FP6-2003-INCO-DEV-2 No. 015111

GUAVAMAP

Improvement of guava: Linkage mapping and QTL analysis as a basis for marker-assisted selection

Instrument: STP
Priority: A.3.2

FINAL REPORT

Period covered: 01.12.2005 – 31.05.2009 **Date of preparation: August 22, 2009**

Start date of project: 01.12.2005 **Duration: 42 months**

Project coordinator: **Prof. Dr. W. Rohde**
Organization: **MPIZ** **1st draft**

Executive Summary

The strategic objectives of this project were to provide the methodological basis and molecular tools for improving the breeding efficiency in the tropical fruit crop guava by

i) analyzing the biodiversity of guava indigenous to the three producer countries Brazil, Venezuela and Mexico through *in situ* and *ex situ* conservation, characterizing this guava germplasm by DNA marker technologies and agro-morphological descriptors, and using it in selected crosses, and

ii) developing DNA marker-based breeding strategies via marker-assisted selection (MAS) by establishing a molecular linkage reference map for guava, mapping qualitative and quantitative QTLs, associating DNA markers to these QTLs, and developing a Molecular Marker Set (MMS) for breeding applications.

The consortium consisted of the following contractors:

Max Planck Institute for Plant Breeding Research	MPIZ	Germany
Instituto Vasco de Investigación y Desarrollo Agrario	NEIKER	Spain
Centre de Coopération Internationale en Recherche pour le Développement	CIRAD	France
Centro de Investigación Científica de Yucatán	CICY	Mexico
Universidad de los Andes	ULA	Venezuela
Embrapa Semi-Arido	CPATSA	Brazil

Project coordination was based at MPIZ (rohde@mpiz-koeln.mpg.de). MPIZ had subcontracted the Instituto de Investigaciones en Fruticultura Tropical (IIFT, subcontractor S1) in Cuba. General and detailed information on objectives and results is presented by the project website www.neiker.net/neiker/guavamap.

The first objective (guava biodiversity analysis) was pursued by the Latin American partners Brazil, Cuba, Mexico, and Venezuela with Brazil and Mexico belonging to the main producer countries world-wide. The Universidad de Los Andes (ULA) in Mérida (Venezuela) was responsible for the execution of this task. On the topic of biodiversity analyses and germplasm prospection, the partners from Brazil (CPATSA) and Venezuela (ULA) completed the germplasm collections (both for guava and other *Myrtaceae*) and genebank establishment, while the CICY (in collaboration with INIFAP) started the collection of tropical guava in order to complement the genebank of guava accessions adapted to semiarid conditions which are prevalent in the production areas of Northern Mexico. All accessions were characterized by UPOV characters and by molecular analysis using the SSR marker set established by the project through

CIRAD. Important results were obtained with respect to the application of the collected germplasm and hybrids produced by controlled crosses for production: (i) The Brazilian guava accessions, in specific the identification of 11 wild-growing *Psidium* species ("araçá") by partner 6 (CPATSA) as sources of resistance to the devastating root knot nematode *Meloidogyne mayaguensis*, now form the basis for controlled crosses and development of new, nematode-resistant varieties for guava production. This nematode is the major pest disease of guava in Brazil and has destroyed more than 60% of the commercial production in Northeast Brazil. Grafting experiments with nematode-resistant accessions and commercial guava cultivars and experiments on cross-pollination have been initiated, and offer prospects for the development of new, pest-resistant varieties. (ii) The breeding program in Cuba with IIFT (subcontractor S1) was based on an existing germplasm collection. Within the GUAVAMAP project, a total of 25 genotypes (derived from the mapping populations MP1, MP2, and MP3) with low size have been selected for propagation and variety development. All of them are now being replicated in plots for the evaluation of important agronomic traits in order to select new cultivars for production in Cuba. In this context it is noteworthy to mention that an undated UPOV descriptor for guava (in English and Spanish) which serves as the basis for agro-morphological description of accessions and cultivars has been developed by subcontractor S1 in the frame of the project.

The second strategic objective of the GUAVAMAP project related to the molecular characterization of the prospected guava germplasm and complements the agro-morphological analysis of the guava accessions by updated UPOV descriptors. For this purpose and for integration into individual molecular linkage maps and establishment of an integrated, high-density reference linkage map of guava, CIRAD had pursued the massive development of microsatellite (SSR) markers and constructed a SSR guava linkage map of 378 marker loci integrating 153 SSR markers into 11 linkage groups. With respect to the AFLP-based linkage maps, by the end of the project the number of mapped markers increased to 1779 markers (projected: 960-1200 markers) on 3 integrated parental linkage maps derived from the three mapping populations (MPs) with 116 markers (AFLP, SSR, COS) per linkage group available on the three maps. QTL analyses in all three mapping populations included leaf length, leaf width, fruit length, fruit width, internal and external pulp thickness, seed numbers, average seed weight, vitamin C content, acidity, total soluble solids, maturity index, average fruit weight, plant height, and cumulative yields for the years 2005 to 2008. Certain QTLs from different progenies could be co-located or closely linked targeting the same genes that determine a trait.

Dissemination of project results culminated in the organization of the "2nd International Symposium on Guava and Other *Myrtaceae*" (http://www.cicy.mx/eventos/guavasymposium2008) in November 2008 in Mérida and Aguascalientes, Mexico. All publications from oral and poster presentations have been edited by the GUAVAMAP project and are now in press by ISHS as a special issue of *Acta Horticulturae*. The symposia were follwed by practical courses at the CICY (Mérida, Mexico) on biotechnology (2 weeks) and bioinformatics (1 week). From project funds, four fellowships were awarded to young scientists (2 from Mexico, 2 from Cuba) to participate to the

Mérida Symposium anfd the two practical courses course. Partners P6 (CPATSA) and P5 (ULA) will host the 3rd and 4th International Symposium on Guava and Other *Myrtaceae* in Brazil (2011) and Venezuela (2014), respectively, under the auspices of the ISHS. Thus continuation of the international collaboration on guava which was initiated by the EC-funded GUAVAMAP project is warranted beyond the duration of the project.

Section 1
Project objectives and major achievements

The project aimed at improving the utilization of the perennial tropical fruit crop guava (*Psidium guajava* L.). This was achieved by a consortium composed of three European laboratories and three institutions in Latin American producer countries. There were two main activities foreseen: (i) Identification and characterization of new guava genotypes and their utilization in breeding programs according to the individual needs of the countries, and (ii) application of biotechnology in the form of DNA markers by establishing individual molecular linkage maps for three existing mapping populations, aligning these individual maps into a reference guava linkage map with the help of microsatellite markers, mapping QTLs for traits important for breeding, and developing a Molecular Marker Set for marker-assisted selection. In specific, new guava germplasm was prospected and conserved *in situ* and *ex situ*, its agro-morphological characters were recorded according to established descriptors and complemented by molecular analysis using AFLP and SSR DNA markers. These two DNA marker types formed the basis for establishing individual and reference molecular linkage maps in guava for three available mapping populations. These mapping populations furthermore allowed for the mapping of QTLs for important breeding traits and thus provided the basis to develop the Molecular Marker Set. Candidate genes for plant development and resistance were identified and mapped onto the guava reference map. Finally, the project disseminated its progress in the field by an international symposium under the auspices of the ISHS ("2nd International Symposium on Guava and Other Myrtaceae") and associated practical courses in biotechnology and bioinformatics (November-December 2008; Mexico).

The specific objectives were as follows:

1) Biodiversity analysis of guava accessions in Brazil, Venezuela and Mexico via prospecting and the analysis of germplasm collections using the UPOV descriptors and DNA markers (AFLP, SSR)
2) Development of molecular resources and construction of molecular linkage maps for guava
   1.1. Massive development of microsatellite markers (SSRs) for guava.
   1.2. Construction of individual linkage maps for three available guava mapping populations based on AFLP and SSR markers.
   1.3. Isolation and sequencing of candidate genes (for resistance, plant development) and individual COS clones and mapping on the guava linkage maps.
3) Establishment of a guava reference linkage map by combining the maps derived from three available mapping populations.
4) Analysis, mapping and comparison of quantitative trait loci (QTLs) in the different genetic backgrounds of the three mapping populations.
5) Dissemination of results and expertise in bioinformatics and biotechnology by an international symposium and a laboratory course and via the internet.

These objectives were executed via nine work packages (see list below).

Work package No[1]	Work package title	Ld contractor[2]	Person-months[3]	Start month[4]	End month[5]	Deliverable No[6]
WP1	**Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela**	**P5**	115	1	36	D1.1-D1.7
WP2	**Construction of individual AFLP-based guava linkage maps**	**P1**	110	1	36	D2.1-D2.3
WP3	**Generation and screening of microsatellite markers (SSRs) in guava**	**P3**	28	1	18	D3.1-D3.3
WP4	**Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map**	**P2**	64	19	36	D4.1-D4.3
WP5	**Construction of a guava COS library and development of SNP/INDEL markers**	**P1**	24	1	24	D5.1-D5.5
WP6	**Isolation and genetic diversity screening of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes)**	**P1**	24	1	24	D6.1 D6.2 D6.3
WP7	**Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map**	**P1**	51	25	36	D7.1-D7.2
WP8	**QTL analyses for the three guava mapping populations**	**P2**	41	1	36	D8.1 - D8.4
WP9	**Dissemination and transfer of project results**	**P1**	18	12	36	D9.1 - D9.2
	TOTAL		**475**			

[1] Work package number: WP 1 – WP 9.
[2] Contractor responsible for the work in this work package.
[3] The total number of person-months allocated to each work package.
[4] Relative start date for the work in the specific work packages, month 1 marking the start of the project, and all other start dates being relative to this start date.
[5] Relative end date, month 1 marking the start of the project, and all ends dates being relative to this start date.
[6] Deliverable number: Number for the deliverable(s)/result(s) mentioned in the work packages: D1 – D9.

WP1: With respect to this work package, partners P5 (ULA) and P6 (CPATSA) completed their respective collections of wild-growing guava and other *Myrtaceae* within the GUAVAMAP project, but they will pursue further prospecting in their countries beyond project duration. Similarly, in Mexico further prospecting to the south of Mexico to collect tropical guavas has been initiated by the CICY and will be continued beyond the duration of the project. With partner P5, crosses to generate new mapping populations are planned for 2009. Partner P6 has done crosses between wild-growing accessions that show resistance to the root knot nematode *Meloidogyne mayaguensis*. Agro-morphological description of the accessions was completed by partners P5 (ULA) and P6 (CPATSA). DNA has been extracted from accessions (*Psidium guajava* and other *Myrtaceae*) for biodiversity analysis (and the analysis was performed using a standard set of SSR primers (elaborated by partner P3, CIRAD) as well as additional SSR primers. Furthermore ULA has extended the SSR studies on 15 more *Myrtaceae* in order to test the cross-taxa transferability

WP2: With all three mapping populations MP1, MP2, and MP3 (generated by and hosted with subcontractor S1, IIFT, Cuba, since 2001), identification of polymorphic, segregating AFLP, SSR, and COS markers has resulted in the mapping of a total of 1779 DNA markers (projected: 960-1200 markers) on individual parental linkage maps derived from the three mapping populations.

WP3: Partner P3 (CIRAD) has pusued the massive development of SSR markers for guava: A total of 428 functional SSR primers pairs were developed and characterized for polymorphisms on all six guava map parents. The developed SSR primers have been made available to all project partners for integrating SSR markers into the AFLP linkage maps of the 4 parents of mapping populations 2 and 3 (MP2, MP3).

WP4: For mapping population 1 (MP1), CIRAD (partner P3) scored a total of 242 SSR loci and established together with AFLP and COS markers a comprehensive SSR linkage map for the MP1 cross. A set of 376 marker loci (153 SSRs, 222 AFLPs, 1 COS) were mapped onto 11 linkage groups cumulating a length of 1185 cM with an average marker density of 3.6 cM and an avew3rage of more than 10 SSR markers per linkage group.

WP5: Partner P1 (MPIZ) was responsible for the establishment of a cosmid (COS) genomic library for guava using the maternal parent (cv. “Enana roja cubana”) for MP1as the source of DNA. Packaged recombinant DNAs (initial cfu’s: 52,000) were used for successive infection/transformation and picking of single colonies into 384well plates by robots at the MPIZ ADIS service unit. At an estimated guava genome size of $0{,}17 \times 10^9$ bp and average insert size of some 40 kb per COS clone, these COS clones correspond to 6-7 guava genome equivalents. For the generation of SNP/indel primers. DNAs were isolated from randomly picked guava COS clones, sequenced, and primer pairs established.

WP6: Partner P1 (MPIZ) was also responsible for this work package. The cosmid (COS) genomic library of guava was individualized and individual

COS clones were spotted onto membranes for molecular hybridization experiments using molecular probes for the identification of putative genes for development (MADS-, HOMEO-box) and resistance (RGL sequences). In total 117 positive clones for RGL sequences, 37 for MADS-box and 22 for HOMEO-box were identified. These COS clones were characterized by end sequencing (see WP5; randomly picked COS clones) and PCR primers designed for amplification of the 6 DNAs from the parents for MP1, MP2 and MP3 using in the first place agarose gel separation (see WP5). PCR primers were supplied to all partners for mapping on the respective mapping populations.

WP7: This task had started with month 25 of the project (year 3) and was completed until the end of the project (see also WP5 and WP6). Thirteen COS clones showed segregation in the MPs, and were mapped onto the respective linkage maps.

WP8: QTL analyses in all three mapping populations were carried out by partner P2 (NEIKER) based on the data collected and recorded by subcontractor S1 (IIFT. Cuba) and submitted to partner P2 for analysis and mapping. The data included leaf length, leaf width, fruit length, fruit width, internal and external pulp thickness, seed numbers, average seed weight, vitamin C content, acidity, total soluble solids, maturity index, average fruit weight, plant height, and cumulative yields for the years 2006 to 2008. QTL analyses for a total of 16 traits were performed, and comparative QTL analyses were completed.

WP9: Dissemination started with the "1st International Symposium on Guava" in Lucknow, India, and culminated in the "2nd International Symposium on Guava and Other *Myrtaceae*" which was organized by GUAVAMAP under the auspices of the ISHS in November 2008 in Mérida/Aguasdcalientes, Mexico, and attended by participants from 16 countries (Australia, Egypt, Canada, USA, Mexico, Cuba, Costa Rica, Venezuela, Honduras, Brazil, Colombia, Spain, France, Germany, India, and Malaysia) including the three biggest producer countries worldwide India, Brazil, and Mexico. The Mérida symposium was followed by practical courses in biotechnologies and bioinformatics at the CICY (Mérida). From project funds, four fellowships were awarded to young scientists (2 from Mexico, 2 from Cuba) for participation. Continuation of the work on guava was warranted by the fact that 2 GUAVAMAP contractors (CPATSA, Brazil; ULA, Venezuela) volunteered to organize together with the ISHS the 3rd (2011) and 4th (2014), respectively, International Symposium on Guava and Other *Myrtaceae*. At the MPIZ, the GUAVAMAP project will form part of an interactive scientific museum (www.wissenschafts-scheune.de) and installed in 2010 to demonstrate EC-funded international collaboration.

Section 2

Work package progress

Progress with respect to the different work packages and deliverables is being presented in the following by the detailed individual reports of the contractors. For each contractor, involvement in work packages and generation of deliverables are presented.

Improvement of guava: Linkage mapping and QTL analysis as a basis for marker-assisted selection (GUAVAMAP)

FP6-2003-INCO-DEV-2 No. 015111

Partner 1
MPIZ

Prof. Dr. Wolfgang Rohde	(scientist in charge, coordinator)	MPIZ
Mrs. Heike Meier-Nieragden	(project officer)	MPIZ
Dr. Jost Muth	(scientist)	IME
Julia Boike	(technician)	IME
Boris Kommor	(PhD student)	EC
Dr. Lien González	(scientist)	DAAD

Final Report (01.12.2005 – 31.05.2009)

The MPIZ/IIFT-related work packages within the project were as follows (in blue):

Work package No[7]	Work package title	Ld contractor[8]	Person-months[9]	Start month[10]	End month[11]	Deliverable No[12]
WP1	**Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela**	**P5**	115	1	36	D1.1-D1.7
WP2	**Construction of individual AFLP-based guava linkage maps**	**P1**	110	1	36	D2.1-D2.3
WP3	**Generation and screening of microsatellite markers (SSRs) in guava**	**P3**	28	1	18	D3.1-D3.3
WP4	**Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map**	**P2**	64	19	36	D4.1-D4.3
WP5	**Construction of a guava COS library and development of SNP/INDEL markers**	**P1**	24	1	24	D5.1-D5.5
WP6	**Isolation and genetic diversity screening of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes)**	**P1**	24	1	24	D6.1 D6.2 D6.3
WP7	**Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map**	**P1**	51	25	36	D7.1-D7.2
WP8	**QTL analyses for the three guava mapping populations**	**P2**	41	1	36	D8.1 - D8.4
WP9	**Dissemination and transfer of project results**	**P1**	18	12	36	D9.1 - D9.2
	TOTAL		**475**			

Within these tasks, MPIZ (in conjunction with subcontractor S1 and IME) carried out the following tasks:

- To co-ordinate the project

[7] Work package number: WP 1 – WP 9.
[8] Contractor responsible for the work in this work package.
[9] The total number of person-months allocated to each work package.
[10] Relative start date for the work in the specific work packages, month 1 marking the start of the project, and all other start dates being relative to this start date.
[11] Relative end date, month 1 marking the start of the project, and all ends dates being relative to this start date.
[12] Deliverable number: Number for the deliverable(s)/result(s) mentioned in the work packages: D1 – D9.

- To supply reference material and material for mapping populations MP1, MP2 and MP3 to the partners (WP1)
- To generate AFLP markers for the guava mapping population MP1 (WP2)
- To construct a cosmid library for guava, individualize the library, prepare DNA array filters, sequence randomly picked COS clones, and develop SNP/INDEL primer combinations (PCs).
- To identify RGL and homeotic gene sequences by either PCR amplifications or molecular hybridization (WP6)
- To provide PCs for the identified COS clones for mapping
- To co-organize the international guava symposium plus associated laboratory course.
- To establish and update, under the EC project umbrella, a guava-online WEB site at MPIZ

I RESEARCH TASKS, ACTIONS and PROGRESS in the PROJECT

Task 1 (WP1):
Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela
Subcontractor S1 pursued the maintenance of the three guava mapping populations located at Alquízar (Havana Province), characterized the traits according to UPOV (see report subcontractor) and submitted the data for QTL analysis to Partner P2. Furthermore, crude DNA extracts were prepared by S1 from the three MPs and the corresponding parents and provided to the contractors for AFLP and SSR map establishment. Finally, 25 genotypes of small growth habit ("enana") were selected from the mapping populations MP1, MP2, and MP3, and are now field-planted at the IIFT gene bank in a randomized field design for the evaluation of agronomic characters and the identification of new cultivars for commercial production in Cuba.

Task 2 (WP2):
Construction of individual AFLP-based guava linkage maps
This task was completed in year 2 by AFLP analysis of mapping population MP1. IME pursued the adaptation of AFLP analysis on the ABI sequence machine using fluorescence-labeled AFLP primers in order to adopt the analysis to a high-throughput approach. This was only possible through the acquisition of new software. Partner P1 produced a total of 1103 AFLP markers from 119 primer combinations (planned 72) and made them available for linkage mapping to partner P2.

Task 3 (WP5):
Construction of a COS library and development of SNP/indel markers
This task was completed in year 2. A cosmid (COS) genomic library was established for guava using the maternal parent (cv. "Enana roja cubana") for MP1as the source of DNA. Packaged recombinant DNAs (initial cfu's: 52,000) were used for successive infection/transformation and picking of single colonies into 384well plates by robots at the MPIZ ADIS service unit. At an estimated

guava genome size of $0{,}17 \times 10^9$ bp and average insert size of some 40 kb per COS clone, these COS clones correspond to 6-7 guava genome equivalents. Both an original library as well as 1 replicate were picked and stored at –80° for further usage (D5.1 completed). Randomly picked guava COS clones were grown in a 96well plate, bacterial pellets were collected, and DNA was extracted for sequencing at the ADIS unit (MPIZ). Sequences were established during the first year, and primer development was pursued in year 2. These primers (a total of 150 primer combinations) were tested on the 6 parents of the three mapping populations and those dete4cting indels were supplied to tzhe partners for mapping.

Task 3 (WP6):
Candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes)

This task was completed in year 2. PCR reactions on genomic guava DNA with degenerate primers for RGL (resistance gene-like) sequences were performed with set of 16 degenerate RGL primer pairs. Appropriate products were cloned and sequenced. From a comparison to established RGL sequences, 6 individual guava RGL clones were identified.The individualized COS clones were spotted onto membranes for molecular hybridization experiments. Although this task was only due in year 2 of the project, we have directly used the membrane filters in order to identify putative genes for development (MADS-, HOMEO-box) and resistance (RGL sequences). So far, a total of 117 positive clones for RGL sequences, 37 for MADS-box and 22 for HOMEO-box were identified and in part mapped.

Task 4 (WP7):
Mapping of COS clones and candidate genes onto the guava linkage maps
This task was completed at the end of year 2. The list of PCR primers for COS clones and the primers themselves were supplied in the beginning of year 3 to the various partners (P2-P6). This task was completed until the end of the project (see also WP5 and WP6). Thirteen COS clones showed segregation in the MPs, and were mapped onto the respective linkage maps.

Task 5 (WP9):
Dissemination and transfer of project results
As the main event in dissemination, the "2nd International Symposium on Guava and Other *Myrtaceae*" was organizad by the coordinator in collaboration with the CICY and under the auspices of the ISHS. The coordinator together with Dr. G. Fermin (ULA; partner P5) has edited the 50 publications received within the frame of this symposium. This work terminated in July 2009, when all edited papers plus additional information as forwarded to ISHS for final deiting and printing of *Acta Horticulturae*. The Mérida symposium was followed by practical courses in biotechnologies and bioinformatics at the CICY (Mérida). From project funds, four fellowships were awarded to young scientists (2 from Mexico, 2 from Cuba) for participation.

II DELIVERABLES

MPIZ has complied with all deliverables prospected during the duration of the project.

III PROBLEMS and DELAYS

MPIZ fulfilled the tasks of this project. The objectives and expected benefits of this project were fully achieved.

IV TRAINING and SCIENTIST EXCHANGE

Did not apply

V DISSEMINATION (publications, posters, presentations)

- The coordinator participated to the 1st International Guava Symposium in Lucknow (India) in December 2005. He presented the results from the previous bilateral guava project (Germany/Cuba) and outlined the tasks for the GUAVAMAP project funded by the EC under INCO-DEV-2.
- On October 14, 2006, the coordinator presented the GUAVAMAP project at the Universidad de Los Andes (ULA) in Merida (Venezuela).
- During the entire month of November 2006, the coordinator worked with partner P5 (CICY, Merida, Mexico) in the frame of the GUAVAMAP project and gave a presentation on the GUAVAMAP project.
- During 12, 2006 – 2, 2007, 11, 2007 – 2, 2008, and in 10 - 11, 2008, the coordinator worked with partner P5 (CICY, Merida, Mexico) in the frame of the GUAVAMAP project.
- The coordinator presented the GUAVAMAP project during the 2nd International Symposium on Guava and Other *Myrtaceae* in Aguascalientes (Nov. 17, 2008).

Publications:

1. Rodríguez, N., Valdés-Infante, J., Becker, D., Velázquez, B., González, G., Sourd, D., Rodríguez, J., Billotte, N., Risterucci, A.M., Ritter, E. and Rohde, W. (2007): Characterization of guava accessions by SSR markers, extension of the molecular linkage map, and mapping of QTLs for vegetative and reproductive characters.
Acta Horticulturae **735**: 201-215.

Presentations:

1. 1st International Symposium on Guava; December 2005, Lucknow, India.

2. ULA, Merida, Venezuela, October 2006
3. CICY, Merida, Mexico, November 2006
4. Radio interview, Mérida, January 2007
5. INIFAP, Campo Experimental Pabellón, January 2007
6. CICY, Mérida, February 2007
7. UANL, Monterrey, September 2007
8. 2nd International Symposium on Guava, November 2008; Aguascalientes, Mexico
9. CICY, Mérida, November 2008 (subcontractor S1)

Posters:

1. Valdés-Infante, J., Rodríguez, N. N.., Becker, D., Velázquez, B., Sourd, D., Ritter, E. and Rohde, W. 2007. Establishment and saturation of the linkage map and QTL analysis in guava (*Psidium guajava* L.). "Second International Congress on Tropical and Subtropical Fruit Crops" organized by the IIFT in La Habana, Cuba (17 – 21 Sep., 2007).
2. Valdés-Infante, J., Rodríguez, N. N., Becker, D., Velásquez, B., Sourd, D., Espinosa, G., Ritter, E., Risterucci, A. M., Billote, N. and Rohde, W. 2007. Molecular characterization of the Cuban guava germplasm by AFLP and SSR analysis. "Second International Congress on Tropical and Subtropical Fruit Crops", La Habana, Cuba (17 – 21 Sep., 2007).
3. Rodríguez Medina, N. N., Fuentes Fiallo, V. R., Hernández Zaldívar, M. R., Valdés-Infante, J., Velázquez Palenzuela, J. B., Rivero, D., Sourd Martínez, D. G., Rodríguez Rodríguez, J. A., González García, G. and Martínez González, F. 2007. Cuban catalogue of guava (*Psidium guajava* L.) cultivars. "Second International Congress on Tropical and Subtropical Fruit Crops", La Habana, Cuba (17 – 21 Sep., 2007).
4. González, L., Becker, D., Schwarz-Sommer, Zs. and Rohde, W. 2007. Genetic diversity screening in *Psidium guajava* L. of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes). VI Latin American and Caribbean Meeting on Agricultural Biotechnology (REDBIO), Viña del Mar, Chile (22 – 26 October, 2007).
5. Valdés-Infante *et al*.: Simple Sequence Repeats (SSRs) for diversity characterization of guava (*Psidium guajava* L.), 2nd International Symposium on Guava and Other *Myrtaceae*, November 2008, Mexico
6. Rodríguez-Medina, N.N. *et al*.: Individual versus combined data sets for the molecular characterization of Cuban guava (*Psidium guajava* L.) germplasm, 2nd International Symposium on Guava and Other *Myrtaceae*, November 2008, Mexico
7. Gonzales, L. *et al*.: Isolation of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes) from *Psidium guajava* L., 2nd International Symposium on Guava and Other *Myrtaceae*, November 2008, Mexico
8. Rodriguez-Medina, N.N. *et al*.: Illustrated descriptors for guava (*Psidium guajava* L.) characterization, 2nd International Symposium on Guava and Other *Myrtaceae*, November 2008, Mexico

VII Coordination

A. In January 2006 the coordinator called the initial meeting of the project in Havana (Cuba). The partners met from January 30 to February 2, 2006, for the presentation of their research center affiliations, the discussion of the work programme, and coordination of specific activities such as training for partners etc.

B. A draft proposal for the consortium contract was written and sent to the contractors for amendments.

C. The coordinator negotiated the purchase and delivery of laboratory equipment for CPATSA (Partner P6) in April 2006.

D. In October 2006 the coordinator visited partner P5 (ULA, Venezuela), presented the GUAVAMAP project to ULA, and visited the guava gene bank area close to Merida. He discussed in detail the work plan, methodological aspects, and the results established by partner P5.

E. In October 2006 the coordinator visited the gene bank in Alquizar (Havana Province) where subcontractor S1 is hosting the three guava mapping populations.

F. In November 2006 the coordinator joined the CICY in Merida, Mexico (partner P4) for a 4months working period within the project. During November he presented the GUAVAMAP project and held a postgraduate course on “Analysis of biomacromolecules”.

G. In January 2007 the coordinator visited the INIFAP Campo Experimental in Pabellón (Mexico) subcontracted by the CICY for guava germplasm collection and organizer of the satellite symposium in November 2008 in Aguascalientes. The venue in Aguascalientes was visited as well as the gene bank and sites for guava production and processing.

H. In September/October 2007 the coordinator visited the subcontractor S1 (IIFT) in Havana (laboratory) and Alquízar (gene bank), Cuba.

I. In September 2007 the second GUAVAMAP project meeting was held in Mérida (Venezuela) with partner P5 (ULA) who organized the meeting.

J. In November 2007, the coordinator joined the CICY in Merida, Mexico (partner P4) for a 4months working period within the project. During November he held a postgraduate course on “Plant Molecular Biology, Biochemistry and Biotechnology I (PMBBBI)” for three weeks of daily lectures.

K. In January 2008 the coordinator visited the INIFAP Campo Experimental in Pabellón (Mexico) subcontracted by the CICY for guava germplasm collection and organizer of the satellite symposium in November 2008 in

Aguascalientes. The venue in Aguascalientes was visited and details discussed with respect to the international symposium

L. In October 2008, the coordinator joined the CICY to help in the local organization of the symposium (see VIII).

M. In November 2008, the coordinator attended the symposia in Merida and Aguascalientes as convener and participant.

VIII ADDITIONAL COMMENTS

A. 2nd International Symposium on Guava and Other *Myrtaceae*:
During the 2nd International Guava Symposium it was agreed that the following 2 symposia will be co-hosted with ISHS by partner P6 in Brazil (2011) and P5 in Venezuela (2014).

Final Report of Subcontractor S1 (IIFT)

Dr. N. N. Rodriguez (scientist-in-charge)
J. Valdés-Infante
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1. Guava DNA isolation and conservation

Healthy and young leaves from parents and overall hybrids derived from three mapping population (MP1, N6 x Enana Roja Cubana; MP2, Suprema Roja X Enana Roja Cubana, and MP3, Belic L-207 X Enana Roja Cubana) were collected for DNA isolation. Total genomic DNA was extracted from these materials by a modification (Ramírez *et al*., 2004) of the CTAB method described by Doyle and Doyle (1990). The integrity and concentration of isolated DNA was determined by electrophoresis in 0.7% agarose gel and compared to 1kb DNA ladder.

More than 300 DNAs samples were then isolated and stored at -20^{o}C before send it to Partner 1 for redistribution and DNA analysis in different laboratories (SSR and AFLP).

2. Plantation management

After the disaster caused in the south of Havana by hurricane Charlie in August, 2004, plants from the three mapping population were pruned at 50 cm high for plantation re-establishment.

After hurricane Charlie, 2004

After that, guava plantation was NPK-fertilized every spring and water supplied three times per week through localized irrigation. Pests were also controlled. Although flowering and fruiting were not observed during 2005, the guava plants recuperated and were evaluated in the years 2006-2008.

3. Characterization the three mapping population by UPOV descriptors

Morphological characterization was done based on UPOV descriptor for guava (UPOV, 1987). A group from overall characters was selected by relating their

contribution to variability: Attitude of branches; color of stem young shoot; anthocyanin coloration and intensity; shape, length, width, length/width ratio and petiole length from mature leaf; fruit weight, shape fruit uniformity, shape at stalk end (rounded, broadly rounded, truncate, necked and pointed), stalk length, neck diameter, skin color, relief of surface, flesh color, evenness and thickness ratio, calyx cavity diameter, calyx cavity/fruit ratio, seed number, seed weight , acidity (mg of tartaric acid/100g of flesh), TSS (°Brix) and vitamin C content (mg of ascorbic acid/100g of flesh) from fruit, were the variables measured. Also yields, tree high and canopy diameter were evaluated. The characterization program were completed in 2008 and data submitted to partner 1 for QTLs analysis.

4. Hybrids pre-selection for new cultivars in Cuba

Attending to the distinctive morph-agronomic characteristics, heterocigosity level and the location in the different diversity groups formed, four cultivars: 'Enana Roja Cubana' or 'EEA 18-40', 'N6', 'Belic L-207' and 'Red supreme', were selected to develop three crosses using the former as female parent (Valdés-Infante et al., 2003; Rodríguez et al., 2005). The evaluation of these three populations has permitted the selection of 25 genotypes with low size. Qualitative and quantitative variables showed a great variability in this group of plants. In the formers, color of skin, shape at the stalk end, relief of surface and flesh color of the fruits were the most variables. .For quantitative traits, seeds weight and number, flesh thickness, content of vitamin C and acidity showed the highest variation coefficient.

Mean values of yield showed an increase throughout the different years evaluated (Table 1). Maximum values of more than 180 kg/tree were recorded in 2006 and 2007. These are promising results for the selection of elite genotypes as new cultivars with productive potential.

Table 1. Annual yields corresponding to the low size hybrids selected from the crosses 'Enana Roja Cubana' x 'N6', 'Enana Roja Cubana' x 'Belic L-207' and 'Enana Roja Cubana' x 'Red supreme'.

	Anual production (Kg/tree)			
	2003	2004	2006	2007
Mean	2.37	34.00	46.97	65.55
Máx.	10.23	120.34	188.80	181.23
Mín.	0.00	1.40	3.00	9.50
S.D.	3.31	28.73	37.56	41.23
C.V (%)	139.48	84.50	79.95	62.90

Max: Maximum; Min: minimum; S.D: Standard deviation; CV: Coefficient of variation.

All of them have been replicated in plots for the evaluation of agronomic important traits for establishing as new cultivars in Cuba. Also, interesting genotypes because of the low number of seeds and high weight of the fruits were already replicated.

5. New guava descriptors

A new descriptor has been made following the indications from UPOV descriptor (UPOV 1987). Some new traits will be included and qualitative traits will be illustrated, offering photos of the different states of each qualitative variable considered. This document was written in English and Spanish.
The norms for scoring, coding, and recording of descriptor states are the following:

1 The SI system is used. The units to be applied are given in parenthesis in each case.
2 Standard color charts are recommended when it is required. However, the document offers a group of important illustrations to help the election of different states of many variables, including colors.
3 Class for quantitative traits has been established generally, according to the variation observed in the Cuban guava germplasm. This permit the evaluation of quantitative traits in both types: quantitative and qualitative data.
4 Values from 1 to 9 are used to design different sates of each descriptor. This contributes to stablish an electronic database and data processing.
5 Minimum, highly discrimination descriptors are marked with a triangle (►). Among them, is recommended to use the following for grouping accessions:
    - Tree: Branching pattern (descriptor 1.1.7)
    - Fruit: Shape (descriptor 1.3.10)
    - Fruit: Shape at stalk end (descriptor 1.3.11)
    - Fruit: Width of neck in relation to that of fruit (descriptor 1.3.13)
    - Fruit: Apex shape (descriptor 1.3.14)
    - Fruit: Color of skin (descriptor 1.3.16)
    - Fruit: Relief of surface (descriptor 1.3.17)
    - Fruit: Color of flesh (descriptor 1.3.25)
6 Specifications about the evaluation and the unit to be applied are given below of each descriptor.

6. Dissemination of the results

IIFT participated in the following scientific events:

(a) Second International Congress on Tropical and Subtropical Fruit Crops organized by the Research Institute on Tropical Fruit Crops belonging to the Agriculture Ministry of Cuba (17 – 21 Sep., 2007).
The contributions were the following:

- Valdés-Infante, J., Rodríguez, N. N., Becker, D., Velásquez, B., Sourd, D., Espinosa, G., Ritter, E., Risterucci, A. M., Billote, N. and Rohde,W. 2007. Molecular characterization of the Cuban guava germplasm by AFLP and SSR analysis.
- Rodríguez Medina, N. N., Fuentes Fiallo, V. R., Hernández Zaldívar, M. R., Valdés-Infante, J., Velázquez Palenzuela, J. B., Rivero, D., Sourd Martínez, D. G., Rodríguez Rodríguez, J. A., González García, G. and Martínez González, F. 2007. Cuban catalogue of guava (*Psidium guajava* L.) cultivars.

- Valdés-Infante, J., Rodríguez, N. N.., Becker, D., Velázquez, B., Sourd, D., Ritter, E. ans Rohde, W. 2007. Establisment and saturation of the likage map and QTLs analysis in guava (*Psidium guajava* L.).

2. Second GUAVAMAP meeting.

(b) 2nd International Symposium on Guava and Other Myrtaceae. Mérida-Aguascalientes, Nov. 10-18, 2008, México.

- Simple Sequence Repeat: Diversity characterization of guava (*Psidium guajava* L.). Valdés-Infante, J., N.N. Rodríguez, B. Velázquez, D. Rivero, F. Martínez, G. Espinosa; A.M. Risterucci, N. Billotte, D. Becker and W. Rohde.
- Comparison of the polymorphism level, discriminating capacity and informativeness of morph-agronomic traits and molecular markers in guava (*Psidium guajava* L.). Valdés-Infante, J., N.N. Rodríguez, B. Velázquez, D. Rivero, F. Martínez, A.M. Risterucci, N. Billotte, D. Becker and W. Rohde.
- Individual versus combined data set for molecular characterization of Cuban guava (*Psidium guajava* L.) germplasm. Rodríguez, N.N., J. Valdés-Infante, B. Velásquez, D. Rivero, F. Martínez, A.M. Risterucci, N. Billotte, D. Becker and W. Rohde.
- Illustrated descriptors for guava (*Psidium guajava* L.). Rodríguez, N.N., G.A. Fermín, J. Valdés-Infante, B. Velázquez, D. Rivero, F. Martínez, J. Rodríguez, and W. Rohde.
- Genetic resources of guava (*Psidium guajava* L.) in Cuba: germplasm characterization and breeding. Rodríguez, N.N., J. Valdés-Infante, G. González, V. Fuentes, J. Cañizares and W. Rohde.

The following papers were published:

- Valdés-Infante Herrero, Juliette, Narciso N. Rodríguez; Dieter Becker; Bárbara Velásquez, Darío Sourd, Georgina Espinosa y Wolgang Rohde. Microsatellites characterization of guava (Psidium guajava L.) germplasm collection in Cuba. Cultivos Tropicales 28:5-11, 2007.
- Valdés-Infante Herrero, Juliette, Narciso Nerdo Rodríguez, Bárbara Velázquez, Gonzalo González, D. Sourd, J. Rodríguez. Mejoramiento genético del guayabo (*Psidium guajava* L.) en Cuba. Boletín Noticitrifrut, 2007.
- Valdés-Infante, J., Rodríguez, N. N., Becker, D., Velásquez, B., Sourd, D., Espinosa, G., Ritter, E., Risterucci, A. M., Billote, N. y Rohde,W. Caracterización por AFLP y SSR del banco de germoplasma de guayabo (*Psidium guajava* L.) en Cuba. Memorias del II Simposio Internacional de Fruticultura Tropical y Subtropical. La Habana Cuba. ISBN 978-959-296-001-5, 2007.
- Rodríguez Medina, N. N., Fuentes Fiallo, V. R., Hernández Zaldívar, M. R., Valdés-Infante, J., Velázquez Palenzuela, J. B., Rivero, D., Sourd Martínez, D. G., Rodríguez Rodríguez, J. A., González García, G. y Martínez González, F. Catálogo de cultivares de guayabo (*Psidium guajava* L.) en Cuba. Memorias del II Simposio Internacional de Fruticultura Tropical y Subtropical. La Habana Cuba. ISBN 978-959-296-001-5, 2007.

- Valdés-Infante, J., Rodríguez, N. N.., Becker, D., Velázquez, B., Sourd, D., Ritter, E. ans Rohde, W. Establecimiento y saturación del mapa de ligamiento genético y de QTLs para el guayabo (*Psidium guajava* L.) en Cuba. Memorias del II Simposio Internacional de Fruticultura Tropical y Subtropical. La Habana Cuba. ISBN 978-959-296-001-5, 2007.
- Celso V. Pommer, Katia R. N. Murakami, Juliette Valdés-Infante, Narciso N. Rodríguez and W. Rohde. *Breeding Guava. In:* S.M. Jain, P.M. Priyadarshan (eds.), *Breeding Plantation Tree Crops: Tropical Species*. DOI 10.1007/978-0-387-71201-7 3, C° Springer Science+Business Media, LLC 2009. New York. p: 83-120, 2008..

Also, in *Acta Horticulturae* will be published the following:

- Simple Sequence Repeat: Diversity characterization of guava (*Psidium guajava* L.). Valdés-Infante, J., N.N. Rodríguez, B. Velázquez, D. Rivero, F. Martínez, G. Espinosa; A.M. Risterucci, N. Billotte, D. Becker and W. Rohde.
- Comparison of the polymorphism level, discriminating capacity and informativeness of morph-agronomic traits and molecular markers in guava (*Psidium guajava* L.). Valdés-Infante, J., N.N. Rodríguez, B. Velázquez, D. Rivero, F. Martínez, A.M. Risterucci, N. Billotte, D. Becker and W. Rohde.
- Individual versus combined data set for molecular characterization of Cuban guava (*Psidium guajava* L.) germplasm. Rodríguez, N.N., J. Valdés-Infante, B. Velásquez, D. Rivero, F. Martínez, A.M. Risterucci, N. Billotte, D. Becker and W. Rohde.
- Illustrated descriptors for guava (*Psidium guajava* L.). Rodríguez, N.N., G.A. Fermín, J. Valdés-Infante, B. Velázquez, D. Rivero, F. Martínez, J. Rodríguez, and W. Rohde.
- Genetic resources of guava (*Psidium guajava* L.) in Cuba: germplasm characterization and breeding. Rodríguez, N.N., J. Valdés-Infante, G. González, V. Fuentes, J. Cañizares and W. Rohde.

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Molecular characterization of Cuban accessions of guava (*Psidium guajava* L.), establishment of a first molecular linkage map and mapping of QTLs for vegetative characters. J. Genet. & Breed., 57:349-358.

Improvement of guava: Linkage mapping and QTL analysis as a basis for marker-assisted selection (GUAVAMAP)
FP6-2003-INCO-DEV-2 No. 015111

Partner 2: NEIKER

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FINAL REPORT – (PERIOD 1.12.2005 - 31.5.2009)

INTRODUCTION

The strategic objectives of this proposal were to provide the methodological basis and molecular tools for improving the breeding efficiency in the tropical fruit crop guava.
The research work was co-ordinated in detail during frequent contacts (e-mail, telephone) with the co-ordinator and between project partners and during various project meetings.
The work package list for the GUAVAMAP project was as follows for NEIKER:

WP No	Work package title	LC	PM	Start month	End month	Deliverable No
WP1	**Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela**	**P5**	115	1	36 (42)	D1.1-D1.7
WP2	**Construction of individual AFLP-based guava linkage maps**	**P1**	110	1	36 (42)	D2.1-D2.3
WP3	**Generation and screening of microsatellite markers (SSRs) in guava**	**P3**	28	1	18	D3.1-D3.3
WP4	**Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map**	**P2**	64	19	36 (42)	D4.1-D4.3
WP7	**Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map**	**P1**	51	25	36 (42)	D7.1-D7.2
WP8	**QTL analyses for the three guava mapping populations**	**P2**	41	1	36 (42)	D8.1 - D8.4
WP9	**Dissemination and transfer of project results**	**P1**	18	12	36 (42)	D9.1 - D9.2
	TOTAL		**475**			

I RESEARCH TASKS, ACTIONS and PROGRESS in the PROJECT

All the figures and tables we refer in the text are shown on the project web page: Http://www.neiker.net/neiker/guavamap

WP1 Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela

Task 1.6: NEIKER has updated annually the guava database with the contributions received from the different partners. These have been integrated in the WEB page.

Also the relevant UPOV descriptors can be downloaded from this WEB page.

WP2 Construction of individual AFLP-based guava linkage maps *and* WP4 Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map

(For logical reasons we include at this point also the SSR and COS markers in the results and discussions of the linkage maps.)

Linkage maps in the three population were produced annually based on the marker data (AFLP, SSR, COS) received by the participants involved as indicated in their corresponding reports as well as based on our own data. In the extension period we have processed the final marker data (AFLP, SSR, COS) received by the participants.

In all populations polymorphic DNA fragments (AFLPs, SSRs, COS) were scored by the different partners, including NEIKER for MP2, for their presence or absence in parents and F1 progenies. Linkage analysis between marker fragments, estimation of recombination frequencies, and determination of linear order between linked loci including multipoint linkage analysis and the EM algorithm for handling missing data were performed as described (Ritter et al., 1990; Ritter and Salamini, 1996). The MAPRF program (Ritter and Salamini, 1996) was applied for the computational methods. Firstly, linkage groups were constructed based on fragments specific to either parent. Linked fragments were arranged into linkage groups using a minimum, commonly accepted LOD threshold of 3.0 between consecutive markers.

Resources for linkage mapping and observed polymorphisms

Different number of AFLP and SSR primer combinations (PCs) were analysed in the three guava mapping populations. Table 2.7 summarizes the resources used for linkage mapping and the polymorphisms detected in each case.

Finally for **MP1** a total of 1103 segregating AFLP markers obtained from 119 PCs were available for linkage mapping (Table 2.7). A total of 248 markers (22.5%) represented individual markers from Enana while 354 (32.1%) were specific for parent N6. A total of 501 markers (45.4%) were common to both parents. On average 9.3 segregating fragments were obtained per primer combination with a maximum of 21 fragments. A maximum of 9 P1 specific and

11 P2 specific bands were observed in specific primer combinations as well as 19 common fragments.
In addition 237 SSR PCs and 1 COS were analysed in this population which generated 363 allelic fragments. Most of the SSRs represented one locus each. A total of 124 alleles descend from Enana, 158 from N6 and 81 alleles are common to both parents of the mapping population. Thus in total 1106+363 = 1469 AFLP and SSR markers were available for linkage mapping in MP1.

In **MP2** a total of 785 AFLP markers were revealed by analysing 141 primer combinations. A total of 196 markers (25%) were specific for Enana, while 261 (33%) were specific for Suprema Roja. In total 328 common markers (42%) were detected. The average number of segregating fragments per primer combination was 5.8 with a maximum of 16 fragments in one PC. Five P1 specific, seven P2 specific and ten common bands were maximally observed in particular primer combinations.
Moreover, segregation data from 62 SSR /COS PCs were available (12 COS). Some of them were also analysed in the MP1 mapping population. A total of 100 allelic fragments were detected. Fifteen 28 alleles descend from Enana, 40 from Suprema Roja and 33 alleles are common to both parents of the progeny. In this way 885 AFLP, SSR and COS markers were available for linkage mapping.

NEIKER (P2) together with Partner 6 (EMBRAPA) was in charge of AFLP analyses in mapping population MP2. DNA was extracted using the Quiagen DNAeasy Plant Mini Kit following the supplier's instructions. AFLP analysis was performed according to Vos et al. (1995) using *Eco*RI/*Mse*I adapters. Preamplification was performed with one selective nucleotide and specific amplification with 3 selective nucleotides (+1/+3 amplification). AFLP fragments were detected on a LI-COR 4200-S1 DNA sequencer using primers labelled with the fluorescent infrared dye IRD800 (LI-COR, Lincoln, Nebraska, USA). Analysis was performed according to the manufacturer instructions.
In the first year a total of 24 AFLP PCs were analysed by NEIKER (the first 24 PCs in Table 2.3a). Also in the second and third year a total of 24 AFLP PCs were analysed by NEIKER each year.
NEIKER extracted in year2 DNAs from 24 Brazilian acccessions from Partner P6 (Embrapa) and send to Partner P3 (CIRAD) who analysed a subset of each partner with a common set of SSR markers (WP3).
NEIKER performed SSR analyses in the MP2 mapping population. SSR analysis was performed as described by Lebrun et al. 2001 and based on the information given by partner P3. SSR bands were revealed using the LiCor System or on an ABI capillary sequencer according to the manufacturer instructions. In YEAR2 a total of 25 SSR PCs were analysed. However only 19 PCs revealed suitable segregation patterns. Others were monomorphic or revealed unclear bands.
In YEAR3 NEIKER contributed 22 new polymorphic SSRs. Two additional SSR primer pairs did not reveal polymorphic amplification products.

In **MP3** 1163 segregating AFLP markers were obtained from 102 primer combinations. A total of 160 markers (13.8%) represented individual markers

from Enana, 343 (29.5%) were specific for Belic and 660 markers (56.7%) were common to both parents.
In addition segregation data from 100 SSR/COS PCs were available (44 COS). Some of them were also analysed in the other mapping populations. A total of 131 allelic fragments were detected with the 100 PCs. Thirty alleles descend from Enana, 18 from Belic and 83 alleles are common to both parents of the progeny. Thus, in total 1294 AFLP and SSR markers were available for linkage mapping in MP3.
The distributions of individual and common fragments in the progenies indicate that Enana is slightly less heterozygous than the other crossing parents, but that all parents share a considerable gene pool.
On the other hand compared with MP2 more segregating AFLP fragments were observed in populations MP1 and MP3 and particularly more common fragments. Parts of the analyses were performed in these populations on the capillary ABI system which is more sensitive than the LiCor system. Nevertheless, many of these bands showed highly distorted segregation ratios and could not be mapped.

Construction of linkage maps
Using the methodology described above integrated linkage maps were produced in all three mapping populations in the 3rd year. In all cases 11 linkage groups (LGs) were obtained corresponding to the 11 chromosomes of the haploid guava genome.
Characteristics of individual parental and integrated maps of **MP1** are shown in Tables 2.1c and 2.1d. The maps are visualised in Fig. 2.1 a-c.
A total of 304 markers could be integrated into the Enana map. Individual linkage groups of Enana vary between 113 and 219cM in length and contain between 16 and 32 markers each. On average a linkage group was composed of 24.0 Enana-specific markers, and 3.6 common markers, summing up to 27.6 markers per linkage group. Total Enana map length was 2012 cM with an average linkage group length of 183 cM.

A total of 372 markers could be integrated into the N6 map. Individual linkage groups of N6 vary between 94.8 and 219 cM in length and contain between 21 and 34 markers each. On average a linkage group was composed of 30.2 N6-specific markers, and 3.6 common markers, summing up to 33.8 markers per linkage group. Total map length of N6 was 1833 cM.

The integrated map contains 632 markers, has a length of 2179 cM, and an average linkage group length of 198 cM. Individual linkage groups vary between 166 and 233 cM in length and contain between 44 and 71 markers each. On average a linkage group is composed of 23.9 Enana-specific markers, 27.3 N6-specific markers, and 6.3 common markers, summing up to 57.5 markers per linkage group.
In addition, a total of 126 so-called RF0 markers (Table 2.1e) and 146 so-called associated markers (Table 2.1f) were determined (see below). Thus, the actual marker number in the final integrated map of this cross including RF0 and associated fragments is 904 with an average of 82 markers per linkage group.
Compared to previous results (Valdés-Infante et al., 2004; Rodriguez et al., 2007) some changes can be observed with respect to the arrangements of

linkage groups and the markers assigned to these groups. Linkage mapping represents a statistical process and the actual linkage map represents the statistically most likely order. Meanwhile considerably more markers have been added for linkage analyses leading to the mentioned re-arrangements and changes in marker assignments.

Linkage mapping in **MP2** was performed as described in material and methods. Characteristics of individual parental and integrated maps are shown in Table 2.3c. The maps are visualised in Fig. 2.2 a-c. Initially parental linkage maps of eleven linkage groups were established. A total of 172 markers (126 specific and 46 common markers) could be integrated into the Enana map. Individual linkage groups of Enana vary between 129 and 154 cM in length and contain between 11 and 22 markers each. On average a linkage group was composed of 11.5 Enana specific markers, and 4.2 common markers, summing up to 15.6 markers per linkage group. Total Enana map length was 1698 cM, with an average linkage group length of 154 cM.
A total of 197 markers (143 specific and 54 common markers) could be integrated in the Suprema Roja (SR) map. Individual linkage groups of SR vary between 121 and 140 cM in length and contain between 13 and 24 markers each with an average of 17.9 markers. Total map length of SR was 1538 cM.
An integrated linkage map of the MP2 mapping population was produced using as anchor points allelic SSR fragments but also common fragments having recombination values of zero with individual markers from both parents. A total of 27 anchor points were available. This map contains 352 markers, has a length of 1761 cM, and an average linkage group length of 160 cM. Individual linkage groups vary between 146 and 173 cM in length and contain between 26 and 40 markers each. On average a linkage group was composed of 11.5 Enana-specific markers, 12.7 Suprema-specific markers, and 7.7 common markers, summing up to 32 markers per linkage group.
In addition, a total of 27 so-called RF0 markers (i.e: markers which are linked with a recombination frequency (RF) of zero to other mapped markers; Table 2.3d) were mapped, which are not displayed in Fig. 2.2. Furthermore, 74 so-called associated markers were determined (Table 2.3e). These markers do not fit precisely in the existing framework maps (probably due to scoring errors), but they show reduced RF values (<10cM) with other mapped markers and therefore they are “associated” to them. In this way the actual marker number in the final integrated map of this cross including RF0 and associated fragments is 453 with an average of 41 markers per linkage group.

Also in **MP3** eleven linkage groups were established initially in both parents. Characteristics of individual parental and integrated maps are shown in Table 2.5c. The maps are visualised in Fig. 2.3 a-c. A total of 132 markers (90 specific and 42 common markers) could be integrated into the Enana map. Individual linkage groups of Enana vary between 132 and 175 cM in length and contain between 8 and 15 markers each with an average of 12 markers per linkage group. Total Enana map length was 1782 cM, with an average linkage group length of 162 cM. A total of 182 markers (128 specific and 54 common markers) could be integrated in the Belic map. Individual linkage groups of Belic vary between 134 and 178 cM in length and contain between 9 and 25 markers

each with an average of 16.5 markers per linkage group. Total map length of SR was 1710 cM.
An integrated linkage map of the MP3 mapping population was produced using as anchor points allelic SSR fragments and some common fragments having recombination values of zero with individual markers from both parents. A total of 24 anchor points were used.
This integrated map contains 301 markers, has a length of 1895 cM, and an average linkage group length of 171 cM. Individual linkage groups vary between 155 and 199 cM in length and contain between 19 and 39 markers each. On average a linkage group was composed of 8.2 Enana-specific markers, 11.7 Belic-specific markers, and 7.5 common markers, summing up to 27.4 markers per linkage group. In addition, a total of 32 RF0 markers (Table 2.5d) were mapped. Furthermore, 89 associated markers (Table 2.5e) were determined. The actual marker number in the final integrated map of this cross is therefore 422 with an average of 38 markers per linkage group.

Location of SSR markers in population maps (Comparative Mapping)
Several SSR markers were evaluated in common in two or even all three mapping populations. Table 2.7a shows the location of mapped SSR markers in populations MP2 and MP3 and their corresponding location in MP1. From the total of 50 SSR primer combinations analysed in MP2 31 could be integrated into linkage groups. This was also the case for 24 SSR PCs (out of 56) in the MP3 population. In 34 cases involving 32 SSRs also map locations in the MP1 population were available.
Certain associations between LGs of different populations are visible in Table 2.7a. For example four of 6 SSRs mapped to Lg6 in MP2 and two of four SSRs located on Lg9 in MP3 are mapped to Lg5 in MP1. Moreover, two SSR on Lg2 in MP3 and three SSR on Lg2 in MP2 are located on Lg8 in MP1. Other associations between linkage groups could consist of Lg7 in MP1, Lg4 in MP2 and Lg7 in MP3 based on markers mPgCIR176 and mPgCIR99 (Table 2.7a). Also potential associations between Lg1 in MP1 and Lg5 in MP2, between Lg2 in MP1 and Lg3 in MP2, as well as associations between Lg4 in MP1 and Lg11 in MP2 are possible.
However, in many cases SSR markers which are located on the same linkage group in MP1 map to different linkage groups in the other populations. This occurs for example in MP3 where for example three SSR markers from Lg2 in MP1 map to three different linkage groups in MP3.
An explanation for this finding could be that the SSR markers are not single loci markers as it has been observed in several cases in our populations and different loci are targeted in each case (see for example mPgCIR186 and mPgCIR243 in Table 2.7a). SSR primers are designed in regions outside of the repeat motive of the SSR and may be located in genes with multiple copies in the genome or belonging to multi gene families. Several different loci can be amplified in this way. This phenomenon has been also observed in other woody species such as *Pinus* (Ritter et al., 2003). However a “majority principle” can determine the most probable association of linkage groups. This however requires the analyses of sufficient SSR PCs for confirmation. In the future it will be necessary to analyse numerous additional SSR markers for completing map alignments in the different genetic backgrounds of our study.

However, in total there are 904+453+422=1779 markers with 82+41+38=161 markers per linkage group available on the 3 maps, which are partially aligned so that the marker info from one map can be transferred to another map at least at the interval level.

WP7 Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map

This task has started at month 25 according to the technical annex.
In the extension period NEIKER finalised the processing of COS markers (Nº 192 to 203 in Table 2.3b.) A total of 15 COS markers were analysed. Three COS primers, COS99, 152 and 177 did not amplify or produced smear.

In total 57 COS markers analysed by the different partners revealed amplification products in one or the other mapping population. However, only 13 of them could be mapped. They are shown in table 2.7d.

WP8 QTL analyses for the three guava mapping populations

Data records were received in year 1 and year 2 and used for QTL analyses. During the third reporting period the final data records were received from Subcontractor S1 (IIFT) of Partner P1. With these data final QTL analyses were performed in all three mapping populations. The traits include: leaf length (LL), leaf width (LW), fruit length (FL), fruit width (FW), internal and external pulp thickness (PI, PE), seed numbers (SN), average seed weight (WS), vitamin C contents (VC), acidity (AC), total soluble solids (TSS), maturity index (MI), average fruit weight (WF), plant height in February 2008 (HP), yield in 2007 (PR) and cumulative yield 2005 to 2007 (CP). Qualitative and quantitative characters were measured according to UPOV (1987) as described by Rodríguez et al. (2004). QTL analyses were performed using MAPRF and SAS software with the Interval mapping method (Knapp et al. 1990).

Characteristics of the evaluated traits

The characteristics of the evaluated traits in the three populations are shown in Table 2.2a. Average trait values were quite similar in all populations with a few exceptions. Vitamin C content was much lower in population MP1 and seed number was somewhat higher in this population compared to the others. In MP1 also highest average progeny yield, cumulative yield and the maximum yield value were detected, despite of the fact that the parent with highest production was Belic. A remarkable high vitamin C content of 687.4 mg/100g fresh weight was also observed in one progeny genotype of MP2.
In general sufficient variation was available to allow an efficient QTL analysis. The coefficients of variation (CV) depended strongly on the particular trait under evaluation. Lowest CV values ranging from 5.7 to 7.1% were obtained for fruit width in all populations, while the highest values were detected for vitamin C contents in MP2 (65.2%) and MP3 (53.9%) or for yield in MP1 (51.8%). The trait specific CV values were quite similar between progenies with the exception of larger differences for leaf length and smaller CV values for AC and VC in the MP1 population.

Table 2.2a shows also the average characteristics of the four parents of the progenies. Some larger differences with respect to trait expressions can be seen. Average fruit weight is highest for Enana, while Suprema Roja and Belic have the largest vitamin C contents. Belic has also the smallest seed numbers and lowest seed weights.

Also correlation analyses between individual trait values were performed in all progenies (Tables 2.2b; 2.4b and 2.6b; respectively). Several characters were significantly positive correlated in all populations. These include leaf length with leaf width, fruit length with width and with average fruit weight, vitamin C contents with acidity and total soluble solids, and yield with cumulative yield, respectively. Seed number and seed weights showed significantly negative correlations in all populations. PE and PI were positive correlated in MP1, negative in MP2 and were uncorrelated in MP3. Plant height was only in MP1 positively correlated with yield and cumulative yield. Surprisingly average fruit weight was not correlated with yield traits in all progenies.

QTL analyses in mapping population: Enana Roja Cubana x N6

QTL analyses for the 16 traits were performed as described in materials and methods. The detected QTLs for each trait are presented in Table 2.2c. For each QTL the absolute effect, the parental descend, the interval in which the QTL was detected and the map location is indicated. Moreover, the probability for the QTL and the amount of variance explained by the QTL is shown.

A total of 75 QTLs were detected for the 16 analysed characters. Between seven QTLs for seed number and vitamin C content and two QTLs for internal pulp thickness and acidity were detected for each trait. Individual QTLs explained between 2.6 (qLWc) and 18.5% (qSNd) of the variance. Total variance of a trait explained by the sum of all detected QTLs varied between 14.4 for acidity and 55.4% for fruit length between traits. These variance values were normally smaller than the sum of values for individual QTLs due to correlative effects, but for some traits (LL, FL, PI) larger R^2 values were observed, probably due to epistatic effects between QTLs.

A total of 35 QTLs descend from Enana, while 40 QTLs were from N6. Although QTL numbers were similar for each parent large differences exist depending on the particular trait. Both QTLs detected for acidity descend from Enana. The same was the case for four out of five QTLs for PE and five of six QTLs for seed weight. In contrary N6 contributed four out of five MI QTLs and five of six QTLs for average fruit weight (Table 2.2c).

Many of the QTLs are co-located or closely linked to SSRs according to the intervals in which QTLs were detected. This will allow an efficient selection of favourable genotypes through marker assisted breeding in different genetic backgrounds based on SSR markers.

The location of the QTLs on the linkage map of MP1 are visualised in Fig. 2.1d. QTLs for different traits are co-located or closely linked in this figure, reflecting the observed correlations between characters. This was for example the case for several QTLs for fruit weight, fruit length and fruit with (qFLb, qWFb on LG2; qFWa, qWFd on LG3; qFWc, qWFe on LG6; qFLd, qWFf on LG10) Also QTLs for plant height and yield (qHPe and qPRb on LG5), for yield and cumulative yield (qCPd and qPRc on LG 11) and QTLs for leaf length and width (qLLe and qLWe on LG11) were co-located or closely linked on the map (Fig. 2.1d).

Compared to previous results (Valdes-Infante et al, 2004; Rodriguez et al., 2007) some changes can be observed with respect to detected QTL numbers and locations. QTL analyses represent statistical processes which depend on the available marker and phenotypic data. Meanwhile, phenotypic data have been completed for many additional genotypes and are included in the analyses leading to the mentioned changes.

QTL analyses in MP2 and MP3 populations

The same traits as in MP1 were also analysed in the other two mapping populations.

Details on all detected QTLs for each trait are presented in Table 2.4c and Table 2.6c in MP2 and MP3, respectively. QTLs are shown in the figures of the integrated maps in MP2 and MP3 (Fig. 2.2c and Fig 2.3c, respectively).

Table 2.7b summarizes and compares the results of QTL analyses in these progenies. In MP2 a total of 56 QTLs were identified while in MP3 59 QTLs were detected. Compared to MP1 the total QTL numbers are somewhat smaller in these populations which might be due to the reduced marker densities in the corresponding maps, where QTLs with smaller effects might not be detected in larger intervals. This hypothesis is supported by the fact that only in MP1 QTLs with smaller R^2 values are identified (for example qLWc; R^2 =2.6; see Table 2.2c).

In MP2 between six QTLs for internal pulp thickness and total soluble solids and two QTL for each of the traits leaf length, external pulp thickness, plant height and yield were detected. Individual QTLs explained between 4.3 (qHP) and 20.6% (qLW) of the variance. Total variance explained by the sum of all detected QTLs varied between 10.8 for plant height and 48.4% for maturity index between traits. In MP2 21 QTL were contributed by Enana while 35 descended from Suprema Roja. All detected QTLs for fruit width, external pulp thickness and vitamin C contents were from Suprema Roja.

In MP3 between seven QTL for internal pulp thickness and one QTL for acidity, seed weight and yield were detected. Individual QTLs explained between 6.6 (qWSa) and 31.6% (qVC) of the variance. Total variance explained by the sum of all detected QTLs varied between 6.6 for seed weight and 68.3% for external pulp thickness between traits. In MP3 28 QTL descended from Enana and 31 from Belic. Both QTLs for maturity descended from Belic. In contrary the four QTLs for cumulative yield as well as five of the six QTLs for leaf length were from Enana.

Only three QTLs in total were detected for yield in both progenies, but with relative high R^2 values of the individual QTLs. The number of QTLs for cumulative yield was higher with eight detected loci in both progenies. As in MP1 in each population one yield QTL was located in the same interval as a QTL for cumulative yield targeting probably the same gene (see Fig. 2.2c and Fig 2.3c, respectively).

Comparative QTL analyses

Potential associations of linkage groups from different progenies have been inferred above. Considering these associations, also certain QTLs from different progenies could be co-located or closely linked targeting the same gene influencing a trait. However, effects of particular alleles at the QTL locus

have to be taken also into account in different progenies. Considering locations of cross-referenced SSR markers in different progenies, interval lengths and relative positions of QTLs, we found in 8 cases out of 18 groups of candidate QTLs potential associations of analogous QTLs of the same trait from different populations (Table 2.7c). Details can be seen on the mentioned web page.

Molecular Marker Set and Recommendation for MAS breeding plan in guava

In QTL analyses we identified in many cases SSR markers linked to individual QTLs. Such markers are particularly useful since they are highly polymorphic and map usually to identical genomic locations in different genetic backgrounds.

Therefore we propose these markers to constitute the Molecular Marker Set (MMS) and recommend them for marker assisted breeding in guava.

Table 2.7e lists the SSR markers of the MMS and their target QTLs for various useful traits in the different mapping populations. In total we propose 39 SSRs for MP1, 8 for MP2 and 3 for MP3.

Off course the markers of the MMS are also useful for other genetic backgrounds, once the particular allelic configurations have been determined for a marker locus and a QTL.

WP9 Dissemination and transfer of project results

YEAR1: A WEB site had been setup by NEIKER which contains all project results, as specified in the Technical Annex. The address is: www.neiker.net/neiker/guavamap. Several links are implemented. The project web page has been updated with all project results.

Several other dissemination activities were performed during the course of the project which are listed below.

II DELIVERABLES

NEIKER completed all deliverables due in the project:

No	Deliverable name	WP no.	Nature	DL	Delivery date (project month)
D8.1	Database of phenotypic characters	8	D	PU	35 available (see webpage)
D9.2	Symposium and laboratory course	9	D	PU	35 completed
D2.3	Linkage maps of 1340-1800 markers	2	P	PP	42 available (see webpage)
D4.3	Molecular Marker Set	4	P	PP	42 available (see webpage)
D7.2	Map positions of COS clones and candidate genes	7	P	PP	42 available (see webpage)
D8.2	Database with results on QTL analysis	8	D	PU	36 available (see webpage)
D8.3	Database for comparative QTL analysis	8	D	PU	36 available (see webpage)

D8.4	Recommendation for MAS breeding plan in guava	8	D	PU	42 available (see webpage)
D9.1	Update of guava website	9	D	PU	42 done

III PROBLEMS and DELAYS

No significant problems have been detected for the realization of all tasks. The small delay in mapping COS clones and definition of MMS and recommendations for MAS was completed successfully by NEIKER until the end of the project. The objectives and expected benefits of this project were fully achieved.

IV TRAINING and SCIENTIST EXCHANGE

- Dr. Ritter from NEIKER participated to the opening meeting of the GUAVAMAP project organized by Partner 1 in January 2006 at IIFT, La Habana, Cuba.

- NEIKER received during 6 weeks a PhD student from Partner P6 (EMBRAPA) for training in the molecular techniques needed for the project. These include DNA extraction, Preparation of materials for AFLP analyses, AFLP analyses, Gel electrophoresis on the Licor sequencer and using silver-staining and SSR analyses.

- Dr Ritter from NEIKER organised end of November 2006 a PhD course at the University of Partner 5 (ULA). In this course he was teaching during one week theoretical and practical aspects of biodiversity analyses, linkage mapping and QTL analyses including computer sessions. Beside other PhD students all project co-workers from Partner 5 participated in this course.

- Dr. Ritter from NEIKER participated to the project meeting of the GUAVAMAP project organized by Partner 5 in September 2007 at the University of Los Andes (ULA) in Merida, Venezuela.

- At the end of November 2007, Dr. Ritter from NEIKER organised a course with partner P6 at Embrapa-Semiarido in Petrolina (Brazil). During this course he was teaching for one week theoretical and practical aspects of biodiversity analyses, linkage mapping, and QTL analyses including computer sessions. Besides other PhD students from 3 Embrapas and 3 Universities all project co-workers from Partner 6 participated in this course.

- NEIKER received in 2008 during 8 weeks (June to August) a PhD student from Partner P6 (EMBRAPA) for training in the molecular techniques needed for the project.

V DISSEMINATION (publications, posters, presentations)

- A Project WEB page has been established which is available to the public and contains all relevant project results. This web page has been updated annually.

- NEIKER is co-author of the poster presented by CIRAD at the PlantGEMs (Plant Genomics European Meetings) 2006 Conference, the 11-14 October 2006, in Venice, Italy: "Microsatellite markers development: towards a genetic map of guava (*Psidium guajava L.*) by Nansot and *al..*

- NEIKER is co-author of the publication: Rodríguez, N., Valdés-Infante, J., Becker, D., Velázquez, B., González, G., Sourd, D., Rodríguez, J., Billotte, N., Risterucci, A.M., Ritter, E. and Rohde, W. (2006). Characterization of guava accessions by SSR markers, extension of the molecular linkage map, and mapping of QTLs for vegetative and reproductive characters. *Sci. Horticulturae*.

- NEIKER presented project results during the Project Meeting at ULA (Mérida, Venezuela) in September 2008.

- Dr. Ritter from NEIKER participated to the GUAVAMAP symposium in Merida (Mexico) and gave two presentations. NEIKER has submitted the two presentations given during the symposium for publication by ISHS and is co-author of a third publication.

Ritter, E, Rodríguez-Medina, N.N., Velásquez, B., Rivero, D., Rodríguez, J.A., Martínez, F and Valdés-Infante, J. (2009) QTL (quantitative trait loci) analysis in guava. Acta Hoticulturae (in press).

Ritter, E., Herrán, A., Ariz, U., Valdés-Infante, J., Rodriguez-Medina, N.N., Briceño, A., Fermin, G., Sanchez-Teyer, F., O'Connor-Sanchez, A., Muth, J., Boike, J, Santos, C.A., Nunes dos Santos, I.C., Rodrigues, M.A., Risterucci, A.M., Billotte, N., Becker, D. and Rohde, W. .(2009). Comparative linkage mapping in three guava mapping populations and construction of an integrated reference map in guava. Acta Hoticulturae (in press).

Billotte, N., Lepitre, V., Nansot, G., Grangeon, R., Pomies, V., Rivallan, R., Risterucci, A.M., Muth, J., Prüfer, D., Valdés-Infante, J., Rodríguez, N., Rohde, W. and Ritter, E.(2009) The microsatellite (SSR)/AFLP reference linkage map of guava. Acta Hoticulturae (in press).

Improvement of guava: Linkage mapping and QTL analysis as a basis for marker-assisted selection (GUAVAMAP)

Proposal FP6-2003-INCO-DEV-2 No. 015111

Partner 3 CIRAD

Dr. Norbert Billotte	CIRAD personnel (scientist-in-charge)
Ange-Marie Risterucci	CIRAD personnel
Ronan Rivallan	CIRAD personnel
MsC students	EC funds

FINAL REPORT - (1.12.2005-31.05.2009)

The work package list for the GUAVAMAP project is as follows (CIRAD participation in blue):

Work package No[13]	Work package title	Ld contractor[14]	Person-months [15]	Start month[16]	End month[17]	Deliverable No[18]
WP1	**Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela**	**P5**	115	1	36	D1.1-D1.7
WP2	**Construction of individual AFLP-based guava linkage maps**	**P1**	110	1	36	D2.1-D2.3
WP3	**Generation and screening of microsatellite markers (SSRs) in guava**	**P3**	28	1	18	D3.1-D3.3
WP4	**Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map**	**P2**	64	19	36	D4.1-D4.3
WP5	**Construction of a guava COS library and development of SNP/INDEL markers**	**P1**	24	1	24	D5.1-D5.5
WP6	**Isolation and genetic diversity screening of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes)**	**P1**	24	1	24	D6.1 D6.2 D6.3
WP7	**Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map**	**P1**	51	25	36	D7.1-D7.2
WP8	**QTL analyses for the three guava mapping populations**	**P2**	41	1	36	D8.1 - D8.4
WP9	**Dissemination and transfer of project results**	**P1**	18	12	36	D9.1 - D9.2
	TOTAL		**475**			

Within the project, CIRAD (Partner 3) is carrying out the following tasks:
1. To advise the SSR analysis for germplam biodiversity evaluation.
2. To extract DNAs from the parents and from progenies of the mapping population MP1
3. To generate functional SSR markers and prescreen them on the three mapping populations
4. To construct an SSR map for MP1
5. To map candidate genes in MP1-derived maps
6. To participate to QTL analysis in MP1 and MMS development
7. To participate to the international symposium and laboratory course at the CICY (partner P4) in November 2008

[13] Work package number: WP 1 – WP 9.
[14] Contractor responsible for the work in this work package.
[15] The total number of person-months allocated to each work package.
[16] Relative start date for the work in the specific work packages, month 1 marking the start of the project, and all other start dates being relative to this start date.
[17] Relative end date, month 1 marking the start of the project, and all ends dates being relative to this start date.
[18] Deliverable number: Number for the deliverable(s)/result(s) mentioned in the work packages: D1 – D9.

I RESEARCH TASKS, ACTIONS and PROGRESS in the PROJECT

WP3: Generation and screening of microsatellite markers (SSRs) in guava

Task 3.1: Generation of functional SSR primers pairs.
Partner P3 (CIRAD) exploited its guava genomic library enriched for (GA)n and (GT)n microsatellite sequences (Risterucci et al., 2005). For this, DNA fragments were cloned into the plasmid vector pGEMT Easy (Promega, Madison, USA), then the ligated products were transformed into Echericia coli by using electroporation-competent XL1-Blue cells (Stratagene, USA) according to the manufacturer's protocol. A set of 1536 bacterial clones were selected and cultivated on selective media in Petri dishes. Cloned DNA was then PCR amplified and the inserts tested by the RFLP technique with 32P-labeled probes of (GA)15 and (GT)15. A total of 1243 clones gave a correct PCR amplification and about 44% (549) of the amplified inserts gave a signal by hybridization. These 549 fragments were sequenced and 448 sequences of good quality were exploited. From these latter, 440 positive ones (i.e. containing effectively a microsatellite sequence) showed an average total length of 378 bp. The sequence analysis and the primers design were performed using the SAT (Ssr Analysis Tool) pipeline of CIRAD. The alignment of the sequences identified 329 unique sequences, singleton (272) or consensus (57) of 2 to 8 homologous fragments (i.e. a redundancy rate of 38%). These unique sequences corresponded to 253 SSR loci of the type (GA)n with 19 (±7) repeats, 72 of the type (GT)n with 10 (±4) repeats, and 22 of tri- or tetra-nucleotide repeats PCR primer pairs were designed for a set of 192 SSR loci: 138 for the type (GA)n, 50 for the type (GT)n and 4 for other types. At the end of Year 1, two sets of 100 polymorphic SSR loci each were delivered to other Partners P2/P6 and P4/P5 for linkage mapping on the MP2 and MP3 crosses respectively. At the end of Year 2, Partner 3 had completed already its Task 3.1 with the development of a total of 307 functional SSR primers pairs which were all tested for their polymorphism on the six guava map parents (detection of polymorphisms, segregation in the respective mapping populations).

In Year 3, Partner 3 worked an additional set of 179 guava SSR sequences not exploited in Year 1 and 2, to increase the number of SSR loci capable to be mapped on MP1, MP2 and MP3. These sequences were analysed and the PCR primers were designed using the SAT (Ssr Analysis Tool) pipeline of CIRAD. Partner 3 checked the PCR functionality and screened the molecular polymorphism of the new 179 SSR primers pairs on the MP1, MP2, and MP3 parents. A total of 121 SSR primers pairs amplified correctly the genomic DNA in guava and are made available.

In summary at the end of the project, Partner 3 developed a total of 428 functional SSR primers pairs and characterized their polymorphism on all six guava map parents. The number of polymorphic SSR loci which can be mapped were equivalent on the crosses MP1, MP2 and MP3, with 253 (60%), 239 (56%) and 221 (52%) respectively. Developed SSR primers are made available to all project partners. **The Task 3.1 is fully completed.**

Task 3.2: Screening of polymorphic SSR loci for a general genetic diversity study

The Task 3.2 was completed by Partner 3 by the delivery of a set of 16 SSR loci (Table 3.2), which are optimal for the genetic diversity study of guava populations (WP1)

WP4: Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map

Task 4.1: Running SSR primers and scoring of alleles in the MP progenies

CIRAD received from Partner P1 a set of genomic DNA samples for 82 progenies of the MP1 (Enana x N6) mapping population. These DNA samples were checked for quality and concentration prior to their molecular analysis. In year 1, Partner 3 analysed the MP1 map parents and progenies using the selected polymorphic SSRs PCR products from SSR amplifications were revealed by Partner 3 according to Roy *et al.* (1996), using the automated infrared fluorescence technology of a Li-Cor IR2 sequencer (Lincoln, Nebraska).

Segregating data could be successfully scored for total of 242 SSR loci (for both Years 1, 2 and 3) were produced for linkage mapping of MP1. An additional set of 891 AFLP markers and of 1 Cosmid (COS) marker provided by Partner 1 (MPIZ) were also used to establish for linkage mapping of the MP1 cross. A SSR-based linkage map of the MP1 cross was constructed under JoinMap ver. 3.0 (van Ooijen and Voorrips, 2001) using the Kosambi function.

A set of 376 marker loci (153 SSRs, 222 AFLPs, 1 COS) were mapped onto 11 linkage groups cumulating a length of 1185 cM *i.e.* an average markers density of 3.6 cM. Additional isolated markers, pairs and small groups of more than 3 markers (totalling about 500 cM) are not be yet mapped and are not shown in that figure.

Task 4.3: Selection of an optimal Molecular Marker Set (MMS) for breeding

The Task 4.3 is completed by Partner 3 with:
- 16 optimal SSR marker loci delivered in Year 2 for genetic diversity analysis.
- 428 SSR marker loci delivered in Years 1, 2 and 3 for linkage mapping, including 253, 239 and 221 SSRs which are/can be mapped on MP1, MP2, MP3 respectively.

Tasks 7.1 and 7.2: Mapping of COS clones and of candidate genes

Partner 3 screened, on the MP1 parents by agarose gel analysis, the polymorphism revealed by 71 PCR primers pairs designed by Partner 1 from 71 COS clones selected in WP 5 and WP6. Only 1 COS locus was polymorphic.

Partner 3 selected that locus for MP1 linkage mapping in WP4. **The Tasks 7.1 and 7.2 are completed.**

Task 8.4: Marker-Assisted Selection plan for guava.

Partners 2 and P3 prepared recommendations for Marker-Assisted Selection (MAS) of guava fruit production, quality and yield stability, which will be part of a further publication to be submitted to the international scientific journal TAG or *Genome*.

Task D9.1: Dissemination and transfer of project results

- Updating of the project WEB page in link with Partner 2
- Final meeting and coordination visits to Partners P1 & P2

References:

Risterucci, A.M., Duval, M.F., Rohde, W. and Billotte, N. (2005). Isolation and characterization of microsatellite loci from *Psidium guajava* L. *Mol. Ecol. Notes* **5**: 745–748

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van Ooijen, J.W. and Voorrips, R.E. (2001). JOINMAP 3.0. Software for the calculation of genetic linkage maps. Plant Research International, Wageningen

II DELIVERABLES

CIRAD complied with all deliverables of the project (CIRAD deliverables in blue):

Del. no. [19]	Deliverable name	WP no.	Lead partici-pant	Estimated person-months	Nature [20]	Dissemination level [21]	Delivery date [22] and status (project month)
D3.1	Sequence information on SSR primers	3	P3	1	P	PP	12 (done) and additional delivery of 128 SSRs
D3.2	Polymorphisms of SSR primers	3	P3	2	P	PP	18 (done) and additional delivery for 128 SSRs
D4.1	MP1 Guava SSR reference linkage map	4	P2	9	P	PP	36 (done)
D4.3	Selection of an optimal Molecular Marker Set (MMS) for breeding	4	P2	1	P	PP	36 (done) for genetic diversity 36 (done) for linkage mapping
D7.1 **D7.2**	Mapping of COS clones and of candidate genes	7	P1	0.5	P	PP	33 done 36 done
D8.4	Marker-Assisted Selection plan for guava.	8	P2	1	P	PU	Done, diffused through next publication
D9.2	(D9.2) Execution of symposium / laboratory course on biotechnology and bioinformatics in Latin America	9	P1	1	R,O	PU	GuavaMap database updating 36, participation to 2nd ISHS International Symposium on *Psidium guava* and other *Myrtaceae*
D0	Year reports	9	P1	0.5	R		12, 24, 36, 42 (completed)

III PROBLEMS and DELAYS

All laboratory tasks were completed successfully by CIRAD until the end of the project.

[19] Deliverable numbers in order of delivery dates: D1 – Dn

[20] Nature of the deliverable using one of the following codes:

R = Report
P = Prototype
D = Demonstrator
O = Other

[21] Dissemination level using one of the following codes:

PU = Public
PP = Restricted to other programme participants (including the Commission Services).
RE = Restricted to a group specified by the consortium (including the Commission Services).
CO = Confidential, only for members of the consortium (including the Commission Services).

[22] Month in which the deliverables will be available. Month 1 marking the start of the project, and all delivery dates being relative to this start date.

IV TRAINING and SCIENTIST EXCHANGE

Year 1:
1. Dr. Billotte and Mr. Risterucci from CIRAD participated to the opening meeting of the GUAVAMAP project organized by Partner 1 in January 2006 at IIFT, La Habana, Cuba.

2. CIRAD corresponded frequently with partners P1 and P2 upon the production and linkage mapping of SSR and AFLP markers for the MP1 mapping population. It has been agreed that CIRAD, in charge of MP1 for SSR linkage mapping, will map (instead of Partner 2) the AFLP markers produced by Partner 1 on this mapping population MP1. This will speed up the SSR and AFLP linkage mapping for MP1.

3. An MsC thesis was produced at CIRAD under the EC project through the tasks 3.1, 3.2 and 4.1, by Mr. Nansot Gregoire, Ms.C. student (URF Sciences, University of Angers, France): "DEVELOPMENT OF MICROSATELLITE MARKERS AND LINKAGE MAPPING OF GUAVA (*Psidium guajava L.*)".

Year 2:
1. Dr. Billotte and Mr. Risterucci from CIRAD participated to the 2nd annual meeting of the GUAVAMAP project organized by Partner 1 in September 2007 at ULA, Merida, Venezuela.

2. A second MsC thesis was produced at CIRAD under the EC project through the tasks 3.1, 3.2 and 4.1, by Mr. Romain Grangeon, Ms.C. student (URF Sciences, University of Angers, France): "Development of microsatellite markers and SSR-AFLP linkage mapping of guava (*Psidium guajava L.*)".

Year 3:
1. Dr. Billotte and Mr. Risterucci from CIRAD participated to the 2nd ISHS International Symposium on *Psidium guajava* and other *Myrtaceae* meeting organized by Partner 1 the 10-12 November 2008 at Merida, Mexico. They made three oral presentations:
- N. Billotte: DNA markers for the analysis of tropical crop Plants
- N. Billotte: The microsatellite (SSR)/AFLP reference linkage map of guava
- A.-M. Risterucci: Development of microsatellite (SSR) markers using the SAT software

The two later presentations will be published in the ISHS *Acta Horticultura* Journal.

2. A third MsC thesis was produced at CIRAD under the EC project through the tasks 3.1, 3.2 and 4.1, by Mr. Vincent Lepitre, Ms.C. student (URF Sciences, University of Angers, France): "SSR-AFLP linkage mapping of guava (*Psidium guajava L.*)".

3. Dr. Billotte and Mr. Risterucci gave training courses at the CICY (Partner 4) laboratory, the 14-18 November 2008 at CICY, on:
- Development of microsatellite (SSR) markers using the SAT software
- Principles and practice of genetic mapping

- Molecular breeding for agronomic traits (example of the drought tolerance in rice)

V DISSEMINATION (publications, posters, presentations)

Data and results obtained by Partner 3 were transmitted to Partner 2 for updating the project homepage. All guava SSR markers and the guava SSR-AFLP reference linkage map of MP1 will be published in 2009 in a Scientific Journal such like TAG or TGG.

Improvement of guava: Linkage mapping and QTL analysis as a basis for marker-assisted selection (GUAVAMAP)

FP6-2003-INCO-DEV-2 No. 015111

Partner 4 CICY

Dr. Lorenzo Felipe Sánchez Teyer	CICY personnel	(scientist-in-charge)
Dr. J.Saul Padilla Ramirez	INIFAP personnel	(Collaborative Scientist)
Dra. Aileen I. O'Connor Sanchez	CICY personnel	(Collaborative Scientist)
MSc. Adriana Quiroz Moreno	CICY personnel	(Technician)
QFB. Miguiel Keb LLanes	CICY personnel	(Technician)
B. Felipe Barredo Pool	CICY personnel	(Technician)
M.C. Alejandra Barraza Morales	EC Funds	
M.Sc. Juan Luis Monrobot	EC Funds	(Technician)
Nina Durán Baeza	EC Funds	
Matilde Ortíz García	EC Funds	(Technician)
Karen Becerril Chi	EC Funds	(Technician)
Alejandra Nuñez Centeno	EC Funds	(Technician)
Dr. Jorge Rubio Piña	EC-Funds	(Technician)

FINAL REPORT (1.12.2005-31.5.2009)

The work package list for the CICY (in blue) was as follows:

Work package No[23]	Work package title	Ld contractor[24]	Person-months [25]	Start month[26]	End month[27]	Deliverable No[28]
WP1	**Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela**	**P5**	115	1	36	D1.1-D1.7
WP2	**Construction of individual AFLP-based guava linkage maps**	**P1**	110	1	36	D2.1-D2.3
WP3	**Generation and screening of microsatellite markers (SSRs) in guava**	**P3**	28	1	18	D3.1-D3.3
WP4	**Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map**	**P2**	64	19	36	D4.1-D4.3
WP5	**Construction of a guava COS library and development of SNP/INDEL markers**	**P1**	24	1	24	D5.1-D5.5
WP6	**Isolation and genetic diversity screening of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes)**	**P1**	24	1	24	D6.1 D6.2 D6.3
WP7	**Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map**	**P1**	51	25	36	D7.1-D7.2
WP8	**QTL analyses for the three guava mapping populations**	**P2**	41	1	36	D8.1 - D8.4
WP9	**Dissemination and transfer of project results**	**P1**	18	12	36	D9.1 - D9.2
	TOTAL		**475**			

1. To identify, collect, and characterize wild-growing guava accessions in Mexico both by agro-morphological descriptors as well as by DNA markers
2. To extract DNAs from the parents and from progenies of the mapping population MP3
3. To generate AFLP markers for the guava mapping population MP3
4. To map SSR and SNP/indel markers on the molecular linkage map(s) for mapping population MP3
5. To construct an SSR map for MP3
6. To map candidate genes in MP3-derived maps
7. To participate to QTL analysis in MP3
8. To organize the international symposium and laboratory course at the CICY (partner P4) in November 2008

[23] Work package number: WP 1 – WP 9.
[24] Contractor responsible for the work in this work package.
[25] The total number of person-months allocated to each work package.
[26] Relative start date for the work in the specific work packages, month 1 marking the start of the project, and all other start dates being relative to this start date.
[27] Relative end date, month 1 marking the start of the project, and all ends dates being relative to this start date.
[28] Deliverable number: Number for the deliverable(s)/result(s) mentioned in the work packages: D1 – D9.

I RESEARCH TASKS, ACTIONS and PROGRESS in the PROJECT

WP1: Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela

The origin of guava (*Psidium guajava* L.) is difficult to locate, however, most authors agree that this fruit crop was originated in the region from the Southeast of Mexico to North of Central America, best known as Mesoamerica, which has been recognized as center of origin and diversity of many species of economic importance (Ortega, *et al*. 2000; Hayes, 1953 cited by Dinesh and Iyer, 2005). At the world level, India is the country with the biggest area cultivated with guavas (150 thousand hectares) and total production of 1.8 million tons (Dinesh and Iyer, 2005). In Mexico, guava is one of the most important fruit crops with 23.9 thousand hectares and an annual production of 250 thousand tons, other important Latin American countries cultivating guava are: Brazil, Venezuela, Cuba, Colombia, etc. Guava is also an important fruit crop in many Asian countries. This fruit crop has gained relevance because its rusticity, heavy fruit load, and the high vitamin C content. The largest compact area producing guava in Mexico is located at the region called “Calvillo-Cañones” in the states of Aguascalientes and Zacatecas with about 50% of the total cultivated area, and this crop represent an active market being, at this days, around of 80 M USD, generating 48,800 direct employments. Most of the guava germplasm cultivated in this region is known as “media china” or “china”. However, several studies have shown that there is a great genetic and morphological variability within and among orchards (Perales, 1993; Padilla *et al*., 2003).

As a part of GUAVAMAP project, partner 4 must to collect and maintain a representative Mexican germoplasm by a germoplasm bank as well as to characterize both morphologically and genetically.

Task 1.1 and 1.3. Prospecting of Guava germoplasm and Gene Bank stablishment (*ex situ* conservation)

For this purposes the *P. guajava* germplasm collection maintained and morphologically characterized (figure 1) by INIFAP includes 113 accessions from 12 states of Mexico, collected from backyards, wild and cultivated areas. Major of this materials were planted during 2005 and 2006. A field trip was realized at the Estado de México during 2007, where about 15 materials were collected, and for Yucatan State on the southwest, collecting only 5 new accessions from backyard on 2009. Seeds of each material was obtained and stored to get plants representing that area of México.

Figure 1. Representative genotypes of Mexican Guava diversity collected and maintained on the *ex situ* germoplasm bank from INIFAP.

A group of guava selections from the Calvillo-Cañones region has been evaluated during the last years. Some of those were characterized and these studies identified contrasting materials with respect to fruit form, fruit size, pulpcolor, fruit yield potential and earliness from flowering to harvest (Padilla *et al*. 2005), thus, seven guava genotypes were chosen as parents to make crosses among them to generate different segregating populations expecting that segregating populations show contrasting characteristics to be analyzed using the QTL technique, which in turn may help to make marker assisted selection in the near future to improve production and quality of guava.

Task 1.5 Establishment of new mapping populations

The seven guava selections used as parents to make the crosses belong to the "*ex situ*" collection of INIFAP located at the Experimental Station of "Los Cañones" in the Zacatecas State (21° 44.7´ N latitude; 102° 58.0´ W longitude and 1508 masl).

During the second half of April 2006, several flower bunds were marked in the seven guava selections to monitor its development. In the first week of May, 2006 just before flower anthesis, buds of mother plants were carefully emasculated by removing the sepal and eliminating stamens leaving only the stigma. Emasculated flowers were covered using waxed bags. Flowers of pollen donor plants were covered early in the morning before they were visited

by bees to avoid contamination from other surrounding trees. After this stage fruits originated from the crosses were tagged and bagged until harvest.
Those fruits were harvested when the external color started to change from green to yellow, then weighted and seeds were obtained from each fruit. Seeds were counted and stored to be planted to generate the populations.
A total of 30 fruits were harvested from the crosses. According to the different combinations among the seven selections it will be possible to obtain up to five populations. It is important to mention that the period from pollination to harvest of guava fruits was different for each material. This period depended on the characteristic of the mother plant. Thus, fruits obtained at Sel-10 were the first (from 127 to 141 days) showing that this material has an early season.The average fruit weight and number of seeds in these two groups of fruits ranged from 27 to 31 g/fruit and from 81 to 138 seed/fruit, respectively. Fruits from Selections 54 and 56 had a period of 160 to 195 days from the day of pollination to harvest showing an intermediate cycle. Average fruit weight and number of seeds was 69 g and 141 seeds in Sel-54, while in Sel-56 were of 107 g and 234 seeds/fruit. Finally, Sel-51 showed the latest period for fruit development (196 to 205 days). However, fruit weight averaged only 56 g and seed number was 160 seed/fruit.
During February of 2007, seeds of guava fruits coming from the crosses were sowed in pots to germinate and to obtain seedlings of all crosses. After 2 months, seedlings of about 15 cm in height were transplanted to bigger plastic bags and collocated in a nursery having 50% shadow for its development.
Currently, a group of 20 F1 plants of each combination (a total of 100 plants) are approximately 1.0 m tall and they are maintained under field conditions.
Plants included in the field collection were pruned at the beginning of 2007 to promote the development of new sprouts and to have enough leaves to be sampled for the DNA analysis. Leaf samples were taken in June, 2007 by personnel of CICY for further processing and extraction of DNA at the biotechnology lab of CICY. A total of 79 plants from different origin were sampled for the DNA Analysis, plus another group of 11 materials were also sampled, including the parents used to make the crosses.
Leaf sampling for DNA extraction and analysis at the biotechnology lab of CICY, was performed in June of 2007. A total of 90 samples were taken, and these included 79 materials of the field collection and 11 materials of the guava selections from where the parents of the crosses were chosen. Study of the genetic diversity of these materials was conducted at CICY.

Task 1.4 Genetic divesity of Mexican guava using molecular markers

DNA extracted was completed from 68 accessions from prospected guava germoplasm shows high quality, quantity and integrity, including Enana and Belic. AFLP markers were applyied using 9 primer combinations and 6 SSR primers among Mexican Guava accessions. For AFLP markers, different number of bands and polymorphism detected was observed among primers, being a total of 335 bands considered for the analysis, showing an average of 81.8% of polymorphism and 37 bands per combination. Range of similarity index observed among regions varies from 0.46 to 0.90 considering all samples. The highest polymorphic level (98%) was observed using the combination AAC-CTT. Considering only Mexican individuals, samples from Michoacán showed the highest range of variation (0.63-0.88) in contrast with

Guanajuato whose range varies from 0.77-0.86. Only 45 common bands were detected among all samples but no specific bands corresponding to each region were observed, analysing only samples from each region separately.
Using 6 primers to amplify SSR regions a total of 79 allels, with an average of 13 allels per locus were observed, varying from 8 to 22 allels depending on the primer pair. Considering all samples, 64.4% of the allels showed heterocigocity.
UPGMA grouping analysis (figure 2), considering AFLP and SSR and including Enana, and Belic, showed two major groups. Group A includes 68% of individuals from all regions; in the case of samples from Zacatecas and San Luis Potosí only appear on this group. For Michoacán, 75% of samples belong to group A, whereas, 72% of samples from Jalisco were contained on group B. Samples remaining were present on both group A and B proportionally. Analyzing the profile of bands, according with dendrogram grouping, it was observed that difference among group A and B, in term of bands, correspond to absence of 13 bands in all samples contained on group B, and the presence of 5 bands only on samples from group B but absent on group A, even those plants from the same origin shows both genotypes.

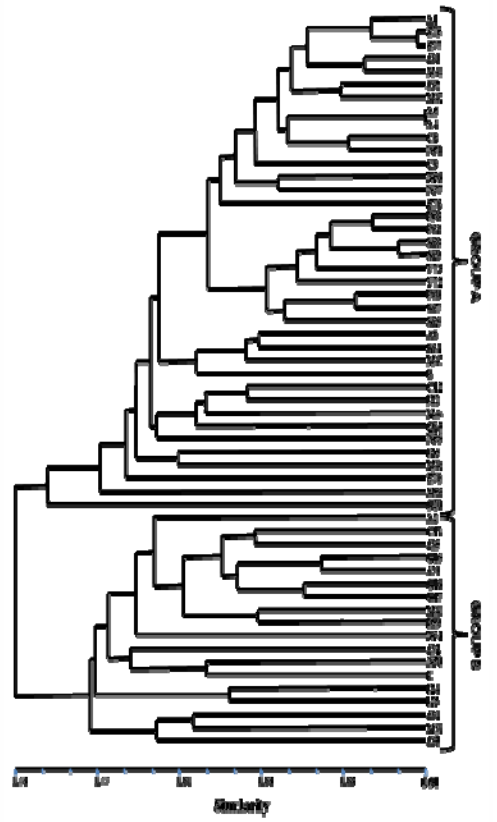

Figure 1. UPGMA dendrogram showing genetic diversity of Mexican guava using AFLP and SSR (Two major groups were observed but no correlation was obtained according with the origin). Letter correspond to origin (State of Mexico) A-Aguascalientes, C-Colima,, G-Guanajuato,, J-Jalisco, M-Michoacán, N-Nayarit, P-Puebla, Q-Queretaro, S-San Luis Potosí , V-Veracruz and Z-Zacatecas. E-Enana and B-Belic, correspond to reference samples used for population mapping purpose.

WP2: Construction of individual AFLP-based guava linkage maps (MP3).

In order to generate markers for mapping prurposes from MP3 guava, 79 segrenats and Enana and Belic (Parental material) were analyzed using AFLP and datas were send to partner 2 for integration onto the linkage mapping of the tree populations. The AFLP profiles were obtained following the protocol of Vos et al., 1995, starting with the preamplification reaction which was done in a final volume of 25 □l containing 37.5 ng of each primer, 2.5 mM MgCl2, 0.2 mM dNTPs, 0.6 U Taq-polimerase and 1 □l of DNA template. In order to verify the amplification reaction, fragments were firstly separated in a 1.5% agorose gel. A portion of the preamplified fragments were then diluted 20 times and used for the selective amplification using primers with 3 selective nucleotides. Reaction products were separated on 5% PAGE and DNA patterns were visualized by silver-staining with the MP3 mapping population, a total of 35 primer combinations were applied in order to obtain useful markers for mapping. This is short of the 48 pcs that is the cumulative number of opcs used in years 1 and 2 of the project. The number of useful markers for mapping derived from AFLP depended of the primer combination and is summarized in Table 1. The primer combination that showed the highest number of putative markers (31 useful bands) for linkage mapping was E57/M56. P4 has completely characterized 297 markers on the basis of size size by comparison to a standard DNA size ladder. In order to analyze gels and to select putative useful markers, different measures were applied:

- A complete area of gel was analyzed
- In those cases were two gels per combination, only non-ambiguos intensity were consider (regions with low intensity on a sigle gel were not consider on both)
- No monomorfic bands among segregant individuals were considered
- Polymorphic bands among segregants, were consider as a putative useful markers when those specific bands were present in less than 50% of individuals

Table 1: Summarize of results Number of bands (ptative markers useful for mapping) per primer combination used on this part of the analysis

Primer combination	Number of informative markers	Primer combination	Number of informative markers
E32/M56	**5**	**E39/M54**	**3**
E35/M52	**6**	**E39/M58**	**4**
E35/M55	**2**	**E40/M52**	**5**
E35/M57	**5**	**E40/M54**	**1**
E38/M57	**3**	**E42/M62**	**2**
E39/M52	**3**	**E56/M57**	**10**
E48/M55	**4**	**E56/M58**	**10**
E49/M55	**30**	**E57/M56**	**31**
E49/M57	**10**	**E57/M58**	**14**
E49/M58	**5**	**E58/M57**	**8**

E50/M55	**5**	**E59/M56**	**5**
E50/M56	**8**	**E59/M57**	**8**
E50/M57	**18**	**E59/M58**	**12**
E51/M57	**7**	**E60/M57**	**6**
E51/M58	**7**	**E61/M56**	**5**
E53/M58	**19**	**E61/M57**	**8**
E55/M57	**6**	**E62/M56**	**7**
E55/M58	**15**		
		Total number of putative markers	**297**

This task was fulfilled at the end of the second year by the sending of data to partner 2.

WP 4: Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map

Task 4.2: Running of 50 SSR pc´s on the guava population

In order to generate markers for mapping prurposes from MP3 guava, 79 segrenats and Enana and Belic (Parental material) were analyzed using SSR and datas were send to partner 2 for integration on the linkage mapping of the tree populations

Microsatellites loci were amplified in a final volume of 20 µl in a mixture containing 10 ng of genomic DNA, 10 pmol of each primer, 1 X PCR buffer, 200 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (invitrogen). Amplification program was 94ºC 3 min, 35 cycles of 94°C 45 s for denature, 55ºC during 1 min for annealing and 72ºC 30 s for extension and 7 min at 72ºC for final extension. Fragments were separated using capillary electrophoresis gel on the automatic sequencer ABI 310, and analyzed using GeneMarker software. On this task P4 run a total of 25 SSR primer combinations using automatic sequencer for all individuals from MP3 and Enana and Belic as a parental material. A binary matrix was sending to partner 2 to integrate into the linkage map. A putative 33 informative alleles were observed as shown on table 2.

PRIMER	SIZE REPORTED	ALLEL REPORTED	Enana	Belic	INFORMATIVE ALELE (IA)	SIZE (OBTAINED)
mPgCIR030	176	A				152
		B	1	0	B	181
mPgCIR032	100	A	1	1		85
		B	1	0	B	93
mPgCIR040	141	A	1	1		129
		B				134
mPgCIR044	243	A	1	1	A	247
mPgCIR048	112	A	1	1	A	94
		B	1	1	B	110
mPgCIR094	254	A	1	0	A	206
		B	1	1		227
mPgCIR091	125	A	1	1	A	103
		B				117
		C	1	1	C	133
mPgCIR099	220	A	1	0	A	197
		B				220
mPgCIR101	110	A				97
		B	0	1	B	107
		C	1	1		109
mPgCIR104	120	A	1	0	A	118
		B	1	1		1120
mPgCIR105	235	A	1	0	A	207
		B	1	1		239
		C				246
mPgCIR137	100	A	0	1	A	96
		B	1	1		100
mPgCIR139	207	A	1	0	A	213
		B	0	1	B	221
		C	1	0		224
		D	0	1		235
mPgCIR151	150	A	1	1	A	149
		B	1	1	B	158
mPgCIR160	116	A	1	1	A	106
		B				120
mPgCIR161	246	A	1	1		249
		B	1	0	B	262
mPgCIR165	124	A	1	1		122
mPgCIR175	104	A	1	0	A	86
		B	1	1		99
						145
mPgCIR 176	151	A	1	0	A	130
		B	1	1		151
						153
mPgCIR183	183	A	1	1	A	158
		B	1	1	B	181
mPgCIR186	224	A	1	0		210
		B	0	1	B	224

WP7. Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map

On this task P4 run a total of 25 primer combinations of COS, visualized on agarose gel were used to analyze 41 individuals from MP3 and Enana and Belic as a parental material.

COS-derived SNP/INDLE bridge markers loci were amplified in a final volume of 20 µl in a mixture containing 10 ng of genomic DNA, 10 pmol of each primer, 1 X PCR buffer, 200 mM dNTPs, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase (invitrogen). Amplification program was 94ºC 3 min, 35 cycles of 94°C 45 s for denature, 55ºC during 1 min for annealing and 72ºC 30 s for extension and 7 min at 72ºC for final extension. Fragments were separated 3% agarose electrophoresis gel and visualized by etidium bromide staining. A binary matrix was prepared considering presence or absence of each locus per segregant individual. Figure 3 shows a COS amplification in on several individuals of MP3.

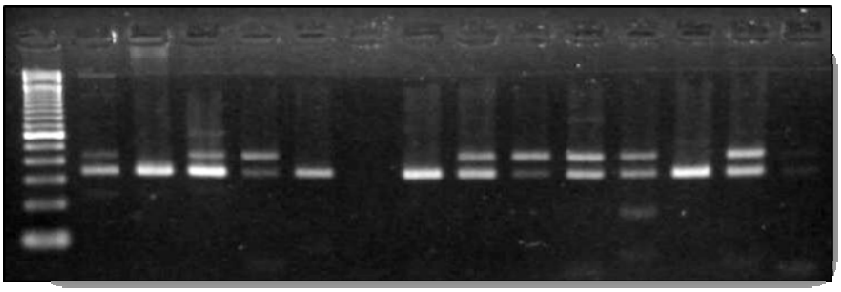

Figure 3. COS amplification on MP3 samples using primer GUAVA-COS 29. DNA ladder correspond to 1Kb.

This task was full filled. P4 run a total of 25 primer combinations of COS, visualized on agarose gel were used to analyze segregants MP3 and Enana and Belic as a parental material.

WP9. Dissemination and transfer of project results

Task 9.2 The CICY will organize the International Symposium at Merida and the following courses on Biotechnology and Bioinformatics. The subcontractor INIFAP (responsible scientist Dr. Saul Padilla) will organize the meeting at Aguascalientes.

This task was successfully fulfilled in Mérida; México on November 10-13, having 35 participants from 17 different counties, mainly academics. A total of 29 talks (including 7 invited speakers) and 13 research poster presentations were showed on a variety of topics related with guava and other *Myrtaceae*. For Aguascalientes satellite symposia on November 17-18 a higer number of participants were recorded, including academics, growers, and government people.

Biotechnology course was offered on November 17-28, 2008 for students from different countries being 11 participants, including 4 fellows (2 Mexican and 2 Cuban).

Bioinformatics course was on December 1-5, 2008 being 14 participants from different countries.

Figure 4. Pictures of each dissemination event.

IV TRAINING and SCIENTIST EXCHANGE

1. Dr. A. O'Connor participated to the opening meeting of the GUAVAMAP project organized by Partner 1 in January 2006 at IIFT, La Habana, Cuba.
2. CICY received Ms. Juliette Valdes-Infante (subcontractor S1; IIFT, Havana, Cuba) for a 2months period (October-November 2006) for AFLP analysis in MP3.
3. The coordinator started his work in the GUAVAMAP project in November 2006.
4. Cuban researchers (Dr. Nerdo Rodríguez, MSc. Juliette Valdéz-Infante, Grecia Montalvo and Maruchi Alonso) were received on Biotechnology Unit at CICY to exchange methodologies and experiences.
5. Dr. Narciso Nerdo Rodrígues from IIFT give a lecture for scientific community of CICY untitled: "Bases Metodológicas para el establecimiento de un programa de Mejoramiento Genético del Guayabo en Cuba".
6. CICY untitled: "Bases Metodológicas para el establecimiento de un programa de Mejoramiento Genético del Guayabo en Cuba".
7. Graduated Student: Pedro Francisco Pech Uc. Thesis dissertation. Instituto Tecnológico Superior de Calkiní. Campeche, Cam. México, 2008.

8. Training Steady on Molecular Markers. PhD. Jorge Rubio Piña. March-May, 2009

V DISSEMINATION (publications, posters, presentations)

Submitted papers:

- Sánchez-Teyer, L.F., Barraza-Morales, A., Keb Llanes, M., Barredo, F., Quiroz-Moreno, A., O´Connor-Sánchez, A. and Padilla-Ramírez, J.S. **Assessment of Genetic Diversity of Mexican Guava Germplasm Using DNA Molecular Markers**. Submitted to Acta Horticulture December, 2008.
- Ritter, E., Herrán, A., Ariz, U., Valdés-Infante, J., Rodriguez-Medina, N.N., Briceño, A., Fermin, G., Sanchez-Teyer, F., O'Connor-Sanchez, A., Muth, J., Boike, J., Santos, C.A., Nunes dos Santos, I.C., Rodrigues, M.A., Risterucci, A.M., Billotte, N., Becker, D. and Rohde, W. **"Comparative linkage mapping in three guava mapping populations and construction of an integrated reference map in guava"**. Submitted to Acta Horticulture December, 2008
- Sánchez-Teyer, L.F., Barraza-Morales, A., Quiroz-Moreno, A., Ortiz-García, M.M., Becerril-Chi K. and Padilla-Ramírez, J.S. **Genetic diversity of mexican guava germplasm evaluated by AFLP and SSR**. ISHS, Submitted to Acta Horticulture June, 2009

Oral presentations

- Sánchez-Teyer, L.F. Barraza-Morales, A. Keb L., Barredo, F., Quiroz-Moreno, A. Padilla-Ramírez, J.S., O´Connor-Sánchez, A. ASSESMENT OF GENETIC VARIABILITY ON MEXICAN GUAVA EVALUATED BY MOLECULAR MARKERS. Second International Symposium on Guava and other myrtacea. Mérida, Yucatán, México. November, 2008.
- Sánchez-Teyer, L.F., Barraza-Morales, A., Quiroz-Moreno, A., Ortiz-García, M.M., Becerril-Chi K. and Padilla-Ramírez, J.S. GENETIC DIVERSITY OF MEXICAN GUAVA GERMPLASM EVALUATED BY AFLP AND SSR. ISHS, Symposium on Molecular Markers in Horticulture. Oregon, USA, July-2009.
-

Poster presentations

- Sánchez-Teyer, L.F. Barraza-Morales, A. Keb L., Barredo, F., Quiroz-Moreno, A. Padilla-Ramírez, J.S. , O´Connor-Sánchez, A. ASSESMENT OF GENETIC VARIABILITY ON MEXICAN GUAVA EVALUATED BY MOLECULAR MARKERS. Second International Symposium on Guava and other myrtacea. Aguascalientes, Ags. México, November, 2008.
- Sánchez-Teyer, L.F. Barraza-Morales, A. Keb L. , Barredo, F., Quiroz-Moreno, A. Padilla-Ramírez, J.S. , O´Connor-Sánchez, A. EVALUACIÓN DE LA DIVERSIDAD GENÉTICA DE LAS GUAYABAS MEXICANAS EVALUADAS POR MARCADORES

MOLECULARES. XXVII Congreso Nacional de Bioquímica. Mérida, Yucatán November, 2008.

References:

Ortega P. R., Martínez A. M. A., y Sánchez G. J. J. 2000. Recursos Filogenéticos Autóctonos. *In*: Recursos Filogenéticos de México para la Alimentación y la Agricultura. Informe Nacional. Servicio de Inspección y Certificación de Semillas y Sociedad Mexicana de Fitogenética, A. C. P. **Ramírez V., R. Ortega P., A. López Alcocer H., F. Castillo G., M. Livera M., F. Rincón S. y F. Zavala G.** (eds). Chapingo, México.

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Improvement of guava: Linkage mapping and QTL analysis as a basis for marker-assisted selection (GUAVAMAP)

FP6-2003-INCO-DEV-2 No. 015111

Partner 5
ULA

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Final Report (1.12.2005 – 31.05.2009)

Summarized Final Report

Within the project, ULA participated in the following activities (all of them already completed. Some, however, will last in time as it will be explained afterwards):

A Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela, and thus, responsible with partners 4 and 6 for deliverables D1.1-D1.7 (tasks for WP1 will be described later):

Year 1:
D1.1 Leaf and fruit material for guava germplasm collected (Done)
D1.4 DNAs isolated from prospected guava germplasm (In progress: it's a continuous process but all samples have been already phenotypically, and many molecularly, analyzed. After GUAVAMAP this work will continue)

Year 2:
D1.1 Leaf and fruit material for guava germplasm collected (Done)
D1.2 UPOV descriptors for prospected germplasm (Done)
D1.3 List of fruit characters (Done)
D1.4 DNAs isolated from prospected guava germplasm (In progress: it's a continuous process but all samples have been already phenotypically, and many molecularly, analyzed. After GUAVAMAP this work will continue)
D1.7 Progenies from selected crosses established (In progress, particularly those by selfing. After GUAVAMAP this work will continue)

Year 3:
D1.5 Phylogenetic (actually, dendograms) trees established (Done)
D1.6 Nursery established (Done, but it's a continuous process. A replicate of the *ex situ* collection will be established at the Jardín Botánico de Mérida. Plants are being cleaned to be transferred to the Jardín Botánico in June 2009).

Year 4: DNA purified from 16 different species of Myrtaceae and used for SSR analysis as well as for sequencing diverse chloroplast genes.

B Contribute with polymorphic AFLP-markers derived from 24 pc's run per year, and whose responsible is partner 1.
Year 1:
Task 2.1 96-120 AFLP-markers derived from 24 pc's for MP3 (Done in 2007)

Year 2 (cumulative):
Task 2.1 192-240 AFLP-markers derived from 48 pc's for MP3 (Done with 2006´s)

Year 3 (cumulative):
Task 2.1 288-360 AFLP-markers derived from 72 pc's for MP3 (Finished in 2008)

C Contribute to the study of the potential polymorphism of 25 chosen SSR loci using primers developed by partner 3.

Year 2:
Task 3.2 Screening of polymorphic 25 SSR loci for a general genetic diversity study on 20 selected samples (Completed for 6 Venezuelan samples)

Year 3:
Task 3.2 Screening of polymorphic 25 SSR loci for a general genetic diversity study on 20 selected samples (Done for the Venezuelan samples AND also applied successfully to 9 different species of Myrtaceae)

Year 4: Applied to 16 different species of Myrtaceae

D Contribute to the integration of SSR markers into individual guava maps and the guava reference linkage map under the responsibility of partner 2.

Years 2 and 3:
Task 4.2 Run approximately 100 functional SSR primer pairs in MP3 in conjunction with partner 4 (Done).

Year 4: Additional primers were used ant this task was finalized in 2009.

E Contribute to the mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map, under the coordination of partner 1

Year 4:
Task 7.1 Mapping of COS clones in MP3 in conjunction with partner 4 (Completed in 2009)
Task 7.2 Mapping of candidate genes in MP3 in conjunction with partner 4 (Done in 2009)

I RESEARCH TASKS, ACTIONS and PROGRESS in the PROJECT

WP1: Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela

Task P5.1.1: Prospecting of guava germplasm and *in situ*, *ex situ* and *in vitro* conservation.

Most of the guava production in Venezuela is based on germplasm imported from Cuba and the Dominican Republic back in the forties. Since then little effort, if any, has been made in Venezuela to improve guava characteristics. Given this fact we concentrated our efforts in recovering and sampling guava samples from remote areas far from commercial orchards. The variability of Venezuelan guavas is astonishingly high, at least from the point of view of observable characteristics. Using the UPOV descriptors for guava from the UPOV organization, we designed a characters sheet where we recorded all characteristics pertinent to the description of guava trees and its leaves, fruits, etc. All sampled trees were registered by GPS and we recommended its preservation to the tree owners (if any). Most of the collected samples were from places where no commercial orchards were nearby. Samples were collected from 80% of Venezuelan states during the years 2005 and 2006. We added samples during the years 2007 and 2008 from the remaining states and only Nueva Esparta (an insular state) and Yaracuy have not been sampled. Special attention was paid to the presence of other myrtaceae close to guava trees sampled for this project. In this regard, our collection includes mostly guavas, but also other plant species that resemble guava or are interesting enough to enrich our collection. So far, we have collected samples from 90% of Venezuelan states.

2005 – 2006

Trips made to collect guava and guava-like samples during 2005 and 2006 were:

Trip One 2005: Southern Venezuela (mostly Gran sabana) in August 2005, where we traveled 4000 Km in a 10 days trip and we collected 28 different samples.

Trip Two 2006: Táchira state in February 2006 (500 Km), 1 day, for 5 samples.

Trip Three 2006: Western Venezuela in March 2006, traveling 2000 Km during 4 days to collect 15 samples.

Trip Four 2006: The most difficult trip was to Amazonas state (Orinoco river) in June 2006, where we traveled >2736 Km (not including the distance covered during four days in the river), during 9 days, and where we collected 45 samples.

Trip Five 2006: Eastern Venezuela during August 2006; 3700 Km were traveled during 11 days that allowed us to collect 34 samples.

2007 - 2008

We finished collecting samples from Venezuela remote areas with the purpose of completing a catalogue of samples coming from the whole country.

Trips made to collect guava and guava-like samples during the year 2008 were as follows:
Trip One 2007: Remote areas from Mérida to Barinas in a 5 days trip walking through an altitudinal gradient that allowed us to cover from 4000 masl to 150 masl.
Trip Two 2007: Táchira state, 2 days.
Trip Three 2007: Some areas non covered before in Mérida state.
Trip Four 2007: Visiting the states of central Venezuela, including Aragua, Carabobo and Yaracuy.
Trip Five 2008: Performed on December covering from Mérida to Zulia sate in very remotes areas that will allow us to sample guavas in cloudy forests not sampled before.

During sample collection we estimated pH, soluble solids, leaves measurements, fruit characteristics, and many others. Once in Mérida, seeds were processed and germinated; some were used for *in vitro* preservation, and herbarium samples were mounted following standard procedures. Finally, some germinated plants have been moved to the guava nursery in Lagunillas (30 min. far from Mérida city).

Briefly, we have collected, until October 2008, 158 samples that have been GPS-recorded, characterized by UPOV descriptors, and each one of them has a herbarium sample. All plants have been or are in the process of germination, while others have been already propagated *in vitro* and/or preserved in the field. Besides that, we have a collection of seeds for all samples.

Task P5.1.2: Fruit characters

For all samples fruits were collected and the following measurements were recorded: diameter, length, color of the cover, color of the flesh, pH, texture, shape and shape uniformity, number of loculi, ^{o}Brix, number of seeds, and the ratio between different measurements. Other data gathered included botanical features of the vegetative part of the plant, ethnobotanical uses of the plant and its parts, data on the area of origin and, when possible, observations about the true origin of the plant according to theirs owners, if any.

Task P5.1.3: Gene bank establishment (*ex situ* conservation)

Some samples have been already brought to the field (San Juan de Lagunillas, Coordinates: N08º31'53'', W71º21'14''), while some others are in the greenhouse or in plastic bags following germination. Some samples have also been propagated *in vitro* for another way of *ex situ* conservation. For this purpose a novel method of regeneration has been successfully assayed (I. Contreras, J. Almeida and C. Valecillos, unpublished).

Currently, the collection comprises 158 samples, of which 101 samples are *P. Guajava* and 57 samples are other species of Myrtaceae. Of the latter,

at least 20 samples have already been identified. This collection includes herbarium vouchers, seeds, DNA samples, and information concerning the origin of the trees. The seeds were aseptically germinated and acclimated in a greenhouse before being transferred to the field. The ex situ collection comprises 50 different samples of *P. Guajava* and 4 samples of other Myrtaceae. Currently the plants are approximately two years old and in a transition to the reproductive stage. The plants established in the field were used to extract nucleic acids for the DNA collection. The phenotypic traits were evaluated and compared with those of the donor plant. Some of the plants exhibiting a promising phenotype were cultivated *in vitro*, using apical and axial meristems as well as nodal segments.

Task P5.1.4: DNA isolation and Biodiversity analysis

We collected guava landraces in an altitude gradient ranging from 0 to 2000 m a.s.l., in a variety of ecosystems, the vast majority of them associated with human settlements, even in the cases of low density. The analyses of 100 samples collected, and evaluated employing phenotypic markers, revealed an elevated degree of polymorphism. The most informative markers, based on degree of variability exhibited, were those related to fruit characters: number of seeds and color of the skin and the flesh. The analyses also reveals a distribution pattern apparently related with certain regions of the country that show more abundance and/or more diversity of phenotypes, which might be related to the reproductive biology of *P. Guajava* (selfing or outcrossing), its biotic and environmental interactions, and anthropic factors. The observed and collected guavas and their broad variability constitute a valuable germplasm resource for the development of genetic improvement programs. Likewise, the variety of phenotypes and their geographical distribution suggest that the north of South America represents the centre of origin of guava; however, this finding requires confirmation through a detailed and comprehensive phylogenetic analysis employing traditional molecular biology tools.

To check for the best primers for biodiversity analysis (see TaskP5.3.2 below), our team, following a matrix of characteristics of the plants already in the field chose 6 samples to assay the SSR primers fo biodiversity. One of the criteria used was origin of the seed donor; i.e., from places of very different ecosystems.

Task P5.1.5: Establishment of new mapping populations

Although the nursery was established in the year 2006, only during the year 2008 were we able to detect plants already flowering. Due to climate change, at least in Venezuela, the rainy season has been longer than expected. In fact, it is still rainy season, when in general, by this time of the year it should be summer, or second dry season. This condition has precluded, we believe, a more extended emergence of flowers.

WP2: Construction of individual AFLP-based guava linkage map

Task P5.2.2: generation of AFLP markers for MP3

In January-February 2006 a first meeting between representatives of all countries involved in the project was held at Havana city, Cuba. Among other things, we received comprehensive information on the financial management of the resources allocated per country to accomplish our scientific goals, besides the primers for the advancement of the guava AFLP-based map.

Once we acquired the equipment for the AFLP analysis we standardized the conditions of first and second PCR. When trying a new pair of primers, amplification products from 10 different samples were run in small gels; if polymorphism was detected a new amplification was performed with all samples in a longer gel (Figure P5.4). So far, we have thoroughly analyzed three pair of primers that detect a high number of polymorphic loci. Some others, more than five, seems not to detect any polymorphism. This task was started in July 2006 since we had to wait for equipment and reagents. We estimate we can produce 10 more informative gels before the end of 2006.

In the year 2007 we analyzed MP3 by AFLP using many different combination of primers and produce the data (48 pcs) for the consolidate years of 2006 and 2007. In the year 2008 we finished the due AFLP analysis of MP3 and all results were sent to Dr. Ritter.

WP3: generation and screening of microsatellite markers (SSRs) in guava

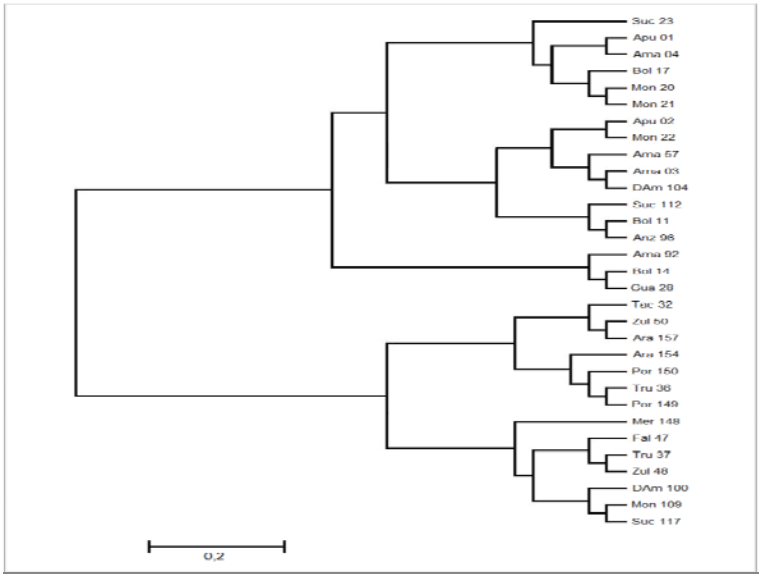
TaskP5.3.2: Screening of polymorphic SSR loci for a general genetic diversity study

From an initial list of more than 100 primers, we selected 25 to assay them in terms of revealing polymorphism for biodiversity analysis. The list of the loci we tried is as shown in Table 2. Primers for each locus were grouped in terms of annealing temperature, and the samples assayed were those mentioned before.

In order to perform the biodiversity analysis, members of the consortium agreed on the II GUAVAMAP meeting held at Mérida, Venezuela in September 2007 that only the 16 most informative primers would be used by all partners with 9 samples from Mexico, Venezuela and Brazil. Venezuela provided DNA from 9 samples to the partners from Mexico and France (Brazil), but only received DNA samples from Mexico (November 14, 2007). However, the DNA received from Mexico was not amenable to perform any kind of experiments due to a very bad quality of the samples. Briefly, the only country that provided DNA for biodieveristy analysis to other members of the consortium was Venezuela.

In the present research, we evaluated the genotypic variability of Venezuelan guava landraces using SSR markers. To attain this goal, 31 guava accessions from different regions of Venezuela were selected, which now belong to a recently established *ex situ* germplasm facility of *Myrtaceae* at Universidad de Los Andes. All samples were evaluated for 16 microsatellite

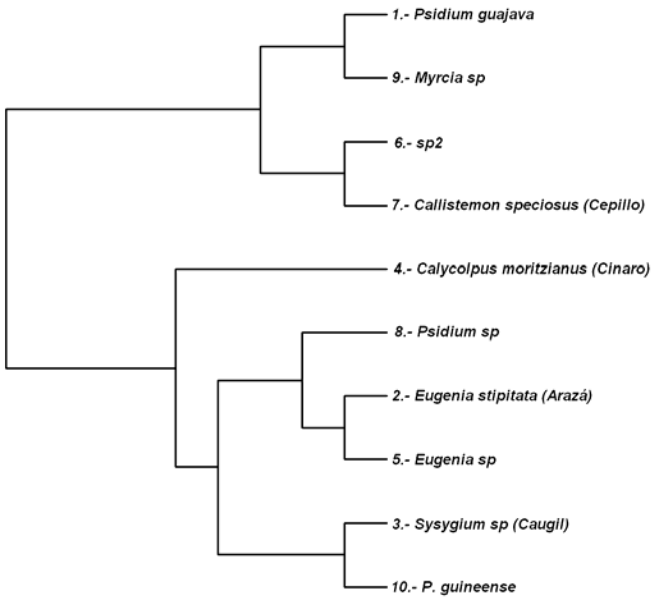
loci, using primers specific for guava. All evaluated loci were highly polymorphic, observing up to 100% of polymorphism and detecting several alleles per locus. These results reveal the great genetic diversity of the natural population of *P. Guajava* in Venezuela, which seems to be associated with its geographical distribution. Finally, these microsatellites allowed us to characterize genetically the accessions of the germplasm bank, which in the future will enable the monitoring, genotyping and selection of specific individuals during the development of coherent programs of genetic improvement.



SSR-derived dendogram of wild Venezuelan guavas according to Nei's similarity using UPGMA (Genetic Data Analysis).

We also tested the primers on a selection of different myrtaceae collected in our trips. The plant samples used in this work were collected in two Venezuelan sharply different and geographically isolated ecosystems. One of these ecosystems is a mountainous region in the Venezuelan Andes, in the state of Mérida, and the other, is located south to the Orinoco River, situated in state of Amazonas, characterized by typical rain forest vegetation. Both regions are ecosystems rich in Myrtaceae species, some of which are shared by both regions, whilst others are exclusive of one of these. Besides the morphological observations, an analysis with more than 16 pairs of guava-derived SSR primers was carried out. All primer combinations were assessed in terms of efficiency of amplification, number of loci, size of the amplification products and degree of polymorphism. Due to the geographical distance between the sampled regions and our research lab, particularly those proceeding from Amazonas, and the low germination efficiency of most of the collected seeds, dry leaf sampled were used as a source of DNA. Successful

amplifications of informative loci validate the transfer of most of the guava-derived SSR primers to other *Myrtaceae* species. This work, however, will be continued to validate our findings including the analysis of other sequences and molecular markers.



Dendogram constructed employing UPGMA. The distances and genetic diversities were estimated with Nei 1978 (Genetic Data Analyses).

WP4: Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map
TaskP5.4.1: running SSR primers and scoring of alleles in the MP progenies

This part of the work was completely done during the year 2008 and all results were sent to Dr. Ritter.

Improvement of guava: **linkage mapping and QTL analysis as a basis for marker-assisted selection (GUAVAMAP)**

FP6-2003-INCO-DEV-2 No. 015111

Partner 6
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Hugo Leonardo Coelho Ribeiro	(CPATSA)
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FINAL REPORT (Dec. 1, 2005 – May 31, 2009)

The work package list for CPATSA (in blue) was as follows:

Work package No[29]	Work package title	Ld contractor[30]	Person-months[31]	Start month[32]	End month[33]	Deliverable No[34]
WP1	**Biodiversity analysis of wild-growing guava in Mexico, Brazil and Venezuela**	**P5**	115	1	36	D1.1-D1.7
WP2	**Construction of individual AFLP-based guava linkage maps**	**P1**	110	1	36	D2.1-D2.3
WP3	**Generation and screening of microsatellite markers (SSRs) in guava**	**P3**	28	1	18	D3.1-D3.3
WP4	**Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map**	**P2**	64	19	36	D4.1-D4.3
WP5	**Construction of a guava COS library and development of SNP/INDEL markers**	**P1**	24	1	24	D5.1-D5.5
WP6	**Isolation and genetic diversity screening of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes)**	**P1**	24	1	24	D6.1 D6.2 D6.3
WP7	**Mapping of COS-derived SNP/INDEL bridge markers, COS clones and candidate genes onto the guava linkage map**	**P1**	51	25	36	D7.1-D7.2
WP8	**QTL analyses for the three guava mapping populations**	**P2**	41	1	36	D8.1 - D8.4
WP9	**Dissemination and transfer of project results**	**P1**	18	12	36	D9.1 - D9.2
	TOTAL		**475**			

Within the project, CPATSA carried out the following tasks:

- To identify, collect, and characterize wild-growing guava accessions in Brazil both by agromorphological descriptors as well as by DNA markers
- To generate AFLP markers for the guava mapping population MP2
- To map SSR and SNP/indel markers on the molecular linkage map(s) for mapping population MP2

[29] Work package number: WP 1 – WP 9.
[30] Contractor responsible for the work in this work package.
[31] The total number of person-months allocated to each work package.
[32] Relative start date for the work in the specific work packages, month 1 marking the start of the project, and all other start dates being relative to this start date.
[33] Relative end date, month 1 marking the start of the project, and all ends dates being relative to this start date.
[34] Deliverable number: Number for the deliverable(s)/result(s) mentioned in the work packages: D1 – D9.

- To organize the second project meeting in August/September 2007 in Brazil
- To participate to the international symposium and laboratory course at the CICY (partner P4) in November 2008

I RESEARCH TASKS, ACTIONS and PROGRESS in the PROJECT

Brazil guava production was 408,283 ton harvested in 18,826 ha in 2002. São Paulo, Pernambuco and Bahia States were responsible for 70% of total production. The São Francisco river valley (SFV), located within the Brazil Northeast, is responsible for 38% of Brazilian production, harvested in 4,263 ha, with a yield of 36.3 ton/ha. Ninety-nine percent of Brazilian guava production is for domestic market, mainly for fresh consumption. Small farmers are the principal growers in the SFV. In the SFV is possible to produce guava with high quality around the year adjusting pruning and irrigation.

The previous guava CPATSA germplasm collection had 22 accessions vegetatively propagated from selected accessions of a collection established by the Pernambuco State Agricultural Research Organization (IPA). The IPA collection was established by seed from many primary introductions from overseas, mainly from the United States. This collection was destroyed by the root-knot nematodes.

Embrapa Semi-Arid (CPATSA) unit is the principal government owned organization within the SFV responsible for conducting agricultural researches with tropical fruits, among of them guava. To guava the main research challenge is to defeat the devastating root knot nematode, which has reduced the guava area in 60%, with a high socio economic impact in the region.

The GuavaMap project was important to establish a *Psidium* germplasm collection from the entire country, in order to preserve the pool gene of guava and aracazeiros and also to characterize for agro-morphological traits and molecular markers. Previous guava collection was not representative of the guava Brazilian diversity because most of the accessions were introduced from the United States. The GuvaMap project was also important to CPATSA to screen for nematode tolerance and some promising genotypes were identified among the guava and aracazeiros accessions. The project was also very important to establish a laboratory to work with molecular markers, mainly AFLP and SSR that will be very important to perform analyses in other plant species and also to train students. It also have to be mentioned that the project was important to CPATSA to participate of the network of guava established in the 2nd international symposium on guava and other Myrtaceae, Merida, Mexico, and also to have access some modern techniques and strategies to apply molecular markers as a tool to develop new fruit cultivars.

WP1:
Biodiversity analysis of wild-growing guava in Brazil

Task 1.1. Prospecting of guava germplasm and *in situ/ex situ* conservation

Material and Methods

***Psidium* germplasm sampling** - prospecting of wild-growing *Psidium* germplasm species was carried out in different eco-geographical zoning regions (EGZRs) of 10 Brazilian States (Fig. 1). An EGZR was defined as a region that has the same vegetation type, soil and altitude, since it was assumed that *Psidium* adaptation might be influenced by these factors. Within a given EGZR the least developed rural population was sampled in order to avoid the prospecting of recently introduced guava germplasm. For the same reasons, sampling was avoided – if possible - within urban areas. For each individual plant *in situ* pictures were taken and GPS geographic coordinates and physical reference sites were also recorded.

***Psidium* germplasm characterization** – guava and araçá plants identified in different ecoregions were characterized for one, 20, 17 and two descriptors of stem, leaves, fruits and seeds, respectively, according to the Protection of New Varieties of Plants - UPOV guidelines.

Relationship between ecogeographic sampling and phenotypic diversity of Brazilian *Psidium* germplasm was carried out based on 35 descriptors of a total 57 UPOV descriptors. Simple percentages were calculated for the applied UPOV guava descriptors, while for others traits, such as leaf and fruit length, leaf and fruit width and spacing of veins of leaf, prior measurements were taken before grouping into a specific descriptor class.

The simple matching coefficient was applied to compute a similarity genetic matrix among the *Psidium* accessions, using the NTSYS software or the proc Distance. The similarity matrix was then used to build a multidimensional scaling graphic and also to build a phenogram tree, according the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method, available in NTSYS software.

Results and Discussion

Ecogeographic region and *Psidium* characterization - Thirty-one ecogeographic regions were sampled in 10 Brazilian States, including Maranhão (MA), Sergipe (SE), Pernambuco (PE), Piauí (PI), Bahia (BA), Rio Grande do Sul (RS), Goias (GO), Rondonia (RO), Amazonas (AM) and Roraima (RR) States (Fig. 1). To date, these *Psidium* germplasm expeditions are the most broadest that have occurred in Brazil and perhaps in countries of the similar extension in the world. A hundred and nine accessions of guava and 34 accessions of araçá from 31 different ecoregions were characterized for 35 UPOV descriptors.

A large majority of araçá accessions presented leaf veins of wide spacing, contrasting with the guava accessions that presented medium to close spacing. Most fruits of araçá accessions were classified as small, while most fruits of guava accessions were grouped into the class of medium. For the flesh fruit color, 91% of araçá were grouped as cream and white, while 58% of guava accessions presented pale pink, pink and dark pink colors. These fruit differences among wild *Psidium* species and guava suggested that the fruit traits have been the most altered trait by artificial selection.

The ecoregions prospected in the Amazonas and Roraima States are located in the upper northern part of South America, where *P. guajava* is

native. Some ecoregions of the Brazil Northeast States were located in the least developed regions of the country, where very ancient *Psidium* accessions have been kept and propagated by slave descents or long-established rural communities.

Clusters of araçá were observed according to the sampled ecogeographic region, as observed for araçá accessions of Bahia (Camamu), Rio Grande do Sul (Pelotas), Goias (Colinas and Goias) and Pernambuco (Escada) (Fig.2). The phenogram tree and MDS graphics presented clusters of guava accession according to some Brazilian States, as observed for accessions sampled in Rondonia (RO), Maranhão (MA), Amazonas (AM) and Goias (GO), but it was not observed clustering by ecogeographic region (Fig.2).

It is expected that capturing rare alleles and enhancement of genetic variability of *Psidium* germplasm could be achieved by sampling accessions according to predefined geographical regions, even to guava germplasm.

WP1:
Biodiversity analysis of wild-growing guava in Brazil

Task 1.3 and 1.2 - Gene Bank establishment and analysis of fruit characters.

Material and Methods

Each sampled accession was propagated by placing 4-5 seeds in a small plastic bag, spraying water each hour. Germination took around 30 days for guava and 45 days for “Araçá”. Six individuals were established at field per *Psidium* accessions, considering spacing of 4.0 m x 4.0 m. Dripping irrigation was adopted not only to irrigate the plants but also to apply fertilizers. All field practices, such as weed control, pruning and pesticide spraying, was done according to local commercial practices.

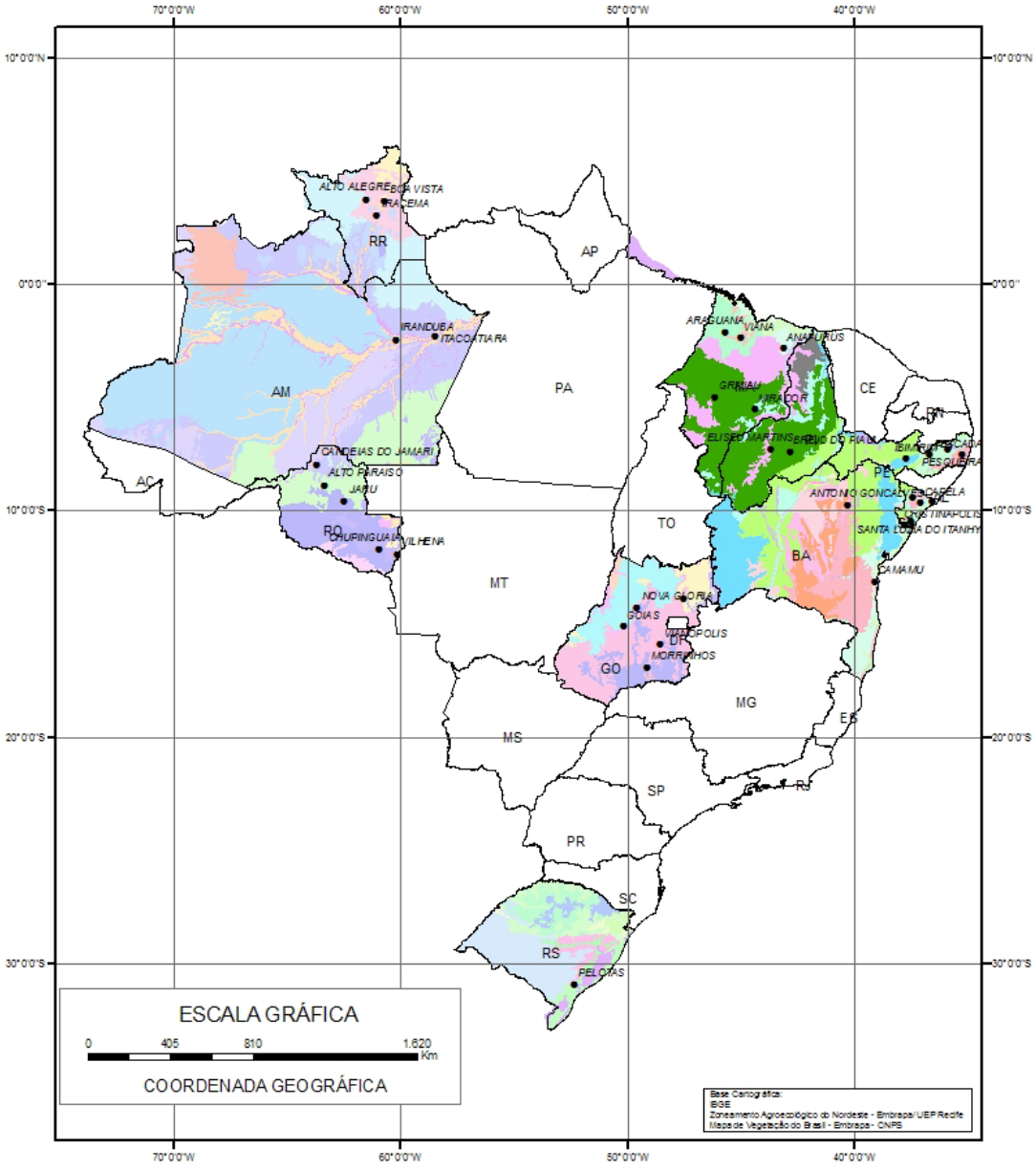

Figure 1. Ecogeographic *Psidium* sampling areas in 10 Brazilian States with definition based on Northeast eco-geographical zoning map (MA, BA, SE, PE and PI) and vegetation maps (RS, GO, RO, AM and RR). Reference cities around an ecoregion were established to facilitate the prospecting activities.

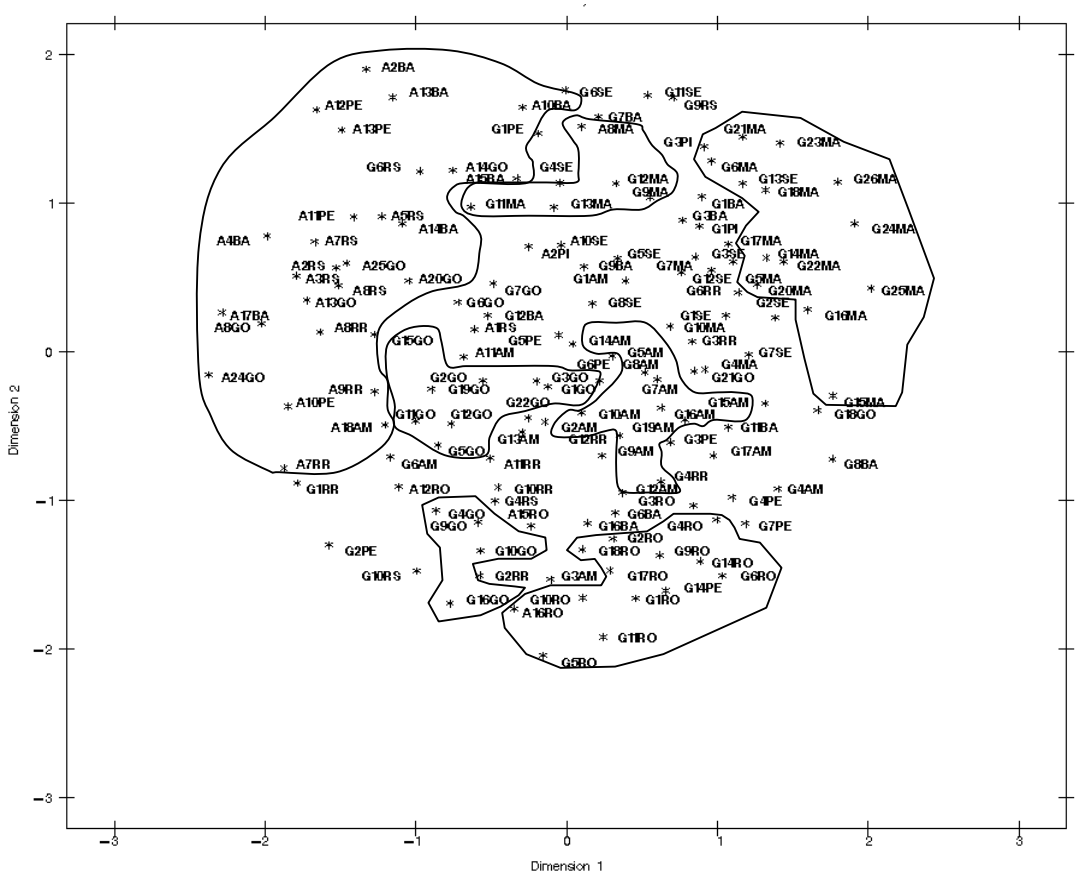

Figure 2. Multidimensional scaling of simple matching coefficient for 143 *Psidium* germplasm (A=araçá – *Psidium* spp., G=guava – *P. guajava*) sampled in different ecogeographic regions of 10 Brazilian States and characterized for 35 categorical UPOV descriptors.

Inflorescence of six individual per accessions were protected with paper bag, before the anthesis to enforce selfing, in order to increase seeds (collected seeds of some accessions are not available to future works), keeping the gene pool of the maternal plant.

Fruits of the first set of the germplasm bank (a hundred-one accessions established in field in February 2007 - total of 61 and July 2007 - total of 40) will be characterized for Vitamin C, total carotenoids, lycopene, minerals (Zn, Fe, Ca and Mg), anti-oxidant activities, total dissolved sugar content and total proteins.

Results

A hundred-fifty-six *Psidium* accessions sampled in ten Brazilian States (Maranhão, Piaui, Sergipe, Pernambuco, Bahia, Rondonia, Amazon, Rio Grande do Sul, Roraima and Goias) were established at Embrapa Experimental Agricultural Station, Petrolina, PE (Fig. 3). Damaged plants, by the nematode *Meloidogyne mayaguensis*, have already been observed in the field. It is expected that an expressive number of accessions will be destroyed

by the nematode. Attempts to identify *Psidium* tolerant accessions are underway to be used as rootstock of the entire germplasm collection. Selfed seeds were harvested to most of *Psidium* accessions what will make possible to re-install the collection in the field or to exchange germsplam. Selfed seeds will be produced to all accessions in 2009.

Preliminary results from fruit characterization have presented the following values: vitamin C – from 409.8 to 20.2 mg/100 g of fresh pulp; proteins – from 0.90 to 0.16%, sugar – from 13.6 to 3.46%, acidity from 1.53 to 0.19% and solids soluble – from 16.25 to 6.8°Brix. Analysis for total carotenoids, lycopene, minerals (Zn, Fe, Ca and Mg) and anti-oxidant activities will be performed in the *Psidium* collection as part of a doctoral thesis.

Figure 3. Growing accessions of the *Psidium* germplasm bank established at the Agricultural Experimental Station of Bebedouro of Embrapa Semi Arid, Petrolina, PE. A: Set 1, B: Set 2. October, 2008.

WP1:
Biodiversity analysis of wild-growing guava in Brazil
Task 1.4 – Biodiversity analysis
Task 1.4.1 DNA Extraction

Material and Methods

Plant material – leaves of three individual of each *Psidium* accessions were collected from the germplasm *Psidium* collection to extract DNA since the maternal DNA samples were not enough and some frozen leaves of maternal plants were damaged by successive thawing.

DNA extraction – leaves of all *Psidium* germplasm bank were frozen with liquid nitrogen and pulverized with a mortar and pestle. DNA was extracted from leaf material by using a (2x) cetyltrimethylammonium bromide (CTAB) extraction protocol of Doyle and Doyle modified to: 6,000 and 10,000 rpm in the first and second centrifugation, respectively; 2.0% beta-mercaptoethanol, and, incubation at 60 ^{o}C for 30 min for all samples. After addition of Tris-EDTA buffer, the DNA solution was treated with RNASE to remove co-isolated RNAs. DNA quantification and integrity was checked in a 0.8% agarose gel, followed by storage at -20 ^{o}C.

Results

It was not observed degraded DNA for the 156 *Psidium* accessions and the samples were diluted to 40 ng/µL and storage at -20 ^{o}C.

WP1:
Biodiversity analysis of wild-growing guava in Brazil
Task 1.4 – Biodiversity analysis
Task 1.4.2 AFLP application and dendogram construction with NTSYS

Material and Methods

Plant material and DNA extraction – as described in section WP1, Task 1.4.1
AFLP reaction and data analysis – Around 200 ng de DNA of each accession was doubled digested in a thermocycler with 0.65 units of *EcoRI* and *MseI*, for 4.0 h, according to the manufacturer temperature recommendations. Ligations of adapters were done in thermocycler conditions with 1.25 units of *T4* DNA Ligase. The pre-amplification reaction was done according to the original AFLP protocol, to a final volume of 15 µL (1.5 µM of *EcoRI* primer, 1.5 µM of *MseI* primer, 0.2 mM of dNTPs, 1x PCR buffer, 2.5 mM of $MgCl_2$, 0.5 units de *Taq* DNA Polimerase and 2 µL of the diluted DNA ligation. The amplification reaction and silver-staining were done as described in section WP2, Task 2.2.

The Jaccard similarity distance was adopted to estimate the distance for each pair of accession individual. The matrix of similarity was then used to build a dendogram tree, according the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method, available in NTSYS software.

Results

A total of 195 AFLP fragments were obtained from fifteen *EcoR1*/*Mse1* primers combination (PC), averaging 13 marker/PC. The co-phenetic correlation was 0.96, indicating that the built dendogram (Fig. 4) was a good representation of the data.

It was observed four clusters of aracazeiro suggesting 1) adaptation to specific ecogeographic region and 2) different species because aracazeiro clusters were separated in the dendogram.

WP1:
Biodiversity analysis of wild-growing guava in Brazil
Task 1.4 – Biodiversity analysis
Task 1.4.3 SSR application and dendogram construction with NTSYS

Material and Methods

Plant material – Genotyping 1: A hundred-ten guava (75) and aracazeiros (35) accessions collected in ten Brazilian States were genotyped with the sixteen SSR makers. All accessions were genotype in two different PAGE glass plates. Genotyping 2: Fifty-two guava and one aracazeiro accessions were chosen to be genotyped with SSR markers, according to the following number by Brazilian States: MA – 07, PI – 03, PE – 6, SE – 6, RO – 5, AM – 6, RS – 4,

GO – 5, RR – 5, and BA – 4. The aracazeiro accession was from BA. The cultivar Paluma was also included.

DNA extraction – as described in section WP1, Task 1.4.1

SSR reaction and data analysis: The PCR conditions were done as recommended by Risterucci et al. (Molecular ecology notes, 2005, v. 5, p. 745-748) with the following modifications: Annealing temperature at 56^{o}C or 60^{o}C, 2.5 mM of $MgCl_2$ and final volume of 10 µL. Total DNA was diluted to10 ng/µL. PAGE and silver-staining was performed as described in section WP2, task 2.2. The 16 polymorphic SSR loci (mPgCIR227, mPgCIR228, mPgCIR229, mPgCIR233, mPgCIR236, mPgCIR242, mPgCIR243, mPgCIR246, mPgCIR247, mPgCIR249, mPgCIR251, mPgCIR252, mPgCIR253, mPgCIR255, mPgCIR256, mPgCIR257) defined to be used by all GuavaMap partners were used according to the described PCR reactions.

The Jaccard similarity distance was adopted to estimate the distance for each pair of accession individual. The matrix of similarity was then used to build a dendogram tree, according the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method, available in NTSYS software

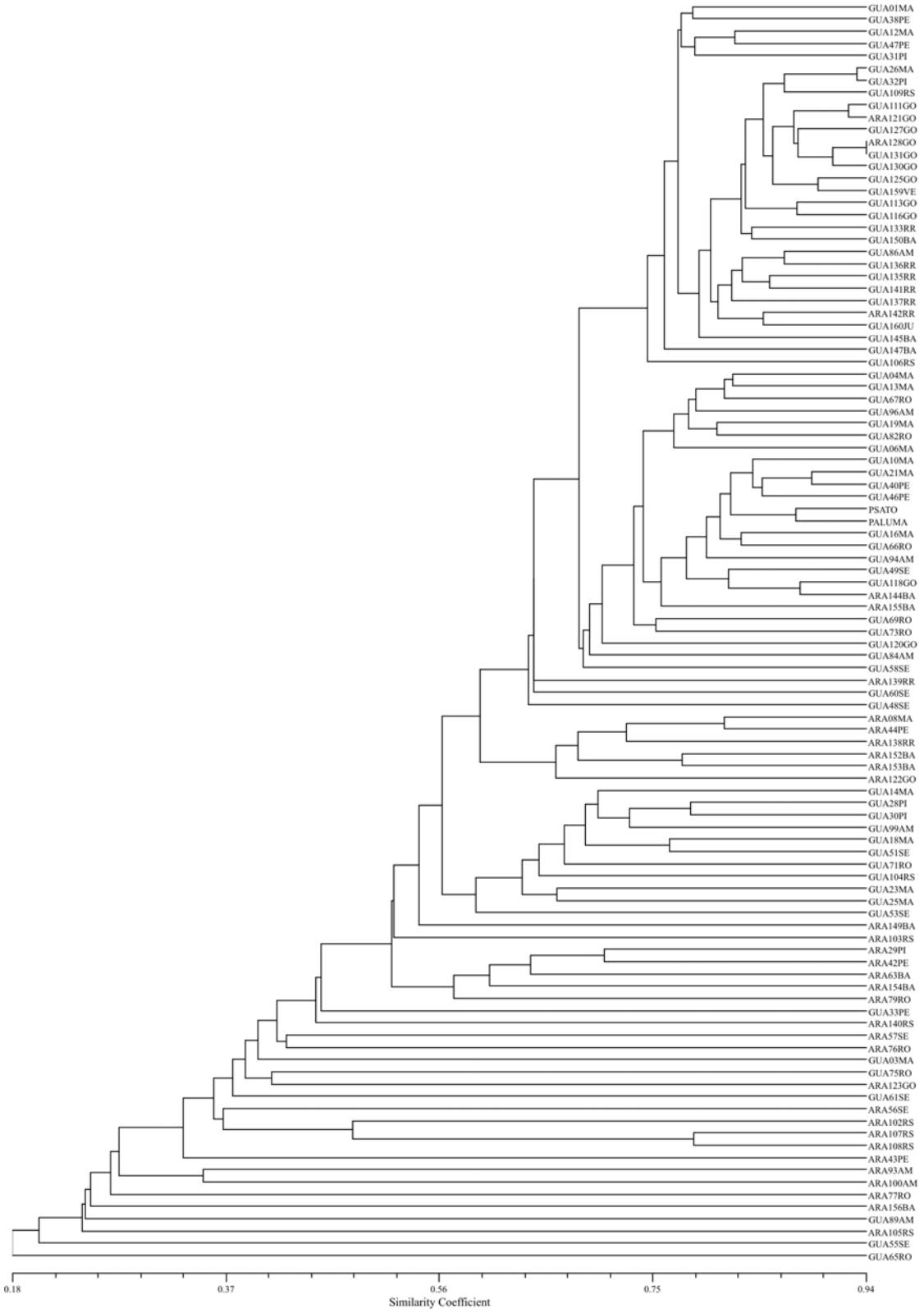
GUA01MA
GUA38PE
GUA12MA
GUA47PE
GUA31PI
GUA26MA
GUA32PI
GUA109RS
GUA111GO
ARA121GO
GUA127GO
ARA128GO
GUA131GO
GUA130GO
GUA125GO
GUA159VE
GUA113GO
GUA116GO
GUA133RR
GUA150BA
GUA86AM
GUA136RR
GUA135RR
GUA141RR
GUA137RR
ARA142RR
GUA160JU
GUA145BA
GUA147BA
GUA106RS
GUA04MA
GUA13MA
GUA67RO
GUA96AM
GUA19MA
GUA82RO
GUA06MA
GUA10MA
GUA21MA
GUA40PE
GUA46PE
PSATO
PALUMA
GUA16MA
GUA66RO
GUA94AM
GUA49SE
GUA118GO
ARA144BA
ARA155BA
GUA69RO
GUA73RO
GUA120GO
GUA84AM
GUA58SE
ARA139RR
GUA60SE
GUA48SE
ARA08MA
ARA44PE
ARA138RR
ARA152BA
ARA153BA
ARA122GO
GUA14MA
GUA28PI
GUA30PI
GUA99AM
GUA18MA
GUA51SE
GUA71RO
GUA104RS
GUA23MA
GUA25MA
GUA53SE
ARA149BA
ARA103RS
ARA29PI
ARA42PE
ARA63BA
ARA154BA
ARA79RO
GUA33PE
ARA140RS
ARA57SE
ARA76RO
GUA03MA
GUA75RO
ARA123GO
GUA61SE
ARA56SE
ARA102RS
ARA107RS
ARA108RS
ARA43PE
ARA93AM
ARA100AM
ARA77RO
ARA156BA
GUA89AM
ARA105RS
GUA55SE
GUA65RO
0.18
0.37
0.56
0.75
0.94
Similarity Coefficient

Figure 4. UPGMA dendogram of Jaccard's coefficient among 103 *Psidium* accessions sampled in ten different Brazilian ecogeographic regions and inferred upon 195 *EcoRI/MseI* AFLP markers. Cophenetic value=0.96.

Results

Among the sixteen SSR markers tested in all *Psidium* accessions only eight presented well defined bands (mPgCIR 233, mPgCIR242, mPgCIR246, mPgCIR249, mPgCIR251, mPgCIR252, mPgCIR255, mPgCIR256, mPgCIR257) in the first genotyping. A hundred and eleven alleles were scored from the eight SSR markers making impossible to correctly identify an allele from one glass plate to another one.

Among the sixteen recommended SSR primers only ten presented bands well defined, suitable for genotyping in the second genotyping: mPgCIR227, mPgCIR242, mPgCIR246, mPgCIR247, mPgCIR249, mPgCIR251, mPgCIR252, mPgCIR255, mPgCIR256, mPgCIR257. Sixty-seven alleles were scored from the ten SSR markers.

It was observed clusters of guava to the States of GO, RR and RO (Fig. 5) suggesting that the sampling by States and ecogeographic regions was a good strategy. It was also observed a mixture of guava accessions independently of the State of origin indicating that guava plants have been freely dispersed from one place to another among the seven sampled Brazilian States (except GO, RR and RO). The aracazeiro accession was placed outside of guava (as an outgroup) in the dendogram. Further investigation will be performed to genotype all Brazilian GuavaMap *Psidium* accessions (a hundred and fifty six accessions)

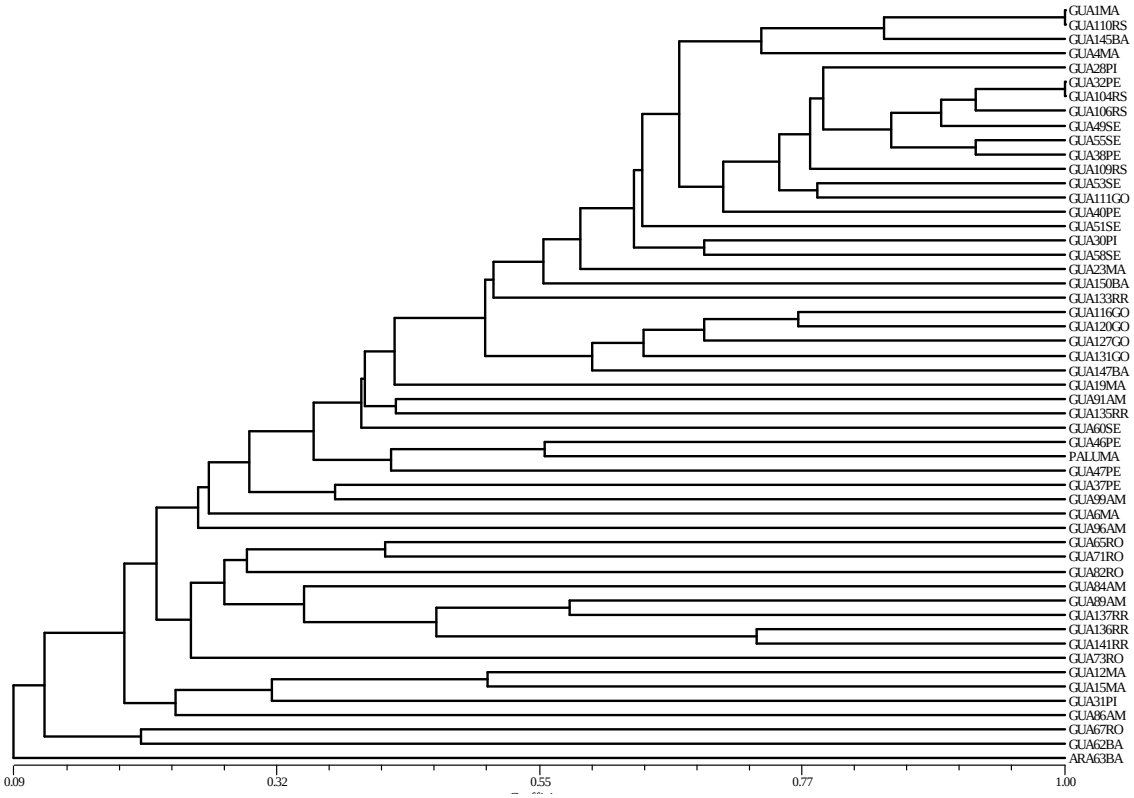

Figure 5. UPGMA dendogram of Jaccard's coefficient among 53 guava and one aracazeiro accessions sampled in ten different Brazilian States inferred upon 67 alleles from ten SSR markers. Cophenetic value=0.86.

WP1:
Biodiversity analysis of wild-growing guava in Brazil

Task 1.5. Establishment of new mapping populations

1.5.1 Screening for tolerance to nematode *Meloidogyne mayaguensis*

Material and Methods

Nematode evaluation of accessions of the *Psidium* germplasm bank: plants were obtained as described in WP 1, task 1.3. a) Without artificial nematode inoculation: plants of accessions were placed in a nursery where the nematode was naturally present and plants were evaluated one year after sowing; b) With artificial nematode inoculation: When plants were 15-20 cm high, they were inoculated with 10.000 eggs/plant (Pi=initial inoculums level), extracted with a blender from infected guava, using NaOCl 0.5%. The pots were arranged in a randomized incomplete block with 10 replicates. Five months after inoculation, the accessions were evaluated according to the scale: 0 = no galls or egg masses, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = over 100 galls or egg masses. Plants presenting an averaged gall and egg mass index of 2 or lower were considered tolerant.

Evaluation of F2 progenies of F1 guava presenting field tolerance to the devastating nematode *Meloidogyne mayaguensis*: progenies were obtained as described in WP 1, task 1.3. Artificial inoculations were done as described in previous item.

Results

Sixty accessions were evaluated to field (nursery) nematode tolerance and ten were selected for further investigation with artificial nematode inoculation because they did not present abnormal roots. Among the ten accessions two guavas from Roraima (RR) presented a mixture of plants tolerant and susceptible to the nematode. These accessions will be subject of further investigations. Aracazeiros accessions from Rio Grande do Sul presented tolerance to the nematode, confirming previous studies performed at Embrapa Cenargen and Embrapa Cpatsa.

Among seventy-four accessions evaluated with artificial inoculation to the nematode only two presented tolerance, the accessions ARA 56 SE and ARA 144 BA. ARA 56 SE is a very small plant that cannot be used as guava rootstock. Plants of ARA 144 BA were used as rootstock of Paluma and Pedro Sato commercial cultivars and they presented incompatibility. Probably this accession did not belong to the *Psidium* genus.

F2 progenies of three guava hybrids (F1) presenting field tolerance to the devastating nematode *Meloidogyne mayaguensis* did not present any resistant progenie: Surubim x Grande Vermelha (140 F2 plants), ECA 1 x Red Selection of Florida (166 F2 plants) and Pentecoste x EEF3 (76 F2 plants) (Fig. 6). These three populations were eliminated because it does not make

sense to establish mapping population that will be destroyed by the nematode when transplanted to field in the experimental station.

Figure 6. Details of roots of guava F2 populations damaged by the nematode *Meloidogyne mayaguensis*.

WP2:
Construction of individual AFLP-based guava linkage maps

Task 2.2. Generation of AFLP markers – mapping population 2

Material and Methods

The pre-amplified samples of the Enana x Suprema Roja mapping population (MP2) were sent by Neiker (Spain) to Cpatsa, Petrolina, PE, to perform AFLP amplifications with selected primer combinations to the MP2.

AFLP amplification reactions were done in a final volume of 10 µl according to the protocol established at Neiker: 0.2 µM of *Eco* Primer, 0.3 µM of *Mse* primer, 0.2 mM of dNTPs, 1x PCR buffer, 2.5 mM of $MgCl_2$, 0.5 units of *Taq* DNA polymerase, and 2.0 µL of pre-amplified DNA. The touch-down thermocycling conditions involved : a) 1 cycle at 94° C for 1 min, b) 13 cycles each one at 94° C for 30 s, 65° C for 30 s, lowered by 0.7° C every subsequent cycle, and 72° C for 1 min, c) 23 cycles each one at 94° C for 30 s, 56° C for 30 s, 72° C for 1 min, d) 1 cycle at 72° C, holding at 4° C. After the PCR reaction 5.0 µl of formamide dye (98% formamide, 10 mM EDTA, 10 mg/L each of bromophenol blue and xylene cyanol) were added to each reaction. The solutions were heated at 95 °C for 4 min and immediately placed on ice before loading onto polyacrylamide sequencing gels.

For PAGE, the larger glass plate (combs with 60 teeth) was treated with 1.0 µL of PlusOne Bind Silane (Amersham Pharmacia Biotech) in 1.0 ml of acidic ethanol (0.5% glacial acetic acid in 95% ethanol). The smaller glass plate was treated with WaterLux (LuxCar acessorios automotivos). Gels (0.4 mm-thick 5.7% polyacrylamide sequencing gels) were prerun at 55 W for 30 min in the sequencing model S3S (Owl separation systems). Fifty-seven samples of 3.5 µL each (plus one of the Fermentas 50 bp ladder) were loaded and the gel was run at 55 W for 2.5 hours. A second gels were done to run the

left 57 individuals (total of 114 samples of the mapping population MP2, plus one of the Fermentas 50 bp ladder. After the run, the gel was cooled on ice before starting the silver-staining procedure. Silver-staining was performed as described by Creste et al., (Plant Molecular Biology Reporter, 2001, 19: 299-306). All gels were scanned with an Epson digitalizer for documentation and future control. An estimated size of AFLP fragment was obtained by inverse mobility method based on regression of known products of the Fermentas 50 bp ladder.

Fluorescent AFLPs were performed at Neiker, Spain, according to specific protocol. The fragment separation was obtained in LI-COR 4200 sequencer, at Neiker.

Results

Two-hundred and ninety-one polymorphic AFLPs bands were scored from 63 primer combinations (PCs) resulting in an average of 4.6 bands/PC. A matrix containing the obtained data was sent to Neiker for establishment of the molecular linkage map of MP2.

Seven fluorescent primer combinations were done at Neiker, by Marciene Rodrigues, and 30 polymorphic bands were obtained for: E43M33 (4 bands), E43M34 (5 bands), E43M31 (1 band), E43M35 (1 band), E43M36 (6 bands), E37M34 (7 bands) and E43M37 (6 bands). The fluorescent AFLP data was left at Neiker (Spain) for establishment of the molecular linkage map of MP2.

WP4:
Integration of SSR markers into the individual guava maps and alignment of these maps into a guava reference linkage map

Task 4.1. Scoring of SSR alleles in the MP2 progenies

Material and Methods

Fluorescent SSRs primers were performed at Neiker, Spain, according to specific protocol. The fragment separation was obtained in a LI-COR 4200 sequencer, at Neiker.

Results

Twenty-four SSRs were screnned at Neiker for: mPgCIR_30, mPgCIR_34, mPgCIR_39, mPgCIR_41, mPgCIR_89, mPgCIR_94, mPgCIR_98, mPgCIR_105, mPgCIR_111, mPgCIR_183, mPgCIR_188, mPgCIR_192, mPgCIR_193, mPgCIR_200, mPgCIR_201, mPgCIR_208, mPgCIR_212, mPgCIR_216, mPgCIR_235, mPgCIR_ 237, mPgCIR_243, mPgCIR_253, mPgCIR_255, mPgCIR_256. Table 1 summarizes the results with nine SSRs that produced polymorphics bands. The data of fluorescent SSRs were left at Neiker for establishment of the molecular linkage map of MP2.

Table 1. Number of bands of nine SSRs primers evaluated in the MP2 population with fluorescent primers at Neiker, Spain.

Primer	E/SR	SR	Bands number

mPgCIR_216	-	+	1
mPgCIR_235	-/+	+/+	2
mPgCIR_256	-/+/+/	+/-/+	3
mPgCIR_30	+/+	-/+	2
mPgCIR_34	+	-	1
mPgCIR_39	+/+	+/-	2
mPgCIR_105	+/+	-/+	2
mPgCIR_193	-/+	+/+	2
mPgCIR_253	-	+	1

II DELIVERABLES FOR THE PROJECT

Task D1.1: Prospecting of guava germplasm in Brazil – Accomplished: A hundred-fifty-six *Psidium* accessions established at Embrapa Experimental Agricultural Station, Petrolina,

Task D1.2: Agromorphological characterization of prospected guava germplasm by UPOV descriptors - Accomplished: accessions characterized for 35 UPOV descriptors. Undergoing activities to characterized for Vitamin C, total carotenoids, lycopene, minerals (Zn, Fe, Ca and Mg), anti-oxidant activities, total dissolved sugar content and total proteins.

Task D1.3: List of fruit characters – accomplished. Undergoing activities for agronomic characterization for fruit yield, number of fruits/plant and pulp percentage.

Task D1.4: Isolation of DNAs from prospected guava germplasm – accomplished

Task D1.5.1: Molecular characterization of prospected germplasm by AFLP markers – accomplished for a hundred-three *Psidium* accessions.

Task D1.5.2: Molecular characterization of prospected germplasm by SSR markers – accomplished for 54 guava accessions.

Task D1.7: Crosses with indigenous guava accessions (lines, varieties) and establishment of F1 progenies – it was not accomplished due to the nematode *Meloidogyne mayaguensis*.

Task D2.2: AFLP primer combinations on MP2 for linkage mapping – accomplished: Two-hundred and thirty-nine polymorphic AFLPs bands were scored from 70 primer combinations.

Task D2.3: SSR primers on MP2 for linkage mapping – partially accomplished: Twenty-four SSRs were screened for linkage analysis.

III PROBLEMS and DELAYS

No technical problem or delays to declare.

IV TRAINING and SCIENTIST EXCHANGE

Rita Mércia Estigarribia Borges visited partner P2 (NEIKER, Spain) for training with DNA extraction, AFLP DNA markers, and silver staining, April, 2006.

Marciene Amorim Rodrigues training at NEIKER to perform fluorescent AFLP and SSR, August, 2008.

Training of undergraduate students with GuvaMap laboratory and field activities:
Jucilene Silva Araújo, Maria Maiany de Oliveira, Hugo Leonardo Coelho Ribeiro, Italo Dourado Teixeira, Yerla Carla Nunes dos Santos, Ednaldo Francisco dos Santos Silva, Juliana Carla da Silva Farias Alves, Valdivia Thais Alves de Lima, Marciene Amorim Rodrigues and Roberta Samra Nunes Lima.

V DISSEMINATION (publications, posters, presentations)

SANTOS, C. A. F.; CASTRO, J. M. C. E. ; Souza, F.F.; VILARINHO, A. A.; FERREIRA, F. R.; PADUA, J. G.; BORGES R.M.E.; RODRIGUES, M.A. 2008. Preliminary characterization of Psidium germplasm in different Brazilian eco-geographic regions. Pesquisa Agropecuária Brasileira, v. 43, p. 437-440. (http://www.scielo.br/scielo.php?pid=S0100-204X2008000300020&script=sci_arttext)

Abstracts in scientific events:
SANTOS, C. A. F.; CASTRO, J. M. C. E. ; SOUZA, F.F.; VILARINHO, A. A.; FERREIRA, F. R.; PADUA, J. G.; CLARET, A. G.; RODRIGUES, M.A. 2008. Prospecting and morphological characterization of Brazilian *Psidium* germplasm. In: 2nd international symposium on guava and other Myrtaceae, 2008, Merida. 2nd international symposium on guava and other Myrtaceae. Merida: CYCI, 2008.
SANTOS, C. A. F.; Souza, F.F.; VILARINHO, A. A.; PADUA, J. G.; RODRIGUES, M. A. 2008. Relationship between ecogeographic sampling and phenotypic diversity of Brazilian *Psidium* germplasm based on categorical descriptors. In: 2nd international symposium on guava and other Myrtaceae, 2008, Merida. 2nd international symposium on guava and other Myrtaceae. Merida: CYCI, 2008.
CASTRO, J. M. C. E.; FLORI, J.E.; SANTOS, C. A. F.; ANTUNES, E.F. 2008. Evaluation of tolerance of *Psidium* species to the Meloidogyne mayaguensis nematode. In: 2nd international symposium on guava and other Myrtaceae, 2008, Merida. 2nd international symposium on guava and other Myrtaceae. Merida : CICY, 2008.
SANTOS, C. A. F.; RODRIGUES, M.A.; RIBEIRO, H.L.C.; OLIVEIRA, M. de O.; ARAUJO, J.; COSTA, T.P.P. 2008. Relações entre amostragem ecogeográfica e diversidade fenotípica em germoplasma de *Psidium* com base em descritores morfológicos. In: II Simpósio brasileiro de recursos genéticos, 2008, Brasília. II Simpósio brasileiro de recursos genéticos. Brasília: Embrapa, 2008.
SANTOS, C. A. F.; RODRIGUES, M.A.; RIBEIRO, H.L.C.; ARAUJO, J. ; COSTA, T.P.P. 2008. Banco de germoplasma de goiabeira e araçazeiros. In: II Simpósio brasileiro de recursos genéticos, 2008, Brasília. II Simpósio brasileiro de recursos genéticos. Brasília: Embrapa, 2008.

VI PLANNING for the future

To complete fruit characterization of germplasm collection for vitamin C, total carotenoids, lycopene, minerals (Zn, Fe, Ca and Mg), total dissolved sugar content and total proteins. To complete SSR analyses to the entire collection, as part of the doctoral thesis of Luiz Claudio Correa, Universidade Estadual de São Paulo, Campus de Botucatu.

To prospect and to collect *Psidium* germplasm in the Central part of Brazil (MG and ES States).

To maintain the germplasm collection as a repository of the Brazilian *Psidium* genetic variability.

To prospect for tolerance to the nematode *Meloidogyne mayaguensis* in order to develop mapping population and to stop the guava field devastation by the nematode.

To submit at least two scientific papers to peer reviewed journals.

To host the 3nd international symposium on guava and other Myrtaceae, at Petrolina, PE, Brazil, in 2011.

Section 3
Consortium management

3.1 Consortium management tasks

In January 2007 INIFAP and the CICY concluded an official contract to subcontract some of the tasks of work package WP1 (collection of wild-growing guava in Mexico, *ex situ* conservation, agro-morphological description, crosses between selected guava accessions) and WP9 (dissemination in form of a symposium/laboratory course; see annex A.IV). The coordinator visited INIFAP in January 2008 to conclude the final preparatory steps for the symposium in Aguascalientes. During the "2nd International Symposium on Guava and Other *Myrtaceae*" (see annex A.I) in Mérida a short GUAVAMAP meeting was held on November 11, 2008, with respect to reporting and project extension.

3.2 Contractors

Changes in the composition of the consortium have not occurred.

3.3 Project timetable and status

An extension of the project for 6 months till May 31, 2009 was granted and the timetable of deliverables adjusted accordingly.

3.4 Problems in consortium management

3.4.1 All partners: Generally the partners do not occupy themselves with the reporting guidelines. In specific, financial reporting was a big problem, both with repect to the deadlines of data submission, the layout of Form C, and the format required by the EC as explanatory notes..

3.4.2 EC financial officer: Although the GUAVAMAP project was late in supplying the management report (see 3.4.2), part of the intolerable retardation in funding has to be attributed to unjustified demands by the EC financial officer. In fact, if the EC financial department cannot come up with a common policy within itself, it should not expect too much from the contractors. Only in the third year of the contract, a template was provided by the then financial officer as a guideline on which information to supply in which format.

ANNEX

A.I Plans for using and disseminating the knowledge

Section 1: Exploitable knowledge

One important aspect of the first project objective ("Analyzing the biodiversity of guava indigenous to the three producer countries Brazil, Venezuela and Mexico", WP1) was the production of hybrid populations for linkage mapping and the possible selection of hybrids that have the potential for the development of varieties according to the needs of the individual countries. In this respect it should be noted that at the gene bank of IIFT (Alquízar, Havana Province, Cuba) which was the subcontractor S1 to MPIZ and hosts the three mapping populations used for linkage mapping in the GUAVAMAP project, attempts on variety development are already under way. The evaluation of the three mapping population had permitted the selection of 25 genotypes of low plant size. All of them are being replicated in plots for the evaluation of agronomic important traits for their introduction as new cultivars in Cuba. Also, interesting genotypes because of the low number of seeds and high weight of the fruits (more than 300 gr.) are evaluated for their potential in industrial processing and direct marketing. Furthermore, CPATSA in Brazil (partner P6) has identified 11 wild-growing *Psidium* species that may serve as rootstocks in the generation of nematode-resistant new cultivars. Nematode attack is the most devastating disease in Brazil and other countries. Thus as an outcome of Work Package 1 the generation of new cultivars for guava production and replanting in nematode-devastated regions is envisioned.

Subcontractor S1 has developed new descriptors for guava (see poster #18) which will form the basis for entries into a future database on guava and other *Myrtaceae* (agro-morphological description), while the Molecular Marker Set (MMS) of SSRs will serve for the molecular analysis. This database is planned to be realized at CIRAD (partner P3).

Section 2: Dissemination of knowledge

Year 1: Already during the first year the project has started dissemination by 1 publication, 3 posters presented at national (Venezuela by ULA) and international congresses (Italy by CIRAD), and lectures given by the coordinator (December 2005: 1st International Guava Symposium in Lucknow, India; October 2006: ULA, Mérida, Venezuela; November 2006: CICY, Mérida, Mexico).

Year 2: These activities were continued in year 2. A total of 5 posters were presented by subcontractor S1 (IIFT) and partner P5 (ULA), respectively during the "Second International Congress on Tropical and Subtropical Fruit Crops" in La Habana, Cuba (17 – 21 Sep., 2007). MPIZ presented part of its specific work during the VI Latin American and Caribbean Meeting on Agricultural Biotechnology (REDBIO), Viña del Mar, Chile (22 – 26 October, 2007). Lectures were given by the coordinator on various occasions such as an invited lecture during the symposium "Biosciences at the Interface of Physics, Chemistry, Biology and Medicine" in Monterrey, Mexico (September 2007).

Year 3: In year 3 the project organized together with the ISHS the **2nd International Symposium on Guava and Other *Myrtaceae*** in Mexico at two locations, Mérida (Biotechnology Part) and Aguascalientes (Guava Production and Processing Part). Furthermore, a biotechnology laboratory course on "*Application of DNA markers for the biodiversity analysis of tropical fruit crops*" was held at the CICY (partner P4) from November 17 – 28, 2008 (the bioinformatics course was held at the CICY from December 1-5, 2008) in Mérida (http://www.cicy.mx/eventos/guavasymposium2008). Copies of the abstract book for the symposia are included in this report.

Extension period: The practical course on bioinformatics was held at the CICY from Dec. 1-5, 2008, with Dr. K. Stueber (MPIZ Cologne) as the instructor. Due to fellowships payed by GUAVAMAP, 4 young scientists (2 from Mexico, 2 from Cuba) could participate to this course. It is anticipated that the ISHS-supported publication in *Acta Horticulturae* of the symposium **2nd International Symposium on Guava and Other *Myrtaceae*** will be available in 2010 latest. Some 50 publications have been received by the 2 editors (G. Fermin, ULA; W. Rohde, MPIZ) and all of them have been edited. They were submitted to the ISHS office at the end of July, 2009, for final editing, layouting and printing. It is expected that this special issue of Acta Horticulturae will appear at the end of 2009.

Cumulative index of dissemination:

Publications:

Rodríguez, N., Valdés-Infante, J., Becker, D., Velázquez, B., González, G., Sourd, D., Rodríguez, J., Billotte, N., Risterucci, A.M., Ritter, E. and Rohde, W. (2007). Characterization of guava accessions by SSR markers, extension of the molecular linkage map, and mapping of QTLs for vegetative and reproductive characters. *Acta Horticulturae* **735**: 201-215.

Posters:

1. Nansot et al., "Microsatellite markers development: towards a genetic map of guava (*Psidium guajava L.*)"; PlantGem (October 11-14, 2006), Italy.
2. C. Valecillos, A. Briceño and G. Fermin: "Estudio de la variabilidad genetica en *Psidium guajava* L. utilizando biomarcadores generales y especificos." VIII Congreso Venezolano de Fruticultura of Venezuela (October 24-27, 2006).

3. Y. Aranguren and G. Fermin: “Germoplama de “guayabas” nativas venezolanas: estudio de variabilidad utilizando marcadores UPOV.” VIII Congreso Venezolano de Fruticultura of Venezuela (October 24-27, 2006).
4. Valdés-Infante, J., Rodríguez, N. N.., Becker, D., Velázquez, B., Sourd, D., Ritter, E. and Rohde, W. 2007. Establishment and saturation of the linkage map and QTL analysis in guava (*Psidium guajava* L.). “Second International Congress on Tropical and Subtropical Fruit Crops” organized by the IIFT in La Habana, Cuba (17 – 21 Sep., 2007).
5. Valdés-Infante, J., Rodríguez, N. N., Becker, D., Velásquez, B., Sourd, D., Espinosa, G., Ritter, E., Risterucci, A. M., Billote, N. and Rohde, W. 2007. Molecular characterization of the Cuban guava germplasm by AFLP and SSR analysis. “Second International Congress on Tropical and Subtropical Fruit Crops”, La Habana, Cuba (17 – 21 Sep., 2007).
6. Rodríguez Medina, N. N., Fuentes Fiallo, V. R., Hernández Zaldívar, M. R., Valdés-Infante, J., Velázquez Palenzuela, J. B., Rivero, D., Sourd Martínez, D. G., Rodríguez Rodríguez, J. A., González García, G. and Martínez González, F. 2007. Cuban catalogue of guava (*Psidium guajava* L.) cultivars. “Second International Congress on Tropical and Subtropical Fruit Crops”, La Habana, Cuba (17 – 21 Sep., 2007).
7. Aranguren, Y. and Fermin, G.: Variabilidad de guayabas nativas en Venezuela por marcadores moleculares y marcadores fenotipicos UPOV. “Second International Congress on Tropical and Subtropical Fruit Crops”, La Habana, Cuba (17 – 21 Sep., 2007).
8. Valecillos, C., Contreras, I., Almeida, J. and Fermin, G.: Establecimiento de bancos de germoplasma para el analisis de variabilidad del gen *plaI* en *Psidium guajava* L. “Second International Congress on Tropical and Subtropical Fruit Crops”, La Habana, Cuba (17 – 21 Sep., 2007).
9. González, L., Becker, D., Schwarz-Sommer, Zs. and Rohde, W. 2007. Genetic diversity screening in *Psidium guajava* L. of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes). VI Latin American and Caribbean Meeting on Agricultural Biotechnology (REDBIO), Viña del Mar, Chile (22 – 26 October, 2007).

2nd Int. Symp. Guava and Other *Myrtaceae* (all posters and lectures will go into print in *Acta Horticulturae*, presumably by 2010 or the end of 2009):

posters continued:

10. Valdés-Infante *et al*.: Simple Sequence Repeats (SSRs) for diversity characterization of guava (*Psidium guajava* L.)
11. Aranguren, Y. *et al*.: Assessment of the variability of Venezuelan guava landraces by microsatellites
12. Aranguren, Y. *et al*.: Variability of Venezuelan landraces of guava by phenotypic markers and geographical origin
13. Valecillos, C. *et al*.: Natural resources conservation: Guava and other *Myrtaceae* germplasm *ex situ* conservation at Mérida, Venezuela
14. Valecillos, C. *et al*.: Cloning and sequencing of the hydroxyperoxide lyase gene and transformation of guava
15. Rodríguez-Medina, N.N. *et al*.: Individual versus combined data sets for the molecular characterization of Cuban guava (*Psidium guajava* L.) germplasm

16. Briceño, A. *et al*.: Assessment of guava-derived SSR to molecularly characterize *Myrtaceae* from two diverse ecosystems in Venezuela
17. Gonzales, L. *et al*.: Isolation of candidate genes for resistance (RGL sequences) and plant development (MADS-box and homeobox genes) from *Psidium guajava* L.
18. Rodriguez-Medina, N.N. *et al*.: Illustrated descriptors for guava (*Psidium guajava* L.) characterization
19. Sánchez-Teyer, L.F. *et al*.: Assessment of genetic diversity of Mexican guava germplasm using molecular markers

Lectures:
1. Sánchez-Teyer, L.F. *et al*.: Assessment of genetic diversity of Mexican guava germplasm using molecular markers (see poster 19)
2. Fermin, G.: On the cultivation of guava in Venezuela
3. Fernandes-Santos, C.A. *et al*.: Prospecting and morphological characterization of Brazilian *Psidium* germplasm
4. Valdés-Infante, J. *et al*.: Comparison of the polymorphism level, discriminating capacity and informativeness of morph-agronomic traits and molecular markers in guava (*Psidium guajava* L.)
5. Fernandes-Santos, C.A. *et al*.: Relationship between ecogeographic sampling and phenotypic diversity of Brazilian *Psidium* germplasm based on categorical descriptors
6. Fermin, G: *Myrtaceae* from Venezuela: diversity, distribution and ethno-botanical aspects
7. Briceño, A. and Fermin, G.: Assessment of guava-derived SSRs to molecularly characterize *Myrtaceae* from two diverse ecosystems in Venezuela
8. Ritter, E. *et al*.: Comparative linkage mapping in three guava mapping populations and construction of an integrated reference map in guava
9. Billotte, N. *et al*.: The microsatellite (SSR) /AFLP reference linkage map of guava
10. Ritter, E. *et al*.: QTL analysis: General aspects and application in guava
11. Fermin, G.: Strategies and molecular markers for the improvement and protection of plants: Cases from Venezuela
12. Rohde, W.: The GUAVAMAP project
13. Rodriguez-Medina, N.N. *et al*.: Genetic resources of guava (*Psidium guajava* L.) in Cuba: germplasm characterization and breeding

Dates	Type	Type of audience	Countries addressed	Size audience	of partner
Dec. 2005	conference	research	international	300	P1
Dec. 2005	media (press)	general public	India		P1
Dec. 2005	publication	research			P1
Aug. 2006	poster	research	international		P3
Nov. 2006	2 posters	research	Venezuela	250	P5
January 2007	Media (press)	General public	Mexico		P1
January 2007	lecture	research, producer	Mexico	70	P1
Sept. 2007	2 posters	research	international	150	P5
Sept. 2007	3 posters	research	international	150	S1
Sept. 2007	conference	research	Mexico	90	P1
October 2007	conferencer	research	international	300	P1
Nov. 2008	conference	research	international	120	P1, P4
End of 2009/beg. 2010	publication	Research, producers	international	World-wide	P1, P5

A.II Status of project

All tasks have been completed.