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Crossbreeding between different geographical populations of the brown dog tick, *Rhipicephalus sanguineus* (Acari: Ixodidae)

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Abstract Brown dog ticks are distributed world-wide, and their systematics and phylogeny are the subject of an ongoing debate. The present study evaluates the reproductive compatibility between *Rhipicephalus sanguineus* ticks from North America, Israel, and Africa. Female ticks of the parent generation were mated with males from the same and alternate colonies. Every pure and hybrid cohort was maintained separately into the F2 generation with F1 females being allowed to mate only with males from the same cohort. The following survival parameters were measured and recorded for every developmental stage: feeding duration and success; engorgement weight, fertility, and fecundity of females; molting and hatching success. Ticks from North American and Mediterranean populations hybridized successfully. The survival parameters of all their hybrid lines were similar to those in pure lines throughout the F1 generation, and F1 adults were fully fertile. Parent adult ticks from the African population hybridized with either North American or Mediterranean ticks and produced viable progenies whose survival parameters were also similar to those in pure lines throughout the F1 generation. However, F1 adults in the four hybrid lines that included African ancestry were infertile. No parthenogenesis was observed in any pure or hybrid lines as proportion of males in F1 generation ranged from 40 to 60 %. Phylogenetic analysis of the 12S rDNA gene sequences placed African ticks into a separate clade from those of the North American or Mediterranean origins. Our results demonstrate that *Rh. sanguineus* ticks from North America and Israel represent the same species, whereas the African population used in this study is significantly distant and probably represents a different taxon.

The findings and conclusions described in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention and the Department of Health and Human Services.

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Introduction

The brown dog tick, *Rhipicephalus sanguineus* (Latreille) is one of the most widely distributed ticks in the world, found globally between 50°N and 30°S latitudes (Walker et al. 2000). This cosmopolitan tick infests domestic and wild mammals, and incidentally man. Its immature stages may infest rodents and other small mammals (Hoogstraal 1985), but canines and especially domestic dogs are the preferred hosts. In addition to being prolific and a menacing ectoparasite of domestic dogs, *Rh. sanguineus* is a vector of several veterinary and human pathogens including *Babesia vogeli, Ehrlichia canis, Hepatozoon canis, Rickettsia conorii, Rickettsia massiliae* and *Rickettsia rickettsii* (reviewed by Dantas-Torres 2008).

Rhipicephalus sanguineus is, however, the most controversial tick species in the genus *Rhipicephalus* (Farid 1996). Following its original description, a number of morphologically and biologically similar species and subspecies have been described around the world. *Rh. sanguineus* remains the type species of the so-called "*Rh. sanguineus* complex" or the *Rh. sanguineus* group, which includes at least five closely related Old World species (Walker et al. 2000). Their differentiation and delineation remain subjects of an ongoing debate (Feldman-Muhsam 1952; Feldman-Muhsam 1968; Pegram et al. 1987a, b, 1989; Farid 1996; Zahler et al. 1997; Baker 1998; Oliveira et al. 2005; Szabo et al. 2005). Morphological identification of brown dog ticks is complicated by intraspecific variability of morphological traits and close similarity to those in related species (Farid 1996; Oliveira et al. 2005) with the result that they are often misidentified (Pegram et al. 1987b; Ioffe-Uspenskiy et al. 1997). Some 18 of the separately described *Rhipicephalus* species have been eventually synonymized with *Rh. sanguineus* (Pegram et al. 1987a, b; Walker et al. 2000). However, recent studies suggested that the resulting combined taxon may actually represent more than one species based on phylogenetic analyses that segregated European and African *Rh. sanguineus* into distinct clades (Szabo et al. 2005; Burlini et al. 2010, Moraes-Filho et al. 2011). These authors also found that *Rh. sanguineus* from Brazil and Asia are genetically closer to tick specimens morphologically identified as *Rhipicephalus turanicus*, than to *Rh. sanguineus* from either North America or Mediterranean.

Yet, the accurate discrimination of species within the *Rh. sanguineus* group is crucial for understanding of the epidemiology and etiology, as well as for control, of the pathogens they transmit. Closely related tick species, and possibly even different populations within a tick species, can differ in their ability to transmit pathogens (Baker 1998; Anderson 2002; Matsumoto et al. 2005) and in the frequency of contacts with humans and domestic animals. This affords medical and veterinary importance to the accurate identification and delineation of tick species. In cases where separation of sibling tick species based solely on morphological traits is difficult, hybridization experiments and molecular markers may be necessary to differentiate between them (Hafez et al. 1981; Zahler et al. 1995; Zahler and Gothe 1997; Baker 1998; Labruna et al. 2009).

Herein, we assess the conspecificity of brown dog ticks (*Rh. sanguineus*) from Africa, Mediterranean, and North America by cross-breeding ticks from representative populations and comparing results of ticks' hybridization to their molecular phylogeny.

Materials and methods

Tick colonies

Three separate laboratory colonies of North American (US), Mediterranean (IS), and African (AF) *Rh. sanguineus* were derived from adult ticks collected off naturally infested dogs in Oklahoma, USA (2001), Israel (2005), and Reunion Island (2009), respectively. The founder ticks were morphologically identified as *Rh. sanguineus* using standard identification keys (Farid 1996; Walker et al. 2000) and molecular methods (Beati and Keirans 2001). Prior to the cross-breeding experiments, ticks were adapted to feeding on New Zealand white rabbits (*Oryctolagus cuniculus*) in all stages as previously described (Troughton and Levin 2007). Ticks from each colony were fed separately from the other colonies to preclude any possibility of mixing prior to the study. Between feedings, ticks were held in incubators under identical conditions of 90 % humidity, 22 °C, and a 16/8 light/dark photoperiod.

Cross-breeding

For the cross-breeding study, three groups of 20 unfed virgin females from each colony were placed on a separate naïve rabbit with twenty males from each of the three colonies (Parental generation—P). This created three homologous and six heterologous crosses (Table 1). Replete females were collected as soon as they completed engorgement and detached from rabbits and the duration of engorgement of each tick was recorded. Females were individually weighed and placed in separate numbered tubes for oviposition. Egg-mass weights corresponding to each female were recorded shortly after completion of oviposition. Subsequently, the blood meal conversion index (BMCI—a measure of tick's capacity to convert its blood meal to eggs) was calculated by dividing the weight of each egg mass by the engorgement weight of the corresponding female. Larval eclosion in each individual egg batch was monitored three times per week for 12 weeks, and the hatch-ability—percentage of hatching eggs—in each batch was determined as described by Drummond et al. (1973).

The ensuing F1 larvae and nymphs from all nine crosses were fed in parallel on separate naïve rabbits. Feeding durations and molting success were recorded for F1 larval and nymphal stages. Twenty of the resulting F1 females from each cross were also placed on a separate naïve rabbit paired with 20 males from the same cohort to assess their fertility and

Table 1 Crossbreeding between three colonies of *Rhipicephalus sanguineus*

	Origin of females		
	Oklahoma, USA (US)	Israel (IS)	Reunion (AF)
Origin of males			
Oklahoma, USA (US)	*US–US*	IS–US	AF–US
Israel (IS)	US–IS	*IS–IS*	AF–IS
Reunion (AF)	US–AF	IS–AF	*AF–AF*

Italicized purebred lines

fecundity. The same parameters of feeding duration, engorgement weight, BMCI, duration of incubation and hatchability of F2 eggs were recorded as for the parental generation.

Phylogenetic analysis

In addition to the crossbreeding experiments, sequences of the mitochondrial 12S rDNA gene for the three purebred *Rh. sanguineus* colonies (US, IS, and AF) were compared. Sequence reactions were performed using an ABI PRISM 3.0 BigDye Terminator Cycle Sequencing kit (Applied BioSystems, Foster City, CA) as recommended by the manufacturer. The amplicons were purified using the Wizard SV gel and PCR clean-up system (Promega, Madison, WI) and sequenced on an Applied BioSystems 3130*xl* genetic analyzer. Sequences were assembled using the DNASTAR Lasergene 8 software package. The National Center for Biotechnology Information (NCBI) Basic Local Alignment Sequence Tool (BLAST) search engine was used to identify homologous sequences. Sequences of the 12S rDNA gene from 18 ticks morphologically identified as *Rh. sanguineus*, *Rh. turanicus,* or *Rh. sanguineus*—like were found in the NCBI GenBank and included in the sequence analysis (Table 2). The closely related *Rhipicephalus camicasi, Rhipicephalus compositus, Rhipicephalus muhsamae, Rhipicephalus pravus, Rhipicephalus simus, Rhipicephalus sulcatus,* and *Rhipicephalus zumpti* were included as outliers. Sequence alignments were performed using ClustalW.

A phylogenetic tree (Fig. 1) was constructed using the neighbor-joining method (Saitou and Nei 1987) based on alignment of 12S rDNA partial sequences (287 bp). The bootstrap consensus was inferred from 1,000 replicates to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and expressed as the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion Option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Statistical analysis

Differences between purebred and hybrid tick lines in feeding, survival, and reproductive parameters were analyzed using χ^2, and analysis of variance (ANOVA). Percentage indices were arcsine-transformed for statistical analyses. Variables were considered significantly different if $P < 0.01$.

Results

In the three purebred colonies, reproductive parameters did not differ significantly between the parental and F1 generations. Therefore, data for all individual pure-bred females in those two generations were combined to increase the statistical power of the analysis (Tables 3 and 5). The duration of larval and nymphal feeding was similar in F1 progenies of all purebred and hybrid crosses with larvae completing engorgement within 3–5 days and nymphs—in 4–7 days. Therefore, these parameters were not used for further comparison of crosses.

Table 2 The origins and Gene Bank accession numbers of mitochondrial 12S rDNA sequences used for phylogenetic analysis

#	Species	Geographic origin	GB accessing number
1	*Rh. sanguineus* IS	Israel	HM138901.1
2	*Rh. sanguineus* OK	Oklahoma, USA	HM138900.1
3	*Rh. sanguineus* AF	Reunion	JQ425164
4	*Rh. sanguineus* Los Angeles	California, USA	HM014443.1
5	*Rh. sanguineus* T6 AZ20	Arizona, USA	HM138903.1
6	*Rh. sanguineus* Portugal	Portugal	FJ536553.1
7	*Rh. sanguineus* Uruguay	Uruguay	AY559843.1
8	*Rh. sanguineus* Argentina	Argentina	AY559841.1
9	*Rh. sanguineus* group KM-2005	Corsica, France	AY947467.1
10	*Rh. sanguineus* Thailand	Thailand	AY987377.1
11	*Rh. sanguineus* Brazil	Brazil	AY559842.1
12	*Rh. sanguineus* strain 32	NA	DQ003004.1
13	*Rh. turanicus* Zambia	Livingstone, Zambia	DQ849232.1
14	*Rh. turanicus* Zimbabwe	Zimbabwe	AF150017.1
15	*Rh. turanicus* isolate T090960	Sicily, Italy	HM014442.1
16	*Rh. turanicus* strain Israel-63	Israel	AF150013.1
17	*Rh. turanicus* strain Fra	France	AF150018.1
18	*Rh. turanicus* isolate 127-tot	NA	AF483244.1
19	*Rh. turanicus* isolate 575-tot	NA	AF483264.1
20	*Rh. camicasi* voucher USNTC-RML 107410	Ethiopia	FJ536556.1
21	*Rh. sulcatus* voucher USNTC-RML 116682	Zambia	FJ536564.1
22	*Rh. compositus*	NA	AF031860.1
23	*Rh. zumpti* South Africa	South Africa	AF150016.1
24	*Rh. muhsamae* v USNTC-RML 116980b	Zambia	FJ536559.1
25	*Rh. simus*	Zimbabwe	AF150019.1
26	*Rh. pravus*	Tanzania	AF150025.1
27	*Rhipicephalus* sp. voucher CAS35	South Africa	FJ536555.1
28	*Rhipicephalus* sp. voucher	Iran	FJ536563.1

NA no data available

Purebred lines

When female *Rh. sanguineus* from three different colonies mated with sympatric males (purebred crosses), the reproductive parameters differed between the three studied populations (Tables 3 and 4). Ticks from the North American colony all engorged between days 7 and 10 after being placed on rabbits. Those from the Israeli and African colonies on average took 1–1.5 days longer to feed to repletion completing their engorgement between days 7 and 13 (Table 3). The overall feeding success of purebred adult ticks from the American and Israeli colonies was similar (95–100 %) and significantly higher than in ticks of African origin ($P = 0.0056$). The weights of replete females in North American, Israeli, and African colonies fluctuated within the ranges of 0.21–0.39, 0.19–0.42, and 0.16–0.25 g, respectively. On average, the engorgement weight of a *Rh. sanguineus* female from the African population was approximately 30 % lower than female engorgement

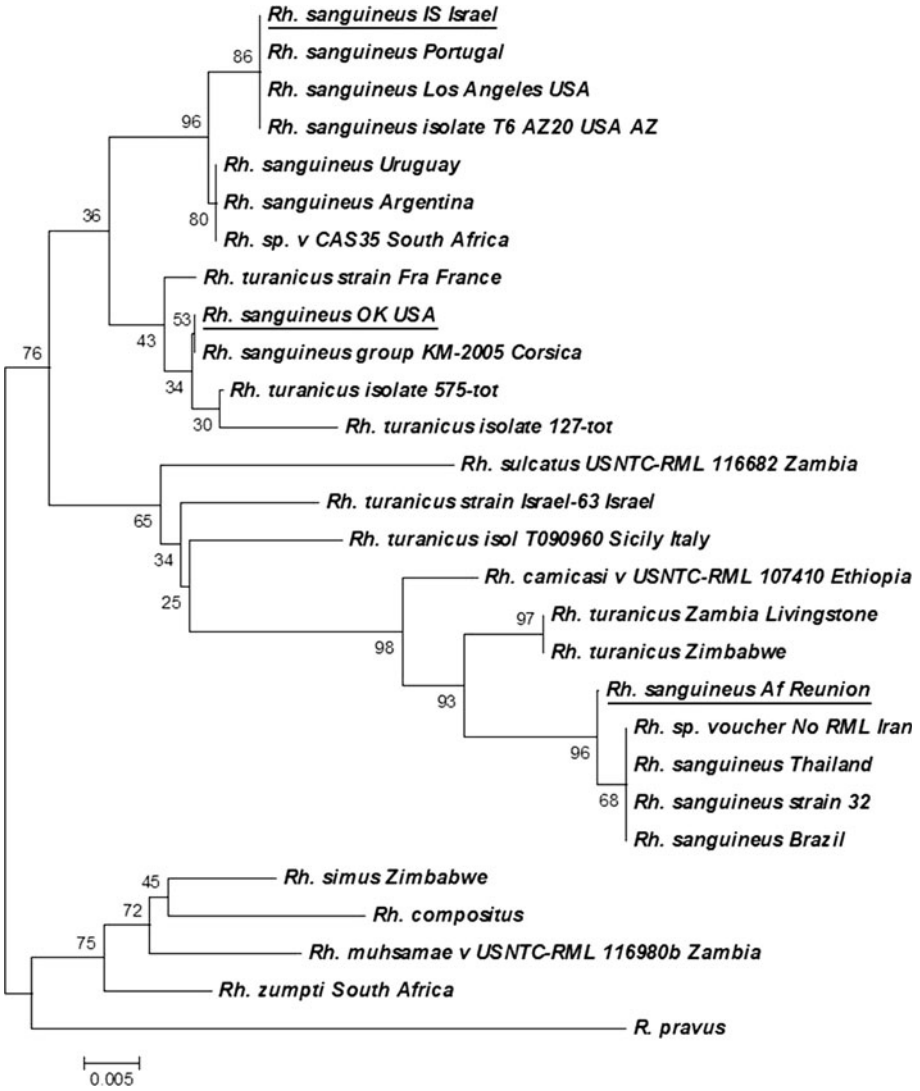

Fig. 1 Neighbor-Joining phylogenetic tree of 12S rDNA partial sequences (287 bp) of ticks morphologically identified as *Rhipicephalus sanguineus* or *Rh. turanicus* from five continents (Table 2). *Rh. camicasi, Rh. compositus, Rh. muhsamae, Rh. simus,* and *Rh. zumpti* were included as out-group. The tree is drawn to scale. Numbers next to the branches represent percentages of replicate trees (out of 1,000 replicates) in which the associated taxa clustered together in the bootstrap test

weights in North American and Israeli colonies ($P < 0.0001$). The BMCI did not differ significantly between the three purebred colonies (Table 3; $P > 0.01$). Consequently, African females oviposited smaller egg-masses than those of North American and Israeli origins.

All purebred engorged females began laying eggs within four to fourteen days after repletion regardless of their origin or the engorgement weight. Eggs laid by individual

Table 3 Reproductive parameters of engorged *Rhipicephalus sanguineus* females from three allopatric populations after homologous and heterologous mating—parental generation

Parental tick combinations	Purebred lines			Hybrid lines					
	♀♀-US	♀♀-IS	♀♀-AF	♀♀-US		♀♀-IS		♀♀-AF	
	♂♂-US	♂♂-IS	♂♂-AF	♂♂-IS	♂♂-AF	♂♂-US	♂♂-AF	♂♂-US	♂♂-IS
Female feeding (days)*	8.18 ± 1.11[a]	9.29 ± 1.80[b]	9.73 ± 1.77[b]	10.00 ± 1.33[b]	8.81 ± 0.98[a]	9.84 ± 0.76[b]	12.17 ± 1.53	9.60 ± 0.80[b]	9.47 ± 0.74[b]
Female feeding success (%)**	100.0 ± 0.0[a]	95.0 ± 9.0[a]	82.5 ± 15.7[b]	95.0 ± 12.9[a]	80.0 ± 23.6	95.0 ± 0.12.9[a]	70.0 ± 27.1	75.0 ± 25.6	75.0 ± 25.6
Engorgement weight (g)*	0.29 ± 0.05[a]	0.31 ± 0.06[a]	0.20 ± 0.03[b]	0.26 ± 0.07[a]	0.26 ± 0.06[a]	0.24 ± 0.07	0.29 ± 0.11[a]	0.19 ± 0.06[b]	0.20 ± 0.03[b]
Preoviposition period (days)*	7.42 ± 1.99[a]	7.81 ± 2.02[a]	8.21 ± 2.43[a]	6.89 ± 1.94[a]	6.00 ± 1.46[a]	9.06 ± 1.48[a]	6.81 ± 1.94[a]	7.53 ± 1.06[a]	8.13 ± 0.35[a]
Engorged females laying eggs (%)**	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	94.4 ± 14.3[a]	86.2 ± 16.8[a]	89.5 ± 18.6[a]	91.7 ± 21.5[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Egg-mass weight (g)*	0.19 ± 0.03[a]	0.19 ± 0.05[a]	0.14 ± 0.03[b]	0.16 ± 0.06[a]	0.14 ± 0.04[b]	0.11 ± 0.07	0.19 ± 0.07[a]	0.13 ± 0.06[b]	0.13 ± 0.03[b]
Blood meal conversion index (%)*	65.4 ± 9.3[a]	60.2 ± 9.5[a]	69.7 ± 9.3[a]	60.5 ± 15.9[a]	52.2 ± 11.1	42.7 ± 27.3	63.0 ± 17.9[a]	61.9 ± 14.1[a]	66.0 ± 10.8[a]
Minimum egg incubation period (days)*	35.73 ± 2.74[a]	36.47 ± 3.23[a,b]	40.30 ± 2.69[b]	37.11 ± 0.83[a,b]	36.07 ± 2.74[a,b]	38.76 ± 5.51[a,b]	38.91 ± 3.27[a,b]	37.13 ± 0.52[a,b]	37.21 ± 1.48[a,b]
Egg hatchability (%)*	93.4 ± 12.6[a]	78.5 ± 29.5[b]	87.3 ± 19.6[a]	80.3 ± 26.3[a]	73.1 ± 29.9	76.0 ± 31.3[b]	54.5 ± 33.3[b]	93.7 ± 12.9[a]	88.3 ± 10.8[a]

* Average ± Standard deviation. Numbers within a row not sharing superscripts are significantly different ($P_{ANOVA} < 0.01$)

** % ± 99 % Confidence interval. Numbers within a row not sharing superscripts are significantly different ($P_{Chi\text{-}squared\ test} < 0.01$)

Table 4 Biological parameters of F1 immature purebred and hybrid progenies of *Rhipicephalus sanguineus* from three allopatric populations

Parental tick combinations	Purebred lines			Hybrid lines					
	♀♀-US	♀♀-IS	♀♀-AF	♀♀-US		♀♀-IS		♀♀-AF	
	♂♂-US	♂♂-IS	♂♂-AF	♂♂-IS	♂♂-AF	♂♂-US	♂♂-AF	♂♂-US	♂♂-IS
Larval molting success (%)*	97.0 ± 0.7[a]	99.1 ± 0.1[a]	97.8 ± 1.1[a]	43.2 ± 8.2	95.4 ± 1.2	84.6 ± 1.9	99.2 ± 0.2[a]	81.1 ± 4.0	88.1 ± 1.9
Nymphal molting success (%)*	77.9 ± 7.6[a]	62.9 ± 8.9[b]	34.6 ± 9.6[c]	91.1 ± 9.9[a]	94.0 ± 4.4	62.4 ± 9.3[b]	89.1 ± 5.8	81.0 ± 7.3[a]	85.8 ± 6.4[a]
Male/female ratio (%)[$]	86/69 (55.5)[a]	59/65 (47.6)[a]	35/22 (61.4)[a]	28/23 (48.3)[a]	78/109 (41.7)[a]	60/53 (53.1)[a]	73/98 (42.7)[a]	96/62 (60.8)[a]	97/72 (57.4)[a]

* % ± 99 % Confidence interval. Numbers within a row not sharing superscripts are significantly different ($P_{\text{Chi-squared test}} < 0.01$)

[$] Proportion of males in each cross is compared to the theoretically expected 50.0 %[a]

females from the American colony hatched after 32–38 days and those produced by Israeli and African ticks required 35–48 days of incubation. On average, the incubation period under 22 °C was significantly shorter in the American colony than in the African colony (Table 3; $P < 0.01$). Eclosion of larvae was observed in every egg batch, but the average proportion of fertile eggs varied significantly between colonies—from 78 % among ticks of Israeli origin to above 90 % among the American *Rh. sanguineus* ($P = 0.0039$) (Table 3).

Following engorgement on NZW rabbits, 97–99 % of purebred larvae successfully molted into the nymphal stage (Table 4). However, purebred nymphs engorging on the same host species had low molting success. The molting success of nymphs from the Israeli colony was significantly lower than those of American origin ($P = 0.0011$) and almost twice as high as nymphs in the African colony ($P < 0.0001$) (Table 4). The male/female ratio among purebred adult ticks did not diverge significantly from the expected 50/50 proportion (Table 4). This confirms that viable progenies in the purebred colonies developed from fertilized eggs, and no parthenogenesis was observed.

Hybrid lines

When North American female *Rh. sanguineus* were fed with Israeli male ticks (US–IS cross), 19 out of 20 females fed to repletion. On average, they required 2 days longer to complete their engorgement ($P < 0.0001$) than those mating with sympatric males (Table 3). Means of engorgement weight, duration of the preoviposition period, the weight of an egg batch and BMCI, as well as proportions of ovipositing females were similar between American females crossbreeding with male ticks of Israeli origin and the purebred colony (Table 3). The average egg hatchability in the US–IS cross was lower than in the purebred American colony but not significantly different ($P = 0.0137$). Larval molting success in the F1 progeny of the US–IS cross was twice as low as that in the purebred parental colonies ($P < 0.0001$); but survival of engorged nymphs was higher, i.e. $P = 0.0270$ in comparison with the American colony, and $P < 0.0001$ if compared to the Israeli colony. The proportion of males in the F1 progeny of the US–IS cross did not deviate from the expected 50/50 ratio (Table 4) confirming that cross-mating resulted in successful fertilization of eggs, and crossbred females did not reproduce parthenogenetically. The resulting F1 adult ticks from the US–IS cross fed successfully and oviposited within the time frame typical for the purebred parental colonies (Table 5). The average weights of both the engorged females and the egg-masses they produced were a little lower than in the parental colonies ($P > 0.01$), but normal viable F2 larvae eclosed from the absolute majority of eggs in every batch (Table 5). These hybrid larvae remained alive and active in an incubator for over four months.

North American female *Rh. sanguineus* mated with African males (US–AF cross) fed to repletion within the same time as period as those in the purebred American colony (Table 3). However, their overall feeding success was low with only 16 out of 20 females successfully engorging ($P = 0.0034$). The average engorgement weight, average duration of the preoviposition period, and the proportion of ovipositing females did not differ significantly from the parameters of a purebred colony (Table 3). On the other hand, BMCI in the US–AF cross was significantly lower ($P = 1.9 \times 10^{-6}$), and consequently, egg batches produced in the US–AF cross were significantly smaller ($P < 0.0001$). Moreover, a significantly lower ($P = 0.0003$) proportion of those eggs produced viable larvae (Table 3). Molting success of immature stages in the US–AF cross was high (Table 4). In fact, significantly more engorged nymphs survived through the molt in the US–AF cross

Table 5 Reproductive parameters* of F1 hybrid *Rhipicephalus sanguineus* females in comparison with those of purebred ticks

Parental tick combinations	Purebred lines			Hybrid lines					
	♀♀-US	♀♀-IS	♀♀-AF	♀♀-US		♀♀-IS		♀♀-AF	
	♂♂-US	♂♂-IS	♂♂-AF	♂♂-IS	♂♂-AF	♂♂-US	♂♂-AF	♂♂-US	♂♂-IS
Female feeding (days)**	8.18 ± 1.11[a]	9.29 ± 1.80[b]	9.73 ± 1.77[b]	7.61 ± 0.78[a]	8.65 ± 0.22[a]	8.30 ± 0.92[a,b]	9.45 ± 2.46[a,b]	12.35 ± 1.35	10.45 ± 0.26[b]
Female feeding success (%)**	100.0 ± 0.0[a]	95.0 ± 9.0[a]	82.5 ± 15.7[b]	90.0 ± 17.7[a]	100.0 ± 0.0[a,b]	100.0 ± 0.0[a]	100.0 ± 0.0[a,b]	100.0 ± 0.0[a,b]	100.0 ± 0.0[a,b]
Engorgement weight (g)**	0.29 ± 0.05[a]	0.31 ± 0.06[a]	0.20 ± 0.03[b]	0.24 ± 0.06[c]	0.25 ± 0.04[c]	0.29 ± 0.05[a]	0.25 ± 0.08[c]	0.16 ± 0.06	0.25 ± 0.07[c]
Preoviposition period (days)**	7.42 ± 1.99[a]	7.81 ± 2.02[a]	8.21 ± 2.43[a]	7.89 ± 2.27[a]	7.60 ± 2.35[a]	8.20 ± 2.21[a]	8.10 ± 2.36[a]	7.80 ± 2.61[a]	7.95 ± 2.39[a]
Females laying eggs (%)**	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]	100.0 ± 0.0[a]
Egg-mass weight (g)**	0.19 ± 0.03[a]	0.19 ± 0.05[a]	0.14 ± 0.03[b]	0.16 ± 0.04[a]	0.16 ± 0.03[a,b]	0.18 ± 0.04[a]	0.16 ± 0.06[a,b]	0.10 ± 0.04	0.16 ± 0.05[a,b]
Blood meal conversion index (%)**	65.4 ± 9.3[a]	60.2 ± 9.5[a]	69.7 ± 9.3[a]	62.7 ± 5.6[a]	52.2 ± 11.1	61.8 ± 4.7[a]	61.6 ± 14.0[a]	56.4 ± 9.3	62.4 ± 5.1[a]
Minimum egg incubation period (days)**	35.73 ± 2.74[a]	36.47 ± 3.23[a,b]	40.30 ± 2.69[b]	34.39 ± 1.91[a]	N/A	36.30 ± 2.83[a,b]	N/A	N/A	N/A
Egg hatchability (%)**	93.4 ± 12.6[a]	78.5 ± 29.5[b]	87.3 ± 19.6[a]	96.9 ± 9.4[a]	0	89.3 ± 18.8[a]	0	0	0

* Average ± Standard deviation. Numbers within a row not sharing superscripts are significantly different ($P_{ANOVA} < 0.01$)

** % ± 99 % Confidence interval. Numbers within a row not sharing superscripts are significantly different ($P_{Chi\text{-}squared\ test} < 0.01$)

than in either purebred colony ($P < 0.0001$). The proportion of males in the F1 progeny of US–AF cross did not deviate significantly from the expected 50/50 ratio (Table 4). The resulting hybrid adults successfully fed to repletion within normal time and all engorged females oviposited (Table 5). Although the weights of the engorged females and the egg batches they produced were somewhat lower than in the corresponding purebred colonies, these were the same as in the crossbred parental females (Table 4) and in the US–IS cross (Table 5). The resulting US–AF egg batches, however, did not produce viable F2 progeny; no larvae eclosed from the 20 egg batches.

When females from the Israeli *Rh. sanguineus* colony were mated with male ticks from the North American colony (IS–US cross), 19 out of 20 females fed to repletion within the same time period as those mating with sympatric males (Table 3). Seventeen (89.5 %) of the engorged females produced egg batches within 7–11 days after repletion. The average engorgement weight of these ticks was significantly lower than in the purebred Israeli colony ($P = 0.0006$). The BMCI was also low, and the average size of resulting egg batches was reduced by almost 50 % compared to the purebred Israeli females ($P = 2.8 \times 10^{-5}$) (Table 3). However, the average proportion of hatching eggs in the IS–US cross was similar to that in the purebred Israeli colony ($P = 0.26$). Larval molting success in the F1 progeny of the IS–US cross was significantly lower than in the purebred parental colonies ($P < 0.0001$); but survival of engorged nymphs was the same as in the Israeli colony. The proportion of males in the F1 progeny of IS–US cross did not deviate from the expected 50/50 ratio indicating that the IS–US cross did indeed produce fertilized eggs (Table 4). The resulting F1 adult ticks from the IS–US cross successfully fed on a rabbit and oviposited within the time frame typical for the purebred parental colonies (Table 5). All other reproductive parameters of these hybrids were also almost identical to those in the purebred Israeli colony ($P > 0.20$), including a normal size of produced egg batches and a high proportion of hatching eggs (Table 5). IS–US hybrid larvae remained alive and active in an incubator for over four months.

Out of 20 Israeli *Rh. sanguineus* females cross-mated with African males (IS–AF cross), only 14 fed to repletion and took 10–14 days to feed. On average, they completed engorgement approximately three days later than those in the purebred Israeli colony ($P = 8.3 \times 10^{-6}$) (Table 3). Nevertheless, they reached similar engorgement weights to the Israeli females mating with sympatric males, and produced egg batches of the same size. All the remaining reproductive parameters in the parental generation were also similar to the purebred colony (Table 3). Molting success of IS–AF hybrid larvae was as high as that of their purebred counterparts, and survival of engorged nymphs exceeded the nymphal molting success in either of the parental purebred colonies (Table 4). The male/female ratio in the F1 progeny of IS–AF cross (73/98) was not significantly different from the expected 50/50 ratio indicating that eggs produced by Israeli females were successfully fertilized by males of African origin (Table 4). The resulting F1 females mating with males from the same cross fed to repletion within the normal time period without a significant delay noticed in the parental generation (Table 5). After engorgement, all females produced egg batches whose average size was only a little smaller than that in the purebred parental colonies. However, these US–AF egg batches did not produce viable F2 progeny (Table 5). Only four individual larvae hatched among the 20 egg batches; and those all died within a few days after eclosion.

When African female ticks were placed on rabbits with male ticks from either the American (AF–US cross) or Israeli colony (AF–IS cross), their feeding success, duration of engorgement, engorgement weights, BMCI, and the average size of egg batches were all similar to those in the purebred African colony (Table 3). High proportions of eggs hatched

in all hybrid batches within normal time periods. The molting success of engorged hybrid larvae in both AF–US and AF–IS crosses was lower than in either parental colonies, but the nymphal molting success was much higher than expected for Israeli and especially African ticks (Table 4). Male/female ratios in AF–US and AF–IS two hybrid lines were 96/62 and 97/72, respectively, which confirmed that African females were fertilized by either American or Israeli males and their progeny was not a result of parthenogenetic production.

In the F1 progeny of the AF–US cross, all 20 females placed on a rabbit fed to repletion but their feeding lasted for 11–14 days—on average 2.5–4 days longer than that of purebred African or American females ($P = 6.3 \times 10^{-7}$) (Table 3). The engorgement weights of these ticks were lower than in parental colonies, though the difference did not reach the level of statistical significance ($P = 0.043$) (Table 5). All engorged females oviposited, yet the BMCI was much lower than in the African colony ($P = 0.0078$), which resulted in production of very small egg batches ($P = 0.0064$) (Table 3). All eggs produced by AF–US hybrid females were infertile as no larvae hatched from the 20 egg batches within 12 weeks after oviposition.

In the F1 progeny of the AF–IS cross, the length of female engorgement was not significantly delayed in comparison with either African or Israeli purebred colonies ($P = 0.26$) (Table 5). All 20 hybrid females successfully fed and reached engorgement weights intermediate between the parental African and Israeli colonies ($P = 0.0098$ and $P = 0.0016$, respectively). The average BMCI was also intermediate between the two parental colonies and all engorged hybrid females laid egg batches of normal size (Table 5). However, these eggs were infertile as only a single larva was observed to attempt eclosion, which was unsuccessful.

Phylogenetic analysis of the purebred ticks

The phylogenetic analysis of partial (287 bp) sequences of the 12S rDNA gene showed 3.2 % divergence between *Rh. sanguineus* from the US and Israeli populations, while the same gene of African ticks differed by 9.2 % from either of those. As a result, North American and Israeli ticks clustered close on the neighbor-joining tree and ticks of African descent represented a separate clade with 74 % bootstrap support (Fig. 1). *Rh. sanguineus* IS grouped together with *Rh. sanguineus* from California (Los Angeles—HM014443.1) and Arizona (HM138903.1), as well as from Portugal (FJ536553.1) with 76 % bootstrap support. *Rh. sanguineus* Oklahoma, US (HM138900.1) appeared closely related to *Rh. sanguineus* from Italy (HM014442.1) and *Rh. turanicus* from France (AY947467.1). *Rh. sanguineus* AF, on the other hand, clustered with *Rh. sanguineus* from Brazil (AY559842.1) and Thailand (AY987377.1), as well as with African ticks from Zambia (DQ849232.1) and Zimbabwe (AF150017.1) morphologically identified as *Rh. turanicus* (Fig. 1). Noticeably, ticks from different countries and continents morphologically identified as *Rh. sanguineus* were intermixed in various phylogenetic clades with those identified as *Rh. turanicus*. This suggests high variability of morphological features in either species and underscores the difficulty of their delineation based solely on morphology.

Discussion

Delineation of species within the *Rh. sanguineus* group has been a controversial and confusing issue with many morphologically similar ticks described in different locations

under different names only to be later synonymized with the *Rh. sanguineus* sensu stricto (Pegram et al. 1987a, b, 1989; Walker et al. 2000). Ticks within this group are known to be competent vectors of viral, bacterial, and protozoan pathogens of humans and domestic animals. As different vector species vary considerably in their ability to acquire, maintain, and transmit particular pathogens, the proper identification of vectors is a prerequisite for studies in biology, ecology, and epidemiology of any vector-borne pathogen. Therefore, clarification of the status and relationships between the members of the *Rh. sanguineus* group presents more than just academic interest.

In accordance with the biological species concept (Mayr 1970), successful interbreeding between organisms from different populations resulting in fertile progeny indicates their conspecificity. Conversely, inability to produce fertile progeny is a result and a sign of reproductive separation between species. Reproductive separation is not usually expected among members of the same species while fruitful hybridization is rare between representatives of different biological species.

Most studies in the possibility of hybridization between closely related tick species have confirmed this concept of reproductive separation. For example, no viable F1 hybrids resulted from cross-mating between *Dermacentor variabilis* and *Dermacentor andersoni* (Oliver et al. 1972), *Amblyomma americanum* and *Amblyomma maculatum* (Gladney and Dawkins 1973), *Rhipicephalus (Boophilus) microplus* and *Rhipicephalus (Boophilus) decoloratus* (Spickett and Malan 1978), or between *Dermacentor marginatus* and *Dermacentor reticulatus* (Zahler and Gothe 1997). Interspecific matings producing infertile F1 hybrids have been reported for *Rhipicephalus (Boophilus) annulatus* and *R. (B.) microplus* (Graham et al. 1972, Davey et al. 1983), *Amblyomma variegatum* and *Amblyomma hebraeum* (Rechav et al. 1982; Clarke and Pretorius 2005), *Ixodes ricinus* and *Ixodes persulcatus* (Balashov et al. 1998), *Ornithodoros papillipes*, *Ornithodoros tartakovskyi* and *Ornithodoros verrucosus* (Balashov 1970). Moreover, reproductive incompatibility between geographically different populations of *Amblyomma cajennense* and *R. (B.) microplus* suggested that these taxa actually represent complexes of different species (Labruna et al. 2009, 2011).

Published examples of hybridization between otherwise "recognized" tick species include an early report from the former U.S.S.R. where *Rh. sanguineus* and *Rh. turanicus* were observed to interbreed and produce fertile progeny (Pervomaisky 1950); and a recent study showing that *Amblyomma gemma* and *A. hebraeum* were genetically compatible producing fully fertile hybrids (Clarke and Pretorius 2005). In both studies, successful cross-mating rendering a fertile hybrid was interpreted as an indication of possible conspecificity of the respective ticks. In addition, conspecificity of *Rh. sanguineus* and *Rh. turanicus* has been suggested based on the results of DNA analysis when *ITS2* sequences of *Rh. sanguineus* from both Azerbaijan and Burkina Faso were found to be identical to that of *Rh. turanicus* from Turkmenistan (Zahler et al. 1997).

It was also proposed that interbreeding might theoretically be possible between recently delineated closely related and morphologically similar ticks (Farid 1996). *Rh. appendiculatus* and *Rh. zambeziensis* are morphologically very similar (Walker et al. 2000) although molecular analysis confirmed that they are separate species (Mtambo et al. 2007). Fertile hybrids were produced by *Rh. zambeziensis* females mating with *Rh. appendiculatus* males, whereas a reciprocal cross resulted in a sterile F1 hybrid (Zivkovic et al. 1986).

Herein, we tested conspecificity of *Rh. sanguineus* from three geographically distant populations by assessing the genetic diversity between them, studying reproduction and survival of ticks under standard laboratory conditions, and comparing the reproductive performance of their crosses.

Recorded reproductive parameters differed between the three tick colonies. Most likely, the noted variations in the duration of egg incubation period and the nymphal molting success reflect how much our standard laboratory conditions diverge from the natural environmental conditions to which particular tick populations have adapted. Inversely, it indicates that environmental requirements vary between the three geographically distant populations, with the African ticks more different in their requirements from ticks of either North American or Israeli origin than those two from each-other.

When pairing female ticks from distinct geographic locations with allopatric males, we considered a possibility of parthenogenetic reproduction. With exception of a few species, females of ixodid ticks feeding in the absence of conspecific males increase their feeding period, have smaller engorgement weights and oviposit none or only a small number of fertile eggs when compared to inseminated females (Oliver 1989). Moreover, parthenogenesis in ticks inevitably results in production of only female offspring. In our study, we did observe delayed engorgement, decreased engorgement weights, and smaller egg masses in some instances of interbreeding. However, all viable F1 progenies (hybrid as well as purebred) contained both males and females, and no gynandromorphs were observed. This shows that all variants of cross-mating in the parental generation resulted in successful egg fertilization with no evidence of parthenogenesis.

Interestingly, results of interbreeding between males from one population with females from another differed from those when origins of males and females were reversed. Although high proportions of cross-mating females in both US–IS and IS–US crosses fed to repletion, engorgement weights in the IS–US cross were lower than in the reciprocal US–IS cross (Table 3). Overall, the American females mating with Israeli males performed almost as well as those fertilized by sympatric males. On the other hand, the decreased engorgement weights in combination with low BMCI and relatively poor egg hatchability resulted in a much smaller number of F1 progeny in the IS–US cross. The US–IS and IS–US crosses also differed in survival and reproductive parameters of F1 hybrids. Molting success of hybrid larvae decreased in both cross-breeding combinations, but in the US–IS cross it was reduced to a greater extent—by more than 50 %—compared to the purebred colonies (Table 4). Notably, the survival of hybrid nymphs in the US–IS cross was higher than in the parental colonies or in the reciprocal IS–US hybrids. Nonetheless, most of the reproductive parameters of US–IS and IS–US hybrid adults of the F1 generation were similar to those in purebred colonies, and both crosses produced normal numbers of F2 larvae (Table 5), which remained alive for over 4 months after eclosion.

Reciprocal cross-mating between North American and African ticks also resulted in successful fertilization. Although the feeding success of crossbreeding females was lower than in those paired with allopatric males, the fed females achieved normal (for the respective source colony) engorgement weight. Reproductive parameters of crossbred African females were essentially the same as in the purebred African colony, while fertilization of American females by African males resulted in significantly reduced fecundity. Survival of F1 hybrid larvae in both crosses decreased, but the molting success of F1 hybrid nymphs again was much higher than in purebred colonies. Contrary to differences between the two crosses observed in parental generation, F1 adults resulting from the US–AF cross blood-fed faster and better than those from the AF–US cross and produced larger egg masses. However, the 40 egg masses laid by the US–AF and AF–US hybrid females produced no viable progeny.

Observed effects of interbreeding between Israeli and African ticks were similar to those described for US–AF and AF–US crosses. Overall, the African females mating with Israeli males performed as well as in the purebred African colony while fertility and

fecundity in the reciprocal cross (IS–AF) were reduced. However, neither parameter decreased as significantly as when North American females cross-mated with African males. All reproductive parameters of F1 hybrids in the IS–AF and AF–IS crosses were similar; they also were at the midway between their parental purebred colonies. And again, no viable F2 progeny resulted from any of the total of 40 hybrid egg masses.

Overall, interbreeding of *Rh. sanguineus* ticks from three geographically distant populations in most cases resulted in reduction of their fecundity, although heterosis apparently caused significant improvement of nymphal molting success in hybrid lines. Nonetheless, hybridization between North American and Israeli *Rh. sanguineus* resulted in fully fertile offspring, while all crosses of either American or Mediterranean ticks with those of African origin invariably resulted in complete infertility of the F1 generation. It appears that in all four F1 hybrid progenies involving parents (either males or females) from the African colony, females remained capable of producing eggs, but the eggs were either not fertilized or failed to develop.

Results of our hybridization experiments confirm conspecificity of Mediterranean and North American populations of *Rh. sanguineus*, which corresponds with conclusions from recent molecular studies (Szabo et al. 2005, Burlini et al. 2010). Reproductive incompatibility is not expected to occur between different populations of a single species. Thus, our results demonstrate that ticks from Reunion belong to a species different from *Rh. sanguineus* of North American and Israeli origins. Akin to our study, crossbreeding between *R. (Boophilus) microplus* from Australia with those from Africa or South America produced viable F1, but F1 adults were infertile (Spickett and Malan 1978, Labruna et al. 2009). This indicated that *R. (Boophilus) microplus* ticks from Africa and S. America represent the same species, while ticks from Australia may be a separate species.

Phylogenetic analysis of partial (287 bp) mitochondrial 12S rDNA gene sequences supports the separation between the brown dog ticks from Reunion and the other two populations by demonstrating deep divisions on a molecular level (Fig. 1). *Rh. sanguineus* AF comprises a separate clade on the phylogenetic tree together with *Rh. sanguineus* from Brazil and Thailand as well as with *Rh. turanicus* from Zambia and Zimbabwe. On the other hand, North American and Israeli *Rh. sanguineus* ticks cluster together with *Rh. sanguineus* from Portugal, France, Uruguay, and Argentina and with *Rh. turanicus* from France. These results concur with a recently published study in phylogeny of the *Rh. sanguineus* group based on mitochondrial 16S rDNA sequences (Moraes-Filho et al. 2011). For our study, we chose to use the mitochondrial 12S rDNA gene because it has been demonstrated to be a good marker for the establishment of genetic relationships among closely related tick species (Szabo et al. 2005; Ketchum et al. 2009; Labruna et al. 2009; Burlini et al. 2010), while deep divergences of the 16S rDNA gene may arise within a single species (Leo et al. 2010). Taken together, sequence analyses of both genes indicate that the taxon *Rh. sanguineus* might represent more than one species.

Thus, we evaluated the reproductive compatibility between three geographically distant populations of *Rh. sanguineus* and found that heterologous crosses between Israeli and North American ticks resulted in highly fertile progeny. The complete reproductive compatibility exhibited in cross-breeding experiments provides an additional evidence of conspecificity between the Mediterranean and North American *Rh. sanguineus* populations.

Our results also showed that ticks from the Reunion population are reproductively incompatible with either the Mediterranean population from Israel or the North American population from Oklahoma. The molecular phylogenetic analysis similarly separated the Reunion population from American and Israeli ticks. Jointly, these results imply that this

particular African population represents a separate species or subspecies. *Rh. sanguineus* population from Reunion was chosen for this study because no other tick species belonging to the *Rh. sanguineus* group have been identified on the island (Walker et al. 2000; Müller et al. 2004). A degree to which the Reunion population is representative of brown dog ticks of the African continent in general requires further detailed assessment. Yet, close relatedness between both the 12S rDNA and 16S rDNA gene sequences of ticks from Reunion to those from Brazil, Thailand, Iran, Zambia, and Zimbabwe indicate that the purported taxon has wide geographical distribution.

Results of our experiments support a hypothesis that at least two different species currently share the name *Rh. sanguineus*. These species need to be redescribed and delineated. Studies involving large number of tick strains from different geographic locations and combining crossbreeding with the molecular approach are needed to accomplish this task.

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