E

BMT/6/3
ORIGINAL: English
DATE: February 4, 2000

UPOV

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

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

Sixth Session
Angers, France, March 1 to 3, 2000

ASSESSMENT OF DISTINCTNESS, UNIFORMITY AND STABILITY OF SUGAR BEET VARIETIES BASED ON AFLP DATA

prepared by experts from Belgium

Assessment of Distinctness, Uniformity and Stability of sugar beet varieties based on AFLP data

JAN DE RIEK, EVELIEN CALSYN, ISABELLE EVERAERT, ERIK VAN BOCKSTAELE & MARC DE LOOSE

J. De Riek ()), Isabelle Everaert, Evelien Calsyn, Erik Van Bockstaele & Marc De Loose, Department for Plant Genetics and Breeding - CLO Gent, Caritasstraat 21, 9090 Melle, Belgium

Introduction

In Europe, until now, variety testing for sugar beets is organised at the national level and is accomplished by specialised sugar beet institutes. The PBR-applicants, the farmers, the sugar industry and the government provide the necessary funding. Because of the lack of UPOV-guidelines for the determination of Distinctness, Uniformity and Stability (DUS) of sugar beet varieties, testing for inscription on national variety lists mingles DUS evaluation with the assessment of the Value for Culture and Use (VCU). Variety testing is expensive due to the large trials at different locations needed for reliable VCU evaluation. Although based on regional diversity in agricultural culture conditions, a standardisation of the different evaluation standards at least for DUS assessment is profitable. This can be partly achieved by adopting general guidelines for evaluation. However, morphology often yields poor descriptors for discrimination of sugar beets. A new approach can be provided by assessment of DUS criteria based on the genetic identity of a variety as estimated by molecular markers. In this study, we aimed to examine different strategies for the evaluation of distinctness, uniformity and stability based on AFLP fingerprints of sugar beet varieties.

Materials and Methods

Plant material

Fifteen sugar beet varieties were included. Seeds of three consecutive seed deliveries were obtained from the KBIVB-Tienen (Belgium) and were identical to what was used in the official variety trials (Table 1). Thirty individual plants per variety per seed delivery were analysed (in total 1350 plants). These plants were processed in blocks of 150 (10 individual plants per variety for a certain seed delivery) through sowing, DNA preparation and AFLP analysis. Blocks were randomised over seed deliveries. Plants were always grown for one month under 16 h day (22°C, 80% relative humidity) and 8 h night (16°C, 80% relative humidity) conditions.

DNA isolation

At harvest, approximately 1 g fresh weight of leaf material was immediately immersed in liquid nitrogen and subsequently lyophilised during 48 h. The dry material was vacuum-packed for storage at -20°C until DNA extraction. Stored material was ground using a Culatti mechanical mill. The DNA isolation protocol was based on the CTAB method by Doyle and Doyle (1987). To 25 mg lyophilised ground tissue, 1 ml CTAB extraction buffer (100 mM Tris-HCl pH 8, containing 2% CTAB, 20 mM EDTA, 1.4 M NaCl, 0.5 mM $Na_2S_2O_5$, 0.4 %   -mercaptoethanol and 1% PVP MW 40000) and RNase (10 U) was added. Samples were incubated for 40 min. at 65°C. Afterwards, samples were homogenised with 1 ml chloroform/isoamylalcohol (24/1) and centrifuged during 15 min. at 10000g. The supernatant was transferred to a fresh tube and the DNA precipitated with 1 ml of ice cold (-20°C) isopropanol. After centrifugation (5000g; 15 min.), the pellet was washed with EtOH (76%) - 0.2 M NaOAc, dried and dissolved in water. DNA concentration and quality was constantly checked compared to a standard series of lambda-DNA on a 1.5% TAE buffered agarose gel after electrophoresis.

AFLP reactions and PAGE

AFLP was performed using the commercially available kit from Perkin-Elmer Biosystems for fluorescent fragment detection (Perkin-Elmer, 1995). *Eco*RI and *Mse*I were used for DNA digestion. Adapter ligation, preselective and selective amplification was performed as in the specified protocols. Selective amplification was done using fluorescent-labelled *Eco*RI-*Mse*I primer combinations with 6 selective bases. Primer combinations used were *Eco*RI-ACA/*Mse*I-CTG (PC1); *Eco*RI-ACT/*Mse*I-CAT (PC2) and *Eco*RI-AGG/*Mse*I-CTT (PC3). PCR amplifications were performed using a Perkin-Elmer 9600. AFLP fragments were separated by PAGE on a ABI Prism 377 DNA Sequencer on 36 cm gels using 4.25% denaturing polyacrylamide (4.25% acrylamide/bisacrylamide 19/1, 6 M urea in 1 X TBE). GS-500 ROX labelled size standard (Perkin Elmer) was loaded in each lane in order to facilitate the automatic analysis of the gel and the sizing of the fragments.

Band scoring and statistical analyses

Genescan 2.1 was used for AFLP data collection and sizing of the fragments. Only the fragments between 70 bp and 450 bp were used for scoring. After export of the Genescan data to Microsoft Access, variations in fragment size (within 1 bp) were assigned to the corresponding categories and a scoring table (1/0) was generated. Using Access queries, marker selection thresholds were set towards signal peak height and marker frequency: average signal peak height of a marker > 120; and frequency of a marker in the whole data set > 0.15 or frequency of a marker for a certain variety within a seed delivery > 0.5 (De Riek *et al.*, 1999). Polymorphic Information Content (PIC) of a marker was calculated as $PIC = 1-[f^2 + (1-f)^2]$ where f is the frequency of a marker in the data set. Calculation of Nei standard genetic distance and standard errors, construction of dendrograms (UPGMA) and bootstrapping, were performed by DISPAN (Pennsylvania State University) and Phylip (University of Washington). Euclidean distances and Jaccard similarities were calculated using MVSP (Kovach Computing). Analysis of molecular variance (AMOVA) was used (Excoffier et al., 1992) on the Euclidean distance matrix

Results and Discussion

Generation of AFLP data

The three AFLP primer combinations used generated approximately 70 to 90 AFLP fragments per reaction. Intense bands, indicative for repetitive fragments, were not observed. The number of fragments generated corresponded to what is reported for primer combinations having an A/T content of the selective nucleotides of 3 and 4 for sugar beet (Hansen et al., 1999). After scoring all plant reactions and applying the marker selection thresholds as defined, in total 696 unique fragments were retained (244 for PC1, 268 for PC2 and 184 for PC3). The distribution of the frequency of appearance of the markers in the data set and the corresponding Polymorphic Information Content (PIC) are shown in Figure 1. Although having a low PIC-value, the group of nearly monomorphic markers (63 markers with f > 0.95) was not excluded from the analysis in order not to overrule the specific absence of a marker in a certain variety. For a similar reason, the frequency of a marker for a certain variety within a seed delivery > 0.5 was set. However, no markers could be identified that were exclusively present or absent in a single variety.

DUS assessment

Firstly, the marker frequency data per variety and/or seed seed delivery were used for classification. Secondly, the genetic structure inferred by varieties and seed deliveries was tested with analysis of molecular variance (AMOVA). At last, individual plant data were input for assignment tests, searching for the most related genotype or group of genotypes.

Classification based on marker frequency data

Starting from marker frequency data, taking all plants of a variety or of a certain seed delivery of a variety as a group, Nei standard genetic distance was used to evaluate the criteria Distinctness and Stability.

The relationship between varieties is shown by Figure 2. The distance matrix this dendrogram is based on is given in Table 2. Three major clusters could be distinguished that are supported by high (> 450) bootstrap values: 1.) 'Avalon' (Delitzsch Pflanzenzucht GMBHP), 'Claudia', 'Victoria' and 'Olivia' (KWS); 2.) 'Opus' (Ulrich Dieckmann), 'Robusta' and 'Orion' (Van Der Have) and 'Winner' (Kuhn); and 3.) 'Pascal' and 'Sakara' (Hilleshög). The varieties 'Jackpot' (Delitzsch Pflanzenzucht GMBHP), 'Nevada' (Agrosem), 'Sfinx' (SES), 'Stratos' (Ulrich Dieckmann) and 'Gerda' (KWS) did not cluster to a specific group. The minimal distance that was observed between varieties belonging to different clusters was 0.0065 (`Claudia` versus `Orion`). In most cases, distances between varieties of different clusters were larger than 0.01. In the first cluster, `Claudia` and `Victoria` appeared to be the most similar varieties (D = 0.0026); in the second cluster, it was `Opus` and `Orion` (D = 0.0020). All observed distances were at least 5 times higher than the standard error on their value.

In a second ordination, the different seed deliveries for each variety were grouped (Fig 3). The matrix of pair wise distances between seed deliveries is not shown. Distances were at least 3 times higher than the standard error on their value. Two random resampling tests were applied on the ordination. Bootstrapping allowed evaluating the reproducibility of the ordination in a statistical way without collecting new data sets. To test the redundancy present in the data set based on 696 markers, random permutation and selection of different subsets of markers was used (Table 3).
Reproducibility as simulated by bootstrapping differed between clusters. Within Cluster 1 the three consecutive seed deliveries were grouped together for 'Jackpot', 'Olivia' and 'Avalon'; for 'Jackpot' (node 15) and for 'Olivia' (node 18) reliable bootstrap values (>80) were obtained. Within Cluster 1 'Claudia' and 'Victoria' were the most similar varieties. Especially the deliveries of '93 and '94 of 'Claudia' and 'Victoria' were very close (D = 0.0031 and 0.0033 respectively). This is in general below the distances obtained between delivery years for each variety (D ranges from 0.0029 to 0.0089). As a result, the resolution obtained with this set of markers was not sufficient to assign the delivery years of each cultivar to the same cluster. 'Claudia-'94' and 'Claudia-'95' were reliably grouped (bootstrap value = 84 for node 2); 'Victoria' was completely dispersed. Random permutation and resampling revealed approximately the same data structure. Nodes with high bootstrap values were already stable using 100 or 200 markers (Table 3).
Different seed deliveries appeared to be much less structured in the second cluster. However, low bootstrap values (around 50 or lower; Table 3) were obtained here. `Nevada`, which was grouped apart from the second cluster using the overall marker frequency data for the three seed deliveries, was tailed now to the second cluster (Fig. 3). The other varieties showed a well-structured grouping between seed deliveries that could even be based on a random selection of 100 markers.
The average standard Nei distance between seed deliveries from the same variety (Table 4) was used to evaluate stability over seed deliveries. Varieties were put in ascending order of average standard Nei distance, showing on top of the list the varieties with more similar seed deliveries. Average distances between seed deliveries from a same variety ranged from 0.0037 to 0.0144. If compared to the distances between varieties (Table 2), for some pair wise distances between varieties grouped to the same clusters, also values in this range were observed. This affirms what was observed in Figure 3 for cluster 1 and 2: no clear delineation of the delivery years within varieties is evident.

Analysis of molecular variance

AMOVA does not allow that structures with more than two levels (e.g. breeders, varieties and seed deliveries) are tested at once. Therefore, two genetic structure designs were applied (Table 5): 1.) attribution of the variation to breeding programmes, and within breeding programmes, to varieties; and 2.) attribution of the variation to varieties, and within varieties, to seed deliveries.
The population pairwise F-statistics matrix (data not shown) revealed an identical data structure as the use of the standard Nei distance; all Fst had a probability that was significant at 0.05 (100 permutations). When testing both genetic structure designs (Table 5), care must be taken in the interpretation of the results because both designs can not be nested. Different breeding programmes accounted for 2.6 % of the total variation; differences among cultivars within breeding programmes for 5.5 % (first design). Major variation remained attributed to the variation within cultivars. Under the second design, taking cultivars as the major level only fitted to 4.5% of the total variation; seed deliveries within cultivars accounted for 9.5 % of total variation. For this design, the variation within seed deliveries was detailed by their sum of squares (Table 6). These values can be used as an estimate for the Uniformity of the seed delivery. As a general conclusion of both AMOVA designs, it can be stated that major variation remains attributed to variation between individual plants within seed deliveries of cultivars; differences among seed deliveries seem to be as important as differences among cultivars or breeding programmes.

Assignment tests on individual plant data

When starting from marker frequencies or in AMOVA, the ordering of genotypes in a certain genetic structure (breeder, variety, and seed delivery) is taken for granted. Such approaches only can give an indirect indication e.g. of mixing of seed lots of related varieties or use of different pollinator lines by a small shift in resemblance between varieties or an increased variation within the variety. Because of the high variation observed within varieties and seed lots, one might consider the calculation of a global distance between accessions as not satisfactory. To overcome this, assignment tests were performed. First, per individual genotype a ranking was made with the 30 most resembling partners. For this, a (1350 x 1350)-resemblance matrix, using the Jaccard coefficient was constructed. Secondly, it was evaluated in what way the 30 most resembling partners were distributed over the total set of different seed deliveries and varieties.

In order to evaluate Distinctness, the distribution for the assignment of individual genotypes to a certain variety is given in Table 7. In the bottom panel the global assignment of the 30 most similar partners is given. Table 7 must be read horizontally: e.g. for all individual genotypes analysed from ‘Opus’, the 30 most similar partners were 649 times tracked back to ‘Opus’ itself, 404 times to ‘Robusta’, 289 times to ‘Orion’, 256 times to ‘Winner’ and so on. Taking this broad assignment using the 30 most similar genotypes, varieties seem to be very diffuse and show similarity to a large set of target accessions. The upper panels of Table 7 show that the distribution of the assignment over the varieties depends on the thresholds imposed on the ranking of plants. Compared to the 30 most similar plants, the number of top 3 or top 10 attributions differed. In general, when reducing the assignment to only the high-ranking genotypes, varieties tend to be less dispersed. Here, a remark must be formulated concerning the way the genotyping over the different seed deliveries was performed. As analyses were always grouped in blocks of ten plants belonging to the same seed delivery, a reduction of the attribution to only the top 3 might cause deviations, as plants are in some cases preferably assigned to individuals of the same block. Taking the top 10 attributions as an example, the assignment test of ‘Sfinx’ and ‘Opus’ are further detailed up to the level of seed deliveries (Table 8 and 9). ‘Sfinx’ was chosen as an example of a variety that is good distinguishable from the others, although it seems to refer to a common genetic pool as e.g. ‘Avalon’, ‘Claudia’, ‘Jackpot’ or ‘Olivia’. From Table 8, it can be observed that the assignment of the individual seed deliveries of ‘Sfinx’ is well structured. There is a preferential attribution to the same seed delivery year but plants are also equally assigned to the other delivery years of the same variety. No such structured assignment could be observed for ‘Sfinx’ to the other varieties in the data set. On the other hand, ‘Opus’ was taken as an example of a variety that cross-attributed to several varieties (Table 9). Although a good year-to-year assignment for the seed deliveries is retained for ‘Opus’, also a structured assignment to ‘Robusta’, ‘Winner’ and to a lesser extent to ‘Orion’ was observed. The other varieties could also be classified according to their degree of cross-attribution according to Table 7 and their appropriate seed delivery attributions (data not shown). ‘Gerda’, ‘Jackpot’, ‘Pascal’, ‘Sakara’, ‘Sfinx’ and ‘Stratos’ appeared to be very typical; ‘Olivia’ and ‘Robusta’ were moderate; ‘Avalon’, ‘Claudia’, ‘Nevada’, ‘Winner’, ‘Opus’, ‘Orion’ and ‘Victoria’ were most cross-attributing. ‘Victoria’, which was the oldest variety in trial, was the most dispersed variety. The order obtained in this way corresponds well to the better-clustered seed deliveries as obtained from the bootstrapping and random resampling experiment (Figure 3); ‘Robusta’ was a little upgraded and ‘Avalon’ was put lower as concluded from the assignment tests. The assignment tests, however, did reveal much better the underlying data structure in order to look for the most similar variety. E.g. ‘Opus’ and ‘Orion’ always appeared to be the closest couple in the ordination based on marker frequencies, this is not directly confirmed by the assignment tests. ‘Robusta’ was the best “target” variety after ‘Opus’ itself. For ‘Orion’, ‘Robusta’ is the best second target far before ‘Opus’.

Uniformity and Stability, if accessed directly from the assignment test as shown in Table 8 and 9 by looking for deviations in the attribution of seed deliveries from the same variety appeared to be little discriminative. Although based on the same observations as for Distinctness, the assessment of Uniformity and Stability is essentially directed to variability within a variety and reproducibility of this variability. So, to assess Uniformity, the average Jaccard similarity between all pairs of plants belonging to the same variety and seed delivery lot was calculated. A ranking was made per year and as an average over the three testing years (Table 10). Stability of a variety over different seed deliveries was interpreted in two ways. First, the average Jaccard similarity was calculated between all pairs of plants belonging to the same variety; secondly, to correct for the overlap with Uniformity, only the average similarity between plants of different delivery years was used (Table 11). In general, the average Jaccard similarity within and between seed deliveries provided a high correlation between the

rankings for Uniformity and Stability (*sensu lato*): varieties that are the most uniform also appeared to be most stable. After correction for the simultaneous Uniformity testing, the ranking for Stability showed major changes for 'Winner' (-5 places) and 'Victoria' (+4).
After AMOVA, Uniformity has been expressed by the means of the specific Sum of Squares per seed delivery (Table 6). Compared to this, in Table 10 quite some rearrangements are observed. 'Olivia' stays in both approaches the most uniform variety; 'Claudia' and 'Jackpot' remain in the top of the listing; in the middle and the bottom part changes are much more drastic: e.g. 'Stratos' (rank 4 - rank 10), 'Avalon' (rank 12 - rank 7). Also before, stability has been expressed as the average standard Nei distance between seed deliveries (Table 4). Again, in Table 11, rearrangements in the ranking are important. 'Olivia', 'Jackpot', 'Claudia' and 'Avalon' are also in Table 4 among the 5 highest-ranking varieties, however, further on the order is not maintained.

Conclusions: value for DUS assessment

Each approach presented is addressing the assessment of DUS criteria by means of AFLP data from a different viewpoint. Distinctness testing could be performed by the three procedures described. The calculation of genetic distances (standard Nei distance from marker frequency data or Fst based on Euclidean distances between genotypes) is very powerful to reveal differences between accessions. All pair wise distances calculated were significant. Due to the major variation within varieties or seed deliveries that is not accounted for when using marker frequency data, the significance testing procedure as provided by the standard errors on Nei distance is probably to sensitive. Surprisingly, the permutation testing in AMOVA taking into account the inside variation also only yielded highly significant Fst-values. Permutation testing in AMOVA concerns the randomisation of individuals in order to test if both sub-populations are drawn from an identical group. The input for AMOVA is a matrix with pair wise distances between individual genotypes. The method does not account for any uncertainty on the pair wise distances itself e.g. due to the choice of a specific subset of markers the distance was calculated on. To circumvent this, bootstrapping and random permutation and selection of different subsets of markers were applied. Both techniques allowed addressing the stability of the obtained groupings as a function of variability imposed by the use of a certain marker subset. Bootstrapping is computational much more feasible and actually reflected well the results from the more extended random permutation and selection procedure. However, both techniques are biased by the composition of the set of varieties in the analysis: seed deliveries of a large set of more closely related varieties are apt to mix up more easily in the groupings. An ideal testing procedure should therefore include the randomisation of individuals between groups and account for uncertainty on the pair wise distances itself.
For Distinctness evaluation assignment tests can offer a useful alternative to extended statistical testing procedures. They appeared to reflect well the results obtained by the previous methods. The computation of the assignment, based on the ranking of individual genotypes to one other, is straightforward, although demanding when large numbers of plants are to be analysed. Although thresholds for distinction can be defined in a clear way, -e.g. 70% of the most similar genotypes must belong to the same variety-, the interpretation of data can also be biased. If DUS-criteria are directly to be set from such assignment tests, one has to carefully consider the set of varieties to be used as a reference framework. New varieties originating from related breeding pools are likely to show a more dispersed assignment than a product from a totally new genetic background. Moreover, in what way thresholds have to be imposed has to be evaluated in the view of the breeding strategy of the hybrid (type of CMS line, use of a line or population as pollinator), seed production and relevance to agricultural performance. However, the intrinsic simplicity of the concept of assignment tests, that has much in common with e.g. the screening for off-types in self-pollinated crops, probably fits best to the current procedures of DUS-testing.

Uniformity and stability testing based on AFLP data appeared to be much more troublesome. Although both parameters can be directly derived from the same data input as for Distinctness testing and their definition is clear (better than or below the level of the reference set), non-consistent results, especially in the middle and bottom parts of the rankings, were observed. Assessment of Uniformity and Stability directly as the average similarity of all pairs of plants within or between specific seed deliveries is the most straightforward as both parameters can be obtained from the same similarity matrix. However, the correlation observed between both parameters might indicate that an element, that is inherent to a specific variety is interfering e.g. the genetic origin of the material or the composition of the components that made the hybrid. Opposite to the granting of

Distinctness, that rewards the creation of a new breed in comparison to all the existing, Uniformity and Stability are much more labels of guaranty for agricultural application. Therefore, they are much closer to VCU evaluation. Moreover, field testing for Uniformity and Stability is also statistically well defined: prove that there are no differences within or between subsequent seed deliveries. Reseeding spare seed from previous years in adjacent plots to the current most easily does this. The most direct benefit of molecular markers in DUS testing currently seems to be for Distinction purposes. A first application that can be accomplished at relatively low cost is its use for prescreening of new applicant varieties and grouping of similar varieties in appropriate field trials.

Table 1: Overview of the varieties tested

Variety	Seed Company	Testing in '93, '94, '95*	Listed on the Belgian Variety list since
Victoria	KWS	C4, C5, C6	1990
Claudia	KWS	C2, C3, C4	1992
Pascal	Hilleshög	C2, C3, C4	1992
Winner	Kuhn	R2, C1, C2	1994
Stratos	Ulrich Dieckmann	R1, R2, C1	1995
Opus	Ulrich Dieckmann	P, R1, R2	1995
Nevada	Agrosem	P, R1, R2	1995
Jackpot	Delitzsch Pflanzenzucht	P, R1, R2	1995
Avalon	Delitzsch Pflanzenzucht	P, R1, R2	1995
Orion	Van der Have	P, R1, R2	1995
Robusta	Van der Have	P, R1, R2	Rejected for listing in 1995
Sakara	Hilleshög	P, R1, R2	1995
Gerda	KWS	P, R1, R2	Rejected for listing in 1995
Olivia	KWS	P, R1, R2	1995
Sfinx	SES	P, R1, R2	1995

* P = preliminary testing year; R1 = first year of registration trials; R2 =second year of registration trials; Cn = n^{th} year of trial for a listed variety

Table 2: Standard Nei genetic distance ($.10^{-2}$) between pairs of varieties (Lower triangle) - standard errors on Nei distances (Upper triangle)

	Avalon	Claudia	Gerda	Jackpot	Nevada	Olivia	Opus	Orion	Pascal	Robusta	Sakara	Sfinx	Stratos	Victoria	Winner
Avalon		0.06	0.26	0.18	0.13	0.13	0.19	0.14	0.21	0.19	0.29	0.20	0.28	0.08	0.17
Claudia	0.45		0.27	0.12	0.12	0.08	0.12	0.09	0.20	0.14	0.27	0.18	0.20	0.04	0.12
Gerda	1.87	1.84		0.34	0.31	0.30	0.35	0.34	0.30	0.36	0.38	0.42	0.30	0.24	0.34
Jackpot	1.09	0.86	2.56		0.13	0.18	0.18	0.17	0.19	0.23	0.27	0.18	0.26	0.13	0.19
Nevada	0.98	0.90	2.12	1.03		0.17	0.10	0.09	0.20	0.13	0.25	0.11	0.21	0.12	0.12
Olivia	0.73	0.58	2.24	1.30	1.27		0.18	0.15	0.25	0.20	0.36	0.22	0.25	0.07	0.20
Opus	1.17	0.79	2.45	1.15	0.72	1.20		0.03	0.19	0.05	0.25	0.18	0.19	0.13	0.05
Orion	0.95	0.65	2.28	1.09	0.68	1.14	0.20		0.21	0.06	0.26	0.17	0.18	0.10	0.05
Pascal	1.47	1.28	2.23	1.26	1.36	1.85	1.46	1.57		0.23	0.18	0.25	0.20	0.17	0.23
Robusta	1.34	0.98	2.36	1.51	1.04	1.41	0.33	0.36	1.76		0.26	0.20	0.20	0.13	0.05
Sakara	1.98	1.80	2.96	1.79	1.89	2.62	1.91	1.94	1.11	1.97		0.29	0.30	0.26	0.25
Sfinx	1.30	1.23	2.88	1.15	0.85	1.31	1.05	1.01	1.61	1.33	1.93		0.28	0.17	0.20
Stratos	1.91	1.39	2.33	1.86	1.56	1.82	1.13	1.23	1.49	1.33	2.07	1.78		0.18	0.20
Victoria	0.54	0.26	1.80	0.88	0.88	0.62	0.83	0.69	1.20	0.97	1.71	1.12	1.10		0.13
Winner	1.22	0.88	2.32	1.16	0.73	1.36	0.30	0.31	1.63	0.32	1.97	1.14	1.29	0.83	

Table 3: Bootstrapping values and numbers for random permutation and selection of different subsets of markers per node of the dendrogram (fig. 3)

Node	Bootstrapping 696 markers	Permutation and selection				
		500 markers	400 markers	300 markers	200 markers	100 markers
1	68	71	63	59	62	39
2	84	93	89	80	64	50
3	100	99	100	100	93	63
4	49	67	58	49	41	25
5	84	93	91	72	62	41
6	51	68	61	39	35	28
7	36	32	39	35	31	18
8	39	47	49	31	31	26
9	72	80	65	68	62	38
10	88	95	89	84	73	52
11	96	99	94	88	69	51
12	95	100	100	100	99	83
13	100	100	100	100	96	75
14	100	100	100	100	98	92
15	100	99	100	98	98	77
16	62	79	76	60	39	18
17	51	75	66	58	39	26
18	94	99	98	91	70	50
19	47	71	64	37	25	12
20	56	74	49	34	27	18
21	85	91	92	82	78	56
22	100	100	100	99	94	66
23	100	100	100	99	95	77
24	97	100	98	94	72	53
25	100	100	100	100	99	84
26	37	69	51	38	21	3
27	42	49	36	27	16	9
28	88	96	93	87	67	44
29	39	53	56	26	12	4
30	38	47	34	21	18	15
31	80	94	89	73	49	12
32	32	44	32	29	15	8
33	66	77	71	59	48	10
34	51	66	49	44	28	9
35	52	87	63	54	32	9
36	28	34	32	21	11	4
37	37	42	38	27	17	8
38	15	27	14	11	10	3
39	23	53	42	30	19	2
40	13	38	27	13	9	4
41	90	97	96	89	56	20
42	44	55	56	42	38	16
43	97	98	98	97	88	58

Table 4: Stability over seed deliveries expressed as the average standard Nei distance between seed deliveries

Variety	Av. St. Nei Distance between seed deliveries
Jackpot	0.00370
Avalon	0.00483
Olivia	0.00497
Claudia	0.00527
Sakara	0.00600
Pascal	0.00637
Robusta	0.00660
Sfinx	0.00683
Winner	0.00737
Nevada	0.00823
Victoria	0.00830
Gerda	0.00860
Orion	0.00960
Opus	0.00963
Stratos	0.01443

Table 5: AMOVA designs and results

Breeding programmes versus cultivars

Source of variation	Degrees of freedom	Sum of Squares	Variance components	Percentage of variation
Among breeding programmes	4	3315.007	1.81060**	2.63
Among cultivars within breeding programmes	10	4033.184	3.81031**	5.54
Within cultivars	1334	83640.191	63.17235**	91.83
Total	1348	90988.382	68.34451	

Cultivars versus seed deliveries

Source of variation	Degrees of freedom	Sum of Squares	Variance components	Percentage of variation
Among cultivars	14	7348.191	3.06447**	4.48
Among seed deliveries within cultivars	30	7537.654	6.46819**	9.46
Within seed deliveries	1304	76102.537	58.81185**	86.05
Total	1348	90988.382	68.34451	

** Significant at 0.01 level, evaluated by 1000 permutations

Table 6: Assessment of uniformity by means of the specific Sum of Squares (AMOVA) per seed delivery

Variety	Seed delivery	Sum of Squares
Olivia	1	1173.415
Stratos	1	1235.483
Jackpot	1	1305.075
Sakara	1	1377.101
Opus	1	1491.219
Robusta	1	1513.600
Victoria	1	1526.037
Claudia	1	1526.893
Avalon	1	1642.899
Winner	1	1769.056
Sfinx	1	1785.993
Pascal	1	1801.558
Orion	1	1824.558
Gerda	1	1852.295
Nevada	1	2010.747
Olivia	2	1379.152
Robusta	2	1437.649
Gerda	2	1514.220
Orion	2	1521.272
Winner	2	1568.335
Opus	2	1604.009
Claudia	2	1625.092
Sfinx	2	1723.335
Jackpot	2	1736.539
Victoria	2	1792.691
Avalon	2	1860.645
Pascal	2	1872.979
Sakara	2	1938.116
Nevada	2	2002.573
Stratos	2	2221.972
Claudia	3	1384.655
Stratos	3	1387.547
Orion	3	1604.791
Victoria	3	1733.574
Avalon	3	1756.147
Gerda	3	1774.087
Olivia	3	1791.702
Jackpot	3	1793.237
Winner	3	1798.356
Nevada	3	1841.554
Pascal	3	1851.793
Sfinx	3	1916.516
Robusta	3	1923.436
Sakara	3	1932.745
Opus	3	1977.891

Variety	Average Sum of Squares over seed deliveries
Olivia	1447
Claudia	1511
Jackpot	1611
Stratos	1614
Robusta	1624
Orion	1649
Victoria	1683
Opus	1690
Winner	1711
Gerda	1713
Sakara	1749
Avalon	1752
Sfinx	1808
Pascal	1841
Nevada	1951

Table 7: Assignment tests per variety (upper panel: 3 most similar plants; middle: 10 most similar plants; lower panel: 30 most similar plants)

	Variety	Avalon	Claudia	Gerda	Jackpot	Nevada	Olivia	Opus	Orion	Pascal	Robusta	Sakara	Sfinx	Stratos	Victoria	Winner
3 most similar plants	Avalon	139	28		18	2	53	2	1		1	1	2	1	2	1
	Claudia	39	126	1	19	2	52	3			4		1	4	11	1
	Gerda			231			3			1		1			1	
	Jackpot	6	7		214	2	9	3	3		2	1	4		3	5
	Nevada	11	11	2	16	107	12	3	13		11		29	1	5	9
	Olivia	22	13		5		215	1	1		2		1		4	1
	Opus	2	10		9	4	17	111	23		37		10	2	4	20
	Orion	12	20		12	9	14	19	88		42	2	5	1	1	32
	Pascal	5	7		16		4	2	1	153	1	32	6	1	2	
	Robusta	6	4		4	3	14	22	22		121		5	2	3	35
	Sakara		1	1		1				26		182	2			2
	Sfinx	6	7		11	8	12	1			2		196		3	1
	Stratos	3	5		3	1	5	7	1	3	4			185	4	4
	Victoria	23	49		29	4	59	6	4	1	4		5	1	81	6
	Winner	3	11		16	5	9	18	20	1	33		7		4	127
10 most similar plants	Avalon	357	116	1	58	6	192	2	8		11	2	16	3	16	9
	Claudia	137	309	1	76	14	229	11	22	2	13	1	17	5	35	12
	Gerda	12	5	658		4	19		2	1	1	1			3	
	Jackpot	25	52		636	11	54	6	9	2	3	3	24		18	12
	Nevada	33	59	2	64	230	48	37	34	1	46	3	91	1	17	43
	Olivia	60	71	1	21	6	662	8	6		6		12		18	3
	Opus	9	35		47	25	56	290	79		142		48	3	16	74
	Orion	43	55	1	50	38	55	77	213		149	2	33	2	18	103
	Pascal	20	31		78	8	32	15	4	400	5	94	24	8	12	12
	Robusta	14	28		13	26	48	82	86		379		24	5	17	118
	Sakara	1	10	1	13	3	1		6	71	5	514	10	2		3
	Sfinx	23	40		39	26	61	28	8	2	13	1	532		8	12
	Stratos	18	29	1	15	6	33	32	12	7	14	1	16	490	18	24
	Victoria	96	176		114	13	184	24	15	4	23	1	25	7	202	27
	Winner	20	56		47	21	38	87	80	5	150	1	32	5	16	312
30 most similar plants	Avalon	788	348	14	199	33	567	35	59	1	47	7	60	5	73	38
	Claudia	404	776	2	339	53	678	73	86	10	81	8	70	15	143	77
	Gerda	132	47	1327	28	6	133	6	8	2	27	3	1	7	47	9
	Jackpot	113	264	2	1548	48	247	58	57	18	29	17	103	3	73	59
	Nevada	121	209	3	203	472	202	152	149	11	150	9	252	6	49	130
	Olivia	256	319	5	163	23	1644	40	34	2	39		82	7	88	33
	Opus	69	178		175	102	221	649	289	5	404	4	164	16	59	256
	Orion	158	221	2	187	111	257	292	509	4	399	8	120	13	74	328
	Pascal	72	130	3	282	44	120	51	30	781	42	269	81	19	37	54
	Robusta	78	150	4	92	91	198	364	299	3	869	7	109	30	50	412
	Sakara	29	52	2	99	25	29	23	22	164	30	1099	79	17	18	15
	Sfinx	114	186		184	105	309	127	65	10	89	11	1058	2	36	68
	Stratos	77	120	4	67	39	198	135	53	15	101	18	82	899	84	82
	Victoria	291	471	9	364	54	564	104	79	14	100	9	106	31	400	120
	Winner	86	208	1	206	103	182	304	277	10	461	9	119	21	73	668

Table 8: Assignment test for 'Sfinx' showing the total numbers for the top 10 attribution to the other varieties in the data set

Variety	Year	Ranking	Avalon			Claudia			Gerda			Jackpot			Nevada			Olivia			Opus			Orion			Pascal			Robusta			Sakara			Sfinx			Stratos			Victoria			Winner		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Sfinx	1	1	1	1								2			1			1																		12	2	5									
Sfinx	1	2	1												1			2	1																	16	1	3							1		
Sfinx	1	3			1		1	1				1	1		1		1	1																		7	4	4						1			
Sfinx	1	4	1	1				1				1		1				1		1																9	2	8									
Sfinx	1	5	1			1	1	1					1								1	2								1						8	3	7								1	
Sfinx	1	6			1	1							1					1	1	1																11		7								1	
Sfinx	1	7						1								1		1		2	1											1				9	2	5				1					
Sfinx	1	8				1		1					1			1		2	1			1					1				2					8		5							1		
Sfinx	1	9	1		1	1		3										1								2			1							4	3	7								1	
Sfinx	1	10				1	1	3					1					1		1	1	1													1	9		5									
Sfinx	2	1													1	1																				4	15	8									
Sfinx	2	2																	1			1														5	12	8				2					
Sfinx	2	3				1	1	1										1	2													1				6	9	7									
Sfinx	2	4						1									1		4			1														3	13	6									
Sfinx	2	5		1	1							1						1		1						1						1				6	5	9									
Sfinx	2	6						1								1	1		2			1			1											7	6	3				1	3				
Sfinx	2	7		1	1			2									2			1		1														6	7	5									
Sfinx	2	8		1		1	1	3				1		1			1				1	1														1	9	4								3	1
Sfinx	2	9										1	1	2	1		3		3		1	1			1							1				5	6	3									
Sfinx	2	10		1		1		2				1		2					1	1		2									2					7	3	6									
Sfinx	3	1											2		1					1																5	2	19									
Sfinx	3	2	1										1																							7	2	16									
Sfinx	3	3			1			2				1	2	1	1					2											1					2	2	13									
Sfinx	3	4											2							4	1															1	5	14									
Sfinx	3	5			1	1												1		3		1			1											4	1	12									
Sfinx	3	6			1								2			1				3		1	1		1											5	3	5									
Sfinx	3	7	1		1	1										1		1		3		1									1	1				2	2	9									
Sfinx	3	8		1				1				1		1			1		1	2		2														4	4	8									
Sfinx	3	9												3	2			1				1										1				6	1	6							1		2
Sfinx	3	10						1					2	1		1				2		3			1											4	4	4									

Table 9: Assignment test for 'Sfinx' showing the total numbers for the top 10 attribution to the other varieties in the data set

Variety	Year	Ranking	Avalon			Claudia			Gerda			Jackpot			Nevada			Olivia			Opus			Orion			Pascal			Robusta			Sakara			Sfinx			Stratos			Victoria			Winner		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Opus	1	1																2			14					3				1	3	2					1					1			2		
Opus	1	2				1	1	1										2	2	1	7			1	1	2				2	1	2										1			1		
Opus	1	3			1	1		1					1		1			2			5	3				3				1	3	1				1		1				1			1		
Opus	1	4			1	1	1	3					1		2			1	1	1	3	1	1			1				1	5											3			2	1	
Opus	1	5				1	1	1				3						2		1	4	3				2				1	2	3						1									
Opus	1	6			1			3				2				1	1	2		1	2	1	1		1	2				1	5	1				2		2								2	
Opus	1	7						1				3			1						5	4			2	3				3	6							2							1	1	
Opus	1	8		1		1						1						3		2	5	2			1	2				2	3	1													1	1	1
Opus	1	9			1							1			1			4			6	2				2				4	4	1						1								1	
Opus	1	10				1						3	1		1	1		3			2	2				1				2	3	3				1		1							1	1	
Opus	2	1	1				1						1		1							14			1	1				1	3							1								2	1
Opus	2	2						1								1		1	2			9	4		1					1	1	2						2							1	2	1
Opus	2	3						1				1		1				2		1	1	6	3		1	2					2	1					1	1	1							2	
Opus	2	4						1												1	1	8	1		1	1				1	2	1				1	1	3			1	1				3	
Opus	2	5				1		1					1								1	8	1		1	2				1	1	3				1		1							2	2	1
Opus	2	6			1							2	2	1		2		2				9	1			1					3							1						1		1	
Opus	2	7					1	1				1	1			1		1	1		1	5	1		1	3					2							3							1	2	1
Opus	2	8			1		1						1	1		2		1			3	6	2			2				1		1				2						2	1		1		
Opus	2	9												1	1	1		2		1	2	6	2		1	1					3	1						4									1
Opus	2	10										1	2		1	1		1	2			8			1	2					3							2								2	
Opus	3	1						1						3								2	18			2					1	1															1
Opus	3	2						1					1	1						1	1	1	11			3					2							1					1		1	2	2
Opus	3	3														1				1		1	11			2					2	4						1		1						1	
Opus	3	4						1											2			4	11			2					3															3	
Opus	3	5				1																4	9		2	1				1	2	1						1				1			2		1
Opus	3	6				1	1					1										1	5			3					3	4						1							1	3	1
Opus	3	7						1					1									2	7		1	2				3		3					1	3							1	2	1
Opus	3	8		1								1		1		1		1			1	3	4		3	2					1	3						1				1			2	1	1
Opus	3	9												2			3	1			1	1	6			2				1	2	1						1				1				2	
Opus	3	10										2	1					2			3	1	6	1	1	2					3							1						1	1	2	

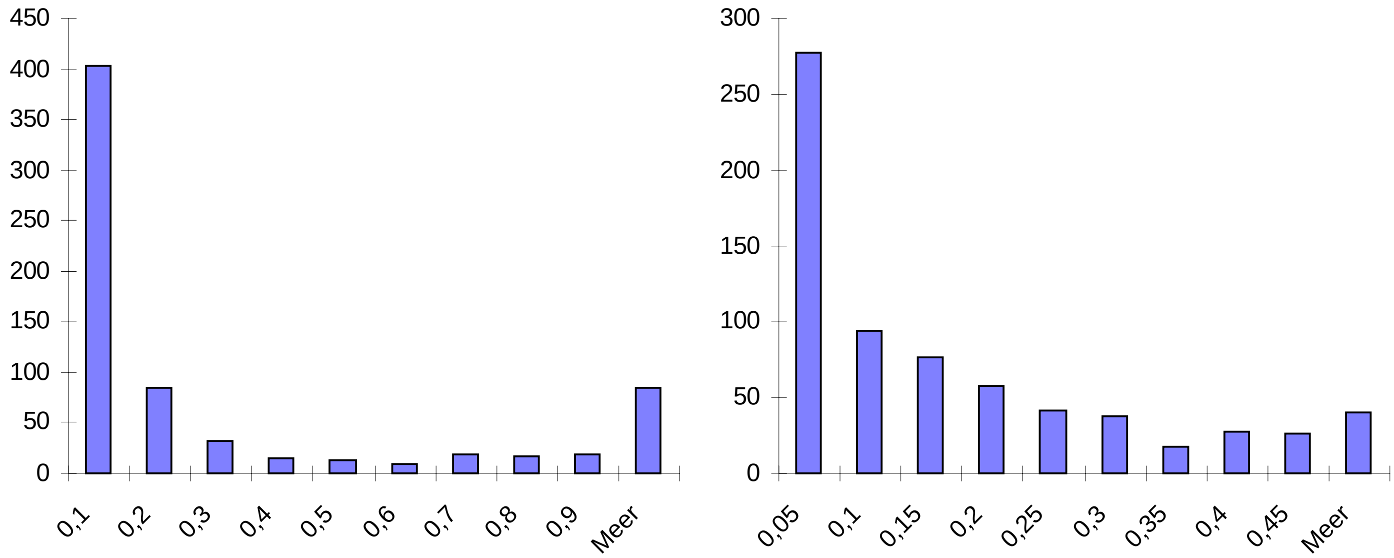
Table 10: Assessment of Uniformity expressed by the average Jaccard similarity between plants of the same seed delivery

Variety	year	Av. Jaccard similarity
Olivia	1	0.684
Jackpot	1	0.673
Stratos	1	0.657
Sakara	1	0.643
Robusta	1	0.627
Opus	1	0.625
Victoria	1	0.622
Claudia	1	0.622
Avalon	1	0.615
Sfinx	1	0.599
Winner	1	0.598
Orion	1	0.574
Pascal	1	0.573
Nevada	1	0.562
Gerda	1	0.552
Olivia	2	0.672
Robusta	2	0.655
Winner	2	0.652
Orion	2	0.643
Gerda	2	0.638
Opus	2	0.630
Jackpot	2	0.628
Claudia	2	0.628
Sfinx	2	0.627
Avalon	2	0.606
Victoria	2	0.603
Nevada	2	0.594
Pascal	2	0.587
Sakara	2	0.578
Stratos	2	0.535
Claudia	3	0.663
Stratos	3	0.645
Orion	3	0.629
Avalon	3	0.627
Jackpot	3	0.621
Olivia	3	0.614
Victoria	3	0.614
Winner	3	0.606
Nevada	3	0.604
Robusta	3	0.598
Sfinx	3	0.598
Opus	3	0.596
Gerda	3	0.595
Pascal	3	0.592
Sakara	3	0.591

Variety	Av. Jaccard similarity for 3 years
Olivia	0.659
Jackpot	0.641
Claudia	0.638
Robusta	0.626
Winner	0.618
Opus	0.617
Avalon	0.616
Orion	0.615
Victoria	0.613
Stratos	0.613
Sfinx	0.608
Sakara	0.605
Gerda	0.595
Nevada	0.587
Pascal	0.584

Table 11: Assessment of Stability: 1.) as the average Jaccard similarity between all plants of the 3 seed delivery years; 2.) idem, but corrected for uniformity

Variety	av. Similarity over 3 production years	after correction for Uniformity
Olivia	0.649	0.665
Jackpot	0.634	0.632
Claudia	0.629	0.646
Robusta	0.616	0.603
Avalon	0.609	0.620
Winner	0.606	0.580
Opus	0.602	0.594
Orion	0.600	0.592
Victoria	0.599	0.607
Sfinx	0.599	0.586
Sakara	0.594	0.588
Stratos	0.586	0.575
Gerda	0.581	0.565
Nevada	0.575	0.556
Pascal	0.575	0.554



Distribution of marker frequencies over the whole dataset

Distribution of the Polymorphic Information Content

Figure 1: Distribution of the marker frequency and the Polymorphic Information Content for the 696 AFLP markers scored on the dataset

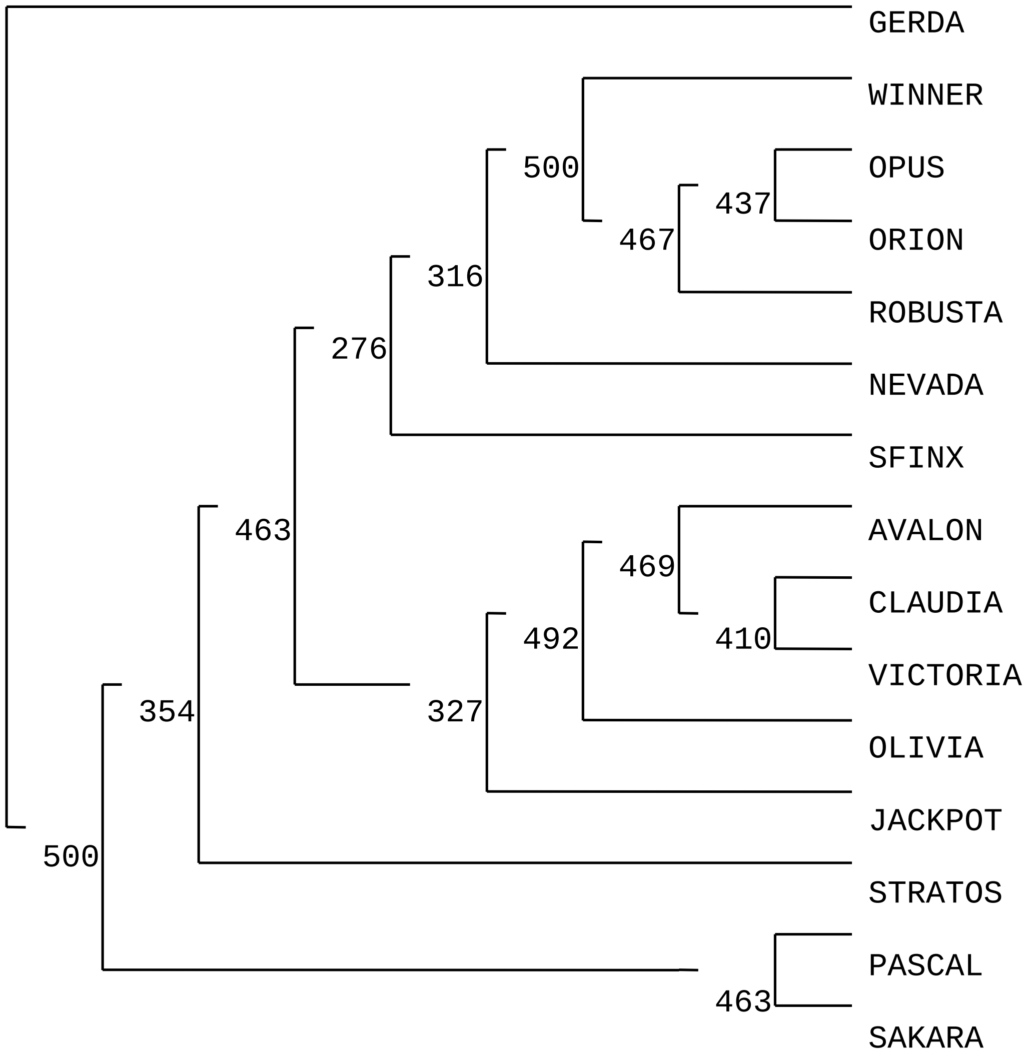


Figure 2: Ordination of the different varieties (standard Nei genetic distance; UPGMA clustering) with indication of bootstrap values on 500 datasets

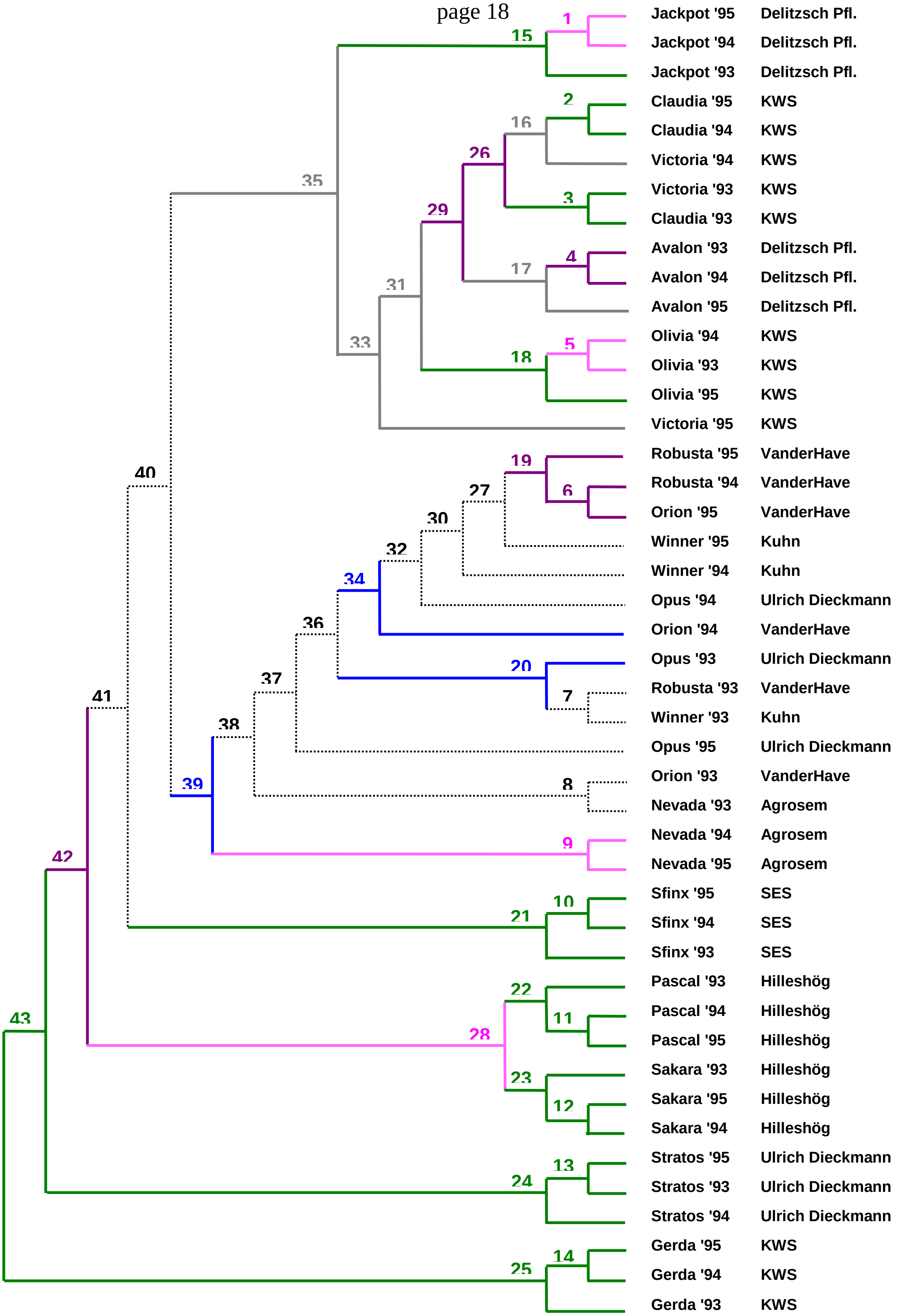


Figure 3: Ordination of the different seed deliveries (standard Nei genetic distance; UPGMA clustering); numbers on the nodes are referring to Table 3

[End of document]