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The Facet of Human Impact: *Solenopsis invicta* Buren, 1972 Spreading around the Atlantic Forest

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Abstract: The present investigation deals with some aspects of the diversity of fire ants (Hymenoptera: Formicidae) in their native range. The Red Imported Fire Ant *Solenopsis invicta* is native to the tropical and subtropical inland territories of South America. In Brazil, it mainly occurs around the Pantanal region and across the Paraguay river, a region composed of grasslands which are seasonally flooded. Recent studies have evidenced this fire ant species is gradually spreading to other regions of Brazil. In the present investigation, we surveyed the molecular diversity of *S. invicta* populations across fragments of Atlantic Forest in São Paulo, Brazil, using mtDNA COI haplotypes. Fire ant nests were sampled along the highways lining the northern and southern slope sides of the mountain range Serra do Mar, SP, Brazil. Four haplotypes were identified (H1–H4), which were assessed for similarity to deposited records by other authors, revealing that the haplotypes H1 and H2 are likely of foreign origin through recent reintroduction via a marine port to the south of the Serra do Mar mountain range. On the other hand, the haplotypes H3 and H4, predominating among the inland samples from the northern side of the mountain range, were most similar to previous records from more central regions of Brazil. Haplotypes clustered into distinct supergroups, further pointing to the occurrence of two separate expansion waves of *S. invicta* in the region. We suggest the obtained pattern indicates the mountain range may function as a geographical barrier deferring gene flow.

Keywords: biological invasion; molecular phylogeny; biogeography; population genetics

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1. Introduction

Currently, there are 191 valid recognized species in the ant genus *Solenopsis* [1], most of which display small monomorphic workers and sparsely populated colonies [2]. In the New World, *Solenopsis* ants typically forage below the undergrowth or in leaf litter [3,4], though some species will also make arboreal nests [5]. The genus is, however, better known for a monophyletic group of about 20 *Solenopsis* species (*S. saevissima* species group [6]), displaying larger polymorphic workers that can construct denser colonies in conspicuous nests above the ground [6]. These are trivially known as “fire ants” both because of their typical red color and the burning sensation brought by their stings [7]. They are highly aggressive ants in the defense of their foraging trails and fragile earthen nests [8,9]. The majority of fire ant species occur in the Neotropical Region, with but five species considered native to the Nearctic Region [10].

Solenopsis invicta Buren, 1972 is the most famous fire ant species, biogeographically originating from the grasslands shared by Paraguay, Bolivia, northeastern Argentina, north Uruguay, and Brazil, including savannas and seasonal wetlands [6]. In Brazil, the natural habitat of *S. invicta* does not approach the Atlantic Forest; notably, they were never recorded from the Dense Ombrophilous Forest. However, previous observations [11] indicate that *S. invicta* is expanding its territory towards denser native vegetation areas (i.e., Dense Ombrophilous Forest) where they have not been observed before.

This species is nowadays one of the most intensively studied animal species because of the abundant economic and ecological damage it distributes worldwide as an invasive pest, seriously impacting agricultural systems and biodiversity [12,13]. Officially designated as the Red Imported Fire Ant (RIFA), *S. invicta* is considered today a top-priority quarantine pest in countries as distant geographically as the United States, China, Australia, and New Zealand [13–15]. The RIFA was apparently first introduced to the southern United States from South America through a seaport at Mobile, Alabama [16], among traded goods [17]. After years spreading further into the United States, RIFA was eventually exported to Australia, China, Taiwan, and elsewhere, as evidenced by reconstructions of their mtDNA phylogeny [14,15,18].

Mitochondrial haplotypes can provide helpful insights into the invasion dynamics of pest species. The formation of a trans- and intercontinental populational network creates a dynamic mosaic of gene flow that can ultimately disrupt intraspecific biodiversity in the native range of a given species [19–22]. For instance, combined analyses of mtDNA and nuclear DNA have demonstrated that multiple *S. invicta* invasions led to a significant loss of molecular diversity among the introduced populations in comparison to native populations [15,23–26]. Knowledge about the molecular diversity of this species in its homeland remains limited, which contributes to a lack of understanding about the processes behind its geographic expansion.

Given the economic and environmental impacts caused by *S. invicta* when invading new territories, it becomes relevant to better understand its geographical expansion into rich biomes such as the Brazilian Atlantic Forest. The objective of the present investigation was to characterize the molecular diversity of *S. invicta* within a pocket of intense car traffic at the outskirts of the Atlantic Forest near the coast of São Paulo State, southeast Brazil. We used COI fragments to identify local *S. invicta* haplotypes in order to better understand their expansion along heavy-traffic highways connecting the interior of the state to the coast, around the marine trade port of São Sebastião (São Paulo State).

2. Materials and Methods

2.1. Collection and Experimental Design

The selected study area included the outskirts of two opposing faces of the mountain range of Serra do Mar, dominated by the Atlantic Forest. The mountains reach around 600 and 850 m, creating a marked division between the lower seacoast and the inland plateau; the two faces of the range are hereafter designated ‘south slope’ and ‘north slope’ (Supplementary Material Figure S1). Local predominant phytophysiognomy was Dense Ombrophilous Forest, characterized by broad-leaved trees, abundant epiphytes, mosses and lichens, characterized as a vegetation adapted to sustain periods of extreme heat and humidity [27]. According to Köppen’s climate classification, the region presents a tropical rainforest climate, with annual precipitation reaching over 2000 mm, and no clearly defined seasonality [28].

Fire ant nests were sampled from the sides of highways lining the forested slopes of the study area. A total of eight nests of *S. invicta* were sampled along the highways SP-99 and BR-101 by the south slope facing the Atlantic Ocean, and ten nests were sampled from the highways SP-70 and SP-88 by the north slope on the continental plateau (Supplementary Material Figure S1). These highways are surrounded by fragments of Atlantic Forest [29] but also include some of the most heavily disturbed, industrialized regions of São Paulo State [30].

The fire ant nests were collected interspaced by 3–25 km from the highway sides, up to a full traveled distance between 40 and 150 km along the selected highways (Supplementary Material Figure S1). To standardize the nest sampling methods, all procedures were performed by the same person. The center of the nest mound containing abundant ant workers was sampled from the surface into a depth of 5 cm using a gardening shovel (geographical coordinates provided in Supplementary Material Table S1). The collected nest fragments were placed inside plastic pots (5 L) previously rimmed on the inside with fluon paint (Dupont, Paulínia, Brazil). Back in the laboratory, the ants were separated from the soil by slow dripping [31]. Obtained ants were fixed in 95% ethanol and stored in a −20 °C freezer. The nests were inspected for the presence of one (monogyny) or more queens (polygyny) and only monogynous nests were observed.

Species identification was later confirmed in the lab with a stereomicroscope, based on the following diagnostic character states from representative major worker specimens: presence of a medial frontal streak, mesonotum convex in lateral view, post-petiole wider than tall from posterior view and with clearly defined surface ridges covering most of the view (see Supplementary Material Figure S2) [6,32]. Vouchers are deposited in the Entomological Collection of the Museu Paraense Emílio Goeldi, Belém, Pará, Brazil under the collection codes MPEG.HHY 03046576–03046593, and also in the reference collection of "Formigas do Alto Tietê" [33].

2.2. Molecular Identification

Solenopsis invicta samples were identified using the DNA barcode technique [34–36] with DNA extracted from two random workers per nest. Total DNA was extracted from the whole body and used to amplify a 910-bp-long fragment of cytochrome oxidase I (COI) gene using the CIJ (COI-RLR) (5′-TTGATTTTTTGGTCATCCAGAAGT-3′) and DDS-COII-4 (5′-TAAGATGGTTAATGAAGAGTAG-3′) [37,38]. When such combination of primers did not amplify the expected fragment, the substitute primer for DDS-COII-4 'Jerry Garcia' (5′-GGGAATTAGAATTTTGAAGAG-3′) [24,39] was used instead, generating a fragment approximately 780 bp long.

The social condition was identified by Multiplex PCR amplification using the set of primers: 26BS (5′ CTCGCCGATTCTAACGAAGGA 3′), 16BAS (5′ ATGTATACTTTAAAGCATTCCTAATATTTTGTC 3′), 24bS (5′ TGGAGCTGATTATGATGAAGAGAAAATA 3′) and 25bAS (5′ GCTGTTTTTAATTGCATTTCTTATGCAG 3′), as described elsewhere [40]. Amplification was performed in reaction tubes with 50 microliters of final volume containing DNA, buffer, MgCl2, dNTPs, primers, and Taq DNA polymerase. The conditions for the amplification reaction were: 1 cycle at 94 °C for 2 min followed by 35 cycles at 94 °C for 15 s, 55 °C for 15 s, 68 °C for 30 s, and a final extension step at 68 °C for 5 min. Additional samples provided by Sanford Porter of the United States Department of Agriculture (USDA) were included as a positive control for polygyny. Electrophoresis of obtained amplicons was conducted and compared with the control polygynous samples.

Sequencing reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Carlsbad, CA, USA/Applied Biosystems, Waltham, MA, USA) in an ABI 3730 DNA Analyzer sequencer (Life Technologies/Applied Biosystems). Samples were edited and verified using BioEdit version 7.2.5 [41] and MEGA 7 [42] softwares. COI sequences were compared to other sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov/genbank, accessed on 15 January 2018) to determine mitochondrial haplotypes. The haplotypes obtained were matched with sequences under accession numbers AY950736.1, AY950742.1, AY950735.1, and EU352608.1 [24,26]. Workers belonging to the same nest had identical COI sequences, supporting the estimated monogynous social structure, thus all subsequent analyses continued using one representative sequence per nest. Low frequency haplotypes were re-sequenced to control for DNA polymerase errors during the amplification. All sequences generated in the present study were deposited to GenBank (accession numbers provided in Supplementary Material Table S1).

2.3. Molecular Diversity and Bayesian Inference

The identification of specific mitochondrial haplotypes and the calculation of haplotype (h) and nucleotide (π) diversity were performed using the DNAsp software [43]. Sequences were split into two groups: (a) samples from the south slope, and (b) samples from the north slope.

Bayesian inference was performed with MrBayes v. 3.2 [44] using the HKY model with a proportion of invariant sites, and the rest was taken from a gamma distribution (HKY + I + G) previously obtained from MrModeltest v. 2.2 [45]. The Markov chain was run for 1,000,000 generations and sampled every 500 generations. Parametric values were determined by discarding 5% of the trees and calculating posterior probabilities using the remaining trees. The generated tree was not rooted, under the assumption that the rate of evolution was approximately uniform across all branches. For this reason, we used the median estimate of the longest path between two taxonomic units, i.e., midpoint rooting [46]. The haplotype network was constructed with Network v. 4.5.1.0 [47] using the median-joining parameter. We also applied the AMOVA test in Arlequin v. 3.5.2.2 to generate the clustering of the haplotypes into supergroups.

2.4. Correlation between Genetic and Geographic Distances

We calculated the correlation between genetic distance and geographic distance for all our samples to estimate the dispersal patterns for *S. invicta* species based on the obtained haplotypes' distribution. The geographical coordinates of the collected nests were transformed into metric distances using the 'rgdal' package [48] of R software [49]. The genetic distance between sequences was calculated using Kimura's two-parameter model of nucleotide substitution [50]. The correlation between genetic and geographic distances was calculated using the Mantel test (based on Spearman's rank correlation, permutations = 2×10^5) in the vegan package of R [51].

3. Results

3.1. Molecular Identification and Diversity

Mitochondrial amplicons were 726 bp long, yielding *S. invicta* haplotypes ranging in 99–100% similarity to sequences deposited in GenBank by previous authors. Our study recovered four different sequences previously described as haplotypes of *S. invicta*, herein termed H1–H4 (Table 1).

Table 1. Haplotypes of *Solenopsis invicta* collected from highways along the border of Atlantic Forest in São Paulo, Brazil, based on 726 bp-long amplicons and sequence similarity at NCBI.

Haplotype (Present Study)	Associated Name at NCBI	NCBI Accession Entry	Type Locality at NCBI
H1	MM34	EU352608.1	Mississipi, EUA
H2		JN808817.1	Corrientes, Argentina
H3	H41	AY950736	Céu Azul, PR, Brazil
H4	H47/H40 *	AY950742.1 AY950735.1	Campo Grande, MS, Brazil Céu Azul, PR, Brazil

* Considered as a single haplotype in the present study, within the obtained amplicon length scope.

The most frequently collected haplotype overall was H3, but haplotypes H1 and H2 were dominant across the south slope of Serra do Mar, facing the coast (Figure 1A), and haplotypes H3 and H4 dominated the north slope of Serra do Mar (Figure 1B). The respective haplotype (h) and nucleotide (π) diversities calculated for sequences retrieved from the north slope were (h ± SD = 0.35 ± 0.159; π = 0.00098 ± 0.00044), and from the south slope (h = 0.75 ± 0.139; π = 0.03139 ± 0.00712).

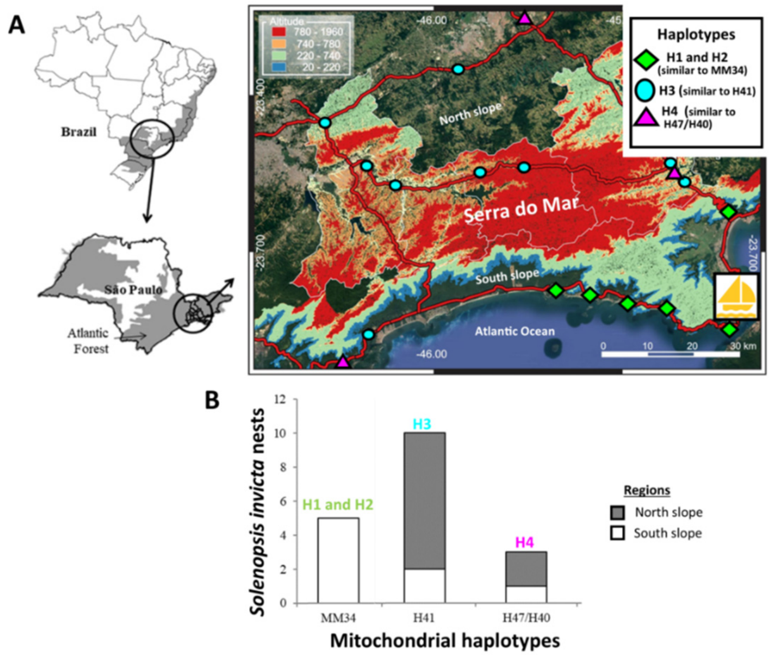

Figure 1. mtDNA COI haplotypes of *Solenopsis invicta* sampled from the north and south faces of the Serra do Mar Mountain range in the state of São Paulo, Brazil. (**A**) Map with sampled haplotype's locations. Red lines indicate the highways used for collections. The elevational gradient in Serra do Mar is indicated by colors, where red corresponds to the highest elevations. A marine port (São Sebastião) is indicated by a yellow boat. (**B**) Distribution of the mitochondrial COI haplotypes obtained.

3.2. Bayesian Inference and Correlation between Genetic and Geographic Distances

Bayesian inference produced a robust phylogenetic tree dividing the mitochondrial haplotypes into supergroup A (composed of haplotypes H1 and H2 from the south slope of Serra do Mar) and supergroup B (composed of haplotypes H3 and H4 from the north slope of Serra do Mar) (Figure 2). Supergroup A was shown to also match previous records from the southern coast of São Paulo (JN808782.1), Argentina (JN808817.1), and the United States (EU352608.1); on the other hand, supergroup B also matched previous records from São Paulo (JN808798.1, JN808783.1), Paraná (southern Brazil, JN808799.1, JN808816.1, AY950734.1) and Pernambuco (northern Brazil, JN808804.1). Other clades obtained contained previous records from southern, southeastern and parts of midwestern Brazil. Genetic and geographic distances were positively correlated according to a Mantel test (r = 0.3532, *p*-value = 0.00183).

Figure 3 presents the haplotype network analysis that compared the different *S. invicta* populations obtained in the study area. The observed genetic distances revealed two supergroups, A and B. Supergroup A included haplotypes H1 and H2 and supergroup B included haplotypes H3 and H4; the genetic difference of haplotypes within each supergroup showed less than 1%, whilst the genetic distance between supergroups was greater than 6% (Figure 3). AMOVA analysis results supported supergroups A and B are different (*p*-value = 0.000), pointing to 98.6% of variance; no difference was found within supergroups (Table 2).

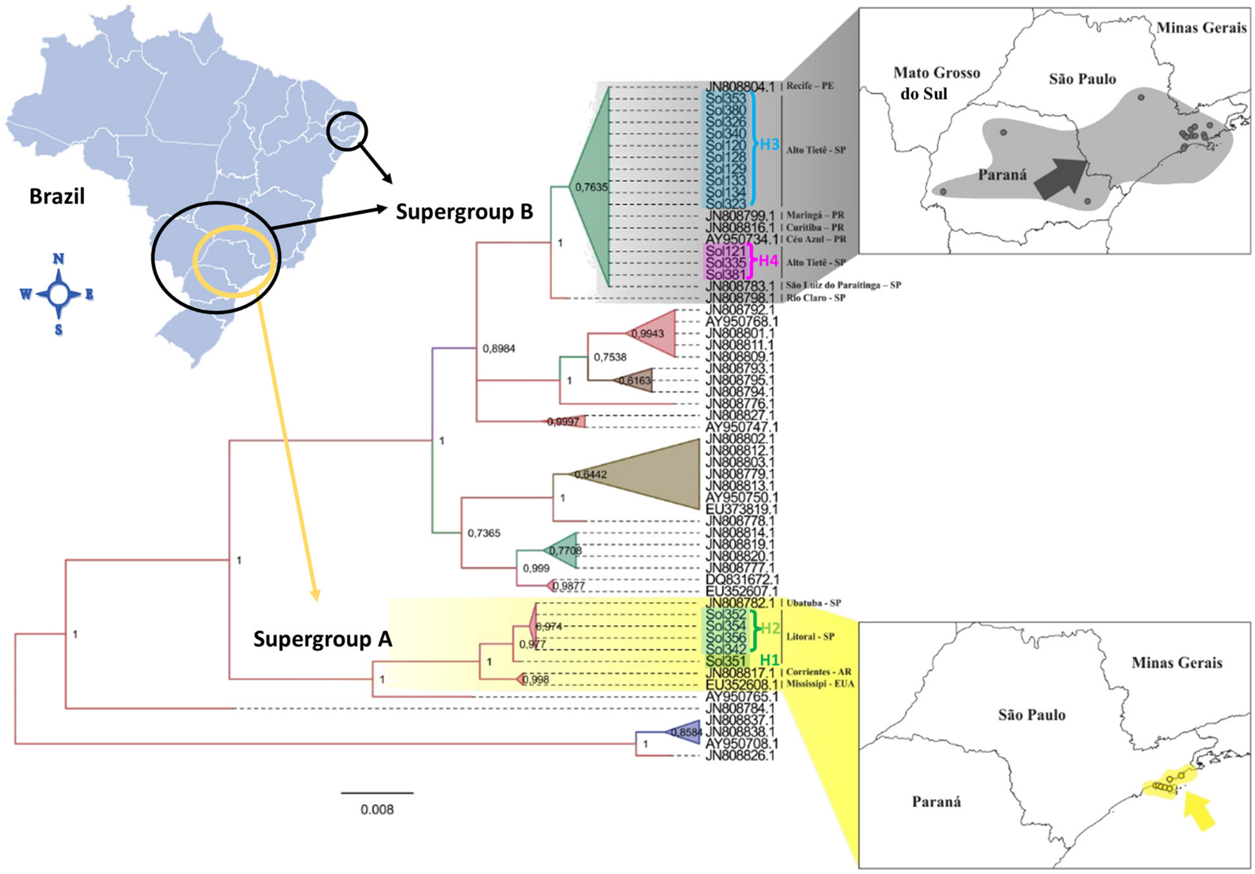

Figure 2. Phylogenetic tree generated by Bayesian inference showing different major lineages of *Solenopsis invicta* found in the south slope (supergroup A) and north slope (supergroup B) of Serra do Mar in the state of São Paulo, Brazil. Supergroup A is shown highlighted in yellow and includes haplotypes H1 and H2. Supergroup B, in gray, includes haplotypes H3 and H4.

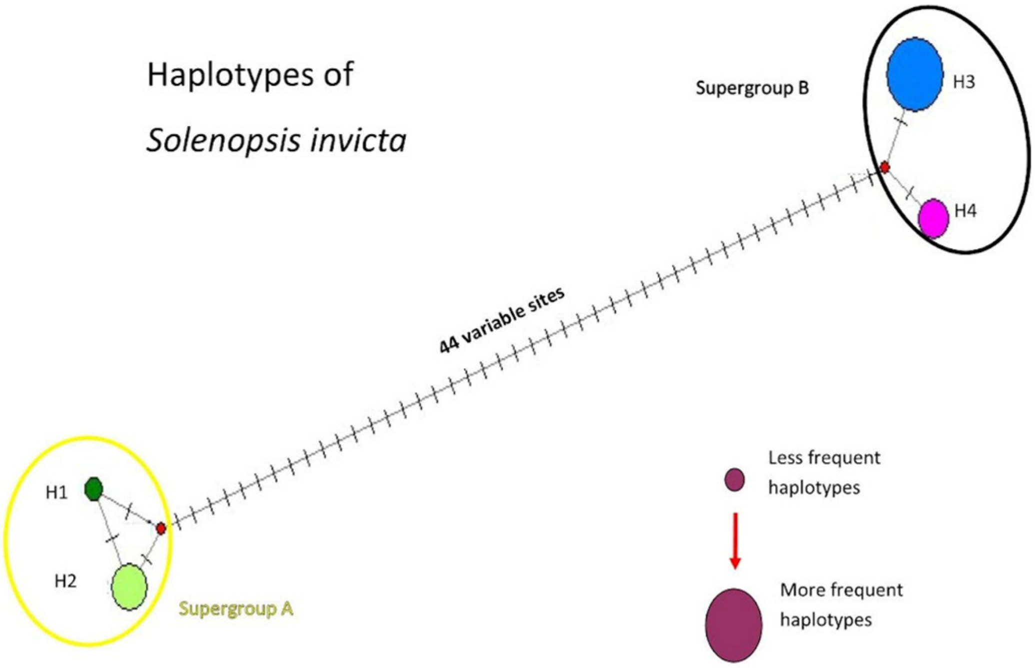

Figure 3. Haplotypes network of Red Imported Fire Ants *Solenopsis invicta* sampled from the north and south faces of the Serra do Mar Mountain range in São Paulo, Brazil. Supergroup A haplotypes

were restricted to the south face of the mountains, and supergroup B haplotypes to the northern face. The haplotype circle size represents the frequency observed, and the whiskers dividing distance lines indicate the numbers of nucleotide differences between haplotypes. Red dots were added by the software, suggesting a hypothetical haplotype.

Table 2. AMOVA results among and within haplotype supergroups A and B of *Solenopsis invicta* fire ant populations sampled from two sides of a mount range in São Paulo, Brazil.

Scope of Variation	Sum of Squares	Variance Components	Percentage Variation	*p*-Value
Between supergroups A and B	328.503	22.72043	98.61733	0.000
Within supergroups A and B	10.381	0.31855	1.38267	
Total	339.333	23.03898		

4. Discussion

In this study, we characterized the molecular diversity of *S. invicta* populations bordering the Atlantic Forest near the seacoast of São Paulo State in Brazil. The study area was selected to contain a significant geographical formation including two well-defined faces of a forested mountain range, which—in principle—cannot be colonized by fire ants. In short, the obtained results indicate a pattern in which imported mitochondrial haplotypes H1 and H2 (supergroup A) colonized the south slope facing the seaside, not far from a maritime port at the city of São Sebastião (São Paulo State, Brazil), while native haplotypes H3 and H4 (supergroup B) were restricted to the north slope facing the continental plateau, likely coming from western Brazil. The fact that H1 and H2 remain limited to the south slope is both indicative of a recent introduction and of the effect of the Serra do Mar range as a geographical barrier delaying the invasive population from expanding into the continent. We further elaborate on this hypothetical scenario below.

The Red Imported Fire Ant is believed to have originated from the western border of Brazil with adjacent countries, spanning grasslands and wetlands in Paraguay, Bolivia, Argentina, and Uruguay [32,52]. The species was first recorded in the state of São Paulo in the late 1980s in the Cerrado biome [53], likely already resulting from an expansion beyond its original natural range. Habitat suitability in disturbed lands, local suppression of specialized predators, and their typical aggressive competitive behavior may have driven a local expansion into regions adjacent to their original territory of natural occurrence [12,20]. Also, their generalist diet habits allow this fire ant species to take advantage of a broad variety of food items [54] while adapting to new foraging ground. As RIFAs display a marked preference for open disturbed environments [55], the fragmentation of natural habitats by pastures is believed to have enabled the rapid expansion of *S. invicta* [56,57].

The molecular diversity of native fire ant populations among Argentina and Brazil is markedly dissimilar, possibly due to the geographical barrier of the Paraná River [38]. On an evolutionary timescale, the establishment of *S. invicta* within Brazil has been demonstrated as recent [52], displaying relatively limited genetic diversity [58]. The molecular diversity of the species within the state of São Paulo has previously been evaluated by different authors along different habitats: around urban areas [7], along highways [46], and in the Atlantic Forest [59]. Fire ant populations across other regions of Brazil (i.e., from the western states of Mato Grosso, Mato Grosso do Sul, or the south states of Paraná, and Rio Grande do Sul) display considerable intrinsic diversity and mtDNA differences [26], enabling a tentative reconstruction of their expansion waves.

In the present study, the haplotypes H1 and H2 closely matched the MM34 *S. invicta* haplotype deposited in the GenBank, which is reported to have invaded the United States from Argentina [26]. These same haplotypes were previously registered in Brazil [46] from the south face of Serra do Mar, not far from the study area (Ubatuba, São Paulo, Brazil; GenBank accession number: JN808782). Thus, the higher frequency of the haplotypes H1 and H2 (related to MM34) lining the south slope towards the maritime port terminal of São Sebastião suggests a recent introduction via cargo ships, as has been suggested by other authors [14] as the main means of *S. invicta* introduction to other different world regions (i.e., Australia, New Zealand, and China). Furthermore, the fact that haplotypes H1 and H2 have been limited to one side of the mountain range indicates it might work as a geographical barrier, slowing their spread. The Paraná River was appointed as a barrier precluding the direct spread of *S. invicta* haplotypes from Argentina into Brazil [38], resulting in split genetic diversity patterns at different levels. The possibility of geographical barriers leaving evolutionary footprints in fire ant haplotypes seems to be an interesting evolutionary mechanism to be confirmed with further inspections beyond mitochondrial genes, including nuclear loci, and possibly in association with other population-specific characteristics.

The haplotypes predominating by the opposite face of the mountain range—H3 and H4—most closely resembled haplotypes H41 and H40/H47 deposited in the GenBank. These were obtained from other regions in Brazil, which is strongly suggestive of a domestic expansion wave [20]. Haplotype H3 (closely related to NCBI H41) was the most frequently collected haplotype overall. The presence of H4 (similar to H40/H47) may further indicate the expansion of *S. invicta* into the region, originating from the south of Brazil (Figure 2).

The fact that a low haplotype diversity was observed among *S. invicta* populations lining the north slope indicates the introduction is recent, considering the typical contraction of genetic diversity in invasive species; this is known as the bottleneck effect [58]. A similar phenomenon was also observed with the ant *Linepithema humile* Mayr, 1868 [60,61] among invasive populations in Africa, North America, Europe, Asia, and Oceania. It is interesting to note that the haplotype diversity was slightly higher lining the south slope of Serra do Mar than in the north slope, though still lower than that observed by other authors at the original habitats of the species [38] and in other invaded areas in Brazil [46]. This is suggestive of a recent introduction of *S. invicta* to the region, where the Serra do Mar range would have barred or at least hitherto delayed introgression among the mitochondrial haplotypes [62,63].

A Mantel test showed a positive correlation between geographic and genetic distances of the *S. invicta* haplotypes sampled in the present study, in agreement with the expected for a process of species expansion and dispersal. The same pattern was also evidenced in previous observations for other species [63,64]. The sampled highways ran by the north and south slopes of Serra do Mar, along the borders of Atlantic Forest fragments. We consider the sampled frequency of *S. invicta* nests as alarming, considering this species is an aggressive and competitive invader [65] that can affect arthropod communities, impacting native biodiversity [66]. Invasive ant species such as *S. invicta* display some adaptations in their behavior and mode of reproduction, which enables them to effectively take over new environments [67] from local ants.

Previous studies [11,68,69] showed that RIFAs are already present in rural areas in Brazil containing fragments of native Atlantic Forest vegetation. In this study, we demonstrate further evidence that *S. invicta* is expanding territory towards the Brazilian Atlantic Forest Domain. There are no reports of relevant economic damage caused by *S. invicta* in Brazil, and it is generally considered a species under control by their natural enemies [53]. However, such natural enemies are bound to be affected by the intense urbanization [70] and fragmentation taking place in the Atlantic Forest [29]. In that sense, *S. invicta* must be monitored over the years, given that the species can have serious negative impacts on biodiversity and ecosystems. Our data suggest that *S. invicta* has expanded through the importation of new haplotypes, likely from a nearby marine terminal port. Insights into the haplotypic diversity of *S. invicta* help us to better understand the expansion of invasive species towards the Atlantic Forest biome, and the roles of anthropic influence and natural geographical barriers, as hereby illustrated by the "Serra do Mar" mountain range. Further information regarding this possibility would be important for the protection and conservation of local biodiversity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15020194/s1, Figure S1: The study area included the outskirts of two opposing faces of the mountain range of Serra do Mar, dominated by the Atlantic Forest.; Table S1: Fire ants collected in the present study.; Figure S2: Photographs of *Solenopsis invicta* (MPEG.HHY03046581).

Author Contributions: Conceptualization, M.S.C.M. and R.F.S.; Data curation, J.M.C.A. and O.G.M.S.; Formal analysis, M.O.R., V.H.N. and R.F.S.; Methodology, D.Y.K., J.M.C.A., R.F.S., V.H.N. and O.G.M.S.; Project administration, M.S.C.M.; Writing of original draft, M.O.R., R.H. and M.S.C.M.; Writing review and editing, M.O.R., E.G.P.F. and A.W.S.H. All authors have read and agreed to the published version of the manuscript.

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