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SYSTEMATIC NOTES OF *ANOPHELES KONDERI* AND ITS FIRST RECORD IN PARANÁ STATE, BRAZIL

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ABSTRACT. We analyzed nuclear (second internal transcribed spacer and white gene) and mitochondrial (cytochrome c oxidase subunit I) data from *Anopheles konderi* collected in the Amazonian states of Acre, Amapá, and Rondônia and the southern Brazilian state of Paraná. This was the first record of *An. konderi* in the state of Paraná. We found a high degree of genetic divergence within the Amazonian region and support for *An. konderi* as a species complex, possibly consisting of 3 species.

KEY WORDS Second internal transcribed spacer, cytochrome c oxidase subunit I, white gene, distribution, new record

INTRODUCTION

The mosquito genus *Anopheles* Meigen contains all the known vectors of human malaria parasites. Many of these malaria vectors belong to cryptic species complexes. In Latin America, 11 species are involved in malaria transmission, and at least 6 of these form cryptic species complexes: *Anopheles aquasalis* Curry (Conn et al. 1993; Maldonado et al. 1997), *An. benarrochi* Cova-Garcia and Lopez (Ruiz et al. 2005; Sallum et al. 2008), *An. oswaldoi* Peryassu (Marrelli et al. 1999a; Sallum et al. 2008), *An. nuneztovari* Gabaldón (Conn et al. 1993; Sierra et al. 2004), *An. albitarsis* Lynch-Arribalzaga (Wilkerson et al. 1995), and *An. triannulatus* Neiva and Pinto (Rosa-Freitas et al. 1998).

Marrelli et al. (1999b) suggested that the *An. oswaldoi* complex consisted of 4 forms based on second internal transcribed spacer (ITS2) ribosomal deoxyribonucleic acid (DNA) sequences from Brazil, Peru, and Venezuela, and they identified 1 of these as *An. konderi* Galvão and Damasceno. This species was originally described in 1942 based on adults, larvae, and pupae collected in Coari, Amazonas State, Brazil (Galvão and Damasceno 1942). However, Lane (1953) considered *An. konderi* to be a synonym of *An. oswaldoi*. Later, Flores-Mendoza et al. (2004) resurrected *An. konderi* from synonymy and designated a neotype. Males of *An. oswaldoi* and *An. konderi* can be distinguished by the shape of apical part of the aedeagus (Flores-Mendoza et al. 2004; Motoki et al. 2007), but it remains difficult to reliably identify adult females. *Anopheles konderi* is known to be sympatric with *An. oswaldoi* s.l. in the southern Amazonian states of Acre (Sallum et al. 2008) and Rondônia (Scarpassa and Conn 2006). Elsewhere *An. konderi* has been reported from the states of Amazonas, Pará, Espírito Santo, São Paulo, Mato Grosso, and Rio de Janeiro in Brazil, as well as in Loreto Department in Peru and Cochabamba State in Bolivia (Flores-Mendoza et al. 2004).

To date, the medical importance of *An. konderi* in malaria transmission is unknown. Experimental infections have indicated that *An. konderi* has lower levels of susceptibility to *Plasmodium vivax* Grassi and Feletti infection than *An. oswaldoi* s.l. (Marrelli et al. 1999a). In the present paper, *An. konderi* is reported for the first time from the state of Paraná, Brazil, and individuals from this state are compared with *An. konderi* from several other states (Acre, Amapá, and Rondônia) in the north of Brazil using data from ITS2, cytochrome c oxidase subunit I (COI) of mitochondrial DNA, and the single-copy nuclear white gene.

MATERIALS AND METHODS

Mosquito collection

The samples used in this study (Table 1) were obtained from either offspring (egg, larva, pupa, and adult) of blood-fed females collected in the field or field-collected larvae and pupae that were reared to adulthood in the laboratory. Freshly emerged mosquitoes were anesthetized with ethyl acetate vapors, and specimens were either kept in separate minute plastic vials in silica gel or were individually frozen at −80°C. The ITS2 sequence of specimen AC18-16 was obtained from GenBank (accession number EU636801). Species identification was based on either male genitalia or 4th-stage larva. Immature stages and male

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Table 1. Taxon ID number, location, and geographic coordinates of specimens used in this study.

Species	Species ID	State, municipality	Geographic coordinates
Anopheles konderi	AC18-16	Acre, Acrelândia	09°68′00″S, 67°13′00″W
An. konderi	AP15(11)	Amapá, Macapá	0°16′17.5″N, 50°53′53.3″W
An. konderi	AP25(1)100	Amapá, Macapá	0°01′60″N, 51°02′60″W
An. konderi	AP25(11)24	Amapá, Macapá	0°01′60″N, 51°02′60″W
An. konderi	RO18(1)6	Rondônia, Monte Negro	10°18′03.5″S, 63°14′09.1″W
An. konderi	PR06(2)13	Paraná, Porto Natal	23°03′46.9″S, 53°37′28″W
An. konderi	PR14(1)9	Paraná, Porto Rico	22°50′60.5″S, 53°21′53.5″W
An. konderi	PR14(3)17	Paraná, Porto Rico	22°50′60.5″S, 53°21′53.5″W
An. konderi	PR14(9)108	Paraná, Porto Rico	22°50′60.5″S, 53°21′53.5″W
An. konderi	PR19(9)1	Paraná, Santa Helena	24°51′46.7″S, 54°21′10.9″W
An. albertoi	MG07(12)-4	Minas Gerais, Frutal	20°01′31.0″S, 49°04′35.4″W
An. arthuri	RO8-1	Rondônia, Monte Negro	10° 16′07.1″S, 63°33′19.4″W
An. strodei	ES09-1	Espírito Santo, Santa Teresa	19°55′0″S, 40°36′0″W

genitalia of the specimens used for DNA extraction were deposited in the Faculdade de Saude Publica-Universidade de São Paulo (FSP-USP) entomological collection as vouchers.

DNA extraction

DNA was extracted from each mosquito specimen following the protocol provided by the QIAgen DNeasy® Blood and Tissue Kit (QIAgen Ltd., Crawley, United Kingdom). Template DNA was retained dry at −80°C in the Laboratório de Sistemática Molecular at Faculdade de Saúde Pública, Universidade de São Paulo, São Paulo, Brazil.

Ribosomal DNA ITS2

The rDNA ITS2 region was amplified using 5.8SF (5′-ATC ACT CGG CTC GTG GAT CG-3′) and 28SR (5′-ATG CTT AAA TTT AGG GGG TAG TC-3′) primers (Sallum et al. 2008). Polymerase chain reaction (PCR) was carried out in a 25-µl aqueous reaction mixture containing 1 µl of DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 µl of dimethyl sulfoxide, 5 pmol of each primer, 200 µM each deoxynucleoside triphosphates (dNTP), and 2.5 U of Taq polymerase (Invitrogen Platinum® Taq DNA Polymerase; Invitrogen Inc., Carlsbad, CA). The PCR amplification profile consisted of a 2-min denaturation at 94°C and 34 cycles at 94°C, 57°C, and 72°C for 30 sec each, followed by a 10-min extension at 72°C. All PCR products were electrophoresed in 1.5% tris-acetate-EDTA (TAE) agarose gels stained with GelRed™ (Biotium Inc., Hayward, CA). ITS2 amplicons initially yielded ambiguous sequence chromatograms, suggesting the existence of intragenomic variation. Amplicons were purified using PEG precipitation (20% polyethylene glycol 8,000/2.5 M NaCl) and then cloned into pGem-T Easy Vector (Promega, Madison, WI). At least 3 positive clones from each individual were sequenced.

COI mitochondrial gene

The COI fragments were amplified using LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAATCA-3′) primers (Folmer et al. 1994). Each PCR reaction was carried out in a 25-µl aqueous reaction mixture containing 1 µl of DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 5 pmol of each primer, 200 µM each dNTPs, and 2.5 U of Taq polymerase (Invitrogen Platinum® Taq DNA Polymerase; Invitrogen). The PCR amplification protocol consisted of a 3-min denaturation at 94°C and 35 cycles at 94°C, 55°C, and 72°C for 1 min each, followed by a 7-min extension at 72°C.

Nuclear white gene

The PCR amplification of the nuclear single copy white gene was conducted using the same primers employed by Bourke et al. (2010). Each PCR reaction was carried out in a 25-µl aqueous reaction mixture containing 1 µl DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 µl of dimethyl sulfoxide, 100 pmol of each primer, 200 µM each dNTPs, and 2.5 U Invitrogen Platinum® Taq DNA polymerase (Invitrogen). The PCR amplification profile consisted of a 3-min denaturation at 94°C and 35 cycles at 94°C for 30 sec, 50°C for 1 min, and 72°C for 2 min each, followed by a 7-min extension at 72°C.

Sequencing

All sequencing reactions were carried out in both directions using ABI Big Dye Terminator Kit version 3.1 (PE Applied Biosystems, Warrington, United Kingdom). For COI and ribosomal DNA, we employed the same primers as those used for PCR, whereas for the white genes, 2 internal primers, W1F (5′-GAT CAA RAA GAT CTG YGA CTC GTT-3′) and W2R (5′-GCC ATC GAG ATG GAG GAG CTG-3′)

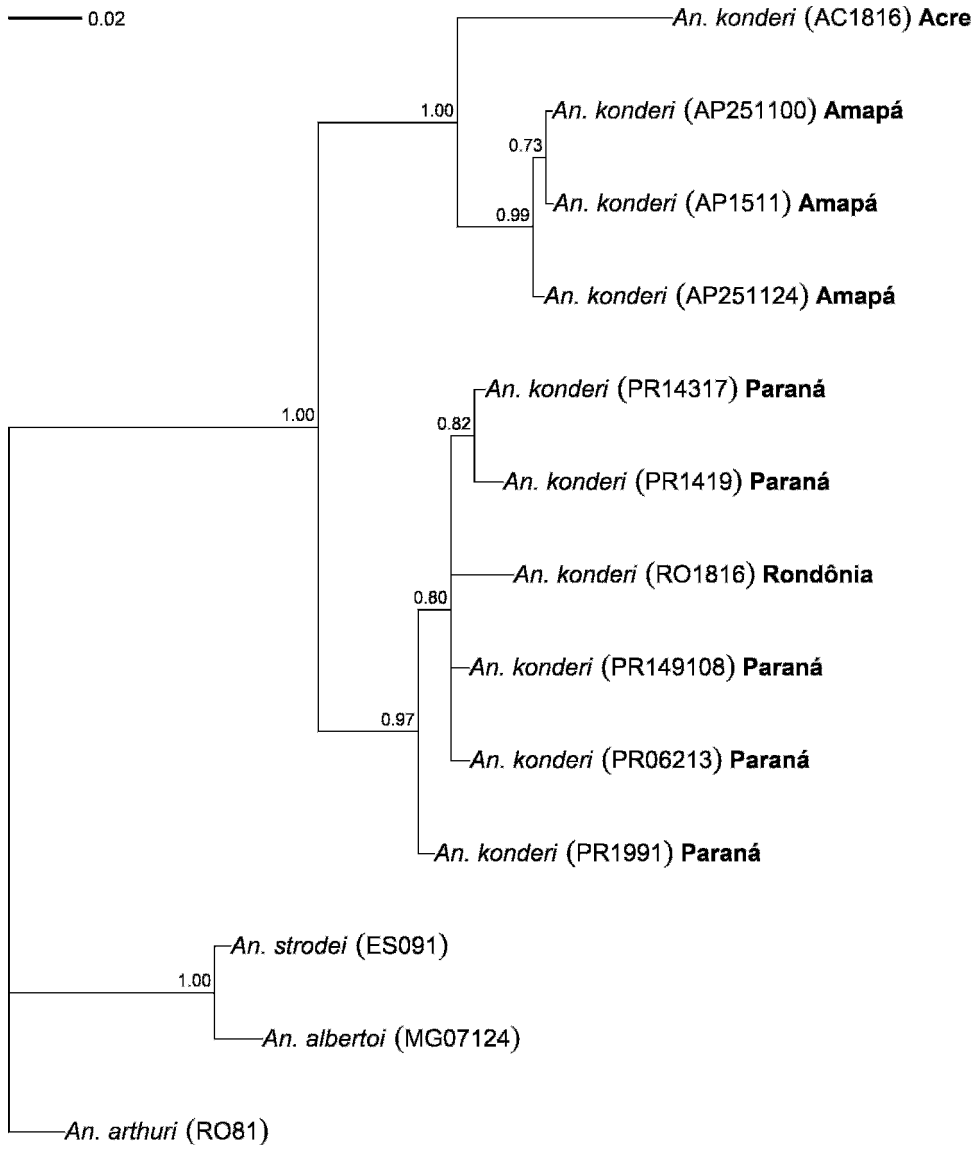

Fig. 1. Bayesian phylogenetic analysis of the mitochondrial cytochrome c oxidase subunit I gene of *Anopheles konderi*. The data were partitioned by codon position with among-partition variation. Numbers at branches indicate Bayesian posterior probability (≥0.70). *Anopheles albertoi*, *An. arthuri*, and *An. strodei* were included as outgroups.

(Bourke et al. 2010), were used. The sequencing reaction consisted of 0.5 µl of Big Dye Terminator Ready Reaction Mix, 3 µl of 5X sequence dilution-buffer (5 mM $MgCl_2$, 200 mM Tris-HCl, pH 9.0), 3.6 pmol of R or F primer, 10 ng of PEG purified PCR product, and the remaining volume was filled with ultrapure water for a total of 10 µl. Sequencing reactions were purified in Sephadex G50® columns (GE Healthcare, Uppsala, Sweden). Sequences were analyzed on an ABI Prism 3130 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA), and edited using Sequencher® for Windows version 4.9 (Gene Codes Corporation, Ann Arbor, MI).

Sequence analysis

Sequences were aligned in CLUSTAL X version 2.0 for MacOSX (Larkin et al. 1997) and optimized manually in MacClade version 4.8 (Maddison and Maddison 2002). The nucleotide divergence was calculated in PAUP* 4.0 using uncorrected *P*-distances and Kimura 2-parameter (K2P) distances. For Bayesian analysis, a parti-

Table 2. Pairwise genetic distances (%) using the Kimura 2-parameter model.

	PR06 (2)13	PR14 (1)9	PR14 (3)17	PR14 (9)108	PR19 (9)1	RO18 (1)6	AP (15)11	AP25 (1)100	AP25 (11)24	AC18-16
PR06(2)13	–									
PR14(1)9	0.80	–								
PR14(3)17	0.50	0.30	–							
PR14(9)108	0.30	0.80	0.50	–						
PR19(9)1	0.50	1.00	0.70	0.50	–					
RO18(1)6	0.70	1.20	0.80	0.70	0.80	–				
AP(15)11	3.40	3.50	3.20	3.40	2.90	3.70	–			
AP25(1)100	3.40	3.50	3.20	3.40	2.90	3.70	0.00	–		
AP25(11)24	3.40	3.50	3.20	3.40	2.90	3.70	0.00	0.00	–	
AC18-16	4.40	4.60	4.20	4.10	3.90	4.40	3.40	3.40	3.40	–

tioning strategy was applied to individual and combined gene sequence data to allow different partitions to have their own model characteristics (composition, rate matrix, and among-site variation) and to allow for among-partition rate variation (APRV). For isolated gene partitions, the ITS2 and white gene was left unpartitioned, while COI could be unpartitioned or partitioned by codon position. For combined genes, the data could be left unpartitioned, partitioned by gene, partitioned by combined ITS2-white gene and COI codon positions, or partitioned by separate ITS2 and white gene and COI codon positions. Optimal evolutionary models for each partition were determined using the default likelihood settings (implementing 56 models and maximum likelihood optimized base trees) and the Akaike Information Criterion in jModelTest (Posada 2008). All possible partitioning schemes for COI, white gene, ITS2, and combined sequence data were then analyzed in MrBayes version 3.1.2 (Ronquist and Huelsenbeck 2003). Each analysis consisted of 2 simultaneous runs repeated 3 times to confirm the convergence of posterior probability distribution. Each run consisted of 12 million steps, where the first 6 million steps were discarded as burn in. It also consisted of 6 heated chains, and adequate mixing was achieved by setting the chain "temperature" to 0.10. The average standard deviation of split frequencies (a measure of the convergence of topology between 2 simultaneous runs) consistently fell below 0.01 in the postburn in samples. Another convergence diagnostic, the potential scale reduction factor, approached the recommended value of 1 for all runs. A consensus tree of postburn in samples for each partitioning scheme was then constructed, using branch support of greater than 70% posterior probability. *Anopheles albertoi*, *An. arthuri*, and *An. strodei* were used as outgroups in all phylogenetic analyses. While all clones were included in the isolated ITS2 analysis, only a single randomly selected ITS2 clone from each individual was included in the combined gene analysis. The best partitioning scheme for isolated and combined genes was then estimated using Bayes factors (BF). These are pair-wise values that represent the relative success of each partitioning scheme at predicting the data (Kass and Raftery 1995). The BF values were calculated according to the formula $BF_{10} = 2(\ln[HM_1] - \ln[HM_0])$, where HM_1 and HM_0 are the harmonic means of the posterior sample of likelihoods (calculated in MrBayes and averaged over 3 runs) of the pair of partition strategies being compared. Positive values of BF_{10} are indicative of support for the partition denoted 1 over the partition denoted 0.

Vouchers

Template DNA from this study was retained in the FSP-USP collection for future reference. Immature stages and male genitalia slides of the same specimens used for DNA extraction were deposited in the FSP-USP entomological collection as vouchers. GenBank accession numbers are ITS 2 (JF437926-JF437964, EU636801, FJ178885, GU226727, FJ178875); mitochondrial COI (JF437965-JF437974, GU226678, GU226688, GU226664); nuclear white (JF437975-JF437984, GU226747, GU226759, GU226730).

RESULTS

Results of morphological comparisons and phylogenetic analyses of the mitochondrial COI, single copy nuclear white gene, and rDNA including the ITS2 sequences support the occurrence of *An. konderi* in Paraná State, and thus represent the first report of the species in the state. Sequence alignments (including the outgroup) yielded 575 base pairs (bp) for ITS2, 732 bp for white (including Exon III, Intron, and Exon IV), and 658 bp for COI, giving a total of 1,965 bp. Among the *An. konderi* sequences, there were 40 variable sites in ITS2, 14 in the white gene, and 43 in COI. Intragenomic differences for ITS2 varied from 0% to 1% (uncorrected *P*-distance). Intraspecific differences calculated using uncorrected *P*-distances varied from 0% to 2.3%. The ITS2 base composition among

Table 3. Model selection for the partitions used in the analyses.

Gene or region[1]	Codon position	jModeltest	Model used
COI		TIM1 + G	GTR + G
	1	TIM1 + G	GTR + G
	2	F81	F81
	3	TIM1	GTR
White		TPM1uf + G	GTR + G
ITS2		TVM + G	GTR + G
White-ITS2		GTR + G	GTR + G
COI-white-ITS2		GTR + G	GTR + G

[1] COI, cytochrome c oxidase subunit I; ITS2, second internal transcribed spacer.

cloned sequences of *An. konderi* from Paraná, Amapá, Acre, and Rondônia states was 28% A, 27% C, 25% G, and 20% T.

The COI base composition across all populations was 29% A, 15% C, 16% G, and 40% T. K2P distances were calculated for COI (Table 2). Differences among the Paraná and Rondônia individuals varied from 0.3% to 1.2%. There was no variation among individuals from Amapá. Differences between individuals from Amapá and those from Paraná and Rondônia varied by 2.9% to 3.7%. The single Acre individual differed from all other *An. konderi* by 3.4% to 4.6%.

Phylogeny

The optimal evolutionary models for the sequence partitions are shown in Table 3. In the absence of the most appropriate rate matrix in MrBayes (i.e., TVM, TIM, and TPM), the most similar rate matrix available was selected (i.e., GTR). For ITS2, the best evolutionary model was found to be TVM + G, but it was replaced by GTR + G in the Bayesian analysis. The best evolutionary model for COI was one that partitioned the data by codon position. For the white gene, the majority of the variation existed within the intron. No variation existed in Exon 3, and only 4 of the 380 sites in Exon 4 were variable. The best model was found to be TPM1uf + G but was replaced with GTR + G. For the combined genes, the best model was one that partitioned the data by COI codon position and separate ITS2 and white gene with APRV (Table 4).

Table 4. Partition schemes for the combined genes, with and without among-partition rate variation.

Partition[1]	APRV[2]	Harmonic mean
None		−4096.63
Gene	−	−4032.00
	+	−4029.88
COI codon position and combined ITS2-white gene	−	−3937.02
	+	−3861.33
COI codon position and separate ITS2 and white gene	−	−3930.23
	+	−3853.12

[1] COI, cytochrome c oxidase subunit I; ITS2, second internal transcribed spacer.
[2] APRV, among partition rate variation.

Analysis of COI (Fig. 1), combined genes, (Fig. 2), and white gene (Fig. 3) showed that individuals from Paraná were closely associated with those from Rondônia. The Bayesian posterior probability (BPP) support for the Paraná/Rondônia clade was 0.97 for the COI (Fig. 1) and 0.98 for combined genes (Fig. 2). For the ITS2 (Fig. 4) there was moderate support for a clade formed by Paraná, Rondônia, and Acre. But 1 of the Amapá clones formed a distinct clade (1.00 BPP), and the remaining clone was sister to the Paraná/Rondônia/Acre clade (0.99 BPP). Individuals from Acre and Amapá formed a strongly supported clade in all but 1 of the gene trees (ITS2), with the former sister to the latter (>0.95 BPP). At the ITS2, individuals from Acre, Rondônia, and Paraná were all found within a distinct clade with 0.89 BPP support. The Paraná/Rondônia and Amapá/Acre clades were reciprocally monophyletic at the COI and the combined gene trees (>0.97 BPP).

DISCUSSION

The findings of this study show that important regional differences exist among *An. konderi* from Brazil. While most populations in this study, such as Acre, Amapá, and Rondônia, were found within the Amazon region, the population from Paraná was ecologically and geographically disparate and was found in a temperate climate in the south of Brazil. Results of all analyses, however, indicate that individuals from Paraná are very closely associated with those from Rondônia, and that the greatest divergence occurs within the Amazon region. In fact the levels of divergence between Acre, Amapá, and Paraná-Rondônia are largely consistent with the 3% threshold adopted by Hebert et al. (2003) for resolving species.

There was generally a high degree of concordance among gene trees in this study. The principal relationships found that the Acre

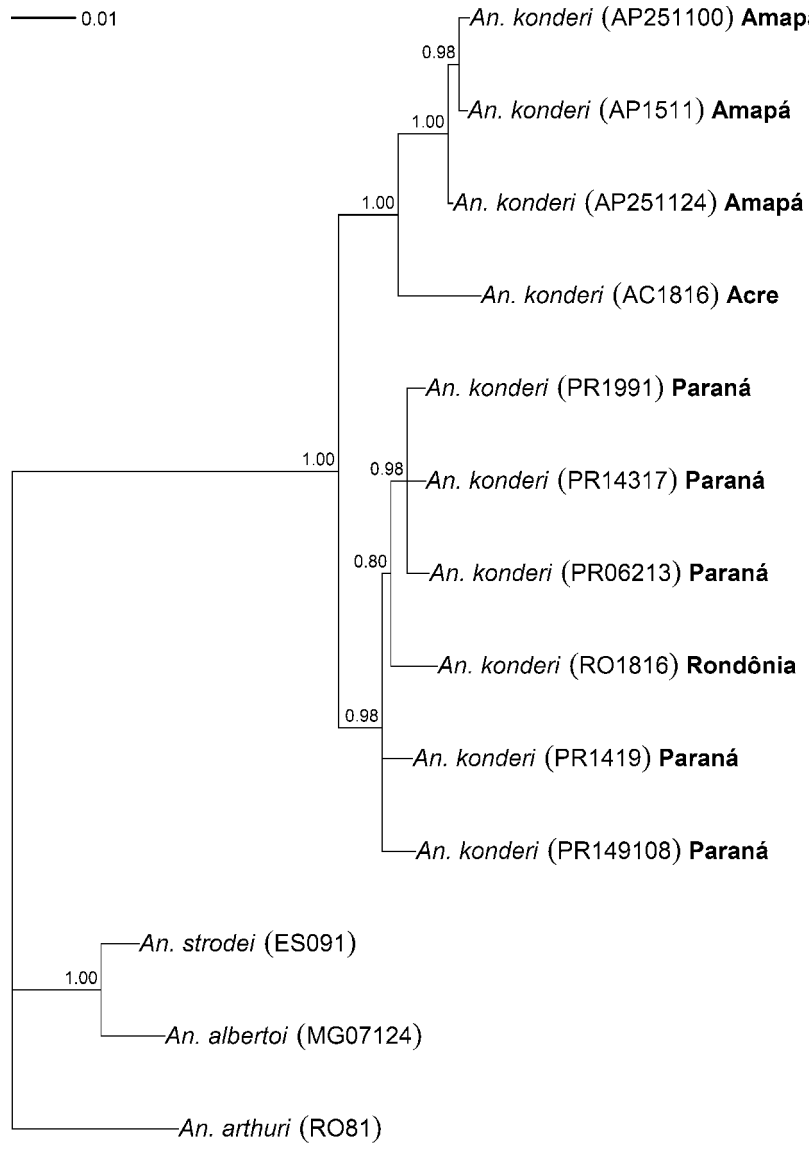

Fig. 2. Bayesian phylogenetic analysis of the combined data sets. The data were partitioned including ITS2, white gene, and COI codon positions with among-partition rate variation. Numbers at branches indicate Bayesian posterior probability (≥0.70). *Anopheles albertoi*, *An. arthuri*, and *An. strodei* were included as outgroups.

individual is a sister to an Amapá clade and that Rondônia and Paraná individuals cluster together. In addition, clearer relationships among these groups in the ITS2 topology, however, were different affected by intragenomic variation. Clones from Acre, Rondônia, and Paraná were all found within a strongly supported group, but high levels of intragenomic variation might explain why we did not observe clustering by geographical origin. Clones from Amapá were largely recovered as a distinct group, with 1 exception (clone AP1C).

The ITS2 gene family normally behaves as a single gene due to the homogenizing effect of concerted evolution (Coleman 2003). However, when mutations rates are higher than the rates of homogenization, intragenomic variation will be observed. Intragenomic variation has also been found in several other *Anopheles* species (Onyabe and Conn 1999; Wilkerson et al. 2004; Fairley et al. 2005; Sallum et al. 2008). Li and Wilkerson (2006) found intragenomic variation at ITS2 to be higher than interspecific variation among species in the *An. albitarsis* species complex. The existence of such variation can be problematic for phylogenetic studies. When constructing phylogenies from a gene, sequences are assumed to be orthologous in nature (their relationship originates from

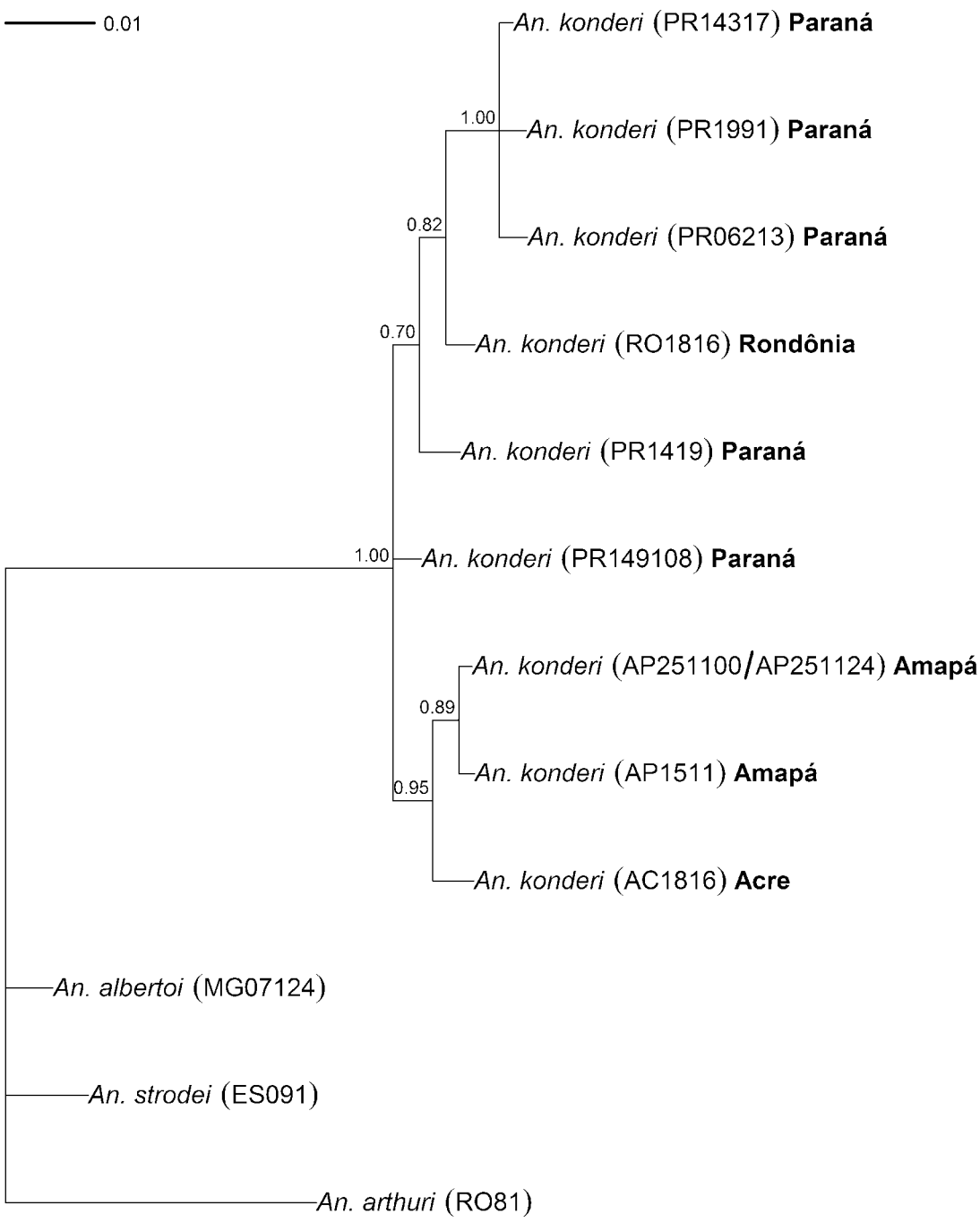

Fig. 3. Bayesian phylogenetic analysis of the nuclear white gene of *Anopheles konderi*. Numbers at branches indicate Bayesian posterior probability (≥0.70). *Anopheles albertoi*, *An. arthuri*, and *An. strodei* were included in the analysis as outgroups.

organismal cladogenesis). The inclusion of paralogous sequences can confound divergence and ultimately lead to incongruent and erroneous phylogenies. We therefore caution against overinterpreting relationships at ITS2 among Paraná, Acre, and Rondônia individuals, where intragenomic variation may have caused the lack of geographic resolution among these individuals.

These findings therefore support the status of *An. konderi* as a complex, possibly consisting of 3 species. However, because of the difficulty in accurately identifying *An. konderi*, the scant

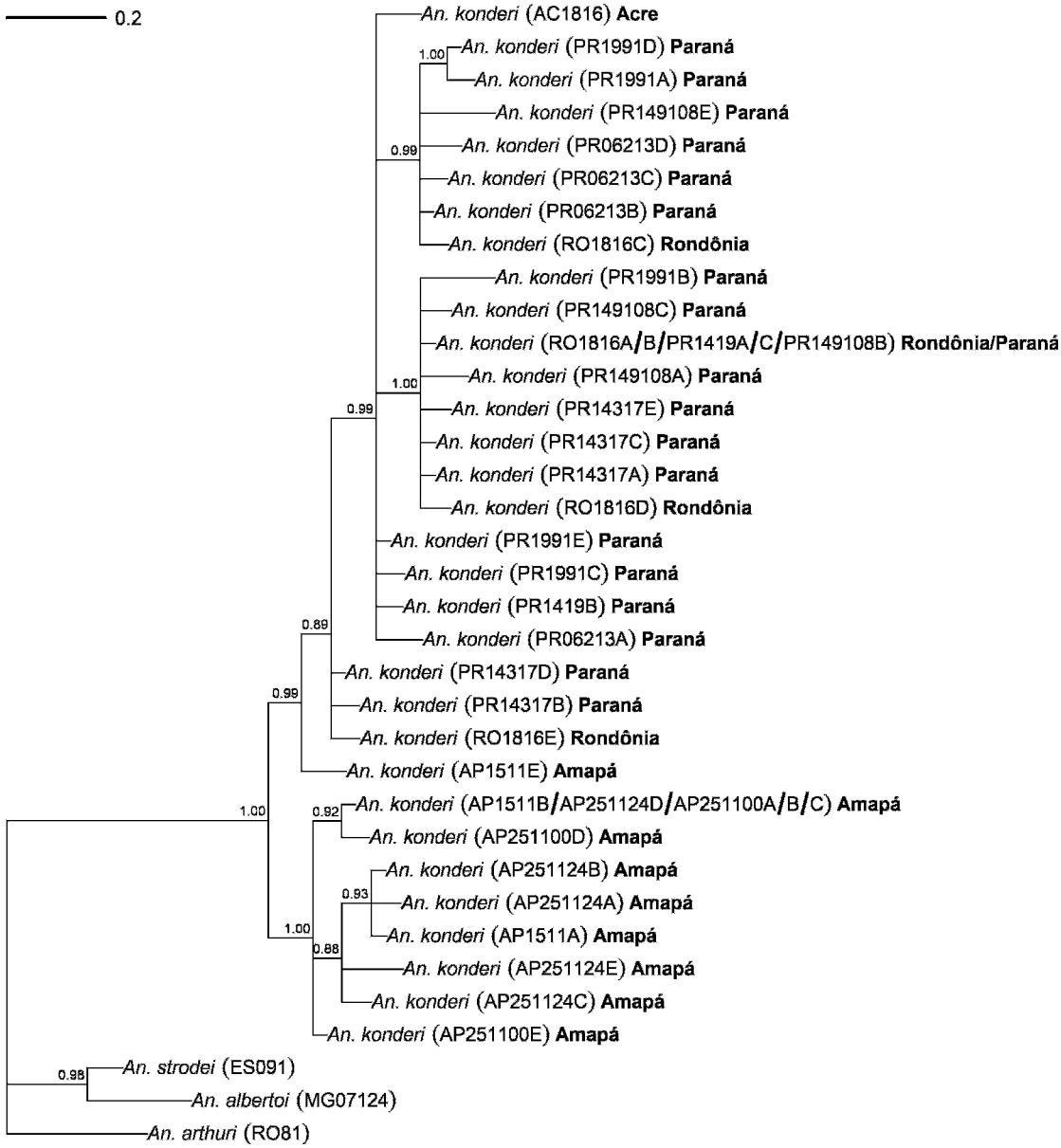

Fig. 4. Bayesian phylogenetic analysis of the second internal described spacer of *Anopheles konderi*. Numbers at branches indicate Bayesian posterior probability (≥0.70). *Anopheles albertoi*, *An. arthuri*, and *An. strodei* were included as outgroups.

information on geographical distributions, and the low number of samples available for this study, we cannot yet provide likely modes of speciation. Although the tentative Amapá and Acre species are only identified from highly restricted localities in the north and south of the Amazon region, respectively, their distributions may yet prove to be sympatric with that of the tentative Paraná-Rondônia species, which now covers more than 1,800 km and includes the 2 main river basins in South America (the Amazon and la Plata). Although the ancestral origin of the Paraná-Rondônia species cannot be inferred from our data, certainly this fluvial species may have undergone relatively uninhibited expansion between the Amazon and la Plata basins, where the Izozog swamp, and Río Parapetí and Río Grande alluvial fans connect both basins (Iriondo and Paira 2007).

In conclusion, our study found strong genetic divergence among populations of *An. konderi* and support for an *An. konderi* species complex. We

propose further sample across its known distribution to further examine genetic divergence and gain a more complete understanding of the species in this complex. Morphological data are also lacking, and data from across life stages may yet support the divergences observed in the current study. Finally, although *An. konderi* is not known to be a vector of malaria, individuals have been experimentally infected with the malaria parasite (Marrelli et al. 1999a). The discovery of new populations of *An. konderi*, such as in Paraná, and phylogenetic discontinuities within *An. konderi* may well have important epidemiological implications.

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