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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

TECHNICAL WORKING PARTY FOR VEGETABLES

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WORK IN RELATION TO THE USE OF MOLECULAR MARKERS

Document prepared by experts from the European Community, Netherlands, Spain and the International Seed Federation

1. The Technical Working Party for Vegetables (TWV), at its fortieth session, held in Guanajuato, Guanajuato State, Mexico, from June 12 to 16, 2006, agreed that it would be useful for experts to provide information on work in relation to the use of molecular markers, in particular in relation to disease resistance. Experts from the European Community (tomato), France (tomato, melon, chicory, shallots), Netherlands (tomato, lettuce, asparagus), Spain (pepper and tomato) and the International Seed Federation (ISF) (tomato in relation to essentially derived varieties) agreed to prepare documents for information and discussion at the forty-first session of the TWV. The information provided by the European Community Netherlands, Spain and ISF is presented in the annexes to this document.

[Annexes follow]

ANNEX I

EUROPEAN COMMUNITY

DEVELOPMENT AND EVALUATION OF MOLECULAR MARKERS LINKED TO DISEASE RESISTANCE GENES FOR TOMATO DUS TESTING (OPTION 1A)

Objectives of the project

The objective of this two-year-long CPVO-funded project between six project partners in the Netherlands (PRI - coordinator, Naktuinbouw), France (GEVES, INRA) and Spain (OEVV, INIA) is to develop and evaluate an option 1(a)[1] approach for the asterisked (obligatory) disease resistance characteristics in the applicable CPVO tomato protocol TP/44/2, namely:

- *Meloidogyne incognita*
- *Verticillium dahliae*
- *Fusarium oxysporum* f.sp. *lycopersici* Race 0
- *Fusarium oxysporum* f.sp. *lycopersici* Race 1
- Tomato Mosaic Virus – Strain 0
- Tomato Mosaic Virus – Strain 1
- Tomato Mosaic Virus – Strain 2
- Tomato Mosaic Virus – Strain 1-2 (subsequently abandoned)

For the development, existing mapping and sequence information is being used. Marker assays are being evaluated for robustness and reproducibility. Results from marker analysis will be compared to phenotypic characterizations using varieties that are in ongoing DUS trial. Conclusions will be drawn and recommendations made on the feasibility of option 1(a)[1].

Results thus far

The first year of the project (2006) has completed the following activities:

1. Literature and database searches
2. Selection of genes
3. Marker assays selection and development of assays
4. Initial evaluation of assays

[1] See document BMT/10/6

The following disease resistance genes have been identified and progress made in the development of the assays:

- *Verticillium* genes Ve1 and Ve2
- Tomato Mosaic Virus Tm1 (linked marker)
- Tomato mosaic Virus Tm2 and $Tm2^2$
- *Melolodogyne incognita* Mi1-2
- *Fusarium* I locus (linked marker)
- *Fusarium* I2 locus

Continuing work

In the second year (2007) of the project, the assays developed will be tested by all the project partners for robustness; DNA from common knowledge tomato varieties used for the development of the tests (at least one resistant and one susceptible variety per test) will be assessed. Assuming that the robustness tests are successful, evaluation of marker assays will be carried out on 20 candidate tomato varieties currently undergoing DUS testing in the three partner countries, as well as a few varieties for which DUS disease tests gave rise to questions in previous years, thereby being to make a direct comparison with the traditional phenotypic characterization (field or laboratory based) for tomato disease resistance.

[Annex II follows}

ANNEX II

PROJECT FOR THE EVALUATION OF THE USE OF DNA TECHNIQUES IN TOMATO

In 2003, a project was carried out with 91 tomato varieties. The 91 varieties were selected in order to cover a large part of the tomato assortment and also to be able to compare different aspects of variety examination. The aspects were:

1. uniformity
2. stability
3. variety typification for DUS (identity and inspection)
4. specific markers (e.g. resistance) – UPOV option 1[2]
5. management of reference collection – UPOV option 2[3]
6. narrowly related varieties (plagiate and EDVs)

The varieties were examined on 70 morphological characteristics from the UPOV Test Guidelines TG/44/10. The varieties were also analyzed using three different molecular techniques, viz. AFLP, STMS and SNP.

For the different aspects of variety examination the following conclusions could be drawn.

Uniformity

Because of cost aspects, the 10 individual samples of the varieties were pooled in the DNA analyses. Therefore, no conclusions could be drawn about uniformity. However, from other experiments it could be concluded that at least 25 % of also hybrid varieties are not uniform on DNA markers.

Stability

For some varieties (open-pollinated (OP) varieties as well as F1-hybrids) maintained by several companies the instability on the morphological level could be confirmed using the DNA techniques. However there were differences in the performance of the techniques. In two cases, SNP was too discriminative, in one case STMS was not discriminative enough. AFLP seems the best technique for this purpose.

Variety typification in groups/types and management of reference collection

The morphological analysis fixed in a dendrogram was compared with the three different DNA analyses also fixed in three different dendrograms.

The general conclusions which could be drawn were as follows:

The STMS technique most frequently supported the morphological analysis. AFLP was a good second best. SNP supported the morphological conclusions less than the other two techniques. It should be noticed, however, that only relatively few SNP markers were used.

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2 See document BMT/10/6

3 "Calibration of threshold levels for molecular characteristics against the minimum distance in traditional characteristics"

Characteristic specific markers

For this purpose varieties with (almost) only difference in resistance were analyzed. In most cases, all three DNA techniques could distinguish the varieties in these cases. Only in one case could no difference be found with STMS and SNP, but AFLP could even distinguish the varieties in that case. Varieties with other than red-colored fruits were also analyzed. In some cases, it could be concluded that the variety with yellow or orange fruit was related to the relative variety with red fruits. In most cases, however, no specific relation could be detected.

Varieties with specific (resistance) characteristics (high lycopene, Leveillula, TYLCV and Oidium resistance) were also analyzed. In most cases the resistant varieties were more distinct from their susceptible versions in DNA analyses than in the morphological analysis. This can be explained by the fact that, with introgression from resistance genes from wild relatives, other DNA with relatively many markers is crossed in.

In one case, no difference was found between two varieties from a different type and with a different resistance. That case illustrates that use of only DNA techniques in variety examination is very risky. In this project no resistance-specific markers were used. That was the subject of a specific Community Plant Variety Office (CPVO) project.

Narrowly related varieties

This is the subject of plagiaristic cases and EDVs. But also support for decisions in DUS on morphological basis by DNA marker techniques. Can a difference in DNA not automatically lead to distinct varieties if they are morphologically identical? The following conclusions could be drawn:

In some cases, no differences were found with DNA-marker techniques, whereas, on the basis of morphological characteristics, varieties were distinct. It seemed that only a narrow genetic difference was the basis for this morphological difference. In these cases of narrowly-related varieties, AFLP was the best technique to differentiate between varieties. STMS and SNP could, in general, discriminate less in these cases. However, with AFLP, some morphologically distinct varieties could not be discriminated. This illustrates again the risk of using only DNA techniques. In some cases, AFLP was even the only technique which could not discriminate between morphologically clearly distinct varieties, whereas, with SNP and STMS differences could be found.

In general, the most important conclusions that could be drawn from this project were as follows.

(1) Various examples show that a decision on the basis of only DNA marker techniques is risky. Combination with a morphological examination is necessary.

(2) Evaluation of stability on the basis of DNA marker techniques has its restrictions because it concerns (mostly) unselected DNA markers.

(3) In cases of relation of varieties (e.g. management of reference collection and support of morphological DUS examination) STMS seems to be the best technique. This techniques supports best what is expected on the basis of traditional morphological DUS research. AFLP is second best and SNP clearly supports morphology at the lowest level.

(4) Questions of narrowly-related varieties and EDVs can best be solved with the AFLP technique.

(5) In cost aspects, DNA techniques are still much more expensive compared to the traditional field trials to carry out DUS research in tomato. Also, because of the relatively large number of resistance characteristics in tomato, the traditional morphological approach in DUS research in tomato poses no problems at the moment.

USE OF DNA MARKERS IN ASPARAGUS

In several cases of inspection and complaints in asparagus (only in male hybrids) the AFLP DNA marker technique was used as the basis to decide.

In general, it could be concluded that this technique was useful in these cases. The DNA pattern of 12 plants of samples of varieties was analyzed with AFLP technique. In general, there were slight differences in the DNA pattern between the individuals within a variety/sample. However, the differences between the varieties were much larger than within the varieties. Almost always, individuals of a variety within one sample clustered in the same cluster. Individuals of samples of the same variety but originating from different trials/locations also always clustered in the same cluster.

By drawing a dendrogram, it could easily be concluded if a complaint of, for example, interchange of samples or doubt of varietal trueness of a sample was justifiable or not.

PROJECT FOR THE USE OF DNA TECHNIQUES TO EXAMINE UNIFORMITY IN LETTUCE

In 2005 and 2006, a project was carried out with the aim of evaluating whether AFLP is a suitable technique to examine uniformity in lettuce and to see whether these molecular data correlate with the morphological data. Lettuce is a uniform crop due to its self-pollination. However, we sometimes see that applications for listing or plant breeders' rights are not sufficiently uniform, i.e. on color, time of maturity, resistances. It is interesting to know whether this insufficient uniformity is based on genetic differences between plants or due to external factors such as moisture at time of planting.

In 2005, 5 lettuce varieties/applications were tested with AFLP. Per variety/application 64 individual plants were sampled. Morphological data from the same samples of the varieties/applications were gathered in trials in 2002-2004. Three AFLP primer combinations were used, they gave 89 markers. Results were presented in a dendrogram and the following conclusions could be drawn:

1	Variety very uniform on morphology	Very uniform on AFLP
2	Variety not uniform on morphology (division of leaf, blistering, color)	Very heterogenic on AFLP
3	Application insufficiently uniform on morphology (heading, size)	Very heterogenic on AFLP, all individuals give a different pattern
4	Application insufficiently uniform on morphology (color)	Heterogenic on AFLP, 8 sub clusters
5	Variety insufficiently uniform on resistance (25% susceptible, 75% resistant), uniform on morphology	AFLP shows one large cluster, but 6 individuals with a different pattern

In 2006, in total, 6 samples of 4 lettuce varieties/applications were tested with AFLP. Two samples were the same as in the 2005 test. Per sample, 64 individual plants were sampled. Morphological data were gathered on the same 64 plants in the trial in 2006. The same AFLP primer combinations were used as in 2005, now giving 70 markers. Results were presented in a dendrogram and the following conclusions could be drawn:

3a	Application insufficiently uniform on morphology (heading, size)	Very heterogenic on AFLP, all individuals give a different pattern
3b	Newly produced seed lot from same application: sufficiently uniform	Heterogenic on AFLP, but less different patterns than in 3a
4	Application insufficiently uniform on morphology (color)	Heterogenic on AFLP, 4 sub clusters
6a	Application insufficiently uniform on morphology (color)	Uniform on AFLP
6b	Newly produced seed lot from same application: insufficiently uniform on morphology (color)	Uniform on AFLP, but 1 individual with different pattern
7	Application insufficiently uniform on resistance, uniform on morphology	Uniform on AFLP

From the results in these two years the following can be concluded:

- Large differences in uniformity of varieties can be observed with AFLP. “Young” varieties/applications may show the largest heterogeneity. Selection of plants and the production of new seed lots give an improvement on morphology and on AFLP pattern.
- If a variety is very uniform on morphology or very clearly not uniform (on many morphological characteristics) this will also be seen in the AFLP results.
- If only AFLP data are used, both too positive as too negative conclusions may be drawn, not corresponding with morphological data.
- No correlation between leaf color and AFLP (the plants of 4, 6a and 6b indicated as off-type on color cannot be identified with AFLP).
- No correlation between resistance and AFLP (5, 7). The same seeds can never be used in a resistance test on seedlings and in an AFLP test (susceptible plants will die).

- AFLP analysis is reproducible, small changes in the method give the same result over years.
- AFLP technique is expensive. The technique has many different steps, is not suitable for high throughput and scoring of data is done by hand. Therefore, less suitable for uniformity studies where a large number of individuals have to be tested.

[Annex III follows]

ANNEX III

ACTUAL SITUATION OF BIOMOLECULAR TECHNIQUES AS SUPPORT OF THE DUS TEST OF CAPSICUM ANNUUM VARIETIES IN SPAIN

There are two lines of work:

1. **Use of a CAPS marker linked to the Tsw gen (resistance to TSWV virus)**
(Palloix and others. Genome 43: 137-142 (2000)
Work developed in the Department of Biotechnology and the UGP of INIA (Madrid) since 2003 to now

2. Use of 27 microsatellites to find genetic polymorphisms in the pepper varieties.
Work developed in the Department of Biotechnology of the Polytechnic University of Valencia in cooperation with INIA since 2004 to now

Use of a CAPS marker linked to the Tsw gen

3. Correspond to the option 1(a)[4] according the UPOV BMT criteria: "*Use of molecular characteristics which are directly linked to traditional characteristics*"

4. Using a pair of primers, a fragment of 568 bp of the DNA is amplified by PCR. The amplification product is treated with a restriction enzyme, giving a pattern of fragments visualized by electrophoresis. There is a very high correlation between certain patterns and the presence of the possible alleles (R resistant, r susceptible). It is a co-dominant marker, so the homozygous can be distinguished from the heterozygous. The genetic distance from the marker to the Tsw locus is estimated by Palloix in 0,87+/-0,62 cM.

5. To increase the consistency of the test, 2 other restriction enzymes are used independently. Each of them gives also well correlated results. (Picture 1)

6. Up to now, we have tested approximately 70 varieties. The results coincide with the applicant declaration except in 2 varieties. We have not compared the results with that of biological resistance tests, because we have not still achieved satisfactory results in the biological test for this virus.

Practical use

7. As it is a case of option 1(a), we predict, from the results, the resistance to TSWV. We give the results an official value, except in the rare cases of no coincidence with the breeder declaration. In these cases, the resistance is not described until the biological test be perfectly achieved. The homozygosity or heterozygosity of the gene expression is not considered for distinctness.

Classify pepper varieties with a set of 27 SSRs markers (microsatellites)

8. Microsatellites are currently recognized as the most useful markers for genotypic identification, because of the repeatability of the results in different labs, and because of the co-dominant markers so that homozygous can be distinguished from the heterozygous.

[4] See document BMT/10/6

9. Our set of markers have been selected from a wider set of 119 markers, 76 of them described in the bibliography and 43 designed by us after consulting the existing genomics data bases of Capsicum.

10. The criteria for screening the markers were:

Reject the markers if any of the following:
- Difficulties in the amplification
- Monomorphism
- Redundant results with another marker
- High variability intra-variety

11. The initial work was developed in a set of 25 varieties (including hybrids and open-pollinated varieties) of the Spanish National List and reference collection, selected to represent the main part of the varietal types. Five plants per variety were individually analyzed.

Assessment of the uniformity of the markers

12. Arbitrarily we established previously:

13. A marker should be considered not uniform if the results were not uniform in more than 4 varieties (out of the 25). The marker should be rejected.

14. The result of a marker in one variety should be considered not uniform if it was different in more than 1 plant (out of the 5).

15. All the 27 markers were uniform enough according to the previously indicated criteria.
- 10 markers were perfectly uniform for the 5 plants of the 25 varieties.
- 5 markers were uniform for all varieties, but in some of them 1/5 plants were different
- 7 markers were not uniform for 1/25 varieties
- 3 markers were not uniform for 2/25 varieties
- 2 markers were not uniform for 3/25 varieties

Assessment of the uniformity of the varieties

16. 10 varieties were perfectly uniform for the 27 markers.
- 5 varieties were uniform for the 27 markers, but 1/5 plants is different for one or more marker
- 5 varieties were not uniform for the 1/27 markers
- 2 varieties were not uniform for the 2/27 markers
- 2 varieties were not uniform for the 3/27 markers
- 1 variety was not uniform for the 4/27 markers

17. The varieties less uniform are open varieties or hybrid of very special types with few varieties belonging to them.

Assessment of the distinctness of the varieties

18. Only one pair of varieties was not distinct. This pair is clearly distinct morphologically.

19. The second phase of the work is to classify a bigger collection of 200 varieties, according to the 27 markers. May be finished at the end of 2007.

20. The third phase of the project (2008, 2009) is to develop new markers to intend achieve the distinctness of the 225 varieties, and to have a high probability of distinguish any other variety.

Practical use of this line of work

21. Currently the work is unfinished and is not possible to foresee the usefulness of this tool.

22. The general idea is to support the field work with a rich information of molecular markers.

23. The calibration of the varietal distances with molecular and morphological markers (option 2) will be done at the end of the second phase, but we do not think that will be very useful.

24. We expect at least the following practical uses:

To check the correct identity of new samples of seeds of the same variety for the maintenance of the reference collection.

To support the selection of the more appropriate varieties for comparison, (basically made by morphological and resistance characteristics).

To support the taking of decisions about distinctness in doubtful cases in DUS testing (frequent in certain groups of this crop)

To support the report about varietal identity of seed samples in cases of complaint.

25. We attach a table and a graphic that give a view of the results of the first phase of the work on a collection of 25 selected varieties.

Picture 1: CAPS marker linked to Tsw gen in Capsicum. Electrophoresis of the digestion product of an amplified fragment of DNA with 3 restriction enzymes in a set of pepper varieties.

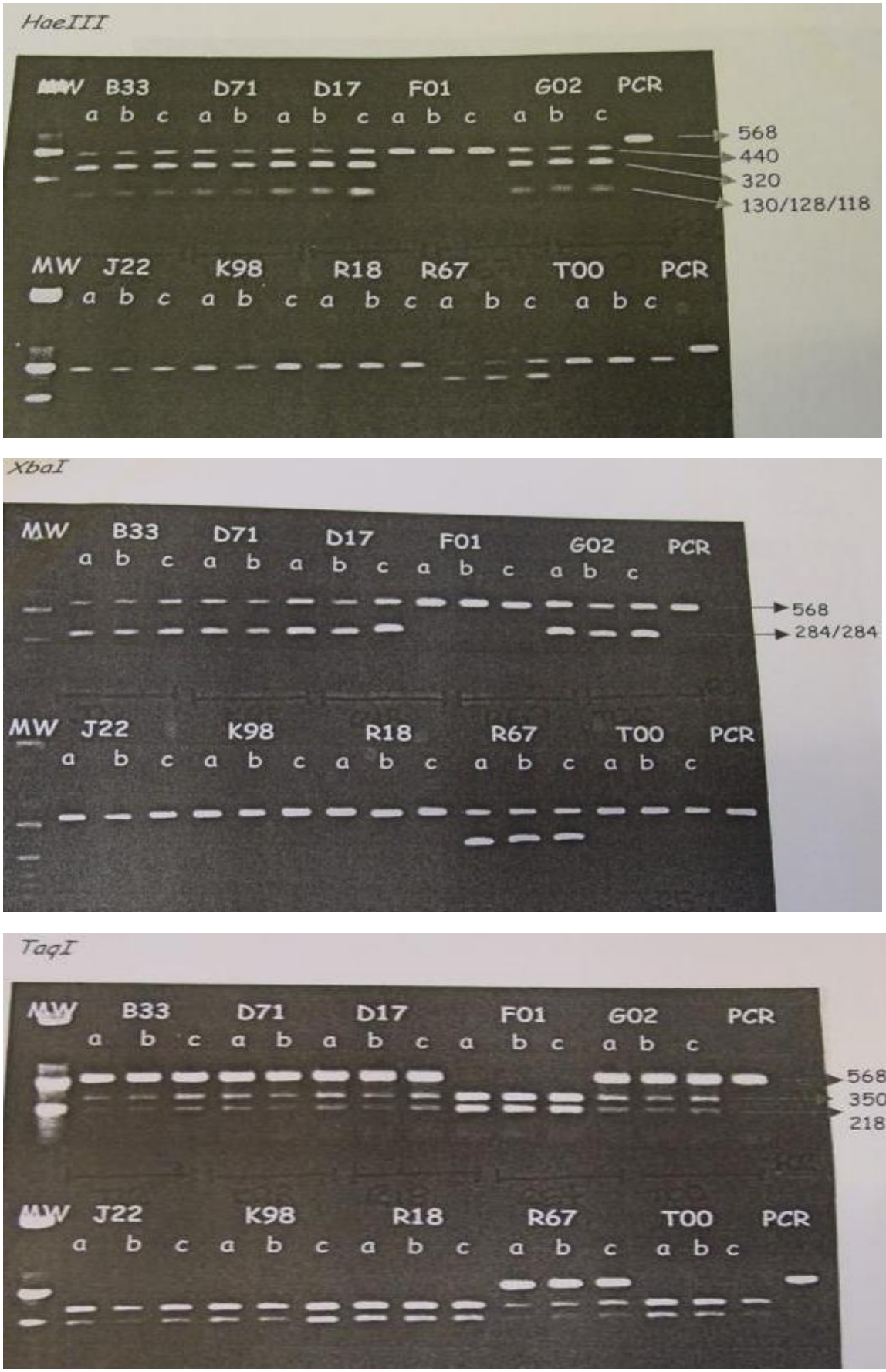
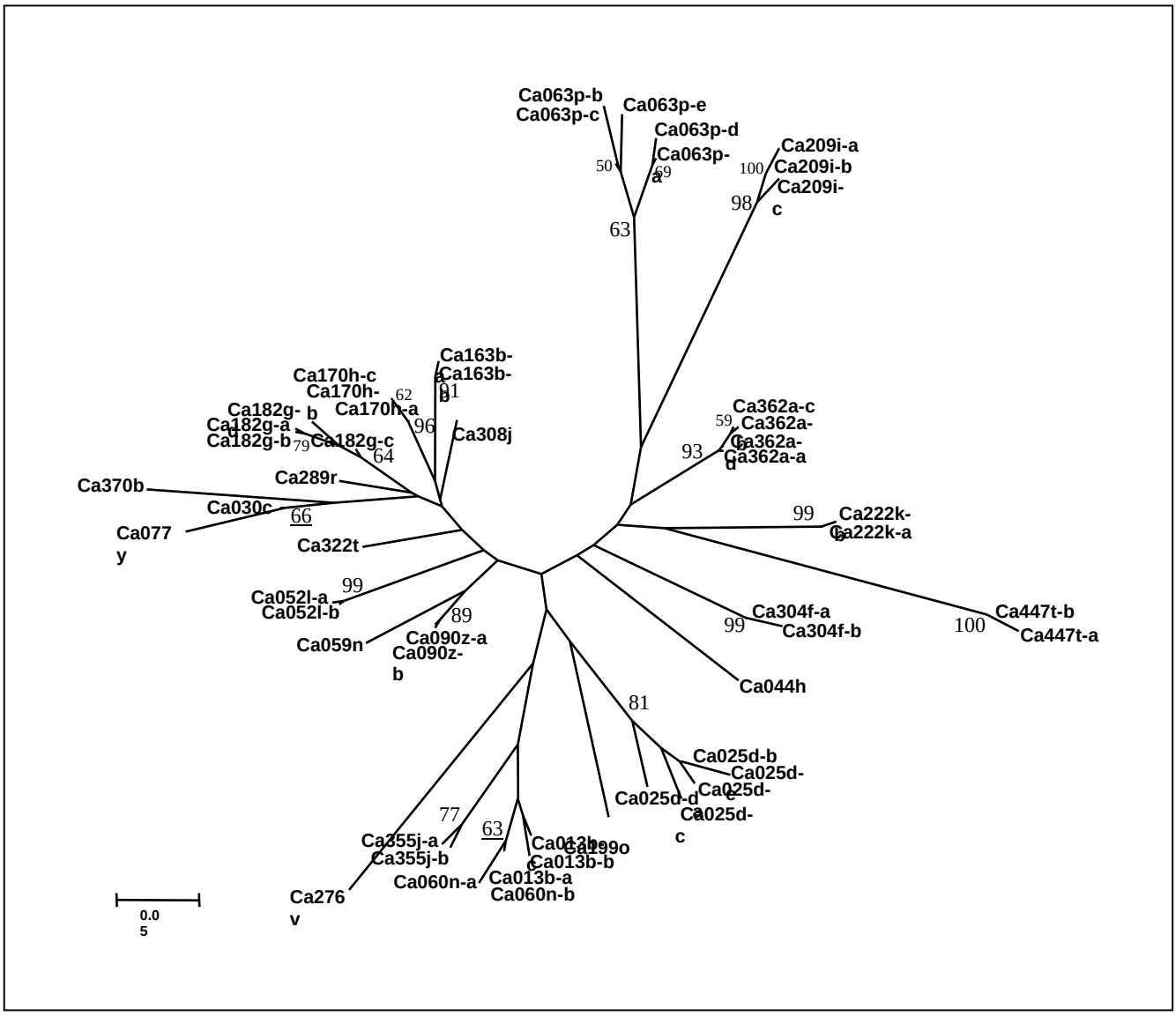


Table 1: Diversity parameter of 27 microsatellite markers analyzed in 5 plants per variety in 25 varieties of *Capsicum annuum*

Marker	Average allelic size (pb)	Alleles Number	Genotypes Number	Genetic Diversity	HETEROZYGOSITY	PIC	Genotype Diversity
E1	189	5	10	0.67	0.27	0.62	0.78
E2	212	2	3	0.11	0.04	0.11	0.15
E3	152	2	2	0.04	0.04	0.04	0.08
E4	193	4	5	0.18	0.07	0.17	0.21
E5	256	3	3	0.15	0.00	0.15	0.15
E6	218	2	3	0.44	0.05	0.34	0.49
E7	204	3	4	0.21	0.15	0.19	0.34
E8	371	2	2	0.04	0.04	0.04	0.08
E9	207	2	3	0.15	0.08	0.14	0.22
E10	182	4	5	0.67	0.27	0.61	0.78
E11	186	2	3	0.04	0.01	0.04	0.05
E12	275	3	5	0.61	0.12	0.54	0.69
E13	212	3	4	0.47	0.14	0.38	0.58
E14	249	2	3	0.48	0.14	0.37	0.60
E15	164	3	5	0.51	0.23	0.41	0.64
E16	315	3	5	0.46	0.28	0.40	0.63
E17	261	2	2	0.10	0.11	0.10	0.19
E18	221	3	4	0.25	0.12	0.23	0.35
E19	132	3	5	0.28	0.14	0.26	0.39
E20	177	3	6	0.39	0.13	0.36	0.47
E21	148	3	4	0.38	0.10	0.32	0.48
E22	265	2	2	0.08	0.00	0.08	0.08
E23	123	2	3	0.40	0.15	0.32	0.52
E24	127	6	10	0.75	0.39	0.72	0.85
E25	220	2	3	0.49	0.21	0.37	0.65
E26	177	5	7	0.33	0.16	0.32	0.43
E27	138	4	7	0.63	0.25	0.56	0.76
Average		3.0	4.4	0.34	0.14	0.30	0.43

Graphic 1: Genetic relationships among the 53 found genotypes. Indicated are the upper 50% values of the *bootstrap* analysis, for 1000 replications. The underline values correspond to nodes common to genotypes of different varieties.



[Annex IV follows]

ANNEX IV

ISF TOMATO STUDY

1. The aim of the tomato study is to design a protocol that would allow a tomato breeder to claim that his proprietary parental line had been misused in the production of a competitor hybrid and that his intellectual property rights were infringed.

2. The situation is as follows: a tomato breeder suspects that a tomato hybrid on the market has been produced with one of his proprietary lines. He has the DNA profiles of the putative infringing hybrid and of his parental line.

3. After discussion among tomato breeding companies, it was decided that tomato hybrids of the Daniela type and their parent lines will be analyzed according to a beforehand agreed set of SSR markers. A tender for the DNA analysis was sent out to several laboratories, out of which TraitGenetics was selected. The participating companies have agreed on a set of 21 selected hybrids and 35 parent lines to be entered in the test. Samples have been sent to the ISF office and under code forwarded to TraitGenetics. Some of the hybrids have the same parent lines. Final results are expected towards the end of 2007.

[End of Annex IV and of document]