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Cross-mating experiments with geographically different populations of *Amblyomma cajennense* (Acari: Ixodidae)

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Abstract The present study evaluated the reproductive compatibility of the crosses between adult ticks of the following three geographically different populations of *Amblyomma cajennense*: State of São Paulo (SP), southeastern Brazil; State of Rondônia (RO), northern Brazil; and Colombia (CO). In addition, crosses between *A. cajennense* ticks from Argentina (AR) and SP ticks were also performed. The Argentinean population (AR) was compatible with SP because their crosses resulted in high % egg hatching (mean values ranging from 71.5 to 93.5%), similarly to all homologous (intrapopulational) crosses. In contrast, the tick populations SP, RO, and CO were shown to be incompatible with each other, since their heterologous (interpopulational) crosses always resulted in very low % egg hatching (range: 0–5%). The F_1 larval offspring derived from some of these females that yielded 5% egg hatching were reared until the F_1 adult stage. In all cases, only adult females molted from engorged nymphs. These F_1 females were likely to be a product of thelytokous parthenogenesis of the SP, RO, and CO females that were used in the heterologous crosses. Reproductive incompatibility is not expected to occur between different populations of a single species. Thus, our results suggest that the taxon *A. cajennense* might be represented by a complex of different species, whereas SP and AR ticks might represent a single species. Further populational genetic studies, coupled with extensive morphological analyses, are needed to clarify and determine a possible complex of valid species that might have been classified under the taxon *A. cajennense.*

Keywords *Amblyomma cajennense* · Biology · Cross-mating · Brazil · Colombia · Argentina

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Introduction

Currently, the taxon *Amblyomma cajennense* (Acari: Ixodidae) represents tick populations of major medical and veterinary importance from southern United States to Northern Argentina (Estrada-Peña et al. 2004). Besides being an important pest to livestock, chiefly horses and cattle, *A. cajennense* is the most common human-biting tick in the Neotropical region (Guglielmone et al. 2006), where it is a major vector of *Rickettsia rickettsii*, the etiological agent of the deadliest spotted fever rickettsiosis of the world (Labruna 2009). Additionally, potentially pathogenic pathogens have been isolated from *A. cajennense*, such as arbovirus (Belle et al. 1980), *Encephalitozoon*-like microsporidian (Ribeiro and Guimaraes 1998), and the spotted fever group agent *Rickettsia amblyommii* (Labruna et al. 2004).

In the past, various taxa described as different species were synonymized with *A. cajennnese,* under the argument that they merely represented intraspecific morphological variations, possibly linked to geographically distinct populations (Aragão and Fonseca 1953; Kohls 1958). Since then, the species *A. cajennense* has been considered to encompass established populations from southern United States to Northern Argentina (Guglielmone et al. 2003), although biological differences between different *A. cajennense* populations have been reported (Guglielmone et al. 1992). More recently, Beati et al. (2007) performed molecular analyses of *A. cajennense* specimens from different American countries, and suggested that *A. cajennense* is a species complex, with each species associated with a particular biogeographical region.

In this context, the present study evaluated the reproductive compatibility of the crosses between four geographically different populations of ticks that have been identified under the taxon *A. cajennense.*

Materials and methods

For the present study, four tick colonies were started with field-collected adult ticks, as detailed in Table 1. These ticks were morphologically identified as *A. cajennense* based on current literature (Barros-Battesti et al. 2006), and reared in the laboratory in order to obtain the next generation of unfed adult ticks, which were used to start experimental tick crosses. Most tick crosses were repeated (replications) with adult ticks of the subsequent laboratory generations of the tick colonies. At all instances, ticks were allowed to feed on

Table 1 Origin of the adult ticks used to start laboratory colonies of *Amblyomma cajennense* for the present study

Country	Locality	Latitude	Longitude	Source	Date	Colony code
Brazil	Gov. Jorge Teixeira (State of Rondônia)	10°38′S	63°29′W	Free-living	April 2005	RO
Brazil	Pirassununga (State of São Paulo)	21°59′S	47°25′W	Horses	August 2005	SP
Colombia	Villeta (Cundinamarca)	05°01′N	74°28′W	Cattle	July 2005	CO
Argentina	El Rey National Park (Province of Salta)	24°41′S	64°36′W	Free-living	September 2009	AR

tick-naïve rabbits (*Oryctolagus cuniculus*), whereas free-living ticks were always held in an incubator set for 25°C, 90% relative humidity, and scotophase. Larval or nymphal infestations were performed inside cotton sleeves glued to rabbit ears, whereas adult ticks were allowed to feed inside cotton sleeves (10 cm diameter) glued (Kamar heat detector adhesive, Kamar, Steamboat Springs, CO, USA) to the shaved dorsum skin of rabbits.

Unfed adults, ca. 30 days old, were sorted to form the infestation male × female crosses. From 2005 to 2009, crosses were made with adult ticks from the same strain (SP × SP, RO × RO, and CO × CO; homologous crosses), and with adult ticks from different strains (SP × RO, SP × CO, RO × SP, RO × CO, CO × SP, and CO × RO; heterologous crosses) (Table 2). Each cross consisted of 10–20 males and 12–25 females per sleeve. Additional sleeves, each containing only 14–20 unfed females from each strain were prepared to be the control of virgin females for each strain. All engorged females recovered from each sleeve (each separate cross) were individually weighed and left in the incubator. The total egg mass produced by each female was weighed and its hatchability was determined as described (Drummond et al. 1973). The egg production efficiency (EPE) was determined as: weight of eggs/weight of the engorged female × 100 (Bennett 1974).

From 2009 to 2010, crosses were made with adult ticks from the same strain (SP × SP, and AR × AR; homologous crosses), and with adult ticks from different strains (SP × AR, and AR × SP; heterologous crosses) (Table 3). Each cross consisted of 10 males and 10 females per sleeve, and procedures for recovery and analysis of engorged females recovered from each sleeve were as described above.

In order to verify successful reproductive compatibility between strains, unfed larvae obtained from each cross (designated as F_1 larvae) were reared separately to the adult stage. The resulting F_1 unfed adults were allowed to infest a rabbit. Recovered engorged females were processed as described above.

Feeding periods of ticks from each cross were compared by the non-parametric Mann–Whitney test, whereas engorged female weights and reproductive parameters (which showed normal distribution) were compared by the Student *t*-test, by using the program Minitab, version 16.0. The proportion of females that successfully fed (number of engorged females in relation to the number of exposed females) or successfully oviposited (number of ovipositing females in relation to the number of recovered engorged females) were compared by the qui-square test or Fisher's exact test, by using the program Epi-Info, version 6. Variables were considered significantly different if $P < 0.05$.

Results

Results of the crosses with the colonies from Brazil (SP and RO) and Colombia (CO) are shown in Table 2. In all crosses (either homologous or heterologous), males and females were observed in copula position (ventral surfaces juxtaposed), although no further effort was done to verify if there were any spermatophore transfer. Fertile females giving rise to large, viable offspring were obtained in all homologous crosses, as shown by high values for egg mass weights, and % egg hatching (mean values varying from 59.3 to 92.1%). In contrast, most of the engorged females obtained in the heterologous crosses yielded non-viable offspring (0% egg hatching), what contributed to significantly lower ($P < 0.05$) mean % egg hatching values, varying from 0.0 to 0.8%. Only 14, 9, 3, 3, and 1 individual females from the crosses SP × CO, CO × SP, CO × RO, RO × SP, and RO × CO, respectively, yielded larvae that hatched from a maximum of 1–5% of the total egg mass. Similarly, 2 engorged females from sleeves containing only CO females (no males) also

Table 2 Results of experimental crosses of adult ticks of *Amblyomma cajennennse* strains from the state of São Paulo, southern Brazil (SP), state of Rondônia, northern Brazil (RO), and from Colombia (CO)

Cross (♂ × ♀)	No. replications^{+}	No. exposed females	No. females that engorged (%)	Feeding period (days)	Engorged female weight (mg)*	No. engorged females that oviposited (%)*	Egg mass weight (mg)*	EPE*	% egg hatching*
SP × SP	3	60	54 (90.0)^{a}	12.0 ± 3.2^{a}	499.5 ± 145.2^{a,c}	52 (96.3)^{a,c,d}	250.1 ± 99.6^{a,c}	47.7 ± 14.7^{a,c}	88.3 ± 12.8^{a}
CO × CO	4	75	71 (94.7)^{a}	10.3 ± 2.8^{b}	627.7 ± 216,1^{b}	69 (97.2)^{a,d}	313.1 ± 153.3^{b}	48.0 ± 15.6^{a,d}	92.1 ± 14.2^{a}
RO × RO	3	55	48 (87.3)^{a,c}	13.4 ± 2.5^{c}	565.5 ± 249.6^{a,b}	48 (100)^{a}	261.4 ± 160.7^{a,b,c}	43.6 ± 11.7^{b}	59.3 ± 38.8^{b}
CO × SP	3	60	52 (86.7)^{a,c}	12.3 ± 2.2^{a}	475.5 ± 196.2^{a,c}	48 (92.3)^{a,b,c,d}	221.8 ± 98.5^{c,e}	44.8 ± 10.7^{b}	0.8 ± 0.3^{c}
CO × RO	3	52	37 (71.2)^{b,e}	12.3 ± 2.1^{a}	803.0 ± 213.3^{d}	34 (91.9)^{a,b,c,d}	403.9 ± 152.4^{d}	46.2 ± 16.1^{a,b}	0.2 ± 0.9^{c,d}
RO × CO	3	62	41 (66.1)^{b,e}	13.2 ± 4.3^{a,c}	528.6 ± 206.2^{a}	33 (80.5)^{b,e,f}	247.8 ± 144.8^{a,c,e}	39.2 ± 23.1^{b,c,e}	0.03 ± 0.2^{d}
RO × SP	2	40	25 (62.5)^{b,e}	12.8 ± 3.7^{a,c}	424.2 ± 188.1^{c}	19 (76.0)^{b,e,f}	179.7 ± 112.4^{e}	39.7 ± 16.6^{b,f}	0.4 ± 1.1^{c,d}
SP × CO	3	60	44 (73.3)^{b,c}	12.3 ± 3.8^{a,c}	560.4 ± 244.3^{a,b}	38 (86.4)^{d,f}	254.1 ± 155.8^{a,b,c}	38.8 ± 17.9^{b,f}	0.7 ± 1.3^{c}
SP × RO	2	40	7 (17.5)^{d}	16.1 ± 7.1^{a,c}	691.2 ± 325.0^{a,b,c,d}	5 (71.4)^{c,e,f}	362.7 ± 2,344^{a,b,c,d,e}	31.9 ± 20.4^{b,d,e}	0.0 ± 0.0
– × CO	2	36	19 (52.8)^{e}	28.6 ± 7.6^{d}	269.7 ± 215.8^{e}	11 (57.9)^{e}	93.3 ± 81.8^{f}	26.1 ± 9.2^{e}	0.5 ± 1.4^{c,d}
– × RO	1	20	1 (5.0)^{d}	42.0 ± 0.0	285.0 ± 0.0	0 (0)	–	–	–
– × SP	1	14	8 (57.1)^{b,e}	19.3 ± 5.2^{e}	180.2 ± 140.7^{f}	4 (50.0)^{e}	78.5 ± 25.7^{f}	28.9 ± 3.4^{e,f}	0.0 ± 0.0

Values followed by different superscript letters in the same column are significantly different ($P < 0.05$)

EPE egg production efficiency (weight of egg mass/weight of the engorged female × 100)

* Values presented as mean ± standard deviation

$^{+}$ Number of infested rabbits

Table 3 Results of experimental crosses of adult ticks of *Amblyomma cajennennse* strains from the state of São Paulo, Brazil (SP), and from Argentina (AR)

Cross (♂ × ♀)	Female data from the original cross^{+}					F_1 adult females from each cross$^{\#}$				
	Feeding period (days)*	Engorged female weight (mg)*	Egg mass weight (mg)*	EPE*	% egg hatching*	Feeding period (days)*	Engorged female weight (mg)*	Egg mass weight (mg)*	EPE*	% egg hatching*
SP × SP	12.8 ± 2.9^{a}	593.2 ± 138.5^{a,b}	325.7 ± 95.6^{a,b}	54.1 ± 5.6^{a}	79.0 ± 16.2^{a}	10.7 ± 1.6^{a}	532.7 ± 101.8^{a}	212.5 ± 69.1^{a}	40.3 ± 10.5^{a}	54.5 ± 15.1^{a}
AR × AR	12.8 ± 1.6^{a}	564.3 ± 112.0^{a}	289.6 ± 112.0^{a}	50.7 ± 15.9^{a}	89.4 ± 11.7^{a,b}	11.8 ± 3.5^{a}	402.0 ± 98.3^{b}	200.1 ± 59.4^{a}	48.5 ± 9.4^{a,b}	77.3 ± 15.7^{b}
SP × AR	10.1 ± 1.1^{b}	708.7 ± 87.1^{b}	396.7 ± 64.0^{b}	55.8 ± 4.3^{a}	93.5 ± 6.3^{b}	10.9 ± 1.4^{a}	782.9 ± 191.5^{c}	412.0 ± 113.1^{b}	52.2 ± 4.2^{b}	81.7 ± 12.6^{b}
AR × SP	11.4 ± 3.7^{a,b}	604.2 ± 228.3^{a,b}	319.1 ± 156.6^{a,b}	50.2 ± 10.3^{a}	71.5 ± 28.6^{a,b}	11.5 ± 2.4^{a}	648.3 ± 110.7^{c}	314.5 ± 52.9^{c}	49.1 ± 6.6^{b}	70.0 ± 22.6^{a,b}

Values followed by different letters in the same column are significantly different ($P < 0.05$)

EPE egg production efficiency (weight of egg mass/weight of the engorged female × 100)

* Values presented as mean ± standard deviation

[+] Each cross consisted of 10 males × 10 females inside a cotton sleeve, which resulted in 9 to 10 recovered engorged females from each cross; the 4 crosses above were done simultaneously on a single rabbit; i.e., 4 cotton sleeves glued to its shaved back

[#] The resultant F_1 larvae of each cross were reared separately using tick-naïve rabbits until the adult stage, from which 12 males and 12 females were infested inside a cotton sleeve on a tick-naïve rabbit, resulting in 11–12 F_1 engorged females from each original cross yielded egg masses with 1% egg hatching. Overall, significantly higher ($P < 0.05$) proportions of females from the homologous crosses successfully fed and oviposited, when compared with ticks from the heterologous crosses. However, values for both female engorged weight and egg mass weight were generally high for both homologous and heterologous crosses, although some significant differences ($P < 0.05$) were observed, including between homologous crosses. Feeding periods were significantly different ($P < 0.05$) between the three homologous crosses (SP, RO, and CO). On the other hand, feeding period of heterologous crosses were similar ($P > 0.05$) to each other and to one or more homologous crosses. Both CO, RO, and SP females exhibited much longer ($P < 0.05$) feeding periods when they fed without males. Although only one engorged female was recovered from the RO group with no males, other 13 exposed females remained attached to the rabbit skin with no marked increasing in their engorgement state for at least 78 days, when these females were manually detached and discarded. Regarding these three groups of females that fed with no males, their engorged and egg mass weights, and EPE were significantly lower ($P < 0.05$) than those observed for females that fed with males.

From four females that had an egg mass with 5% egg hatching (one CO × SP, one RO × SP, and two SP × CO females), their F_1 larval offspring were reared separately in the laboratory until the F_1 adult stage. In all cases, only adult females were obtained from a total of 12–40 engorged nymphs that were obtained from each offspring; i.e., none engorged nymph molted to male. These F_1 females were allowed to feed on rabbits, but only a few (2–5) engorged females were recovered from each of the four infestations. These F_1 females fed for 6–26 days (mean: 13.3 ± 6.1 days), weighed 68.1–797.0 mg (mean: 312.4 ± 237.6 mg), and only 6 females (the heaviest ones) oviposited 32.6–349.2 mg of eggs (mean egg mass: 167.3 ± 98.8 mg), but no egg developed into larva. In parallel, the F_1 larval offspring of homologous crosses (SP × SP, CO × CO, and RO × RO) were also reared to the adult stage, but in this case, both male and female ticks were obtained.

Results from the crosses with the colonies from Brazil (SP) and Argentina (AR) are shown in Table 3. Fertile females giving rise to large, viable offspring were obtained in all homologous and heterologous crosses (% egg hatching values varying from 71.5 to 93.5%). Generally, the two homologous (SP × SP, and AR × AR) and heterologous (SP × AR, and AR × SP) crosses yielded statistically similar ($P > 0.05$) feeding and reproductive values. From these four crosses, the F_1 larval offspring were reared separately until the adult stage, which also had its reproductive performance evaluated. While F_1 adult females from both homologous and heterologous crosses were highly fertile, with % egg hatching values varying from 54.5 to 81.7%, F_1 females from the heterologous crosses tended to have a higher reproductive performance, as demonstrated by significantly higher ($P < 0.05$) engorged female and egg mass weights.

Discussion

This study evaluated the reproductive compatibility between four geographically distinct populations of *A. cajennense*. While populations SP (from southeastern Brazil), RO (from northern Brazil), and CO (from Colombia) were shown to be incompatible with each other, the Argentinean population (AR) was compatible with SP. Recently, Mastropaolo et al. (2010) reported reproductive incompatibility between two geographically distinct populations of *A. cajennense* from Argentina, namely a population from Copo National Park, and the same AR population evaluated in the present study. Reproductive incompatibility is not expected to occur between different populations of a single species. Thus, our results suggest that the taxon *A. cajennense* might be represented by a complex of different species, as previously speculated by other authors (Guglielmone et al. 1992; Beati et al. 2007; Mastropaolo et al. 2010). In fact, preliminary genetic analysis indicated notable genetic divergence between *A. cajennense* ticks within its distribution area (Beati et al. 2007). Further populational genetic studies, coupled with extensive morphological analyses, are needed to clarify and determine a possible complex of valid species that might have been classified under the taxon *A. cajennense.*

To the best of our knowledge, before the recent study of Mastropaolo et al. (2010), experimental studies on interspecific mating with ticks of the genus *Amblyomma* have been restricted to Gladney and Dawkins (1973), who cross-mated *Amblyomma maculatum* with *Amblyomma americanum*. The present results of the crosses between SP, RO, and CO ticks were quite similar to the results reported by Gladney and Dawkins (1973). When *A. maculatum* males were confined with *A. americanum* females inside infestation sleeves glued to hosts, the two sexes were seen in copula position, and females successfully engorged, detached, and oviposited large egg masses similarly to females from conspecific (homologous) infestations. However, % egg hatching ranged from 0 to at most 5% for *A. americanum* females mated by *A. maculatum* males, similarly to the egg masses derived from the heterologous crosses between SP, RO, and CO of the present study. Part of the F_1 larvae derived from crosses between *A. maculatum* × *A. americanum* was reared to the adult stage, always resulting in ticks phenotypically identified as females (Gladney and Dawkins 1973). The same result was observed in the present study, that is, only females were observed when heterologous crosses-derived F_1 larvae (CO × SP, RO × SP, and SP × CO) were reared to the adult stage. While Gladney and Dawkins (1973) reported malformations, such as the presence of gynandromorphs, fewer than the normal number of legs (7 or less) or “curly legs”, and the presence of “nymphal-adult intermediates” among some of their F_1 hybrid females, in the present study at least four F_1 females derived from SP × CO had seven legs, and one F_1 female derived from CO × SP had a “curly” atrophic leg (data not shown). In addition, both in the present and in the study of Gladney and Dawkins (1973), when some of these F_1 females were allowed to feed without males, they produced no offspring.

Gladney and Dawkins (1973) also reported that *A. americanum* virgin females, when allowed to feed in the absence of males, increased their feeding period, had smaller repletion weights, and oviposited none or only few fertile eggs when compared to females mated with conspecific males. Very similar results were observed in the present study, when SP, RO, or CO virgin females fed without males. Thus, as concluded by Gladney and Dawkins (1973), the few larvae produced by these females were a product of thelytokous parthenogenesis. Similarly, the few larvae produced by the interspecific crosses were rather a product of parthenogenesis than a successful fertilization of *A. americanum* females by *A. maculatum* males (Gladney and Dawkins 1973). The same conclusion can be applied to the present study; i.e., larvae generated by heterologous crosses between SP, RO, and CO adult ticks are likely to be a product of parthenogenesis, as corroborated to the fact that all F_1 adult ticks were phenotypically females. Finally, the main biological difference between virgin females, and females from the heterologous crosses were the significantly shorter feeding periods and larger engorged weights of the later females; what indeed indicate that SP, RO, and CO females copulated with males during heterologous infestations, although this copulation was not fertile, as observed by Gladney and Dawkins (1973) for *A. maculatum* and *A. americanum.*

Interestingly, the crosses between SP and AR ticks were highly fertile, similarly to homologous crosses. In addition, F_1 adults derived from heterologous crosses (both SP × AR and AR × SP) showed higher reproductive performance than the F_1 adults derived from homologous crosses (both AR × AR, and SP × SP). This superiority could be linked to the obvious heterosis that was generated by crossing two geographically distant populations of a given tick species, as previously reported for many other animal groups and plants (Veitia and Vaiman 2010). Thus, our results suggest that SP and AR ticks represent a single species. On the other hand, if SP, RO, and CO ticks represent different tick species, our results show that their reproductive incompatibility, a condition possibly related to genetic dissimilarity, has contributed to species integrity. In addition, other factors such as specific preferences for certain bioclimatic zones might also have contributed to species integrity.

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