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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS
GENEVA

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA PROFILING IN PARTICULAR

Twelfth Session
Ottawa, Canada, May 11 to 13, 2010

USE OF A MOLECULAR MARKER BASED SYSTEM FOR IDENTIFICATION OF VARIETIES IN BRAZIL

Document prepared by an expert from Brazil

WORKING GROUP ON BIOCHEMICAL AND MOLECULAR TECHNIQUES AND DNA PROFILING IN PARTICULAR

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Use of a molecular marker-based system for identification of varieties in Brazil

Soybean and Rice

Document prepared by :
Luís Pacheco
SNPC/MAPA - Brazil

PVP in Brazil

✓ Breeder Testing System

✓ PVP Office – technical staff:

1 Coordinator

6 Examiners

Examination of applications and Granting of Plant Breeder’s Rights

✓ 1 Laboratory – Live Samples Storage (Seed or DNA)

2 Experts

✓ Independent Test Lab

DNA fingerprints of PBR and NLi varieties

Support for identification of varieties

Identification of Genetic Profiles of SSR markers in DNA

✓ Objectives - **Identification** of varieties

Enforcement of Seed Law (cooperative work within Departments of MAPA)
Seed Certification
PBR and NLi Varieties
Comparison of "new" and "old" varieties
Post control

✓ Control samples

SNPC provided **coded** samples
Doubled samples from SNPC
Negative Control – water / Positive Control – laboratory control sample
2 DNA extractions (bulks of 50 young leaves)
Genetic Analysis by 2 different staff in different days – minimize human error

✓ SSR Markers

Highly informative
Extensively validated in scientific literature

Statistical Analysis

✓ Loci polimorphism analysed through PCR

Primers marked with fluorescence blue (FAM), green (HEX) and yellow (NED)

✓ Alleles Detection

High resolution Capillary Electrophoresis

DNA automated sequencing – ABI Prism 3100

✓ Size of Alleles

Estimated by algorithm “Local Southern” – Software Genotyper

Discrete allele sizes - Least Squares Minimization Algorithm – Allelobin

Di, tri and tetranucleotides – variations of 1,5 pb between different runs and 0,5 pb in the same run – positive control in all tests allows to identify deviations

✓ Genetic Similarity

Genetic distance between pairs of entries – NTSYSpc 2.10z

Diagonal Matrix of Genetic Distances – UPGMA

Dendogram of Genetic Distances - NTSYSpc 2.10z

Soybean and Rice - Importance in Brazil

- **Soybean** - most important agricultural commodity
  Area – 27,1 million ha
  Yield –57,1 million tons
  745 varieties in NLi
  543 protected by PBR:
    - ~ **50** new varieties protected/year
    - ~ **1/3** of the titles granted in 1 year by SNPC
- **Rice –** one of most important foods
  Area – 2,9 million ha
  Yield – 12,6 million tons
  231 varieties in NLi
  77 protected by PBR

Numbers of Samples x markers

Soybean: 556 Samples x 15 SSR loci

Rice: 79 Samples x 16 SSR loci

SOYBEAN

SSR Markers - Soybean

- BARC-Satt002 Sequência simples repetitiva de trinucleotídeo; grupo de ligação D2
- BARC-Satt005 Sequência simples repetitiva de trinucleotídeo; grupo de ligação D1
- BARC-Satt042 Sequência simples repetitiva de trinucleotídeo; grupo de ligação A1
- BARC-Satt045 Sequência simples repetitiva de trinucleotídeo; grupo de ligação E
- BARC-Satt038 Sequência simples repetitiva de trinucleotídeo; grupo de ligação G
- BARC-Satt030 Sequência simples repetitiva de trinucleotídeo; grupo de ligação F
- BARC-Satt009 Sequência simples repetitiva de trinucleotídeo; grupo de ligação N1
- BARC-Satt70 Sequência simples repetitiva de trinucleotídeo; grupo de ligação B2
- BARC-Satt100 Sequência simples repetitiva de trinucleotídeo; grupo de ligação C2
- BARC-Satt114 Sequência simples repetitiva de trinucleotídeo; grupo de ligação F
- BARC-Satt586 Sequência simples repetitiva de trinucleotídeo; grupo de liagação F1
- S45035 “Kunitz trypsin inhibitor gene” SSR de dinucleotídeo; inform. de mapa indisponível
- GMABAB - sequência simples repetitiva de dinucleotídeo; grupo de ligação N1
- BARC-Satt431 Sequência simples repetitiva de trinucleotídeo; grupo de ligação J
- BARC-Satt216 Sequência simples repetitiva de trinucleotídeo; grupo de ligação D1

Tabela 1. Perfil genético multiloco de amostras de soja fornecidas pelo LADIC/MAPA, analisadas em 15 locos microssatélites. São apresentados os valores em pares de base (pb) de cada um dos dois alelos observados em cada loco nas amostras analisadas, após conferência dos genótipos em regime de análise de prova e contraprova.

SATT005	1	181.58	181.58	**SATT038**	1	168.17	168.17	**SATT586**	1	216.64	216.64
SATT005	2	178.51	178.51	**SATT038**	2	171.20	171.20	**SATT586**	2	203.40	203.40
SATT005	3	156.86	156.86	**SATT038**	3	168.05	168.05	**SATT586**	3	210.15	210.15
SATT005	4	156.90	156.90	**SATT038**	4	168.20	168.20	**SATT586**	4	199.84	199.84
SATT005	5	153.88	153.88	**SATT038**	5	168.14	168.14	**SATT586**	5	210.25	210.25
SATT005	6	178.51	178.51	**SATT038**	6	168.14	168.14	**SATT586**	6	210.23	210.23
SATT005	7	153.85	153.85	**SATT038**	7	168.26	168.26	**SATT586**	7	210.30	210.30
SATT005	8	153.81	153.81	**SATT038**	8	168.23	168.23	**SATT586**	8	196.88	196.88
SATT005	9	153.74	153.74	**SATT038**	9	168.03	168.03	**SATT586**	9	203.33	210.09
SATT005	10	156.89	156.89	**SATT038**	10	168.04	168.04	**SATT586**	10	210.18	210.18
SATT005	11	153.67	153.67	**SATT038**	11	171.18	171.18	**SATT586**	11	210.21	210.21
SATT005	12	156.92	156.92	**SATT038**	12	168.10	168.10	**SATT586**	12	210.23	210.23
SATT005	13	153.82	153.82	**SATT038**	13	168.07	168.07	**SATT586**	13	235.74	235.74
SATT005	14	154.32	154.32	**SATT038**	14	168.18	168.18	**SATT586**	14	236.39	236.39
SATT005	15	156.98	156.98	**SATT038**	15	168.14	168.14	**SATT586**	15	210.27	210.27
SATT005	16	144.28	144.28	**SATT038**	16	168.29	168.29	**SATT586**	16	200.12	200.12
SATT005	17	156.99	156.99	**SATT038**	17	171.37	171.37	**SATT586**	17	235.81	235.81
SATT005	18	156.91	156.91	**SATT038**	18	171.13	171.13	**SATT586**	18	199.98	210.04
SATT005	19	153.85	153.85	**SATT038**	19	168.00	168.00	**SATT586**	19	210.13	210.13
SATT005	20	153.85	153.85	**SATT038**	20	168.03	168.03	**SATT586**	20	203.24	203.24
	⋮				⋮				⋮		
SATT005	550	156.95	156.95	**SATT038**	550	167.57	167.57	**SATT586**	550	235.42	235.42
SATT005	551	157.03	157.03	**SATT038**	551	188.94	188.94	**SATT586**	551	209.99	209.99
SATT005	552	178.97	178.97	**SATT038**	552	167.58	167.58	**SATT586**	552	209.98	209.98
SATT005	553	135.46	135.46	**SATT038**	553	167.58	167.58	**SATT586**	553	199.83	199.83
SATT005	554	153.65	153.65	**SATT038**	554	167.60	167.60	**SATT586**	554	203.23	203.23
SATT005	555	153.80	153.80	**SATT038**	555	167.61	167.61	**SATT586**	555	196.61	196.61
SATT005	556	153.68	153.68	**SATT038**	556	167.61	167.61	**SATT586**	556	203.23	203.23
SATT005	Gen	156.88	156.88	**SATT038**	Gen	171.19	171.19	**SATT586**	Gen	235.68	235.68
SATT100	1	141.72	141.72	**SATT009**	1	217.78	217.78	**SATT431**	1	231.48	231.48
SATT100	2	141.70	141.70	**SATT009**	2	239.87	239.87	**SATT431**	2	231.54	231.54
SATT100	3	138.59	138.59	**SATT009**	3	221.10	221.10	**SATT431**	3	231.59	231.59
	⋮				⋮				⋮		

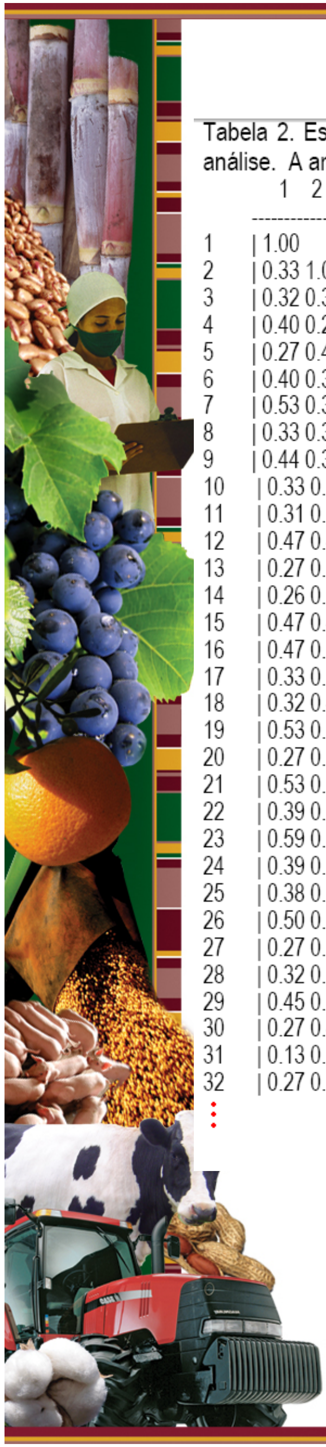
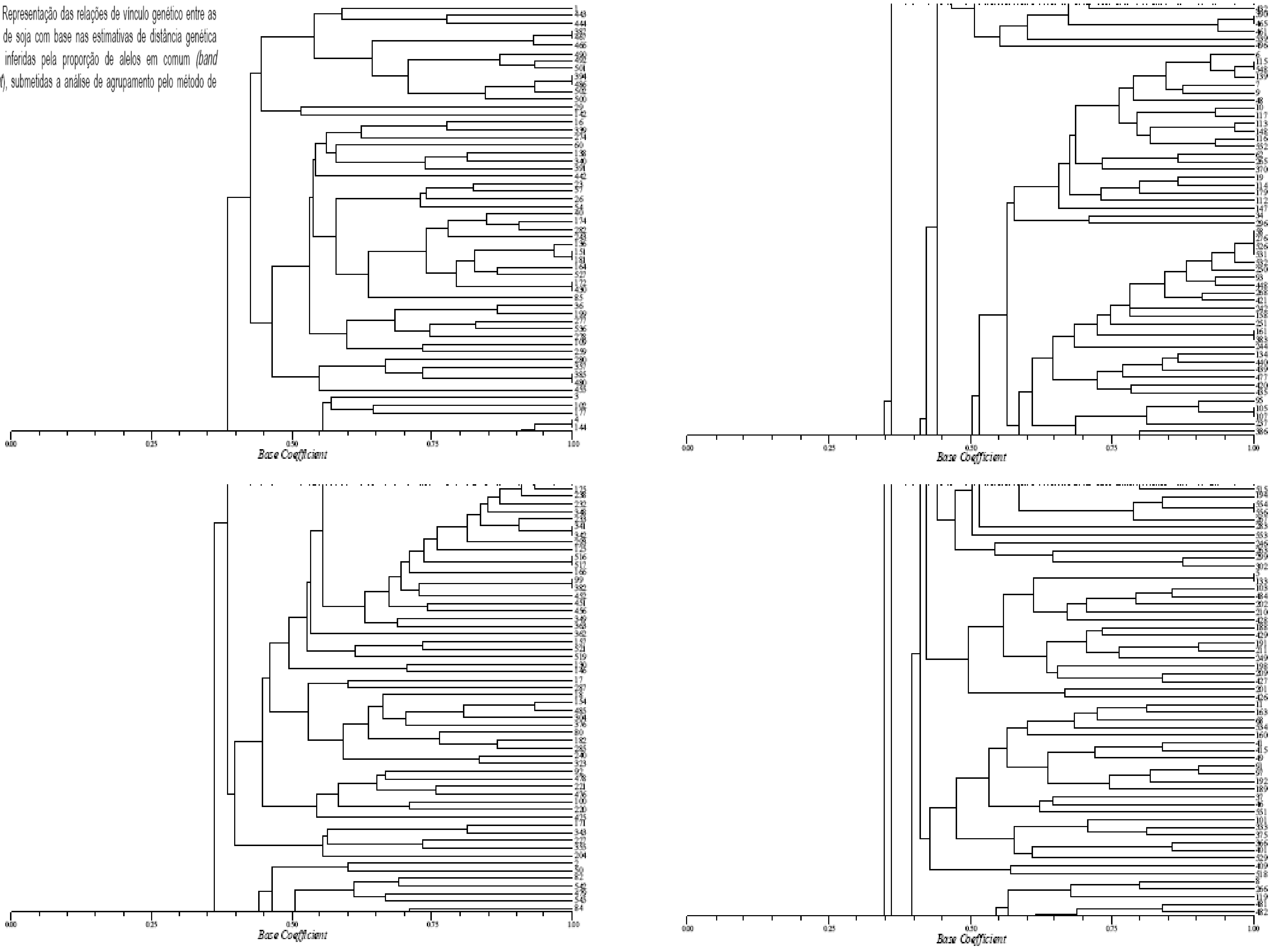
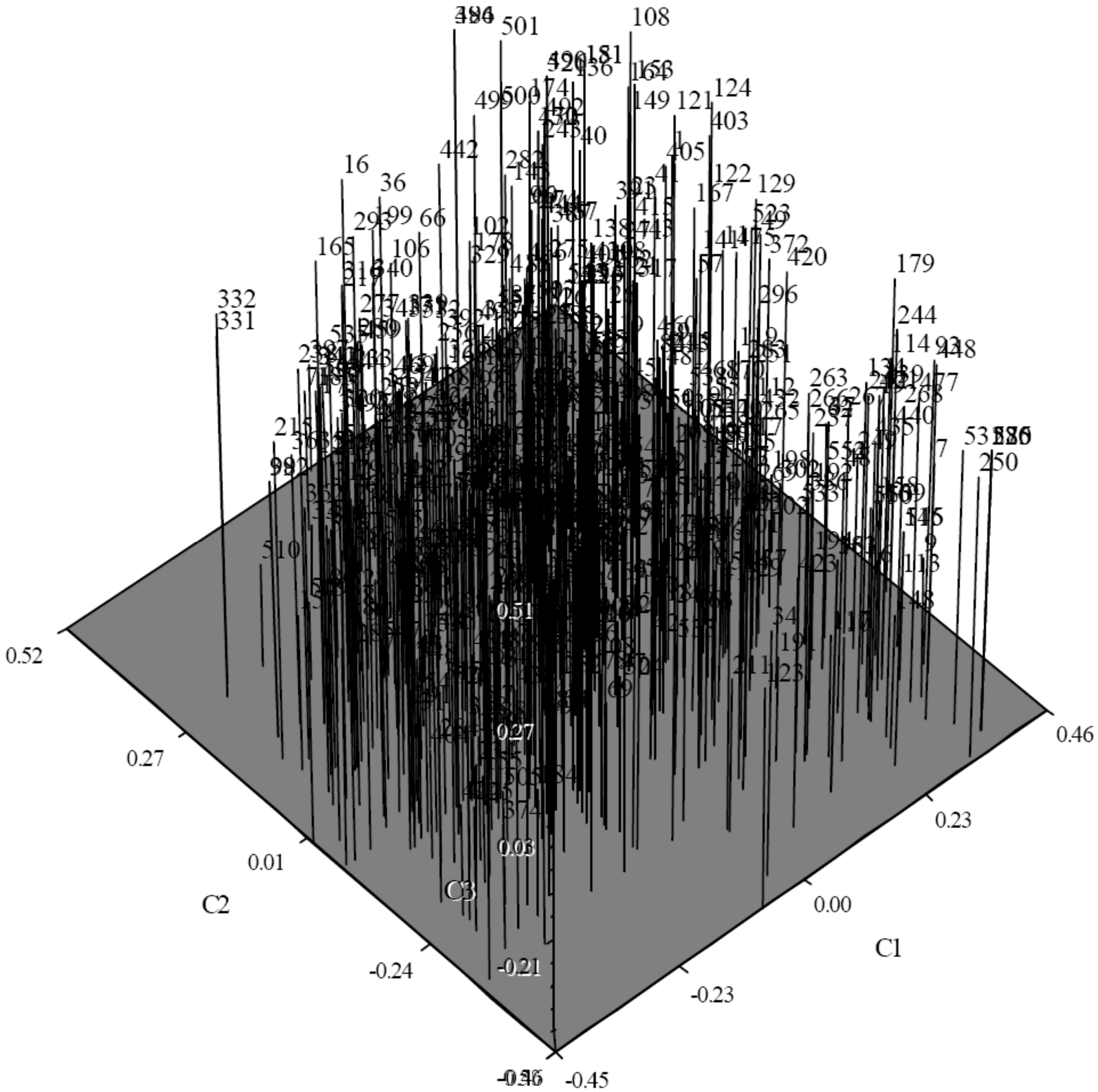


Tabela 2. Estimativas de distância genética par-a-par com base na proporção de alelos em comum (*band coefficient*) entre as 555 amostras de soja análise. A amostra de soja "Genomax" também foi incluída na análise.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	⋯
1	1.00																																
2	0.33	1.00																															
3	0.32	0.32	1.00																														
4	0.40	0.27	0.65	1.00																													
5	0.27	0.47	0.32	0.27	1.00																												
6	0.40	0.33	0.39	0.33	0.53	1.00																											
7	0.53	0.33	0.45	0.47	0.60	0.80	1.00																										
8	0.33	0.33	0.39	0.40	0.53	0.67	0.67	1.00																									
9	0.44	0.38	0.36	0.38	0.56	0.81	0.88	0.69	1.00																								
10	0.33	0.20	0.39	0.33	0.47	0.80	0.73	0.60	0.75	1.00																							
11	0.31	0.38	0.42	0.31	0.38	0.44	0.50	0.44	0.53	0.44	1.00																						
12	0.47	0.40	0.58	0.53	0.40	0.47	0.60	0.47	0.50	0.53	0.56	1.00																					
13	0.27	0.20	0.26	0.27	0.53	0.33	0.47	0.40	0.44	0.33	0.38	0.27	1.00																				
14	0.26	0.19	0.25	0.26	0.52	0.39	0.45	0.39	0.42	0.32	0.36	0.26	0.97	1.00																			
15	0.47	0.40	0.58	0.53	0.40	0.47	0.60	0.47	0.50	0.53	0.56	1.00	0.27	0.26	1.00																		
16	0.47	0.33	0.52	0.60	0.33	0.20	0.33	0.27	0.25	0.13	0.31	0.40	0.40	0.39	0.40	1.00																	
17	0.33	0.53	0.32	0.40	0.53	0.33	0.47	0.33	0.38	0.33	0.31	0.40	0.47	0.45	0.40	0.47	1.00																
18	0.32	0.45	0.38	0.39	0.52	0.39	0.39	0.32	0.30	0.32	0.36	0.39	0.32	0.38	0.39	0.45	0.65	1.00															
19	0.53	0.27	0.45	0.47	0.47	0.67	0.87	0.53	0.75	0.67	0.63	0.73	0.40	0.39	0.73	0.33	0.33	0.26	1.00														
20	0.27	0.13	0.13	0.07	0.33	0.40	0.40	0.40	0.50	0.40	0.25	0.20	0.53	0.52	0.20	0.27	0.33	0.26	0.33	1.00													
21	0.53	0.20	0.19	0.20	0.13	0.33	0.33	0.27	0.44	0.33	0.38	0.40	0.27	0.26	0.40	0.33	0.13	0.13	0.47	0.40	1.00												
22	0.39	0.13	0.19	0.19	0.26	0.19	0.32	0.26	0.36	0.19	0.30	0.32	0.39	0.38	0.32	0.32	0.19	0.19	0.39	0.45	0.58	1.00											
23	0.59	0.29	0.34	0.41	0.29	0.47	0.47	0.35	0.50	0.41	0.50	0.53	0.24	0.23	0.53	0.53	0.29	0.40	0.53	0.35	0.59	0.40	1.00										
24	0.39	0.39	0.31	0.39	0.32	0.26	0.39	0.19	0.30	0.26	0.36	0.39	0.32	0.31	0.39	0.39	0.71	0.50	0.39	0.19	0.19	0.38	0.34	1.00									
25	0.38	0.19	0.30	0.31	0.31	0.50	0.69	0.44	0.65	0.50	0.29	0.38	0.63	0.61	0.38	0.25	0.44	0.24	0.63	0.63	0.38	0.48	0.33	0.42	1.00								
26	0.50	0.31	0.48	0.38	0.38	0.44	0.44	0.38	0.41	0.38	0.41	0.50	0.19	0.18	0.50	0.50	0.31	0.42	0.44	0.38	0.38	0.42	0.67	0.36	0.29	1.00							
27	0.27	0.33	0.32	0.33	0.33	0.33	0.33	0.33	0.50	0.40	0.38	0.47	0.40	0.39	0.47	0.33	0.33	0.26	0.40	0.40	0.47	0.39	0.35	0.26	0.31	0.31	1.00						
28	0.32	0.32	0.38	0.39	0.39	0.32	0.32	0.26	0.24	0.32	0.24	0.52	0.39	0.44	0.52	0.39	0.58	0.50	0.39	0.26	0.19	0.38	0.29	0.69	0.42	0.36	0.32	1.00					
29	0.45	0.39	0.38	0.26	0.26	0.45	0.45	0.32	0.48	0.45	0.48	0.52	0.19	0.19	0.52	0.26	0.32	0.31	0.45	0.26	0.45	0.25	0.57	0.38	0.30	0.42	0.32	0.19	1.00				
30	0.27	0.40	0.32	0.33	0.33	0.27	0.27	0.20	0.19	0.27	0.31	0.47	0.33	0.39	0.47	0.33	0.60	0.52	0.33	0.13	0.20	0.26	0.18	0.65	0.31	0.25	0.27	0.84	0.26	1.00			
31	0.13	0.20	0.39	0.33	0.27	0.27	0.20	0.33	0.31	0.33	0.25	0.40	0.20	0.19	0.40	0.20	0.27	0.26	0.27	0.33	0.47	0.39	0.29	0.26	0.31	0.31	0.53	0.39	0.19	0.20	1.00		
32	0.27	0.33	0.32	0.40	0.27	0.47	0.47	0.40	0.56	0.53	0.63	0.53	0.27	0.26	0.53	0.20	0.40	0.26	0.60	0.27	0.40	0.32	0.35	0.52	0.38	0.25	0.53	0.32	0.45	0.40	0.40	1.00	
⋮																																	⋱

Figura 2. Representação das relações de vínculo genético entre as amostras de soja com base nas estimativas de distância genética par-a-par inferidas pela proporção de alelos em comum *(band coefficient)*, submetidas a análise de agrupamento pelo método de *UPGMA*.




0.52
0.27
0.01
-0.24
C2
0.51
0.27
0.03
-0.21
-0.46
C3
0.46
0.23
0.00
-0.23
-0.45
C1

Results - Soybean

✓ No subdivisions of samples in clusters

clear genetic similarities between some groups of samples BUT
apparent narrow genetic base of brazilian soybean germplasm limits
precise reconstruction of relations of genetic linkages of all accessions

✓ Group of selected markers allowed differentiation of samples

✓ Soybean: autogamous and diploid - bulk samples

genotype in each loci is tippically homozigote (one allele duplicated)
When two distinct alleles are observed: evidence of residual heterozigose or mixed lines – in this case, additional tests are needed

✓ Genetic Similarity - conclusions (based on the multiloci genetic profiles)

525 unique genetic profiles – 94,77% of samples
Undistinguishable samples
Very similar samples
Evaluated case by case – EDVs, parentals, siblings, etc.

RICE

SSR Markers - Rice

- **RM252** Seqüência repetitiva em tandem de dinucleotídeo CT; cromossomo 4
- **RM224** Seqüência complexa repetitiva em tandem do tipo (AAG)8(AG)13; cromos. 11
- **OG44** Seqüência repetitiva em tandem de dinucleotídeo AG; cromossomo 3
- **RM248** Seqüência repetitiva em tandem de dinucleotídeo CT; cromossomo 7
- **OS19** Seqüência repetitiva em tandem de dinucleotídeo AG; cromossomo 6
- **OG106** Seqüência repetitiva em tandem de dinucleotídeo AG; cromossomo 9
- **OG81** Seqüência repetitiva em tandem de dinucleotídeo AG; cromossomo 1
- **RM263** Seqüência repetitiva em tandem de dinucleotídeo CT; cromossomo 2
- **RM420** Seqüência repetitiva em tandem de tetranucleotídeo AAAT; cromossomo 7
- **RM418** Seqüência repetitiva em tandem de trinucleotídeo ATT; cromossomo 7
- **RM475** Seqüência repetitiva em tandem de tetranucleotídeo TACT; cromossomo 2
- **RM171** Seqüência repetitiva em tandem de tetranucleotídeo GATG; cromossomo 10
- **RM481** Seqüência repetitiva em tandem de dinucleotídeo CAA; cromossomo 7
- **OG61** Seqüência repetitiva em tandem de dinucleotídeo AG; cromossomo 6
- **RM335** Seqüência repetitiva em tandem de trinucleotídeo CTT; cromossomo 4
- **RM105** Seqüência repetitiva em tandem de trinucleotídeo CCT; cromossomo 9

Tabela 1. Perfil genético multiloco de amostras de arroz fornecidas pelo LADIC/MAPA, analisadas em 16 locos microssatélites. São apresentados os valores em pares de base (pb) de cada um dos dois alelos observados em cada loco nas amostras analisadas, após conferência dos genótipos em regime de análise de prova e contraprova.

Loco	LADIC	alelo 1	alelo 2	Loco	LADIC	alelo 1	alelo 2
RM248	1	99.83	99.83	**RM335**	1	113.71	113.71
RM248	2	83.36	83.36	**RM335**	2	136.86	136.86
RM248	3	83.41	83.41	**RM335**	3	158.61	158.61
RM248	4	93.65	93.65	**RM335**	4	99.93	99.93
RM248	5	99.82	99.82	**RM335**	5	146.01	146.01
RM248	6	89.69	99.86	**RM335**	6	113.55	113.55
RM248	7	99.86	99.86	**RM335**	7	99.67	99.67
RM248	8	89.55	89.55	**RM335**	8	146.12	146.12
RM248	9	83.10	93.63	**RM335**	9	108.11	108.11
RM248	10	99.83	99.83	**RM335**	10	113.69	113.69
RM248	11	89.45	89.45	**RM335**	11	146.23	146.23
RM248	12	81.05	99.79	**RM335**	12	99.93	146.26
RM248	13	89.46	89.46	**RM335**	13	99.58	99.58
RM248	14	83.42	83.42	**RM335**	14	158.63	158.63
RM248	15	83.38	83.38	**RM335**	15	155.42	155.42
RM248	16	89.53	89.53	**RM335**	16	145.98	145.98
RM248	17	99.78	99.78	**RM335**	17	140.02	140.02
RM248	18	89.68	89.68	**RM335**	18	146.18	146.18
RM248	19	89.64	89.64	**RM335**	19	99.70	99.70
RM248	20	89.63	89.63	**RM335**	20	146.18	146.18
	⋮				⋮		
RM248	69	89.69	89.69	**RM335**	69	146.17	146.17
RM248	70	89.67	89.67	**RM335**	70	146.10	146.10
RM248	71	89.71	89.71	**RM335**	71	146.09	146.09
RM248	72	89.65	89.65	**RM335**	72	146.17	146.17
RM248	73	89.68	89.68	**RM335**	73	146.16	146.16
RM248	74	89.64	89.64	**RM335**	74	146.18	146.18
RM248	75	99.85	99.85	**RM335**	75	99.61	99.61
RM248	76	79.13	79.13	**RM335**	76	139.88	139.88
RM248	77	93.79	93.79	**RM335**	77	99.68	99.68
RM248	78	89.69	89.69	**RM335**	78	99.69	99.69
RM248	Genomax	93.69	93.69	**RM335**	Genomax	108.12	108.12
OG44	1	150.17	150.17	**RM420**	1	184.03	184.03
OG44	2	163.01	163.01	**RM420**	2	183.87	183.87
OG44	3	150.29	150.29	**RM420**	3	184.05	184.05

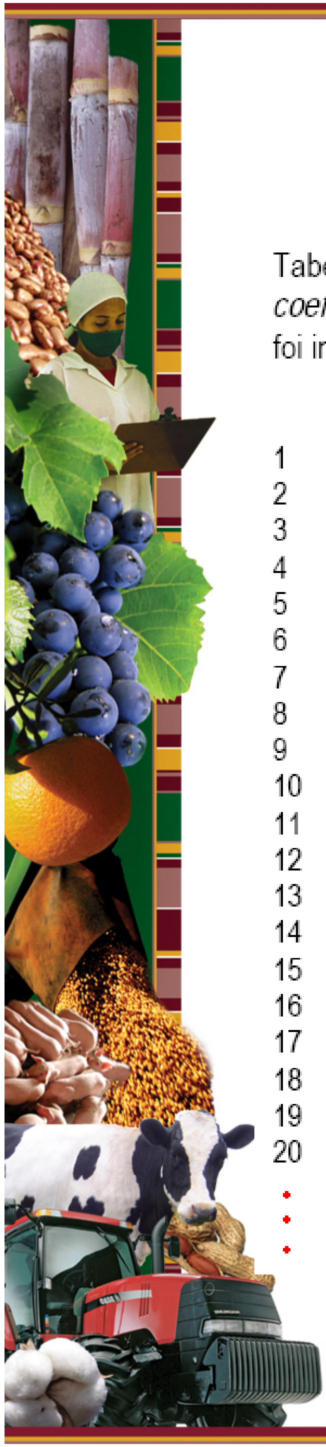
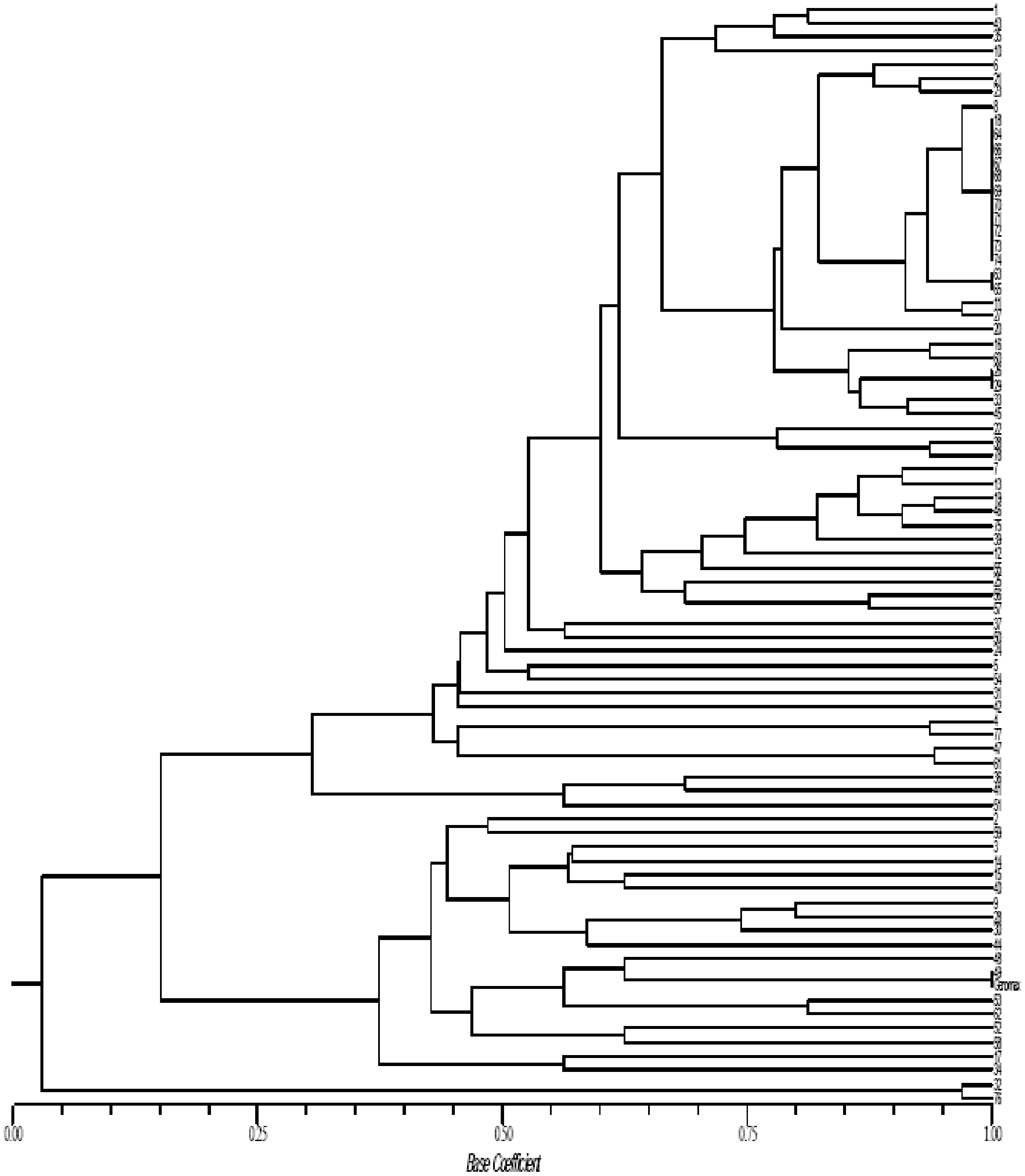
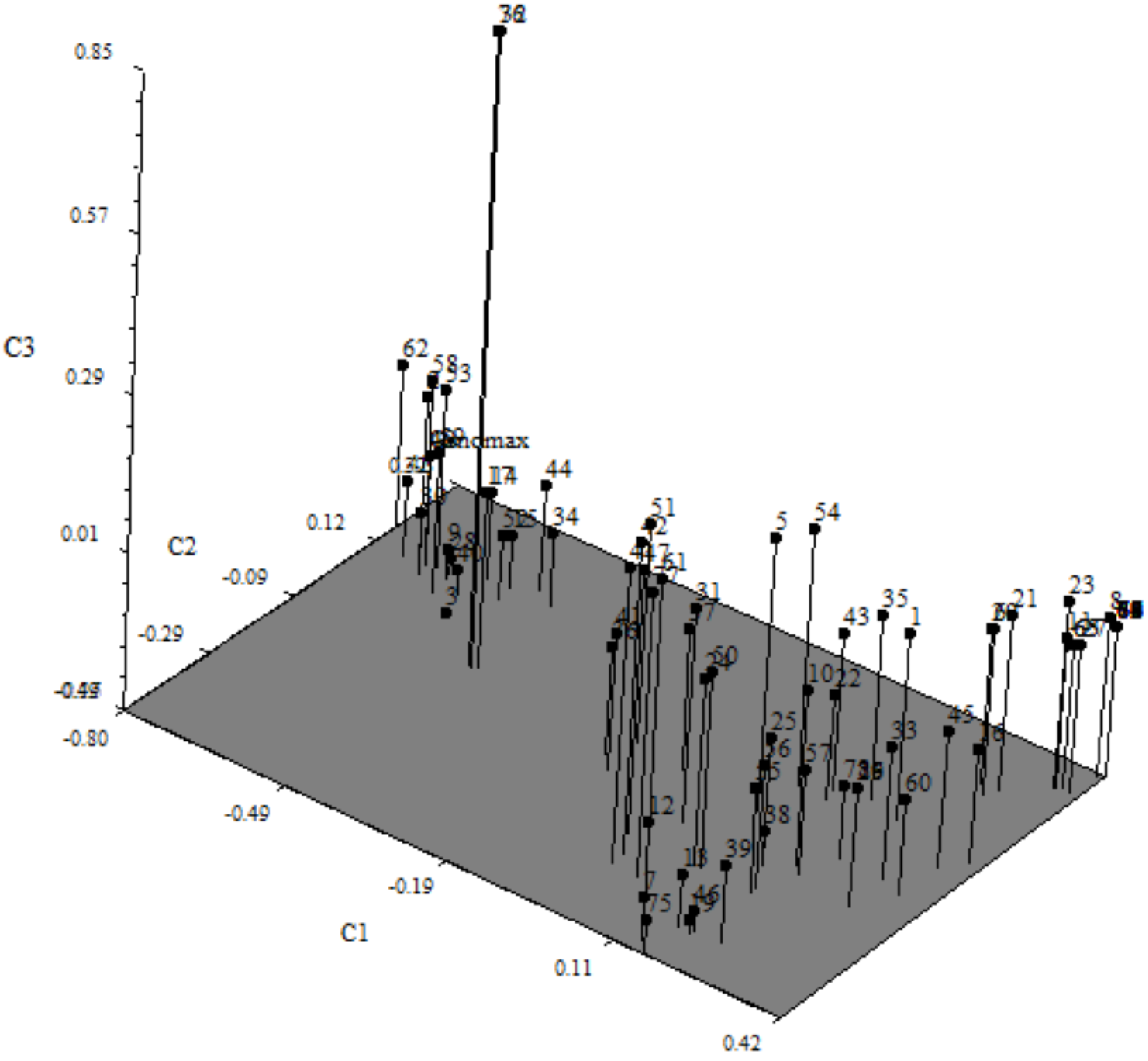


Tabela 2. Estimativas de distância genética par-a-par com base na proporção de alelos em comum (*band coefficient*) entre as 78 amostras de arroz utilizadas na análise. A amostra de arroz "Genomax" também foi incluída na análise.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	…
1	1.00																				
2	0.06	1.00																			
3	0.23	0.40	1.00																		
4	0.44	0.06	0.11	1.00																	
5	0.50	0.13	0.11	0.50	1.00																
6	0.85	0.06	0.17	0.30	0.42	1.00															
7	0.55	0.06	0.28	0.42	0.42	0.53	1.00														
8	0.67	0.06	0.17	0.36	0.55	0.76	0.53	1.00													
9	0.17	0.40	0.53	0.11	0.11	0.17	0.22	0.17	1.00												
10	0.74	0.06	0.21	0.40	0.46	0.72	0.61	0.61	0.16	1.00											
11	0.67	0.06	0.17	0.36	0.48	0.82	0.53	0.88	0.17	0.61	1.00										
12	0.46	0.10	0.24	0.41	0.46	0.45	0.75	0.50	0.19	0.57	0.50	1.00									
13	0.44	0.06	0.23	0.44	0.38	0.48	0.91	0.55	0.17	0.51	0.55	0.67	1.00								
14	0.19	0.56	0.57	0.06	0.13	0.18	0.18	0.18	0.46	0.17	0.18	0.15	0.13	1.00							
15	0.13	0.50	0.57	0.06	0.13	0.18	0.24	0.24	0.51	0.17	0.18	0.21	0.19	0.56	1.00						
16	0.69	0.06	0.23	0.44	0.56	0.73	0.67	0.85	0.17	0.69	0.85	0.62	0.69	0.19	0.19	1.00					
17	0.19	0.31	0.29	0.06	0.25	0.24	0.30	0.18	0.46	0.23	0.18	0.26	0.25	0.31	0.50	0.19	1.00				
18	0.69	0.06	0.17	0.38	0.50	0.79	0.55	0.97	0.17	0.63	0.91	0.51	0.56	0.19	0.25	0.88	0.19	1.00			
19	0.48	0.06	0.28	0.42	0.36	0.53	0.88	0.59	0.22	0.61	0.59	0.75	0.91	0.18	0.24	0.73	0.24	0.61	1.00		
20	0.63	0.06	0.23	0.38	0.50	0.67	0.48	0.79	0.23	0.63	0.79	0.46	0.50	0.19	0.25	0.81	0.13	0.81	0.55	1.00	
⋮																					⋱


0.00
0.25
0.50
0.75
1.00
Base Coefficient


36
0.85
0.57
C3
0.29
0.01
-0.45
-0.80
C2
0.12
-0.09
-0.29
-0.49
-0.19
0.11
0.42
C1
62
58
53
Penomax
44
17
34
51
5
54
31
43
35
1
21
23
8
50
10
22
25
16
57
45
33
60
12
38
39
13
7
75
46
3
9

Results - Rice

- ✓ Subdivisions of samples in two clusters

  probably subgroups indica (irrigated) and japonica (dry lands),
  except 2 samples (LADIC 32 and 76) - hybrids between the two subgroups?

- ✓ Group of selected markers allowed differentiation of samples

- ✓ Rice: autogamous and diploid - bulk samples

  genotype in each loci is tippically homozigote (one allele duplicated)
  When two distinct alleles are observed: evidence of residual heterozigose or mixed lines – in this case, additional tests are needed

- ✓ Genetic Similarity - conclusions (based on the multiloci genetic profiles)

  66 unique genetic profiles – 83,54% of samples
  Undistinguishable samples (genetic similarity > 0,95)
  Very similar samples
    Evaluated case by case – EDVs, parentals, siblings, etc.

Practical uses of the system

- PVP enforcement and Seed Inspection
- Identification of characteristics
- Identify mixture in seed samples
- To check purity of DNA samples
- To Check declared parental information
- Resulting in improvement of the effectiveness and efficiency of the DUS testing process

Example

BRS Carla
Depigmentation due to
high temperature and
hydric stress

BRS Celeste
Depigmentation due to
high temperature and
hydric stress

BRS Celeste - typical (black) and altered color of hilum

BRS Carla - typical (brown) and altered color of hilum

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Rice

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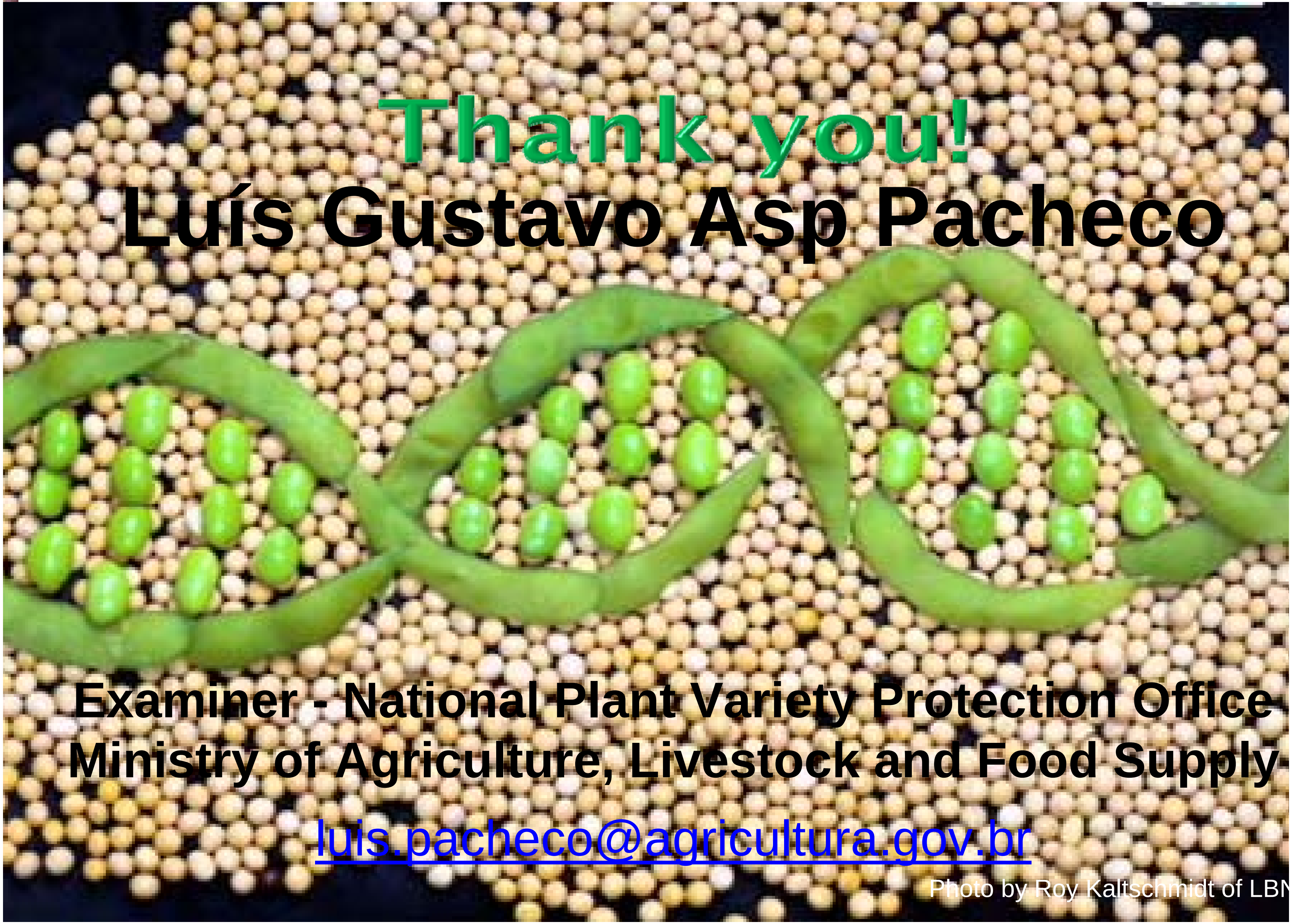

Thank you!

Luís Gustavo Asp Pacheco

Examiner - National Plant Variety Protection Office
Ministry of Agriculture, Livestock and Food Supply

luis.pacheco@agricultura.gov.br

Photo by Roy Kaltschmidt of LBN