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Relationship between *Psidium* species (Myrtaceae) by resistance gene analog markers: focus on nematode resistance

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ABSTRACT. Guava (*Psidium guajava* L.) crop is severely affected by the nematode *Meloidogyne enterolobii*. Native *Psidium* species have been reported as sources of resistance against this nematode. Knowledge on the molecular relationship between *Psidium* species based on plant resistance gene analogs (RGA) can be useful in the genetic breeding of guava for resistance to *M. enterolobii*. In this study, RGA markers from conserved domains, and structural features of plant R genes, were employed to characterize *Psidium* species and establish genetic proximity, with a focus on nematode resistance. SSR markers were also applied owing to their neutral nature, thus differing from RGA markers. For this, species reported as sources of resistance to *M. enterolobii*, such as *P. cattleianum* and *P. friedrichsthalianum*, as well as species occurring in the Atlantic Rainforest and susceptible genotypes, were investigated. In 10 evaluated *Psidium* species, high

interspecific genetic variability was verified through RGA and SSR markers, with intraspecific variation in *P. guajava* higher with SSR, as was expected. Resistant species were clustered by RGA markers, and differential amplicons among genotypes resistant and susceptible to *M. enterolobii* were identified. Knowledge on the molecular relationships between *Psidium* species constitutes useful information for breeding of the guava tree, providing direction for hybridization and material for rootstocks. Additionally, the genetic relationship between native species, which have been little studied, and *P. guajava* were estimated by RGAs, which were confirmed as important markers for genetic diversity related to pathogen resistance.

Key words: *Meloidogyne enterolobii*; Resistance gene analog marker; Guava; SSR

INTRODUCTION

Meloidogyne enterolobii is a harmful nematode of the guava crop (*Psidium guajava* L.) (Pereira et al., 2009). Although genetic resistance to this nematode has not been reported in commercial guava genotypes, different studies have identified resistant species in the genus *Psidium*, such as *P. cattleianum* and *P. friedrichsthalianum* (Biazatti, 2013; Freitas et al., 2014; Souza et al., 2015). However, the genus *Psidium* comprises approximately 100 species (Landrum and Kawasaki, 1997), of which the majority are little known, as respective studies are still lacking. The identification and characterization of resistant *Psidium* species may benefit the genetic breeding and development of new technologies for guava production, both by characterizing sources of resistance and by identifying species useful as rootstocks for *P. guajava*. Strategies using the so-called resistance gene analogs (RGA), molecular markers based on plant resistance genes (R genes), constitute one unexplored alternative that may be used for this purpose.

R genes are crucial for disease responses, and their products are important for pathogen inhibition (Sekhwal et al., 2015). These genes are of a qualitative nature (Kushalappa and Gunnaiah, 2013) and are subject to monogenic inheritance, which facilitates their introgression into commercial cultivars. Moreover, owing to their large effect, they constitute targets for breeding, which increases interest in the identification and characterization of sources of resistance. A large number of R gene products is known, and they encode proteins with conserved domains (Sekhwal et al., 2015). Studies have investigated the domains of these proteins to identify genes providing resistance to different pathogens in crops (Lei et al., 2014) and to predict R genes from sequenced genomes using bioinformatic approaches (Sekhwal et al., 2015).

The conserved domains most commonly found in these proteins are as follows: leucine-rich repeats (LRR); nucleotide-binding sites (NBS); leucine zipper (LZ); coiled coil (CC); interleukin-1 receptor-homologous protein-protein interaction domain [Toll/Interleukin-1receptor (TIR)]; transmembrane domains (TM); nuclear localization signals (NLS); amino acid (WRKY); and kinase domains (Sekhwal et al., 2015). The NBS domain contains three strictly ordered motifs; a P-loop or kinase-1a, kinase-2, and kinase-3a, which are critical for the binding of various ATP/GTP proteins (Tameling et al., 2002). Conserved domains are also described within NBS and LRRs, and include the hydrophobic domains GLPLAL and RNBS,

with the latter consisting of RNBS-A, -B, and -C motifs (Dinesh-Kumar et al., 2000; Tameling et al., 2002; Amaral et al., 2006). GLPLAL is a highly conserved domain found in NBS-LRR resistance genes (Lu et al., 2011), which has also been described as a transmembrane motif of the NBS domain (Baek and Choi, 2013).

Plant RGAs can be grouped as either nucleotide-binding sites (leucine-rich repeat [NBS-LRR]) or as transmembrane leucine-rich repeats (TM-LRR). The NBS-LRRs (classified into two major groups: TIR-NBS-LRRs and CC-NBS-LRRs) specifically target pathogenic effector proteins inside host cells, termed an effector-triggered immunity (ETI) response. The TM-LRRs are main components of the first-line plant immune response triggered by microbial elicitors in the extracellular space (Sekhwal et al., 2015). For resistance to nematodes, R genes in both groups have been described. The genes *Mi-1* and *Hero A* in tomato encode proteins containing a CC-NBS-LRR domain (Milligan et al., 1998; Ernst et al., 2002), and the gene *Ma* in *Prunus cerasifera* encodes proteins containing a TIR-NBS-LRR domain (Claverie et al., 2011). In soybean, the genes *rhg1* and *Rhg4* confer resistance to cyst nematode and encodes proteins containing extracellular LRR transmembrane domains (Meksem et al., 2001), as does the gene *Hs1pro-1* of sugar beet (Cai et al., 1997).

The conserved protein domains permit degenerate primers to be used in studies aiming to identify resistance genes in different species (Vieira et al., 2016); the so-called RGA markers. In this context, the main objective of this study was to characterize *Psidium* species using genomic regions associated with pathogen resistance in plants using RGA markers from the NBS, GLPLAL, and RNBS-B domains. For this, nematode-resistant and -susceptible species were employed. In addition, microsatellites, considered as neutral markers, were applied to expand the information on the genetic proximity of the species. In this way, the present work aims to study distinct species using molecular tools in order to characterize and estimate the genetic relationships between *Psidium* species with a focus on nematode resistance.

MATERIAL AND METHODS

Plant material

Genotypes were selected with the aim of representing species and plants with known resistance or susceptibility to *M. enterolobii*, as well as *Psidium* species not yet studied. Twenty plants were used, comprising 10 *Psidium* species (Table 1). Ten *P. guajava* genotypes were included: four cultivars - Paluma, Pedro Sato, Kumagai, and Século XXI (susceptible to *M. enterolobii* according to the literature) - and six wild genotypes found to have low *Meloidogyne* reproduction factor values after phenotypic evaluation (Silva, 2013). The remaining species were included in order to investigate *M. enterolobii* resistance as reported in the literature, such as *Psidium friedrichsthalianum*, *Psidium cattleianum*, and *Psidium myrtoides* (Biazatti, 2013; Freitas et al., 2014). In addition, species collected in the Atlantic Rainforest biome of the state of Espírito Santo (ES - Brazil) without known information on pathogen resistance were included as follows: *Psidium brownianum*, *Psidium guineense*, *Psidium oblongatum*, *Psidium ovale*, *Psidium sartorianum,* and *Psidium* sp. Wild species were collected, identified, and deposited in the RB herbarium collection, with duplicates for the herbaria VIES and CVRD (See: http://sweetgum.nybg.org/science/ih/). Commercial seedlings were obtained from the plant nursery FRUCAFÉ, located in Linhares (ES - Brazil). Since it has been recently shown that the incompatibility of genome size and chromosome number may be related to a lack of

success using wild species as rootstocks (Souza et al., 2015), information was also sought regarding the ploidy of the species, aiming to aggregate knowledge that may direct studies on their utilization (Table 1).

Table 1. Description of the genotypes used in this study.

Species	Genotype/Abbrev.	Reaction to *M. enterolobii*	Chromosome number (2n)[9]
P. guajava	cv. Paluma (P)	Susceptible[1]	22
P. guajava	cv. Pedro Sato (PS)	Susceptible[1]	22
P. guajava	cv. Kumagai (K)	Susceptible[1]	22
P. guajava	cv. XXI Century (C)	Susceptible[1]	22
P. guajava	ES1	Resistant[2]	22
P. guajava	ES2	Resistant[2]	22
P. guajava	PR1	Resistant[3]	22
P. guajava	PR2	Resistant[3]	22
P. guajava	PR3	Resistant[3]	22
P. guajava	MG	Resistant[3]	22
P. friedrichsthalianum	*P.f.*	Resistant[4,5]	22, 44
P. myrtoides	*P.m.*	Resistant[6,7]	88
P. cattleianum I	*P.c.*I	Resistant[1,5,6,7]	44, 46, 55, 58, 66, 77, 82, 88
P. cattleianum II	*P.c.*II	Resistant[1,5,7]	44, 46, 55, 58, 66, 82,88
P. guineense	*P.g.*	Resistant[8]/ Susceptible[1,5,7]	22, 44, 55
P. ovale	*P.ov.*	-	-
P. oblongatum	*P.o.*	-	-
P. brownianum	*P.b.*	-	-
P. sartorianum	*P.s.*	-	-
Psidium sp.	*P.*sp.	-	-

[1]Miranda et al., 2010; [2]Vieira, 2011; [3]Silva, 2013; [4]Genotype with resistant phenotype provided by EMBRAPA/CENARGEM; [5]Freitas et al., 2014; [6]Genotypes with resistant phenotype identified by the research group; [7]Marques et al., 2012; [8]Costa et al., 2012; [9](Ellshoff et al., 1995; Costa and Forni-Martins, 2007; Coser et al., 2012; Souza et al., 2015).

Molecular analysis

The genomic DNA of each plant was extracted from young leaves by the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1990). The integrity and concentration of DNA samples were verified by electrophoresis on 0.8% agarose gel stained with ethidium bromide and by spectrophotometry. To identify polymorphisms related to pathogen resistance in *Psidium* species, RGA markers were used, which derived from degenerate primers of the P-loop motif from the domains NBS (S2 and F1), GLPLAL (As1, As2, As3, and LM637), and RNBS-B (R1) (Kanazin et al., 1996; Leister et al., 1996; Yu et al., 1996; Chang et al., 2009) (**Table S1**).

Combinations of these RGA primers were amplified in a final volume of 20 µL containing 1X Master Mix (*Fermentas*), 30 ng DNA, 0.4 µM each primer, and 1.0 U Taq DNA polymerase. The reactions were subjected to initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 40°C, and 2 min at 72°C, with a final extension of 10 min at 72°C. The polymerase chain reaction (PCR) products were separated by gel electrophoresis on 1.4% agarose, stained with ethidium bromide and visualized under ultraviolet (UV) light. Twenty-eight combinations of the RGA primers S2, As1, As2, As3, LM637, F1, and R1 were evaluated before hand; those showing best amplification quality were selected for use in the study as follows: As3+R1, As1+As2, As2+As2, As1+F1, S2+S2, LM637+LM637, As2+LM637, As1+LM637, As1+As1, and As3+As3. The detected polymorphisms were converted to a binary data matrix to identify dissimilarities among the genotypes using the index of simple coincidence. This index was selected to consider coincidences (1-1; 0-0) and disagreements (0-1; 1-0) in the same way. Clustering analysis of species and primers with similar amplification profiles was performed using

each obtained matrix and the un weighted pair group method with arithmetic mean (UPGMA). The data were represented by a heatmap, whereby the default color gradient sets the lowest value in the heatmap (0 - absence) to yellow and the highest value (1 - presence) to blue. Heatmaps are well-suited for the visualization of large volumes of multidimensional data, and can be used to identify clusters of rows with similar values, which are shown as areas of similar color. The analysis was performed using the software R.

As RGA markers are derived from conserved regions within plant genomes, analyses were also carried out with hypervariable genomic regions using 10 microsatellite markers common to the selected species (Tuler et al., 2015), aiming to comparatively evaluate the species. The following markers were employed: mPgCIR98 and mPgCIR256 (annealing temperature = 50°C), mPgCIR416, mPgCIR16, mPgCIR347, mPgCIR11, mPgCIR13, mPgCIR109, mPgCIR187, and mPgCIR392 (annealing temperature = 55°C) (Tuler et al., 2015). The reaction was performed in a final volume of 15 µL containing 60 ng DNA, 0.3 µM each primer, 1X KCl buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP (deoxynucleotide triphosphates), and 1.0 U Taq DNA polymerase. The program used included 4 min at 94°C, followed by 35 cycles of 45 s at 94°C, 1 min at the annealing temperature, and 1 min at 72°C, with a final extension of 8 min at 72°C. The PCR products were separated and visualized on 6% polyacrylamide gel electrophoresis stained with ethidium bromide and photographed under UV light. Subsequently, values for polymorphic information content (PIC) and observed heterozygosis (H_0) per primer were estimated. Data obtained for alleles of each individual were subjected to dissimilarity index analysis using the weighted index. Based on the dissimilarity values, UPGMA grouping was performed using the software R.

RESULTS

Analyses of RGAs

The RGA markers amplified 419 fragments in the 20 evaluated genotypes. Only four fragments were conserved in all species (derived from the primer combinations As1+As2, As2+As2, As1+F1, and As1+As1) (Table 2 and **Table S2**), with 15 fragments occurring in at least 18 genotypes. Species-specific fragments totaled 154, the greatest numbers of which were detected for *P. guajava*, *Psidium* sp., and *P. ovale,* and the smallest number for *P. cattleianum* (Table 3 and **Table S2**).

Table 2. Description of the amplicons generated by 10 combinations of RGA markers in *Psidium* species.

Combination of *primers*	All species				*Psidium guajava*		
	Na	Nam	%p	Bp	Na	Nam	%p
As3 + R1	37	0	100	270-2250	15	11	26.7
As1 + As2	54	1	98.2	120-2200	15	8	46.7
As2 + As2	28	1	96.4	550-3000	12	11	8.3
As1 + F1	9	1	88.9	250-1400	4	1	25.0
S2 + S2	46	0	100	250-2600	16	8	50.0
LM637 + LM637	50	0	100	240-2300	18	6	33.3
As2 + LM637	67	0	100	190-2400	25	14	36.0
As1 + LM637	46	0	100	220-2500	14	9	35.7
As1 + As1	34	1	97.1	600-2500	12	10	16.7
As3 + As3	49	0	100	270-2400	15	10	33.3
Total	419	4	99.0		146	88	39.7

Na = number of amplicons; Nam = number of monomorphic amplicons; %p = percentage of polymorphic amplicons; bp = variation in amplicon size (base pairs).

Table 3. Number of amplicons per species and number of amplicons specific to each *Psidium* species, amplified by 10 combinations of RGA primers.

Species	Na	Nasp	%sp
P. cattleianum	112	3	2.68
P. myrtoides	113	7	6.19
P. guineense	86	10	11.63
P. brownianum	91	11	12.09
P. friedrichsthalianum	96	13	13.54
P. sartorianum	99	17	17.17
P. oblongatum	99	18	18.18
P. guajava	146	29	19.86
P. sp.	114	24	21.05
P. ovale	84	21	25.00
Total		154	

Na = number of amplicons; Nasp = number of amplicons specific to the species; %sp = percentage of amplicons specific to the species.

For *P. guajava* plants, a reduced number of amplicons (146) and percentage of polymorphism (39.7%) were detected (Table 2). Nevertheless, 14 of those fragments were observed only in genotypes resistant to *M. enterolobii*, of which six were also identified in at least one of the source-of-resistance species *P. cattleianum, P. friedrichsthalianum,* and *P. myrtoides*. The genotypes ES1, ES2, PR1, PR2, PR3, and MG of *P. guajava* possessed fragments common with this species (**Table S3**).

Ample genetic diversity among species was indicated by the dissimilarity, which varied from 0.64 (between *P. guajava* and *P. myrtoides*) to 0.92 (between *P. cattleianum* I and *P. ovale*), with a mean value of 0.78. The mean dissimilarity among *P. guajava* genotypes was much lower (0.46). *Psidium guajava* and *P. guineense* presented a higher number of common amplicons, thus constituting a group (Figure 1). In the same way, the species *P. friedrichsthalianum, P. myrtoides,* and *P. cattleianum* I and II, which are known to be resistant, were grouped, showing similar amplification patterns. The others species presented differential amplification patterns and were not clustered. For *P. guajava*, the genotypes Paluma and Século XXI were grouped, as were MG, PR2, PR3, Pedro Sato, and Kumagai, and ES1, ES2 and PR1.

Based on the amplification profiles of the studied species, the RGAs were distributed in four relevant groups (G) (Figure 1 - vertical grouping; **Table S2**). The first group (G1) was composed of the most highly conserved amplicons in the species. The groups G2 and G3 comprised species-specific amplicons; those predominantly of *P. guajava* were clustered in G2, and those specific to each *Psidium* species in G3, reflecting a divergent pattern of amplification, with amplification specificity observed for some species. In G5, amplicons common to species constituting sources of resistance to *M. enterolobii* (*P. cattleianum, P. myrtoides,* and *P. friedrichsthalianum*) were observed.

Analyses of microsatellite markers

The 10 microsatellite markers used in this study amplified 60 alleles, with three to nine alleles per locus. High values were found for the observed heterozygosity (H_0) (0.25 to 0.79, mean 0.59) and PIC (0.23 to 0.75, mean 0.54). However, these parameters were lower when considering only the genotypes of *P. guajava*. Here, 26 alleles were identified, with one to five alleles per locus, and lower H_0 and PIC, which was zero for the monomorphic markers mPgCIR16, 416, 187, and 392. The species *P. cattleianum, P. friedrichsthalianum,*
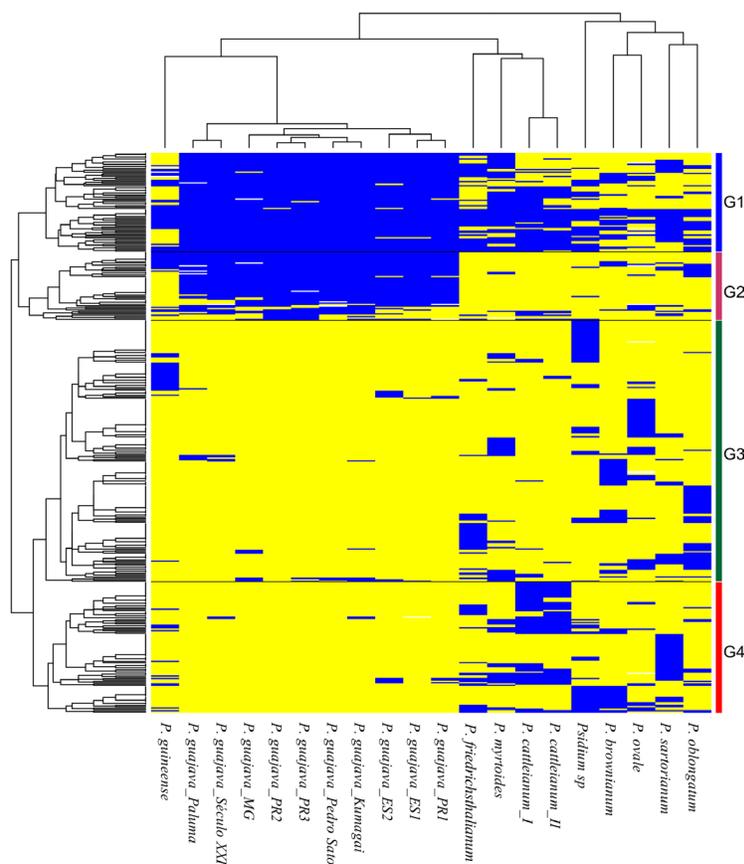
Figure 1. Heatmap showing the amplification profile of the resistance gene analog (RGA) markers in each species; grouping of analyzed species of the genus *Psidium* (horizontal grouping); grouping of RGA amplicons (vertical grouping; G = group). Blue and yellow bands represent the presence and absence of amplification, respectively.

P. guineense, *P. sartorianum,* and *Psidium* sp. presented SSR (Simple Sequence Repeat) with more than two alleles per *locus* (**Table S4**).

SSR markers corroborated the high genetic diversity between genotypes and species. The dissimilarity varied from 0.08 (between ES1 and PR1) to 0.91 (between Século XXI and *P. oblongatum*), with a mean of 0.67. With SSRs, the mean dissimilarity value was greater (0.60) among the genotypes of *P. guajava* than that obtained with RGA markers (0.47). The mean dissimilarity level among the species was similar between the two markers (0.75 for SSR and 0.78 for RGA). However, the clustering obtained with SSR data differed from that based on RGA. The species *P. guajava, P. guineense, P. oblongatum,* and *P. ovale* were grouped, as well as *P. cattleianum* I, II, and *Psidium* sp., and *P. brownianum*, *Psidium* sp., and *P. friedrichsthalianum. Psidium sartorianum* was not grouped (Figure 2). Considering only the genotypes of *P. guajava*, the cultivars Pedro Sato and Kumagai were grouped, as well as Paluma and Século XXI. The genotypes ES1 and PR1, and MG, and ES2 and PR2 (considered resistant) formed a group, with PR3 forming an independent group.
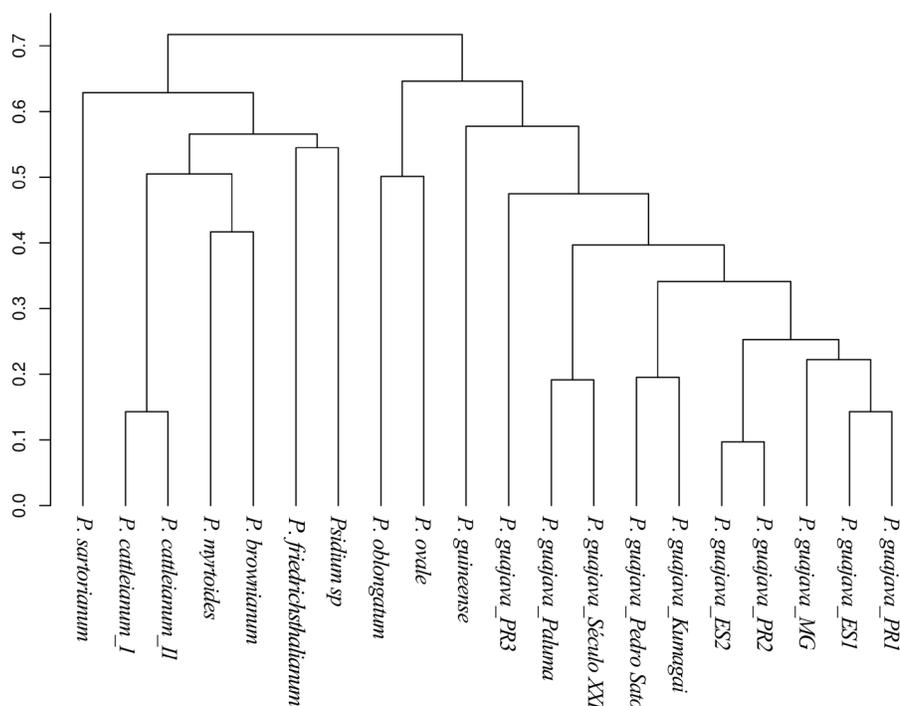
Figure 2. Grouping of genotypes and species based on 60 alleles obtained from 10 SSR (Simple Sequence Repeats) markers conserved in all species.

DISCUSSION

This study revealed high variability of *Psidium* species related to the NBS domains, allowing inferences to be made about the divergence of genomic regions associated with resistance in these species. This variability, related to the number of species-specific amplicons detected by the RGA markers, demonstrates the importance of using different species in breeding aiming at disease resistance, as resistance genes may be present in only one or few species. The genetic diversity of *Psidium* species shown hereby RGA markers represents a collection of random R genes conferring resistance to different pathogens in these species. Such characterization of the gene pool of wild relatives is useful for the management of genetic resources. Additionally, species-specific amplicons can be explored, taking advantage of their potential as markers for use in the introgression of R genes in *P. guajava* by interspecific hybridization. Conversely, the existence of common amplicons in the species suggests these RGAs were conserved in genome regions during evolution. Therefore, these regions represent good candidates for sequencing in different species to help detect R genes in *Psidium*, and for the development of molecular markers for the genus. These amplicons may contribute to the localization of expressed genes in linkage maps and to the identification of markers associated with resistance.

The high variability among species was also characterized by the high values of H_0 and PIC obtained by the SSR markers. For *P. guajava* individuals, lower variability was observed

by both RGA and SSR markers in relation to those obtained considering all species. However, the intraspecific variability of *P. guajava* identified by SSR was higher than that identified by RGA markers, revealing conservation of the RGA compared to the SSR. This reinforces the conserved nature of RGA markers (Sekhwal et al., 2015), which differs from the highly polymorphic nature of SSR.

Even with lower intraspecific variation of *P. guajava*, differential fragments were detected in genotypes reported as resistant, which may be related to resistance to *M. enterolobii*. The majority of these fragments were amplified by primers based on the GLPLAL region (As1, As2, As3 and LM637). This region has been described as a domain located between the LRR and NBS domains (Dinesh-Kumar et al., 2000), and as a motif of the NBS domain (Baek and Choi, 2013). Its high conservation among species (Leister et al., 1996; Lu et al., 2011) highlights the potential use of this region inbreeding programs of guava trees aimed at pathogen resistance. The four most common fragments among resistant genotypes were derived from the genomic regions GLPL, RNBS, and NBS. These fragments only amplified in sources of resistance to *M. enterolobii* and in genotypes of *P. guajava* classified as resistant to this nematode, suggesting that these regions could be further explored for their potential as markers for resistance to this nematode and for conversion to cleaved amplified polymorphic sequence (CAPS) and sequence-characterized amplified region (SCAR) markers for use in marker-assisted selection.

The polymorphisms generated by RGA markers revealed similarities among species with regards to resistance to *M. enterolobii*, as seen by the grouping patterns obtained with these genotypes; however, with SSR markers, the resistant species were dispersed across the groups. In this way, the clustering of *P. cattleianum, P. myrtoides*, and *P. friedrichsthalianum* may be related to the amplification of regions related to resistance, as these species have been reported to be resistant in different studies (Miranda et al., 2010; Marques et al., 2012; Biazatti, 2013; Freitas et al., 2014). Conversely, the grouping of *P. guajava* and *P. guineense* by RGA markers is consistent with that described in the literature; there is evidence for the existence of nematode resistance genes in these species, since susceptible and resistant genotypes have been reported (Miranda et al., 2010; Costa et al., 2012; Silva, 2013) and large variation in the reproduction factor has been reported for *P. guajava* (Miranda et al., 2010, 2012; Scherer et al., 2012; Silva, 2013). *Psidium guineense* and *P. guajava* display many morphological similarities (Costa and Forni-Martins, 2007), and in the present study, both behaved comparably with the two employed markers. In addition, *P. guineense* is a tetraploid species and *P. guajava* is a diploid species (Coser et al., 2012; Souza et al., 2015). Together with the results presented here, those data suggest evolutionary proximity. More phenotypic evaluations should be carried out in these species, with the aim of identifying intraspecific resistance. Moreover, similarity between both species may explain the successful generation of interspecific hybrids of *P. guajava* x *P. guineense*; the same has not been reported for the species *P. cattleianum* and *P. friedrichsthalianum* (Costa et al., 2012), which diverged the most from *P. guajava* by both SSR and RGA markers.

The larger divergence of *P. cattleianum* and *P. friedrichsthalianum* to *P. guajava* may also interfere in their utilization as rootstocks. Compatibility of species in the grafting process depends on morphological, physiological, and biochemical factors, which are influenced and determined by genetic factors as well as by ploidy incompatibility (Souza et al., 2015). Freitas et al. (2014) obtained 50% compatibility when grafting *P. guajava* cv. Paluma in *P. cattleianum* and in *P. friedrichsthalianum*, with physiological compatibility in the field only observed with

P. friedrichsthalianum. Biazatti (2013) and Robaina et al. (2015) did not achieve compatibility of *P. cattleianum* with *P. guajava* cv. Pedro Sato and cv. Paluma, respectively. Owing to the difficulty in the grafting process, the identification and isolation of genes related to resistance in these species may help to better understand pathogen resistance, and to develop strategies for introgression of these genes in guava trees.

The detection of more than two alleles by SSR loci for *P. cattleianum*, *P. friedrichsthalianum*, *P. guineense,* and *P. myrtoides* corroborates the polyploid nature of these species (Costa and Forni-Martins, 2007; Souza et al., 2015) and indicates the same nature for *P. sartorianum*, for which no previous information regarding ploidy is available. Conversely, a diploid nature can be suggested for the species *P. ovale*, *P. brownianum*, *P. oblongatum,* and *Psidium* sp., which presented a maximum of two alleles per locus. This aggregates the important knowledge obtained from this work, as ploidy difference is one of the aspects that may hamper the attainment of rootstocks for *P. guajava* (Souza et al., 2015). Further studies on resistance screening and rootstock usage of these species for *P. guajava* are recommended. From another perspective, polyploid species were verified to present generally lower number of specific amplicons, therefore, having more common regions with the most distant species by RGA markers. Hence, they differ from *P. guajava*, a diploid, which exhibited one of the highest numbers of specific amplicons, along with *P. ovale*, *P. brownianum*, *P. oblongatum,* and *Psidium* sp. Moreover, those species presented the greatest genetic divergence in relation to *P. guajava* based on RGA data, which were shown through the obtained groupings and the dissimilarities among the species. Together, these data suggest divergence in the sequences of diploid species. The hypothesis is thus raised that, in diploids, a larger number of mutations has accumulated in these regions, since they might be more ancient and associated with the origin of the polyploids via autopolyploidy or allopolyploidy, then leading to greater homogenization of the genomes in those regions.

Besides *P. guineense*, *P. oblongatum* and *P.ovale* were the species closest to *P. guajava* by SSR markers, presenting similarity with the cultivars Paluma and Século XXI, which are often cultivated in Brazil. However, based on RGA markers, those species displayed high divergence to *P. guajava*, which suggests the presence of differential resistance genes in relation to those of the guava tree. *Psidium ovale* is a bushy species with many morphological differences to *P. guajava*, while *P. oblongatum* has morphological similarity, with large fruits similar to the guava (Tuler, 2014). Further research is necessary to study the responses of these species to different diseases that affect *P. guajava*, particularly *M. enterolobii*, and to investigate hybridization with *P. guajava*.

The wild species *P. sartorianum*, *P. oblongatum*, *P. brownianum*, *P. ovale,* and *Psidium* sp. presented high variability based on RGA and SSR markers. These species occur in fragments of the Atlantic Rainforest in the state of Espírito Santo (ES - Brazil) (Tuler, 2014; Tuler et al., 2015). Today, the state's vegetation is distributed in isolated remnants of different sizes. This organization justifies the high dissimilarities among the above-mentioned native species, and indicates that they may present conserved resistance genes not yet identified in other species, as well as other traits of interest for the breeding of guava trees.

Subgroups in the grouping identified by RGA markers indicate that these markers were able to differentiate between species. Previous studies with these markers evaluating germplasm attest to their potential for the detection of origins and relationships among the genotypes (Chen et al., 1998, 2009). Evidence for this was demonstrated in the grouping of *P. guajava* cultivars, where Pedro Sato and Kumagai, as well as Paluma and Século XXI, were clustered, the latter being the result of a crossing between Paluma and Supreme 2 (Costa and

Pacova, 2003). A similar cluster of cultivars was observed using SSR data, and also indicates low diversity of cultivars, as reported by Coser et al. (2012). Planting cultivars with a narrow genetic basis is risky for any culture, especially for perennial species owing to their increased vulnerability to environmental changes, i.e., biotic and abiotic stresses that may lead to loss of crops, as observed for guava trees attacked by *M. enterolobii* (Pereira et al., 2009).

In the literature, *P. guajava* genotypes, though susceptible, vary greatly in the reproduction factor parameter (Miranda et al., 2010, 2012; Scherer et al., 2012), indicating the existence of genetic factors involved in the nematode interaction. However, resistance in the species has been little reported so far, with few cases cited in the literature and no confirmation in the field (Miranda et al., 2012; Silva, 2013). In the present study, the genotypes previously selected in the species were used as resistant genotypes. Although different tests still need to confirm such resistance, the clustering of *P. guajava* genotypes that are considered resistant was verified with both markers, with greater divergence revealed by RGA markers for the genotypes MG, ES2, ES1, PR1, and PR2.

The present study included the molecular characterization of *Psidium* species, some of which have little or no previous molecular description, providing important information for guava tree breeding programs. Furthermore, genetic variability of *Psidium* genotypes is evaluated using molecular markers, constituting the first description of RGA analysis in these species. The studied genotypes exhibit different genetic behavior, and can be useful for crossings, as rootstocks, or for the selection of genes of interest for guava breeding. The identification of differential genomic fragments in *Psidium* with resistance to *M. enterolobii* was accomplished, and common regions of RGAs were demonstrated in 10 *Psidium* species. This information has potential to be used for the development of markers of R genes for the breeding of *P. guajava*. Additionally, two DNA markers in genotypes that are susceptible and resistant to the nematode were compared. The differential RGAs identified are a valuable resource for the identification of R genes and the development of RGA molecular markers for the construction of genetic maps, and may also be useful for improving disease resistance. Hence, this work provides useful information for breeding programs of guava tree, especially with regard to resistance to *M. enterolobii*. This study also provides molecular evidence for studies of the plant-nematode interaction, an area in which there has been a delay compared to studies of other pathogens.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Sequences of RGA and SSR (Simple Sequence Repeats) primers amplified in *Psidium* species.

Table S2. RGA fragments amplified in *Psidium* spp. Presence (1) and absence (0) of fragment.

Table S3. RGA fragments amplified in *Psidium guajava* genotypes resistant to *Meloidogyne enterolobii* and absent in susceptible genotypes.

Table S4. Number of alleles amplified in *Psidium* spp. using SSR markers.