



## STRATEGIES AND PRACTICAL ASPECTS IN GUAVA (*PSIDIUM GUAJAVA* L.) BREEDING: 12 YEARS OF EXPERIMENTS

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**Abstract:** The process of plant breeding of perennial species is quite different from annual species due to several particular biological aspects. In addition, information about this process in adjacent cultures, such as the guava exemplified here, is scarce and is of great value to any breeder. In this review, we seek to share as many questions as we deem relevant about our guava genetic improvement program, addressing aspects of overlapping generations, long reproductive cycle, phenotype expression at different ages in the same individual, and biotechnological tools. These issues brought about the need for greater rigor and precision due to: repeated assessments over time on selected genotypes over several years; comparison of genotypes from different generations, which nested in time implies different environments; relevance of the individual representing a genotype as a unit of selection; and availability of data related to the survival rate of plants over age, time and generations, requiring more elaborate statistical methods. Finally, in addition to culture-specific practical issues, we brought an example of our approach to try to introduce favorable genes for resistance to disease in guava.

**Keywords:** perennial crops, interspecific crosses, genetic gain.

### Introduction

Guava (*Psidium guajava* L.) is a fruit highly appreciated in Brazil, which ranks sixth in production worldwide after India, Indonesia, Mexico, China, and Pakistan. Average guava production in these countries is 500,000 tons, with the exception of India, which produces 12 times more (FAO 2020). In Brazil, guava is cultivated mainly by small producers, with an average yield of 26 t/ha, generating over one billion Brazilian real in production value (IBGE 2020).

This tropical fruit stands out for its characteristic flavor, which provides the generation of various by-products such as jellies, jams, juices, syrups, sweets, ice creams, etc. Recently, in addition to biometric assessments, research has looked into the use of parts of the guava plant with some beneficial property-in particular, aqueous extracts of the plant's leaves. Among these properties, antibacterial activity is highlighted, especially on gram-negative bacteria such as *E. coli* and *P. mirabilis* (Malegowd et al., 2017; Selvam et al., 2020).

In Madagascar, another typical tropical region of guava, the skin and leaves are widely used for the treatment of diabetes. One study proposed to determine whether the properties of guava had indeed any action, considering a possible future application in the treatment of type-2 diabetes (Beidokhtia et al., 2020). The authors concluded that the leaves and skin can be used as a natural source of alpha-glucosidase and alpha-amylase inhibitors, improving glucose uptake in muscle cells while increasing triglycerides in adipocytes.

These recent studies on uses of the plant's properties go further in their investigations, ranging from the presence of low-molecular-weight proteopoly saccharides with potent anti-inflammatory and immunomodulatory effects in the seeds (Hsiao-Chien and Jin-Yuarn, 2020) to even a possible anticancer effect against colorectal cancer by angiogenesis inhibition in leaf extracts (Bronwyn et al., 2020). Another interesting property found in the plant that may be close to being used on a large scale in tropical regions such as Brazil is its promising larvicidal activity against *Aedes aegypti* larvae. This is one of the main vectors of human pathogens, including the dengue and chikungunya viruses, two public health problems with a large number of affected individuals and which have major impacts on the economy (Alves et al., 2017). Its control is notably challenging, and synthetic insecticides are one of the most common methods of vector control, but their use raises a number of environmental issues. Therefore, the application of natural products constitutes an effective, easily biodegradable, and low-cost alternative.

In view of its many possible beneficial uses, guava seems to be a promising crop in full expansion, as in the last five years the market has grown 52% in Brazil alone (FAO 2020), having not yet reached its full potential. The main reason for the possibility of expansion is related to the low number of cultivars available and adapted to the producing regions, with only 17 cultivars being registered with the Ministry of Agriculture, Livestock, and Supply (MAPA) and available. To try to fill this market gap, breeders are challenged to generate new high-yielding, pest- and disease-

resistant cultivars that adapt to producing regions and, especially, that are accepted by the consumer market. However, the breeding process of guava, like other perennial plants, is very long due to the plants' life cycle. This challenge must be overcome in the shortest possible time by optimizing the breeding process.

A very clear example of this demand occurred in the northern region of Rio de Janeiro, Brazil, where there was a productive and industrial center focused on guava. In the early 2000s, a disease decimated the entire local production chain in a few years. This disease has been described as "guavadecline", which is caused by a fungus  $\times$  nematode  $\times$  plant interaction. Because no commercial cultivar exists that is resistant to the disease, production was eradicated.

Researchers from the State University of Northern Rio de Janeiro (UENF) identified this gap and quickly started a program for the breeding of the genus *Psidium*. In the first decade already, initial results included the identification of a source of resistance to the disease, involving interspecific crosses (*Psidium guajava*  $\times$  *Psidium guineense*). Since then, researchers have sought new strategies to incorporate this resistance into new cultivars (Miranda et al., 2010). The guava breeding program has expanded its objectives, ranging from targeted crosses to germplasm characterization, advanced statistical techniques for predicting breeding values, and use of cutting-edge technology in molecular markers. This review aims to explore aspects related to guava breeding, using the guava breeding program at UENF as an example.

### **Botanical aspects related to breeding**

Guava belongs to the genus *Psidium*, of the family Mirtaceae. This family comprises over 70 genera and 2,800 species, of which 110 to 130 are native to Tropical and Subtropical Americas. Thanks to this considerable genetic diversity, it is to be expected that many traits can still be included in new cultivars, as in the case of nematode resistance, which was incorporated into guava from *P. guineense*. This prospect is also reinforced by the availability of several closely related species that generate fertile

hybrids. However, from the breeding standpoint, here begins the first barrier of perennial species, which is the time required for the stages of seed collection, synchronization of species reproduction cycles, crosses, and generation and evaluation of hybrids.

On the other hand, management of this plant is easier compared with large species. This is because the plant is a small shrub (Koller, 1979), reaching 3 to 6 m in height. If well-trained, and with planned pruning, time is gained in cultivation practices and fruit harvest. Leaves are opposite, have an elliptical-oblong shape, and fall off after maturation. Flowers are white, hermaphroditic, and sprout in isolated buds or groups of two or three buds, always at the leaf axils and on shoots that appear on young branches.

Hermaphrodite flowers are another advantage for the breeder, as monoecious plants dispense with the need to “double” the experimental area to have male and female plants. Considering that the spacing adopted is usually  $1.5 \times 3$  m, the experimental area could easily be scaled to large sizes that would require a lot of skilled labor for management. Guava also features cross-pollination that varies between plants, typically ranging between 25.7 and 41.3%. Insects are responsible for carrying out these crosses, with the honeybee (*Apis mellifera*) being the main pollinating agent (Soubihe Sobrinho and Gurgel, 1962).

Guava fruits are berries that vary in shape, pulp color, and size depending on the cultivar. For the industry, the most important trait is yield, which is correlated with fruit weight, which usually ranges between 150 and 400 g. In Brazil, red- and white-flesh-colored cultivars are registered, the red varieties being the most common. The production of white-flesh fruits is uncommon due to their low market acceptance, particularly for fresh consumption. Fruit flavor may vary between cultivars/genotypes mainly due to pH, acidity, and brix degree. These variables may constitute a hindrance to breeding programs because they are poorly correlated, making indirect selection difficult. In addition, their measurement is complex and time-consuming.

Fruiting begins between the second and third years after vegetatively propagated seedlings are transplanted as cuttings to the definitive location (orchard). Although it may not be the longest among perennial species, this period must be taken into consideration in the breeding program, as there will always be a need for crossing and seed germination to test individuals. Pruning (control of the number and size of branches) is an important cultivation treatment in guava, as it interferes not only with the agility of other treatments but also with flowering. In general, four and six forks of the main trunk of the plant are kept, keeping the plant up to two meters in height. Flowering occurs between 45 and 70 days after pruning, starting at the axils of new branches, while fruit set takes place approximately 90 days after pruning (Soubihe Sobrinho, 1951).

It should be emphasized that when plants are artificially crossed, flower-bud and fruit abortion rates are higher. As a consequence, breeders require almost an entire life cycle of annual species just to know whether the number of pollinated fruits was sufficient. Again, more plants could be used for the crosses, but the experimental area easily scales to large proportions. Regarding the number of plants, the breeder still needs to plan well how many and which genotypes they will use in the experiments, since there is great genetic diversity available. Although some botanical aspects highlighted here facilitate common practices in breeding programs when aligned with cultivation practices, a long time must still be invested in the evaluation of the traits.

### **Germplasm**

In Brazil, few institutions maintain *Psidium* germplasm banks. These are: Agronomic Institute of Campinas - IAC; Agricultural Research Corporation of Pernambuco- IPA; Agricultural Research Center of the Semi-Arid Tropics - CPATSA; Faculty of Agricultural and Veterinary Sciences - FCAV/UNESP; Agricultural Development Company of Bahia - EBDA; and Brazilian Agricultural Research Corporation- EMBRAPA Clima Temperado. These institutions develop studies aimed at

agronomic characterization, except for EMBRAPA Clima Temperado, which conducts research focusing on the morphological, biochemical, and molecular approaches. In total, 324 accessions of *P. guajava* and 174 accessions of *P. guineense* are cataloged.

There is also a considerable amount of genetic material of the genus *Psidium* native to some regions of Brazil. Plants can be found growing spontaneously almost throughout the territory; however, in the Atlantic forest, “cerrado”, and “pantanal” biomes, their frequency is higher (Salis et al., 2006). This native genetic material encompasses several traits, such as robustness, resistance to pests and diseases, and even flooding tolerance. Searching for these materials may be an interesting alternative strategy, since, in addition to being already adapted to a certain environment, they possess traits of interest to be incorporated into commercial cultivars. Nonetheless, most individuals found natively are not *P. guajava*, but *P. cattleianum*, or *P. guineense* (Franzon et al., 2009; Manica, 2000; Vieira et al., 2010).

These species can be crossed, generating fertile hybrids with *P. guajava*, and can be easily introduced into a breeding program, as seen in studies aimed at incorporating nematode resistance into commercial cultivars (Gomes et al., 2017). Both *P. cattleianum* and *P. guineense* are native to southern South America and have dispersed well enough in tropical and subtropical climates, reaching as far south as Mexico, and also in Hawaii, where *P. cattleianum* became a weed (Brandão et al., 2002). A better description of the occurrence of some of the hundreds of *Psidium* species is given in Franzon et al. (2009).

### **Psidium breeding**

In Brazil, the main varieties emerged from informal selection made by producers of Japanese origin who selected and maintained clones in their orchards, whose fruits exhibited desired qualities. This selection carried out by the producers generated important cultivars, such as Kumagai, Ogawa, Pedro Sato, and Sassaoka. Despite the great genetic variability existing in spontaneous guava populations in Brazil, the

introduction of improved genetic material from Australia, the United States of America, and India has greatly contributed to the breeding of this species in Brazil. This introduction of new cultivars began in 1976 at the Faculty of Agricultural and Veterinary Sciences of São Paulo State University, where a breeding program produced the cultivars Rica and Paluma (Pereira and Nachtigal, 2002).

The breeding methods applied to the crop are basically selection and hybridization. To achieve success in a plant breeding program, breeders must possess basic information on the inheritance of the main agronomic traits to be improved, as well as the genetic divergence available for improvement (Pereira and Nachtigal, 2002). In general, active breeding programs are based on the identification of some genetic material that already comprises desirable traits, which proceeds directly to Value for Cultivation and Use (VCU) tests, followed by registration and release of the new cultivar. Parallel to this, controlled artificial pollination can be achieved with crosses between plants that show traits of interest to the generation of new cultivars.

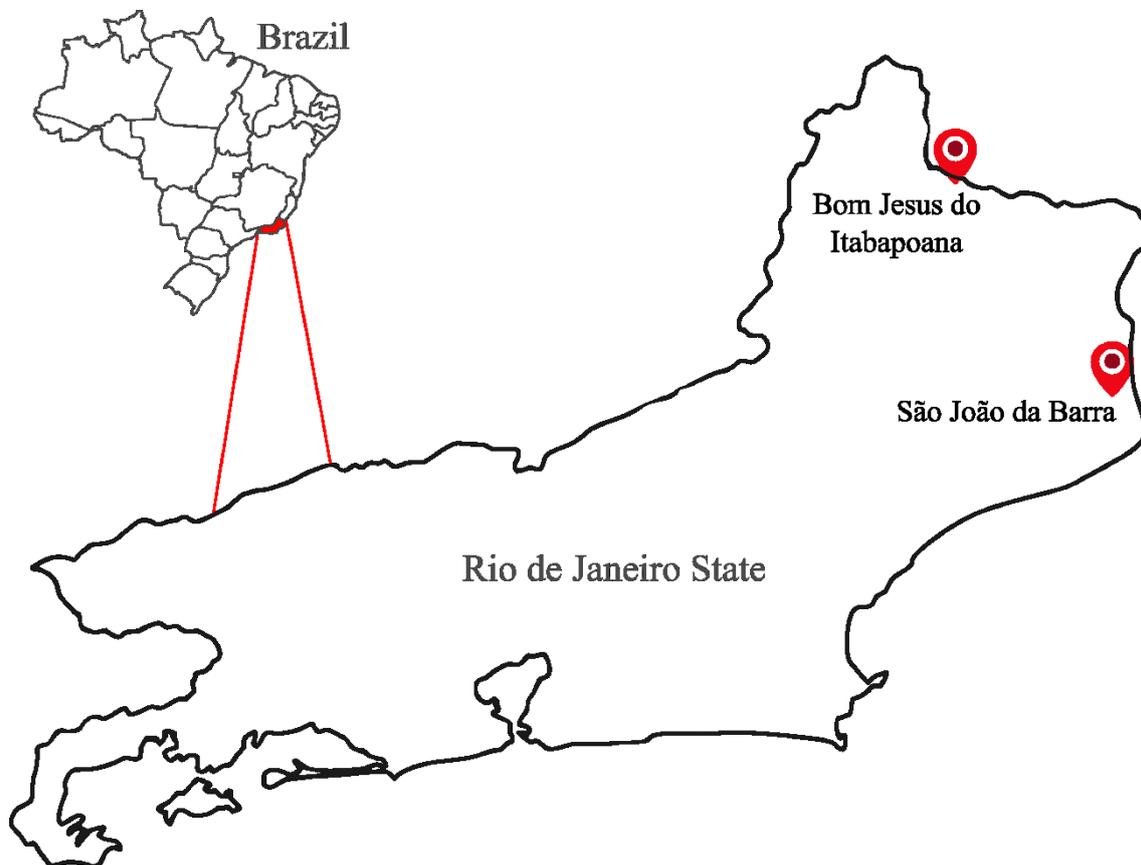
These crosses can be intervarietal or interspecific, and are generally made in simpler experiments, without undergoing several selection cycles with classic methods such as mass selection, stratified selection, among others. This dynamic has been followed because there are few available cultivars registered with relevant information for producers to choose, but there is a demand. Thus, breeders gather some basic information about the material, evaluate their adaptability and stability in possible favorable environments, and register the genetic material.

Generally, materials with traits such as the following are registered: average fruit weight greater than 150g, ellipsoid shape, few and small seeds, green-yellow skin, pink or red flesh, high vitamin C content, and pleasant taste and aroma. These characteristics may vary slightly, as the final market is divided in two: one that uses the fruits for processing industrialized foods and another for fresh fruit consumption.

There are other active guava breeding programs. One example is the State University of Northern Rio de Janeiro, which is currently developing research on mapping resistance to the nematode *Meloidogyne enterolobii* (associated with the guava decline disease) in a segregating population of *Psidium* sp. and marker-assisted selection. There is also a program at the Federal University of Espírito Santo, which has been developing works involving genetic diversity, chemical compounds of interest, biological activity of *Psidium* sp., identification, and characterization of *Psidium* species present in the Atlantic Forest, sensory analysis, and applied biotechnology.

## *Psidium* breeding UENF

Considering the high potential of the guava fruit and the low availability of cultivars that meet the needs of producers, UENF started a guava breeding program aiming at the selection of individuals with unknown traits that would serve as support for the generation of better, high-yielding hybrids adapted to the environment of the north and northwest regions of Rio de Janeiro. The first study started with the investigation of 20 accessions of *Psidium guajava* derived from open pollination, in the municipalities of São João da Barra and Bom Jesus do Itabapoana- RJ (Figure 1).



**Figure 1.** Collection sites of the first accessions of the guava breeding program at the State University of Northern Rio de Janeiro.

This study sought cytogenetic information by evaluating meiotic behavior based on normal (tetrads) and abnormal (triads, dyads, and monads) meiotic products. It was found that cell division was generally normal, and that guava is diploid with 11 pairs of chromosomes ( $2n = 2x = 22$ ) and pollen viability greater than 90% (Costa

Itayguara and Forni-Martins, 2006). This information was important for planning crosses, as individuals with different ploidy levels could be used, possibly generating infertile individuals or problems with other molecular techniques. Pollen information was also important for planning crosses (Pessanha et al., 2011).

Genetic diversity was also evaluated using RAPD markers with 28 primers, which generated 157 bands. In this study, marker information was used and multivariate statistical techniques were applied using the Nei and Li index (Nei and Li, 1979) with UPGMA hierarchical clustering and the Toucher optimization method. This work was important for targeting the initial crosses, indicating which individuals to select, and demonstrated that artificial crosses were possible. With the crossing of nine individuals, the seeds generated 17 full-sib families of *Psidium guajava* with wide genetic variability and a considerable degree of heterozygosity.

Within these families, 26 physicochemical and morpho-agronomic traits were evaluated in 138 individuals. This characterization work provided an early identification of promising genotypes for the continuity of the program (Campos et al., 2013). Even in these early stages, advanced statistical approaches such as the use of artificial intelligence were already used. A neural network based on the Kohonen model (Kohonen, 1982) was used with an input matrix of  $n$  accessions and  $m$  traits ( $138 \times 26$ ), with the output neuron defining cluster classes to determine the possible genetic variability in the families (Campos et al., 2016). These studies confirmed that there was still sufficient genetic diversity to proceed with the program and validate the previous steps, as well as to generate information about the individuals deriving from the targeted crosses.

Simultaneously, statistical methods were applied with Mixed Models using REML/BLUP estimators and predictors to determine the breeding values and genetic parameters at different production times (Quintal et al., 2017). The selection index of Mulamba & Mock (Mulamba and Mock, 1978) also proved to be the most suitable for the guava crop among several available approaches in the literature used in other species. This index provided the highest accuracy values, and the first production season showed the greatest gains. This would already indicate that selection based on the first production can be a viable alternative for the crop and that there might be indications of low stability in individuals regarding the environment (considered the production season) or possible cycles of high/low production.

The prediction of genetic gain between and within progenies was also investigated (Paiva et al., 2016). In this study, the focus was to identify possible genotypes for industrial purposes, adopting fruit number and weight as the main traits. The classic selection index by Smith and Hazel (Smith, 1936; Hazel, 1943) was the selection criterion that showed the best results in terms of gains, thus being indicated as the most adequate selection strategy. Another important contribution of this study was investigating repeatability coefficients of traits related to guava production. By associating the adaptability and stability of the progenies with the genetic gains between and within the families of several crops, we found that four production seasons can be used to generate descriptive models of these guava families. This information is particularly useful for planning future crosses and the time needed to acquire reliable information on the species.

These families were kept in the program and evaluated each season to generate more data with greater reliability. Using data from nine years of this population, the researchers sought to employ statistical methods in the genetic analysis of these individuals to select methods and models that can be applied in future populations in order to optimize and reduce the time needed to select promising individuals. At this stage of the program, the Bayesian methodology and covariance structures were applied (Silva et al., 2020).

As can be seen, the program has been developing two basic types of breeding populations: one using crosses in pairs, in which full-sib families are genetically evaluated; and the other using self-pollination systems for the formation of inbred families. Table 1 shows the narrow-sense heritability values based on the mean of the families, for the three populations under study. The inbred families show great potential, with clear increases in heritability for the two estimators. In the field experiments, there is an increase in the homogeneity of plants within the inbred lines; considerably greater earliness of the plants; and a clear increase in the phenotypic values of the main study traits: fruit weight, number of fruits, and total production.

**Table 1.** Estimates of genetic parameters in different populations of *P. guajava*.

Trait	Parameter	Full-sibs (1)	Full-sibs (2)	S <sub>1</sub> families
Fruit weight	$h^2_a$	0.37	0.46	0.32
	$h^2_{mp}$	0.71	0.85	0.89
Number of fruits	$h^2_a$	0.15	0.18	0.62
	$h^2_{mp}$	0.34	0.3	0.79
Total production	$h^2_a$	0.16	0.31	0.60
	$h^2_{mp}$	0.54	0.56	0.77

Full-sibs (1)= first generation of families obtained, Full-sibs (2)= second generation of families obtained, S<sub>1</sub> families = first generation of inbreeding between the selected genotypes: Sources: Ambrosio et al. 2020, Bezerra et al. 2020, Quintal et al. 2017.

This demonstrates the potential for using genotypes derived from self-pollination as a strategy to develop new guava cultivars. The genotypes now developed are in the S<sub>2</sub> family evaluation phase. Future studies point to the development of diallel crosses for purposes of estimating combining ability and the effects of intraspecific heterosis, as a way of developing single-hybrid cultivars.

Innovative statistical approaches were used again at this stage, and the Bayesian methodology proved to be superior to the frequentist approach. In this study, there were situations of unintentional incomplete data, and even with complete data, the use of *a priori* information in the model provided by the Bayesian approach generated more accurate information. This approach was useful both in an attempt to avoid penalizing families with a smaller number of individuals, some of whose data were lost due to external reasons that were not inherent to the genetic basis of the plant. With a larger set of data on the families, the repeatability of the variables indicated that four harvests would allow for an efficient selection, as it optimizes the process by reducing the time required for each cycle.

Because the guava crop is known for having a mixed reproductive system, the goal was to obtain two new populations: the first from crosses between the best individuals, and the second by self-pollination of the individuals. A population of 11 guava full-sib progenies from biparental crosses was generated, whose components of variance, individual heritability, progeny selection accuracy, and overall mean of the populations were estimated using the REML/BLUP method to assess the individuals' development (Santos et al., 2020). The procedure proved to be efficient, generating individuals that

showed traits with values superior to the overall mean of their parents, and the strategy of obtaining full-sib families was confirmed to be efficient, providing significant gains in the breeding process. The genetic characterization of this population is being performed with the use of microsatellite markers (in progress).

The other population of individuals resulting from self-pollination was evaluated to identify superior individuals via truncation selection (Ambrósio et al., 2021). To determine whether inbreeding depression occurred, a study of inbreeding depression based on seed attributes using S<sub>0</sub>, S<sub>1</sub>, and S<sub>2</sub> families is being developed (in progress).

To date, 10 years have been invested in this breeding program. However, in parallel, other approaches and attempts to incorporate new traits into the genetic material are being studied. Among them, one of great importance and which showed promising results is the incorporation of resistance to the nematode that causes guava decline, the main problem for regional producers.

### Breeding aiming at resistance to guava decline

The southeast region of Brazil is the second largest guava producer in the country; however, in the last two decades, there has been a reduction of production areas due to the occurrence of guava decline. The death of the plants was caused by parasitism by *Meloidogyne enterolobii* combined with *Fusarium* sP., which became a threat to guava production. This disease causes symptoms such as galls on the roots, loss of fine roots, leaf yellowing, defoliation of the entire shoot, and, consequently, plant death. About one third of the cultivated area was infested, inevitably leading to the destruction of orchards (Gomes et al., 2012).

The impact caused by guava decline in Brazil disrupted almost the entire guava production chain in centers with significant production, with an estimated loss of US\$ 61 million (Pereira et al., 2009). Several studies were developed in search of efficient methods to control this pathogen (Carneiro et al., 2007) and discovered moderate resistance in the species *P. friedrichsthalianum* and resistance in three accessions of *P. cattleyanum*, suggesting that the use of these resistant genotypes for the formation of rootstocks would be an important alternative in the management of the disease. However, cv. Paluma, the main cultivar used by producers in Brazil, showed incompatibility when grafted onto *P. cattleyanum* rootstock (Robaina et al., 2015). Considering the market demand for alternatives that would enable the cultivation of the fruit, the program opened a new line of work with *P. cattleyanum* accessions. This *Psidium* species was found close to orchards and at infested sites without showing the symptoms of the disease, thus being considered resistant to the nematode *Meloidogyne enterolobii*.

Twenty-seven accessions of *P. cattleyanum* were then collected and evaluated for genetic variability along with 10 *P. guajava* genotypes using ISSR markers (Oliveira et al., 2014). The study also investigated traits referring to the phenological phases, from the establishment of the floral buds to fruit harvest. Some *P. cattleyanum* genotypes genetically close to *P. guajava* were identified, once again using multivariate techniques with Ward-MLM and UPGMA. A possible interspecific hybridization was observed.

A total of 696 crosses were performed using *P. cattleyanum* and *P. guineense* as donors of resistance genes. After the H<sub>1</sub> population was established, experiments were conducted with inoculation of *M. enterolobii* (Gomes et al., 2017). The study evaluated reproduction, number of leaves, plant height, shoot fresh weight, root fresh weight, and root volume, in addition to 14 multicategorical morphological descriptors, according to a document from the International Union for the Protection of New Plant Varieties - UPOV. The most successful cross was between *P. cattleyanum* and *P. guajava* (Almeida, 2017).

Once the hybrids that were resistant to the nematode *M. enterolobii* were identified, genetic characterization was carried out using SSR markers from 94 individuals and their respective parents (Santos et al., 2020). This study sought to select the genotypes genetically closest to *P. guajava* for generation advancement, to obtain individuals with productive potential and resistance to guava decline. The next steps will involve similar and parallel evaluations of the program for those individuals with possible resistance. After identifying an individual with the desirable traits of *P. guajava* as well as the resistance of *P. cattleyanum*, VCU tests will be carried out and a new cultivar will be registered.

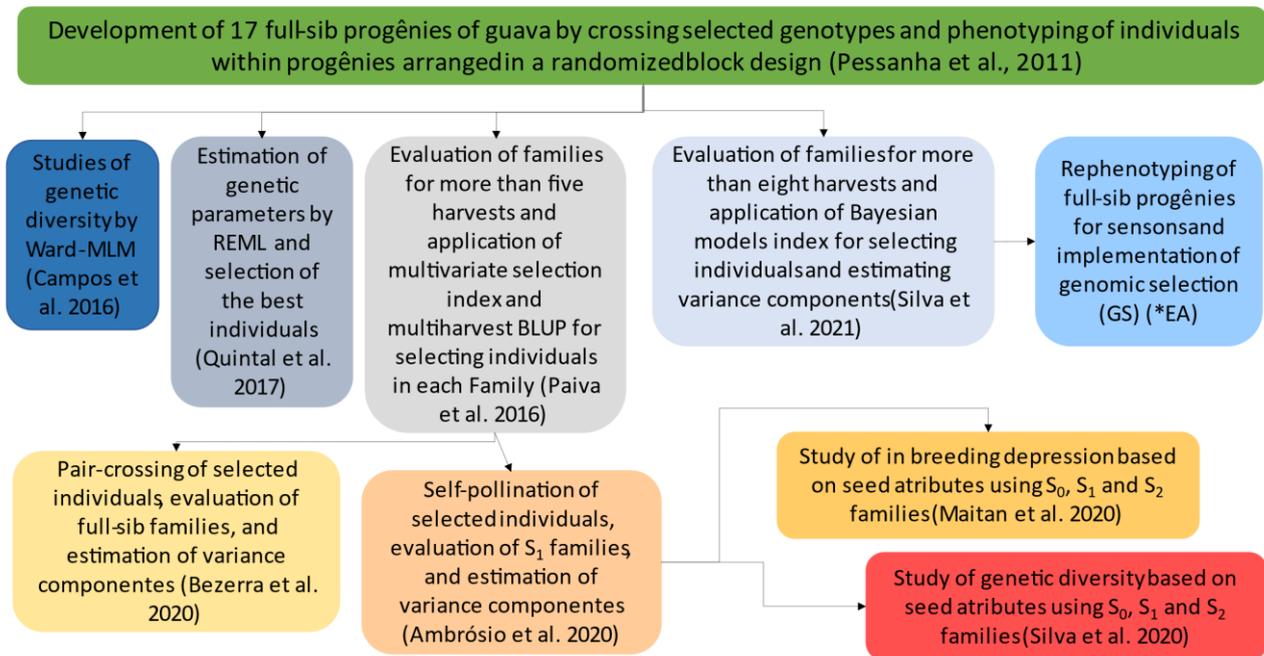
The program can be briefly described in the flowchart below (Figure 2). Some unpublished data were also included for a better understanding of the program's strategy.

Overall, the selection works developed by the guava breeding program at UENF resulted in the selection and cloning of ten genotypes that offer advantageous productivity traits, which are being tested for stability and adaptability for cultivation in the region. Additionally, resistance can be incorporated into the breeding population to obtain tolerant genotypes that can be evaluated for other agronomic traits.

### Future prospects

Guava breeding programs have undertaken germplasm prospection, which can serve the consumer market with tests of Value for Cultivation and Use, and registered new cultivars. In parallel with this, these programs have used more complex genetic designs and crosses in an attempt to incorporate new traits into future cultivars.

Perennial-fruit breeders generally seek to reduce selection cycles to optimize the release of new cultivars. In this context, the integration between classical breeding that employs traditional crossing and selection techniques and breeding that uses molecular biotechnology and applications of informatics has brought great advances, revealing a permanent change.



**Figure 2.** Flowchart of the guava breeding program developed at UENF.

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