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ORIGINAL PAPER

Comparative analysis of germ cells and DNA of the genus *Amblyomma*: adding new data on *Amblyomma maculatum* and *Amblyomma ovale* species (Acari: Ixodidae)

Fredy Arvey Rivera-Páez[1,2] · Bruno Rodrigues Sampieri[3] · Marcelo Bahia Labruna[4] · Renata da Silva Matos[1] · Thiago Fernandes Martins[4] · Maria Izabel Camargo-Mathias[1]

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Abstract Among tick species, members of the subfamily Amblyomminae have received special attention, since they serve as vectors for pathogens such as *Rickettsia* spp. and display cryptic species complexes that make their taxonomical classification challenging. *Amblyomma ovale*, *Amblyomma maculatum*, and other species of the genus *Amblyomma* have shown a long history of taxonomic controversies. Spermiotaxonomy has proved to be a valuable tool in the solution of systematic conflicts in Metazoa that can aid molecular and external morphological analyses in ticks and, overall, provide more robust analyses and results. With this in mind, this study included histological analyses of the reproductive system of the species *A. ovale* and *A. maculatum*, as well as the description of morphohistological characters of the male reproductive system of ticks of the genus *Amblyomma*, in order to evaluate these characters within the current clustering proposals. In addition, 16S rDNA and COI (mitochondrial) molecular markers were used to study the genetic relationships of the species. The results show that the tick male reproductive system and its germ cells contain useful candidate characters for taxonomical analyses of Ixodida.

Keywords *Cryptic species* · Spermiotaxonomy · Ticks · Histology · Molecular markers

The original version of this article was revised: The data in Table 3 were misaligned. Correct table alignment is presented here.

✉ Maria Izabel Camargo-Mathias
micm@rc.unesp.br

[1] Departamento de Biologia, Instituto de Biociências, UNESP - Universidade Estadual Paulista, Avenida 24-A, 1515, Bairro Bela Vista, Rio Claro, SP CEP13506-900, Brazil

[2] Grupo de Investigación GEBIOME, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas, Calle 65 No. 26-10 Apartado Aéreo 275 Manizales, Caldas, Colombia

[3] Universidade Estadual de Campinas, Museu de Zoologia, R. Charles Darwin s/n, cidade universitária, Campinas, SP, Brazil

[4] Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo-USP, Av. Prof. Orlando Marques de Paiva, 87, CEP 05508-000, Cidade Universitária, São Paulo, SP, Brazil

Introduction

Tick (Ixodidae) systematics has been discussed for decades, and periodically, the phylogeny of several groups is revisited through new analytical tools. Among the most known tick subfamilies, Amblyomminae has received special attention, since it harbors cryptic species that, until recently, were considered polymorphic by authors that followed the analyses of Hoogstraal and Aeschlimann (1982). Some important revisions have been made using molecular and biogeographical phylogeny techniques such as those developed by Black and Piesman (1994), Burger et al. (2012), Beati et al. (2013), and Nava et al. (2014), which confirmed the polyphyletic origin of the subfamily, its current distribution in the American continent, and the real taxonomic status of some species of medical and veterinarian importance, such as the *Amblyomma cajennense* sensu lato complex.

The *Amblyomma maculatum* group includes the following species: *A. maculatum* (Koch, 1844); *Amblyomma neumanni* (Ribaga, 1902); *Amblyomma parvitarsum* (Neumann, 1901); *Amblyomma tigrinum* (Koch, 1844); and *Amblyomma triste* (Koch, 1844) (Camicas et al. 1998). Camicas et al. (1998) clustered them within the *Amblyomma ovale* group that includes *A. ovale* and *Amblyomma aureolatum*, in the revisited version of the subgenus *Anastosiella*, originally proposed by Santos Dias (1963).

The taxa *A. maculatum*, *A. triste*, and *A. tigrinum* were described as valid species by Koch (1844), based on adult specimens from the USA, Uruguay, and Brazil, respectively. However, Neumann (1899) considered that these three taxa belonged to a single species, namely *A. maculatum*, which was adopted by subsequent authors, until the study of Kohls (1956), who revalidated the three species based on morphological characters. Since then, although considered to be separate species, the identification of these tick species has been controversial, in particular the distinction between *A. maculatum* and *A. triste* (Estrada-Peña et al. 2005; Guglielmone et al. 2013; Lado 2015; Mendoza Uribe and Chavez Chorocco 2004; Mertins et al. 2010). Lado (2015), based on different mitochondrial and nuclear molecular markers, suggests that *A. triste* should be synonymized with *A. maculatum*.

On the other hand, *Amblyomma aureolatum* (Pallas, 1772) and *Amblyomma ovale* (Koch, 1844) have been described as the "*ovale*" complex by Aragão and da Fonseca (1961). These authors redescribed the male and female of both tick species, discussed the systematics, and convincingly supported the use of the name *A. aureolatum* and *A. ovale* (Guglielmone et al. 2003).

Against this backdrop, this study aimed to reanalyze the systematics and phylogenetic positions of representatives of Amblyominnae subfamily, including the species *A. ovale* and *A. maculatum* collected in Colombia, adding new data related to their reproductive system and germ cell morphology given the association of this species with epidemiological issues and public health in North and South America (Ferrari et al. 2012; Guglielmone et al. 2003; Lado et al. 2015; Nava et al. 2008). Furthermore, this study compiled the results obtained until now, based on the descriptions by Sampieri et al. (2014, 2016a, b), on the anatomy and morphology of the male tick reproductive system, mainly the genus *Amblyomma* with occurrence in Brazil and Colombia.

A list of useful characters in taxonomic analyses was obtained from the results generated in these studies adding the new data obtained here which were contrasted with the molecular data generated from the analysis of the mtDNA COI and 16S rDNA gene sequences, in order to confirm if the male reproductive system and its germ cells are a source of candidate characters for tick systematics.

Materials and methods

Study species and molecular evaluation

During the months of August 2014, January and August 2015, and May 2016, ticks of the species *A. ovale* and *A. maculatum* were directly collected from dogs (*Canis familiaris*) in the municipalities of Saldaña (3° 55′ 36″ N, 74° 58′ 33″ W) and Ibagué (04° 23′ 50″ N, 75° 8′ 12″ W) in the department of Tolima, Andean region of Colombia. The ticks were taxonomically identified based on their external morphology, using a light microscope (Leica M205C stereomicroscope), following Aragão and da Fonseca (1961), Jones et al. (1972), and Estrada-Peña et al. (2005). In addition, specimens from each species were prepared for scanning electron microscopy (SEM) (Hitachi Scanning Electron Microscope model TM3000), according to the techniques described by Corwin et al. (1979).

Following the morphological identification, a molecular assessment was performed for the species *A. ovale* and *A. maculatum*, as well as for the species analyzed in terms of their reproductive system by Sampieri et al. (2014, 2016a, b), being these: *A. aureolatum*, *A. triste*, and *Amblyomma sculptum* (Berlese, 1888) (Ixodidae, Amblyomminae). The species *Rhipicephalus sanguineus* s.l. (tropical linage) and *Ornithodoros rostratus* (Aragão, 1911) were used as outgroups I and II, respectively. In the same way, the reproductive system of *A. ovale* and *A. maculatum* (Colombia) were processed and analyzed by histological techniques as described in Sampieri et al. (2016a, b).

Specimens of each species, *A. aureolatum*, *A. triste*, *A. sculptum*, *A. ovale*, *A. maculatum*, and *Rhipicephalus sanguineus* s.l., were individually processed for the molecular analyses. For this purpose, DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol, and tested by two PCR protocols, one targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene and the other the 16S rDNA gene. For the COI gene PCR, we used primers LCO1490 (F) 5-GGTCAACAAATCATAAAGATATTGG-3 and HCO2198 (R) 5-TAAACTTCAGGGTGACCAAAAAATCA-3 (Folmer et al. 1994). For the 16S rDNA gene, we used primers F 5′-CTGCTCAATGATTTTTTAAATTGCTGTGG-3′ and R 5′-CCGGTCTGAACTCAGATCAAGT-3′ (Norris et al. 1996). In addition to the *Amblyomma* species used for the morphological studies, the species *A. cajennense* s.s. (Brazil) and *A. mixtum* (Colombia) were also amplified, as representatives of the "*cajennense*" complex.

The PCR products were visualized on horizontal 1% agarose gels with 1X TBE pH 8.0 running buffer at 110 V/50 mA, stained with SYBR Safe® dye and photo-documented on a GelDoc-It®2310 Imager (UVP). The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen®), according to the manufacturer's instructions, and sent to Macrogen Inc. (South Korea) for DNA sequencing. The sequenced fragments were analyzed and edited using Geneious Trial v8.14 (Drummond et al. 2009) and Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). In addition, the sequences were searched by MegaBlast against the public databases and deposited in Genbank and Barcode of Life Data Systems (BOLD). The sequences for each gene

were aligned using ClustalW (Thompson et al. 1997), included in the program MEGA version 7 (Tamura et al. 2013). Intraspecific nucleotide divergences were estimated with the program MEGA, using the Kimura 2 Parameter distance model (K2P; Kimura 1980). Species confirmation was carried out through a similarity analysis, based on maximum likelihood (ML), with the K2P model and 1000 bootstrap replications, using the program MEGA.

Morphohistology and matrix construction

For this study, we initially used the morphological characters of the male reproductive system obtained by Sampieri et al. (2014, 2016a, b). The characters were centered on three representative species of the genus *Amblyomma*: *A. aureolatum* (A), *A. triste* (B), and *A. sculptum* (C), and the species *Rhipicephalus sanguineus* s.l. (tropical lineage) and *Ornithodoros rostratus* were used as outgroup 1 (GE1) and outgroup 2 (GE2), respectively. In addition to the previously mentioned species, the reproductive system of *A. ovale* (D) and *A. maculatum* (E) were analyzed through histological techniques. Since these specimens were collected in situ while feeding on the blood of their natural hosts and their feeding was interrupted, it was not possible to describe the complete morphohistology of their reproductive system, given that the system's development is directly related to feeding.

Candidate characters with relevant variations were obtained from the observation of the morphology and ultramorphology of the male reproductive system of these species, and were coded based on the character state, such as apomorphic = 1 and plesiomorphic = 0. Then, a character matrix containing the taxa, characters, and character state for each species (0 or 1) was constructed. We determined the qualitative Jaccard index based on 10 morphological characters (Moreno 2001); furthermore, we used cluster analysis of the single linkage based on the Jaccard index in order to compare the segregation pattern of the species included in the study. The cluster analysis was performed using the software PAST 3.11 (Hammer et al. 2001).

This study was submitted and approved by the Ethical Committee in Animal Use (CEUA) from the Biological Sciences Institute of UNESP, Rio Claro, SP, Brazil, number 017/2012, Protocol 1422.

Results

Study species and molecular assessment

The morphological analyses showed that the ticks collected from dogs in Colombia were most similar to *A. maculatum*, as indicated by the scutal ornamentation, coxal spur patterns, and presence of tubercles at the postero-internal angle of all festoons, except for the middle one (Fig. 1a–c). Although, the *A. maculatum* specimens from Colombia typically were shown to have a pair of stout ventral spurs on the distal extremity of metatarsi II, III, and IV (Fig. 1c). The determination of *A. ovale* was carried out based on the scutal ornamentation patterns in the females, marginal grooves of the males (incomplete in *A. aureolatum* and complete in *A. ovale*), and differences in spurs of coxa I in both sexes.

The molecular analyses of the 403 bp 16S rDNA gene fragment and 480 bp COI gene fragment showed topologically similar phylogenetic trees (Fig. 2a, b) and allowed a clear determination of the study species, with the exception of *A. maculatum* and *A. triste*, which showed interspecific genetic distances too small to be considered different species, ranging between 0.0 and 2.1 for 16S and between 0.0 and 5.2 for COI, with the highest values found for *A. maculatum*

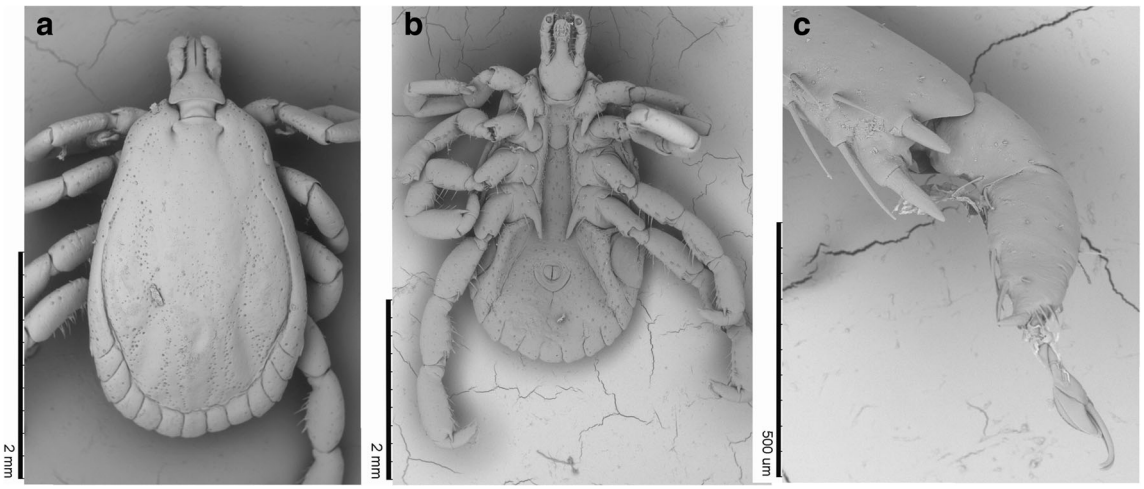

Fig. 1 *A. maculatum* specimens from Ibagué- Colombia. **a** Male dorsal view *A. maculatum.* **b** Male ventral view *A. maculatutum*. **c** Spines on metatarsus IV of female *A. maculatum*

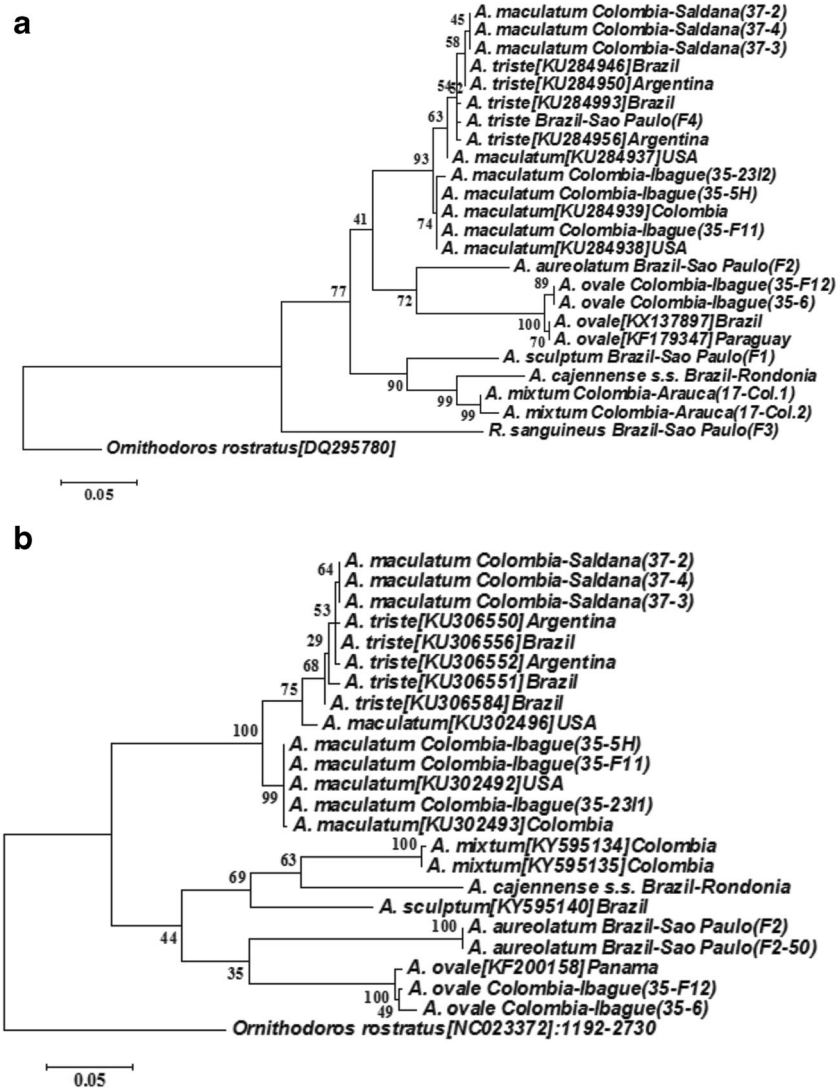

Fig. 2 **a** Maximum likelihood (ML) tree using the sequences of the mtDNA 16S rDNA gene. **b** Maximum likelihood (ML) tree using the sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene

(Tables 1 and 2). These interspecific genetic distances allowed us to cluster the species belonging to the *A. cajennense* s.l., *Amblyomma maculatum*, and *Amblyomma ovale* complexes (Fig. 2a, b). For the genus *Amblyomma*, interspecific genetic distances ranged from 6.1–23.8 for 16S and 16.2–26.9 for COI, excluding the differences found between *A. triste* and *A. maculatum*, which ranged between 0.3–2.1 and 0.2–5.2 for 16S and COI, respectively (Tables 1, 2).

Table 1 Kimura 2 parameter (K2P) distances (in percentage) for the mtDNA 16S rDNA gene

Tick species	*A. ovale*	*A. aureolatum*	*A. maculatum*	*A. triste*	*A. cajennense* s.s.	*A. mixtum*	*A. sculptum*	*R. sanguineus*	*O. rostratus*
A. ovale	0.0–0.9								
A. aureolatum	14.6–14.9	–							
A. maculatum	14.6–15.3	12.5–13.9	0.0–2.1						
A. triste	15.3–16.0	13.5–14.2	0.3–2.1	0.0–0.9					
A. cajennense s.s.	23.4–23.8	18.6	15.3	15.0–16.0	–				
A. mixtum	20.6–22.6	15.3–16.8	13.1–14.9	13.1–15.6	6.1–7.4	1.2			
A. sculptum	19.0–20.1	15.6	15.3	15.6–16.4	13.2	10.8–12.1	–		
R. sanguineus	28.7–29.1	24.5	22.5–23.7	23.3–23.7	26.7	26.2–27.4	24.5	–	
O. rostratus	34.0–35.0	35.9	27.4–27.8	27.8–29.1	38.4	35.4–37.3	32.7	36.9	–

Table 2 Kimura 2 parameter (K2P) distances (in percentage) for the mitochondrial cytochrome c oxidase subunit I (COI) gene

Tick species	*A. ovale*	*A. aureolatum*	*A. maculatum*	*A. triste*	*A. cajennense* s.s.	*A. mixtum*	*A. sculptum*	*O. rostratus*
A. ovale	0.8–1.7							
A. aureolatum	20.4–21.2	0.0						
A. maculatum	18.9–21.5	25.1–26.0	0.0–5.2					
A. triste	18.9–20.1	25.0–25.7	0.2–5.2	0.2–1.3				
A. cajennense s.s.	21.1–21.7	25.7	26.2–26.9	25.4–26.6	–			
A. mixtum	20.0–20.9	23.3–23.6	20.2–21.1	20.2–21.1	16.2–16.4	0.2		
A. sculptum	22.0–22.3	21.6	21.4–22.2	21.6–21.9	17.0	16.9–17.1	–	
O. rostratus	28.6–29.2	31.8	27.7–30.2	29.5–29.9	32.8	32.8	27.4	–

Morphohistology

The male reproductive system of *A. maculatum* and *A. ovale* studied here showed a basic morphology similar to other species of the genus and the family Ixodidae. For both species, it was not possible to observe if the testicles were connected to the distal region. However, both species showed germ cells organized in packets lined with a simple epithelium along the testicles up to a certain spermiogenesis developmental stage.

In *A. maculatum*, only two spermatid developmental stages were observed: (a) the initial developmental stage (sp I), where no cell limits are evident and the nucleus is round, occupying most of the cytoplasm (Fig. 3a, b) and (b) mature spermatids (spm) in the interior of the seminal vesicles that display clear cell limits, with the central region of the cytoplasm enclosing the membranous complex, a filiform, and extensive nuclear process; caudal region without constriction, with a round pole; and anterior region with a round rimless operculum (Fig. 3c).

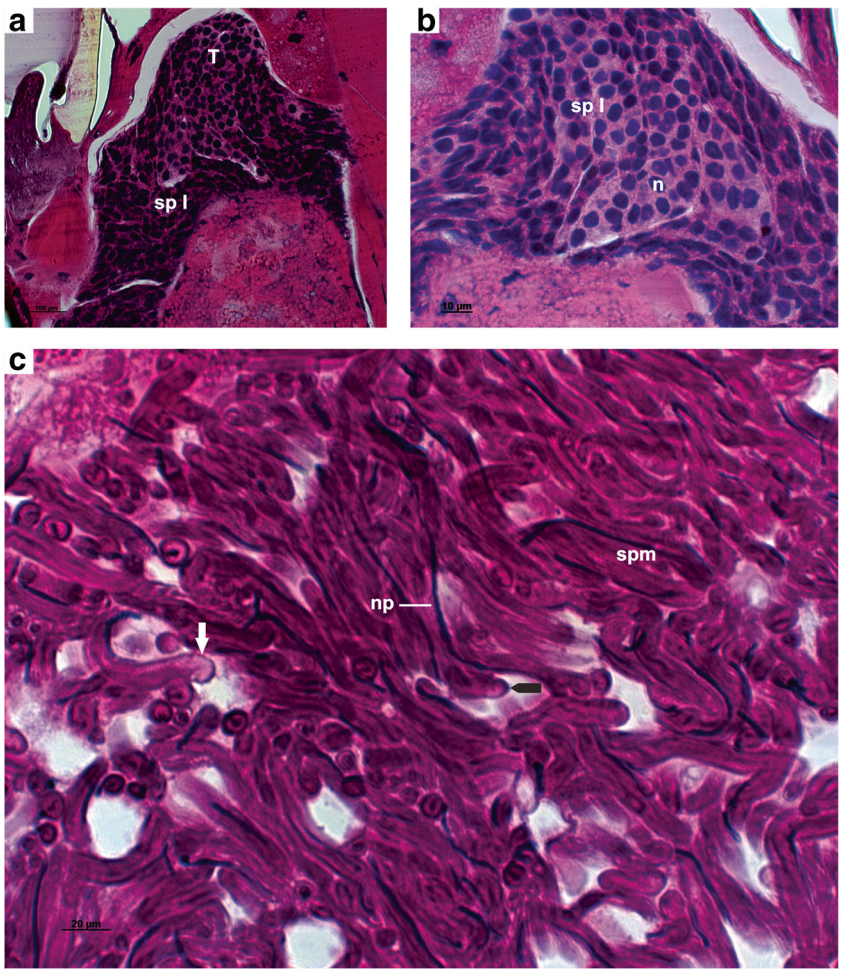

Fig. 3 Histological sections of the *Amblyomma maculatum* reproductive system (Ibagué - Colombia). **a**–**c** Testis housing spermatids I (sp I). Mature spermatids (spm) exhibiting a round operculum (arrow)

In *A. ovale*, several spermiogenesis stages were observed, for a total of six morphologically distinct stages. The first stage displayed an identical morphology to that described for *A. maculatum* (Fig. 4a, b). In the second stage (sp II), the cells begin a hypertrophy process with a cytoplasmic enlargement and no evident cell limits (Fig. 4c). The spermatid cell limits were observed only by the third development stage (sp III), indicating the beginning of formation of membranous cisternae. In this stage, the nucleus is enlarged and round, with one of two visible nucleoli, and the presence of cytoplasmic bridges between cells (Fig. 4d). The most marked morphological alterations can be observed at stage IV, in which cell limit enlargement is evident, the spherical cell shape becomes amoeboid, the nucleus becomes compact, and the cytoplasm is very heterogeneous (Fig. 4e, f). The next stage, sp V, is characterized by cell elongation and membrane cisternae fusion, as well as a marked condensation and elongation of the nucleus, which at this moment is referred to as the nuclear process; in addition, there is a total loss of spermatocyte organization (Fig. 4g).

The sixth and last stage (sp VI) of spermiogenesis exhibits a typical tick spermatic cell: elongated and filiform,

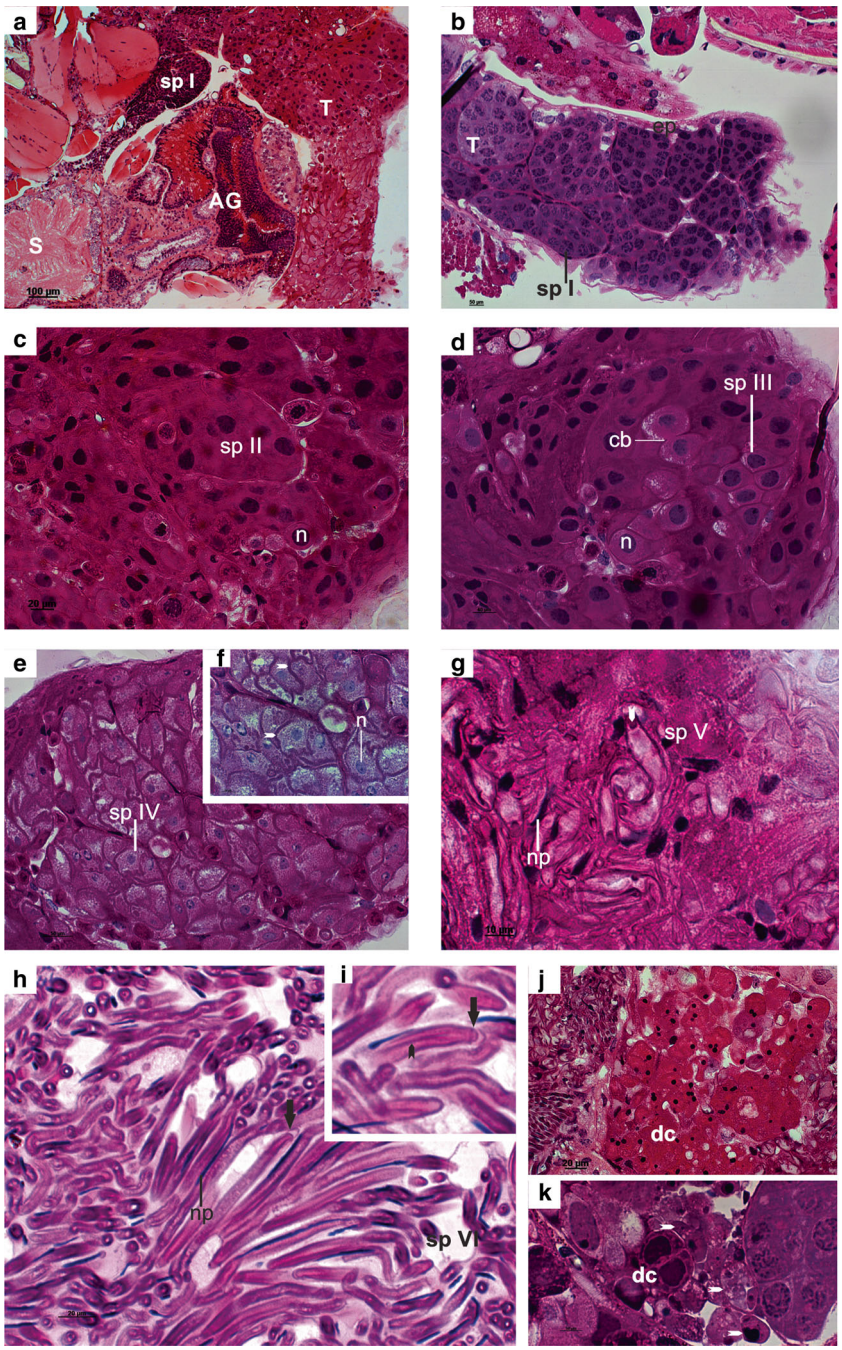

Fig. 4 Histological sections of the *Amblyomma ovale* reproductive system (Saldaña - Colombia). **a** Overview of the reproductive system, highlighting the accessory glad complex (AG) and testis (T) housing spermatocysts and germ cells in different stages. **b–d** Spermatocysts housing spermatids I (sp I), II (sp II), and III (sp III). **e**, **f** Spermatid IV (sp IV) going through hypertrophy and cisternae formation (arrowhead). **g** Spermatid V (sp V) characterized by the elongation process. **h**, **i** Spermatids VI (sp VI) matured, highlighting the nuclear process (np) and the operculum (arrow). **j**, **k** Spermatids undergoing degeneration process (DC). cb = cellular bridges; n = nucleus; S = singanglium

with a nuclear process highly stained by hematoxylin and positioned in the cellular cortex, with a membranous complex in the center of the cytoplasm that longitudinally crosses the spermatid. Furthermore, sp VI of *A. ovale* characteristically shows a cone-shaped operculum with a rim at its base, evident in histological sections, with a tail-like non-constricted posterior cell region and cone-shaped pole (Fig. 4h, i). In *A. ovale* and *A. maculatum*, many degenerating germ cells were observed, which displayed autophagy and varied apoptosis stages that included apoptotic bodies (Fig. 4j, k).

Character selection for matrix construction

The species *O. rostratus* (Argasidae), used as outgroup, has plesiomorphic characters, since it is known that representatives of the family Argasidae show basal characters and it is likely that the ancestor that gave rise to Ixodidae was similar to the members of this family. Therefore, the characters chosen and the stages used in this study were (1) testicle anatomy—single testicle (0), paired testicles with connection (1), and individualized paired testicles (1); (2) seminal vesicle—lateral disposition (0) and dorsal (1); (3) spermatogenesis—finalized at the immature stage (0) and finalized at the adult stage (1); (4) cytoplasmic bridges—absent (0) and present (1); (5) nucleus shape and chromatin condensation in the spermatid with cisternae formation—round with centralized condensed chromatin (0) and oval with peripheral condensed chromatin (1); (6) number of stages of membranous cisternae formation in spermatids—three (0) and two (1); (7) nuclear process formation—not evident (0) and evident (1); (8) operculum rim—present (0) and absent (1); (9) midline constriction of the tail-like in mature spermatid—presente (0) and ausente (1); and (10) nucleus shape in mature spermatid—filiform (0) and spiral (1).

The data gathered is included in Table 3, which displays the following results: (a) characters 1 and 2 are apomorphic for species A–E and GE1; (b) character 3 shows a synapomorphy between all taxa, except for B; (c) characters 4 and 6 cluster species A–D and GE1; and (d) characters 5, 8, 9, and 10 do not show any evident clustering and will be discussed below. The qualitative

Table 3 Morphological characters matrix used for evaluation of the male reproductive system of ticks

Taxons \ *Character*	1	2	3	4	5	6	7	8	9	10
A	1	1	0	1	1	1	1	0	0	1
B	1	1	1	1	1	1	1	1	0	1
C	1	1	0	1	1	1	1	0	1	0
D	1	1	0	1	0	1	1	0	1	0
E	1	1	0	--	--	--	1	1	1	0
GE1	1	1	0	1	1	1	0	1	1	1
GE2	0	0	0	0	0	0	0	0	0	0
	ABCDE	**ABCDE**	**ACDE**	**ABCD**	**ABC**	**ABCD**	**ABCDE**	**BE**	**CDE**	**AB**

GE *Grupo Externo*, ***A*** *A. aureolatum*, ***B*** *A. triste*, ***C*** *A. sculptum*, ***D*** *A. ovale*, ***E*** *A. maculatum*

Table 4 Jaccard's index of similarity—morphological characters used for evaluation of the male reproductive system of ticks

	A	B	C	D	E	GE1	GE2
A	1						
B	0.66	1					
C	0.66	0.43	1				
D	0.54	0.33	0.81	1			
E	0.40	0.40	0.75	0.75	1		
GE1	0.54	0.54	0.54	0.43	0.55	1	
GE2	0.17	0.05	0.17	0.25	0.16	0.11	1

GE *Grupo Externo*, ***A*** *A. aureolatum*, ***B*** *A. triste*, ***C*** *A. sculptum*, ***D*** *A. ovale*, ***E*** *A. maculatum*

Jaccard index showed that the analysis of the 10 morphological characters allowed to differentiate each of the species studied, since the similarity coefficient is always less than 1 (Table 4). The cluster analysis of the single linkage based on the Jaccard index, in order to compare the segregation pattern of the species included in the study, clustered the five species of the subfamily Amblyomminae, excluding the species *Rhipicephalus sanguineus* s.l. (tropical lineage) and *Ornithodoros rostratus*, used as outgroup 1 (GE1) and outgroup 2 (GE2), respectively (Fig. 5).

Discussion

The external morphology of *A. ovale* and *A. maculatum* corresponded to that proposed by Aragão and da Fonseca (1961), Estrada-Peña et al. (2005) and Jones et al. (1972). The 16S and COI genes analyses confirmed the current taxonomic proposals, supporting the separation of the subfamilies *Amblyominnae* and *Rhipicephalinae* and of the species complex within the genus *Amblyomma*. The 16S and COI genes indicate that *A. maculatum* and *A. triste* may in fact be a single species with a wide geographic distribution, as has been proposed by other authors (Estrada-Peña et al. 2005; Lado 2015). However, it is noteworthy that the intraspecific genetic distance of *A. maculatum* is the highest of all the species studied, which could indicate that the external morphological evaluation has its limitations, and many individuals that are characterized as *A. maculatum* can actually correspond to other species of the "*maculatum*" complex. This can only be confirmed through experimental crosses between species of the complex from different localities.

The taxonomical analysis performed in this study presents important information regarding the phylogeny of the ticks of the genus *Amblyomma*, which has been the center of constant controversies and revalidations in the past years. Despite the evident need for a greater sample size of taxa for the genus, as well as a broader character selection for cladogram construction, several homology hypotheses are informative and consistent with the well-established clusters. The novel aspect of this study is the implementation of morphological characters not used until now, in order to understand the relationships between tick species. As the studies by Sampieri et al. (2016a, b) have shown, spermiotaxonomy is a promising tool for separating Ixodida species, and their phylogeny can benefit from the information generated through this analysis and the understanding of tick morphology and ultrastructure of the reproductive system and its germ cells.

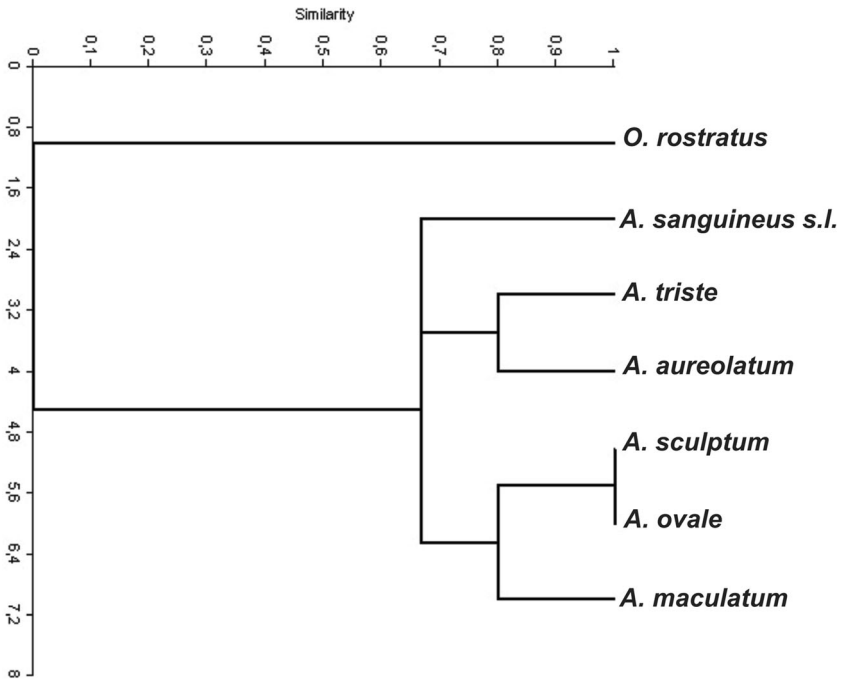

Fig. 5 Cluster analysis of the single linkage with Jaccard distance in order to compare the segregation pattern of the species included in the study. Characters (1, 2, 7–10). **A. sculptum* is different from *A. ovale* in character 5 (character not included)

This species separation is possible by analyzing characters 8, 9, and 10 (Table 3), which do not generate plausible clustering hypotheses, since in all three cases, the species *R. sanguineus* was clustered with *Amblyomma* species. For character 8, *A. sculptum*, *A. ovale*, and *A. aureolatum* were excluded from the cluster, while for character 9, *A. triste* and *A. aureolatum* were excluded, and for character 10, *A. sculptum*, *A. ovale*, and *A. maculatum* were not part of the cluster. In view of this, the three characters (8—presence of operculum rim, 9—midline constriction of the tail-like region, 10—nuclear process morphology) can be used together to analyze the ultramorphology of mature spermatids of each species and allow their separation, since these characters, along with operculum shape (unique to each species studied to date), can aid in confirming two or more closely related species. This information allows us to suggest that *A. maculatum* (Colombia) and *A. triste* (Brazil), belonging to the "*maculatum*" complex in the Americas, are representatives of different species, according to our morphohistological study (Similarity 0.4) (Table 4); however, these results must be complemented with studies at the ultrastructural level.

Characters 1 and 2 cluster taxa A–E and GE1, thereby clustering all of the species of the genus *Amblyomma* and *R. sanguineus* and suggesting that these are a synapomorphy of the family Ixodidae. In a similar manner, the homology proposed by characters 4 and 6 most likely clusters this family, since they only exclude taxon E (due to the lack of information on these characters for this taxon). In the case of character 3, the only species excluded from the cluster is *A. triste*, which is questionable whether this character is eligible for this type of analysis. An incorrect interpretation of the studies of Sampieri et al. (2016b) could have occurred regarding the presence of spermatocytes II in cell division in adults of *A. triste* (which is why we concluded that the first analysis of character 3 was not eligible for cladistic analysis). Character 5 suggests an improbable clustering and could have been observed from morphological differences generated from technical artifacts.

Character 7 suggests an interesting clustering, since taxa A–E are included, and it shows the formation of a nuclear process that could probably be a synapomorphy of the subfamily Amblyomminae. In this analysis, the characters observed from previously published studies and the data presented herein show the relevance of identifying novel characters for research in taxonomy and phylogeny, since family- and subfamily-level clustering is possible in these cases and, in the same way, the separation of cryptic species can be complemented with this information (Table 4) and cluster characters (1, 2, 7–10) (Fig. 5).

For a spermiotaxonomy robust analysis, a wide sampling and experimentation is required, but still can be a useful tool in solving problems of this nature when DNA analysis is ambiguous or not conclusive, as in the case of the comparison between *A. maculatum* and *A. triste*.

Although this is a preliminary study, the data obtained indicate that the morphology and ultrastructure of the male reproductive system in ticks can generate eligible characters for establishing homology hypotheses and cladistic studies. A robust phylogeny for Ixodida can be constructed through detailed studies of these characters, making use mainly of ultrastructural analyses and relating ticks with other Parasitiformes with widely studied reproductive systems and spermatozoids, thus contributing to an integrative taxonomy of this group.

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