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High gene flow in *Amblyomma ovale* among fragments of Brazilian Atlantic rainforest

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Abstract

The genetic structure of populations of the tick *Amblyomma ovale* from five distinct areas of the Brazilian Atlantic rainforest was evaluated via DNA sequencing and associated with the presence of domestic dogs acting as hosts at the edge of forest fragments. Ticks were collected from domestic dogs and from the environment between 2015 and 2017. Four collection areas were located in the surroundings and within the Serra do Mar State Park, São Paulo State (23°37'21"S, 45°24'43"W), where dogs were

bimonthly monitored along two years using camera traps and GSM trackers. To determine the spatial limits of genetic structure, ticks collected upon dogs living near the Serra do Baturié, Ceará State (4°15'40"S, 38°55'54"W) were included as well. A total of 39 haplotypes of 16S rRNA and *Cox* 1 mitochondrial genes sequences were observed, with 27 of them coming from areas within the Serra do Mar State Park. No haplotype was shared between the Serra do Mar and the Serra do Baturié indicating isolation of tick populations at the scale of 2,000 km. Although three different haplotype lineages of *A. ovale* occurred within the Serra do Mar State Park, no genetic structure was found across the study sites within this park, suggesting high tick gene flow across a range of 45km. Monitoring data from domestic dogs and wild carnivores showed that these species share the same habitats at the forest edge, with dogs playing a likely limited role in tick dispersal. Our findings have important implications for understanding the genetic structure of wide spread *A. ovale* along Brazilian rainforest remnants, which can further be associated to tick-borne infectious agents, such as *Rickettsia parkeri*, and used for predicting future patterns of tick diversity in the Brazilian Atlantic rainforest.

Introduction

By definition, a host represents most of the ecological requirements for a parasite. This dependency often demands specific adaptations that can lead to population divergence and even speciation when parasites exploit different host types (Dupraz et al., 2016; McCoy et al., 2013; De Meeus, 2012). The recent application of molecular techniques to parasitic organisms has provided insightful information on their biology and ecology, which has been useful to measure parasite population structuring and to approach the

factors associated with divergence, such as spatial isolation and host type. However, understanding the relative importance of these factors in structuring populations can be particularly challenging when parasites have broad distributions and use several host species to complete the life cycle (Dupraz et al., 2016; Mazé-Guilmo et al., 2016; Nadolny et al., 2015). This is frequently the case for ticks, where two or more phylogenetically distant hosts may be used for blood feeding in immature and adult life stages. For example, hard ticks (Ixodidae family) often need a minimum of two distinct host species for complete development: commonly one host type for larval and nymphal stages and a different one for the adult phase (Krawczak et al., 2016a; Martins et al., 2016; Ogrzewalska et al., 2012). Still, specificity for a particular host can vary. Moreover, there is frequently a range of different potential host species that can be exploited during each developmental stage. This complex life cycle provides these ectoparasites the chance to maintain populations across different ecological conditions and to disperse at different spatial scales (Krawczak et al., 2016b; Mazé-Guilmo et al., 2016; Ogrzewalska et al., 2012).

The phylogenetic relationships within the *Amblyomma* genus has been widely discussed and revised over the last decades, especially the branch which includes *Amblyomma ovale* (Murgas et al., 2013; Aragão and Fonseca., 1961). While the geographic distribution of this tick extends across the Neotropical region from central-northern Argentina into the Nearctic region of Mexico, with a few records from the United States and Caribbean (Guglielmone et al., 2003), the knowledge on its life cycle has still some significant gaps, mainly in relation to host use (Krawczak et al., 2016b; Martins et al., 2012). Although small rodents are considered to play an important role for larvae and nymphs of *A. ovale* under natural conditions (Szabó et al. 2013), wild birds have been reported to be parasitized in some regions of Brazil (Ramos et al. 2015; Luz and

Faccini, 2013; Ogrzewalska et al., 2009). In addition, *Gallus gallus* was found to be an adequate host for immature stages under laboratory conditions (Martins et al., 2012). These results suggest that birds may play a role in maintaining immature of this tick in natural populations and, as a consequence, may also disperse specimens across geographic locations (Luz and Faccini, 2013; Ogrzewalska et al., 2009). In turn, adults of *A. ovale* are considered to prefer wild carnivores but may frequently use dogs, especially if they are in contact with forest habitats (Maturano et al., 2015; Szabó et al., 2013; Martins et al., 2012; Sabatini et al., 2010; Ogrzewalska et al., 2009).

The Brazilian Atlantic rainforest is considered a hotspot of biodiversity, with one of the world's most rich environments in terms of species (Ribeiro et al., 2009). It is also one of the most threatened biomes on the planet. Its original range stretched along the eastern Brazilian coast and covered 131,546,000 hectares (Frigeri et al., 2014; Verdade et al. 2004). In contrast, if taking into account forest fragments above 100 hectares in size, only 8.5% of its original surface remains currently (Castilho et al. 2012). If forest patches above three hectares are considered then this proportion increases to 12.5% (Schnell et al. 2013). Seventy percent of the Brazilian population currently lives among the remaining forest patches of this biome (IBGE, 2017). To help conserving the remaining native areas, several protected areas were created. One of these is the Serra do Mar State Park (IUCN category II), located on the seacoast of São Paulo State, with 332,000ha spanning across and around 25 cities (Gigliotti and Santos, 2013). Currently, this is the largest continuous remaining fragment of the Brazilian Atlantic rainforest. Given the high level of deforestation and perturbation of this natural rainforest ecosystem, the capacity of forest patches to sustain large mammals such as wild carnivore populations is limited, and access of domestic animals (i. e. dogs) into the forest area has notably increased (Frigeri et al. 2014). As consequence, contact with

parasites typically associated with wildlife is expected (Frigeri et al., 2014; Sabatini et al., 2010). Indeed, over the last decade, a *Rickettsia parkeri* strain Atlantic rainforest-causing rickettsiosis has emerged in eastern Brazil, where it is primarily transmitted by *A. ovale* ticks (Krawczak and Labruna, 2018; Krawczak et al., 2016a; Szabó et al., 2013). This emergence could be directly linked to an increased human exposure to ticks brought via domestic dogs entering Atlantic rainforest patches, one of the natural habitats of *A. ovale* (Szabó et al., 2013; Spolidorio et al., 2010; Labruna et al. 2007). The use of different alternative hosts along the forest edge may also result in a divergence between wild and domestic parasite populations, leading to associated changes in parasite ecology and, in the case of ticks, in the frequency and circulation of associated pathogens.

To investigate gene flow between wild host populations and dogs within forest patches and among the forest edge, between 2015 and 2017 we collected specimens of *A. ovale* upon dogs and directly from the environment in five remnants of Atlantic rainforest. Four of these areas were located on the boundaries of the Serra do Mar State Park, where dogs were monitored bimonthly through camera traps and GPS/GSM trackers. A fifth population was used to estimate the degree of long-range gene flow and included the analysis of ticks collected upon dogs living in the edge of an Atlantic rain forest fragment in Guaramiranga municipality, another preserved area of Atlantic rainforest located at northern limit of the biome, near 2,000 km away from the Serra do Mar State Park.

Methods

Study area

The study comprised four areas (A1-A4) within a section of the Serra do Mar State Park in the municipality of Caraguatatuba, along the northern coast of São Paulo State, in southeastern Brazil (Figure 1). This municipality is 48,510 ha wide, with a population of 116,786 inhabitants living between the coast and the forest (IBGE, 2017). Social and economic inequality is significant, and the use of the forest by the local population reflects this situation. As an external location to the Serra do Mar State Park, we included ticks collected in Serra do Baturié (A5), located in a Brazilian Atlantic rainforest fragment in Ceará State, northeast Brazil (Figure 2). A straight line between Areas 1-4 and Area 5 is 2,325 km long.

Within the Serra do Mar State Park, samples were obtained from four different areas (Figure 1), three (A1 - A3) at the park edge, where people and dogs live in close contact with the forest, and one completely within the park (A4). A1 was emplaced within a poor community characterized by subsistence plantations and hunting, 15 km away from A2. This last area was located into a low-income community as well yet without economic exploitation of the forest and separated from A3 by 15 km. A3 was represented by a middle-income residential zone, where inhabitants use the forest for leisure activities. Finally, A4 was located inside the limits of the park, in an area where dogs supposedly had no access, and the human dwellers correspond exclusively to the Park staff; this area was 3 km away from the following area (A2).

The areas where ticks were collected directly from vegetation were selected according to the following criteria: topographic gradient (from sedimentary plains to low and high slope mountain), altitude below 300 m, and accessible trails. The presence of dogs with access to forest edges was also an inclusion criterion for the selected areas.

Dog sampling and collection of free-living ticks

We sampled a cohort of 20 dogs, including individuals older than one year only, and born and raised in the region as described elsewhere (Pinter et al., 2008). In each of areas A1 – A3, 20 dogs were sampled every two months, comprising a total of 60 dogs sampled on six occasions between June 2015 and April 2016. During each visit, dogs were examined for the presence of ticks.

Free-living ticks were collected in A4, inside the Serra do Mar State Park, where dogs supposedly had no access. To reach this area, a 3-km trail was walked in the direction of the forest core in April 2017. The ticks found on the vegetation were collected along the trail using drag sampling (Arzua et al., 2006).

All adult ticks collected were preserved in 70% ethanol and taken to the Laboratory of Parasitic Diseases of the School of Veterinary Medicine and Animal Science of the University of São Paulo, where they were morphologically identified to species level following Barros-Battesti et al. (2006). Collecting procedures were previously approved by the "Instituto Chico Mendes (ICMBio-SISBio permit 42793), "Companhia Ambiental do Estado de São Paulo" (CETESB – SP) and by the Ethics Committee of the School of Veterinary Medicine of the University de São Paulo (certificate number 6441270114).

Telemetry data

Six Global System for Mobile Communication (GSM) GPS-TK102B (CU-TEK®) trackers were used to track dog movements, two in each of areas A1–A3. The trackers were placed on the collars of dogs allowed to move freely and that were thought to use the forest (unrestricted dogs). Each device remained attached to the dog for a one-week

period, after which the devices were installed on other selected dogs from the same area. The devices were monitored weekly from October to December 2015. During the monitoring period, the devices were programmed to send a SMS message with the geographic coordinates every 30 minutes, from 6 pm to 8 am. This time was selected because this was the period when dogs were unrestricted and could possibly use the forest.

Camera trap records

From December 2015 to August 2016, six HD Auto-Focus Nature View camera traps (BUSHNELL®) were installed along Serra do Mar State Park trails used by GPS-tracked dogs and used to record animal usage along these paths. Two cameras were installed in each of areas A1–A3, one on the forest edge and other at least 300 m further into the forest. Every 25 days, batteries were changed and videos and photographs were downloaded. In addition, photographic record obtained by two camera traps installed in A2 was provided by other researchers working in the same area and period (unpublished data by Mora et al., 2017).

Analyses

DNA isolation and PCR amplification

Genomic DNA of ticks was isolated using the DNeasy Tissue kit (Qiagen, Valencia, CA, EUA) following manufacturer's instructions. Each tick was processed individually and two regions of mitochondrial DNA (16S rRNA and c*ox*1 genes) were amplified by PCR (see AI in Supplementary materials for details) (Black and Piesman, 1994; Simon

et al., 1994). The amplified products were sequenced in both directions (forward and reverse) using the Sanger method (Eurofins Genomics, Courtaboeuf, France).

Genetic analyses

To ensure sequence quality and confirm species identifications, we manually verified the sequence chromotographs and checked sequence similarity to other sequences deposited in GenBank using BLASTN (Basic Local Alignment Search Tool-Nucleotide). The sequences of both genes were then aligned, edited manually using MEGA software (version 6.0.5), and concatenated for each individual (Tamura et al., 2013). Two files containing the alignments for each gene fragment were also created. For the three files, haplotypes were defined using DNAsp version 5.10.01 (Librado and Rozas, 2009). Then, we performed the genetic analyses on each file.

A median-joining algorithm (MJ), based on parsimony criteria (Bandelt et al., 1999) using Network version 5.0.0.0 (fluxusengineering.com) was employed to visualize the frequency and spatial distribution of haplotypes. Genetic diversity (haplotype and nucleotide) within each *A. ovale* population was estimated using ARLEQUIN software version 3.5.1.2. (Excoffier et al., 1992). To assess the genetic neutrality of gene sequences, we used Tajima's D and Fu's Fs (Fu, 1997; Tajima, 1989), with 1,000 random permutations, as implemented in ARLEQUIN.

The partition of genetic variance among sampled sites was estimated using an Analysis of Molecular Variance (AMOVA) with ARLEQUIN software version 3.5.1.2. (Excoffier et al., 1992). To determine how genetic diversity was structured without a geographic constraint *a priori*, we also performed a Spatial Analysis of Molecular Variance (SAMOVA). This analysis identifies the spatial scale of a genetically

homogeneous population, estimating the number of *K* clusters based on the maximized intergroup genetic variance - Fct index (Dupanloup et al., 2002).

To evaluate genetic divergences between *A. ovale* populations from Ceará and São Paulo States, two unrooted Neighbor-Joining trees were constructed for 16S rRNA and *cox1* genes, and percentages of genetic identities were calculated upon the resulting alignments using Geneious R9 (Kearse et al. 2012).

Results

Tick sampling

Nineteen, 23 and 18 dogs were monitored in A1, A2, and A3, respectively. A total of 190 *A. ovale* specimens were collected directly from these animals: 155 in A1, 18 in A2, and 17 in A3. All ticks from A2 and A3, and a subset of 20 adult ticks from A1, were submitted to molecular analyses. A total of 13 adult *A. ovale* were collected from the environment in A4 and were also used in molecular analyses. In A5, a total of 32 individuals of *A. ovale* were collected from dogs living in the surroundings of Serra do Baturié in Ceará.

Telemetry data

A total of 12 dogs were monitored, four per area (A1-A3). Of these, two dogs were recorded inside the forest (in A1 and A3) (Figure 1). The other two dogs seemed to roam only around the urban areas. At times, when the dogs were in the GSM shaded areas, it was not possible to register their position. Telemetry devices were previously

tested on edge and core areas of the forest. In both areas the devices failed to transmit data in determined points (probably by the density of vegetation), therefore avoiding an accurate calculation of overall use of forest areas by tagged dogs.

Photographic Records

During the monitoring period, camera traps recorded 2,855 photographs and 156 videos. Thirty-eight of these files registered medium or large mammals (≥1 kg); 22 recorded the following species of carnivores: *Eira barbara, Nasua nasua, Leopardus pardalis, Leopardus* sp*., Puma concolor* and *Canis lupus familiaris*. Cameras installed at the forest edge (cameras E1 and E3, Figure 1) recorded dogs in all studied areas. In A1, records of dogs were also obtained by the camera installed into the forest (camera I1, Figure 1). People were also recorded walking in the forest alongside their dogs in A1 and A3, particularly in the morning for A1 (AM acronym, Figure 1) and during the weekends or holidays for A3. *Leopardus pardalis, Leopardus* sp*.,* and *E. barbara* were recorded by the camera installed at the forest edge (camera E3, Figure 1) only 100 m away from houses, where dogs were also recorded, demonstrating an overlap of ranges in different periods: wild carnivores at night and dogs during the day. *Puma concolor* was also recorded in A1 and A2 by cameras installed inside the forest (cameras I1 and I2, Figure 1).

Genetic analyses

A total of 97 adult *A. ovale* ticks were submitted to molecular analyses, from which we obtained 87 partial sequences of the 16S rRNA gene (275 bp), 78 of the *cox*1 gene (436

bp), and concatenated arrangements (711 bp) for 70 ticks (Table 1). The *cox*1 gene fragment yielded higher polymorphism than the 16S rRNA gene, with 54 and 22 variable sites, respectively (Table 2). Haplotype and nucleotide diversity were high within all tick populations (Table 3).

A total of 39 unique haplotypes were observed when both mitochondrial gene sequences were combined. Of these, 27 were observed in samples from A1–A4. None of the 12 observed haplotypes from A5 were shared with the Caraguatatuba samples (A1–A4). However, haplotype sharing was observed among the Caraguatatuba populations (A1–A4).

The Network showed the presence of four well-supported haplotype lineages, where the lineage from A5 was completely distinct from those of A1-A4 (Figure A2). The lineages from A1-A4 were separated from the A5 lineage by more than 40 mutational steps (Figure 2). The spatial distribution of haplotypes from the three lineages in A1-A4 was completely mixed (Figure 2).

No deviation from neutrality was found at the two genes examined for either Tajima's D or Fu's F test ($p > 0.05$) suggesting no evidence of selection on the genes or recent population expansion (Table 3).

In agreement with network, AMOVA results showed that within Caraguatatuba, only 0.17% of the variation could be attributed to the interpopulation level; no barrier to gene flow seems to occur among these populations (Table 4). When A5 is included this value rises to 85.95% of the molecular variation.

The spatial analysis (SAMOVA) also indicated strong geographic isolation between Caraguatatuba (A1–A4) and Ceará (A5) samples ($F_{CT} = 0.87335$), coinciding with the

AMOVA results. Again, no significant differences were found among A1 to A4 suggesting no small scale geographic structure.

Nucleotide sequence identity for both 16S rRNA and *cox1* haplotypes observed in Serra do Baturié (Ceará) population (A5) ranged between 95.6 – 100%. Identities for the same genes within Serra do Mar State Park populations (A1-A4) varied identically. Remarkably, comparisons between Serra do Baturié and Serra do Mar State Park populations showed nucleotide identities lower than 95.6%, reaching 92.0% in some cases (see Figure 3 A2 Supplementary materials for details.).

Discussion

In Brazil, *A. ovale* has a unique life history. This species is one of the most widely distributed ticks parasitizing carnivores in their adult stages and associated to rodents and other small warm-blooded vertebrates in their immature stages. However, in the state of São Paulo (southeastern Brazil), *A. ovale* is not found above 300 m of altitude (Barbieri et al., 2015). These characters make this tick a parasite highly dependent on their hosts for interpopulational dispersal and consequent gene flow in subtropical areas. In this study, we found that *A. ovale* has high intra-population genetic diversity and high gene flow at a scale of tens of kilometres (within Caraguatatuba City), but can show strong structure at higher spatial scales (Ceará State to Sao Paulo State).

Evolutionary distances measured among haplotypes within populations were small, suggesting that tick diversification has (relatively) occurred locally with some potential mixing of different populations at some point in time. Additionally, population densities are high and stable enough to maintain this diversity over time. This type of population stability is also supported by the neutrality tests, which do not suggest recent extreme

shifts in population demography. Moreover, comparisons of nucleotide identities between Serra do Baturié and Serra do Mar State Park *A. ovale* (<95.0%) suggest that this species could be split into two putative sibling species.

Monitoring data from the dogs (telemetry and camera trap records) showed that these domestic animals only rarely enter the inner parts of large forest fragments, in opposition to what has been previously reported by other authors (Butler and Bingham, 2000; Galetti and Sazima, 2006). Despite a low sample size of dog tracks, this data and camera traps suggest that these animals are mainly restricted to forest edges and go deeply into the forest only when accompanied by their owners. In contrast, camera trap records clearly demonstrated the presence of wild carnivores in anthropized areas, including those areas where dogs were recorded. Thus, *A. ovale* ticks are likely brought to the forest edge by wild carnivores and rodents, and then use dogs as an alternative host, without the establishment of distinct populations exploiting exclusively domestic hosts. Indeed, this hypothesis is supported by a lack of genetic structure between Areas A1-A3 (where only dogs were sampled) and A4 (where ticks were sampled from the vegetation). The SAMOVA did not detected evidence of genetic structuring within populations, a fact that is not unexpected if distinct host-associated populations exist in sympatry. This hypothesis is also the most parsimonious explanation of high genetic difference between Caraguatatuba and Ceará populations. Thus, it is likely that wild animals with limited dispersal distances due to high forest fragmentation are responsible for tick dispersal.

In contrast to what has been suggested for several *Amblyomma* species inhabiting Atlantic rainforest ecosystems (Ogrzewalska et al., 2015, 2012, 2011), the complete isolation of tick populations from Serra do Mar State Park and Serra do Baturié suggest that birds have an irrelevant role in the spread of larvae and nymphs of *A. ovale*.

However, this assumption should be confirmed in a larger scale study, with a higher number of sites and complementary molecular techniques.

Due to its steep and relatively narrow coastal plain, Caraguatatuba was forgotten during the Brazilian economic cycles, and for that reason, some Atlantic rainforest fragments remained well preserved (Mouret, 2017). As a consequence, Serra do Mar State Park is currently one of the largest ecological reserves for native Atlantic fauna in Brazil. In addition to this fact, high vagility of wild carnivores may explain haplotype diversity (and expected high effective population size) and high gene flow of *A. ovale* in the region. However, more subtle genetic differences among populations within the Serra do Mar State Park cannot be completely discarded until an analysis using polymorphic genetic markers such as single-nucleotide polymorphism (SNP) or microsatellites are applied. A diversity of factors may change across these areas that could alter the genetic composition of tick populations, including the presence and abundance of different host species. The effect of these factors on the genetic structure of *A. ovale* and its evolutionary trajectory will depend on the flexibility of the tick to successfully use different environments and host species (Dupraz et al., 2016; McCoy et al., 2013; De Meeus, 2012). What is clear from our work is that forest fragmentation can play a major role in isolating tick populations and may have direct implications for the circulation of tick-borne disease agents, such as *R. parkeri*. Future work on this tick using more accurate genetic markers is now required for determining finer scale structure among contiguous forest regions, the relation to host and habitat type, and whether the extreme isolation observed at a larger spatial scale (>2,000 km) corresponds to a general pattern of this tick species when sampling isolated forest fragments. This data will become utterly important if we are to understand the epidemiology of tick-borne pathogens and the evolutionary trajectory of tick diversity.

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Table Caption

Table 1 - *Amblyomma ovale* populations used in this study, with collection localities, population abbreviations, host species and the number of ticks used in genetic (16S rRNA and *cox*1 genes) analyses. F: female; M: male.

Table 2: Genetic indexes of *Amblyomma ovale* population diversity for the 16S rRNA, *cox*1 and concatenated gene fragments.

Table 3: Selective neutrality of *Amblyomma ovale* involving 70 nucleotide sequences (16S rRNA + *cox*1) and a total of 708 positions

Table 4: AMOVA partitioning of genetic variation of mitochondrial haplotypes among sampling areas and between forest fragments, and among sampling areas within Caraguatatuba.

Figure Captions

Figure 1 - Maps of monitored areas with the tracking locations of dogs monitored by GPS collar during the period of monitoring and location of cameras traps from 2015 to 2016. Caraguatatuba–SP, Brazil. Legend: E = forest edge; I = Inside forest.

Figure 2 – Geographic distribution of *Amblyomma ovale* haplotypes (H1-H39) and the haplotype network indicating the mutational steps among the different haplotype lineages. Different colors refer to different haplotype lineages and the relative size of the pie chart is proportional to the frequency of each lineage within the site.

Supplementary materials

A1 Supplementary materials: Table - Primers used in the amplification of the partial sequences of the *Amblyomma ovale* genes and the mitochondrial.

A2 Supplementary materials: Figure 3 – Gray-scale heatmap matrixes calculated upon the alignments of 275 bp partial fragments of 16S rRNA (A) and cox1 (B) genes from Serra do Baturié (A5) and Serra do Mar State Park (A1 – A4) populations of *A. ovale*. In each tree, Serra do Baturié sequences are denoted with orange and Serra do Mar State Park sequences with blue branches. Evolutionary relationships were inferred using Tamura-Nei genetic distance model. Support values obtained after 1000 bootstrap replications are indicated for main branches. Resulting trees were midpoint-rooted and transformed to cladrograms

TABLES

Table 1 - *Amblyomma ovale* populations used in this study, with collection localities, population abbreviations, host species and the number of ticks used in genetic (16S rRNA and *cox*1 genes) analyses. F: female; M: male.

Area	State /Park	host	Number of ticks male-female	Number of sequences 16s	Number of sequences COI	Number of concatenated sequences
1	SP/ Serra do Mar State Park	19 dog	10M-10F	17	13	13
2	SP/Serra do Mar State Park	24 dog	11M-7F	18	13	9
3	SP/Serra do Mar State Park	18 dog	8M-6F	14	13	12
4	SP/Serra do Mar State Park	Free living	5M-8F	9	9	9
5	CE/Serra do Baturié	dog	9M-23F	29	29	27
	total		97	87	77	70

SP = São Paulo; CE = Ceará

Table 2: Genetic indexes of *Amblyomma ovale* population diversity for the 16S rRNA, *cox*1 and concatenated gene fragment.

	16S rRNA				*Cox*1				16S rRNA + *cox*1			
Pop.	NS	H	Hd±SD	k	NS	H	Hd±SD	k	NS	H	Hd+SD	k
A1	11	9	0.882±0.003	3.7059	10	6	0.769±0.010	4.6923	21	10	0.949±0.002	8.6026
A2	10	11	0.918±0.002	3.1169	13	7	0.731±0.017	5.1282	22	9	1.000±0.002	9.6389
A3	10	10	0.933±0.002	2.8286	12	4	0.423±0.027	2.5897	22	8	0.909±0.004	5.6061
A4	9	7	0.867±0.011	3.2000	14	3	0.511±0.027	4.3556	23	7	0.917±0.008	8.1111
A5	17	9	0.653±0.007	1.9793	13	8	0.687±0.008	4.1453	13	12	0.875±0.002	2.3077
Total	22	28	0.910±0.00025	5.4865	54	20	0.809±0.001	0.0430	71	39	0.962±0.001	25.044

NS: number of variable sites; H: number of haplotypes; Hd ± SD: Haplotype diversity and standard deviation; k: mean number of nucleotide differences.

Table 3: Selective neutrality of *Amblyomma ovale* involving 70 nucleotide sequences (16S rRNA + *cox*1), and a total of 708 positions.

Pop.	S	Ps	Θ	π±SD		D	Fs
A1	21	0.029619	0.009545	0.012100+/-	0.006845	1.164245	-1.27792
A2	22	0.031030	0.011417	0.013595+/-	0.007931	0.943172	-2.90406
A3	22	0.031030	0.010275	0.007907+/-	0.004662	-1.018854	-0.74595
A4	23	0.032394	0.011919	0.011424+/-	0.006758	-0.205787	-1.49974
A5	13	0.018336	0.004757	0.003255+/-	0.002055	-1.066686	-5.10633
Total	71	0.100282	0.020812	0.035374+/-	0.018372	2.358450	-2.08980

Pop = Populations for area, S = number of segregating sites, Ps = S/n, $\Theta = p_s/a_1$, π±SD= nucleotide diversity and standard deviation, D= the Tajima test statistic and Fs = Fu's Fs test.

Table 4: AMOVA result, intra and inter- *Amblyomma ovale* populations mitochondrial genes 16S and COI variation.

Source of variation	d.f	Sum of square	Componentes of variation	Percentage of variation
Among populations (A1 –A4)	3	11.898	0.00668Va	0.17
Within populations (A1-A4)	39	151.893	3.89470Vb	99.83
Total	42	163.791	3.90138	
Among populations (A1 –A5)	4	682.150	12.70751 Va	81.95
Within populations (A1-A5)	65	181.893	2.79836 Vb	18.05
Total	69	864.043	15.50586	

d.f. = degrees of freedom.

Figure1
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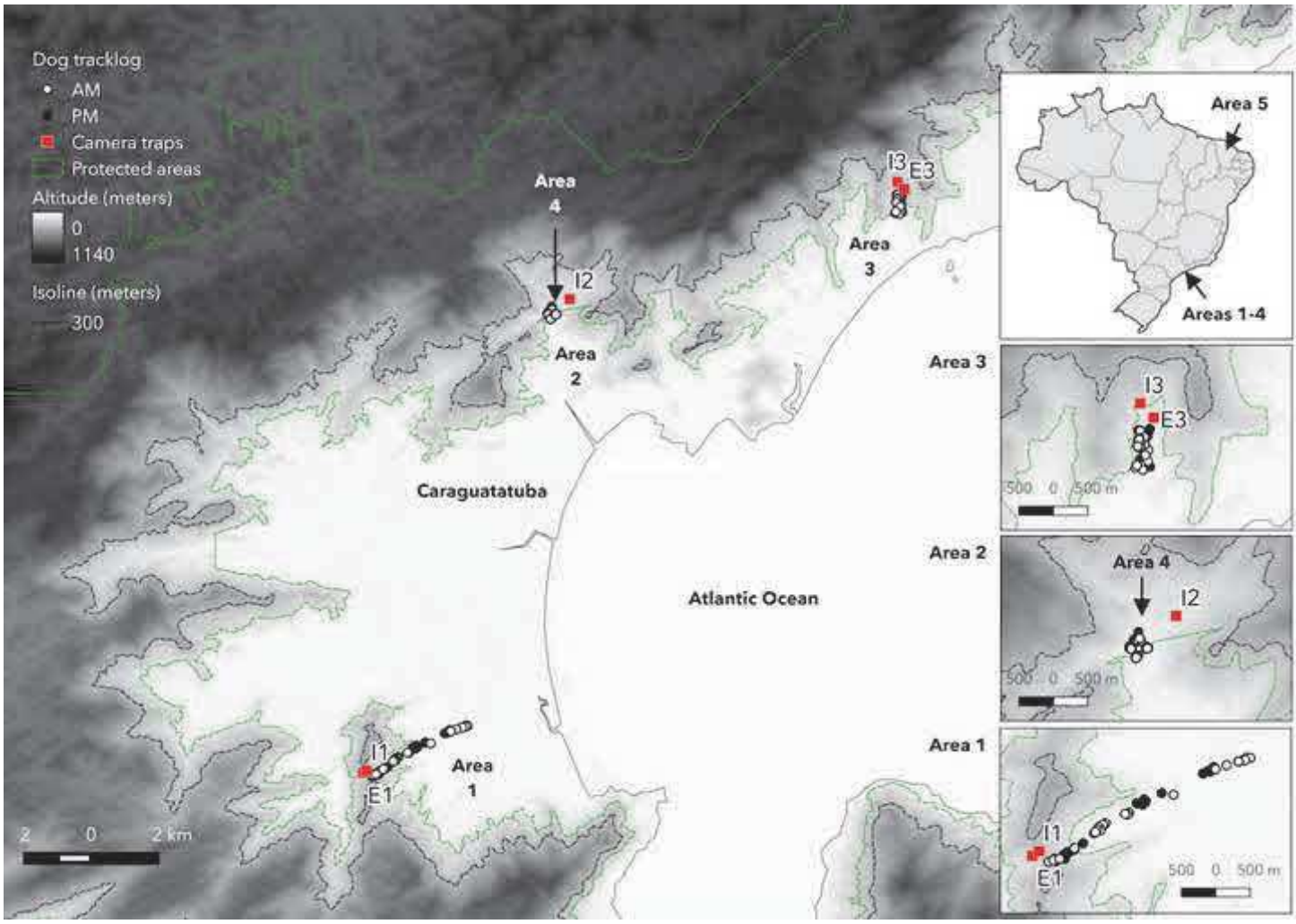

Figure2
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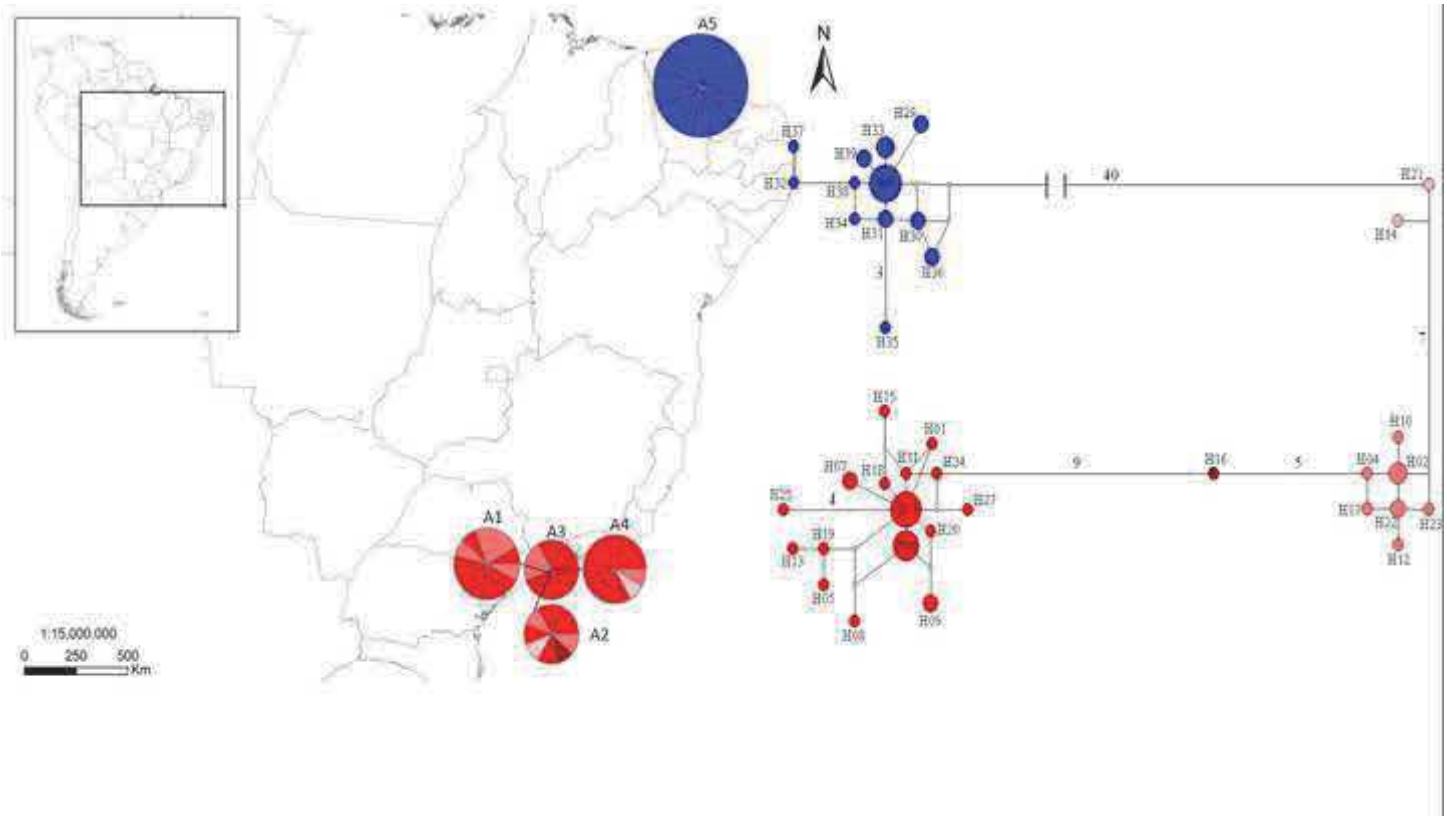
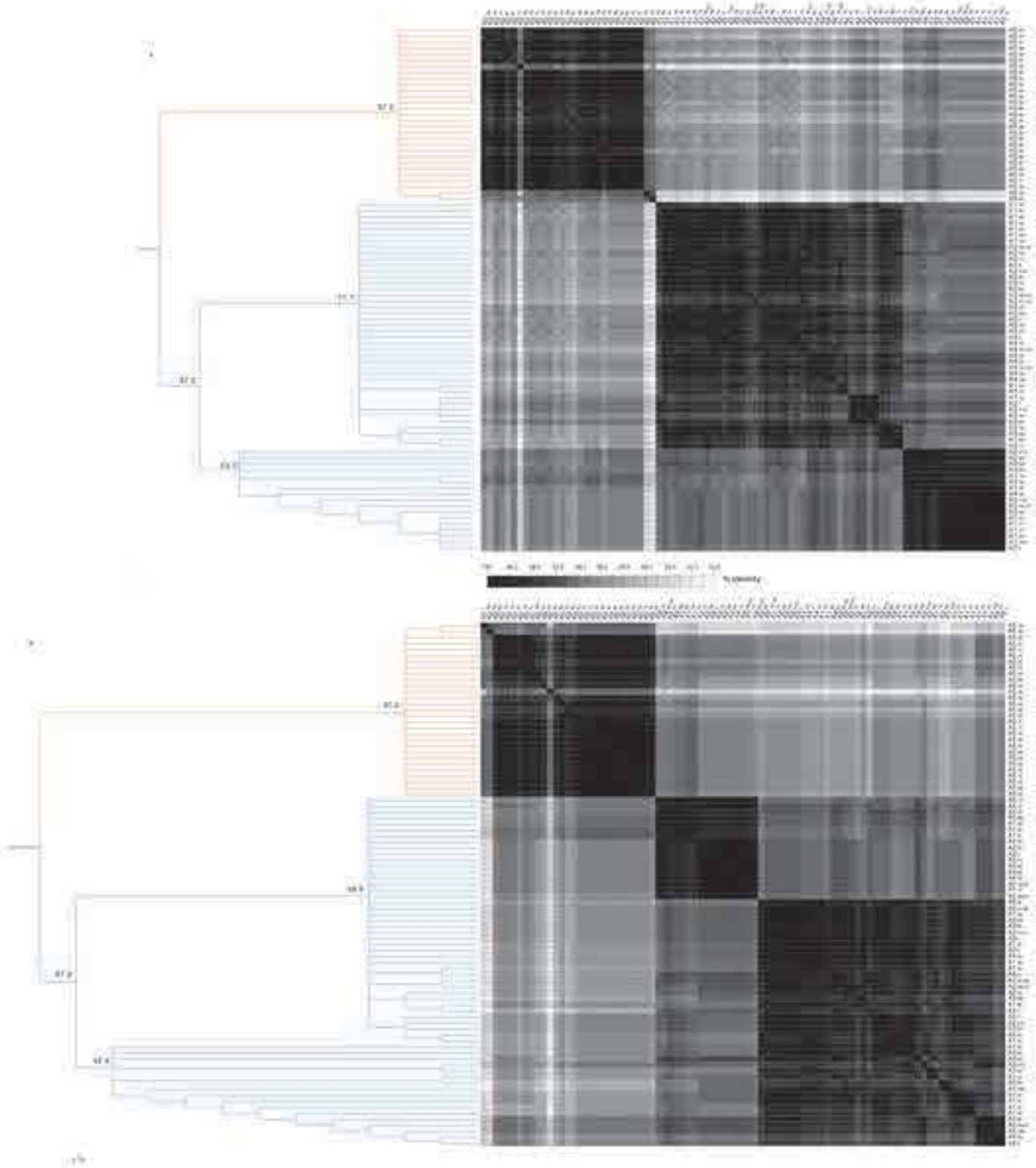


Figure3

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