers uniquely amplify species in the Oswaldoi complex. The primer for species B has a unique sequence, but the primer for species A anneals to a region in the ITS2 that is not unique to specimens of

An. oswaldoi collected in Cochabamba; three ITS2 clones of *An. oswaldoi sensu lato*, whose sequences are available in GenBank, have an identical sequence in this region of their ITS2 (see Fig. 2.1). Our multiplex PCR, therefore, does not distinguish between these sequence variants among the Oswaldoi complex. Whether these sequence variants represent one or more species is presently not known.

The sequencing protocol used in this study did not address intra-individual variation, but provides the consensus ITS2 sequence found in the hundreds of copies of rDNA units present in an individual. Past studies have shown that intra-individual sequence variations exist in the ITS2 of some members of the subgenus *Nyssorhychus* (Marrelli et al. 1999, Onyabe and Conn 1999, and Wilkerson and Li unpublished data). If such sequence variants in the ITS2 are relatively rare (e.g., Porter and Collins 1991, Fritz et al. 1994, Fritz 1998, Scott et al. 1993) then they should not affect the production of a single diagnostic amplicon for a species using PCR. Hypothetically, however, such sequence variants may be more common or even fixed in isolated populations within a species. If the sequence variant is included within the region of a species specific primer, then the primer's effectiveness at producing a species specific amplicon may be compromised for particular populations.

Because of the ecological complexity of the Neotropics and the high diversity of anopheline species, a number of authors have argued that the epidemiology and control of malaria can only be understood and managed effectively at the regional level (Rubio-Palis and Zimmerman 1997, Tadei et al. 1998, Lounibos and Conn 2000). The multiplex PCR developed here is useful, at the very least, as a taxonomic tool that has regional utility in an area of Bolivia that has endemic malaria. We have used this multiplex PCR

to identify larvae and puape from 56 sampling sites and have identified one or both species (A and/or B) from 26 of these sites (Fritz unpublished data). Species B is one of the two most common species present in the Chapare Valley, particularly in the foothills (the other being *An. trinkae*), and represented 18% of all anophelines identified from the Chapare and Carrasco valleys in a recent survey (n = 2,700) (G. Fritz unpublished data). Although the vector status of species A and B are unknown, *An. oswaldoi sensu lato* is considered an important vector of *Plasmodium* spp. in other areas of South America (Hayes et al. 1987, Klein et al. 1991a, b, Rubio-Palis et al. 1992, Branquinho et al. 1993, 1996). The multiplex PCR described in this study, then, will simplify the task of identifying species in the Oswaldoi complex and facilitate studies that aim to elucidate the basic biology, ecology and behavior of these species.

2.6 Tables and Figures

```
AF055072    GAACGCATATTGCGCATTGCACGACTCAGTGCGATGTACACAT  43
Sp. B       ------------------------------------------
Sp. A       ------------------------------------------
AF055069    ------------------------------------------

AF055072    TTTTGAGTGCCCACATTCACCGCAGAACCAACTAGCATAGCCC  86
Sp. B       -----------------------------------AG-T-G
Sp. A       ----------------------------------------G
AF055069    ----------------------------------------G

AF055072    ACGAAAGCTTTGCTGCGTACTGATGATTTGATTGGCCCCGTGC 129
Sp. B       T---G-------------------------A--------
Sp. A       T-------------------------.-G---A--AT----
AF055069    T-------------------------.-G---A--AT----

AF055072    CAGTCAAGCATTGAAGGACTGTGGCGTGGTGGGTGCACCGTGT 172
Sp. B       --A---------------------------------------
Sp. A       --AC--------------------------------------
AF055069    --AC--------------------------------------

AF055072    GTGTGTCGTTGCTTAATACGACTTCATTCTCTGGTATCACATC 215
Sp. B       ----------------------.-------------------
Sp. A       ---C------------------.-------------------
AF055069    ---C------------------.-------------------

AF055072    TGGAGCGGGCTATCCAGTCACAATCCCCAGCGACATGTGCAGG 258
Sp. B       ----------------------------------A
Sp. A       ---------------------------------C-------A
AF055069    ---------------------------------C-------A

AF055072    TAT...AGCCCCGATGTGGAGGACCA.....TCCTCCCTCAAA 301
Sp. B       --AGGT--------------------..AG.------------
Sp. A       --.....-------------------CCATA------------
AF055069    --.....-------------------CCATA------------

AF055072...GCCAATGTGATACACATCAACAGAGAGAGA  CCAAACGTACC 344
Sp. B       -------C--------C-------------  -----------
Sp. A       ---C------------C----C--------..-----------
AF055069    ---C------------C----C--------GA-----------

AF055072    CTGAAGCAACGGTATGCGCACACGAGTGCAACTCATTGAAGCG 387
Sp. B       -----------.-------------------------------
Sp. A       -----------.------------C-----G------------
AF055069    -----------.------------C-----G------------

AF055072    CGCACGATCGAAAGAGAACCGATCAAGTGGGCCTCAAATAAT 429
Sp. B       ----------------------------------------
Sp. A       ----------------------------------------
AF055069    ----------------------------------------
```

Fig. 2.1. Sequence alignment of the ITS2 and flanking regions of the 5.8s and 28s rRNA genes of Sp. A, Sp. B, and *An. oswaldoi* specimens from Genbank that are the closest sequence matches to Sp. A and Sp. B, AF055069 and AF055072, respectively. The ITS2 begins at approximately position 61 and ends approximately 15 bp from the end of the sequence shown. A dash indicates identity with the sequence given for *An. oswaldoi* AF055072 and a period indicates a gap introduced to maintain alignment. Underlined regions represent species specific primer annealing sites.

Table 2.1 Collection data for Sp. A and Sp. B samples sequenced for ITS2 from Cochabamba, Bolivia.

Species	Site Code -Sample #	Province, Location	Coordinates
Sp. A	CC1M03-A	Carrasco, Carmen Coni	S 16° 53' W 065° 10'
	CC1M03-5		
	CC1M03-10		
	CC1M03-34		
Sp. B	AB1A-22	Chapare, Ambrosia River	S 17° 00' W 065° 39'
	AB1A-30		
	AB1A-14		
	SR1M03-25	Chapare, San Rafael	S 17° 03' W 065° 29'
	CM1M03-A	Chapare, Cristal Mayu	S 16° 59' W 065° 37'
	CM1M03-1	River	
	PS1M03-1	Chapare, Padresama River	S 17° 01' W 065° 29'
	PS1M03-4		
	JP1M03-7	Chapare, Jatum Pampa	S 17° 00' W 065° 34'

Table 2.2 Collection localities for specimens tested with *An. oswaldoi* A and B specific primers.

Species	Collection Location	Coordinates	No. tested
An. albimanus	United States, Florida, Lab Colony, USDA,	Not known	1
An. albitarsis	Venezuela, Zulia, Rio Socuaro	8°54'N, 72°38'W	1
An. aquasalis	Suriname, Paramaibo	5°50'N, 55°11'W	1
An. argrytarsis	Brazil, Ceara, Ubjara	30°53'S, 40°54'W	1
An. bennarochi	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. braziliensis	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. darlingi	Bolivia, Beni, Guayaramirín	10°51'N, 65°21'W	1
An. deanorum	Brazil, Rondonia, Costa Marques	12°26'S, 64°18'W	1
An. evansae	Brazil, Rio de Janeiro	23°47'S, 43°49'W	1
An. galveoi	Brazil, Sao Paulo, Pariquera Acu	Not known	1
An. konderi	Brazil, Rondonia, Costa Marques	12°26'S, 64° 18'W	2
An. marajoara	Bolivia, Cochabamba, Chapare Valley	17°58'S, 64°49'W	1
	Venezuela, Cojedes	Not known	1
An. nuneztovari	Brazil Roraima, Boa Vista, Pará Belem	2°49'N, 60°40'W	1
	Brazil Roraima, Boa Vista, Pará Belem	1°24'S, 48°26'W	1
An. oswaldoi A	Bolivia,Cochabamba, Chapare Valley	16°53'S, 65°11'W	1
	Brazil, Pará, Urucuri	Not known	1
An. rangeli	Bolivia, Cochabamba, Chapare Valley	17°14'S, 64°23'W	2
An. oswaldoi B	Bolivia, Cochabamba, Chapare Valley	16°59'S, 65°37'W	2
	Bolivia, Cochabamba Chapare Valley	17° 01'S, 65° 29'W	2
An. strodei	Bolivia, Cochabamba, Chapare Valley	16°59'S, 65°11'W	2
An. triannulatus	Bolivia, Cochabamba, Chapare Valley	17°14'S, 64°23'W	2
An. trinkae	Bolivia, Cochabamba, Chapare Valley	16°58'S, 65°22'W	1

Table 2.3 Species specific primers annealing to the ITS2 of two species of South American anopheline mosquitoes in the subgenus *Nyssorhynchus.*

Species	ITS2 Primer Sequence (5'- 3')	Amplification Product (bp)
An. oswaldoi A	CTT TGA GGG AGG ATA TGG	335
An. oswaldoi B	CTA CCT TAT CTG CAC ATG	298

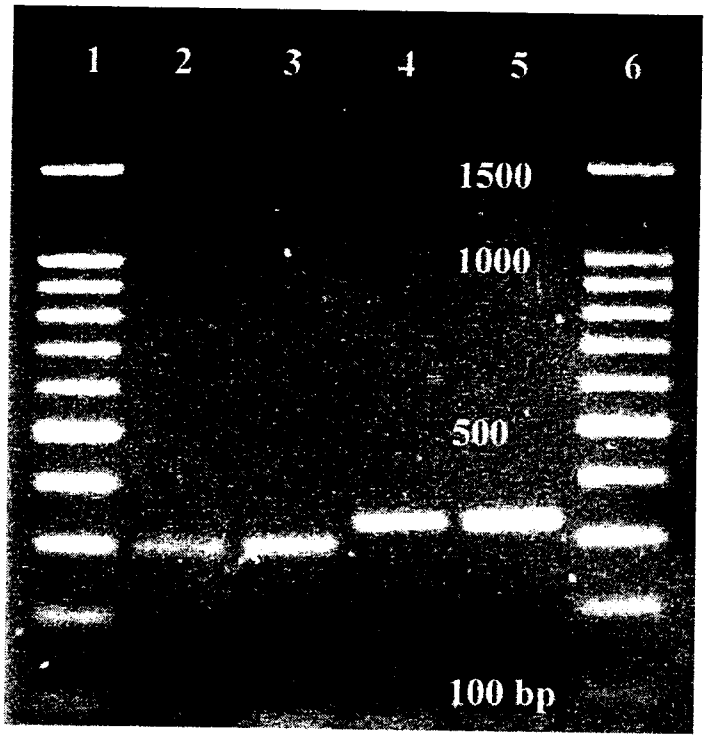

Fig. 2.2 Species specific amplicons for *An. oswaldoi* B (lanes 2 and 3) and *An. oswaldoi* A. (lanes 4 and 5). Lanes 1 and 6 = 100 bp DNA ladder.

2.7 References Cited

Beckingham, K. 1982. Insect rDNA. The cells Nucleus, vol. X, pp. 205-269. In H. Busch and L. Rothblum [eds.], Academic, New York.

Branquinho, M. S., C. B. Taipe-Lagos, R. M. Rocha, D. Natal, J. M. S. Barata, A. H. Cochrane, E. Nardin, R. S. Nussenzweig and J. K. Kloetzel. 1993. Anophelines in the State of Acre, Brazil, infected with *Plasmodium falciparum, P. vivax,* the variant *P. vivax* VK247 and *P. malariae.* Trans. R. Soc. Trop. Med. Hyg. 874: 391-394.

Branquinho, M. S., M. S. Araujo, D. Natal, M. T. Marrelli, R. M. Rocha, A. L. Taveira and J. K. Kloetzel. 1996. *Anopheles oswaldoi* and important potential malaria vector in Acre, Brazil. Trans. R. Soc. Trop. Med. Hyg. 90: 233.

Charlwood, J. D. and D. Edoh. 1996. Polymerase chain reaction used to describe larval habitat use by *Anopheles gambiae* complex (Diptera: Culicidae) in the environs of Ifakara, Tanzania. J. Med. Ent. 33(2): 202-204.

Cornel, A. J., C. H. Porter, and F. H. Collins. 1996. Polymerase chain reaction species diagnostic assay for *Anopheles quadrimaculatus* cryptic species (Diptera: Culicidae) based on ribosomal DNA ITS2 sequences. J. Med. Ent. 33(1): 109-116.

Crabtree, M. B., H. M. Savage, and B. R. Miller. 1995. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. Am J. Trop. Med. Hyg. 53(1): 105-109.

Deane, L. M. 1988. Malaria studies and control in Brazil. Am. J. Trop. Med. Hyg. 38(2): 223-230.

de Arruda, M., M. B. Carvalho, R. S. Nussenzweig, M. Maracic, A. W. Ferreira, and A. H. Cochrane. 1986. Potential vectors of malaria and their different susceptibility to Plasmodium falciparum and Plasmodium vivax in northern Brazil identified by immunoassay. Am. J. Trop. Med. Hyg. 35:873-881.

Faran M. E. 1980. Mosquito studies (Diptera: Culicidae) XXXIV. A revision of the Albimanus Section of the subgenus *Nyssorhynchus* of *Anopheles.* Contrib. Am. Entomol. Inst. (Ann Arbor) 15: 1-215.

Faran, M. E. and K. J. Linthicum. 1981. A handbook of the Amazonian species of *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae). Mosq. Syst. 13: 1-81.

Fritz, G. N., Engman, S. Rodriguez, R., and Wilkerson R. C. 2004. Identification of four vectors of human *Plasmodium spp.* by multiplex PCR: *Anopheles rangeli, A. strodei, A. triannulatus,* and *A. trinkae* (Diptera: Culicidae: *Nyssorhynchus*) J. Med. Ent. 41(6): 1111-1115.

Fritz, G. N. 1998. Sequence analysis of the rDNA internal transcribed spacer 2 of five species of South American human malaria mosquitoes. J. of Seq. and Map. 8(4): 215-221.

Fritz, G. N., J. Conn, A. Cockburn, and J. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). Mol. Biol. Evol. 11(3): 406-416.

Goriup, S., and J. H. Pull. 1988. Field research in the conext of malaria control, pp. 1742-1764. In W. H. Wernsdorfer and I. McGregor (eds.), Malaria: principles and practices of Mariology, vol. II. Churchill Livingston, New York.

Harbach R. E. 1994. Review of the internal classification of the genus *Anopheles* (Diptera: Culicidae): the foundation for comparative systematic and phylogenetic research. Bulletin of Entomological Research 84: 331-342.

Haworth, J. 1988. The global distribution of malaria and the present control effort, pp. 1379-1420. *In* W. H. Wernsdorfer and I. McGregor (eds.), Malaria: principles and practices of marlariology, vol. II Churchill Livingston, New York.

Hayes, J., G. Calderon, R. Falcon and V. Zambrano. 1987. Newly incriminated anopheline vectors of human malaria parasites in Junin Department, Peru. J. Am. Mosq. Cont. Ass. 6: 700-707.

Klein, T. A., J. B. P. Lima and M. S. Tada. 1991. Comparative susceptibility of anopheline mosquitoes to *Plasmodium falciparum* in Rondônia, Brazil. Am. J. Trop. Med. Hyg. 44: 598-603.

Linthicum, K. J. 1988. A revision of the Argyrytarsis section of the subgenus *Nyssorhynchus* of *Anopheles* (Diptera: Culicidae). Mosq. Syst. 20: 99-271.

Lounibos, L. P. and J. Conn. 2000. Malaria vector heterogeneity in South America. Amer. Ent. 46: 238-249.

Lounibos, L. P., R. C. Wilkerson, J. E. Conn., L. J. Hribar, G. N. Fritz, and J. A. Danoff-Burg. 1998. Morphological, Molecular, and Chromosomal Discrimination of Cryptic *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae) from South America. J. Med. Ent. 35: 830-838.

Marrelli, M. T., R. S. Malafronte, C. Flores-Mendoza, R. Lourenco-De-Oliveira, J. K. Kloetzel, and O. Marinotti. 1999. Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi* (Diptera: Culicidae). J. Med. Entomol. 36(6): 679-684.

Miller, B. R., M. B. Crabtree, and H. M. Savage. 1996. Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. Insec. Mol. Bio. 5(2): 93-107.

Oaks, S. C., V. S. Mitchell, G. W. Pearson, and C. C. J. Carpenter (eds.) 1991. Malaria. Obstacles and opportunities. National Academy of Science Press, Washington, DC.

Onyabe, D. Y., and J. E. Conn. 1999. Intragenomic heterogeneity of ribosomal DNA spacer (ITS2) varies regionally in the neotropical malaria vector *Anopheles nuneztovari* (Diptera: Culicidae). Ins. Mol. Biol. 8: 435-442.

Peyton, E. L., R. C. Wilkerson, and R. E. Harbach. 1992. Comparative analysis of the subgeners *Kertesia* and *Nyssorhynchus* of *Anopheles* (Diptera: Culicidae). Mosq. Syst. 24: 51-69.

Porter, C. H. and F. H. Collins. 1991. Species diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 45: 271-279.

Póvoa, M. M., R. A. Wirtz, R. N. L. Lacerda, M. A. Miles, and D. Warhurst. 2001. Malaria vectors in the municipality of Serra do Navio, State of Amapá, Amazon Region, Brazil. Mem. Inst. Oswaldo Cruz 96:179-184.

Rubio-Palis, Y., R. A. Wirtz and C. F. Curtis. 1992. Malaria enotomological inoculation rates in Western Venezuela. Acta Tropica 52: 167-174.

Rubio-Palis, Y., H. Zimmerman. 1997. Ecoregional classification of malaria vectors in the Neotropics. J. Med. Ent. 34(5): 499-510.

Silva do Nascimento, T. F., R. Lourenco-de-Oliveira 2002. Anopheles halophylus, a new species of the subgenus *Nyssorhynchus* (Diptera: Culicidae) from Brazil. Mem. do Inst. Osw. Cruz. 97(6): 801-811.

Scott, J. A., W. G. Brogdon, and F. H. Collins. 1993. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am. J. Trop. Med. Hyg. 49: 520-529.

Tadei, W. P., B. Dutary-Thatcher, J. M. M. Santos, V. M. Scarpassa, I. B. Rodrigues, and M. S. Rafael. 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. Amer. J. Trop. Med. Hyg. 59:325-335.

Thompson, J. D., D. G. Higgins, T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acid. Res. 22: 4673-4680.

Wilkerson, R. C., T. V. Gaffigan, J. B. Lima. 1995. Identification of species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). Mem. Inst. Oswaldoi Cruz 90: 721-732.

Wilkerson, R. C., T. J. Parsons, D. G. Albright, T. A. Klein, M. J. Braun. 1993. Random amplified polymorphic DNA (RAPD) markers readily distinguish cryptic mosquito species (Diptera: Culicidae): *Anopheles*). Insect Mol. Biol. 1:205-211.

CHAPTER 3

Identification of *Anopheles* (*Nyssorhynchus*) *marajoara* (Diptera: Culicidae) in Bolivia using PCR and a restriction endonuclease

3.1 Abstract

Anopheles Nyssorhynchus marajoara is a member of the Albitarsis cryptic species complex and has been shown to be a primary vector of human *Plasmodium* in parts of Brazil. We report the first record of *An. marajoara* in the department of Cochabamba, and confirm its presence in the department of Santa Cruz, Bolivia. We also describe a PCR protocol producing a single amplicon (~334 bp) of a section of the internal transcribed spacer 2 specific for members of the Albitarsis species complex; the PCR protocol did not amplify the DNA of 17 other species in the subgenus *Nyssorhynchus*. Digestion of the amplicon with the restriction endonuclease Bfa-I produces two fragments specific to *An. marajoara* in Bolivia.

3.2 Introduction

Anopheles marajoara Galvão and Damasceno is in the subgenus *Nyssorhynchus*, which includes most of the primary and secondary vectors of malaria in South America (e.g., de Arruda et al. 1986, Goriup and Pull 1988, Hayes et al. 1987, Haworth 1988, Branquinho et al. 1993, Lounibos and Conn 2000). Though historically thought to be unimportant in the transmission of malaria, *An. marajoara* has been incriminated recently as a major vector in Amapá state (Conn et al. 2002) and Boa Vista, Brazil (Conn et al., personal communication).

Anopheles marajoara is one of four species belonging to a cryptic complex, (Albitarsis complex), which also includes *An. albitarsis* A, *An. albitarsis* B, and *An. deaenorum* (Narang et. al. 1993, Wilkerson et al. 1995a, 1995b). Aside from the larvae of *An. deaneorum*, larvae, pupae and adults of this complex are not distinguished reliably using morphological keys. Although initially resolved by RAPD-PCR in 1995 by Wilkerson et al. (1995a, 1995b) the four species in this complex can now be identified by a series of PCR reactions using different pairs of primers that anneal to the internal transcribed spacer 2 (ITS2) (Li and Wilkerson, personal communication).

An. marajoara was described in 1942 from specimens collected on the island of Marajo in Pará, Brazil (Galvão and Damasceno 1942). Subsequent reports of *An. marajoara* reported for Colombia, Panama, Venezuela, Costa Rica, Honduras, Trinidad, the Guianas, and Bolivia indicated this species had a very broad distribution (Linthicum 1988). In Bolivia, *An. marajoara* has been reported from the departments of Beni and Santa Cruz (Linthicum 1988). These records, and others reported for *An. marajoara* in S. America, however, are questionable since they were obtained prior to the recognition of

the Albitarsis species complex. In this study, we report the first record of *An. marajoara* in the department of Cochabamba, and confirm its presence in the department of Santa Cruz, Bolivia. We also describe a PCR that identifies any life stage of an unknown anopheline mosquito as belonging to the Albitarsis species complex. Subsequent digestion of the amplicon with a restriction endonuclease resolves *An. marajoara* in Bolivia. This PCR protocol differs from the recently reported technique by Li and Wilkerson (personal communication) insofar as specimens do not require pre-identification to the Albitarsis complex prior to PCR for specific diagnosis. We chose the ITS2 spacer sequence to develop a species diagnostic for *An. marajoara* for the following reasons: complete sequences of this spacer are available in GenBank for all four species in the Albitarsis complex as well as for 17 other species in the subgenus Nyssorhynchus (of the 27 Nyssorhynchus species currently identified), rapid rates of sequence divergence between closely related species that otherwise show little genetic divergence (Fritz et al. 1994, Fritz 1998, Fritz 2004, Crabtree et al. 1995, Charlwood and Edoh 1996, Miller 1996), and multiple copies of the ITS2 increasing PCR amplification efficiency.

3.3 Materials and Methods

Collection and Preliminary Identification of Specimens

Mosquito larvae and pupae were collected from 56 sites in the Chapare and Carrasco Valleys, in the departments of Cochabamba and Santa Cruz, in May 2003 as part of an ecological study on anopheline larvae in this region of Bolivia. Sites positive for *An. albitarsis* sensu latu were identified from link-reared specimens from each

location using the key of Linthicum (1988). Specimens from each site were also stored in 90% ethanol for subsequent sequencing and PCR-based identification.

Sequencing and Primer Design

Whole mosquito DNA was isolated using DNeasy kits (Qiagen Inc., Valencia, CA) following manufacturer instructions for the isolation of DNA from animal tissue. The ITS2 was amplified by PCR using: 2 µl of DNA, ½ µl of TaKaRa Ex Taq DNA polymerase, 5 µl 10X Ex Taq Buffer, 4 µl of dNTP Mixture (2.5 mM each) all obtained from Takara Bio Inc. (Shiga, Japan), 33.5 µl H_2O, 2.5 µl of the 5.8s (5' - TGTGAACTGCAGGACACATG - 3') and 28s (5'- ATGCTTAAATTTAGGGGGTAGTC - 3') (Porter and Collins 1991) conserved primers at 40 ng/µl and using a Hybaid PCR Express Thermal Cycler (Thermo Electron Corp.). The thermocycler temperature profile was: 1 cycle of 1 min at 94°C, 30 sec. at 65°C, and 30 sec. at 72°C, 28 cycles of 30 sec. at 94°C, 30 sec. at 65°C, and 30 sec at 72°C, and 1 cycle of 30 sec. at 94°C, 30 sec. at 65°C, and 5 min. at 72°C. Amplicons were subsequently purified using the Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA) according to manufacturer instructions for a microcentrifuge.

Forward and reverse cycle sequencing reactions of the ITS2 were done with a CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Inc., Fullerton, CA) following the manufacturer's recommendations for sequencing from a double stranded template. Sequencing was completed using a CEQ 2000 dye terminator sequencer (Beckmand Coulter, Inc., Fullerton, CA). Forward and reverse sequences were aligned using Sequencher™ 3.0 (Gene Codes Corp., Ann Arbor, MI) and saved as text format files.

A primer specific to the Albitarsis species complex was designed by aligning ITS2 sequences of all four members of the complex (Li and Wilkerson, personal communication) using CLUSTALW (Thompson et al. 1994) along with other species of the subgenus *Nyssorhynchus* (available on GenBank) including: *An. dunhami, An. strodei, An. trinkae, An. triannulatus, An. rangeli, An. galvaoi, An. darlingi, An. albimanus, An. aquasalis, An. nuneztovari, An. evansae, An. konderi, An. argyritarsis, An. braziliensis , An. dunhami, An. oswaldoi sensu lato,* and *An. rondoni*. ClustalW alignments were manually optimized and a primer chosen that annealed to a sequence in the ITS2 unique to the Albitarsis species complex. PCR reactants and thermocycler temperature profiles were optimized by standard protocols and chosen to give easily resolved amplicons on 2% gels.

The primer specific to the Albitarsis species complex was complexed with the 28s conserved primer (above) and tested on the DNA from 19 species in the subgenus *Nyssorhynchus*, which included: *An. trinkae, An. triannulatus, An rangeli, An. strodei, An. aquasalis, An albimanus, An. darlingi, An evansae, An. marajoara, An. albitarsis, An. nuneztovari, An. galveoi, An. deanorum, An. bennarochi, An. konderi, An. braziliensis, An. argrytarsis,* and *An. oswaldoi sensu lato* (Table 3.1). These specimens had been link-reared and identified using morphological keys (Faran 1980, Faran and Linthicum 1981, Linthicum 1988) or obtained from laboratory colonies.

Restriction Enzyme Digest

A restriction endonuclease cut site, specific to the ITS2 of *An. marajoara* in Bolivia, was located by sequence aligment (using CLUSTALW, Higgins 1993) with all three other members of the Albitarsis cryptic species complex. These sequences were

obtained and their specific identities were confirmed by personal communication with Richard Wilkerson, Walter Reed Biosystematics Unit, Smithsonian Institution, Wash., D. C., and they are available on GenBank (Fig. 3.1).

Prior to restriction enzyme digestion, PCR amplicons were purified by the addition of 7 µl of 8M potassium acetate, centrifugation at 4°C for 15 min., supernatant transfer to a 0.5 ml eppendorf tube and incubation overnight at -15°C in 150 µl of 95% ETOH. After incubation, the DNA was centrifuged at 20,000 x g for 15 min at 4°C and the resulting pellet washed twice with 100 µl of 70% ETOH followed by two centrifugations at 20,000 x g for 2 min. The resulting pellet was vacuum dried and re-suspended in 40 ul of 1X #4 buffer (New England Biolabs, Beverly, MA). Twelve µl of suspended DNA was then subjected to an overnight digestion at 37°C with 1/2 µl of Bfa-I (C/TAG) restriction endonuclease (New England Biolabs, Beverly, MA).

3.4 Results

Five specimens identified as *An. albitarsis sensu lato*, and collected at four locations in Bolivia (Table 3.2), were sequenced for the ITS2 (Fig. 3.1) and found to be *An. marajoara* when aligned with sequences available for the Albitarsis species complex. Sequence alignments of the Albitaris species complex with those of all other available *Nyssorhynchus* species indicated a region (5'- TTT GAT AGA CCC CGT GTC-3') that was unique to species in the Albitarsis complex (Fig. 3.1). As expected, a primer that anneals to this site, complexed with a conserved primer annealing to the 28s (see Methods section), produced a single amplicon in species of the Albitarsis complex only (Fig 3.2). Optimized PCR reaction protocols for 50 ul amplifications included: 5 ul of

10X buffer, 8 ul of 1.25 mM DNTPs, ½ µl of taq. polymerase at 5 U/µl, 21.0 µl of H_2O, 6 µl of 25 mM MgCl, 2.5 µl of each of the two primers at 40 ng/µl. The Hybaid thermocycler (Thermo Electron Corp.) temperature profile was: 30 cycles of 94° C for 1 min., 64° C for 30 sec., and 72° C for 1 min. Amplification products were electrophoresed on 1.5% gels and visualized with ethidium bromide stain (Fig. 3.2). The PCR produced a single (~334 bp), easily resolved amplicon subsequently confirmed on 170 specimens of *An. marajoara* from five sites in Bolivia, one *An. marajoara* from Brazil, and other members of the Albitarsis complex (Tables 3.1 and 3.3)

A restriction enzyme cut site was identified as unique to *An. marajoara* and due to a single nucleotide transversion (T-A) (Fig. 3.1). Digestion of amplicons with the restriction enzyme Bfa-I cut those of *An. marajoara* at the species specific sequence (Fig. 3.1) producing two expected fragments of ~202 bp and ~138 bp (Fig. 3.2). These two fragments were not observed when amplicons of the three other members of the species complex were subjected to digestion with Bfa-I (data not shown); these specimens included *An. deaneorum* from Ariquemes, Rondonia, Brazil, *An. albitarsis* A from Sao Paulo, Brazil, and *An. albitarsis* B from Iraquera, Bahia, Brazil (Table 3.1). Clones of *An. marajoara* from Brazil (Li and Wilkerson, personal communication) indicate that the point mutation creating the species specific cut site (Fig. 3.1) may be polymorphic in at least some populations of this species. We amplified and Bfa-I digested the amplicons of 170 *An. marajoara* collected from five collection sites in the departments of Cochabamba and Santa Cruz, Bolivia and of one specimen from the Matto Grosso, Brazil (Table 3.3, Fig. 3.3); all specimens produced the two diagnostic fragments indicative of *An.*

marajoara. Digestion of the ITS2 of a single specimen of *An. marajoara* from Manaus, Brazil, however, did not produce the expected two-fragment pattern of DNA.

The five locations in the Chapare/Carrasco valley region (Fig. 3.3) that we chose for testing the diagnostic PCR protocol were sites that had relatively high densities of *An. albitarsis* s.l. (as determined by link-reared voucher specimens). At all sites, larvae were found inhabiting sunlit flooded grassland, ponds or marshy areas in agricultural lands including cattle ranches.

3.5 Discussion

Since *An. marajoara* has now been shown to be an important vector of human *Plasmodium* in some parts of Brazil (Conn et al. 2002), its presence in the eastern valleys of Cochabamba and adjacent region of Santa Cruz is noteworthy. Of the 16 provinces in Cochabamba, the Chapare and Carrasco have historically had the highest malaria incidence rates (e.g., between 8.1 to 19 recorded infections per 1000 individuals between 1996-97, Velasco and Soriano 1998). The Chapare/Carrasco valley system is also of particular interest to malariologists because it exemplifies the changing nature of tropical rainforest with concomitant changes in the epidemiology of malaria. Most of the Chapare/Carrasco valley is now secondary forest or cultivated lands peppered by subsistence farmers, large-scale agricultural monoculture, and a high proportion of migrant workers. Human disturbed rainforests may exhibit a five-fold increase in anopheline densities over undisturbed forests (Tadei et al. 1998).

Ruibo-Palis and Zimmerman (1997) reported *An. albitarsis s.l.* as a common anopheline in savannah ecoregions of S. America, and Conn et al. (2002) found this

species increasing in relative abundance where land was deforested and converted to agriculture. Consistent with these studies, the locations where we found high densisites of *An. marajoara* were agricultural lands or grazing pastures cleared of forest. Larvae were collected in sunlit, marshy areas within the transition zone between the eastern piedmont of the Chapare/Carrasco valley system and the savannah grasslands of the department of Santa Cruz (Fig. 3.3). This region, devoid of cattle ten years ago, now has large tracts of grassland, and a number of cattle ranches. Our data suggests that *An. marajoara* may be increasing in frequency in this rapidly changing region of Bolivia.

As part of a study on the ecology of anopheline larvae in the Chapare/Carrasco valley of Cochabamba, we have been developing specific primers for a number of anopheline taxa collected in this region, thus enabling the identification of all life stages. Unlike the PCR protocol described by Li and Wilkerson (personal communication) for identifying species in the Albitarsis complex, our PCR protocol does not require prior identification of specimens to the complex. All stages of development of any unidentified anopheline can now be diagnosed for membership in the Albitarsis complex. In a two-step process, our protocol also separates *An. marajoara* from other members in the complex.

Li and Wilkerson (personal communication) reported that *An. marajoara* from Brazil exhibits substantial intragenomic sequence variation for the ITS2. Cloned ITS2 sequences of multiples specimens from Brazil show that the T-A transversion (underlined in Fig. 3.1) in the C/T<u>A</u>G restriction site is polymorphic in *An. marajoara.* If the polymorphism for this restriction site is present in all individuals, then our protocol should identify all members of this species. We cannot rule-out the possibility, though,

that there are individuals or populations fixed for an ITS2 sequence devoid of the Bfa-I cut site. The inability of Bfa-I to cut the ITS2 of a single specimen of *An. marajoara* from Manaus, Brazil (Table 3.2) suggests there may be such instances. Nevertheless, our data in Bolivia indicates that most, if not all, *An. marajoara* have the Bfa-I cut site; of 170 specimens analyzed in five locations, all had the Bfa-I cut site. Since no specimen identified as belonging to the Albitarsis complex lacked the Bfa-I cut site, our data also suggest that other members of the Albitarsis complex may be absent or rare from this region of Bolivia.

Accurate and quick molecular methods to identify anopheline mosquitoes, such as the one described in this study, have led to their recent use in field studies. Charlwood and Edoh (1996), for instance, used PCR to describe larval habitats used by the *An. gamibiae* complex in Tanzania, and (Fritz and Paudel, unpublished data) described larval habitat and species distributions in the Chapare/Carrasco provinces for *An. trinkae*, *An. triannulatus*, *An. rangeli, An. strodei*, *An. oswaldoi*, and an undescribed species using two multiplex PCR (Brelsfoard et al. in prep, Fritz et al. 2004). Combinations of several multiplex PCR should allow quick and accurate identification of all or most species in any given area, enabling more comprehensive studies to be initiated on the basic biology, behavior and transmission dynamics of vectors of human *plasmodium* spp. Because of the ecological complexity of the Neotropics, the rapidly changing environment due to human disturbance, and the high diversity of anopheline species, a number of authors have argued that the epidemiology and control of malaria is best understood and managed at the regional level (Rubio-Palis and Zimmerman 1997, Tadei et al. 1998, Lounibos and Conn 2000). The PCR diagnostic developed in this study, though not necessarily

applicable to other regions of S. America, is a useful taxonomic tool that has regional utility in an area of Bolivia with endemic malaria.

3.6 Tables and Figures

Table 3.1 Collection localities for specimens in the subgenus *Nyssorhynchus* tested with the complex specific primer for the ITS2 of the Albitarsis complex.

Species	Collection Loc.	Coordinates	No. tested
An. albimanus	United States, Florida, Lab Colony, USDA	Not known	1
An. albitarsis A	Venezuela, Zulia, Rio Socuaro	8°54'N, 72°38'W	1
	Brazil, São Paulo, 6 km SW Registro,	24°36'S, 47°53'W	2
An. albitarsis B	Brazil, Bahia, Itaquara	Not Known	2
An. aquasalis	Suriname, Paramaibo	5°50'N, 55°11'W	1
An. argrytarsis	Brazil, Ceara, Ubjara	30°53'S, 40°54'W	1
An. bennarochi	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. braziliensis	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. darlingi	Bolivia, Beni, Guayaramirín	10°51'N, 65°21'W	1
An. deanorum	Brazil, Rondonia, Costa Marques	12°26'S, 64°18'W	1
	Brazil, Rondonia, Ariquemes	Not known	1
	Brazil, Guajara Mirim	10°50'S, 65°20'W	1
An. evansae	Brazil, Rio de Janeiro	23°47'S, 43°49'W	1
An. galveoi	Brazil, Sao Paulo, Pariquera Acu	Not known	1
An. konderi	Brazil, Rondondia, Costa Marques	12°25'S, 64°18'W	1
An. marajoara	Bolivia, Cochabamba, Chapare Valley	17°10'S, 64° 16'W	2
	Bolivia, Cochabamba, Chapare Valley	17°58'S, 64° 49'W	1
	Brazil, Mato Grosso, Peixoto de Azevedo	Not known	1
	Brazil, Amazon, Manaus	2°53'S, 60°15'W	1
An. nuneztovari	Brazil Roraima, Boa Vista, Pará Belem	2°49'N, 60°40'W	1
	Brazil Roraima, Boa Vista, Pará Belem	1°24'S, 48°26'W	1
An. oswaldoi s. l.	Bolivia,Cochabamba, Chapare Valley	16°53'S, 65°11'W	2
	Brazil, Pará, Urucuri	Not known	1
	Bolivia, Cochabamba, Chapare Valley	16°59'S, 65°37'W	1
	Bolivia, Cochabamba, Ichilo	17°10'S, 64°16'W	2
An. rangeli	Bolivia, Cochabamba, Chapare Valley	17°14'S, 64°23'W	1
	Bolivia, Cochabamba, Chapare Valley	16° 55'S, 65°23'W	1
An. strodei	Bolivia, Cochabamba, Chapare Valley	17°01'S, 64°53'W	1
	Bolivia, Cochabamba, Chapare Valley	17°02'S, 64°51'W	1
An. triannulatus	Bolivia, Cochabamba, Chapare Valley	17°14'S, 64°23'W	1
	Bolivia, Cochabamba, Chapare Valley	17°12'S, 64°30'W	1
An. trinkae	Bolivia, Cochabamba, Chapare Valley	16°58'S, 65°22'W	1
	Bolivia, Cochabamba, Chapare Valley	16°57'S, 65°19'W	1

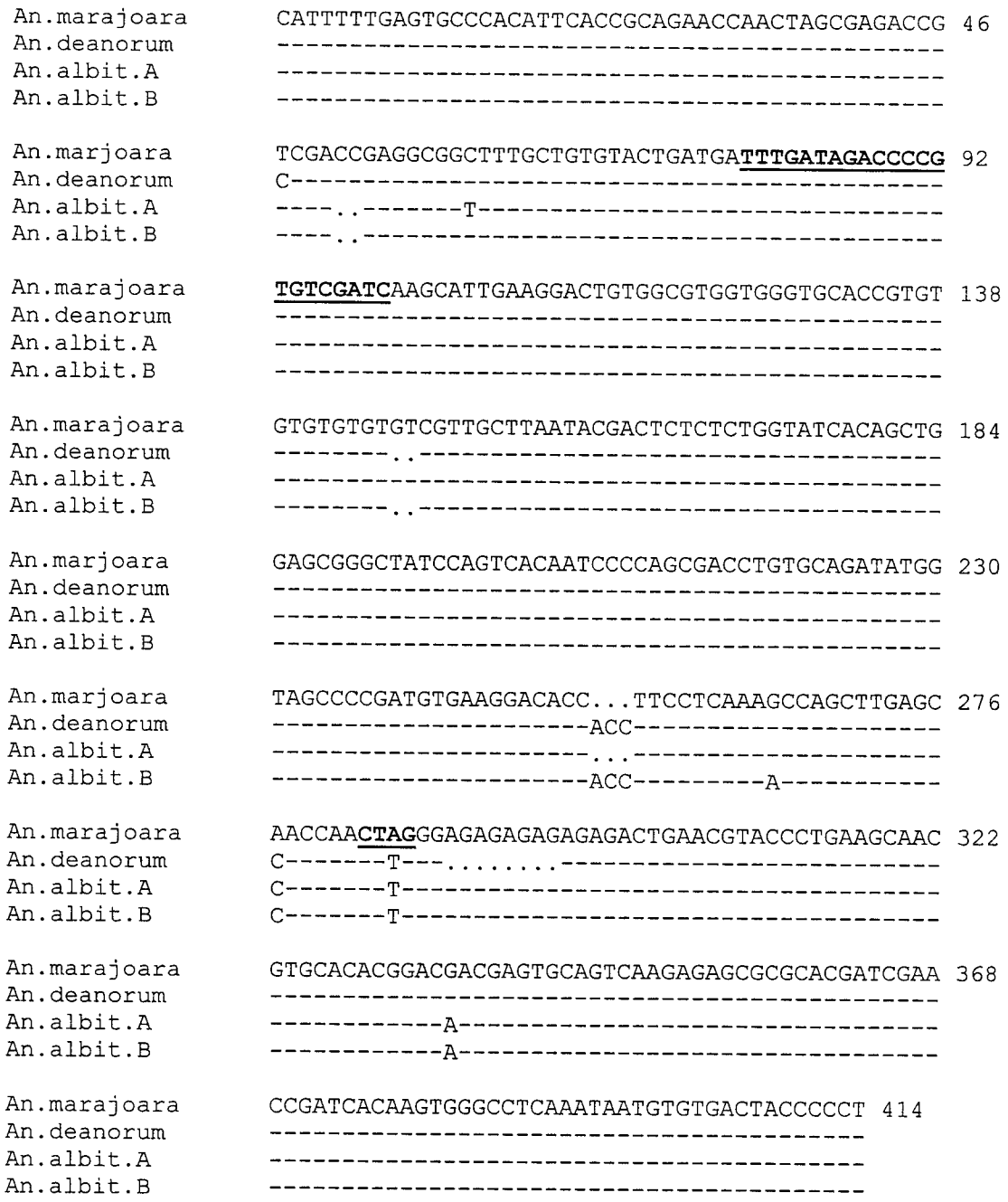

Fig. 3.1 Sequence alignment of the ITS2 and partial flanking regions of the 5.8s and 28s rRNA genes of *An. albitarsis* A, *An. albitarsis* B, *An. deanorum*, and *An. marajoara* (consensus sequence from 3 sites in Bolivia). The ITS2 begins at position 21 and ends 30 bp from the end of the sequence. A dash indicates identity with the sequence given for *An. marjoara* and a period indicates a gap introduced to maintain alignment. Underlined regions signify Bfa-I (C/TAG) restriction endonuclease cut site and Albitarsis Complex specific primer annealing site.

Table. 3.2. Collection data for samples of *An. marajoara* sequenced for the ITS2 from Bolivia.

Site Code–Sample Number	State, Province	Locality	Coordinates
RL1M03-3	Cochabamba, Carrasco	Rio Lagrimas	17° 09'S, 064° 37'W
VI1M03-A	Cochabamba, Carrasco	Valle Ivirza	17° 07'S, 064° 55'W
IV5M03-15	Cochabamba, Carrasco	Ivirgarzama	17° 01'S, 064° 50'W
PG1M03-1 PG1M03-40	Santa Cruz, Ichilo	Pto. Grether	17° 10'S, 064° 16'W

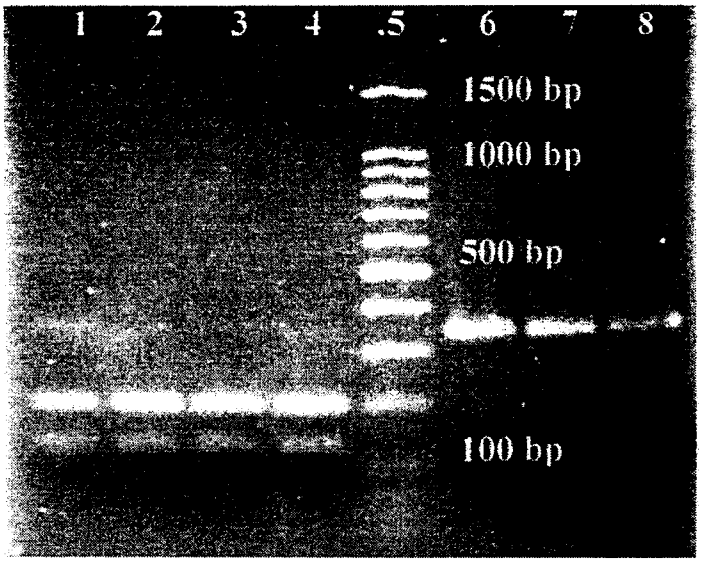

Fig. 3.2. Restriction enzyme digest with Bfa-I of albitarsis complex amplicon distinguishing *An. marajoara* (lanes 1-4). Lane 5 = 100bp ladder. Albitarsis complex specific amplicon (lanes 6-8).

Table 3.3. Collection localities and number of *An. marajoara* specimens identified by using Albitarsis complex specific primer and Bfa-I restriction enzyme.

Country, state, province	**Locality**	**Site Code**	**Coordinates**	**No.tested**
Bolivia				
Cochabamba,Carrasco	Chasqui	CQ1M03	17° 58'S, 64° 49'W	37
Cochabamba, Carrasco	Ivivirgarzama	IV5M03	17° 01'S, 64° 50'W	40
Cochabamba, Ichilo	Puerto Grether	PG1M03	17° 10'S, 64° 16'W	47
Cochabamba, Carrasco	Rio Lagrimas	RL1M03	17° 09'S, 64° 37'W	6
Cochabamba, Carrasco	Valle Ivirza	VI1M03	17° 07'S, 64° 55'W	40
Brazil				
Mato Grosso	Peixoto de Azevedo	Not known	Not known	1

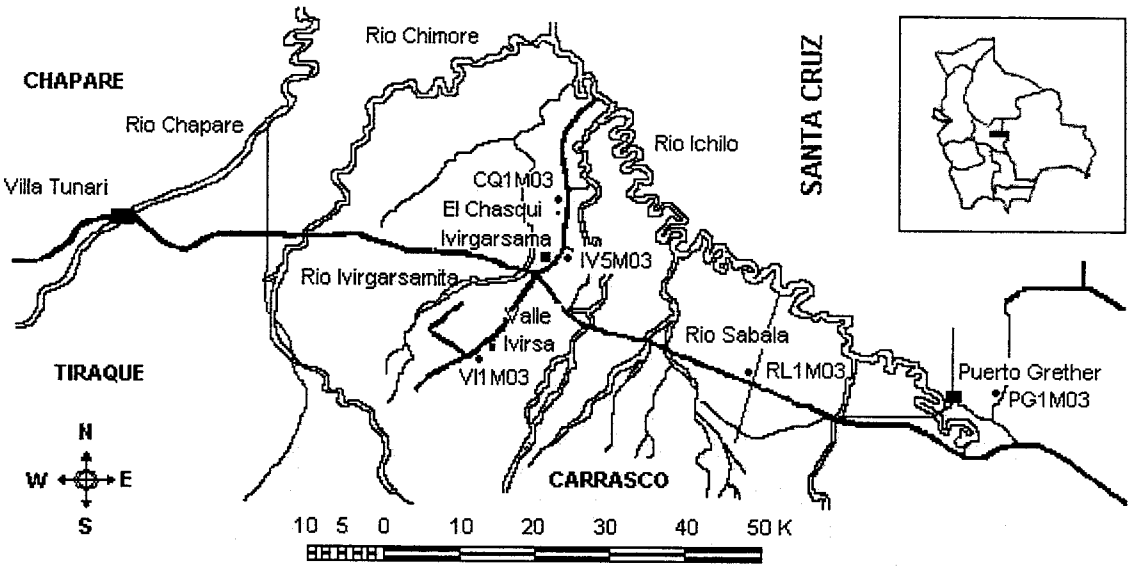

Figure 3.3. Locations in the Chapare/Carrasco valley region where specimens of *An. marajoara* were collected and tested with diagnostic PCR protocol. Small closed circles with site codes (see Table 3) represent collection sites. Labeled rectangles represent the cites of Villa Tunari, Ivivirgarsama, Valle Ivirsa, El Chasqui, and Puerto Grether.

3.7 References Cited

Branquinho, M. S., C. B. T. Lagos, R. M. Rocha, D. Natal, J. M. S. Barata, A. H. Cochrane, E. Nardin, R. S. Nussenzweig, and J. K. Kloetzel. 1993. Anophelines in the state of Acre Brazil infected with *Plasmodium faciparum, P. vivax,* the variant *P. Vivax* VK247 and *P. malariae.* Trans. Soc. Med. Hyg. 87: 391-394.

Charlwood, J. D. and D. Edoh. 1996. Polymerase chain reaction used to describe larval habitat use by *Anopheles gambiae* complex (Diptera: Culicidae) in the environs of Ifakara, Tanzania. J. Med. Ent. 33: 202-204.

Conn, J. E., R. C. Wilkerson, M. N. O. Segura, R. T. L. De Souza, C. D. Schlichting, R. A. Wirtz, and M. M. Póvoa. 2002. Emergence of a new neoptropical malaria vector facilitated by human migration and changes in land use. Am. J. Trop. Med. Hyg. 66: 18-22.

Crabtree, M. B., H. M. Savage, and B. R. Miller. 1995. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. Am J. Trop. Med. Hyg. 53: 105-109.

de Arruda, M., M. B. Carvalho, R. S. Nussenzweig, M. Mracic, A. W. Ferreira, And A. H. Cockrane. 1986. Potential vectors of malaria and their different susceptibility to *Plasmodium faciparum* and *Plasmodium vivax* in northern Brazil identified by immunoassay. Am. J. Trop. Med. Hyg. 35: 873-881.

Faran, M. E. 1980. Mosquito studies (Diptera: Culicidae) XXXIV. A revision of the Albimanus section of the subgenus *Nyssorhynchus* of *Anopheles.* Contrib. Am. Entomol. Inst. (Ann Arbor). 15: 1-215.

Faran, M. E. and K. J. Linthicum. 1981. A handbook of the Amazonian species of *Anopheles (Nyssorhynchus)* (Diptera: Culicidae). Mosq. Syst. 13: 1-81.

Fritz, G. N., J. Conn, A. Cockburn, and J. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). Mol. Biol. Evol. 11: 406-416.

Fritz, G. N. 1998. Sequence analysis of the rDNA internal transcribed spacer 2 of five species of South American human malaria mosquitoes. J. of Seq. and Map. 8: 215-221.

Fritz, G. N., S. Engman, R. Rodriguez, and R. C. Wilkerson. 2004. Identification of Four vectors of human *Plasmodium* spp. by multiplex PCR: *Anopheles rangeli, An. strodei, An. triannulatus,* and *An. trinkae* (Diptera: Culicidae: *Nyssorhynchus*). J. Med. Ent. 41: 1111-1115.

Galvao, A. L. A. and R. G. Damasceno. 1942. Sobre um novo anofelino da Ilha de Marajo, *Anopheles (N.) marajoara* n. sp. Ann. paulist. Med. Cir. 44: 424-427.

Goriup, S., and J. H. Pull. 1988. Field research in the context of malaria control, pp. 1741-1764. . *In* W. H. Wernsdorfer and I. McGregor (eds.), Malaria: principles and practices of marlariology, vol. II Churchill Livingston, New York

Haworth, J. 1988. The global distribution of malaria and the present control effort, pp. 1379-1420. *In* W. H. Wernsdorfer and I. McGregor (eds.), Malaria: principles and practices of marlariology, vol. II Churchill Livingston, New York.

Hayes, J., G. Calderon, R. Falcon, and V. Zambrano. 1987. Newly incriminated anopheline vectors of human malaria parasites in Junin Department, Peru. J. Am. Mosq. Contr. Assoc. 3:418-422.

Linthicum, K. J. 1988. A revision of the argyritarsis section of the subgenus *Nyssorhynchus* of *Anopheles* (Diptera Culicidae). Mos. Syst 20: 98-271.

Lounibos, L. P. and J. Conn. 2000. Malaria vector heterogeneity in South America. Amer. Ent. 46: 238-249.

Miller, B. R., M. B. Crabtree, and H. M. Savage. 1996. Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. Insec. Mol. Bio. 5: 93-107.

Narang, S. K., T. A. Klein, O. P. Perera, J. B. Lima, and Amazonia Toda Tang. 1993. Genetic evidence for the existence of cryptic species in the *Anopheles albitarsis* complex in Brazil: allozymes and mitochondrial DNA restriction fragment length polymorphisms. Biochem. Gen. 31: 97-111.

Porter, C. H. and F. H. Collins. 1991. Species diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 45: 271-279.

Rubio-Palis, Y. and R. H. Zimmerman. 1997. Ecoregional classification of malaria vectors in the Neoptropics. J. Med. Ent. 34: 499-510.

Tadei, W. P., B. D. Thatcher, J. M. M. Santos, V. M. Scarpassa, I. B. Rodriguez, and M. S. Rafeal. 1998. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. Am. J. Trop. Hyg. 59: 325-335.

Thompson, J. D., D. G. Higgins, T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acid. Res. 22: 4673-4680.

Velasco, M. J., J. C. S. Soriano. 1998. Analasis descriptivo de la situacion epidemiological de la malaria en el departamento de Cochabamba. Revista Medica 2: 5-10.

Wilkerson, R. C., T. V. Gaffigan, J. B. Lima. 1995a. Identification of species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). Mem. Inst. Oswaldoi Cruz 90: 721-732.

Wilkerson, R. C., T. J. Parsons, T. A. Klein, T. V. Gaffigan, E. Bergo, J. Consoli. 1995b. Diagnosis by random amplified polymorphic DNA polymerase chain reaction of four cryptic species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* (Diptera: Culicidae) from Paraguay, Argentina and Brazil. J. Med. Ent. 32: 697-704.

CHAPTER 4

Sequence analysis of the rDNA internal transcribed spacer 2 and PCR identification of *Anopheles fluminensis* (Diptera: Culicidae: *Anopheles*) in Bolivia

4.1 Abstract

Anopheles fluminensis is a member of the Arribalzagia Series in the subgenus *Anopheles*. We report the first record of this species in the department of Cochabamba, Bolivia. This species was sampled from two locations in the foothills of the eastern Andes Mountains that border the Chapare Valley. Larvae were collected in fast flowing, shaded streams at the edges of rocky pools. We provide the first sequence data for the rDNA of *An. fluminensis*, the internal transcribed spacer 2 (ITS2) flanked by short sections of the 5.8s and 28s rDNA genes. The ITS2 of *An. fluminensis* includes three large repeats (approx. 125 bp). We describe a primer and PCR diagnostic for identifying this species in the Chapare Valley, Bolivia.

4.2 Introduction

Anopheles fluminensis was described by Root (1927) from the State of Rio de Janeiro, Brazil and is a member of the Arribalzagia Series of the Laticorn Section in the subgenus *Anopheles* (Reid and Knight 1961). The Arribalzagia Series contains 24 species (Wilkerson and Peyton 1990). Within this series, *An. fluminensis* is morphologically very similar to *An. mediopunctatus* (Lutz) (Root 1927, Lounibos et al. 1997), which in turn has recently been shown to comprise a cryptic complex of three species (Wilkerson and Sallum 1999, Sallum et al. 1999). Although not currently considered an important vector of human *Plasmodium* spp. (Cequeira 1961, Gorham et al. 1973) specimens identified as near *An. fluminensis* have been incriminated as vectors of human malaria in eastern Peru (Hayes et al. 1987, Rubio-Palis and Zimmerman 1997). In addition, *An. mediopunctatus*, which may be confused with *An. fluminensis*, has been shown to be vector competent for both *P. vivax* and *P. falciparum* and incriminated as a vector in Brazil (Klein et al. 1991a & b, Povoa et al. 2000).

An important first step in understanding the epidemiology of malaria in S. America is accurate species identification, particularly to differentiate vector and non-vector species (Wilkerson et al. 1995, Rubio-Palis and Zimmerman 1997, Fritz 1998, Conn et al. 2002, Fritz 2004). Over a decade ago Oaks et al. (1991) opined that the single most important laboratory technique that should be developed and transferred to field studies on malaria was simple and inexpensive methods to differentiate anopheline mosquitoes. Currently, the only way to differentiate species of the Arribalzagia Series is by examining morphology of various life stages. For instance, Wilkerson and Peyton (1990) emphasized the use of costal wing spots to develop standardized nomenclature for

the Arribalzagia series. Other studies have used morphological characteristics of male genitalia, and immature life stages (i.e. egg. larva, and pupa) (Lounibos et al. 1997, Sallum et al. 1999, Wilkerson and Sallum 1999), all of which require time-intensive sample preparation. The advent of DNA-based techniques for discriminating organisms, particularly the polymerase chain reaction (PCR), now provide methodologies that are quick and more accurate means of identifying all stage of development. No molecular based methods exist, however, for identifying any of the members of the Arribalzagia Series.

In this study we report the first sequence of the rDNA internal transcribed spacer 2 (ITS2) of a species in the Arribalzagia Series, the first report of this species in the Department of Cochabamba, Bolivia, and we describe a PCR protocol for identifying any stage of this species in this region of Bolivia. A diagnostic primer was developed from sequences of the ITS2. This spacer was chosen for a species diagnostic because it is flanked by highly conserved ribosomal RNA coding regions (18s 28s, and 5.8s) that occur in tandem repeat arrays with the ITS2. In addition, rDNA spacers diverge rapidly, exhibit homogenization and concerted evolution and, therefore, are useful for differentiating closely related species that otherwise exhibit little genetic divergence (Fritz 1994, Crabtree et al. 1995, Charlwood and Edoh 1996, Miller 1996, Fritz 2004).

4.3 Materials and Methods

Collection and Identification of Specimens

Specimens of *An. fluminensis* examined in this study were collected in May 2003 and May 1996 from two sample sites in Cochabamba, Bolivia (Table. 4.1). Collection

sites were located in the Chapare Valley near the San Rafael River in the piedmont ecoregion of the eastern Andes foothills (Fig. 4.1) Several individuals were link reared from each site in order to obtain larval and pupae exuviae, and pinned adults. Identities of link-reared offspring were determined by morphological keys by Gorham et al (1973) for larvae and pinned adult females, and adult female identifications were confirmed by Richard C. Wilkerson (Walter Reed Biosystematics Unit, Smithsonian Institute). Larval and pupal samples were stored in 90% ETOH until used for morphological identifications, sequencing, and PCR based identifications.

Sequencing and Primer Design

Single larval or adult mosquito DNA was isolated using Qiagen DNeasy kits (Qiagen, Inc., Valencia, CA) following the instructions for the isolation of DNA from animal tissues. The ITS2 region was amplified by the polymerase chain reaction (PCR) using primers that annealed to conserved regions of the 5.8s (5'-TGTGAACTGCAGGACACATG – 3') and 28s (5'-ATGCTTAAATTTAGGGGGTAGTC-3') (Porter and Collins 1991), using a Hybaid PCR Express Thermocycler (Thermo Electron Corp.) with the following temperature profile: 1 cycle of 1 min at 94°C, 30 sec. at 65°C, and 30 sec. at 72°C, 28 cycles of 30 sec. at 94°C, 30 sec. at 65°C, and 30 sec. at 72°C, and 1 cycle of 30 sec. at 94°C, 30 sec. at 65°C, and 5 min. at 72°C. Amplifications were found to be sufficient for use in cycle sequencing using 2 µl of DNA after isolation with DNeasy kits in a 50 µl reaction, containing the following reagents: ½ µl of TaKaRA Ex Taq, 5 µl of 10X Ex Taq Buffer, 4 µl of dNTP Mixture (2.5 mM of each) (all obtained from Takara Bio Inc. (Shiga, Japan), 33.5 ul of H_2O, and 2.5 µl of each of the two primers at 40 ng/ul (5.8s and 28s).

Amplicons were subsequently purified using Qiaquick PCR purification kits (Qiagen Inc., Valencia, CA) according to manufacturer's instructions for a microcentrifuge.

Forward and reverse consensus sequences of the ITS2 were obtained by using primers that anneal to conserved regions of the 5.8s and 28s. Sequencing reactions were accomplished using a CEQ Dye Terminator Cycle Sequencing Kit (DTCS) (Beckman Coulter Inc., Fullerton, CA) following manufacturer's recommendations for sequencing from a double stranded template. All sequencing was completed using a Beckman Coulter 2000 sequencer (Beckman Coulter Inc., Fullerton, CA). Forward and reverse sequences were subsequently aligned using Sequencher^{TM} 3.0 (Gene Codes Corp., Ann Arbor, MI) and manually checked for optimal alignment. Two specimens of *An. fluminensis* were sequenced from site MY1M03 (Fig. 4.1, Table 4.1) where we had identified this species based on morphological characteristics of larvae and adults, using keys by Gorham et al. (1973). A species specific primer was designed by aligning available ITS2 sequences from a sequence database (Genbank) for members of the subgenus *Anopheles* including *An. eiseni, An. mattogrossensis, An. mediopunctatus, An. peruassui*, and *An. quadrimaculatus*, using CLUSTALW (Thompson et al. 1994). In addition, the primer sequence was compared by alignment to multiple members of the subgenus *Nyssorhynchus* including *An. dunhami, An. strodei, An. trinkae, An. triannulatus, An. rangeli, An. galvaoi, An. darlingi, An. marajoara, An. albimanus, An. nuneztovari, An. evansae, An. konderi, An. argyritarsis, An. braziliensis, An. deanorum, An. albitarsis, An. albitarsis* B, and *An. rondoni* and *An. oswaldoi sensu lato.* Furthermore, the chosen primer sequence was subjected to an automated search function (i.e. "blasted") to find homologous sequences on a sequence database (Genbank) to

confirm the primer's exclusiveness for the targeted species. The primer was optimized using standard protocols and chosen to give an easily resolved amplicon on 2% gels stained with ethidium bromide. The species specific primer was paired with a primer that annealed to the 5.8s, and tested on its target species, and subsequently tested on samples of DNA from seven members of the subgenus *Anopheles* and 16 members of the subgenus *Nyssorhynchus* (Table 4.2).

Restriction Enzyme Digest

Because the ITS2 sequence of *An. fluminensis* was found to contain three large, tandem repeats, we tested for the presence of these repeats in field samples of *An. fluminensis* by endonuclease digestion (Apek-I, New England Biolabs, Beverly, MA) of a unique restriction site found in each repeat. The complete ITS2 amplified by PCR reaction outlined above was first purified using Qiagen Qiaquick PCR purification kits (Qiagen, Inc., Valencia, CA) following manufacturer's instructions. However, in the last step of the Qiagen kit we suspending DNA in 40 µl of 1X #3 buffer supplied by New England Biolabs (Beverly, MA) instead of buffer supplied with the Qiagen PCR purification kit, to maximize enzyme digest. Twelve µl of the purified DNA was subjected to an overnight digestion at 75 °C with ½ µl Apek-I, followed by electrophoresis of 10 µl of cut DNA on a 3% agarose gel stained with ethidium bromide. Field samples tested with Apek-I included nine individuals from MY1M03 and six from SR1-A (Fig. 4.1, Table 4.1).

4.4 Results

Larvae of *An. fluminensis* were found at two sites on the eastern foothills (piedmont ecoregion) of the Andes Mountains that lead into the Chapare Valley (Fig. 4.1). The aquatic sites were fast-flowing, shaded streams, and larvae were collected at the edges of rocky pools, often in the vegetation, debris, or among roots that border these pools.

ITS2 sequence was obtained from within 4-12 bp of the primers that annealed to the 5.8s and 28s regions flanking the ITS2. Boundaries of the 5.8s and 28s regions were estimated by comparison with those determined for ITS2 sequences for *An. mediopunctatus* and *An. quadrimaculatus* available on Genbank (Accession numbers: AF462379 and U32550, respectively). The ITS2 begins at position 53 (Fig. 4.2) and ends at position 637, and is approximately 584 nucleotides. The ITS2 of *An. fluminensis* was found to contain three large repeats (Figs. 4.2, 4.3, 4.4) each containing a single unique restriction endonuclease (Apek-I) cut site (Fig. 4.2 and 4.4). The first repeat, counting from the beginning of the ITS2, is approximately 124 bp in length, while the second and third are 127 bp, expanded by 3 bp insertions (Fig. 4.3). Digestion of the ITS2 amplicon with Apek-I was expected to produce four fragments of DNA of the following approximate sizes: ~143, ~144, ~223, and ~170 bp. However, the two smallest fragments because of similar sizes could not be differentiated; therefore, producing only three fragments on an agarose gel (Fig. 4.5).

A single species specific primer (5'-GAC CAC CAA AAG AGT CGG-3') was chosen for amplifying a single amplicon in *An. fluminensis* of ~225 bp (Fig. 4.5), when combined in a PCR with the 5.8s primer found in the conserved 5.8s rDNA gene. The

primer sequence was not present in its presumptive close relative, *An. mediopunctatus*, nor in any of the other species tested in the subgenus *Anopheles* or *Nyssorhynchus*. The location for the primer was chosen so that it did not anneal within any of the repeats. The rationale for this choice was to prevent ambiguous amplicons of different sizes in individuals of *An. fluminensis* that may have variable numbers of repeats in the ITS2. Nevertheless, Apek-I digestion of all field collected samples produced the expected three banded pattern of the correct sizes in gel electrophoresis (Fig. 4.5).

Optimized 50 ul PCR reactions included: 5 ul of 10X buffer, 8 ul of DNTP mix at 1.25 Mm, ½ µl of taq. Polymerase at 5 U/µl, 21.0 µl of H_2O, 6 µl of MgCl at 25 mM, 2.5 µl of each of the two primers at 40 ng/µl (*An. fluminensis* species specific and 5.8s). We examined various program parameters, especially the annealing temperature to optimize amplifications. A Hybaid PCR express thermocycler (Thermo Electron Corp.) was used for all PCR with the following program: 29 cycles of 30 sec. at 94° C, 30 sec. at 60° C, and 30 sec. at 72° C, and once cycle of 30 sec. at 94° C, 30 sec. at 60° C, with a final extension step of 2 min. at 72° C. Amplification products were electrophoresed on 1.5% gels and visualized with ethidium bromide stain. PCR reactions with *An. fluminensis* species specific and 5.8s primers produced no unexpected amplicons when tested on other members of the subgenus *Anopheles* and *Nyssorhynchus* (data not shown).

4.5 Discussion

Since its description, *An. fluminensis* has been reported in Trinidad (Rawling 1993), Colombia (Quinones et al. 1987), Peru (Morales-Ayala 1971), Argentina (Mitchell and Darsie 1985) and various locations in Brazil (e.g., Milward de Andrade 1962,

Barbosa et al. 1993, Lounibos et al 1997, Guimareas et al. 2000). Although Gorham et al. (1973) list *An. fluminensis* as present is Bolivia, no reference is provided. The Mosquito Catalog of the World, managed by the Walter Reed Biosystematic Unit, also lists *An. fluminensis* as present in Bolivia, but none of the references provided for its known distribution mention this species with regard to Bolivia. Consequently, we know of no published records of *An. fluminensis* in Bolivia. We assume, therefore, that this is the first confirmed report of *An. fluminensis* in Bolivia. Its presence in the eastern Andean piedmont is consistent with its ecoregional classification by Rubio-Palis and Zimmerman (1997).

The ITS2 of *An. fluminensis* is unusually large, since the majority of anophelines sequenced to date have this spacer in the range of 350bp to 400bp in length. The three repeats in the ITS2 suggest that one or two duplications led to its unusual size. A BLAST search of this repeat in GenBank did not show similarity to any other sequences; suggesting the origin of the repeat is within the ITS2 itself. Since we did not sequence clones of the ITS2, but rather obtained the consensus sequence through PCR of whole body DNA, we cannot rule-out the possibility that intraindividual variation exists for the number of repeats present in rDNA units. When amplifying the ITS2, the presence of two faint, additional amplicons of lower molecular weight suggests intraindividual variation in repeat number, but this possibility will need to be confirmed with the sequencing of clones.

The ITS2 of *An. fluminensis* is also unusual for having large repeats. Large repeats of varying size have been reported in the rDNA spacers of species in the genera *Daphnia, Aedes, Drosophila, Rattus,* and *Xenopus* (Labhart and Reeder 1984, Simeone

et al. 1985, Murtifand and Rae 1985, Cassidy et al. 1986, Park and Fallon 1990, Crease 1993). Perera et al. (1998) also reported a 36-nucleotide repeat in the intergenic spacer of *Anopheles aquasalis* Curry. Large repeats, however, have never been reported in any species of *Anopheles* for the rDNA internal transcribed spacers. The only reports of repeats in transcribed spacers have been of small repeat regions usually comprising short di-nucleotide repeats often associated with microsatellite regions (Park and Fallon 1990, Porter and Collins 1991, Fritz et al. 1994, Fritz et al. 1998, Wilkerson et al. 2004). Whether or not the large repeats found in *An. fluminensis* affect transcription or RNA processing is not known, but conformational studies on the repeats of the ITS2 in tick (Mureell 2001) suggest that repeat copy does not affect function.

To date, *An. mediopunctatus* has been verified by Wilkerson and Sallum (1999) as occurring only in the coastal regions of São Paulo, Rio de Janeiro and Paraná, Brazil. The species specific primer developed in this study was not tested against any members of the Arribalzagia Series. However, when tested on seven members of the subgenus *Anopheles* and 16 members of the subgenus *Nyssorhynchus* no amplicons were produced. Additionally, when blasted on genbank the primer sequence was not homologous with any sequence of anopheline mosquito, including its presumed close relative, *An. mediopunctatus*. Of the 24 species in the Arribalzagia Series, only one other species has been sequenced for the ITS2. Thus, it is possible that the primer developed in this study anneals to other species in this Series. Nevertheless, no other members of the Arribalzagia Series have ever been collected in Cochabamba, though extensive collecting in the Chapare Valley has occurred over the past 20 years. We think, therefore, that the PCR diagnostic developed in this study has, at least, regional utility and will facilitate

studies that aim to elucidate the basic biology, ecology, and behavior of this species in Bolivia.

4.6 Tables and Figures

Table 4.1. Larval collection data for *An. fluminensis* specimens collected in the Chapare Valley, Cochabamba, Bolivia.

Site Code	Location	Habitat	Elevation (m)	Coordinates
MY1M03	Muyurina	Intermittent mountain stream, partly shaded.	500	17° 03.552''S, 065° 29.924''W
SR1-A	San Rafael River	Intermittent mountain stream partly shaded, lined by grasses.	360	17° 03.888''S, 065° 29.442''W

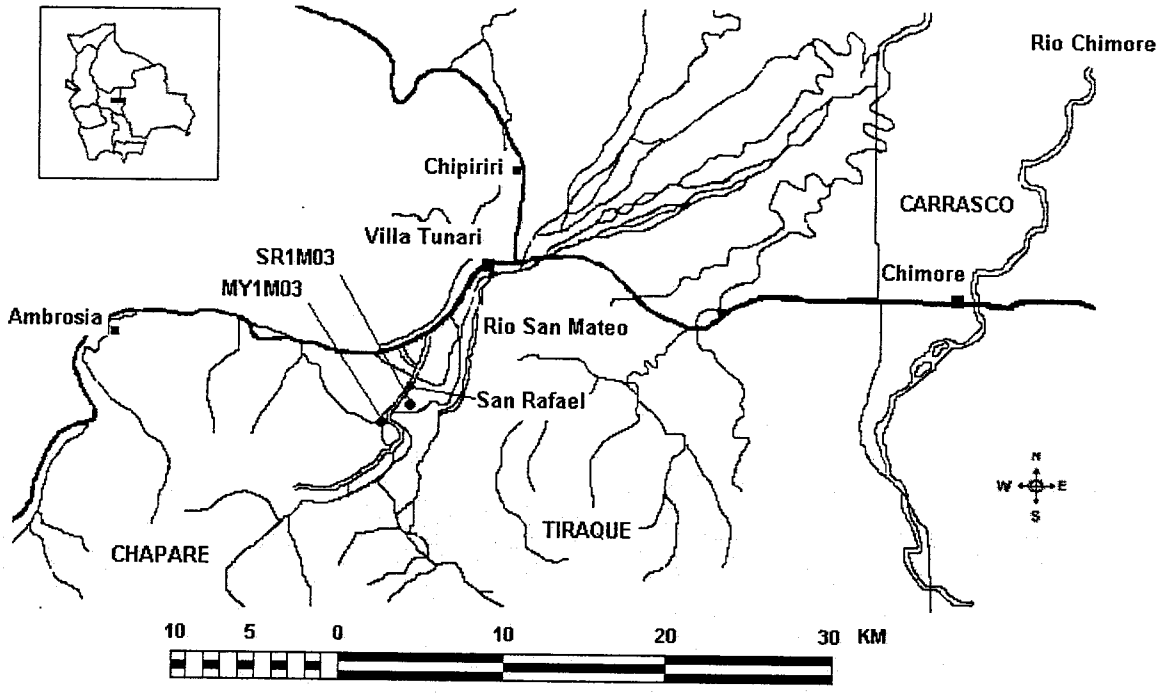

Figure 4.1. Map of the Chapare/Carrasco Valley region where *An. fluminensis* was collected. Solid circles with site codes (SR1M03 and MY1M03) represent collection sites for An. fluminensis (see Table 4.1). Rectangles represent the cities of Ambrosia, San Rafael, Villa Tunari, .Chipiriri, and Chimore.

Table 4.2. Collection localities for specimens in the subgenera *Nyssorhynchus* and *Anopheles* tested with species-specific primer.

Species	Collection Location	Coordinates	No. tested
Subgenus *Nyssohynchus* spp. tested			
An. albimanus	United States, Florida, Gainsville, Lab Colony, USDA	Not known	1
An. albitarsis	Brazil, Iguape	24°44'S, 47°35'W	1
An. aquasalis	Suriname, Paramaibo	5°50'N, 55°11'W	1
	Brazil, Marajo Island	Not known	1
An. bennarrochi	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. darlingi	Bolivia, Beni, Guayaramirín	10°51'N, 65°21'W	1
An. deanorum	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. evansae	Brazil, Rio de Janeiro	23°47'S, 43°49'W	2
An. konderi	Brazil, Rondonia, Costa Marques	12°25'S, 64°18'W	1
An. marajoara	Venezuela, Cojedes	Not known	1
	Bolivia, Cochabamba, Chapare Valley	17°58'S, 64°49'W	2
An. nuneztovari	Brazil, Roraima, Boa Vista	2°49'N, 60°40'W	1
	Venezuela, Barinans, Boconoito	Not Known	1
An. oswaldoi s.l.	Brazil, Pará, Urucuri	Not known	1
	Bolivia, Cochabamba, Chapare Valley	16°53'S, 65°11'W	3
	Bolivia, Cochabamba, Chapare Valley	16°59'S, 65°37'W	2
An. rangeli	Bolivia, Cochabamba, Chapare Valley	16°56'S, 65°23'W	1
An. strodei	Bolivia, Cochabamba, Chapare Valley	17°02'S, 64°51'W	1
An. triannulatus	Bolivia, Cochabamba, Chapare Valley	17°12'S, 64°30'W	1
An. trinkae	Bolivia, Cochabamba, Chapare Valley	16°57'S, 65°19'W	1
Subgenus *Anopheles* spp.			
An. earlei	United States, Minnesota, Champlin, Elm Creek Park	45°10'N, 93°25'W	1
An. franciscanus	United States, California, El Dorado County, Cameron	39°16'N, 123°33'W	1
An. freeborni	United States, California, Yolo County, Knights Landing	38°47'N, 121°43'W	1
An. hermsi	United States, California, San Mateo County, Jasper Ridge Preserve	37°24'N, 122°13'W	1
An. occidentalis	United States, California, Alameda County, Coyote Hills	39°58'N, 120°26'W	1
An. perplexis	United States, Florida, Lake Panasofkee	Not known	1
An. punctipennis	United States, California, Lake Vera	39°18'N, 121°01'W	1

GCATATTGCGCATCGTGCGACACAGCTCGATGCACATATCTTTGAGAGTCCATAC 55

TTGACATAGTCAAACTACGGTTGTCTGGGCGCAAGCTCGGACACTACCGTGCATA 110

TTGGGGTGGCGGCGCCTACTCGGCGTCGTAGCCCTTAAAATCCCTGTGGAGCGTG 165

TTCACCGACTCTTTTGGTGGTCTCTCGTCACCAAGTGG**GCAGC**GGTGGCCGGCAC 220

CCTCACCTCTTCACCGAGCCGTTGAGATGTGTTCGGTCACACATACGCTGATCAC 275

GACGAGCGTCCTTAAGGTGGTGGACTGCCCATGGAAGGGAGCTCTTGGTCGTCTC 330

TCGTCACCAAAAGTGG**GCAGC**GGTGGCCTGCACCTCTCACCTCTTCACCGAGCCG 385

TTGAGATGTGTACGGTCACGCATACGCTGACCACGACGAGCGATCATAAGGTGGT 440

TGACTACCTCCTGATACGAGCTCTTGGTCGTCTCTCGTCACCAAAAGTGG**GCAGC** 495

GGTGGCCTGCACCTCTCACCTCTTCACCGAGCCGTTGAGATGTGTTCGGTCACGC 550

ATACGCTGATCACGACGAGCGACCAAAAGGTGGTACCAAGAATTATGTATGGGTA 605

TGGTACCGAAACGAACGCTCCCTTCGGTGGAGTTTATGAGCAGT 649

Fig. 4.2. ITS2 sequence and flanking regions of the 5.8s and 28s rRNA genes of *An. fluminensis*. The underlined regions represent the repeat units. Bolded sections of sequence represent Apek-I digest sites.

```
1st   ACTCTTTTGGTGGTCTCTCGTCACCAA..GTGGGCAGCGGTGGCCGGCA 219
2nd   GAG--C-----C---------------AA----------------T--- 362
3rd   GAG--C-----C---------------AA----------------T--- 506

1st   CC.CTCACCTCTTCACCGAGCCGTTGAGATGTGTTCGGTCACACATACG 267
2nd   --T-------------------------------A-------G------ 411
3rd   --T-------------------------------T-------G------ 555

1st   CTGATCACGACGAGCGTCCTTAAGGTGGT 296
2nd   ----C-----------AT-AT-------- 440
3rd   ----T-----------AC-AA-------- 584
```

Fig. 4.3. Sequence alignment of repeat units within the ITS2 of *An. flumeninsis.* Dashes represent identical sequence with the 1st repeat unit and dots represent gaps introduced to maintain alignment. The numbering scale for the nucleotides to the right of the sequences coincides with the scale in Fig. 4.1.

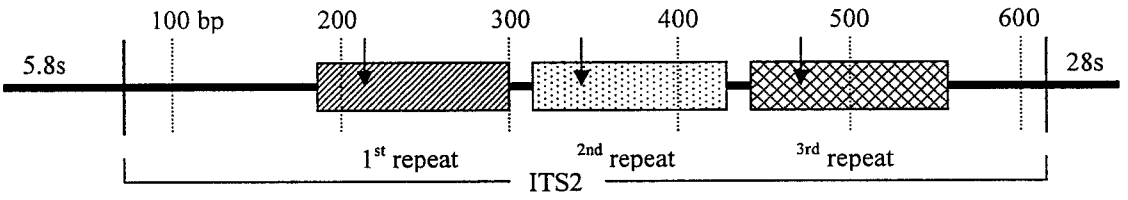

Fig. 4.4. Restriction map for the ITS2 of *An. fluminensis* with Apek-I. The ITS2 is outlined on the bottom of the figure and flanked by the two conserved genes (5.8s, 28s). Repeat regions are represented by the rectangles with different shading patterns, labeled 1st repeat, 2nd repeat, and 3rd repeat, respectively from left to right. Dotted lines with numbers above them seperate 100 bp segments, that coincide with the numbering in Fig. 4.1. Arrows represent approximate Apek-I cut sites unique to each repeat unit.

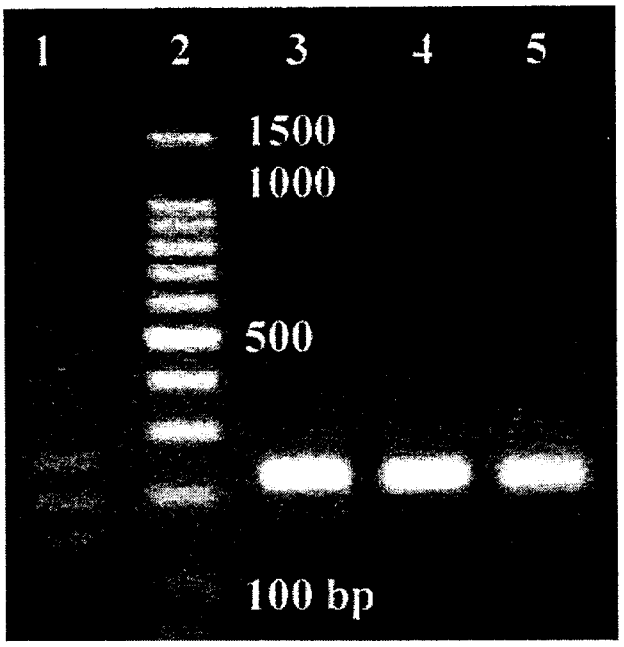

Fig. 4.5. Restriction enzyme digest with Apek-1 of *An. fluminensis* ITS2 amplicon (Lane 1) and species specific amplicon for *An. fluminensis* (Lanes 3-5). Lane 2 = 100 bp DNA ladder.

4.7 References Cited

Barbosa, O. C., U. Teodoro, A. L. Lozovei, V. La Salvia Filho, R. P. Spinosa, E. Maurício de Lima, M. E. M. C. Ferreira. 1993. Nota sobre culicídeos adultos coletados na região sul do Brasil. Rev. Saúde Pública. 27(3): 214-216.

Cassidy B. G., H. F. Yang-Yen, L. I. Rothblum. 1986. Transcriptional role for the nontranscribed spacer of rat ribosomal DNA. Mol. Cell. Biol. 6: 2766-2773.

Cerqueira, N. L. 1961. Distribuicão geográfica dos mosquitos da Amazônia. Rev. Bras. Ent. 10: 111-168.

Charlwood, J. D. and D. Edoh. 1996. Polymerase chain reaction used to describe larval habitat use by *Anopheles gambiae* complex (Diptera: Culicidae) in the environs of Ifakara, Tanzania. J. Med. Ent. 33(2): 202-204.

Conn, J. E., R. C. Wilkerson, M. N. O. Segura, R. T. L. de Souza, C. D. Schlichting, R. A. Wirtz, M. M. Póvoa. 2002. Emergence of a new neotropical malaria vector facilitated by human migration and changes in land use. Am. J. Med. Hyg. 66(1): 18-22.

Crabtree, M. B., H. M. Savage, and B. R. Miller. 1995. Development of a species-diagnostic polymerase chain reaction assay for the identification of *Culex* vectors of St. Louis encephalitis virus based on interspecies sequence variation in ribosomal DNA spacers. Am J. Trop. Med. Hyg. 53(1): 105-109.

Crease, T. J. 1993. Sequence of the intergenic spacer between the 28s and 18s rRNA encoding genes of the crustacean, *Daphnia pulex*. Gene 134: 245-249.

Fritz, G. N., J. Conn, A. Cockburn, and J. Seawright. 1994. Sequence analysis of the ribosomal DNA internal transcribed spacer 2 from populations of *Anopheles nuneztovari* (Diptera: Culicidae). Mol. Biol. Evol. 11(3): 406-416.

Fritz, G. N. 1998. Sequence Analysis of the rDNA internal transcribed spacer 2 of five species of South American human malaria mosquitoes. J. Sequencing and Mapping 84: 215-221.

Fritz, G. N., Engman, S. Rodriguez, R., and Wilkerson R. C. 2004. Identification of four vectors of human *Plasmodium spp.* by multiplex PCR: *Anopheles rangeli, A. strodei, A. triannulatus,* and *A. trinkae* (Diptera: Culicidae: *Nyssorhynchus*) J. Med. Ent. 41(6): 1111-1115.

Gorham, J. R., C. J. Stojanovich, H. G. Scott. 1973. Illustrated key to the anopheline mosquitoes of western South America. Mos. Sys. 5(2): 97-156.

Guimarães, A. E., R. Pinto de Mello, C. M. Lopes, C. Gentile. 2000. Ecology of Mosquitoes (Diptera: Culicidae) in areas of Serra do Mar State Park, State of São Paulo, Brazil. I-monthly frequency and climatic factors. Mem. Inst. Osw. Cruz. 95(1): 1-16.

Hayes, J., G. Calderon, R. Falcon, and V. Zambrano. 1987. Newly incriminated anopheline vectors of human malaria, parasites in Junin Department, Peru. J. of Am. Mosl Contr. Assoc. 3: 418-422.

Klein, T. A., J. B. P. Lima, M. S. Tada, and R. Miller. 1991a. Comparative susceptibility of anopheline mosquitoes in Rondonia, Brazil to infection by *Plasmodium vivax.* Am. J. Trop. Med. Hyg. 44:463-470.

Klein, T. A., J. B. P. Lima, and M. S. Tada. 1991. Comparative susceptibility of anopheline mosquitoes to *Plasmodium falciparum* in Rondonia, Brazil. Am. J. Trop. Med. Hyg. 44:598-603.

Labhart R., R. Reeder. 1984. Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of *Xenopus laevis*. Gene 134: 245-249.

Lounibos, L. P., D. Duzak, J. R. LinleyU, R. Lourenco-de-Oliveira. 1997. Egg Structures of *Anopheles fluminensis* and *Anopheles shannoni.* Mem. Inst. Osw. Cruz. 92(2): 221-232.

Miller, B. R., M. B. Crabtree, and H. M. Savage. 1996. Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. Insec. Mol. Bio. 5(2): 93-107.

Milward de Andrade, R. 1962. Distribicão de Anophelinos pr município cidades e vilas do setado de Pernambuco Brasil. (Diptera: Culicidae). Rev. Bras. Malariol. Doencas Trop. 14(1-2): 145-58.

Mitchell, C. J., R. F. Darsie Jr. 1985. Mosquitoes of Argentina, Part II geographic distribution and bibliography (Diptera: Culicidae). Mos. Syst. 17(4): 279-286.

Morales-Ayala, F. 1971. A list of the mosquitoes of Peru (Diptera: Culicidae). Mosq. Syst. Newsletter. 3(3): 138-145.

Murrell, A., N. J. H. Campbell and S. C. Barker. 2001. Recurrent gains and losses of large (84-109 bp) repeats in the rDNA internal transcribed spacer 2 (ITS2) of rhipicephaline ticks. Ins. Mol. Biol. 10: 587-596.

Murtif, V. L., P. M. M. Rae. 1985. In vivo transcription of rDNA spacers in *Drosophila.* Nucl. Acids Res. 13: 3221-3239.

Oaks, S. C., V. S. Mitchell, G. W. Pearson, and C. C. J. Carpenter (eds.) 1991. Malaria. Obstacles and opportunities. National Academy of Science Press, Washington, DC.

Park, Y., A. M. Fallon. 1990. Mosquito ribosomal RNA genes: characterization of gene structure and evidence for changes in copy number during development. Ins. Biochem. 20(1): 1-11.

Perera, O. P., A. F. Cockburn, S. E. Mitchell, J. Conn, J. A. Seawright. 1998. Species-specific repeat units in the intergenic spacer of the ribosomal RNA cistron of *Anopheles aquasalis* Curry. Am. J. Trop. Med. Hyg. 59(5): 673-678.

Porter C. H., F. H. Collins. 1991. Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles Hermsi* (Diptera: Culicidae). Am. J. Trop. Med. Hyg. 45(2): 271-279.

Póvoa, M. M., A. N. M. da Silva, C. C. B. dos Santos, M. N. de O. Segura., R. L. D. Machado. 2000. Malaria transmission. Ciencia e Cultura. 52(4/5): 208-212.

Quiñones P., M. L., M. F. Suárez A., G. A. Fleming. 1987. Distribución y bionomía de los anofelinos de la costa pacífica de Colombia. Colombia Medica 18(1): 19-24.

Rawling, S. C. 1993. The malaria situation in the Caribbean region. W. I. Med. 42: 134-136.

Reid, J. A. and K. L. Knight. 1961. Classification within the subgenus *Anopheles* (Diptera, Culicidae). Ann. Trop. Med. Parasit. 55:474-488.

Rubio-Palis, Y., R. H. Zimmerman. 1997. Ecoregional classification of malaria vectors in the Neoptropics. J. Med. Ent. 34(5): 499-510.

Sallum, M. A., R. C. Wilkerson, O. P. Forattini. 1999. Taxonomic study of species formerly identified as *Anopheles mediopunctatus* and resurrection of *An. costai* (Diptera: Culicidae). J. Med. Ent. 36(3): 282-300.

Simeone, A., A. La Volpe, L. Boncinem. 1985. Nucleotide sequence of complete ribosomal spacer of *Drosophila melanogaster*. Nucl. Acid. Res. 13: 1089-1101.

Thompson, J. D., D. G. Higgins, T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl. Acid. Res. 22: 4673-4680.

Willkerson, R. C., and E. L. Peyton. 1990. Standardized nomenclature for the costal wing spots of the genus *Anopheles* and other spotted-wing mosquitoes (Diptera: Culicidae). J. Med. Entomol. 27: 207-224.

Wilkerson, R. C., T. V. Gaffigan, J. B. Lima. 1995. Identification of species related to *Anopheles* (*Nyssorhynchus*) *albitarsis* by random amplified polymorphic DNA-polymerase chain reaction (Diptera: Culicidae). Mem. Inst. Oswaldoi Cruz 90: 721-732.

Wilkerson, R. C., M. A. M. Sallum. 1999. *Anopheles* (*Anopheles*) *forattinii*: a new species in series Arribalzagia (Diptera: Culicidae). J. Med. Ent. 36(3): 345-354.

Wilkerson, R. C., J. F. Reinert, C. Li. 2004. Ribosomal DNA ITS2 sequences differentiate six species in the *Anopheles crucians* complex (Diptera: Culicidae). J. Med. Ent. 41(3): 392-401.