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Genetic Structure of Amblyomma Cajennense (Acari: Ixodidae) Populations Based on Mitochondrial Gene Sequences

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GENETIC STRUCTURE OF *AMBLYOMMA CAJENNENSE* (ACARI: IXODIDAE) POPULATIONS BASED ON MITOCHONDRIAL GENE SEQUENCES

by

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(Under the Direction of Dr. Lorenza Beati)

ABSTRACT

Amblyomma cajennense (Fabricius, 1787) (Acari: Ixodidae) is a common tick species that has a large geographic distribution from the southern regions of the United States (Texas), to the Caribbean Islands, Central, and South America. This tick is a vector of the agent of Brazilian spotted fever, an often fatal disease in South America. Throughout its geographic range, populations of *A. cajennense* have shown differences in ecological adaptation while feeding on a variety of hosts ranging from livestock, birds, and humans. In order to examine the taxonomic status and phylogeographic evolution of this species, we analyzed mitochondrial 12S rDNA, control region (d-loop), and cytochrome oxidase II gene sequences of *A. cajennense* specimens collected in eight different localities. The results showed that our samples are grouped in five strongly supported monophyletic lineages, each corresponding to geographically or ecologically distinct populations. The strong phylogenetic structure indicates that *A. cajennense* may actually be a species complex in need of thorough morphological reassessment.

INDEX WORDS: *Amblyomma cajennense*, 12S rDNA, Control region (d-loop), Cytochrome oxidase II, Brazilian spotted fever, Cryptic species, Phylogeography, Genetic structure

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CHAPTER 1

INTRODUCTION

Taxonomic information

Amblyomma cajennense (Fabricius, 1787), commonly known as the Cayenne tick, is a common tick species infesting livestock and a number of other vertebrates including humans and birds (Borges et al., 2002; Oliviera et al., 2003; Lopes et al., 1998; Rojas, Marini and Coutinho, 1999). This tick has a wide distribution, ranging from the southern regions of the United States to the Neotropics. Because this tick is so widespread, taxonomists have been studying it since the late 18th Century and several names have been applied to it as shown below

Synonymy list

Acarus cajennensis Fabricius, 1787
Ixodes cajennensis (Fabricius, 1794)
Ixodes crenatus Say, 1821
Amblyomma tenellum Koch, 1844
Amblyomma mixtum Koch, 1844
Amblyomma sculptum Berlese, 1888
Ixodes herrerae Dugés, 1891
Amblyomma parviscutatum Neumann, 1899
Amblyomma versicolor Nuttall & Warburton, 1908
Amblyomma tapiri Tonelli-Rondelli, 1937
Amblyomma finitimum Tonelli-Rondelli, 1937

The above synonymy list shows that this species has been extensively studied and indicates that its taxonomy over the centuries has been controversial. *Amblyomma cajennense* was first described and named by Fabricius from a tick collected in Cayenne, the capital of French Guiana. Later, morphologically similar species were established by Koch (1844). These were later synonymized with *A. cajennense* by Neumann (1899, 1911) and Robinson (1926). Tonelli-Rondelli (1939) still believed that Neumann and

Robinson overlooked important characteristics and pointed out morphological differences evident in geographically distinct populations of *A. cajennense*. She recognized 6 valid independent species, which are: *A. cajennense*, *A. tenellum* (Mexico), *A. mixtum* (Mexico), *A. sculptum*, *A. tapiri* (French Guiana), and *A. finitimum* (French Guiana) (Tonelli-Rondelli, 1939). Aragão and Fonseca (1953) synonymized all of Koch's and Tonelli-Rondelli's species with *A. cajennense.* Although there is now a general consensus in applying all these names to refer to *A. cajennense*, minor morphological and major ecological differences indicate that this widely distributed tick species may include genetically diverse populations (Guglielmone et al., 1992).

Geographic distribution

The distribution of *A. cajennense* ranges from the southern regions of the United States (Texas), to the Caribbean Islands, Central, and South America as shown in FIG. 1 (Estrada-Peña, et al., 2004). Thus far, *A. cajennense* has not been found either north of latitude 27°N or south of latitude 29ºS (Estrada-Peña et al., 2004).

The geographic range of *A. cajennense* has been found to be limited by temperature. (Estrada-Peña et al., 2004).

FIG. 1: Known distribution of *Amblyomma cajennense* (•) ranging from Texas to northern Argentina. Modified from Estrada-Peña et al., 2004.

Low temperatures in mountainous areas such as the Mexican Sierra Madre and in the Andes have been shown to be an obstacle for the establishment of this arthropod (Estrada-Peña et al., 2004). Differences in ecological adaptation among *A. cajennense* populations have been noted (Estrada-Peña et al., 2004). This tick is known to survive in regions with very different ecological conditions (FIG. 2), spanning from arid grasslands to tropical forests.

Host Associations

The geographic distribution of a tick species is usually determined by host distribution and dispersal. *Amblyomma cajennense* is a three-host tick with equines as the principal host (Borges et al., 2002; Oliveira et al., 2003) that maintains the population in domestic environments (Labruna et al., 2002). However, this arthropod, and its immature stages in particular, have been found on a wide range of hosts including wild and domestic ungulates, birds (Rojas et al., 1999) and humans (Lopes et al., 1998), showing a relatively low level of host specificity. Carried by domestic animals (transported by humans) or by birds, *A. cajennense* may have dispersed across long distances and over land barriers, such as the Andes Mountains, large rivers (Amazon), and oceans (Caribbean), and other inhospitable habitats (Estrada-Peña et al., 2004).

Economic impact

This arthropod is an important vector for pathogenic organisms such as *Rickettsia rickettsii* the bacterium that causes Rocky Mountain Spotted Fever, which is called Brazilian spotted fever in Brazil, (Aragão, 1936; Dias and Martins, 1939; Sangioni et al., 2005) and possibly Venezuelan equine encephalomyelitis virus (Linthicum et al., 1991). This tick species has also been implicated in the transmission of the unknown causative

agent of human Lyme disease in Brazil although the occurrence of this disease in South America is still controversial (Castagnolli et al., 2003). These diseases are important to both humans and livestock (Lopes et al., 1998).

Moreover, infestations of cattle by these ticks can lead to decreased weight, reduced milk production, malnutrition and starvation (Teglas et al., 2005). When the ticks bite, they leave small perforations in the hide in which flies such as the screwworm (*Cochliomyia hominurorac*) or secondary screwworm (*C. macellaria*) may subsequently lay their eggs causing further damage to the hides. The hides of these cattle are a major source of income in poor rural areas and such infestations hurt the local economics and lead to further deterioration of living conditions for humans (Teglas et al., 2005). For the development of preventative measures, it is crucial to ensure that the taxonomy of these ticks is fully understood.

Ticks and population genetics studies

There are several molecular tools used currently in the study of intraspecific tick phylogenetics. Among the most frequently used methods are phylogenetic analysis of nuclear genes with a high mutation rate, such as the internal transcribed ribosomal spacer (ITS2) (Rich et al., 1997; Zahler et al., 1995; Zahler and Goethe, 1997) or of the mitochondrial genome, typically looking at the 12S rDNA, 16SrDNA or the Cytochrome oxidase I gene (Barker and Murrell, 2004). However, recent studies using other genes in the mitochondria have shown much potential for the study of phylogeny of ticks (Barker and Murrell, 2004). Also, nuclear microsatellite loci have been found to be effective tools in order to detect genetic variability within tick species (McCoy and Tirard, 2000; de Meeûs et al., 2002; McCoy et al., 2003).

Main Hypothesis

Amblyomma cajennense has a large distribution range and we therefore, could expect to find a relationship between geographic and genetic distances. Land and water barriers such as the Andes Mountains, the Atlantic, and large Amazonian rivers would further isolate populations, decrease the genetic flow of *A. cajennense,* and increase genetic differentiation.

Conversely, one could also imagine that, given the preferred association between *A. cajennense* and domestic animals, this tick may have accompanied humans throughout the subcontinent, therefore, maintaining continuous gene flow among tick populations. Transport of immature stages by migrating birds would also contribute to homogenous genetic populations.

Purpose of study

Compare the following scenarios:

Hypothesis 1: *Amblyomma cajennense* is carried by hosts (domestic ungulates and birds) over large geographic distances, thus leading to weak geographic genetic structure.

Hypothesis 2: *Amblyomma cajennense* occurs in ecologically highly diverse areas, with populations isolated from each other by barriers, and is subdivided into genetically divergent populations.

In order to evaluate these hypotheses we have studied the genetic variability of three *A. cajennense* mitochondrial gene sequences in tick samples collected in eight countries representing diverse ecological areas of the neotropics and Mexico.

CHAPTER 2

MATERIALS AND METHODS

Sample Collection

The samples used in this study were collected in Peru, Brazil, Ecuador, French Guiana, Argentina, Venezuela, Mexico and Costa Rica. Samples were collected in 6 of the 8 biomes where *A. cajennense* is known to occur. The biomes that were represented in this study were:

(1) Tropical grasslands / Savannahs

(2) Tropical humid forests,

(3) Tropical dry forests / woodlands,

(4) Temperate grasslands,

(5) Sub-tropical / temperate rain forests / woodlands, and

(6) Mixed mountain systems (FIG. 2). Table 1: shows the sample locations and the biomes that were represented at the collection sites. The samples were stored and shipped in vials containing 70% ethanol. Some of the samples were obtained from the US National Tick Collection at Georgia Southern University,

FIG. 2: The geographic range of A. cajennense overlapped with corresponding biomes. Sample areas used in this study are represented by (•). Modified from maps 3&4 of United Nations Environment Programme, 2007.

Statesboro, GA. Appendix A shows the location and who collected the specimens used in this study. The outgroups chosen for this study were *Amblyomma imitator* and *Amblyomma americanum*. *Amblyomma americanum* (lone star tick) is distributed throughout the southeastern and south central United States and has been associated with "Southern tick-associated rash illness" (STARI) (Masters et al., 2008). *Amblyomma imitator* (North America-Texas) is morphologically similar to *A. cajennense* seeming to "imitate" the look of *A. cajennense* (Kohls, 1958). Appendix B lists all of the samples extracted and sequenced for this study.

Table 1: *Amblyomma cajennense* were collected from the following countries and the biomes of the sampled areas.

Country	Location	Biome
Ecuador	Quito	Border of tropical humid forests, and mixed mountain systems
Costa Rica	Guanacaste	Tropical dry forests / woodlands
Mexico	Veracruz	Tropical humid forests
French Guiana	Cayenne	Tropical humid forests
Peru	Jaen	Mixed mountain systems
Venezuela	National Park	Tropical dry forests / woodlands
Argentina	Yungas	Border of tropical humid forests and tropical dry forests
	Chaco Serrano	Tropical dry forests / woodlands
	Chaco Occidental	Temperate grasslands
Brazil	São Paulo	Sub-tropical / temperate rain forests / woodlands
	Corumba	Tropical grasslands / savannas
	Rio de Janeiro	Tropical humid forests
	Minas Gerais	Sub-tropical / temperate rain forests / woodlands
	Rondônia	Tropical humid forests

DNA Extraction

DNA extractions for all tick samples were carried out individually by using DNAeasy Tissue Kit (Qiagen, Inc., Chatsworth, CA) and according to a protocol modified from Beati and Keirans (2001). A small sample on the posterior-lateral idiosoma of each tick was excised and the tick was placed in a 1.5 ml Eppendorf vial containing 180 µl of ATL buffer, and 40 µl of Proteinase K (14.3 mg / ml). It was

vortexed until thoroughly mixed and then placed in a 56°C dry bath for 24 hours (Beati and Keirans, 2001).

A volume of 20 µl of AL buffer was added; the vials was vortexed and kept at 72ºC for 10 minutes. Absolute ethanol (240µl) was added and the vials were vortexed immediately. The solution was transferred to a Qiagen column. The clean tick cuticle was left in the Eppendorf tube and absolute ethanol was added to preserve the sample until it could be mounted on a slide for future morphological analyses.

The vials were centrifuged at 13,750 g (10,000 rpm) for 1 min. The collection tube was discarded and the centrifuge column containing the DNA was placed in a fresh collection tube. Two washes with 500 µl of AW1 and AW2 buffers followed. The DNA was eluted in a total of 60 µl H_2O and stored at + 4°C.

PCR Analysis

The mitochondrial gene sequences of the 12S rDNA, cytochrome oxidase II (COII), and control region or d-loop were amplified and sequenced using primers listed in Table II (Beati and Keirans, 2001; and Beati, unpublished data). Table III lists the composition of the PCR amplification mixtures and PCR programs.

DNA electrophoresis was carried out on 1% agarose gels stained with ethidium bromide and in 0.5 x Tris Borate-EDTA (TBE) buffer. The gels were run at 300 volts for approximately 20 minutes. The DNA fragments were then examined under UV light. A digital image was taken of each gel. Positive samples were selected for further purification and sequencing.

Table 2: Lists the gene position of the PCR amplification mixture and the primers used.

Gene	Approx. gene length	Position in the *R. sanguineus* mitochondrial genome (GenBank:NC_002074)	Approx. length of PCR product	Position of primers in the *R. sanguineus* mitochondrial genome (GenBank:NC_002074)	Primer		
12SrRNA	686 bp	8063-8749	340 bp	8103 - 8123 (in 12S)	T2A	Forward	5'-AAAGAGTGACGGGCGATATGT-3'
				8478 - 8459 (in 12S)	T1B	Reverse	5'-AAACTAGGATTAGATACCCT-3'
Dloop	304 bp	8750-9054	440 bp	8597 - 8617 (in 12S)	Dloop3-1x	Forward	5'-TAACCGCTGCTGCTGGCACAA-3'
				9078 - 9060 (in tRNA-Ile)	Dloop4-1x	Reverse	5'-TAACCCTTTATTCAGGCAT-3'
Cytochrome Oxidase II	675 bp	2690-3365	600 bp	2644 - 2667 (in Cytochrome oxidase I)	CitoXIIF	Forward	5'-TCAGAACATTCTTTCAATCAAAAT-3'
				3288 - 3265 (in cytochrome oxidase II)	CitoXIIR2	Reverse	5'-CCACAAATTTCTGAACATTGACCA-3'

Table 3: PCR mixtures and programs for 12S rDNA, Control Region (dloop) and Cytochrome Oxidase II.

	12S		Dloop		COII	
Kit	Master		Triple		Triple	
PCR Mix	dH_2O	10.3µl	dH_2O	14.5µl	dH_2O	14.5µl
	Taq buffer*	2.50µl	HIFI	2.50µl	HIFI	2.50µl
	Taq master enhancer	5.00µl	$MgCl^2$	2.50µl	$MgCl^2$	2.50µl
	$MgCl^2$	1.50µl	Primer dloop3-1x	1.25µl	Primer CitoXIIF	1.25µl
	Primer TIB	1.25µl	Primer dloop3-1x	1.25µl	Primer CitoXIIR2	1.25µl
	Primer T2A	1.25µl	dNTP's	0.25µl	dNTP's	0.25µl
	dNTP's	0.50µl	Taq DNA Polymerase	0.25µl	Taq DNA Polymerase	0.25µl
	Taq DNA Polymerase	0.20µl	sample	2.50µl	sample	2.50µl
	sample	2.50µl				
PCR Program	Initial Denaturation for 5min at 94ºC		Initial Denaturation for 5min at 93ºC		Initial Denaturation for 5min at 93ºC	
	5 Cycles of:		8 Cycles of:		5 Cycles of:	
	Denaturation for 25sec at 94ºC		Denaturation for 20sec at 93ºC		Denaturation for 20sec at 93ºC	
	Annealing for 35sec at 50ºC		Annealing for 25sec at 65ºC -1.5ºC/cycle		Annealing for 30sec at 55ºC -1.0°C/cycle	
	Elongation for 30sec at 68ºC		Elongation for 45sec at 72ºC		Elongation for 1min at 72ºC -0.2ºC/cycle	
	30 Cycles of:		10 Cycles of:		10 Cycles of:	
	Denaturation for 25sec at 94ºC		Denaturation for 20sec at 93ºC		Denaturation for 20sec at 93ºC	
	Annealing for 30sec at 53ºC		Annealing for 30sec at 53ºC -0.4ºC/cycle		Annealing for 45sec at 50ºC -0.4ºC/cycle	
	Elongation for 30sec at 70ºC		Elongation for 45sec at 70ºC -0.2ºC/cycle		Elongation for 45sec at 70ºC -0.4ºC/cycle	
	Final Elongation for 5min at 70ºC		17 Cycles of:		20 Cycles of:	
	4ºC ? End		Denaturation for 20sec at 93ºC		Denaturation for 20sec at 93ºC	
			Annealing for 35sec at 51ºC		Annealing for 55sec at 46ºC	
			Elongation for 40sec at 69ºC		Elongation for 1min at 67ºC	
			Final Elongation for 5min at 69ºC		Final Elongation for 5min at 67ºC	
			4ºC ? End		4ºC ? End	

* 2.5x Taq reaction buffer (with 125 mM KCl, 75 mM Tris-HCl pH 8.4, 4 mM Mg^{2+}, 0.25% Nonidet-P40)

DNA Purification

DNA was purified in preparation for DNA sequencing. Qiagen MinElute™ Spin Columns (Hilden, Germany) were used according to the manufacturer's protocol. PB buffer (100 µl) and 20 µl of the amplified sample were mixed and placed into a column. The columns were centrifuged at 13,750 g for 75 seconds. PE buffer (735µl) was added to the column and then centrifuged again at 13,750 g for 75 seconds. Purified DNA was eluted from the column with 30 µl of H_2O and was refrigerated.

Sequencing

DNA sequencing was performed at the University of Washington High-Throughput Genomics Unit. Purified amplified products (5 ng / ul) mixed with one of the two primers (two reactions / sample) were sent to this facility.

Assembly

Sequences were sent from the University of Washington through ftp. The two strands of each sequence were verified for accuracy and assembled into a contig by Sequencher 4.5, Gene Codes Corporation (Ann Arbor, MI). Primer sequences were removed and a FASTA file was created for each assembled contig (The FASTA format is utilized by the programs used for alignment and analysis).

Alignment

The program MacClade 4.08 (Sinauer Associates, Inc., Sunderland, MA) was used to align the sequences for each gene (Maddison and Maddison, 2002). The ribosomal 12S rDNA and Dloop sequences were aligned manually and according to secondary structure (Beati and Keirans, 2001). COII is a protein-encoding gene. Therefore, in order to align COII, the sequences were translated into amino acids using

MacClade 4.08. The amino acid sequences helped in realigning the DNA sequences by codons, along their reading frames. Phylogenetic analyses for these molecular data sets were inferred by maximum parsimony (MP) using PAUP 4.0. (Swofford, 2002). Sequence distances were evaluated by maximum likelihood (by using the substitution model best matching the data) based on the MP tree with the best maximum likelihood score. In PAUP all MP analyses were achieved using a heuristic search. Gaps were treated as missing for the analyses of all three genes. Branch supports (MP) were calculated by bootstrap analysis (1,000 replicates) and were considered to be resolved if $\geq$70% (Hillis and Bull, 1993). When more than one optimal tree was found by MP, a strict consensus tree was generated. Bayesian analysis of the alignments was performed by using MrBAYES (v3.1) (Huelsenbeck, 2000; Huelsenbeck and Ronquist, 2001). Bayesian Markov Monte Carlo analyses were performed by running simultaneously four chains (2 runs) for 1,000,000 replicates and by using the nucleotide substitution model selected by MrBayes. Trees were sampled every 100 iterations. Topologies that had been saved before the likelihood values stabilized were discarded from the final sample (25% burning). A 50% majority-rule consensus tree of the remaining sampled trees was performed in PAUP, and posterior probability values recorded for each branch. All these analyses were first completed separately for 12S rDNA, cytochrome oxidase II, and the control region (Dloop). A combined analysis of the concatenated sequences of the 3 genes was also performed.

CHAPTER 3

RESULTS

Phylogenetic Analysis

12S rDNA:

The alignment of the 12S rDNA sequences (90 total) resulted in a matrix of 357 characters. The stem portions of the 12S rDNA secondary structure are functionally constrained and therefore conserved and easy to align. Although more variable, the loop regions were also fairly easy to align. The alignment was reduced to a total of 30 unique haplotypes for phylogenetic analysis.

The Peruvian samples had 8 haplotypes (total sequences), Ecuadorian 5, Mexican 3, Argentina 5, Costa Rican 2, Brazil 5, and French Guiana 2 (Appendix B). Identical haplotypes were found in Ecuador, Costa Rica, and Mexico. The 3 Venezuelan haplotypes were identical to each other and to one of French Guiana. The other haplotypes were region-specific.

Distances between sequences are shown in Table IV. The sequence divergence within geographically close samples was very low (0.1-4.8%) when compared to the distance between samples from different localities (10.8-24.0%). The sequences of the outgroups, two clearly distinct *Amblyomma* species, only differed from each other by (10%). These data also show that differences between samples from Ecuador, Costa Rica and Mexico were minimal (0.1-1.2%). Similarly samples from French Guiana /Venezuela/ Rondônia (0.1-0.6), and those from Brazil (Rio) and Yungas were very close (1.6-1.9%). The Minas Gerais sequences were related to the Brazilian samples but show a slightly higher level of divergence (4.8%).

Table 4: Maximum likelihood distances (%) between 12S rDNA sequences

	Clade I Peru	Clade II Argentina (Chaco)	Clade III Brazil (Coastal)	Clade IV French Guiana	Clade V Mexico	Outgroups
Clade I	0.1-0.6					
Clade II	18.3-20.9	0.3-0.6				
Clade III	16.0-21.5	17.0-20.7	0.3-4.8			
Clade IV	18.1-21.8	17.5-19.5	10.8-13.2	0.1-0.6		
Clade V	19.9-24.0	17.4-19.7	7.6-11.2	7.6-8.4	0.1-1.2	
Outgroups	20.3-27.5	25.4-27.5	17.1-27.7	24.4-27.6	23.5-30.4	10

The aligned matrix was first analyzed by MP and contained 90 informative characters. A heuristic search produced 4 equally parsimonious trees (length=174; CI=0.787; RI=0.938). Their strict consensus is shown in Figure 3. Bootstrap values are shown above tree branches. Thicker lines represent lineages supported by ≥ 70% bootstrap. All *A. cajennense* are clustered in a monophyletic clade. The basal lineage among *A. cajennense* is monophyletic and groups all Peruvian samples (Clade I). The next diverging branch includes all Argentinean ticks from the Chaco area (Clade II). The remaining sequences are included in a monophyletic clade (Clade VI), which is subdivided in 3 strongly supported, but unranked, lineages: one includes the Brazilian samples from São Paulo, Rio de Janeiro, Minas Gerais, and Mato Grosso do Sul (Corumba), and the Argentinean from Yungas province (Clade III). Within Clade III, the Yungas haplotypes cluster with one of the São Paulo haplotypes (Clade IIIa). The second clade encompassed the sequences from French Guiana, and Rondônia (Brazil). The third clade is composed of the Mexican, Costa Rican and the Ecuadorian samples (Clade V).

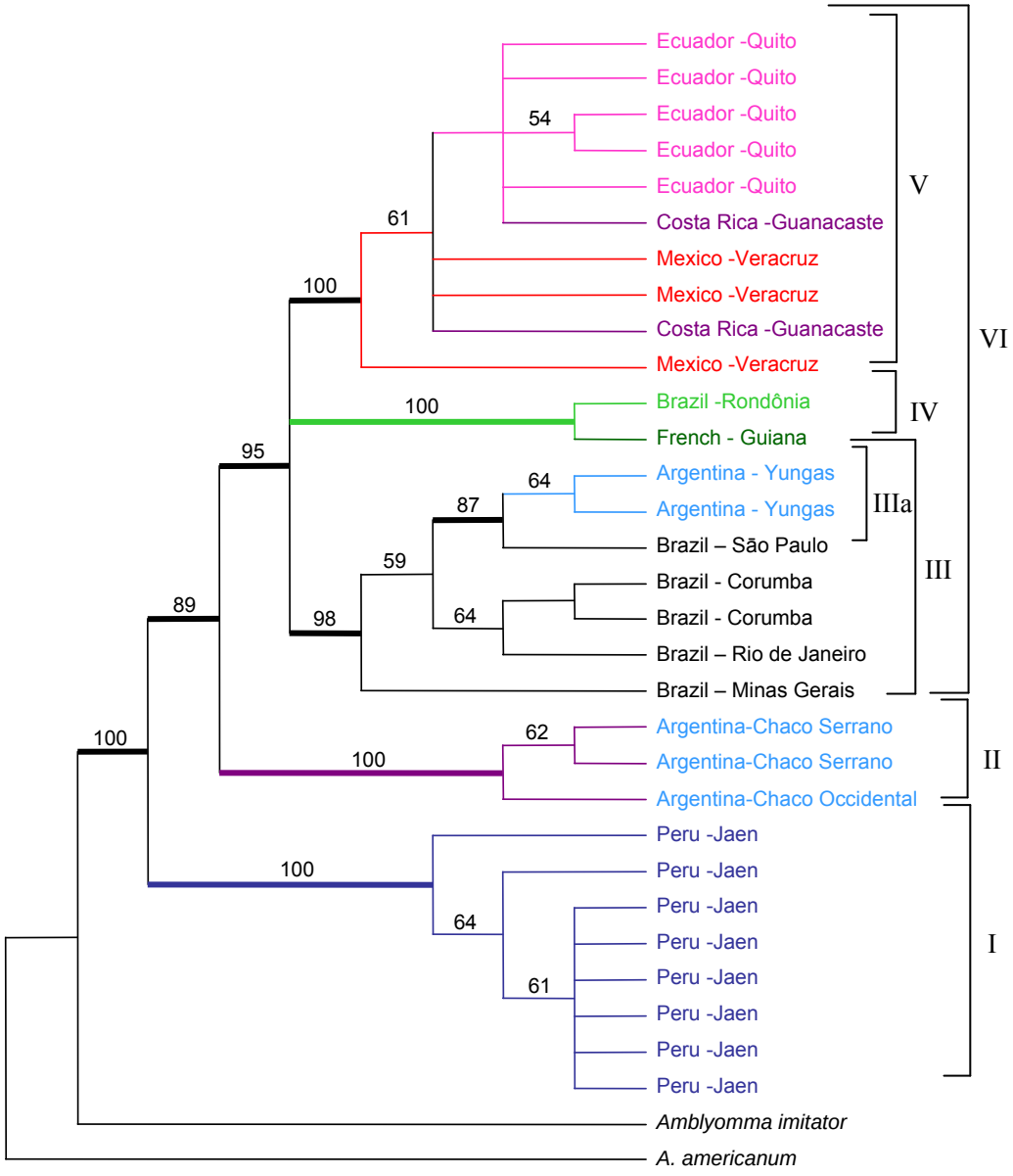

FIG. 3: Strict consensus tree of *Amblyomma cajennense* 12S rDNA gene sequences.

The Bayesian analysis (MB) and MP reconstructions show similar basal topologies with the Peruvian branch diverging first (Clade I), followed by the Chaco lineage (Clade II). However, within the next diverging clade MB, unlike MP, resolves some additional relationships. Clade III diverges first, followed in order of divergence by Clade IV and V. However, support for a monophyletic Clade IV-V is weak.

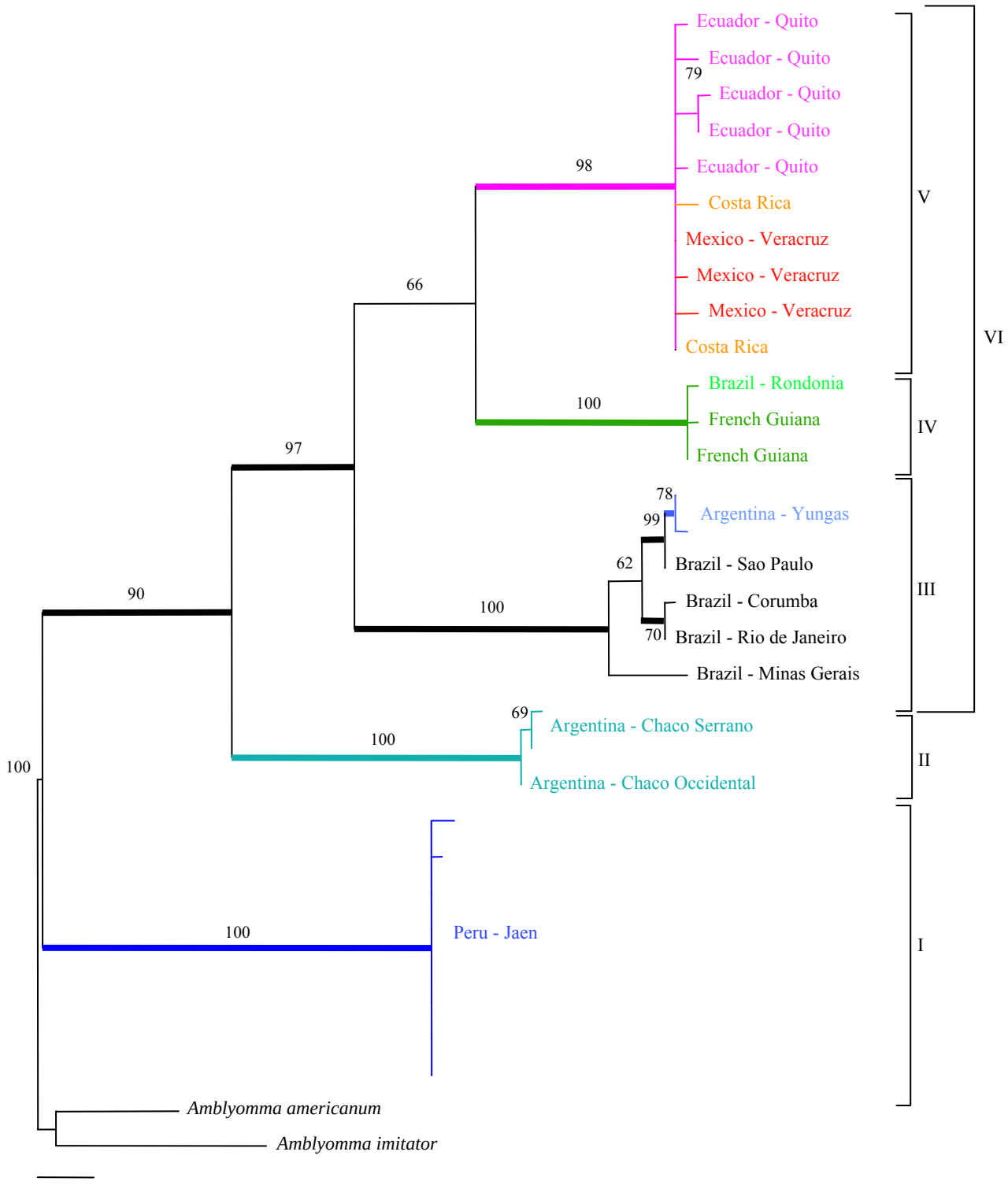

FIG. 4: Bayesian phylogeny of *Amblyomma cajennense* 12S rDNA gene sequences.

D-loop

The alignment of the D-loop sequences resulted in a matrix of 415 total characters (79 parsimony informative). The sequences were organized into unique haplotypes (22 total haplotypes). The structure of the control region gene in invertebrates is known to include a highly variable region. The approximately 154 bp long variable region of the sequences of *A. cajennense* was so variable that it was impossible to align it in any acceptable way and was therefore discarded prior to phylogenetic analysis. The remaining sequence of DL was relatively more conserved and easy to align manually.

The Peruvian samples have 3 haplotypes, Ecuadorian 1, Mexican 1, Argentina 7, Brazil 8, Venezuela 1, and French Guiana 1 (Appendix B). Identical haplotypes were found in Ecuador, Costa Rica, and Mexico. The other haplotypes were region-specific.

Distances between sequences are shown in Table V. The sequence divergence within geographically close samples was very low (0.1-5.8%) when compared to the distance between samples from different localities (11.6-23.1%). The sequences of the outgroups, two clearly distinct *Amblyomma* species, only differ from each other by (14.9-15.2%). These data also show that differences between samples from Ecuador, Costa Rica and Mexico are minimal (0.2-0.5%). Similarly samples from French Guiana /Venezuela/ Rondônia (0.1-3.6%), and those from Brazil (Rio) and Yungas are also very close (1.6-1.9%). The Minas Gerais sequences related to the Brazilian samples but show a slightly higher level of divergence (5.8%).

The aligned matrix was first analyzed by MP and contained 79 informative characters. A heuristic search produced 16 equally parsimonious trees (length=197; CI=0.711; RI=0.881). Their strict consensus is shown in Figure 4. Bootstrap values are

Table 5: Maximum likelihood distances (%) between D-loop sequences

	Clade I Peru	Clade II Argentina (Chaco)	Clade III Brazil (Coastal)	Clade IV French Guiana	Clade V Mexico	Outgroups
Clade I	0.1-3.6					
Clade II	18.4-18.8	0.2-0.5				
Clade III	19.4-21.3	15.5-17.1	0.2-5.8			
Clade IV	23.1-23.9	16.4-17.6	11.7-13.4	0.2-1.1		
Clade V	19.7-20.2	15.3-16.1	13.4-14.7	11.6-12.9	0.2-0.5	
Outgroups	17.4-18.6	22.4-23.7	18.4-19.9	16.1-17.7	18.0-19.1	14.9-15.2

shown above tree branches. Lineages supported by ≥ 70% bootstrap are represented by thicker lines. All *A. cajennense* on the MP tree are clustered in a monophyletic clade and subdivided into 3 strongly supported, but unranked, lineages (FIG. 4): Clade I, II and VI. These data cannot resolve basal relationships between the Peruvian and the Chaco samples. As for 12S rDNA, clade VI was also a polytomy of 3 strongly supported clades. One branch was composed of the Mexican, Costa Rican and Ecuadorian sequences. The second branch included a basal lineage of the French Guiana samples and a monophyletic branch that contained the Venezuelan and the Rondônia haplotypes. The third lineage was split in a Minas Gerais clade and a clade which assembles clade III sequences. As for the 12S rDNA tree, the Yungas haplotypes cluster with the Atlantic Brazilian haplotypes.

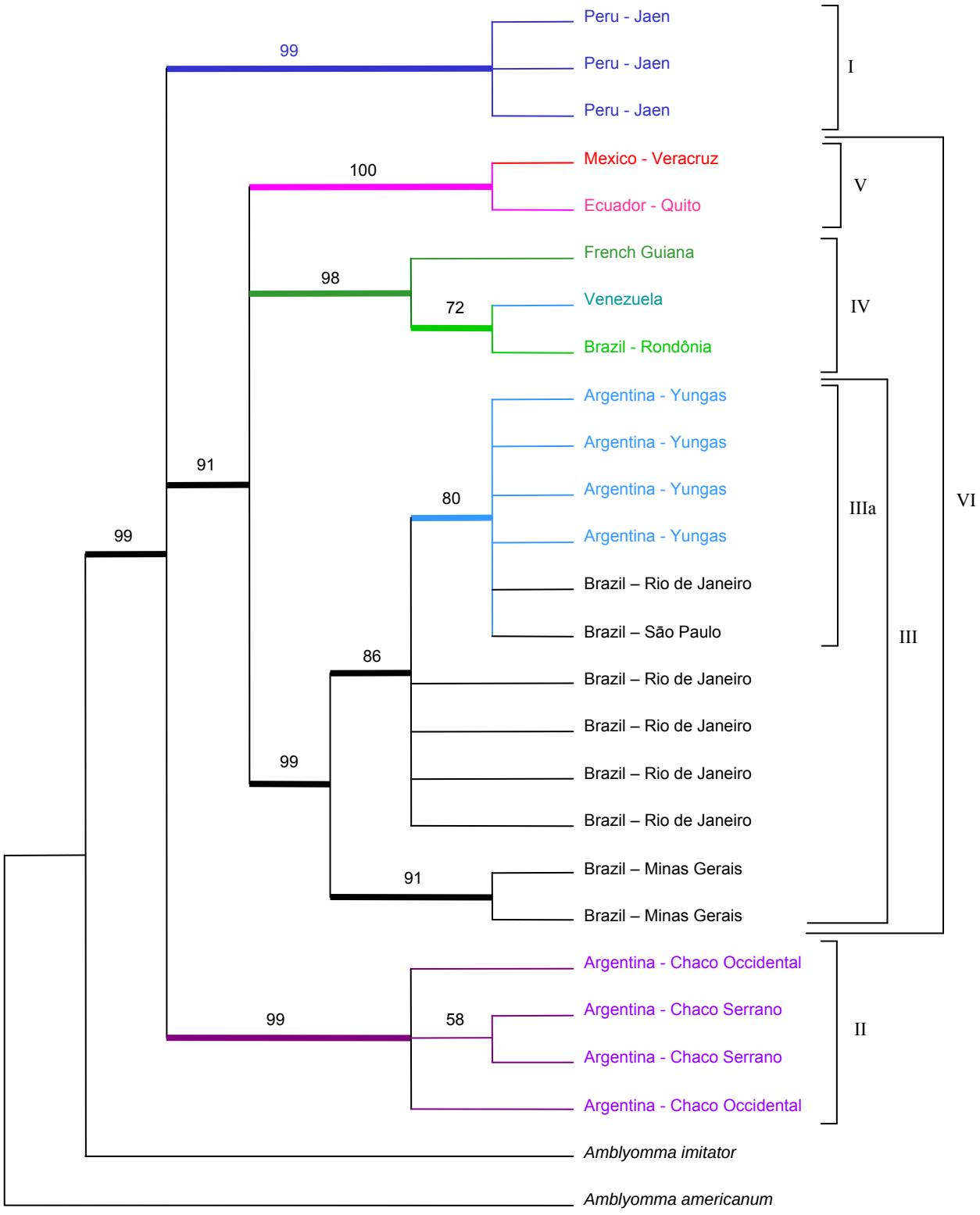

FIG. 5: Strict consensus tree of *Amblyomma cajennense* D-loop gene sequences.

The MB DL analysis resulted in a tree with better resolution than that of the MP tree (FIG. 5). Among the resolved clades, the Peruvian lineage is basal (clade I) followed, as was the case for 12S rDNA, by the Chaco district haplotypes. Within the next clade, again, the branching order of the three monophyletic clades is not resolved.

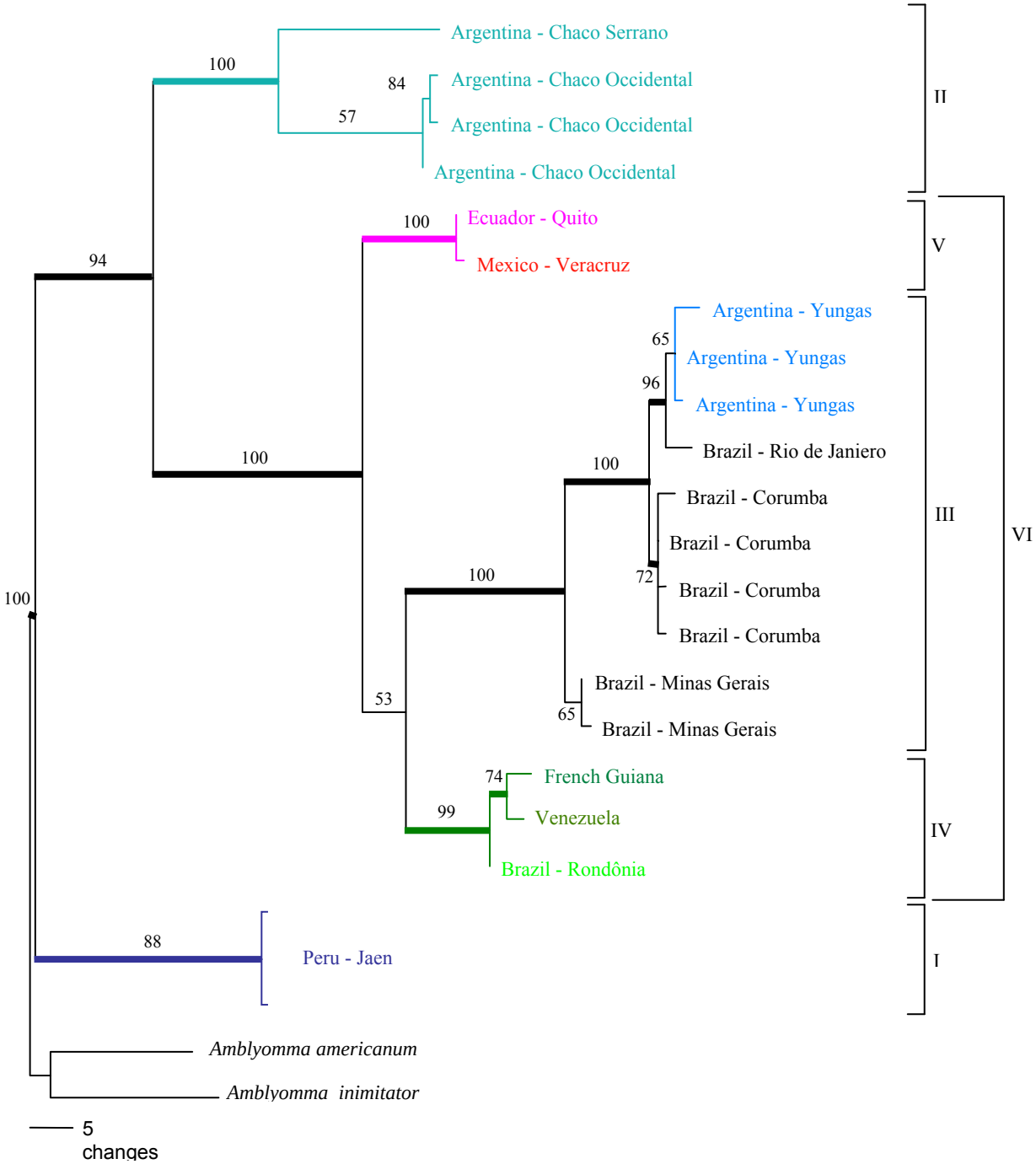

FIG. 6: Bayesian phylogeny of *Amblyomma cajennense* D-loop gene sequences.

COII

The alignment of the COII sequences resulted in a matrix of 565 total characters (177 parsimony informative). The sequences were organized into unique haplotype (22 total unique haplotypes). The Peruvian samples have 2 haplotypes, Ecuadorian 1, Mexican 2, Argentina 6, French Guiana 4, and Brazil 7 (Appendix B). Identical haplotypes were found in Ecuador and Mexico. The other haplotypes were region-specific.

Distances between sequences are shown in Table VI. The sequence divergence within geographically close samples is very low (0.1-5.6%) when compared to the distance between samples from different localities (9.4-16.8%). The sequences of the outgroups, two clearly distinct *Amblyomma* species, only differed from each other by (12.5%). These data also show that differences between samples from Ecuador, Costa Rica and Mexico are minimal (0.1-0.3%). Similarly samples from French Guiana /Venezuela/Rondônia (0.3-0.5%), and those from Brazil (Rio) and Yungas are also very close (1.6-1.9%). The Minas Gerais sequences related to the Brazilian samples but show a slightly higher level of divergence (5.6%).

Table VI: Maximum likelihood distances (%) between COII sequences

	Clade I Peru	**Clade II Argentina (Chaco)**	**Clade III Brazil (Coastal)**	**Clade IV French Guiana**	**Clade V Mexico**	**Outgroups**
Clade I	0.3-0.5					
Clade II	9.4-11.2	0.5-1.1				
Clade III	16.8-21.6	16.5-21.7	1.0-5.6			
Clade IV	14.3-16.4	13.3-15.5	9.5-14.7	1.0-1.3		
Clade V	14.1-15.7	12.9-14.0	11.0-14.5	7.3-8.8	01.-0.3	
Outgroups	20.3-30.0	15.5-16.3	18.4-21.1	20.5-29.0	17.0-24.8	12.5

The aligned matrix was first analyzed by MP and contained 177 informative characters. A heuristic search produced 16 best equally parsimonious trees (length=364; CI=0.713; RI=0.819). Their strict consensus is shown in Figure 7. Bootstrap values are shown above tree branches. Thicker lines represent lineages supported by ≥ 70% bootstrap. All *A. cajennense* on the MP tree are clustered in a monophyletic clade and subdivided into 2 strongly supported, ranked, lineages (FIG. 6). The basal clade included the Peruvian and the Chaco lineages (clades I & II). The second clade included all remaining haplotypes (clade VI). Clade III was subdivided in 2 monophyletic sister groups. One branch included the Brazilian samples (São Paulo & Minas Gerais) and the other included Argentinean (Yungas) and Brazilian Rio de Janeiro sequences. The second branch was also composed of two strongly supported groups: the Rondônia region sequences, and one containing a basal Ecuadorian/Costa Rican lineage and the Mexican sequences.

For COII the ML and MB reconstructions show similar topologies (FIG. 7). All *A. cajennense* on the ML tree were clustered as in the MP tree in a monophyletic clade and subdivided into 2 sister lineages. Relationships between clades was fully resolved. The basal lineage was split into two well resolved groups, the Peruvian and the Chaco districts. The next clade is constituted by two lineages: one included clade III and clade V (clade IV nested), whereas the other included the Yungas and coastal Brazilian haplotypes.

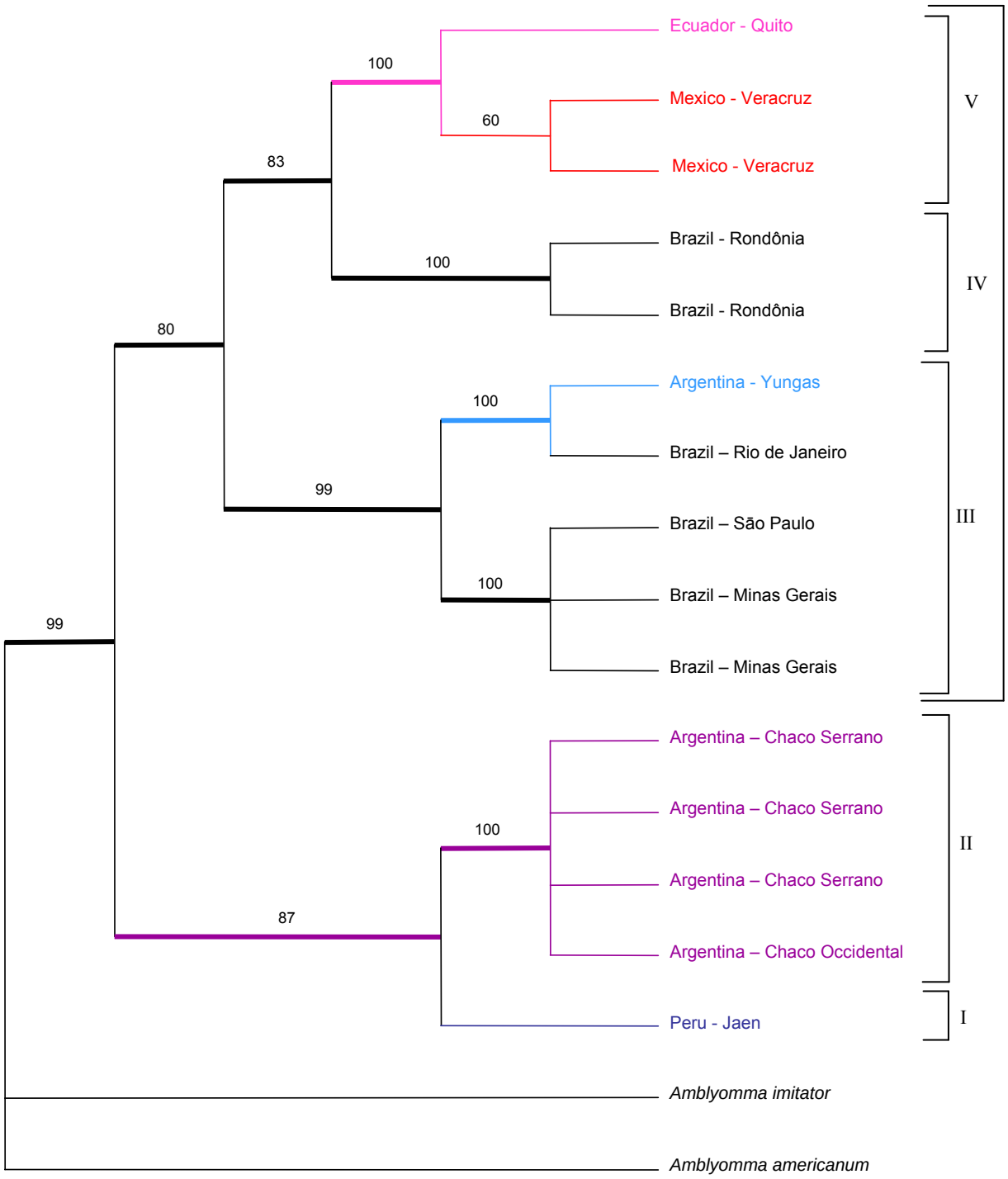

FIG. 7: Strict consensus tree of *Amblyomma cajennense* COII gene sequences.

In general, if discrepancies were found when comparing results obtained by analyzing different genes. They were always related to the branching order between the

Peru and the Chaco lineages and/or to the relationships between the three main branches in Clade VI.

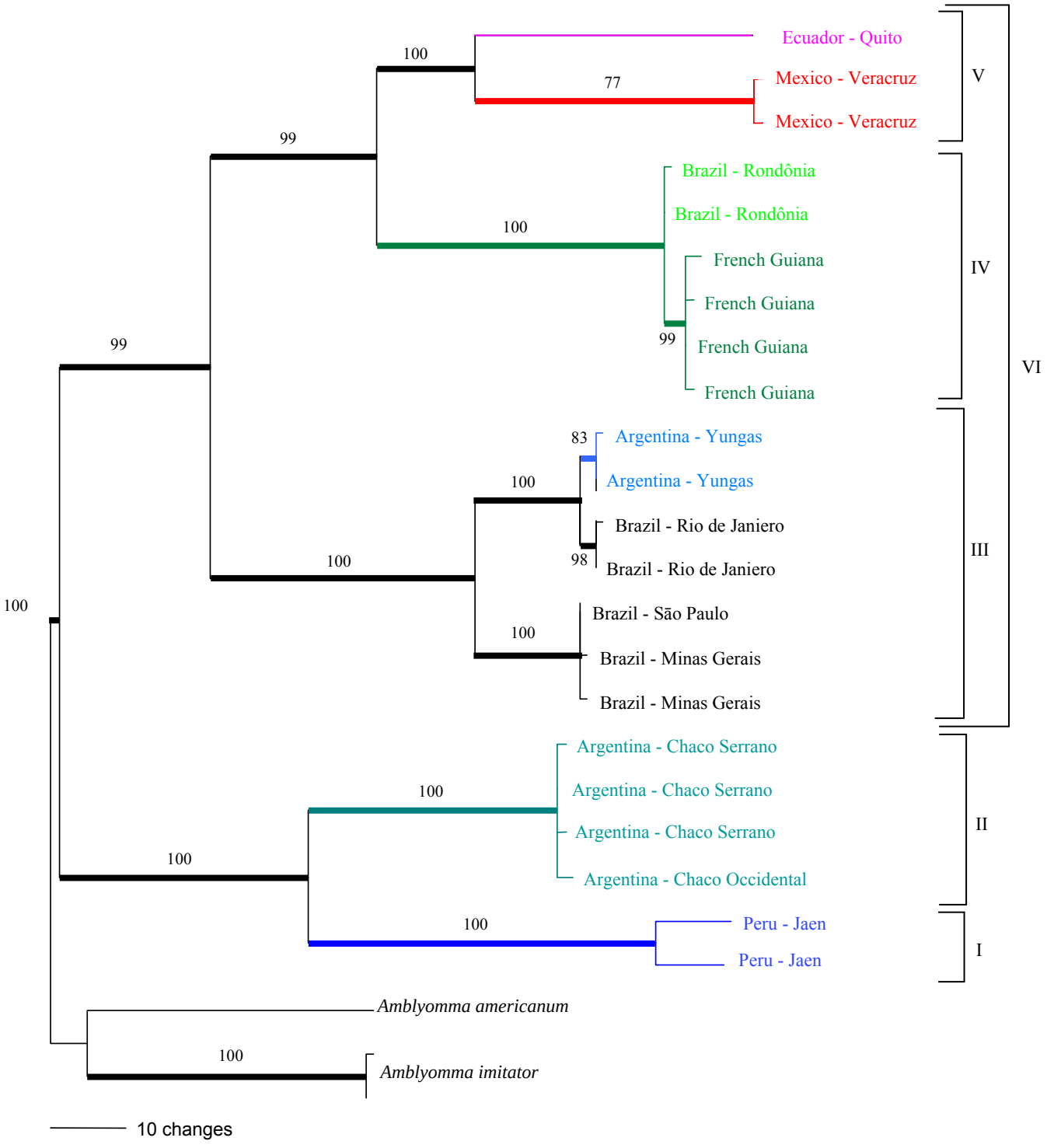

FIG. 8: Bayesian phylogeny of *Amblyomma cajennense* COII gene sequences.

Concatenated analysis

After concatenating the sequences for all available samples, we obtained a data matrix of 1314 bp (330 parsimony informative). The MP analysis of the concatenated data matrix resulted in 2 equally short trees (length 726; CI = 0.726; RI = 0.828). Their strict consensus (FIG. 9) shows that all sequences clustered in a monophyletic clade. The branching pattern of the lineages diverging first, the Peruvian and the Chaco branches, is not resolved whereas the relationships between the remaining branches are well resolved. The three main clades were subdivided in two sister groups, one including the Yungas/Brazilian Atlantic sequences, with the Minas Gerais sequences being basal, and the other being separated into two monophyletic groups, the Ecuador/Mexico/Costa Rica lineage and the French Guiana/Rondônia/ Venezuela clade. The overall structure of the MB tree (FIG. 10) is identical to that of the MP tree. Nevertheless, after the Bayesian analysis of the Peruvian and Chaco lineages are strongly clustered in a basal monophyletic clade. The concatenated analysis shows the same topology than that of the COII sequences.

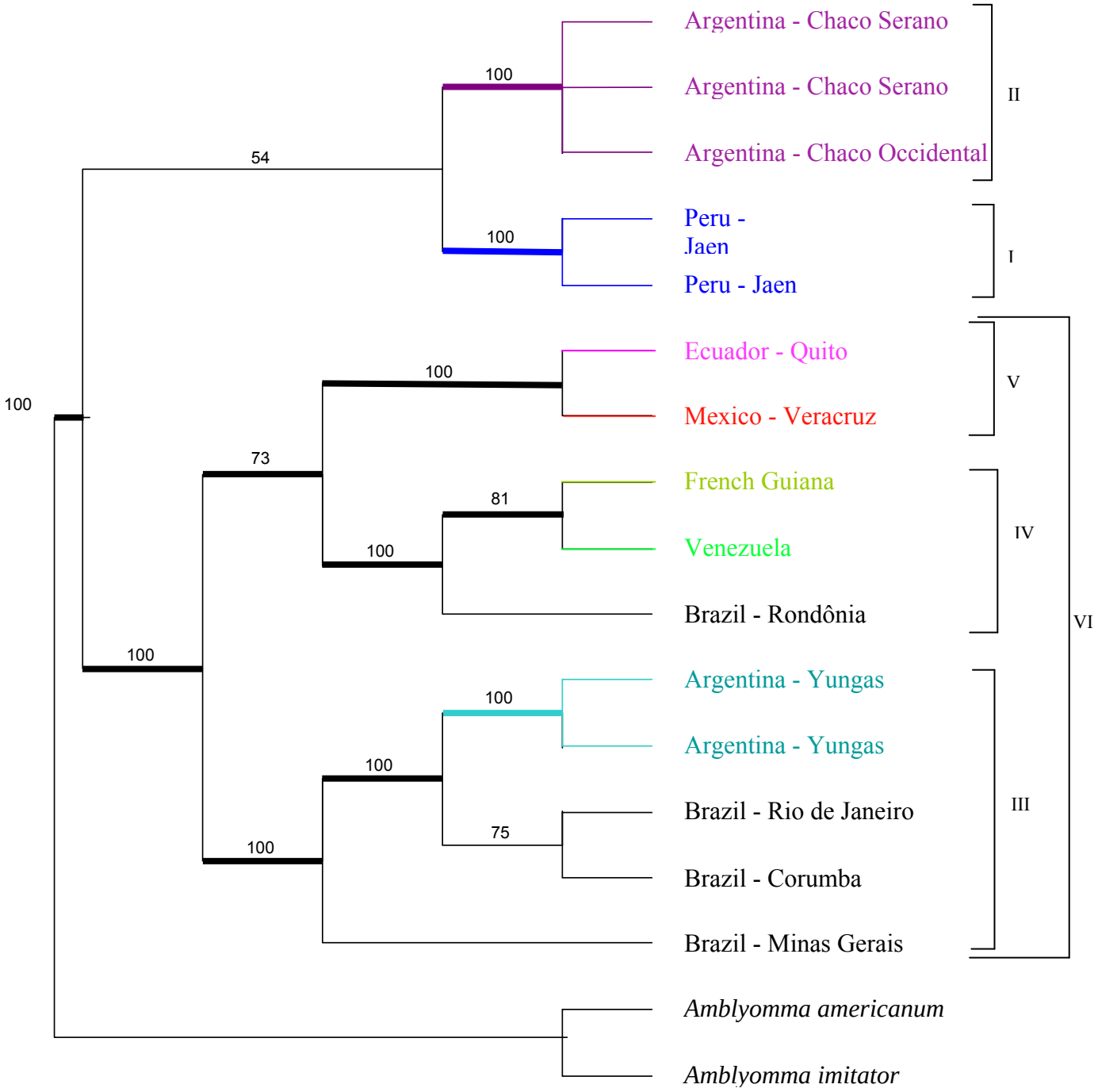

FIG. 9: Concatenated strict consensus of *Amblyomma cajennense* gene sequences.

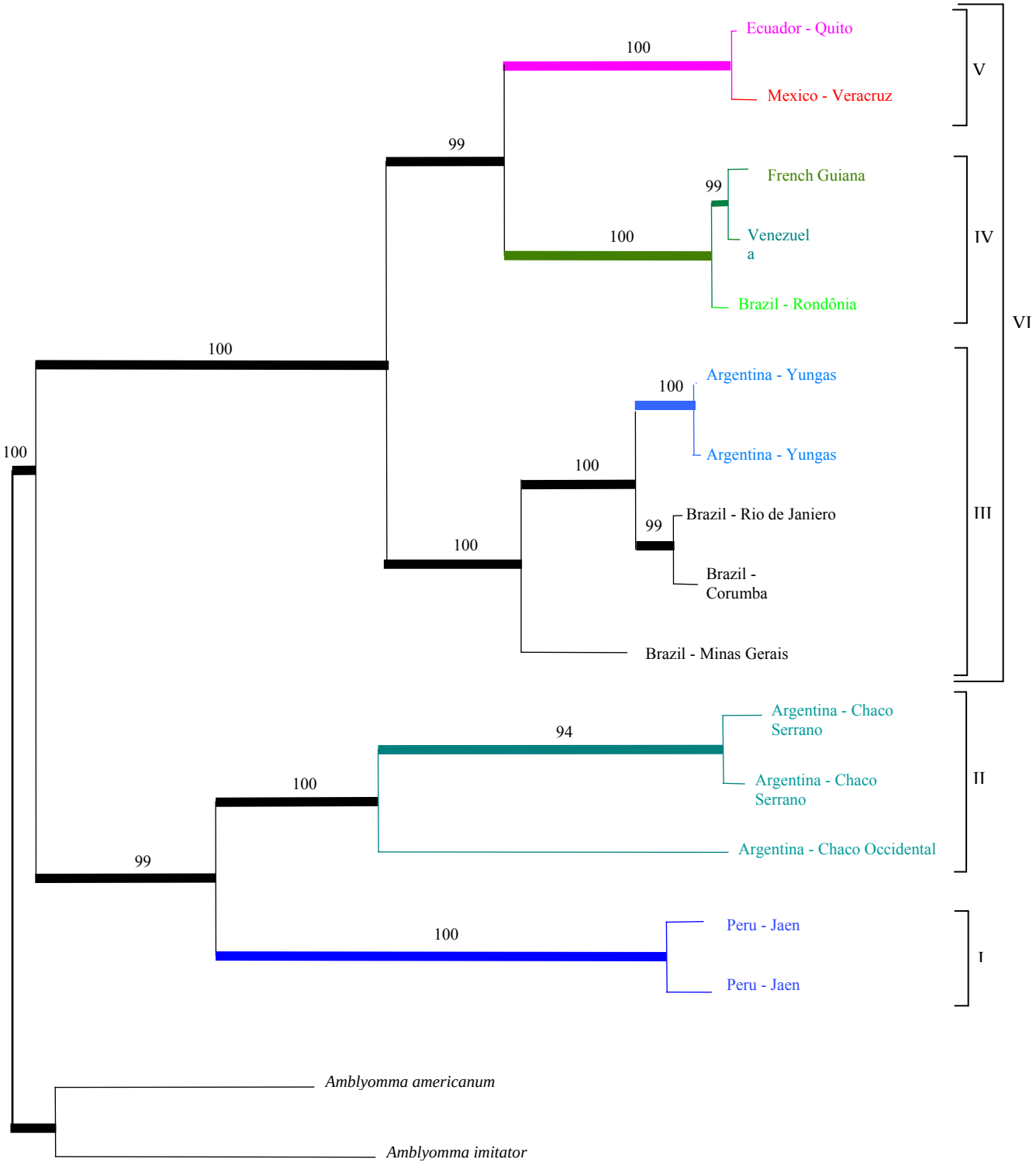

FIG. 10: Concatenated bayesian phylogeny of *Amblyomma cajennense* gene sequences.

CHAPTER 4

CONCLUSIONS

Sampling

The main focus of this study was to determine whether or not *A. cajennense* is genetically structured. We studied the genetic variability of three *A. cajennense* mitochondrial gene sequences in tick samples collected in eight locations representing diverse ecological biomes throughout South & Central America including Mexico. For each locality, if at all possible, we analyzed several samples in order to compare diversity within and between localities. The sampling was limited by availability. The ticks used in this study were collected in Mexico, Costa Rica, Venezuela, Ecuador, French Guiana, Peru, Brazil and Argentina. Unfortunately, DNA could not be obtained from samples from additional countries or ecological areas (USA-Texas, Colombia, Bolivia, Jamaica). However, the specimens used in this study represent the diversity in ecosystems considered to be suitable for *A. cajennense.* Samples were collected in 6 of the 8 different biomes where *A. cajennense* was known to occur. The only two biomes not investigated in this study were mixed island systems (Caribbean islands) and temperate broad-leaf forests (southern-central Florida). These two biomes should be sampled in future to determine whether there are, in fact, other genetic lineages found in these areas.

Choice of gene sequences

In this study we studied the genetic diversity of *A. cajennense* by analyzing phylogenetically three mitochondrial gene sequences, the 12S rDNA, the control region, and the cytochrome oxidase II. Mitochondrial genes sequences are commonly used for the evaluation of the relationships between and within tick species (Barker and Murrell,

2004; Norris et al., 1996; Beati and Keirans, 2001). The mitochondrial genes are maternally inherited, and thus evolve at a faster rate than that of nuclear genes (Norris et al., 1996). This faster evolution provides better resolution where investigating closely related taxa. The 12S rDNA gene for example, has been observed to evolve rapidly and has been used in a number of population genetic studies, including this one, for this very reason (Norris et al., 1996).

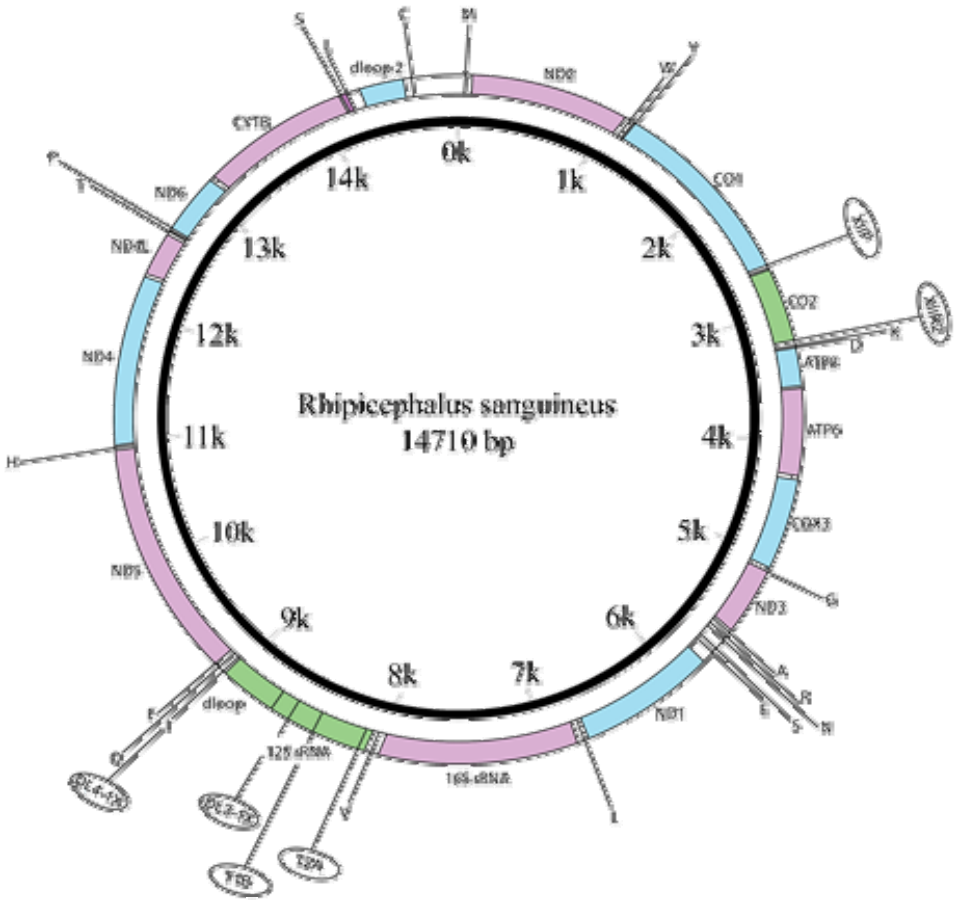

FIG. 11: Mitochondrial genome of *Rhipicephalus sanguineus* 12S rDNA, D-loop and COII and the corresponding primer used for *A. cajennense*.

The mitochondrial genome of most tick species that have been studied thus far are typically 14 kb long. The genome is circular, with 37 genes including 13 protein encoding genes (e.g., cytochrome oxidase II), 2 ribosomal RNA genes (12S rDNA) and a non coding regulatory control region (dloop) (Black and Roehrdanz, 1998).

The genes in this study were also chosen because of their location on the mitochondrial genome (FIG. 11.). The 12S rDNA and the control region are located next to one another, while the cytochrome oxidase II gene is on the opposite side of the genome. Because of possible introgression of mitochondrial genetic material ("invasion" of a mitochondrial genome by DNA from foreign mitochondria), it is always advisable to use more than one mitochondrial gene in any phylogenetic analysis (Dowling and Secor, 1997). The genes should be chosen from different sections of the mitochondrial genome. If for example the 12S rDNA and the contiguous D-loop genes had been inserted through introgression into the *A. cajennense* genome, their phylogenies would be similar to each other but be totally differed from that of the COII trees. All our phylogenetic trees show congruent topologies, which would argue against possible introgression events in these genes. Nevertheless, knowing that the analysis of mitochondrial and nuclear gene sequences sometimes result in non congruent phylogenies, it is certainly useful, in future, to also sequence a fast evolving nuclear gene of these specimens in order to see if its analysis corroborates our data.

Phylogenetic analyses

Good resolution. With the three genes, phylogenetic analyses show good overall resolution with most bootstrap and posterior probability values exceeding 70%. This

indicates that the genes chosen in this study were informative at the required taxonomic level.

Congruent reconstructions in each gene when comparing the two different analysis methods (MP vs. MB). For all our genes, the topology and level of resolution of the trees were very similar when comparing MP and MB. The fact that different algorithms result in the same branching patterns indicate that, for each data set, we have a good proportion of informative characters and that the results cannot be altered by simply changing the analysis method.

Congruent reconstructions when comparing different genes. Analyses with the three genes result in fairly similar topologies. If contradictions are found, they are all related to non-resolved lineages. The trees obtained with the 3 genes all show that *A. cajennense* is subdivided in the same 5 monophyletic, strongly supported clades. The clades have a distinct geographic distribution and are characterized by mutually exclusive haplotypes, with no apparent overlap. However, we have not sampled in the regions between areas occupied by each of these clades and can, therefore, not know whether or not there are areas where these groups may occur in sympatry. The MB analysis of the concatenated gene sequences resulted in a fully resolved phylogeny with one basal lineage subdivided in two monophyletic branches, the Peruvian and Chaco clades. The next diverging clade includes the Brazilian/Yungas samples, whereas the most recently evolving clade contains the two sister lineages: French Guiana/Venezuela/Rondônia and Ecuador/Costa Rica/Mexico. By combining the 3 gene data sets, the minor weaknesses observed in the separate phylogenies were compensated and this emphasizes the effect of increasing the number of informative characters for better phylogenetic results.

In terms of ecology, lineages that first diverge from the root are the Peruvian and the Chaco sequences. It would appear that the basal lineages in our phylogeney are better adapted to dry climatic environments such as the dry mountainous shrubland regions of the interandean valleys of Peru and the dry grasslands of the southern Chaco regions of Argentina. The third group includes specimens collected from the tropical dry, humid and subtropical forest areas ranging from northern Argentina (Yungas), through southern Brazil (Rio de Janeiro, São Paulo, Mato Grosso, and Minas Gerais). The fourth lineage includes the tropical humid and tropical grasslands of the Rondônia region of Brazil, French Guiana and the Venezuela group. The last lineage includes the tropical humid/dry forests of Ecuador, Costa Rica and Mexico.

Future studies

Evidence of our experiment suggests that there are at least five geographically distinct populations of *Amblyomma cajennense*. However, it is as yet unknown whether these groups qualify as cryptic species (species that are morphologically identical but cannot interbreed). Our data show that sequence divergences between these groups are much higher than genetic variability within groups. Furthermore, genetic distance between the two outgroup species (clearly distinguishable taxa) is often less important than that found between our clades. Although genetic distances cannot be used as such for species definitions, they provide support for the hypothesis that the clades correspond in fact to different species.

In order to further investigate this issue, the morphology of ticks belonging to the five clades should be thoroughly reassessed. When conducting this experiment, the ticks were not pulverized to obtain their DNA, but instead, cut in such a way that the cuticle

was kept intact for further morphological examination. By examining the cuticles from these ticks, there could potentially be phenotypic features that are unique for any of the five lineages – features ignored by the taxonomists who decided that this is merely a taxon with high morphological intraspecific polymorphism. Preliminary observations of these cuticles have already indicated that there are, in fact, unique phenotypic characteristics unique to each clade.

Another way of determining whether or not these are different species would be to establish colonies for each clade and then do cross-breeding experiments. Again, some of these experiments are under way and are already showing incompatibility between some of these groups.

In conclusion, analyses of mitochondrial gene sequences have revealed the occurrence of genetic structure in *A. cajennense* which is compatible with cryptic speciation. Preliminary data (morphology and cross breeding experiments) are confirming our results.

These findings are important because they help to resolve an old taxonomic question and because they may explain why some *A. cajennense* populations are known to transmit *R. rickettesii* (Brazil – Atlantic coast and Argentina – Yungas) and others are either infected with non-pathogenic *rickettsiae* (Ecuador) (Greg Dasch, CDC personal communication) or not infected at all (Argentina – Chaco). These data emphasize the importance of thorough systematic analysis when studying vectors of public health interest.

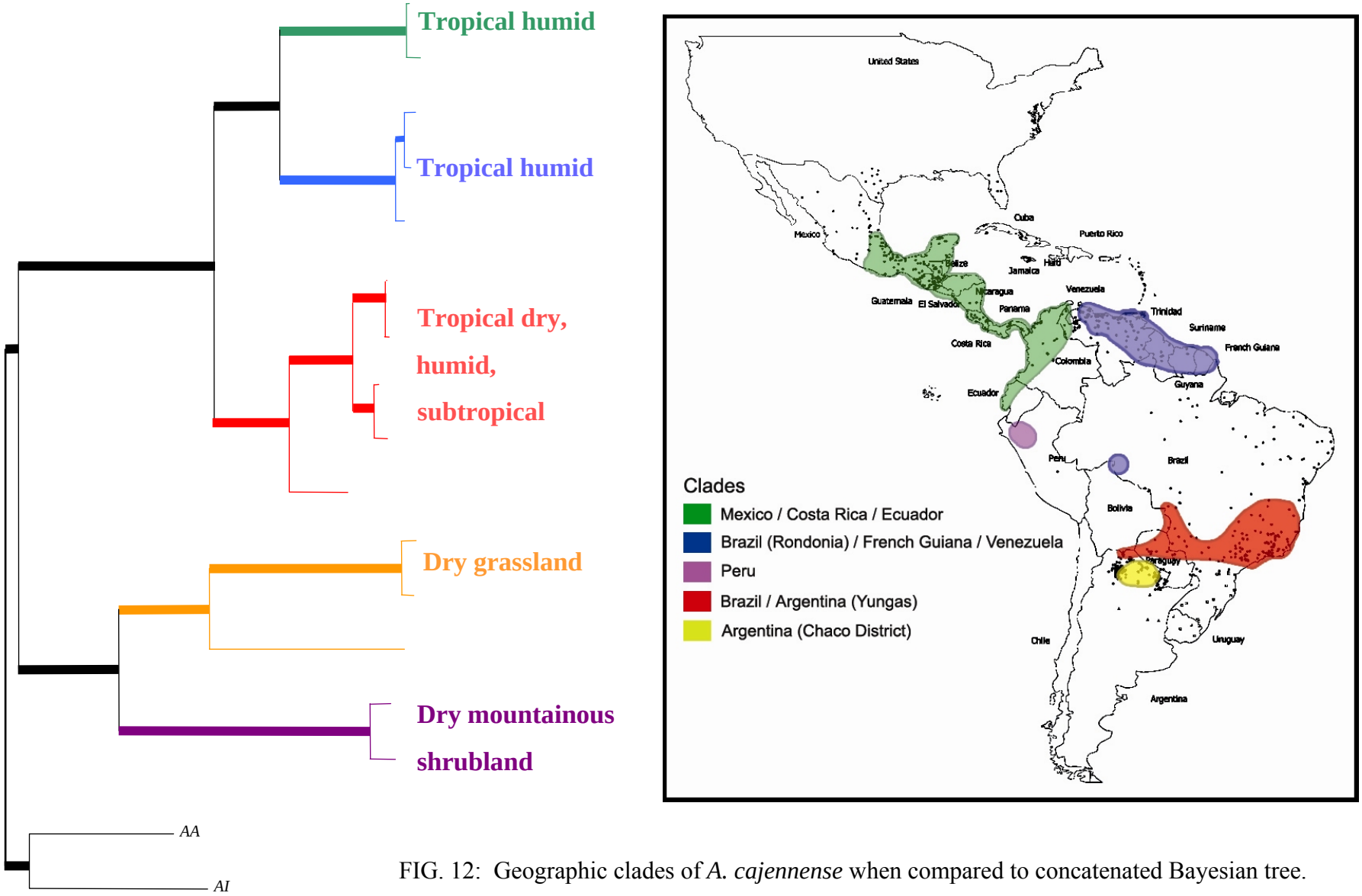

FIG. 12: Geographic clades of *A. cajennense* when compared to concatenated Bayesian tree.

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APPENDIX A:

Location and collectors of samples used in this study

Country	Locations	Collector
Costa Rica	Guanacaste	Lorenza Beati
Mexico	Veracruz	Carmén Guzmán Cornejo
French Guiana	Cayenne	Lorenza Beati
Peru	Jaen	Abraham Cáceres
Venezuala	National Park	IAP-USNTC
Argentina	Yungas Chaco Serrano Chaco Occidental	Alberto Guglielmone
Brazil	Sao Paulo Corumba Rio de Janeiro Minas Gerais Rondonia	Darcí Barros-Battesti, Marcelo Labruna & IAP-USNTC
Ecuador	Quito	Lee Cohnstaed, Rommy Terán, Renato León, Lorenza Beati & IAP-USNTC*

*Institute of Arthropodology & Parasitology - US National Tick Collection

APPENDIX B:

Samples collected in Central and South America used in this study.

Sample #	Collection #	Country	vial #	sex/stage	extraction date	12S	dloop	CcO II
1		**Peru**	15	f*	n/a	-		
2		**Peru**	15	f*	n/a	-		
3		**Peru**	15	f*	n/a	-		
4		**Peru**	15	f*	n/a	-		
5		**Peru**	15	m*	n/a	-		
6		**Peru**	15	m*	n/a	-		
7		**Peru**	15	m*	n/a	-		
8		**Peru**	15	m*	n/a	+		
9		**Peru**	15	m	n/a	+		
10		**Peru**	15	f	n/a	+	-	
11		**Peru**	15	m	n/a	+	-	
12		**Peru**	15	m	n/a	+	-	
13		**Peru**	15	f	n/a	+	+	+
14		**Peru**	15	f	n/a	+	+	+
15		**Peru**	17	m	n/a	-		
16		**Peru**	17	m	n/a	+		
17		**Peru**	17	m	n/a	+		
18		**Peru**	17	m	n/a	+		
19		**Peru**	17	m	n/a	+		
20		**Peru**	17	m	n/a	-		
21		**Peru**	17	m	n/a	-		
22		**Peru**	17	m	n/a	-		
23		**Peru**	17	f	n/a	+		+
24		**Peru**	17	f	n/a	+		
25		**Peru**	17	f	n/a	-		
26		**Peru**	17	f	n/a	+	+	
27		**Peru**	17	m	n/a	-		
28		**Peru**	17	m	n/a	+		
29		**Peru**	17	m	n/a	+		
30		**Peru**	17	m	n/a	-		
31		**Peru**	17	*	n/a	-		
32		**Peru**	17	*	n/a	-		
33		**Peru**	17	*	n/a	+		
34		**Peru**	17	*	n/a	-		
35		**Peru**	17	*	n/a	+		
36		**Peru**	17	*	n/a	+		
37		**Peru**	17	*	n/a	+		
38		**Peru**	17	*	n/a	+		
39		**Peru**	17	f	n/a	+	+	+
40		**Peru**	17	f	n/a	+	+	+
41		**Peru**	17	f	n/a	+	+	+
42		**Peru**	17	f	n/a	-	-	

43		**Peru**	17	m	n/a	+	+	+
44		**Peru**	17	m	n/a	+	+	+
45		**Peru**	17	m	n/a	+	+	+
46		**Peru**	17	m	n/a	+	+	+
47		**Peru**	17	f	1/12/2006	+	-	
48		**Peru**	17	f	1/12/2006	+	+	+
49		**Peru**	17	f	1/12/2006	-		
50		**Peru**	17	f	1/12/2006	+	-	
51		**Peru**	17	m	1/12/2006	+	-	
52		**Peru**	16	m	1/12/2006	+	+	+
53		**Peru**	16	m	1/12/2006	+	-	
54		**Peru**	16	m	1/12/2006	+	+	+
55		**Peru**	16	f	1/13/2006	+	-	
56		**Peru**	16	f	1/13/2006	+	-	
57		**Peru**	16	f	1/13/2006	+	+	+
58		**Peru**	16	f	1/13/2006	+	-	
59		**Peru**	16	m	1/13/2006	-		
60		**Peru**	16	m	1/13/2006	+	+	+
61		**Peru**	16	m	1/13/2006	+		
62		**Peru**	16	m	1/13/2006	+		
63		**Peru**	17	f	1/18/2006	-		
64		**Peru**	17	f	1/18/2006	-		
65		**Peru**	17	f	1/18/2006	-		
66		**Peru**	17	f	1/18/2006	+	+	+
67		**Peru**	17	f	1/18/2006	+		
68		**Peru**	17	m	1/18/2006	+	+	+
69		**Peru**	17	m	1/18/2006	-		
70		**Peru**	17	m	1/18/2006	+	-	
71		**Peru**	17	m	1/18/2006	+	-	
72		**Peru**	17	m	1/18/2006	+	+	+
73		**Peru**	17	m	1/18/2006	+	-	
74		**Peru**	17	m	1/18/2006	+	+	+
75		**Peru**	17	m	1/18/2006	+	+	+
76		**Peru**	17	m	1/18/2006	+	+	+
77		**Peru**	17	m	1/18/2006	+	+	+
78		**Peru**	17	m	1/18/2006	+	+	+
79		**Peru**	16	m	1/19/2006	+	+	+
80		**Peru**	16	m	1/19/2006	-		
81		**Peru**	16	m	1/19/2006	-		
82		**Peru**	16	m	1/19/2006	+		
83		**Peru**	16	m	1/19/2006	+		
84		**Peru**	16	m	1/19/2006	+		
85		**Peru**	16	m	1/19/2006	+		
86		**Peru**	16	m	1/19/2006	+		
87		**Peru**	16	m	1/19/2006	+		
88		**Peru**	16	m	1/19/2006	+		
89		**Peru**	16	m	1/19/2006	+		
90		**Peru**	16	m	1/19/2006	+	+	+

91		**Peru**	16	m	1/19/2006	+	+	+
92		**Peru**	16	m	1/19/2006	+		
93		**Peru**	16	m	1/19/2006	+		
94		**Peru**	16	m	1/19/2006	+		
95		**Peru**	16	f	1/25/2006	+	+	+
96		**Peru**	16	m	1/25/2006	+		
97		**Peru**	16	m	1/25/2006	+		
98		**Peru**	16	m	1/25/2006	-		
99		**Peru**	16	m	1/25/2006	-		
100		**Peru**	16	m	1/25/2006	-		
101		**Peru**	16	m	1/25/2006	-		
102		**Peru**	16	m	1/25/2006	-		
103		**Peru**	16	m	1/25/2006	+		
104		**Peru**	16	m	1/25/2006	+		
105		**Peru**	16	m	1/25/2006	+		
106		**Peru**	16	m	1/25/2006	+		
107		**Peru**	16	n	1/25/2006	+		
108		**Peru**	16	n	1/25/2006	+		
109		**Peru**	16	n	1/25/2006	+		
110		**Peru**	16	n	1/25/2006	+		
111		**Peru**	16	n	1/25/2006	+		
112		**Peru**	16	n	1/25/2006	+		
113		**Peru**	16	n	1/25/2006	+		
114		**Peru**	16	n	1/25/2006	+		
115		**Peru**	16	n	1/25/2006	+		
116		**Peru**	16	n	1/25/2006	+		
117		**Peru**	16	n	1/25/2006	+		
118		**Peru**	17	m	1/26/2006	+		
119		**Peru**	17	n	1/26/2006	+		
120		**Peru**	17	n	1/26/2006	+		
121		**Peru**	17	n	1/26/2006	-		
122		**Peru**	17	n	1/26/2006	-		
123		**Peru**	16	n	1/26/2006	-		
124		**Peru**	16	n	1/26/2006	+		
125		**Peru**	16	n	1/26/2006	+		
126		**Peru**	16	n	1/26/2006	+		
127		**Peru**	16	n	1/26/2006	+		
128		**Peru**	16	n	1/26/2006	+		
129		**Peru**	16	n	1/26/2006	+		
130		**Peru**	15	n	2/1/2006	+		
131		**Peru**	15	n	2/1/2006	+		
132		**Peru**	15	n	2/1/2006	+		
133		**Peru**	15	n	2/1/2006	+		
134		**Peru**	15	n	2/1/2006	+		
135		**Peru**	15	n	2/1/2006	+		
136		**Peru**	15	n	2/1/2006	+		
137		**Peru**	15	n	2/1/2006	+		
138		**Peru**	15	n	2/1/2006	+		

139		**Peru**	15	n	2/1/2006	+		
140		**Peru**	15	n	2/1/2006	+		
141		**Peru**	15	n	2/1/2006	-		
142		**Peru**	15	n	2/1/2006	+		
143		**Peru**	15	n	2/1/2006	+		
144		**Peru**	15	n	2/1/2006	-		
145		**Peru**	15	n	2/1/2006	+		
146		**Peru**	15	n	2/1/2006	+		
147		**Peru**	15	n	2/1/2006	+		
148		**Peru**	15	n	2/1/2006	+		
149		**Peru**	15	n	2/1/2006	+		
150		**Peru**	15	n	2/1/2006	+		
151		**Peru**	15	n	2/1/2006	+		
152		**Peru**	15	n	2/1/2006	+		
153		**Peru**	15	n	2/1/2006	-		
1		**Ecuador**	1	f	5/23/2006	-		
2		**Ecuador**	1	f	5/23/2006	+	-	
3		**Ecuador**	1	f	5/23/2006	+	-	
4		**Ecuador**	1	f	5/23/2006	+	-	
5		**Ecuador**	1	f	5/23/2006	+	-	
6		**Ecuador**	1	f	5/23/2006	+	+	+
7		**Ecuador**	1	f	5/23/2006	+	+	+
8		**Ecuador**	1	f	5/23/2006	+	+	+
9		**Ecuador**	1	f	5/23/2006	+	+	+
10		**Ecuador**	1	f	5/23/2006	-		
11		**Ecuador**	1	m	5/23/2006	-		
12		**Ecuador**	1	m	5/23/2006	-		
13		**Ecuador**	1	m	5/23/2006	-		
14		**Ecuador**	1	m	5/31/2006	-		
15		**Ecuador**	1	m	5/31/2006	+	+	+
16		**Ecuador**	1	m	5/31/2006	+	+	+
17		**Ecuador**	1	m	5/31/2006	+	+	+
18		**Ecuador**	1	m	5/31/2006	+	+	+
19		**Ecuador**	1	n	5/31/2006	-		
20		**Ecuador**	1	n	5/31/2006	+		
21		**Ecuador**	1	n	5/31/2006	+		
22		**Ecuador**	1	n	5/31/2006	+	-	
23		**Ecuador**	1	n	5/31/2006	+	+	-
24		**Ecuador**	1	n	5/31/2006	+	+	-
25		**Ecuador**	1	n	5/31/2006	+	+	-
26		**Ecuador**	1	n	5/31/2006	+	+	+
27		**Ecuador**	1	n	5/31/2006	+	+	+
28		**Ecuador**	1	n	5/31/2006	+	+	+
29		**Ecuador**	1	n	5/31/2006	+	+	+
30		**Ecuador**	1	n	5/31/2006	+	+	+
31		**Ecuador**	1	n	5/31/2006	+	+	+
32		**Ecuador**	1	n	5/31/2006	+	+	+
33		**Ecuador**	1	n	5/31/2006	+	+	+

34		**Ecuador**	1	n	5/31/2006	+	+	+
35		**Ecuador**	1	n	5/31/2006	+	+	+
36		**Ecuador**	1	n	5/31/2006	+	+	+
37		**Ecuador**	1	n	5/31/2006	+	+	+
38		**Ecuador**	1	n	5/31/2006	+	+	+
39		**Ecuador**	1	n	5/31/2006	+	+	+
40		**Ecuador**	1	n	5/31/2006	+	+	+
41		**Ecuador**	1	n	5/31/2006	+	+	+
42		**Ecuador**	1	n	5/31/2006	+	+	+
43		**Ecuador**	1	n	5/31/2006	+	+	+
44		**Ecuador**	1	n	5/31/2006	+	+	+
46		**Ecuador**	2	m	6/5/2006	+		
47		**Ecuador**	2	n	6/5/2006	+		
48		**Ecuador**	2	n	6/5/2006	+		
49		**Ecuador**	2	n	6/5/2006	+		
50		**Ecuador**	2	n	6/5/2006	+		
51		**Ecuador**	2	n	6/5/2006	+		
52		**Ecuador**	2	n	6/5/2006	+		
53		**Ecuador**	2	n	6/6/2006	+		
54		**Ecuador**	2	n	6/6/2006	+		
55		**Ecuador**	2	n	6/6/2006	+		
56		**Ecuador**	2	n	6/6/2006	+		
57		**Ecuador**	2	n	6/6/2006	-		
58		**Ecuador**	2	n	6/6/2006	+		
59		**Ecuador**	2	n	6/6/2006	-		
60		**Ecuador**	2	n	6/6/2006	-		
61		**Ecuador**	2	n	6/6/2006	+		
62		**Ecuador**	2	n	6/6/2006	+		
63		**Ecuador**	2	n	6/6/2006	+		
64		**Ecuador**	2	n	6/6/2006	-		
65		**Ecuador**	2	n	6/6/2006	+		
66		**Ecuador**	2	n	6/6/2006	+		
67		**Ecuador**	2	n	6/6/2006	+		
68		**Ecuador**	2	n	6/6/2006	+		
69		**Ecuador**	2	n	6/6/2006	+		
70		**Ecuador**	2	n	6/6/2006	-		
71		**Ecuador**	2	n	6/6/2006	+		
72		**Ecuador**	2	n	6/6/2006	+		
73		**Ecuador**	2	n	6/6/2006	+		
74		**Ecuador**	2	n	6/6/2006	+		
75		**Ecuador**	2	n	6/6/2006	+		
76		**Ecuador**	3	m	6/15/2006	+	+	+
77		**Ecuador**	3	m	6/15/2006	+	+	+
78		**Ecuador**	3	m	6/15/2006	+	+	+
79		**Ecuador**	3	n	6/15/2006	+		
80		**Ecuador**	3	n	6/15/2006	+		
81		**Ecuador**	3	n	6/15/2006	+		
82		**Ecuador**	3	n	6/15/2006	+		

83		**Ecuador**	3	n	6/15/2006	+		
84		**Ecuador**	3	n	6/15/2006	+		
85		**Ecuador**	3	n	6/15/2006	+		
86		**Ecuador**	3	n	6/15/2006	+		
87		**Ecuador**	3	n	6/15/2006	+		
88		**Ecuador**	3	n	6/15/2006	-		
89		**Ecuador**	3	n	6/15/2006	+		
90		**Ecuador**	3	n	6/15/2006	+		
91		**Ecuador**	3	n	6/15/2006	+		
92		**Ecuador**	3	n	6/15/2006	+		
93		**Ecuador**	3	n	6/15/2006	+		
94		**Ecuador**	3	n	6/15/2006	-		
95		**Ecuador**	3	n	6/15/2006	+		
96		**Ecuador**	3	n	6/15/2006	+		
97		**Ecuador**	3	n	6/15/2006	+		
98		**Ecuador**	3	n	6/15/2006	+		
99		**Ecuador**	3	n	6/15/2006	-		
100		**Ecuador**	3	n	6/15/2006	+		
101		**Ecuador**	3	n	6/15/2006	+		
102		**Ecuador**	3	n	6/15/2006	+		
103		**Ecuador**	3	n	6/15/2006	+		
104		**Ecuador**	3	n	6/15/2006	+		
105		**Ecuador**	3	n	6/15/2006	-		
106		**Ecuador**	3	n	6/15/2006	-		
107		**Ecuador**	3	n	6/15/2006	-		
1		**Costa Rica**	1	n/a	n/a	+	+	+
2		**Costa Rica**	1	n/a	n/a	+	+	+
3		**Costa Rica**	1	n/a	n/a	+	-	
4		**Costa Rica**	1	n/a	n/a	+	+	+
5		**Costa Rica**	1	n/a	n/a	+	+	+
6		**Costa Rica**	1	n/a	n/a	+	+	+
7		**Costa Rica**	1	n/a	n/a	+	-	
8		**Costa Rica**	1	n/a	n/a	+	-	
9		**Costa Rica**	1	n/a	n/a	+	-	
10		**Costa Rica**	1	n/a	n/a	+	+	-
1		**French Guiana**	1	n/a	n/a	+	-	+
2		**French Guiana**	1	n/a	n/a	+	+	-
3		**French Guiana**	1	n/a	n/a	+	+	+
4		**French Guiana**	1	n/a	n/a	+	+	+
1		**Mexico**	1	F	10/21/2006	+	+	-
2		**Mexico**	1	F	10/21/2006	+	+	-
3		**Mexico**	1	M	10/21/2006	+	+	+
4		**Mexico**	1	M	10/21/2006	+	+	+
5		**Mexico**	1	M	10/21/2006	+	+	+
6		**Mexico**	1	N	10/21/2006	+	+	+

7		**Mexico**	1	N	10/21/2006	+	+	-
8		**Mexico**	1	N	10/21/2006	+	+	-
9		**Mexico**	1	N	10/21/2006	+	+	+
10		**Mexico**	1	N	10/21/2006	+	+	+
11		**Mexico**	1	N	10/21/2006	+	+	+
12		**Mexico**	1	N	10/21/2006	+	+	+
1	123780	**Argentina**	1	F	2/20/2007	+	+	+
2	123780	**Argentina**	1	M	2/20/2007	+	+	+
3	123780	**Argentina**	1	M	2/20/2007	+	+	+
4	123780	**Argentina**	1	M	2/20/2007	+	+	+
5	123784	**Argentina**	2	M	2/20/2007	+	+	+
6	123784	**Argentina**	2	F	2/20/2007	+	+	+
7	123784	**Argentina**	2	F	2/20/2007	+	+	+
8	123782	**Argentina**	3	F	2/20/2007	+	+	+
9	123782	**Argentina**	3	F	2/20/2007	+	+	+
10	123782	**Argentina**	3	M	2/20/2007	+	+	+
11	123781	**Argentina**	4	M	2/20/2007	+	+	+
12	123779	**Argentina**	5	M	2/20/2007	+	+	-
13	123779	**Argentina**	5	F	2/20/2007	+	+	+
14	123783	**Argentina**	6	M	2/20/2007	+	+	+
15	123783	**Argentina**	6	M	2/20/2007	+	+	+

APPENDIX C:

CLADE 1 — **Haplotypes**

Location	Samples	12SrRNA	Dloop	COII
Peru	Peru_13	1	-	-
	Peru_44	1	1	-
	Peru_45	-	1	-
	Peru_95	-	1	-
	Peru_14	2	1	-
	Peru_46	2	1	-
	Peru_52	2	1	-
	Peru_75	2	1	-
	Peru_60	2	1	-
	Peru_39	2	-	-
	Peru_92	2	-	-
	Peru_26	3	-	-
	Peru_78	3	3	-
	Peru_77	3	1	3
	Peru_91	3	1	-
	Peru_66	3	1	-
	Peru_54	3	1	-
	Peru_41	4	-	-
	Peru_43	4	1	-
	Peru_74	5	-	-
	Peru_57	5	1	-
	Peru_79	5	1	-
	Peru_68	6	6	-
	Peru_76	7	-	-
	Peru_72	7	7	-
	Peru_90	8	1	-

CLADE 2 — **Haplotypes**

Location	Samples	12SrRNA	Dloop	COII
Argentina (Chaco Occi)	Argentina_1	17	17	-
	Argentina_2	18	18	18
	Argentina_3	18	5	20
	Argentina_4	18	10	-
Argentina (Chaco Serr)	Argentina_5	19	19	19
	Argentina_6	19	19	19
	Argentina_7	19	19	19
	Argentina_11	19	19	-

CLADE 3

Location	Samples	Haplotypes 12SrRNA	Dloop	COII
Argentina (Yungas)	Argentina_8	20	20	-
	Argentina_9	20	11	20
	Argentina_12	20	12	12
	Argentina_13	20	13	13
	Argentina_14	20	14	-
	Argentina_10	21	21	21
	Argentina_15	21	21	21
Brazil (Sao Paulo)	SPF1	24	8	4
	SpM10	25	-	-
	SPM1	-	25	-
	SPM2	-	-	4
	SPM5	-	-	4
Brazil (Minas Gerais)	dlc22	-	8	-
	MGM4	24	-	-
	MGF8	24	-	-
	MGF2	-	27	8
	MGF3	-	-	9
Brazil (Rio de Jinero)	SAF5	25	-	-
	Saf7	25	-	-
	Saf6	26	-	-
	SAFF2	-	23	-
	dlw14	-	23	-
	SAFM2	-	26	-
	dlw15	-	29	-
	dlw16	-	30	-
	dlw17	-	31	-
	dlw18	-	32	-
	SAFF2	-	5	-
	SAFF4	-	5	-

CLADE 4

Location	Samples	Haplotypes 12SrRNA	Dloop	COII
Brazil (Corumba)	Amcaj122954	26	-	-
	Amcaj122965	22	-	-
Brazil (Rondonia)	ROF1	-	22	-
	ROF4	-	22	-
	ROFF2	-	-	6
	ROFF1	-	-	7
Venezuela	Amcaj47831	23	-	-
	47831 Venezuela	-	28	-
French Guiana	F.G. drag	23	-	-
	FG03	-	24	-
	FG20081	-	-	33
	FG20082	-	-	33
	FG20083	-	-	33
	FG20084	-	-	33
	FG20085	-	-	33
	FG20086	-	-	33
	FG20087	-	-	33
	FG20088	-	-	33
	FG20089	-	-	33

CLADE 5

Location	Samples	12SrRNA	Dloop	COII
Ecuador	Ecuador_6	9	9	9
	Ecuador_7	9	9	9
	Ecuador_76	9	9	9
	Ecuador_77	9	9	9
	Ecuador_78	9	9	9
	Ecuador_26	9	9	-
	Ecuador_27	9	9	-
	Ecuador_28	9	9	-
	Ecuador_29	9	9	-
	Ecuador_30	9	9	-
	Ecuador_31	9	9	-
	Ecuador_32	9	9	-
	Ecuador_33	9	9	-
	Ecuador_34	9	9	-
	Ecuador_35	9	9	-
	Ecuador_36	9	9	-
	Ecuador_37	9	9	-
	Ecuador_39	9	9	-
	Ecuador_40	9	9	-
	Ecuador_8	9	-	9
	Ecuador_9	9	-	9
	Ecuador_17	9	-	9
	dlw5-Ecuador	-	9	-
	dlw7-Ecuador	-	9	-
	Ecuador_16	-	-	9
	Ecuador_15	9	-	1
	dlw6-Ecuador	-	2	-
	Ecuador_38	9	2	-
	Ecuador_42	9	2	-
	Ecuador_18	10	9	9
	Ecuador_41	11	9	-
	Ecuador_43	12	9	-
	Ecuador_44	13	2	-
Costa Rica	Costa Rica_01	-	9	-
	Costa Rica_02	-	9	-
	Costa Rica_04	-	9	-
	Costa Rica_05	-	9	-
	CRIIBF	9	-	-
	CRXF	9	-	-
	CRXIBN	9	-	9
	CRXIIBF	-	-	9
	CRXIIBF2	-	-	9
	CRVIIIA	14	-	9
	CRXIIB12	14	-	-
	CRIXII	14	-	-
	CRBF12	14	-	-
	Costa Rica_06	-	4	-
	CRXIBF12	21	-	-
Mexico	Mexico_3	14	9	14
	Mexico_5	14	9	14
	Mexico_6	14	9	14
	Mexico_11	14	9	-
	Mexico_15	-	-	9
	Mexico_4	14	2	2
	Mexico_12	14	16	-
	Mexico_10	16	9	-
	Mexico_9	15	15	-
	dlw20	-	33	-