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Research article

Occurrence of 15 Haplotypes of *Linepithema micans* (Hymenoptera: Formicidae) in Southern Brazil

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Abstract

The ant genus *Linepithema* is widely known, thanks to the pest species *Linepithema humile* (Mayr), which is easily mistaken for *Linepithema micans* (Forel) due to their morphological similarity. Like *L. humile*, *L. micans* is associated to the main grapevine pest in Brazil, *Eurhizococcus brasiliensis* (Wille), also known as ground pearl. Therefore, the present study uses mtDNA fragments to expand the knowledge of haplotype diversity and distribution of *L. micans* in the state of Rio Grande do Sul (Brazil), to understand the genetic differences of the populations identified in this study. We identified 15 haplotypes of *L. micans* spread across different localities. Twelve of these haplotypes were new for the species. The high haplotype diversity uncovered in Rio Grande do Sul (Brazil) for this species was predictable, as *L. micans* is in its native environment. Additional studies that take gene flow into account may reveal interesting aspects of diversity in these populations.

Key words: mitotype, ground pearl, Rio Grande do Sul

The ant genus *Linepithema* Mayr is well-known around the world thanks to the Argentine ant, *Linepithema humile* (Mayr), which is widely distributed and considered invasive and a pest in many countries. Bolton (2016) recognized 20 valid species in this genus, distributed in Central and South America and some countries in Africa, Europe, and Oceania.

For many years, *L. humile* (Hymenoptera: Formicidae) was considered prevalent and the only species of this genus (Gallotti 1976, Soria and Gallotti 1986, Botton et al. 2010) in areas infested by *Eurhizococcus brasiliensis* (Wille) (Hemiptera: Margarodidae) that is considered the main grapevine pest in Brazil (Gallotti 1976, Soria and Gallotti 1986, Botton et al. 2010). However, Sacchett et al. (2009) identified many species of ants, including *Linepithema micans* (Forel 1908), in the vineyards of the Serra Gaucha region that were infested by *E. brasiliensis*. This result was confirmed in the Brazilian state of Rio Grande do Sul by Martins et al. (2012) using molecular techniques. This high visibility of *L. humile* as the main pest likely stems from the great similarity between the workers of the two species, which causes errors arising from morphology-based identifications (Wild 2007).

The association of *E. brasiliensis* with *L. micans* has generated problems for viticulture, especially in southern Brazil (Nondillo et al. 2013, 2016). One of the important aspects of the survival of *E. brasiliensis* is its interaction with ants that harvest the expelled sugar excretions ("honeydew") in a mutualistic association, in which both the ant and hemipteran pests are benefited (Sacchett et al. 2009, Nondillo et al. 2013). A significant increase in the population size of *E. brasiliensis* and *L. micans* occurs when they are associated, demonstrating the importance of this ant species in the establishment of the scale on the roots of grapevine plant (Nondillo et al. 2013, 2014, 2016). So far, the use of toxic baits to manage *L. micans* to reduce vineyard infestation by scales has yielded promising results (Nondillo et al. 2014). However, the ecological interactions of *L. micans* with *E. brasiliensis* and its role in their dispersal are not fully known. Understanding the diversity and distribution of this species in its natural environment is essential to properly control ground pearl infestations (see Nondillo et al. 2016). In this sense, it is important to learn how diverse *L. micans* is in its natural environment, because the systematics of this group is complex (Wild 2007). Furthermore, the ability of *L. micans* to adapt to different conditions and ecological roles may be explained by its close phylogenetic relationship with *L. humile* (Wild 2009). This raises the possibility that this species may also display eruptive population dynamics in its native environment (Martins et al. 2012).

Sequencing mtDNA fragments is a frequent tool to distinguish different haplotypes and to understand evolutionary processes. Martins et al. (2012) characterized mitochondrial fragments (partial

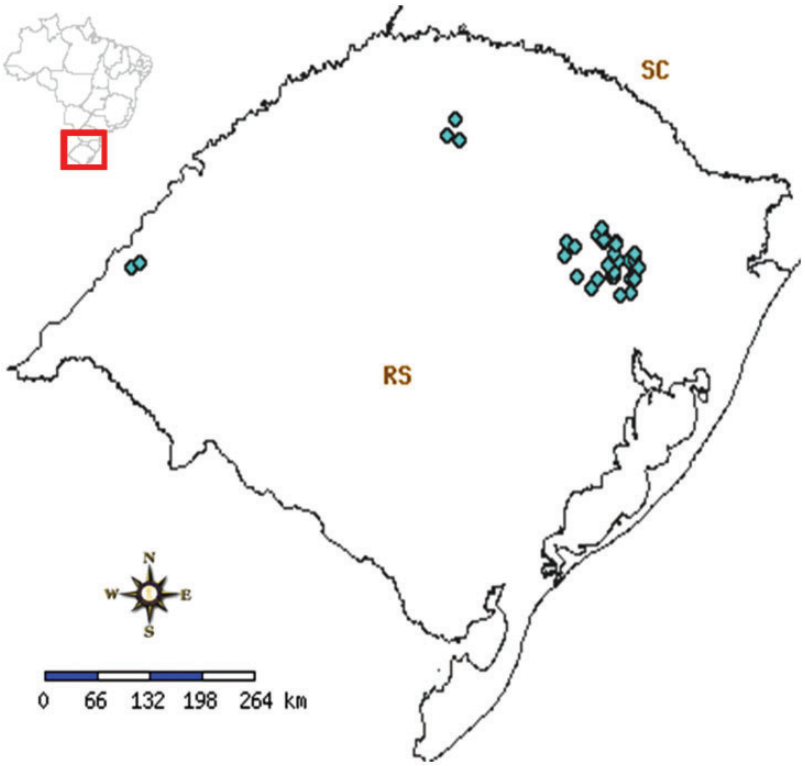

Fig. 1. Collection sites of *L. micans* for the present study.

COI, tRNAleu, and partial COII) in populations of *L. micans* in southern Brazil and identified three different haplotypes. The present study aims to expand the knowledge of haplotype diversity and distribution in the region of state of Rio Grande do Sul (Brazil), in an attempt to understand the genetic differences of the populations identified in this study.

Materials and Methods

Study Areas

Ants were collected from grapevines that were infested with ground pearl in the following grape-producing municipalities in the state of Rio Grande do Sul, Brazil: Antônio Prado, Caxias do Sul, Dois Lajeados, Farroupilha, Flores da Cunha, Garibaldi, Monte Belo do Sul, Nova Pádua, Pinto Bandeira, Sarandi, and Veranópolis (55 properties in total, Fig. 1).

There were two collections per vineyard block between 2008 and 2011, one in spring/summer and the other in autumn/winter. The ants were collected using underground pitfall traps designed by Morini et al. (2004) with slight modifications. The traps consisted of a set of two empty, black, plastic pipes (3.3 cm diameter by 5.0 cm height) connected by a 5.0-cm string, with a cap and 21 lateral holes (3 mm). The string was connected to the bottom of the plastic pipe to be able to locate the traps in the soil.

Two kinds of food attractants were used as bait for the ants in the traps. In one of the containers we used cotton wool soaked with a diluted honey solution (70%) and in the other, sardines preserved in vegetable oil. The baits were placed inside the lid of each container. The pitfall traps were placed in the soil to a depth of ~20 cm and were left in the field for a period of 24 h, when they were collected and brought to the laboratory for sorting. After sorting, the ants were stored in vials containing 70% alcohol to be identified later.

Species Identification

The ants were separated into morphospecies, and one specimen of each was mounted following the protocol described by Longino (2000). The ants were identified to subfamily (Bolton 2003) and genus (Bolton 1994). Morphological identification was performed in two steps: at the level of morphospecies, by comparison to specimens deposited in the collection of the Alto Tietê Myrmecology Lab at Universidade de Mogi das Cruzes (Brazil), and at the level of species, by Alex Wild (University of Texas/Austin). *Linepithema micans* was found in 35 of the 55 vineyard blocks sampled; the sampling locations are shown in Fig. 1 and listed in Table 1.

Genetic Analyses

All the ants collected within each property were treated as a single *L. micans* population, both because of their territorial behavior, which is unlikely to allow other subtypes of the same species to forage nearby, and because they are polydomous, i.e., they inhabit numerous nests spread over several meters, sometimes even kilometers. Thus, the high incidence of *L. micans* in vineyard makes it impossible to establish other species of ants (Nondillo, personal observation). Therefore, the analysis included 35 populations of *L. micans* in total. For each population, DNA was extracted from 10 whole individuals, randomly selected. The ants were stored in 80% alcohol and chilled to −20 °C from the date of collection until the extraction. The extractions followed the protocol of Martins et al. (2012).

The GoTaq Hot Start Polymerase kit (Promega) was used for DNA amplification, carried out in a Veriti thermal cycler (Applied Biosystem). The protocol for each amplification reaction was adjusted to 5.0 µl GoTaq Flexi Buffer; 1.0 µl dNTPs (2 mM); 2.5 µl $MgCl_2$ (25 mM); 1.5 µl forward primer (ANT-F 5′-ATTCATTCTT ATCTTGAAATATTATTTC-3′) and 1.5 µl reverse primer (ANT-R 5′-TTCATAAGTTCAGTATCATTGGTG-3′) (Martins et al. 2007),

Table 1. *Linepithema micans* collection sites for the present study, with their corresponding haplotypes

Haplotype	Sequences	Locality	Geographic coordinates
H1	Dlbedin	Dois Lajeados	29° 01.354′ S, 51° 50.522′ W
	DIscussel	Dois Lajeados	28° 58.398′ S, 51° 48.358′ W
	DLdalmas	Dois Lajeados	28° 56.838′ S, 51° 50.630′ W
	CSdallegrave	Caxias do Sul	29° 17.838′ S, 51° 13.827′ W
	FCsmiderle	Flores da Cunha	29° 04.505′ S, 51° 14.246′ W
	MBSbragagnollo	Monte Belo do Sul	29° 09.743′ S, 51° 38.862′ W
	APlovatel	Antônio Prado	28° 50.729′ S, 51° 17.713′ W
	Gagostini	Garibaldi	29° 14.811′ S, 51° 38.334′ W
	HM026683 (Martins et al. 2012)	Bento Gonçalves	29°09.0′ S, 51° 31.0′ W
H2	Dltoni	Dois Lajeados	28° 57.0′ S, 51° 47.0′ W
	Fmoroni	Farroupilha	29° 07.682′ S, 51° 24.394′ W
	Vceppo	Veranópolis	28° 57.224′ S, 51° 31.292′ W
	Vpertile	Veranópolis	28° 58.192′ S, 51° 29.425′ W
H3	PBcomiatto	Pinto Bandeira	29° 07.883′ S, 51° 27.265′ W
	CSmarchesini	Caxias do Sul	29° 14.923′ S, 51° 14.376′ W
	APfalavigna	Antônio Prado	28° 52.673′ S, 51° 13.971′ W
H4	PBgiovanini	Pinto Bandeira	29° 08.818′ S, 51° 26.276′ W
	Vtedesco	Veranópolis	28° 57.343′ S, 51° 31.374′ W
	APfaraon	Antônio Prado	28° 50.277′ S, 51° 22.928′ W
	NPalessi	Nova Pádua	29° 01.863′ S, 51° 19.881′ W
H5	Spotrich	Sarandi	27° 51.289′ S, 53° 01.390′ W
	Scótica	Sarandi	27° 53.706′ S, 52° 58.838′ W
	CStonietto	Caxias do Sul	29° 13.123′ S, 51° 15.237′ W
H6	Scorno	Sarandi	27° 51.943′ S, 53° 01.199′ W
	HM026685 (Martins et al. 2012)	Pinto Bandeira	29°07.0′ S, 51° 26.0′ W
H7	CSlklering	Caxias do Sul	29° 14.477′ S, 51° 14.363′ W
	Csdklering	Caxias do Sul	29° 17.308′ S, 51° 14.255′ W
	FCmascarello	Flores da Cunha	28° 59.552′ S, 51° 13.308′ W
H8	Frobetti	Farroupilha	29° 12.597′ S, 51° 26.884′ W
H9	FCpelizer	Flores da Cunha	28° 57.910′ S, 51° 13.516′ W
	FCcasagrande	Flores da Cunha	29° 04.766′ S, 51° 15.051′ W
H10	FCventurini	Flores da Cunha	29° 01.683′ S, 51° 12.983′ W
H11	Vrmazzarollo	Veranópolis	28° 57.122′ S, 51° 31.559′ W
H12	Vmmazzarollo	Veranópolis	28° 58.616′ S, 51° 30.044′ W
H13	NPcamana	Nova Pádua	29° 02.885′ S, 56° 21.191′ W
H14	NPtatto	Nova Pádua	29° 01.723′ S, 51° 16.160′ W
H15	HM026684 (Martins et al. 2012)	Pelotas	31°44.0′ S, 52° 19.0′ W

both at 6 pmol; 0.15 µl GoTaq Hot Start; 3.0 µl resuspended DNA; and q.s. to 2.5 µl with autoclaved Milli-Q water. The program of the thermal cycler was adjusted to 95 °C for 7 min (initial denaturation), 35 cycles at 94 °C for 1.5 min, 43 °C for 2 min, and 70 °C for 3 min, and a final extension at 65 °C for 7 min. The results of the amplifications were analyzed in 1% agarose gel.

For some populations, it was necessary to perform nested PCR because the amount of material generated by the amplification was not enough for sequencing. For this reason, a 1:9 dilution of the first PCR product was used as the template DNA for a second PCR reaction.

The PCR reactions were purified with the GFX PCR DNA kit (GE Healthcare) following the protocol of the manufacturer, quantified using NanoDrop 2000 (Thermo Scientific), and directly sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an automatic sequencer 3130 Genetic Analyzer (Applied Biosystems) using capillary electrophoresis.

The consensus sequences generated were manually edited with BioEdit (Hall 1999) and aligned using ClustalW (Thompson et al. 1994). After the sequences were confirmed by similarity comparison against the NCBI database, the coding regions (COI and COII) were analyzed separately through the ORF Finder (Open Reading Frame Finder) tool of NCBI (http://www.ncbi.nlm.nih.gov/) to confirm the desired fragment. The tRNA genes were identified using tRNAscan-SE 1.21 (Lowe and Eddy 1997). The numbers and positions of the synonymous and nonsynonymous mutations (deletions, substitutions, and insertions) were identified using DAMBE (Xia and Xie 2001). A median-joining haplotype network was constructed with Network 4.5.1.0 (Bandelt et al. 1999). Haplotype (h) and nucleotide (r) diversity were estimated with DNAsp v5 (Librado and Rozas 2009).

The geographic coordinates of the colonies were transformed into metric distances using the rgdal routine (Bivand et al. 2013) in R software (R Core Team 2015). The genetic distances (by nucleotide) between each pair of sequences (Table 2) were calculated using the Kimura 2-parameter model (Kimura 1980) in PAUP 4.0 (Swofford 1993). The correlation between genetic and geographic distances was calculated with the Mantel test using the vegan routine in R (Oksanen et al. 2015).

Results and Discussion

Thirty-four out of the 35 colonies sampled were successfully sequenced. We obtained a 489-bp consensus sequence, with 150 bp corresponding to COI, 60 bp to leucine tRNA, and 271 bp to COII.

Table 2. Genetic distances based on paired nucleotide differences among *L. micans* haplotypes

	H1[a]	H2	H3	H4	H5	H6[a]	H7	H8	H9	H10	H11	H12	H13	H14	H15[a]
H1[a]	xxx														
H2	3	xxx													
H3	1	4	xxx												
H4	4	1	5	xxx											
H5	4	3	5	4	xxx										
H6[a]	2	1	3	2	2	xxx									
H7	1	2	2	3	5	3	xxx								
H8	22	23	22	24	24	22	23	xxx							
H9	1	4	1	5	5	3	2	22	xxx						
H10	3	6	4	7	7	5	4	23	4	xxx					
H11	1	4	2	5	5	3	2	23	2	4	xxx				
H12	3	2	4	3	3	1	4	21	4	6	4	xxx			
H13	3	6	4	7	7	5	4	23	4	2	4	6	xxx		
H14	1	2	2	3	3	1	2	23	2	4	2	2	4	xxx	
H15[a]	6	5	7	6	6	4	7	26	7	9	7	5	9	5	xxx

[a] Haplotypes previously found by Martins et al. (2012).

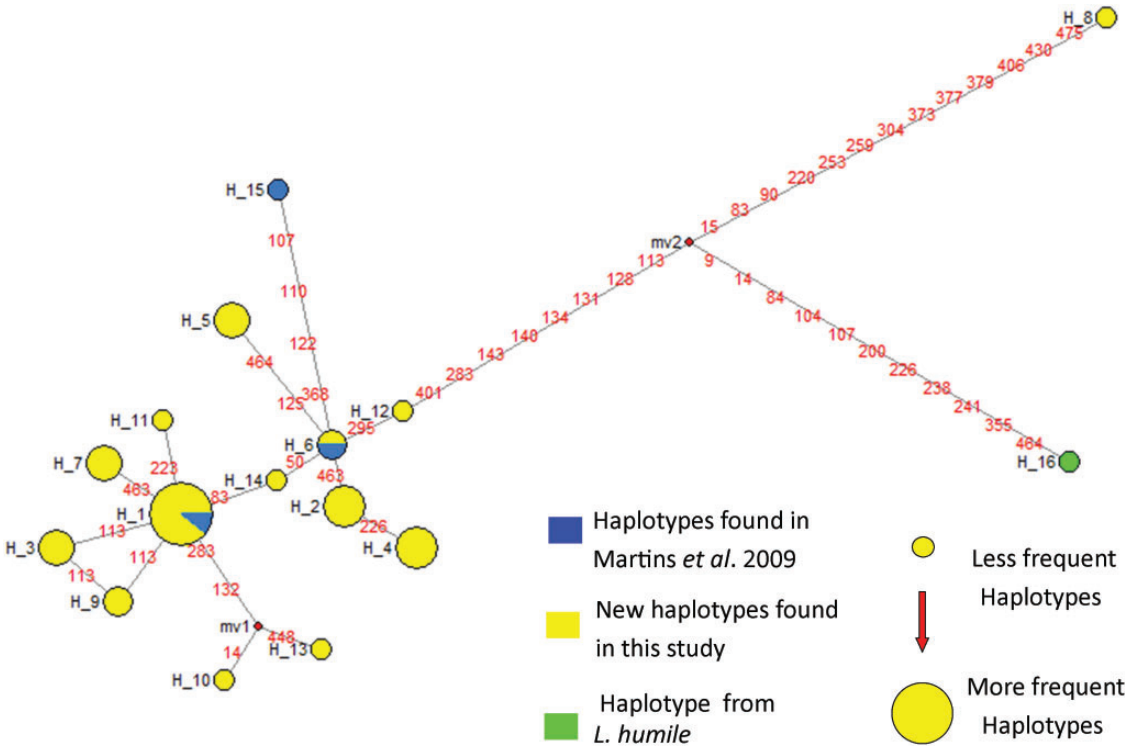

Fig. 2. *Linepithema micans* haplotype network in Rio Grande do Sul, Brazil. The haplotypes analyzed by Martins et al. (2012) are indicated in blue, and the haplotypes found in this study are indicated in yellow. *Linepithema humile* haplotype (retrieved from GenBank AF147050) used as outgroup is indicated in green. The haplotype size represent the frequency found, and red numbers represent the sites where there are nucleotide differences between haplotypes. The MV1 point in red was added by the program as hypothetical haplotype.

Nucleotide frequencies were 0.374 for A, 0.143 for C, 0.075 for G, and 0.426 for T, corroborating the high A + T frequency for insects (Simon et al. 1994). All samples were distributed into 15 distinct haplotypes; of these, 12 were new for the species. The new findings were deposited in GenBank under accession numbers KM211952–KM211965. For comparison, the haplotypes from Martins et al. (2012) were also added to the analyses. The leucine tRNA was identical to the one described by Martins et al. (2012) for *L. micans*. No variation was found among the additional colonies analyzed, therefore this gene is conserved in this species.

Haplotype Network

A network analysis identified 15 *L. micans* haplotypes in Rio Grande do Sul, Brazil (Fig. 2; Table 2). Note how all haplotypes of *L. micans* are distinct from *L. humile*. We identified 12 new haplotypes in the present study. H1 and H6 were detected at multiple locations. The H1 haplotype, which had already been found in Bento Gonçalves (Martins et al. 2012), was detected in Dois Lajeados, Caxias do Sul, Flores da Cunha, Monte Belo do Sul, Antônio Prado, and Garibaldi. The H6 haplotype, previously found in Pinto Bandeira (Martins et al. 2012), now has an additional record in

Sarandi. In contrast, H15 is still confined to Pelotas, as previously observed by Martins et al. (2012). There is no relationship between haplotype variation and geographic location, because haplotypes were shared among different localities. It is also interesting to note that H8, from Farroupilha, was genetically very distant from the other haplotypes. Despite being very different from the others, H8 cannot be considered a separate species, because the delimitation of this species is too complex to be analyzed using a single gene (Green 1996), but we cannot exclude the possibility. Recent studies have adopted the so-called integrative taxonomy approach (Schlick-Steiner et al. 2010, Darienko et al. 2015), which consists of analyses that include more genes (e.g. nuclear genes) and behavioral, natural, and morphological data, among others, to confirm the hypothesis.

Based on genetic distance measured by nucleotide differences, the most variable haplotype (5.31%) is H8, from the Farroupilha colony (Table 2). With the exception of H8, the maximum difference between pairs of all the other haplotypes was 1.86%, which is an expected value for intraspecific diversity of *L. micans* (Hebert et al. 2003). The nucleotide differences between haplotypes H1, H6, and H15 were also analyzed, because these had already been identified by Martins et al. (2012). These haplotypes had the same rate of variation as the others.

Moreover, there is no correlation between the genetic distances and the geographic locations of the colonies using the Mantel test ($r = 0.007$, $P = 0.206$). The lack of correlation may be related to the presence of different ant mitotypes in the neighboring locations. The common association of *L. micans* with grapevines (Sacchett et al. 2009) could explain this widespread distribution, because seedlings of this plant are sold throughout the state, which may facilitate the dispersal of this species to new locations.

The high haplotype diversity observed in Rio Grande do Sul, Brazil (h = 0.905, or 0.911 after excluding H8), was expected for *L. micans*, because this species was in its native environment (Tsutsui et al. 2001). Hence, we believe that the great diversity confirmed by the present study reflects the fact that the species is native to the region. This also gives *L. micans* the ability to settle in the vineyard, and does not allow other species of ants to associate in the region. More studies that take gene flow into account may reveal interesting aspects of diversity in these populations.

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