Trypanosomatids have a wide variety of hosts and can infect invertebrate and vertebrate animals of virtually all orders. These organisms are second only to nematodes as the invertebrates that have the widest range of hosts and geographical distribution (Stevens et al. 2001, Simpson et al. 2006). Trypanosomatids include heteroxenous genera with alternating cycles between invertebrate hosts (usually hematophagous arthropods) and vertebrates (Trypanosoma, Leishmania, and Endotrypanum; Hoare 1972).

The genus Leishmania comprises ~30 different species that infect mammalian orders and are transmitted by various blood-sucking invertebrates of the family Psychodidae, which are distributed in tropical and subtropical regions (Ashford 2000, Desjeux 2004). These parasites can infect humans and cause different clinical symptoms, of which visceral leishmaniasis is the most severe.

The species that cause visceral leishmaniasis are Leishmania (Leishmania) donovani, in India, Bangladesh, Nepal, and Pakistan; Leishmania (Leishmania) infantum in the Mediterranean (Europe and Africa); and Leishmania (Leishmania) chagasi in different countries of the Americas (New World; Lainson and Hoare 1972). The taxonomy of the species causing visceral leishmaniasis in the Americas was recently revised and it was suggested that the species name Leishmania (Leishmania) infantum chagasi should be used (Shaw 2006).

The vertebrate hosts include humans, domestic dogs (that are considered to be the main reservoir), and wild mammals. The main wild reservoirs are wild canids (Deane and Deane 1954, 1955; Lainson et al. 1969; Courtenay et al. 2002; Lainson and Rangel 2005), cats (Savani et al. 2004, Dahroug et al. 2010), rodents (Lainson and Rangel 2005), opossums of the genus Didelphis (Travi et al. 1994, Lainson and Rangel 2005), and bats (De Lima et al. 2008).

The species of the genus Trypanosoma that parasitize mammals have been divided into the groups Salivaria and Stercoraria, according to their development in the invertebrate host, and consequently the route of elimination of infective forms by the vector. The Stercoraria group includes the subgenera Schizotrypanum (type species Trypanosoma cruzi), Herpetosoma (Trypanosoma lewisi), and Megatrypanum (Trypanosoma theileri) (Hoare 1972). Only Schizotrypanum has a monophyletic group, although there is still some controversy in this regard. The subgenera Herpetosoma and Megatrypanum have been shown to be polyphyletic, thus confirming that the traditional taxonomic criteria are insufficient for classifying the trypanosomatids.
panosomes into subgenera (Stevens et al. 2001, Maia da Silva et al. 2004, Rodrigues et al. 2006). Other than T. cruzi, which infects humans and wild and domestic mammals, only a few species of trypanosomes of bats are classified in the Schizotrypanum subgenus and are morphologically indistinguishable (Hoare 1972).

In this study, we conducted a survey of the diversity of parasites of the genus Trypanosoma and Leishmania in small mammals and domestic animals in the Corrego do Veado Biological Reserve and its surroundings, in the state of Espírito Santo, through serological, parasitological (isolation), and molecular techniques, with traditional markers used for groups.

Materials and Methods

Study Area and Small Mammals Caught. The Corrego do Veado Biological Reserve (2,382 ha) is located in the municipality of Pinheiros (40° 08' 45” S, 18° 20' 33” W) in the northern part of the state of Espírito Santo and is the last remnant of the Atlantic Rainforest in the region. Deforestation and the creation of private farmland properties in the surrounding area have increased significantly due to the growth of regional agricultural production. Agriculture in this region is based on extensive grazing areas, along with plantations of coffee, fruit (papaya, passion fruit, and coconut), and sugarcane (serving alcohol factories that supply power plants located in the region; Fig. 1).

Sherman traps were used to catch small mammals and bats were caught using mist nets (7 by 3 m) in two campaigns in June and November 2012, each lasting for 15 d. The small mammals and bats that were caught were anesthetized (ketamine and xylazine), and blood samples were collected by means of heart puncture. Animals were identified using identification keys and original descriptions (Vizotto and Teddei 1973). Some animal specimens that could not be identified at the species level during the field collection were euthanized, fixed, and deposited in the Mammal Collection in the department of Biological Sciences, Federal University of Espírito Santo. The animals were caught and handled in accordance with the recommendations of the Brazilian Institute for the Environment and Renewable Natural Resources.

Blood samples were collected from all the dogs and horses that were present in the surroundings of the Biological Reserve and were also included in the study. Some risk factor variables (age, hunting activity, and sex) were assessed.

Isolation of Trypanosomatids. To detect trypanosomatid parasites, blood samples from wild and domestic animals and popliteal lymph node aspirates obtained through fine-needle puncture in dogs were inoculated into Vacutainer tubes containing a biphasic medium consisting of 15% sheep red blood cells as the solid phase (blood agar base), overlain by liquid liver infusion tryptose (LIT) medium supplemented with 20% fetal bovine serum (Marcili et al. 2009a,b). The culture was incubated at 28°C and grown in LIT medium for DNA preparation, and the isolates were cryopreserved in liquid nitrogen in the Brazilian Trypanosomatid Collection (Colecção Brasileira de Tripanossomatídeos, CBT), in the Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, University of São Paulo, Brazil. Blood samples were fixed in ethanol (primary samples) for molecular detection.

Serological Diagnosis of L. infantum chagasi. Blood samples were collected from the jugular or brachial vein of the dogs and sera were separated and stored at −20°C until tested. An indirect fluorescent antibody test (IFAT) was used for detection of anti-L. infantum chagasi antibodies, with a cutoff value of 1:40. Promastigote forms of the L. infantum chagasi CBT 96 strain were used as the antigen as described by Ferrer et al. (1995). L. infantum chagasi IFAT-positive sera were tested at twofold serial dilutions until the last positive dilution, and rabbit anticanine IgG conjugate (Sigma-Aldrich, St. Louis, MO) was used in both tests. Positive and negative control sera were added to each slide.

Molecular and Phylogenetic Analysis. DNA for culture samples was extracted from the trypanosome cultures using the phenol–chloroform method, and primary samples were purified using the Wizard DNA Clean-Up System (Promega, Madison, WI). The DNA samples were subjected to the conventional polymerase chain reaction (PCR) for trypanosome barcoding on a fragment of ~900 bp of the V7V8 SSU rDNA (Marcili et al. 2009a,b) and gGAPDH gene (Hamilton et al. 2004). PCR products of the expected size were purified and sequenced in an automated sequencer (ABI Prism 310, Applied Biosystems, Foster City, CA). The nucleotide sequences generated were deposited in GenBank (Table 1).

The sequences obtained were aligned with sequences previously determined for other trypanosomatid species available in GenBank (Table 1) using ClustalX (Thompson et al. 1997) and were adjusted manually using GeneDoc (Nicholas et al. 1997). The barcoding sequences were used to construct a phylogenetic tree using maximum parsimony, as implemented in PAUP version 4.0b10 (Swofford 2002) with 500 bootstrap replicates. Bayesian analysis was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001) with 1,000,000 replicates. The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability.

Results

In total, 154 small mammals were caught in the area. They were found to be distributed among four orders and 25 species. The largest number of individuals caught belonged to the Order Chiroptera, with 138 individuals in four families (Phyllostomidae, Vespertilionidae, Thyropteridae, and Emballonuridae; Table 2). In the surroundings of the Biological Reserve, 47 dogs and 20 horses were sampled on 22 private properties.

Serological analysis was performed only on the domestic animals because it was only possible to draw small amounts of blood from the small mammals...
and because conjugates and positive controls were not available for all species or groups. All the dogs (47) and horses (20) were seronegative for anti-*L. infantum chagasi* antibodies. Furthermore, the domestic animals did not present skin lesions.

Blood cultures on the domestic animals and cultures on popliteal lymph node aspirates from the dogs were negative for both parasite genera, thus corroborating the serological findings. Among the wild mammals, the prevalence through blood cultures was 10.38% (16), but only 5.84% (9) in relation to established cultures. All the positive blood cultures were from the Order Chiroptera, and the prevalence for this order was 11.54 and 6.52% in relation to positive and established cultures, respectively. The morphology relating to all the positive blood cultures was compatible with the genus...

Fig. 1. Geographical origin of small mammals and domestic animals caught in the Espírito Santo state, Pinheiros county.
Trypanosoma and the Phylllostomidae and Vespertilionidae bats families. The established cultures (isolates) were cryopreserved in CBT, while some positive blood cultures from Myotis nigricans (Schinz) (1), Desmodus rotundus Geoffroy (1), Carollia perspicillata (4), and Saccopteryx bilineata (Temminck) (1) were not established. The few epimastigote forms in both CBT and LIT were compatible with the Schizotrypanum subgenus, from D. rotundus, while all others showed morphology similar to the Schizotrypanum subgenus (Table 2). Screenings on primary samples (blood) from all the domestic animals and from 107 wild mammals were negative for trypanosome DNA barcoding, including the individuals with positive blood culture, probably due to low parasitemia levels.

The phylogenies based on trypanosomatid DNA barcoding (V7V8) of the SSU rDNA gene sequences obtained in this study were compared with sequences in GenBank, and assessments were done using maximum parsimony and Bayesian analysis and identical topologies. The isolates were grouped as Trypanosomatids and corroborated the V7V8 topology (Fig. 2B). The gGAPDH gene was also obtained from bat isolates and the Trypanosoma cruzi marinkellei subgenus (Taeniodontinae) (1).

Table 1. Trypanosome isolates, host, and geographic origin and sequences of SSU rDNA and gGAPDH used for phylogenetic analysis

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<tr>
<th>Trypanosomatid species</th>
<th>Isolate code</th>
<th>Host</th>
<th>Geographic origin</th>
<th>Accession No.</th>
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a Sequences determined in this study and deposited in GenBank are underlined and bold.

Brazilian states: ES, Espirito Santo; SP, Sao Paulo; RO, Rondonia; GO, Goias; MT, Mato Grosso; BA, Bahia; MS, Mato Grosso do Sul; AM, Amazonas; RN, Rio Grande do Norte.
The Atlantic Rainforest is the original forest type throughout the territory of the state of Espírito Santo. It is the biome with the highest degree of degradation and is currently restricted to remnants. The main diseases caused by trypanosomes, that is, visceral leishmaniasis and American trypanosomiasis, are related to processes of deforestation and colonization. These processes became accelerated through economic growth that started in the early 20th century. The growing process of deforestation of areas of this biome has enabled growth in the numbers of cases of visceral leishmaniasis and American trypanosomiasis.

In the state of Espírito Santo, visceral leishmaniasis has been found to be distributed in central-northern region, especially in places bordering endemic areas (Falqueto et al. 2009). However, there is no record of occurrences of the main vector, *Lutzomyia longipalpis* França, in these areas (Pinto et al. 2010). Most reports on *L. infantum chagasi* in dogs have consisted of serological data, except in the municipality of Pancas, where the parasite was isolated (Falqueto et al. 2009). The serological analyses were performed using a rapid test for *Leishmania* parasites (Grimaldi Jr. et al. 2012), and cross-reactivity with other trypanosomatids may have occurred. In addition, *Leishmania braziliensis* has been reported through serological and parasitological evaluations to be present in horses, dogs, and vectors (Falqueto et al. 1986, 1987, 1991, 2003; Rocha et al. 2010). We did not find any serological, parasitological, or molecular data about the presence of *Leishmania* or *Trypanosoma* parasites in domestic animals.

In the Corrego do Veado Biological Reserve, 25 species were caught, but 16 of them were bats. Other Atlantic Rainforest fragments in the state of Espírito Santo presented greater diversity of small mammals (Moreira et al. 2005), in comparison with the number found in the Corrego do Veado Biological Reserve. This was probably related to habitat heterogeneity, which is a major factor in determining the occurrence and distribution of small non-flying mammals (Vera y Conde and Rocha 2006). Illegal hunting and burning are determinants of diversity loss in the Corrego do Veado Biological Reserve. Furthermore, there were only two capture campaigns lasting 15 days each, and it is likely that a different sample design with monthly collections would add a greater number of species in the area.

The only group found to be positive in parasitological approaches was the chiropteran hosts. This was probably because of the low numbers of other groups caught and the high degree of dispersion of bats. The prevalence assessed through blood cultures were comparable with those of other Atlantic Rainforest areas and biomes (Cavazzana et al. 2010, Marcili et al. 2013). *T. dionisii* is the only trypanosome species that has been isolated in the Atlantic Rainforest biome (Cavazzana et al. 2010). However, exceptionally, *T. marinkellei* was isolated in the Corrego do Veado Biological Reserve from *Myotis nigricans*.

*T. marinkellei* is strongly associated with phyllostomid bats (Marinkelle 1976, Cavazzana et al. 2010, Marcili et al. 2013) and is distributed in the Pantanal, Cerrado, and Amazonia biomes in Brazil (Cavazzana et al. 2010, Marcili et al. 2013). The results from the current study demonstrated that the occurrence of *T. marinkellei* in the Atlantic Rainforest and in Vespertilionidae bats (*M. nigricans*) in the Atlantic Rainforest biome, for the first time.

### Discussion

The Atlantic Rainforest is the original forest type throughout the territory of the state of Espírito Santo. It is the biome with the highest degree of degradation and is currently restricted to remnants. The main diseases caused by trypanosomes, that is, visceral leishmaniasis and American trypanosomiasis, are related to processes of deforestation and colonization. These processes became accelerated through economic growth that started in the early 20th century. The growing process of deforestation of areas of this biome has enabled growth in the numbers of cases of visceral leishmaniasis and American trypanosomiasis.

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### Table 2. Hosts species and hemoculture positivity of small mammals examined in this study

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<th>Order</th>
<th>Genus</th>
<th>Species</th>
<th>Examined/positive HE</th>
<th>No. of individuals</th>
<th>Totala</th>
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<td>Platyrhinus</td>
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<td>Saccopteryx</td>
<td>bilineata</td>
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<td>Sturnira</td>
<td>lilium</td>
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<td></td>
<td>Thyroptera</td>
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<td>sp.</td>
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<td>incanus</td>
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<td>sp.</td>
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<td>Carnivora</td>
<td>Naussa</td>
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<td>Holochilus</td>
<td>brasiliensis</td>
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<tr>
<td>Total</td>
<td>21</td>
<td>25</td>
<td>154/16</td>
<td>9</td>
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* Total of isolates established and cryopreserved in Coleção Brasileira de Tripanossomatídeos (CBT).
In the animals sampled, the absence of Leishmania and Trypanosoma species that are pathogenic for humans does not rule out the existence of these parasites in this area. Furthermore, the loss of biodiversity and the fragmentation of habitats may increase the potential risk and dynamics of parasite infection, probably through population density or suitable reservoirs.

The Atlantic Rainforest, which was the original forest cover of the territory of the state of Espírito Santo, is one of the biomes with the highest degree of degradation in the world and is currently restricted to small isolated forest remnants, surrounded by agricultural farmland. Owing to increasing deforestation process in the remaining areas of Atlantic Rainforest, studies conducted in these areas provide the knowledge of parasite diversity or detect parasites that can accelerate the loss of host biodiversity.

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Fig. 2. (A) Phylogenetic trees inferred by parsimony based on V7–V8 SSU rDNA gene sequences of 47 trypanosomes and Trypanosoma rangeli as outgroup (868 characters, 150 parsimony informative). (B) Dendrogram inferred by parsimony analyses based on gGAPDH sequences from 22 Schizotrypanum trypanosomes sequences (517 characters, 50 parsimony informative). Numbers at nodes are the support values for the major branches (bootstrap or posterior probability) derived from 500 replicates, respectively, for maximum parsimony and Bayesian analyses.
ical and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats. Int. J. Parasitol. 40: 345–355.


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