First evidence of *Anaplasma platys* in *Rhipicephalus sanguineus* (Acari: Ixodida) collected from dogs in Africa

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ABSTRACT

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A total of 27 ticks, comprising *Rhipicephalus sanguineus* (Latreille) (n = 21), *Haemaphysalis leachi* (Andouin) (n = 4) and *Haemaphysalis paraleachi* (Camicas, Hoogstraal & El Kammah) (n = 2) were recovered from two clinically healthy female dogs in the Democratic Republic of the Congo. DNA of *Anaplasma platys* was detected in a female *R. sanguineus*, using primers derived from the 16S rRNA gene, which amplify members of the family Anaplasmataceae . *Anaplasma platys* DNA was also detected in the blood of one of the dogs. Phylogenetic analysis based on partial sequences of the 16S rRNA, the *glt*A and the g*ro*EL genes ranged the detected agent within the *Anaplasma* clade. This is the first reported detection of *A. platys* in ticks in Africa. This finding raises the question of the possible involvement of *R. sanguineus* in *A. platys* infection of dogs.

Keywords: *Anaplasma platys*, dogs, *glt*A, *gro*EL, 16S rRNA

INTRODUCTION

Rhipicephalus sanguineus (Latreille), the brown dog tick, also known as the kennel tick, was believed to be native to Africa. However, this tick is now found world-wide between the latitudes 50 °N and 30 °S due to commerce in dogs, which are the preferred hosts for all its life stages. *Rhipicephalus sanguineus* is the biological vector of human pathogenic bacteria, such as the *Rickettsia conorii* strains recognized as the aetiologic agents of Mediterranean spotted fever (MSF), Israeli typhus and Indian tick typhus (Raoult & Roux 1997). *Rhipicephalus sanguineus* has been confirmed as the vector of *Babesia canis* (Christophers 1907), *Hepatozoon canis* (Nordgren & Craig 1984) and *Ehrlichia canis* (Donatien & Lestoquard 1937). The role of this tick in the transmission of *Anaplasma platys*, the causative agent of canine infectious cyclic thrombocytopenia (CICT), is discussed by Simpson, Gaunt, Hair & Kocan (1991) and Inokuma, Raoult & Brouqui (2000).

Methods based on analysis of DNA sequences are very efficient tools for the detection and characterization of pathogenic agents in vectors and their

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hosts (Higgins & Azad 1995; Parola, Beati, Cambon, Brouqui & Raoult 1998). Their advantages rely mainly on the fact that they are relatively fast and do not necessitate a previous isolation of the agent. Molecular analysis, particularly the 16S rRNA gene sequence comparison, is now widely used for the identification and classification of microorganisms (Woese 1987; Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa & Rurangirwa 2001). Ehrlichial species were grouped into three monophyletic groups based on their 16S gene sequences: the *Ehrlichia canis* group, the *Ehrlichia phagocytophila* group, and the *Ehrlichia sennetsu* group (Walker & Dumler 1996). This classification has been recently reorganized based on the 16S rRNA, the *gro*ESL, and the surface protein gene sequences into four distinct clades: *Anaplasma* (including the *Ehrlichia phagocytophila* group, *Ehrlichia platys* and *Ehrlichia bovis*), *Ehrlichia* (including *Cowdria ruminantum*), *Wolbachia,* and *Neorickettsia* (including *Ehrlichia sennetsu* and *Ehrlichia risticii*) (Dumler *et al.* 2001). Due to the similarity between the 16S rRNA gene sequences of members of the family Anaplasmataceae, new genes are required for resolution of ehrlichial species. Protein encoding genes such as the ankyrin (*ank*) gene (Massung, Owens, Ross, Reed, Petrovec, Bjoersdorff, Coughlin & Murphy 2000), the heat shock protein (*gro*ES and g*ro*EL) gene (Sumner, Storch, Buller, Liddell, Stockham, Rikihisa, Messenger & Paddock 2000), and the citrate synthase (*glt*A) genes (Inokuma, Brouqui, Drancourt & Raoult 2001) have been used in phylogenetic analysis of different ehrlichial species. The present study reports the detection of *A. platys* in *R. sanguineus* removed from dogs in the Democratic Republic of the Congo (D.R.C.) using three different genes, the 16S rRNA, the *glt*A gene and the g*ro*ESL operon.

MATERIALS AND METHODS

Dogs and ticks

Two female mixed-breed Midget Spaniels of 2 and 4 years of age were inspected for tick infestation during regular veterinary control. The dogs were living together in the residential square of Kinshasa, the capital of the D.R.C. Ticks were removed mainly from the ears. Upon arrival in the laboratory, they were kept at room temperature for one week to allow digestion of the blood meal. All the ticks were carefully identified by means of morphological identification keys, and then kept at –70 °C until investigated.

Tick and dog blood DNA extraction

Before the extraction procedure, individual ticks were disinfected by immersion into 70 % ethanol for 5 min, rinsed in sterile water and dried with sterile filter paper. They were then cut into small pieces which were subjected to DNA extraction using the QIAamp tissue kit procedure (QIAGEN, GmbH, Hilden Germany) in accordance with the manufacturer's instructions. The DNA of lice from a laboratory colony fed on an uninfected rabbit was similarly extracted together with the tick DNA to serve as an extraction blank control. DNA was eluted in a final volume of 200 ml and kept at –20 °C until investigated. DNA was extracted from blood samples of the dogs by the QIAamp blood kit procedure (QIAGEN) following the manufacturer's instructions, and eluted in a final volume of 200 µℓ. The samples were kept at –20 °C until tested by polymerase chain reaction (PCR).

PCR screening of tick and blood DNA for the presence of bacterial pathogens

The DNA from the individual dogs was screened by PCR for the presence of *Rickettsia*, *Bartonella*, *Borrelia* and *Francisella* parasites using specific primers for each bacterium as described in Parola & Raoult (2001). For members of the family Anaplasmataceae, amplification was performed using primers 16SEHR.D and 16SEHR.R (Table 1) as has previously been described (Parola *et al.* 1998). Furthermore, primers specific to *A. platys* were used to amplify the 16S rRNA gene from the ticks by the technique described by Inokuma *et al.* (2000).

All amplifications were carried out in a Peltier model PTC-200 thermal cycler (MJ Research, Inc, Watertown, MA, USA). For PCR reaction, 7.5 µℓ of DNA template (undiluted and 10-fold diluted) was added to a 17.5 µℓ of master mixture consisting of 10 pmol of each primer, 0.5 U of ELONGase mix enzyme (GibcoBRL), 20 mM concentration of each deoxynucleoside phosphate and 1.8 mM of $MgCl_2$. Two negative controls were included in the reaction: DNA from uninfected lice and master mix using sterile water instead of DNA template. As positive control DNA extracted from *Neorickettsia sennetsu* (*E. sennetsu*, Dumler *et al.* 2001) obtained from Y. Rikihisa, Ohio State University, Colombus, OH, USA was used. The amplification programme consisted of a first denaturation step at 94 °C for 4 min followed by 39 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C for 90 s and a final 7 min extension at 68 °C. The amplification product was visualized under UV illu-

mination after electrophoresis migration on a 1% TBE agarose gel stained with ethidium bromide.

Amplification of the *gro*EL and the *glt*A genes

Primers were designed based on the alignment of *gro*ESL sequences of *A. platys* from databank (GenBank accession number AY008300) and *A. platys* sequences provided by H. Inokuma (unpublished data 2002). The genes were amplified in two steps using the primers Plhsp1f/Sqhsp3r and Sqhsp2f/Plhsp1r, respectively. Primer sequences and positions are listed in Table 1. The amplification conditions were the same as the foregoing except that annealing was performed at 54 °C for a period of 45 s, and the final volume of the reaction mixture was 50 µℓ.

The partial *glt*A gene was amplified using forward primers PlgltA1f and reverse primers PlgltA1r based on the alignment of *A. platys glt*A gene sequences provided by H. Inokuma (unpublished data 2002). Primer sequences and respective positions are listed in Table 1. The annealing temperature was 55 °C and the annealing time was 45 seconds. The amplification was performed in a 50 µℓ reaction mixture.

Sequencing and phylogenetic analysis

Before sequencing reactions, PCR products were purified by the QIAquick PCR purification kits (Qiagen, GmbH, Hilden Germany) according to the manufacturer's instructions. Sequencing reactions were carried out by the fluorescence-labelled dideoxynucleotide technology using the dye terminator cycle sequencing kit (Perkin-Elmer). Sequencing products were separated and collected by the means of an Applied Biosystem automatic sequencer model 3100 (Perkin-Elmer).

The 16S rRNA gene was sequenced with the same primers used for amplification. Primers used for sequencing the *gro*ESL and the *glt*A genes are listed in Table 1.

Sequences were edited using the Autoassembler (version 1.4; Perkin Elmer) package.

Multiple alignment with the 16S rRNA, the *gro*ESL and the *glt*A gene sequences of other members of the family Anaplasmataceae from Genbank was performed with the Clustal W programme (Thompson, Higgins & Gibson 1994, at http://npsa.pbil.ibcp.fr/). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* 2.1 version (Kumar, Tamura, Jakobsen & Nei 2001). Phylogenetic trees were inferred from multiple sequence alignment after removing all gaps, using the neighbor-joining and maximum parsimony methods (MEGA 2.1 version). Distance matrix was calculated using the Kimura-2 parameters. Five hundred cycles of bootstrap replicates were used to estimate the node reliability of the phylogenetic trees (Felsenstein 1985).

GenBank accession numbers

The sequences obtained in this study have been deposited under Genbank accession numbers AF478131, AF478129 and AF478130 for the 16S rRNA, the *gro*ESL and the *glt*A genes, respectively. Accession numbers to *glt*A, *gro*EL sequences of the organisms used in this study are listed in Table 2.

RESULTS

In total, 27 adult ticks were removed from the two dogs. The ticks were identified as *Rhipicephalus sanguineus* (Latreille, 1806) (eight males and 13 females), *Haemaphysalis leachi* (Andouin, 1826) (four females) and *Haemaphysalis paraleachi* (Camicas, Hoogstraal & El Kammah 1983) (two females).

A single product of approximately 345 bp was amplified and sequenced with primers derived from the 16S rRNA gene of the family Anaplasmataceae in a single partially fed female *R. sanguineus*. A PCR product of the same size was yielded from positive control. This fragment showed 99 % identity to that of *A. platys* from Genbank (AF286699). Furthermore, a product of 678 bp was amplified and sequenced with primers specific to *A. platys* in the ticks. A BLAST search for this fragment revealed a 99 % identity with *A. platys* strains from GenBank (AF286699).

Amplification of the *gro*ESL operon with primers Plhsp1f/Sqhsp3r and Plhsp1r/Sqhsp2f yielded single fragments of 840 bp and 1277 bp, respectively. Sequencing and assemblage of these products yielded a 1600 bp long fragment excluding primer regions. Sequence comparison revealed that a G in *A. platys*-RDC was substituted by an A in *A. platys* at position 117 (of AY044161) leading to a substitution of a lysine by a glutamic acid at position 9 from the putative start codon of the *gro*EL gene of *A. platys* AY044161 in the amino acid sequences.

Using primers PlgltA1f and PlgltA1r, a fragment of 1302 bp of the *glt*A gene was amplified and se-

TABLE 1 Primers used for amplification and sequencing the groESL and the gltA genes of *Anaplasma platys*-RDC, and their corresponding orientation and positions

Primers in 5'-3' orientation		Used for[a]	Orientation[b]	Position[c]
*gro*ESL gene	Plhsp1f TAG TGA TGA AGG AGA GTG AC	A	F	1
	Plhsp1r GTC AAC CAC TAC GGC ATT C	A	R	1670
	Sqhsp1f GTG ATG AAG GAG AGT GAC AT	A, S	F	3
	Sqhsp1r CAA CCA CTA CGG CAT TCA AG	S	R	1669
	Sqhsp2f GAA GTA GCT AGG GCT AAG G	A, S	F	393
	Sqhsp4r CCT TAG CCC TAG CTA CTT C	S	R	411
	Sqhsp3f AGG CCT CTG CTC ATC ATC G	S	F	822
	Sqhsp3r CGA TGA TGA GCA GAG GCC T	A, S	R	840
	Sqhsp4f TAG CGA AGC TAT GTG GCG G	S	F	1201
	Sqhsp2r CCG CCA CAT AGC TTC GCT A	S	R	1219
				Position[d]
gltA gene	PlgltA1f TTG GAT ATT GGG TAA CGC TG	A	F	224
	PlgltA1r CAC TTC TTC CGG GTA TAC CAC	A	R	1532
	SqgltA1f GGA TAT TGG GTA ACG CTG TA	S	F	226
	SqgltA1r CTT CTT CCG GGT ATA CCA C	S	R	1530
	SqgltA2f ATCCTT CAG GAG AGA TGC TC	S	F	613
	SqgltA3r GAG CAT CTC TCC TGA AGG AT	S	R	633
	SqgltA3f CAC ATG GAG GTG CCA ACG A	S	F	1117
	SqgltA2r TCG TTG GCA CCT CCA TGT G	S	R	1035
				Reference
16S RNA gene	16SEHR.D GGTACCYACAGAAGAAGTCC	A	F	Parola *et al.* 2001
	16SEHR.R TAG CAC TCA TCG TTT ACA GC	A	R	

[a] A amplification, S sequencing
[b] F forward primer, R reverse primer
[c] Position of *Anaplasma platys* AY008300
[d] Position of *Anaplasma centrale gltA* gene AF304141

TABLE 2 Genbank accession numbers of organisms used in the study

Organisms used	GenBank accession no.	
	gltA	*groEl*
Anaplasma platys	Not available	AY008300
Anaplasma phagocytophila HGE agent	AF304136	AF172163
A. phagocytohila (Ehrlichia equi)	AF304137	AF173988
A. phagocytophila	AF304138	U96735
A. marginale strain Florida	AF304140	AF165812
A. marginale strain Idaho	AF304139	Not available
A. centrale	AF304141	Not available
Ehrlichia canis	AF304143	U96731
Ehrlichia chaffeensis	AF304142	L10917
Ehrlichia muris	AF304144	AF210459
Ehrlichia sp. from *Ixodes ovatus*	AF304145	AB032712
Ehrlichia (Cowdria) ruminantium	AF304146	U13638
Neorickettsia (ehrlichia) risticii	AF304147	Af206299
Neorickettsia (Ehrlichia) senetsu	AF304148	AF060197
Neorickettsia helminthoeca	AF304149	Not available
Rickettsia prowazekii	L38987	Y15783
Bartonella henselae	J01619	U78514

>*Anaplasma platys* (AY044161)

V	F	G	S	V	S	L	K	F	E	R	L	E	M	A	N	T	V	35
GTT	TTT	GGT	TCT	GTT	AGT	TTA	AAG	TTT	GAG	AGG	TTA	GAA	ATG	GCA	AAT	ACG	GTT	107

>*Anaplasma platys*-RDC (AF478129)

V	F	G	S	V	S	L	K	F	E	R	L	E	M	A	N	T	V	35
GTT	TTT	GGT	TCT	GTT	AGT	TTA	AAG	TTT	GAG	AGG	TTA	GAA	ATG	GCA	AAT	ACG	GTT	107

>*Anaplasma platys* AY044161

V	T	G	**K**	V	L	D	K	S	I	R	E	V	V	R	T	L	E	53
GTC	ACG	GGT	**AAG**	GTG	TTA	GAT	AAG	TCG	ATT	AGG	GAA	GTG	GTA	CGA	ACC	CTG	GAA	161

>*Anaplasma platys*-RDC (AF478129)

V	T	G	**E**	V	L	D	K	S	I	R	E	V	V	R	T	L	E	53
GTC	ACG	GGT	**GAG**	GTG	TTA	GAT	AAG	TCG	ATT	AGG	GAA	GTG	GTA	CGA	ACC	CTG	GAA	161

>*Anaplasma platys* (AY044161)

D	A	V	G	C	T	A	G	P	K	G	L	T	V	A	I	S	K	71
GAC	GCT	GTT	GGG	TGT	ACT	GCC	GGA	CCG	AAA	GGG	CTT	ACT	GTT	GCT	ATT	AGC	AAG	215

>*Anaplasma platys*-RDC (AF478129)

D	A	V	G	C	T	A	G	P	K	G	L	T	V	A	I	S	K	71
GAC	GCT	GTT	GGG	TGT	ACT	GCC	GGA	CCG	AAA	GGG	CTT	ACT	GTT	GCT	ATT	AGC	AAG	215

FIG. 1 Substitution of nucleotides in the sequences of *Anaplasma platys* strains

quenced. This fragment revealed a 99 % identity to *A. platys,* GenBank accession number AB058782.

Phylogenetic trees inferred from the 16S rRNA, the *gltA* and the *gro*ESL by both neighbour-joining and the maximum-parsimony methods showed similar topology. *Anaplasma platys*-RDC falls within the *Anaplasma* group based on the three genes (Fig. 2).

Sequencing of the product amplified from the blood of one dog with primers derived from the 16S rRNA gene of the Anaplasmataceae yielded a 345 bp fragment which showed 99 % identity with *A. platys* from GenBank (AF286699). No amplification was yielded from the negative controls. DNA of *Rickettsia*, *Bartonella, Borrelia* and *Francisella* was not detected in the ticks.

DISCUSSION

The presence of *A. platys* in *R. sanguineus* ticks removed from dogs in the D.R.C., and the detection of the same agent in a blood sample of one of the infested dogs are reported in this study. Primers specific to *A. platys* were used to partially amplify the 16S rRNA gene. The strain was further characterized by amplification and sequencing of two other genes: the g*ro*ESL operon and the *glt*A genes. Sequence analysis of these genes place the detected agent within the *Anaplasma* clade (Dumler *et al.* 2001). The *gltA* gene showed longer tree branches than the *gro*ESL operon, as previously observed (Inokuma *et al.* 2001).

Anaplasma platys is a parasite of canine platelets, and dogs naturally infected with this agent do not necessarily show clinical signs. However, severe canine ehrlichiosis has been reported to occur in mixed infection of *E. canis* and *A. platys* in China (Hua, Yuhai, Shide, Yang, Bohai, & Xiangrui 2000). The report on natural and experimental infection of dogs with this agent suggests the presence of more virulent strains in Greece (Kontos 1994). *Anaplasma platys* has been reported in France (Beaufils 1985), in Japan (Kawahara, Ito, Suto, Shibata, Rikihisa, Hata & Hirai 1999), in Taiwan (Chang, Chang, Lin, Pan & Lee 1996), in the United States (Matthew, Ewing, Murphy, Kocan, Corstvet & Fox 1997; Kordick, Breitschwerdt, Hegarty, Southwick, Colitz, Hancock, Bradley, Rumbough, Mcpherson & Maccormack 1999), and more recently in Australia (Brown, Martin, Roberts & Aitken 2001). These findings overlap with the geographical distribution of *R. sanguineus,* which is associated with dogs. *Anaplasma platys* was suspected to be transmitted to dogs by *R. sanguineus* (Woody & Hoskins 1991). However, Simpson, Gaunt, Hair & Kocan (1991) failed to demonstrate by light and electron microscopy the ability of *R. sanguineus* to transmit *A. platys* during

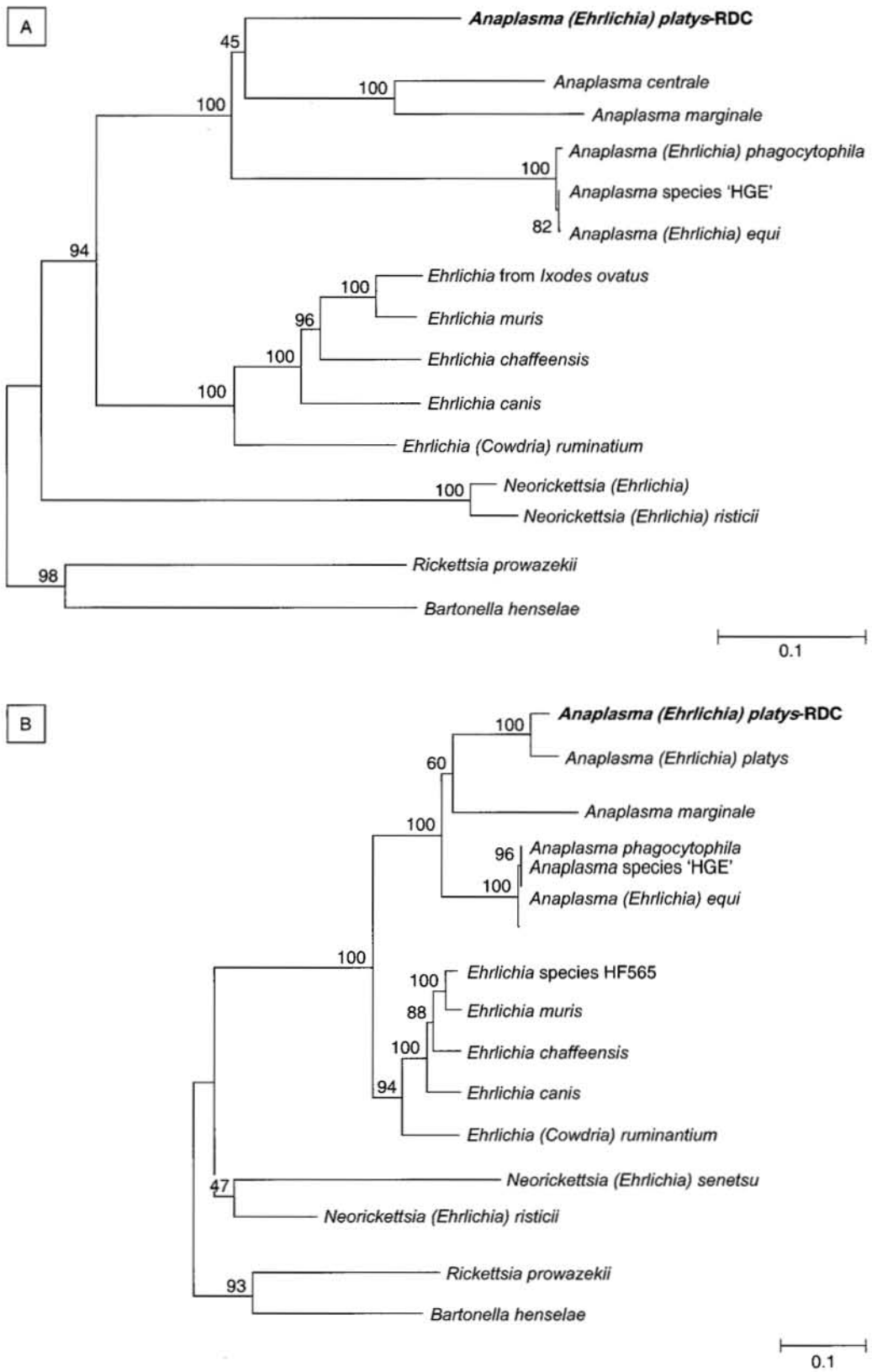

FIG. 2 Phylogenetic trees inferred from the citrate synthase (*glt*A) [A] and the heat shock protein (*gro*ESL) [B] genes of *Anaplasma platys* (= *Ehrlichia platys*, Dumler *et al.* 2001) with the neighbor-joining method. Bootstrap values calculated using p-distance parameters are represented on the top of branches. To assess the tree node reliability 500 bootstrap replicates were used. Scale bars represent 10 % of nucleotide divergence

experimental transmission. On the other hand, in a study conducted by Kontos (1994), *R. sanguineus* was recorded on all dogs naturally infected with *A. platys*. The first association between *A. platys* and ticks was reported from the Prefecture of Okinawa, Japan (Inokuma *et al.* 2000). In the present study, we demonstrated the presence of *A. platys* DNA in the ticks and in the blood of one of the dogs. Because the ticks from the two dogs were combined in a single sample it was not possible to determine from which dog the positive tick was removed. Nevertheless, the detection of *A. platys* in a single *R. sanguineus* among all the ticks collected from the dogs suggests the involvement of ticks in the dissemination of *A. platys* infection. Moreover, *A. platys* was not detected in other tick species removed from the dogs. The failure to detect the agent in ticks during experimental transmission (Simpson *et al.* 1991) could be caused by limited sensitivity of the method used for detection (i.e. microscopy).

In conclusion, this work reports the first molecular detection of *A. platys* in Africa. The identity of the agent was confirmed by use of three different genes. However, since ticks are known to retain host/pathogen DNA throughout blood meal processing, further investigation, including culturing the organism and xenodiagnosis, are required to demonstrate the exact role played by *R. sanguineus* in the dissemination of *A. platys*.

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