Identification of *Anopheles (Nyssorhynchus) albitarsis* complex species (Diptera: Culicidae) using rDNA internal transcribed spacer 2-based polymerase chain reaction primes

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Anopheles (Nyssorhynchus) marajoara *is a proven primary vector of malaria parasites in Northeast Brazil, and* An. deaneorum *is a suspected vector in Western Brazil. Both are members of the morphologically similar Albitarsis Complex, which also includes* An. albitarsis *and an undescribed species,* An. albitarsis "B". *These four species were recognized and can be identified using random amplified polymorphic DNA (RAPD) markers, but various other methodologies also point to multiple species under the name* An. albitarsis. *We describe here a technique for identification of these species employing polymerase chain reaction (PCR) primers based on ribosomal DNA internal transcribed spacer 2 (rDNA ITS2) sequence. Since this method is based on known sequence it is simpler than the sometimes problematical RAPD-PCR. Primers were tested on samples previously identified using RAPD markers with complete correlation.*

Key words: *Anopheles albitarsis* - species complex - polymerase chain reaction identification - malaria - internal transcribed spacer 2

The Neotropical *Anopheles (Nyssorhynchus) albitarsis* complex currently consists of four morphologically similar species, at least one of which, *An. marajoara* Galvão & Damasceno, is an important malaria vector in the state of Amapá, Brazil (Conn et al. 2002). Prior to Linthicum's (1988) revision of the Argyritarsis Section, which includes the Albitarsis Complex, the only species of the four currently recognized species considered valid was *An. albitarsis* Lynch-Arribalzaga. Linthicum (1988) resurrected a second species, *An. marajoara*, from synonymy and reviewed the taxonomic history of *An. albitarsis* and related species. He also synonymized *An. domesticus* Galvão & Damesceno with *An. marajoara* and declared *An. allopha* Peryassu to be a *nomen dubium*. Subsequently, Rosa-Freitas (1989) described a third species, *An. deaneorum*, also a potential malaria vector in the state of Rondônia in Western Brazil (Klein et al. 1991a,b), and Wilkerson et al. (1995a,b), using random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), discovered another as yet undescribed species, *An. albitarsis* species "B". Many suspected *An. albitarsis*

s.l. to be a species complex because of its wide distribution and great morphological and behavioral variability. Various methods were used to make sense of the *An. albitarsis* puzzle, including chromosomes, allozymes, mitochondrial DNA restriction fragment length polymorphisms (mtDNA RFLPs), RAPD-PCR, morphology, and behavior (Kreutzer et al. 1976, Rosa-Freitas & Deane 1989, Rosa-Freitas et al. 1990, Narang et al. 1993, Wilkerson et al. 1995a,b). Wilkerson et al. (1995a,b), using RAPD-PCR, demonstrated the existence of the four currently recognized species (but see Lehr et al. 2005) and found 2-4 diagnostic RAPD primers for each that serve to identify individuals from throughout their ranges. RAPD-PCR has been the only method to discriminate among the four with certainty since only *An. deaneorum* can be reliably separated morphologically, and then only in the larval stage. The species are distributed in Central and South America as follow: *An. albitarsis* (Southern Brazil, Northern Argentina, Paraguay), *An. deaneorum* (Northern Argentina to Western Brazil), *An. marajoara* (Brazil, Venezuela, Colombia, Southern Central America), and *An. albitarsis* B (South, Central, and Eastern Brazil). While reproducible in any given lab that has standardized the technique, RAPD-PCR can be unreliable as an identification method when differing extraction protocols, reagents, and PCR machines are used. In addition, the above method (Wilkerson et al. 1995a,b) can require two or more reactions to make an identification. Because of the medical importance of the complex we sought a means, other than RAPD-PCR, to carry out identifications in support of epidemiological, ecological, and population genetics studies.

With the above objective in mind, we investigated the rDNA ITS2 for species-specific sequence variation. Ribosomal DNA has been utilized for systematics and phylogenetics studies in a wide variety of organisms (e.g. Hillis & Dixon 1991, Miller et al. 1997 for Diptera). The

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
AUG 2005		**00-00-2005 to 00-00-2005**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Identification of Anopheles (Nyssorhynchus) albitarsis complex species (Diptera: Culicidae) using rDNA internal transcribed spacer 2-based polymerase chain reaction primes	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Walter Reed Army Institute of Research,Department of Entomology,503 Robert Grant Ave.,Silver Spring,MD,20910-7500	

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	**Same as Report (SAR)**	**6**	
unclassified	**unclassified**	**unclassified**			

functional regions that produce the ribosomes are highly conserved, but the transcribed (ITS1 and ITS2) and non-transcribed spacer (intergenic spacer, IGS) regions have high interspecific and low intraspecific variability, making them useful for explaining relationships of recently diverged species, and also useful as a basis for PCR identification of morphologically similar species. ITS2 sequences usually allow unambiguous species identifications in closely related *Anopheles* species (reviewed by Collins & Paskewitz 1996). Other examples since the above review include: *An. (Ano.) quadrimaculatus* complex (Cornel et al. 1996); *An. (Cellia) maculatus* complex (Torres et al. 2000); *An. (Cel.) dirus* complex (Walton et al. 1999); *An. (Cel.) fluviatilis* complex (Manonmani et al. 2001); *An. (Ano.) bancroftii* group (Beebe et al. 2001); *An. (Cel.) punctulatus* group (Beebe et al. 1999); *An. (Cel.) funestus* and related species (Hackett et al. 2000); and, *An. (Ano.) cruciaris* complex (Wilkerson et al. 2004). Rarely, the ITS2 sequences among cryptic species are too close to allow PCR-based identification. The only published example is *An. (Ano.) inundatus* Reinert (as C2) and *An. (Ano.) diluvialis* Reinert (as C1) of the *An. quadrimaculatus* complex (Cornel et al. 1996).

We describe here development of rDNA ITS2 primers based on cloned sequence (Li & Wilkerson, unpublished data). This technique makes possible reliable identifications for those studying ecology, behavior and malaria transmission in relation to these species.

MATERIALS AND METHODS

Source of specimens - Specimens used for testing of PCR primers are the same as those reported in Wilkerson et al. (1995a,b). These represent samples of the four species from widely separated localities as described in the introduction and include the type localities for the three named species. Additional specimens from subsequent studies were also tested from Venezuela (Rubio-Palis et al. 2004), Trinidad, Boa Vista (state of Roraima, Brazil) and localities in the state of Amapá, Brazil. Localities and coordinates can be found in Table I and in the above references.

Cloning - Locality and other information for specimens used to obtain clones, as well as Genbank accession numbers, are given in Table I (unpublished data, Li and Wilkerson). These clones are the basis for the species-specific primers described here. Detailed findings will be published elsewhere.

Species identification - Peyton et al. (1992) provide a definition of *An.* subgenus *Nyssorhynchus*. Adult female *Nyssorhynchus* mosquitoes were first placed in the Albitarsis Complex by the following combination of characters (Linthicum 1988): hindtarsomere 5 without a basal dark band, vein 1A mostly pale-scaled (Argyritarsis Section), sternum I with a distinct row of white scales on each side and, posterolateral scale tufts beginning on segments III or IV. The presumed sister species to the Albitarsis

TABLE I

Collection localities, type localities, number of clones, and GenBank accession numbers for specimens used in cloning of rDNA ITS2 of species belonging to the *Anopheles (Nyssorhynchus) albitarsis* complex

Species	Code	Country	State	Locality (type locality)	Coords	Clones	GenBank accession nrs
albitarsis	BR504(8)	Brazil	Paraná	nr Guaira	24°04' S 54° 15' W	15	AY828320-AY828323
albitarsis	AR7(8)	Argentina	Buenos Aires	Baradero (type locality)	33° 48' S 59° 30' W	41	AY828334-AY823336
albitarsis B	BR019(12)	Brazil	Ceará	Paraipaba	3° 25' S 39° 13' W	10	AY828324-AY828327
albitarsis B	BR/SP 500(1)	Brazil	São Paulo	near Registro	24° 37' S 47° 53' W	28	AY828337-AY828338
marajoara	BR026(12)	Brazil	Amazonas	Manaus	2° 53' S 60° 15' W	15	AY828328-AY828329
marajoara	BR/R001(10)	Brazil	Pará	Marajó Is. (type locality)	1° 00' S 49° 30' W	37	AY828339-AY828340
marajoara	COJ9	Venezuela	Cojedes	Finca "Rosa Blanca"	9° 36' N 68° 54'W	9	AY828344-AY828345
marajoara	COJ10	Venezuela	Cojedes	Finca "Rosa Blanca"	9° 36' N 68° 54'W	4	AY828346-AY828347
marajoara	BR4	Brazil	Roraima	Boa Vista	2° 45' 28" N 60° 42' 18" W	4	AY828348-AY828350
marajoara	PIS9	Brazil	Amapá	N of Amapá	Not known	7	AY828351-AY828353
marajoara	ITB13763	Brazil	Pará	Near Itaituba	Not known	3	AY828354
deaneorum	BR/R007(15)	Brazil	Rondônia	Guajará Mirim (type locality)	10° 50' S 65° 20' W	11	AY828330-AY828333
deaneorum	AR2(3)	Argentina	Corrientes	90 km W Posadas	Not known	8	AY828341-AY828343
deaneorum	AR3(4)	Argentina	Corrientes	Corrientes	27° 30' S 58° 48' W	26	AY828341-AY828343

Complex, *An. braziliensis* (Chagas), has the rows of white scales on sternum I but the posterolateral tufts begin on segment II. Additional *An. braziliensis* differences can also be found in the adult female, male genitalia, and pupal and larval stages (Linthicum 1988). Species determinations were then made using RAPD-PCR as described in Wilkerson et al. (1995a,b) and corroborated with the primers described here.

DNA isolation - DNA was isolated from individual adult mosquitoes by phenol-chloroform extraction as described in Wilkerson et al. (1993).

PCR amplification - Conserved primers flanking the ITS2 region correspond to conserved sequences in the 5.8S and 28S ribosomal subunits of *An. quadrimaculatus* complex species (Cornel et al. 1996): ITS2F 5'-TGTGAACTGCAGGACACATGAA-3' (forward) and ITS2R 5'-ATGCTTAAATTTAGGGGGTAGTC-3'(reverse). These, in conjunction with single species-specific primers (Table II), designed from cloned sequence (Table I), were used to identify the species. Fragment sizes listed in Table II include the ITS2 (Cornel et al. 1996) and 134 base pairs from the flanking 5.8S and 28S rDNA subunits. Primer beginning position numbers shown in Table II refer to ITS2 sequence (without flanking sequence) as reported by Li & Wilkerson in Genbank (Table I).

Reactions were carried out in a total volume of 50 µl using the PCR buffer (GeneAmp 10× Buffer II) supplied with AmpliTaq DNA polymerase (Applied Biosystems, CA). Final reaction concentrations were: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.0 mM MgCl2; 1 mg/ml BSA; 0.25 mM each of dATP, dCTP, dGTP, and dTTP; universal and species-specific primer concentration as shown in Table II; 0.2-4.0 ng template DNA (1/100 of DNA from entire adult mosquito)/reaction; and 1.25 U of Amplitaq/reaction. A Perkin Elmer Applied Biosystems 9700 thermocycler was used for PCR with the following parameters: initial denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at primer-specific temperatures shown in Table II for 30 s, and elongation at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR product was separated on a 2.5% agarose gel and visualized with ethidium bromide stain. Fragment sizes were estimated by comparison to molecular weight standards provided by lambda DNA digested by *Hind*III, and phiX174 DNA digested with *Hae* III (Sigma, St. Louis, MO).

RESULTS AND DISCUSSION

Based on sequence derived from cloning of ITS2 (Li & Wilkerson, unpublished data, Table I) we designed a series of six ITS2 internal primers that can be used for identification of the four species in the complex (Table II). The six primers are used for single species identifications or, in the case of primers albCD and albABD, for groups of species. Single species-specific primers are run with both ITS2F and ITS2R primers. The combination of the ITS2F and ITS2R primers, along with a species-specific primer, gives a diagnostic band in the presence of target DNA template, but results only in an ~ 490 bp band, representing the entire ITS2 and flanking regions, if the target spe-

TABLE II

Species-specific primers, fragment sizes, annealing temperatures, and primer concentrations for identification of the four species in the *Anopheles albitarsis* complex. All are forward primers except albC, which is reverse

Primer designation	Primer sequence	Primer beginning position on ITS2 [a]	Universal primer	Species identified	Annealing temp. °C	Band size (bp) [b]	ITS2F primer concentration (pmol/50 µl)	ITS2R primer concen-tration (pmol/50 µl)	Species-specific primer concen-tration (pmol/50 µl)
albCD	5'-AACTAGCGAGACCGTCGACC-3'	16	ITSII/R	C, D	56	? 390	5	10	10
albABD	5'-CGATGTGAAGGACACCACCCT-3'	220	ITSII/R	A, B, D	60	? 190	5	10	10
albB	5'-TTCCTCAAAACCAGCTTGAGCA-3'	239	ITSII/R	B	60.5	? 170	5	10	10
albD	5'-GAACCAACTAGCGAGACCGC-3'	11	ITSII/R	D	56	? 390	5	10	10
albC	5'-CCACAGTCCTTCAATGCTTGATT-3'	102	ITSII/F	C	64	194	10	5	10
albA	5'-AGACCGTCGAGAGGCGGT-3'	24	ITSII/R	A	56	? 390	5	10	10

A: *An. albitarsis*; B: *An. albitarsis* B; C: *An. marajoara*; D: *An. deaneorum*. Primer position is in the ITS2, not the total amplicon. Length of amplified bands include 134 bases in the 5.8S and 28S rDNA subunits; *a*: see text for explanation; *b*: slight variation in size is due to indels and microsatellite regions, see text for explanation.

cies DNA is absent. In all reactions, including those that give a species diagnostic band, the ~ 490 bp band can be expected. Note that it is intermittent when in combination with the species-specific primers as a result of competition for PCR components. Our primers were not tested on other *An. (Nyssorhynchus)* but, based on ITS2 sequence generated by us (unpublished data), and that in Genbank, no homologous annealing sites are apparent. The possibility of false positives is precluded however by the initial morphological identification. A reaction producing only the complete ITS2 band should result if a non-target species, either a member of the Albitarsis Complex or another *Nyssorhynchus*, was tested. Lack of any band indicates absence of or degraded DNA. Since sequence differences available for primer design were few we obtained spurious results if we used a multiplex of species-specific primers (data not shown). Because of this only one species-specific primer can be used per reaction. For the same reason, the reaction conditions presented here should be duplicated as closely as possible. The ABI 9700 PCR machine used by us was calibrated shortly before testing the primers, and we therefore believe that the temperatures reported here are accurate.

Four primers allowed identification of single species and two serve to identify combinations of species (Table II). By following Fig. 1 it is possible to identify an unknown specimen that was first morphologically identified as belonging to the Albitarsis Complex. Primer albCD, with ITS2F and ITS2R, will either yield an ~ 390 bp frag-

ment (often also with a ~ 490 bp fragment, see above) indicating the presence of either *An. marajoara* or *An. deaneorum*; or it yields only the ~ 490 bp fragment representing the entire ITS2 amplicon (Fig. 2), indicating the presence of *An. albitarsis* or *An. albitarsis* B. As can be seen in Fig. 1 an additional primer (always with both universal primers) is then chosen depending on the result: albC for *An. marajoara* (Fig. 3); or albB for *An. albitarsis* B (Fig. 4). The apparent negative results for *An. deaneorum* and *An. albitarsis* are actually positive if judged by the first reaction, i.e. it has to be one or the other. If desired, a further confirmation reaction can be carried out for these two species; albD for *An. deaneorum* (Fig. 5), or albA for *An. albitarsis* (Fig. 6). In addition, primer albABD can be used to identify three species (Fig. 7), *An. albitarsis*, *An. albitarsis* B, and *An. deaneorum*. The two-step process may not be necessary if one can make a presumptive identification based on locality, e.g. only *An. marajoara* has been found north of the Amazon River, or morphology, head hair 3-C is multibranched only in *An. deaneorum*.

Species-specific primers were tested with complete correlation on all specimens in our collection that had previously been identified using RAPDs, i.e. 99 *An. albitarsis*, 56 *An. albitarsis* B, 407 *An. marajoara*, and 41 *An. deaneorum*. *An. marajoara* from north of the Amazon River is of particular interest since Lehr et al. (2005) hypothesize the existence of an additional species ("E") in the Albitarsis Complex from Venezuela and the state of
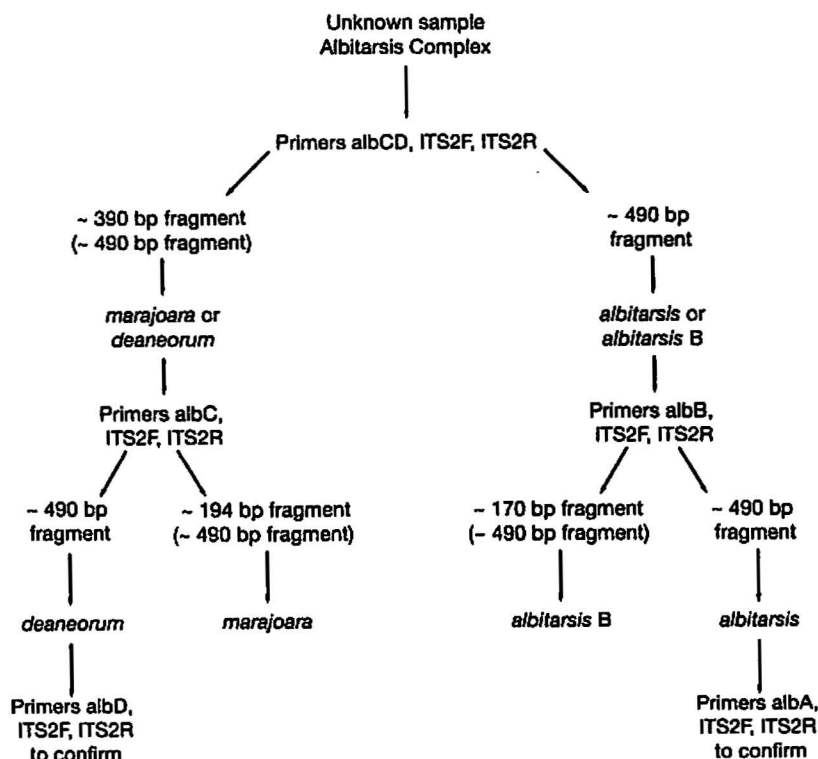

Fig. 1: scheme for identification of *Anopheles albitarsis* complex unknowns. Specimens first identified morphologically as members of the complex then require two reactions to reach a positive identification. ITS2F and ITS2R primers are included as positive controls. These produce a ~490 bp fragment which does not always appear because of competition for template in species-specific reactions and it is therefore indicated below by parentheses.

Roraima, Brazil. This finding was not corroborated by us with ITS2 sequence or through identifications using the technique described here.

Recently, Merritt et al. (2005) also developed a method for identification of *An. marajoara* based on the presence of the fourth intron of the *white* gene, which is absent in the other members of the complex. We sequenced this fragment of *white* gene for a subsample of the specimens used here and agree with their results (data not shown).

Our unpublished findings (Li & Wilkerson, see Table I Genbank accession numbers) show intragenomic variability in the ITS2 of these four species. Our primers were designed so that this variability would have no affect on PCR results. Note that because of indels and variable microsatellite regions PCR product size can vary, e.g. Fig. 7, *An. deaneorum*, "D" (see Genbank accessions in Table I).

It is known that *An. marajoara* is a significant malaria vector, at least in Northeastern Brazil, but the status of the other species is poorly understood. The ITS2-based PCR protocol presented here provides a means for vector ecologists, malaria epidemiologists and control personnel to positively identify members of the Albitarsis Complex.
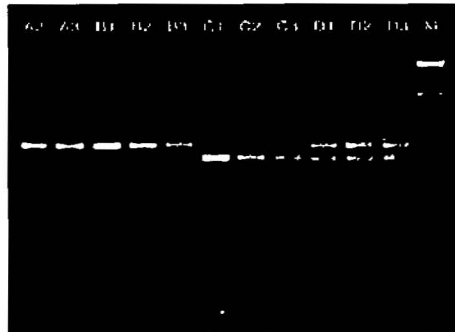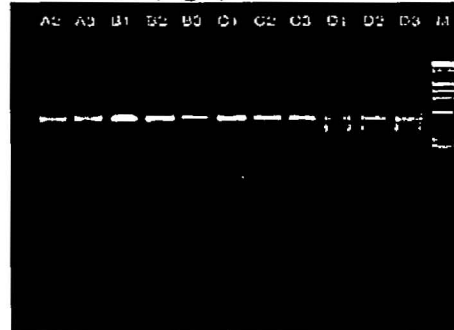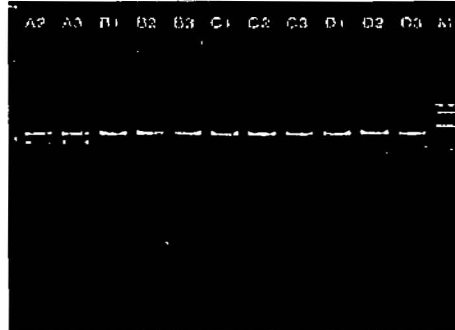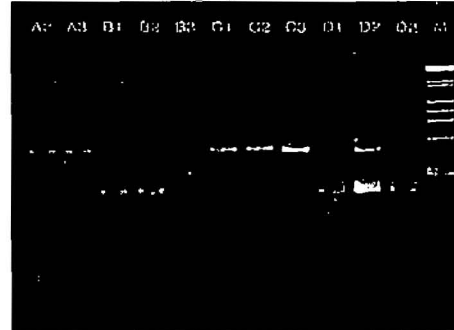

Primer albCD (Fig.2)
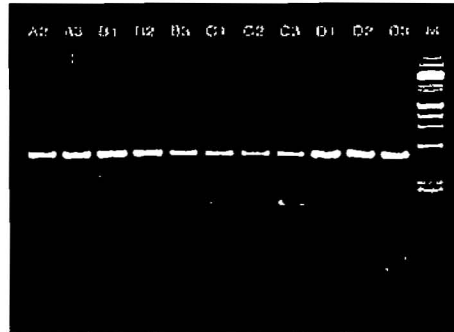
Primer:albC (Fig.3)
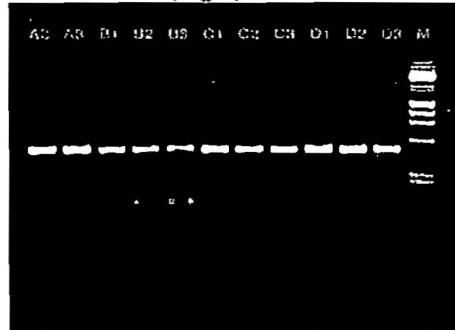
Primer:albB (Fig.4)

Primer: albD (Fig.5)

Primer: albA (Fig.6)

Primer: albABD (Fig.7)

Fig. 2: *Anopheles marajoara* and *An. deaneorum*-specific ITS2 amplification with primers albCD, ITS2F, and ITS2R. A: *An. albitarsis*, B: *An. albitarsis* B, C: *An. marajoara*, D: *An. deaneorum*. Larger fragment (~ 490 bp) is the entire ITS2 amplicon, smaller fragment (~ 390 bp) is diagnostic for the two species. Fig. 3: *An. marajoara*-specific ITS2 amplification with primers albC, ITS2F, and ITS2R. Larger fragment (~ 490 bp) is the entire ITS2 amplicon, smaller fragment (194 bp) is diagnostic for *An. marajoara*. Fig. 4: *An. albitarsis* B-specific ITS2 amplification with primers albB, ITS2F, and ITS2R. Larger fragment (~ 490 bp) is the entire ITS2 amplicon, smaller fragment (170 bp) is diagnostic for *An. albitarsis* B. Fig. 5: *An. deaneorum*-specific ITS2 amplification with primers albD, ITS2F, and ITS2R. Larger fragment (~ 490 bp) is the entire ITS2 amplicon, smaller fragment (390 bp) is diagnostic for *An. deaneorum*. Fig. 6: *An. albitarsis*-specific ITS2 amplification with primers albA, ITS2F, and ITS2R. Larger fragment (~ 490 bp) is the entire ITS2 amplicon, smaller fragment (390 bp) is diagnostic for *An. albitarsis*. Fig. 7: *An. albitarsis*, *An. albitarsis* B, and *An. deaneorum*-specific ITS2 amplification with primers albABD, ITS2F, and ITS2R. Larger fragment (~ 490 bp) is the entire ITS2 amplicon, smaller fragment (~ 190 bp) is diagnostic for the three species. Note apparent double band for *An. deaneorum* which is probably the result of the variable microsatellite regions.

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