Disclaimer: unless otherwise agreed by the Council of UPOV, only documents that have been adopted by the Council of UPOV and that have not been superseded can represent UPOV policies or guidance.

This document has been scanned from a paper copy and may have some discrepancies from the original document.

———

Avertissement: sauf si le Conseil de l'UPOV en décide autrement, seuls les documents adoptés par le Conseil de l'UPOV n'ayant pas été remplacés peuvent représenter les principes ou les orientations de l'UPOV.

Ce document a été numérisé à partir d'une copie papier et peut contenir des différences avec le document original.

———

Allgemeiner Haftungsausschluß: Sofern nicht anders vom Rat der UPOV vereinbart, geben nur Dokumente, die vom Rat der UPOV angenommen und nicht ersetzt wurden, Grundsätze oder eine Anleitung der UPOV wieder.

Dieses Dokument wurde von einer Papierkopie gescannt und könnte Abweichungen vom Originaldokument aufweisen.

———

Descargo de responsabilidad: salvo que el Consejo de la UPOV decida de otro modo, solo se considerarán documentos de políticas u orientaciones de la UPOV los que hayan sido aprobados por el Consejo de la UPOV y no hayan sido reemplazados.

Este documento ha sido escaneado a partir de una copia en papel y puede que existan divergencias en relación con el documento original.

UPOV

BMT/3/4
ORIGINAL : English
DATE : July 25, 1995

INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

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

Third Session

Wageningen, Netherlands, September 19 to 21, 1995

THE USE OF DNA-BASED MARKERS FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTING IN OILSEED RAPE AND BARLEY

Document prepared by experts from the United Kingdom

THE USE OF DNA-BASED MARKERS FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTING IN OILSEED RAPE AND BARLEY

David Lee, James C. Reeves and Robert J. Cooke

NIAB, Cambridge, U.K.

Introduction.

This is the second report to the UPOV BMT of progress made in a research project underway at NIAB to investigate DNA profiling techniques for potential use in DUS testing of oilseed rape (*Brassica napus*) and barley (*Hordeum vulgare*). This research programme aims to develop and evaluate suitable methods for assessing the extent of DNA polymorphism in these crop species and to provide information on the relative usefulness of various techniques for the molecular characterisation and identification of crop cultivars (varieties). The approach adopted was outlined in UPOV paper BMT/2/8 (1994) but briefly the work has concentrated on probe-based methodology using RFLPs and amplification-based PCR methods such as RAPDs. BMT/2/8 presented data from RFLP analysis and preliminary results of some RAPD analyses in rape.

The present paper includes further data from RFLP and RAPD analysis in oilseed rape and some RAPD analyses in barley. Methods for data evaluation and utilisation are considered, and the results obtained from RFLP and RAPD analyses are compared. Data relating to the uniformity of DNA profiles in rape are also presented and various issues relating to the use of profiling methods in DUS testing are discussed, along with an indication of the direction for future development.

Materials and Methods

Methods for DNA extraction, RFLP analysis and RAPD analysis have been given previously (BMT/2/8, 1994).

Data evaluation was carried out either manually, by scoring gels or autoradiographs visually for the presence/absence of bands from a particular probe-enzyme combination or primer, or using the software 'GelCompar' (from Applied Maths), which uses digitised images of gels or autoradiographs and allows for normalisation of tracks and gels and automated comparisons and analyses.

Results

1. Oilseed rape

1.1 RFLPs

Our previous paper to the BMT reported the use of several multi-copy probes, including some simple sequence repeats. Of the probes that have been examined, two were found to be particularly useful in terms of their discriminating power, expressed in terms of the percentage of all of the different pair-wise comparisons between a set of cultivars. On that basis, using a gel scoring strategy that defined all of the bands that were present in the collection of cultivars following restriction and probing and classifying each cultivar in terms of the presence/absence of bands at defined places on the gel, the probe pN180 produced a 96.6% separation and the probe pN216 a 93.4% separation of 62 cultivars, following digestion with *Hind*III. In combination, a 99.2% separation was achieved.

The data from gels can also be used to produce dendrograms or cluster analyses, that demonstrate the relationships between cultivars (Figures 1-3). Analysis of the data from the two probes in combination (Figure 3) clusters the cultivars into two main groups, which correspond to winter and spring types.

1.2 RAPDs

There are several reports in the literature that RAPDs are susceptible to changes in analytical conditions and are thus somewhat unreliable. However, by attention to detail, by the use of good quality DNA and by suitable choice of reagents (for example, we have found that in our hands the enzyme 'Amplitaq' is less sensitive to changes in $MgCl_2$ concentration than native Taq polymerase), it is possible to minimise these variations. Thus within a laboratory, repeatable and useful banding patterns can be produced using RAPDs.

We have screened a number of primers and taken the five most useful ones (i.e. those showing most clear repeatable polymorphisms) for further study using 50 cultivars of oilseed rape. Table 1 summarises the separation rates in terms of different pair-wise comparisons achieved using these five primers.

In contrast to the RFLP data, which were scored manually from autoradiographs, these data have been produced using gel imaging and the 'GelCompar' program to scan the gel images and match bands from different tracks automatically (see below). As we are still evaluating this software, and visual inspection of the gels does not completely agree with the computerised interpretation, the separation rates should be viewed with some caution. Nonetheless, it is clear from Table 1 that RAPD analysis can readily discriminate between oilseed rape cultivars.

TABLE 1. The use of RAPDs in oilseed rape. The separation rates achieved using five different primers with a collection of 50 cultivars are given. The asterisks refer to the band acceptance criteria judged to be most appropriate in each case (see 1.3 below).

% Separation Rates at Different Band Acceptance Criteria (a)						
Primer	**30:1**	**20:1**	**10:1**	**5:1**	**5:5**	**5:10**
6	98.4	99.0	99.5	99.8*	98.9	93.7
18	91.1	93.2	97.4	98.3	97.7*	94.0
33	92.5	98.7	99.5	99.8*	99.4	96.9
62	89.3	96.2	99.8*	99.9	99.5	97.1
70	96.9	98.9	99.8	100*	100	97.2

(a) - band acceptance criteria given as % above background : % minimum area;
* - indicates optimum separation based on the best grouping of the 100bp markers at the most inclusive settings (see below).

1.3 Use of 'GelCompar' to set band acceptance criteria

Although the visual assessment of gels and the manual scoring of profiles to produce binary (+/-) matrices of gel patterns (see BMT/2/8, 1994) is a reliable and relatively straightforward means of data analysis, it is rather cumbersome and time consuming and is not feasible for the analysis of either a large number of cultivars or a large number of gels. Thus we have been investigating the possibility of using automated gel evaluation, data capture and processing using the software programme 'GelCompar'. One of the features of this programme is that various criteria can be set for the acceptance of the presence of a band. This is particularly important in RAPDs since the bands produced vary greatly in intensity, probably reflecting copy number differences of the target sequences, the extent of homology between primers and targets and heterogeneity within the analysed populations. This variability makes the scoring of bands difficult, especially for those bands which are faint, and it is not straightforward deciding when a band is present or not (incidentally, this is a problem commonly encountered in other gel electrophoretic procedures). With the software, criteria can be set which a band must meet before it is accepted. In essence, these criteria are (i) the band must exceed a certain percentage of the whole pattern in intensity, as measured by the area under the gel scan; (ii) the band must exceed a certain threshold above the background value.

The effects of altering these criteria on the separations obtained are illustrated in Figures 4-6, using data from one RAPD primer as an example. When using the software, we have empirically adopted the strategy of choosing the band acceptance criteria that produce the optimum clustering of the 100bp molecular weight markers

(e.g. Figure 4). It will be seen from Figures 4-6 that altering the band acceptance level above background progressively from 10% to 30% affects the clustering of the markers and reduces the separation rate between cultivars from 99.9% to 89.3%. The effect of reducing the background threshold is to increase the number of bands which are accepted for inclusion in the analysis. In turn, this has the effect of increasing the separation rates, since they are based on more data points.

Again these rates must be cautiously viewed, but the principle remains valid (see also Table 1 for results with other primers). However, there are some difficulties with the use of the software (see Discussion) and we are still evaluating the most appropriate criteria for band acceptance and for optimising separations.

1.4 Uniformity

The question of the uniformity of DNA profiles within a cultivar has been addressed by the analysis, using RAPDs, of 10 individual plants from a number of oilseed rape cultivars. Some of the results are summarised in Table 2 and show clearly that there is variation within cultivars for their RAPD profiles when using certain primers.

TABLE 2. Uniformity of RAPD profiles in four (A-D) cultivars of oilseed rape, from the analysis of 10 single plants of each cultivar.

Cultivar	Primer	No.of different RAPDs profiles	Ratio
A	6	1	-
A	33	4	7:1:1:1
A	62	2	9:1
A	70	3	6:3:1
B	62	4	7:1:1:1
B	6	5	4:3:1:1:1
B	70	10	1:1:1:1:1:1:1:1:1:1
B	33	7	3:2:1:1:1:1:1
C	33	2	8:2
D	33	1	-

Results for cultivar A using primer 33 are also shown in Figure 7. The four banding patterns found within the 10 plants can be seen (track pairs 1, 2, 4 and 6).

The implications of such results for the uses of profiling techniques in DUS testing are discussed below.

2. Barley

2.1 RFLPs

Although no data are presented in this paper, work to date has shown very little polymorphism between the 36 cultivars studied using a range of cereal probes. This work is continuing using more probes. NIAB has access to a large panel of probes

from the JI Centre (Norwich), and a number of cDNA probes in particular will be examined, since this represents an opportunity to assess polymorphism arising from the expressed regions of the genome. This has been considered as important by some in previous discussions within the BMT.

2.2 RAPDs

As with RFLPs, RAPD analysis of barley cultivars has revealed relatively little polymorphism using the primers so far tested. Improved band separations and enhanced polymorphism can be achieved by pre-digestion of DNA prior to amplification (R. Koebner, pers comm.). Even so, only moderate rates of separation between the 36 cultivars have been obtained to date (see Table 3), although these can be improved by combining data from more than one primer (see Figure 8 and Table 3).

TABLE 3. The use of RAPDs in barley. The separation rates achieved using three different primers with a collection of 36 cultivars, separately and in combination, are given.

Primer	% separation
UCD60	24
ATC43	67
G19	64
3 combined	95

Discussion

1. RFLPs/RAPDs

Both RFLPs and RAPDs produce high rates of separation between cultivars of oilseed rape, although it must be remembered that the 'GelCompar'-generated separation rates to date are probably over-estimates. There is less useful polymorphism in barley. With care, both techniques can produce reliable and repeatable results within the same laboratory.

The cluster analyses produced by RFLP analysis separate rapeseed cultivars into groups which are equivalent to the winter and spring types (see Figure 3). This is in contrast to the situation with RAPDs (for example cf. Figure 4) where any division by type is less apparent. Hence it might be argued that RFLPs are more truly reflecting some underlying, genetically based, relationship. There are valid reasons why this might be so. With RFLPs, a band present at the same place on a gel in two cultivars is likely to represent true band homology. However, in RAPDs, there is no *a priori* reason to believe that two corresponding bands are homologous, rather they just have the same molecular weight. Thus band sharing in RAPD profiles does not necessarily indicate genetic similarity. The clusters obtained by combining data from several RAPD primers would probably better reflect true relationships and we are investigating methods for achieving this optimally using 'GelCompar'.

2. Gel Evaluation

Although programs such as 'GelCompar' offer considerable potential advantages for automated gel recording and data manipulation, there are certain limitations, at least in our hands, which remain problematic. For instance, the relative intensities of the bands within a track can be altered by changing the contrast/brightness parameters of the gel imaging device, and this can in turn affect the interpretation of the same gel, with the same band acceptance criteria, on different scans. Band alignment across tracks is also a problem. Even with the use of a carefully chosen set of internal bands, the program can mis-align bands, thus artificially increasing the separation rate achieved. It is possible to increase the tolerance level for band matching, but this may again lead to the wrong alignment of bands that are genuinely different. 'GelCompar' also has problems in differentiating between bands that are close together, but nonetheless can be resolved visually.

Notwithstanding these problems, the program is consistent with its analysis, i.e. it will produce the same set of bands from the same scan using the same set of band acceptance criteria. The fact that these criteria can be altered is an interesting feature, as it provides the possibility of objective and automated gel interpretation, which would be of considerable significance in the DUS testing context. The software also allows for the compilation of databases of profiles from different gels, and for sorting and storage of data. Such databases might find uses in cultivar registration outside of straightforward DUS testing. For these reasons, we are continuing our investigation of the uses of 'GelCompar' and other software packages.

3. Uniformity

The work presented above (Figure 7, Table 2) clearly demonstrates that oilseed rape cultivars can be heterogeneous in their DNA profiles as determined using RAPDs. Whilst this is not an entirely unexpected finding, it does raise potential difficulties in the use of DNA profiling for DUS testing, if the criteria presently used for assessment of uniformity were maintained. The problems are analogous to those presented by the use of protein/isozyme electrophoresis in allogamous or partially allogamous crops, in that the genetic structure of cultivars of such crops means that there is an inherent variability within them, such that they will not be completely homogeneous in discontinuous characters such as isozyme or DNA profile when examined on a plant by plant basis . There are a number of ways of approaching this problem:

(i) it could be decided that this lack of uniformity precludes the use of such profiling techniques;

(ii) it could be accepted that the level of non-uniformity exhibited by currently registered cultivars (which would need to be determined systematically and empirically) represented a baseline, which candidates in the future would not be allowed to exceed;

(iii) it could be suggested that from a certain date, all future candidates would have to be uniform for the particular profiling character;

(iv) it could be accepted that the repeatability (i.e. stability) of the differences between cultivars is more important than the insistence on plant to plant uniformity. Thus if the variability within a cultivar, as estimated either by single plant analysis or by a bulk analysis, is maintained from generation to generation (is stable) then this could be accepted as evidence of sufficient uniformity within that cultivar. This proposition would be recognising that the examination of uniformity is at least partly to ensure that the distinguishing features of a cultivar are maintained during multiplication and commercialisation. Hence it is stability rather than uniformity *per se* which is essential.

This last point would represent a change in the philosophy underlying aspects of DUS testing, but might be biologically, as well as practically, desirable. The insistence on complete uniformity of DNA profile within cultivars, in addition to being in all probability difficult to achieve, is also of doubtful biological and agronomic value. Profiling techniques are ideally suited to the rapid assessment of stability, since different generations can be screened and compared side by side on the same gel.

Concluding Remarks

Our research to date clearly demonstrates that both RFLPs and RAPDs possess considerable powers of resolution, particularly for cultivars of oilseed rape, and thus their uses within the DUS testing context need to be carefully evaluated. Notwithstanding doubts about the reproducibility of RAPDs and difficulties in the automated and objective evaluation of gels, it is apparent that both techniques are highly discriminating, convenient, relatively rapid, suitable for side by side comparisons of samples and can be carried out at any time of the year. These represent considerable advantages over most of the morphological characters currently used in DUS tests and it is difficult to believe that a morphological character with such attractive features would not be treated very seriously as a potential DUS tool.

It has to be recognised that there are serious reservations in some quarters about the use of profiling techniques for cultivar registration, which seem to revolve around problems connected with insufficient coverage of the genome and lack of knowledge of the genetic control of the marker(s). However, it is very doubtful that any of the currently used morphological characteristics fulfil such criteria either and so, judged against the same standards as other DUS characters, molecular markers must still be seen as being potentially advantageous and worthy of further study. It may be that these problems, along with difficulties in standardisation, eventually preclude the use of e.g. RAPDs for cultivar registration, but there would still be important applications of the techniques in, for instance, verification of identity and checking stability (perhaps in the certification context). The databases and clusters produced by profiling may also be utilised in areas that are somewhat peripheral to DUS testing itself, but are nonetheless essential in their own right, such as the grouping of existing and candidate cultivars and selection of appropriate controls.

The problems thus far raised against the use of DNA profiling may be diminished by the use of the 'second generation' techniques such as sequence tagged site microsatellites, which may also be easier to score reliably. We intend to investigate the

uses of microsatellites in oilseed rape in a subsequent project. There are also concerns about the uniformity of DNA markers of all types, but as outlined above (Discussion), such difficulties can be addressed, albeit in a manner which requires something of a shift in the current philosophy underlying uniformity and stability measurements.

Thus in conclusion, we believe that the use of DNA-based markers in DUS testing is feasible and that more research is needed to investigate (a) other profiling methods such as microsatellites, (b) automated methods of gel evaluation, recording etc, (c) applications of databases of DNA profiles within the DUS context, (d) appropriate means to utilise DNA markers within the cultivar registration system.

Acknowledgement. This work is supported by a grant from MAFF.

Figure Legends

Figure 1. Cluster analysis (UPGMA) of oilseed rape cultivars from DNA bands produced by the hybridization of a genomic clone (pN180) to HindIII digests.

Figure 2. Cluster analysis (UPGMA) of oilseed rape cultivars from DNA bands produced by the hybridization of a genomic clone (pN216) to HindIII digests.

Figure 3. Cluster analysis (UPGMA) of oilseed rape cultivars from combined data from clones pN180 and pN216 (HindIII digests).

Figure 4. Cluster analysis (UPGMA) of oilseed rape cultivars from RAPD analysis using primer 62, with band acceptance criteria of 10% of total area and 1% above background. The separation rate is 99.8%.

Figure 5. Cluster analysis (UPGMA) of oilseed rape cultivars from RAPD analysis using primer 62, with band acceptance criteria of 20% of total area and 1% above background. The separation rate is 96.2%.

Figure 6. Cluster analysis (UPGMA) of oilseed rape cultivars from RAPD analysis using primer 62, with band acceptance criteria of 30% of total area and 1% above background. The separation rate is 89.3%.

Figure 7. RAPDs profiles of 10 individual plants (in pairs), along with a bulk DNA preparation (B) from those 10 plants, of oilseed rape cultivar A (as in Table 2), using primer 33. Note the heterogeneity of RAPD profile within this variety - there are four profiles within this variety using this primer (pairs 1, 2, 4 and 6).

Figure 8. Cluster analysis (UPGMA) of 35 barley cultivars, using the combined data from three RAPD primers. The separation rate is 95%.

pN180 (HindIII)
Entries: 62
Clustering: UPGMA

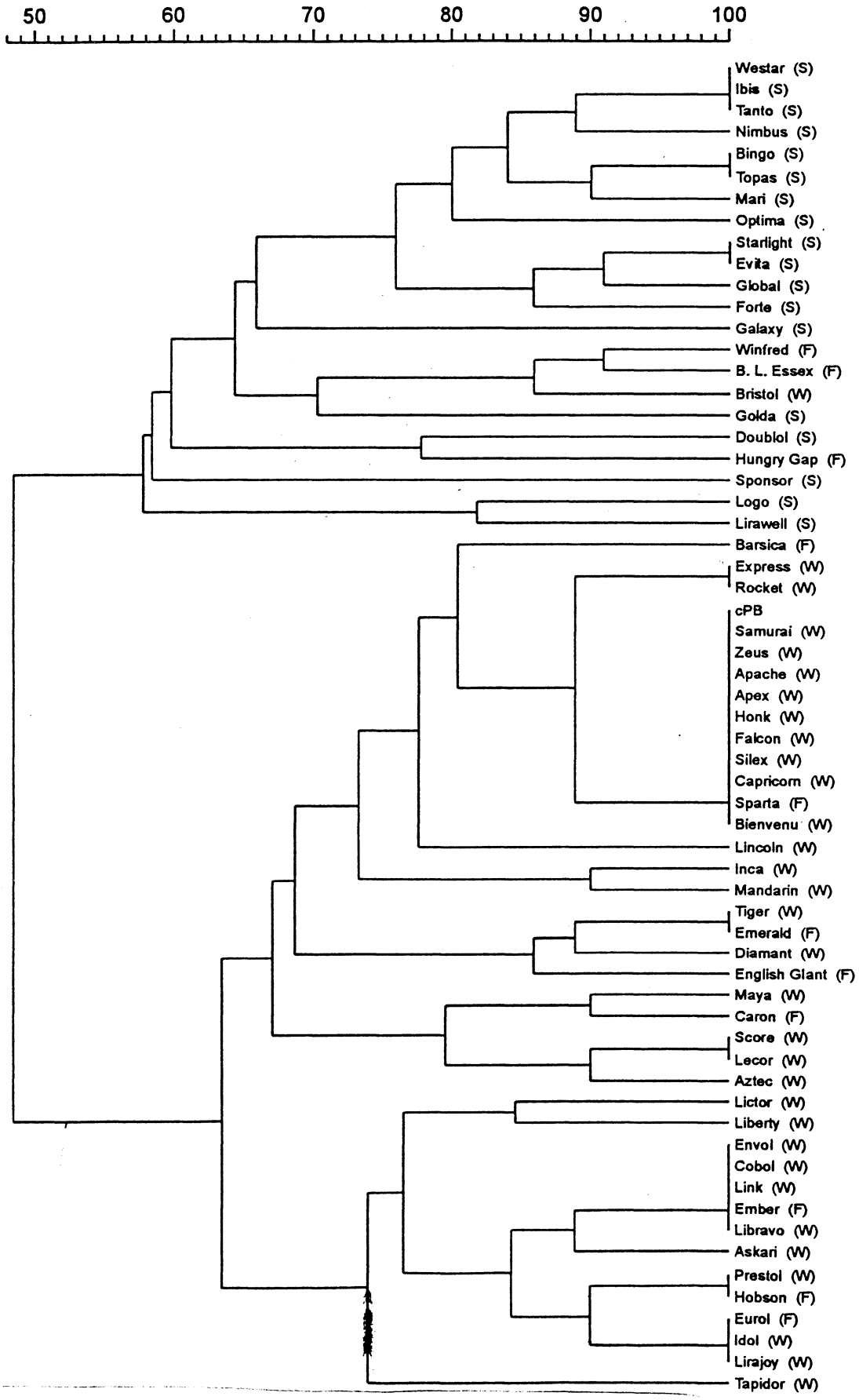


Figure 1. Cluster analysis (UPGMA) of oilseed rape cultivars from DNA bands produced by the hybridization of a genomic clone (pN180) to HindIII digests.

pN216 (HindIII)
Entries: 62
Clustering: UPGMA

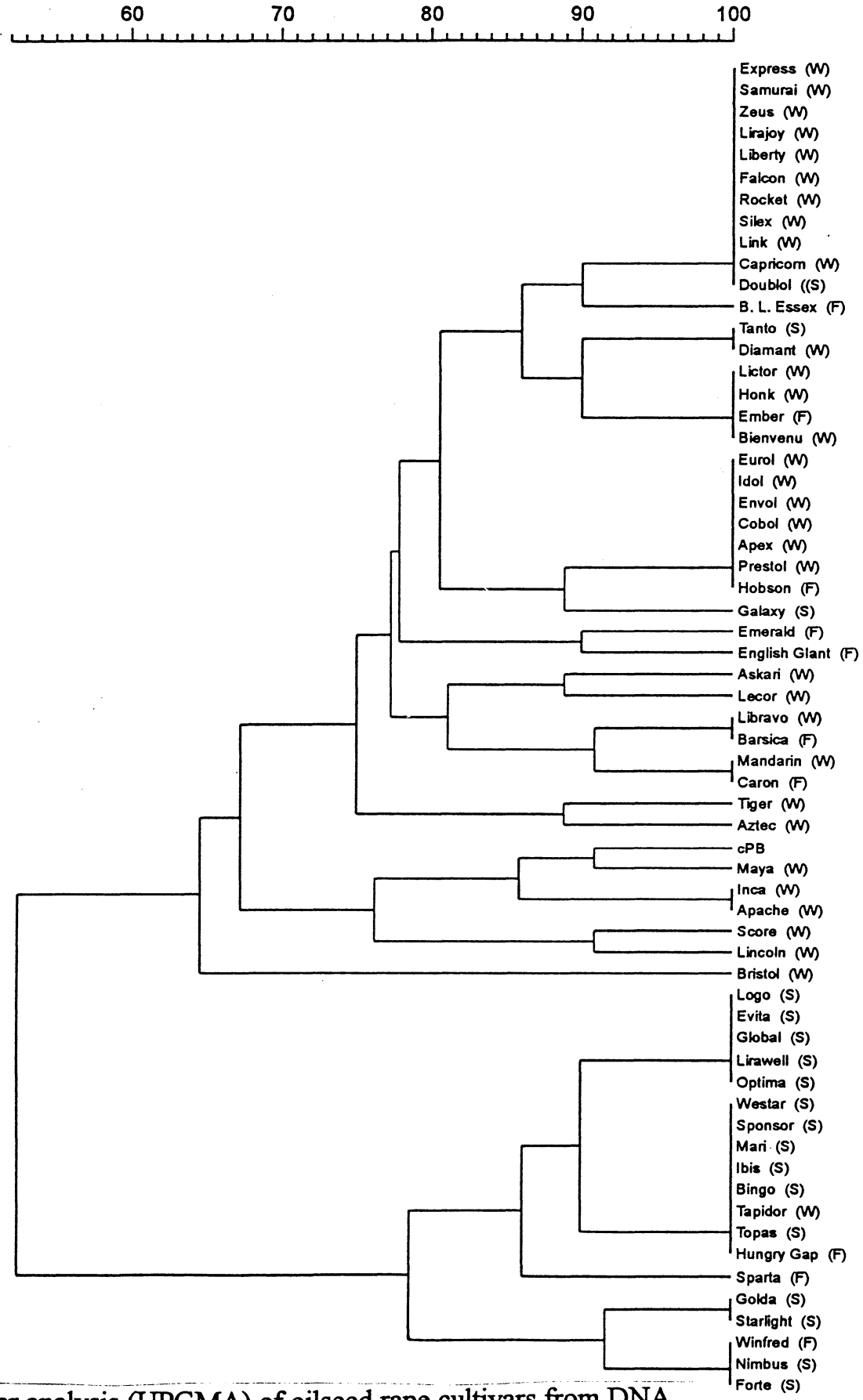


Figure 2. Cluster analysis (UPGMA) of oilseed rape cultivars from DNA bands produced by the hybridization of a genomic clone (pN216) to HindIII digests.

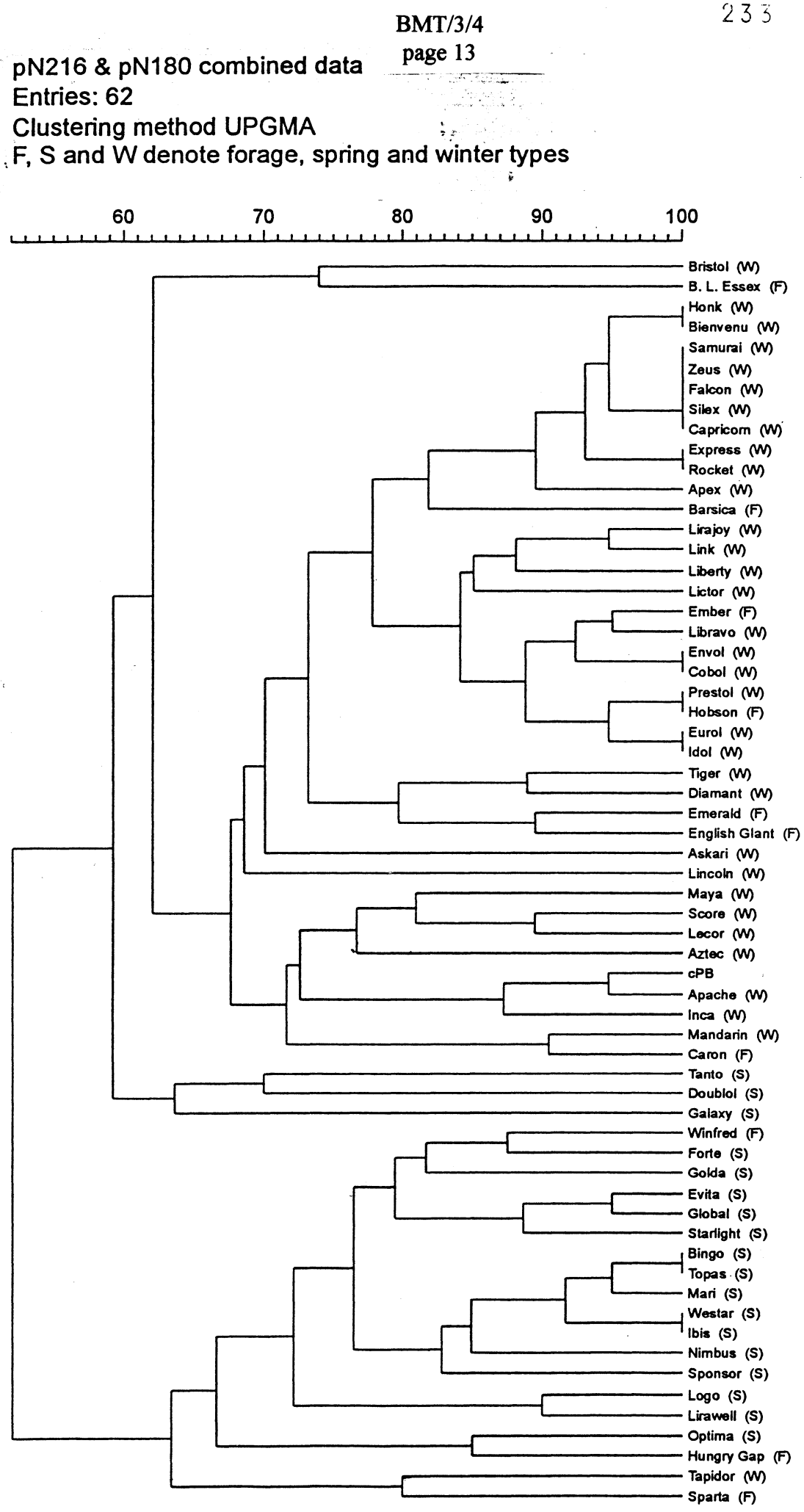


Figure 3. Cluster analysis (UPGMA) of oilseed rape cultivars from combined data from clones pN180 and pN216 (HindIII digests).

Figure 4. Cluster analysis (UPGMA) of oilseed rape cultivars from RAPD analysis using primer 62, with band acceptance criteria of 10% of total area and 1% above background. The separation rate is 99.8%.

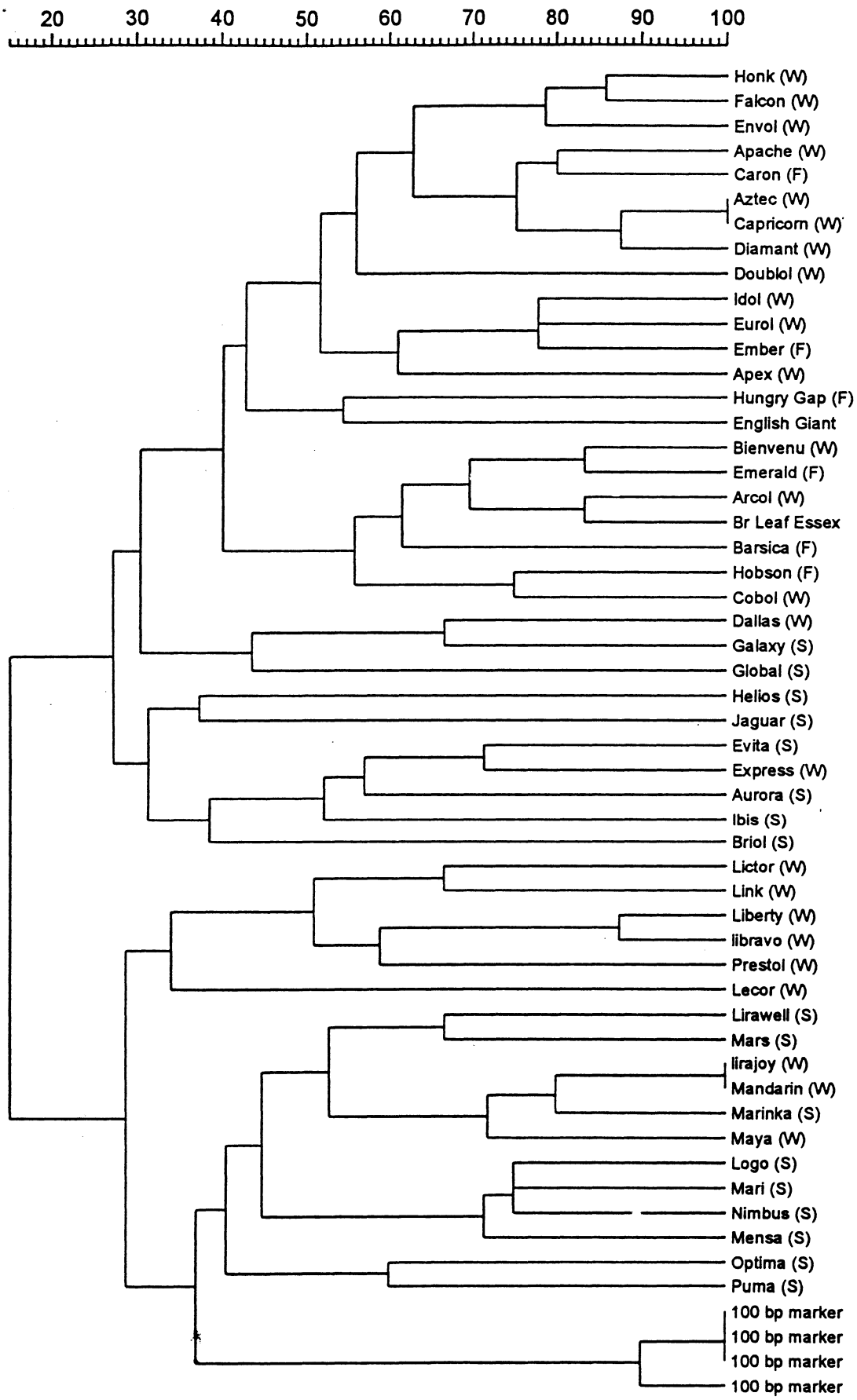


Figure 5. Cluster analysis (UPGMA) of oilseed rape cultivars from RAPD analysis using primer 62, with band acceptance criteria of 20% of total area and 1% above background. The separation rate is 96.2%.

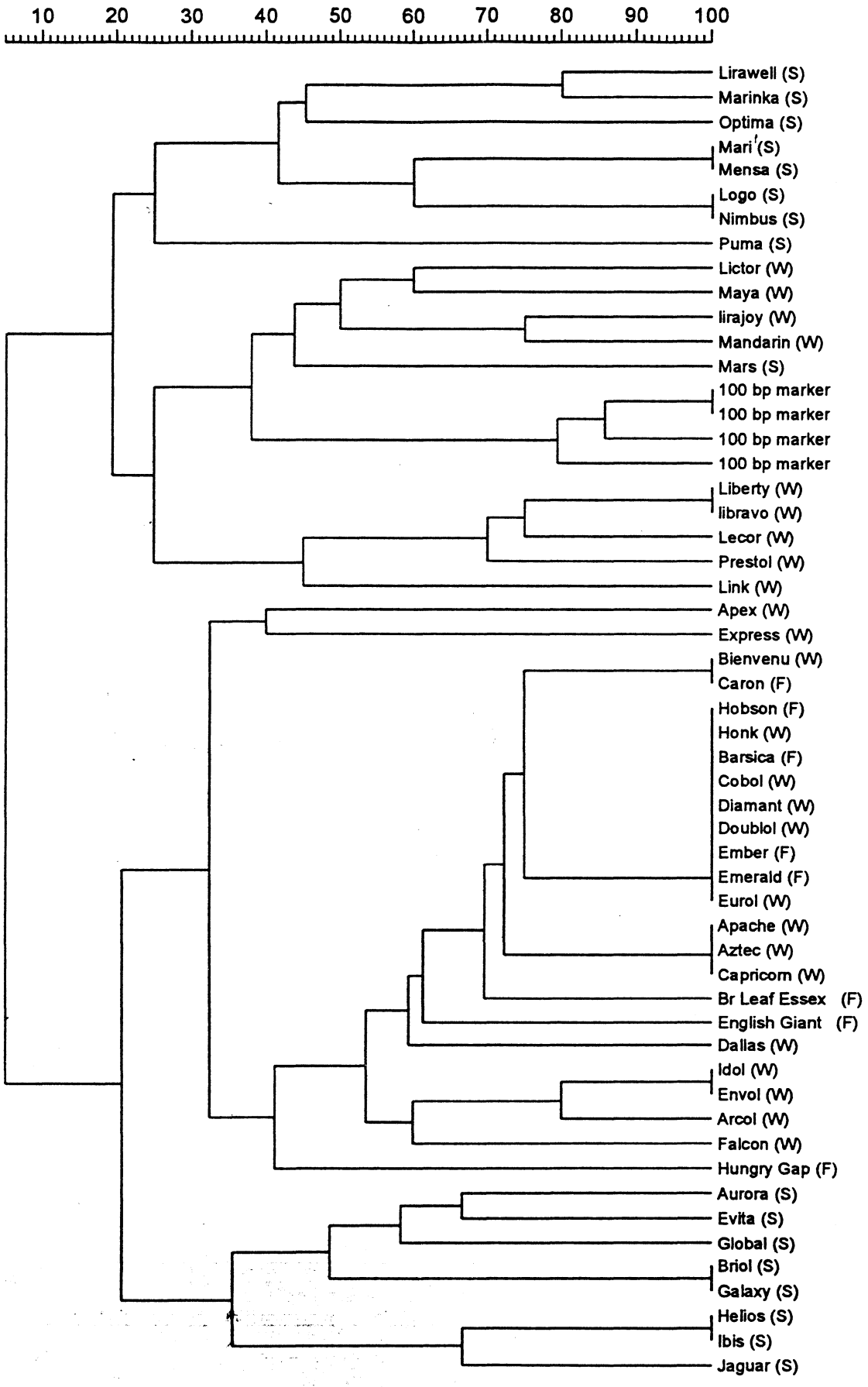
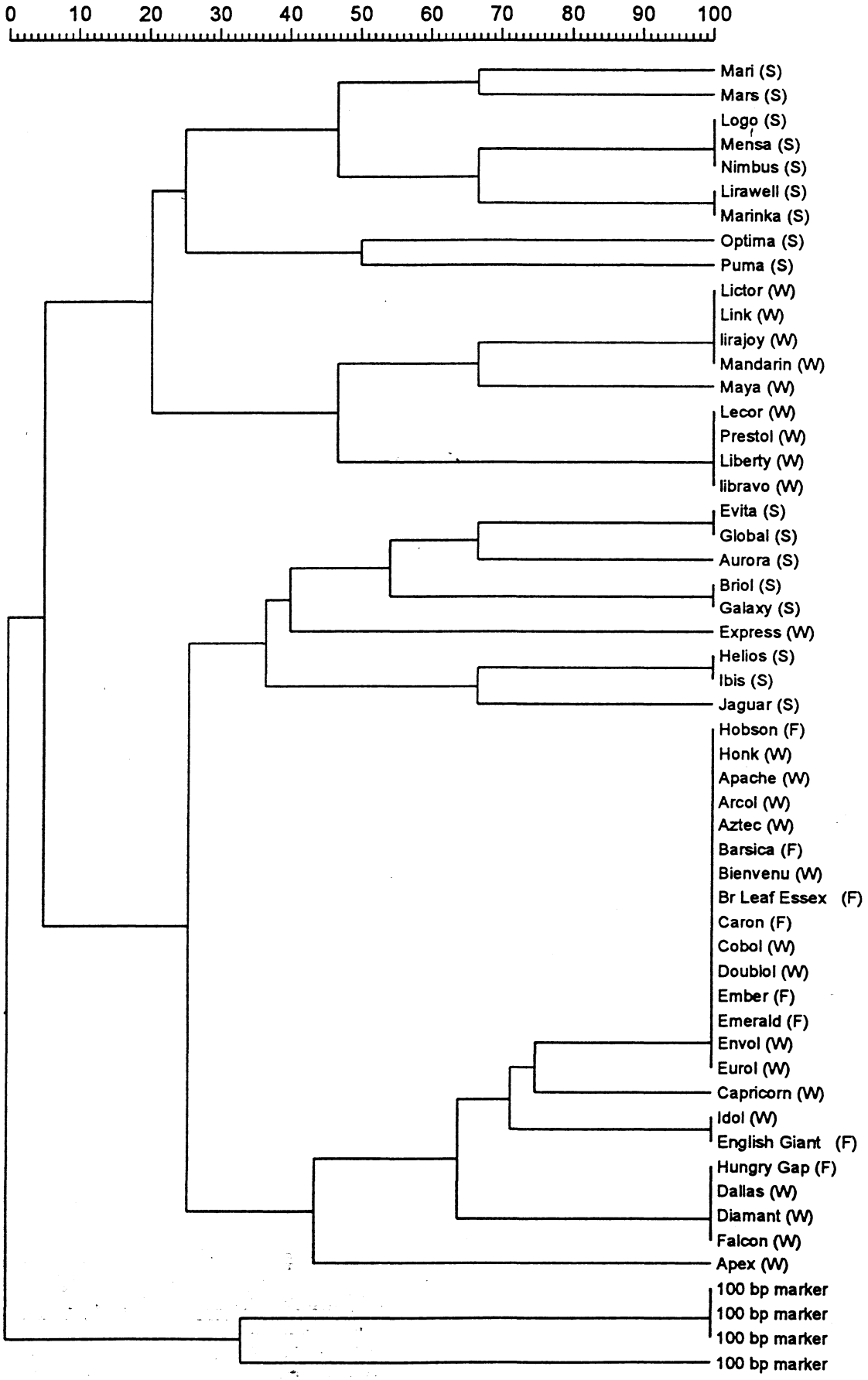


Figure 6. Cluster analysis (UPGMA) of oilseed rape cultivars from RAPD analysis using primer 62, with band acceptance criteria of 30% of total area and 1% above background. The separation rate is 89.3%.



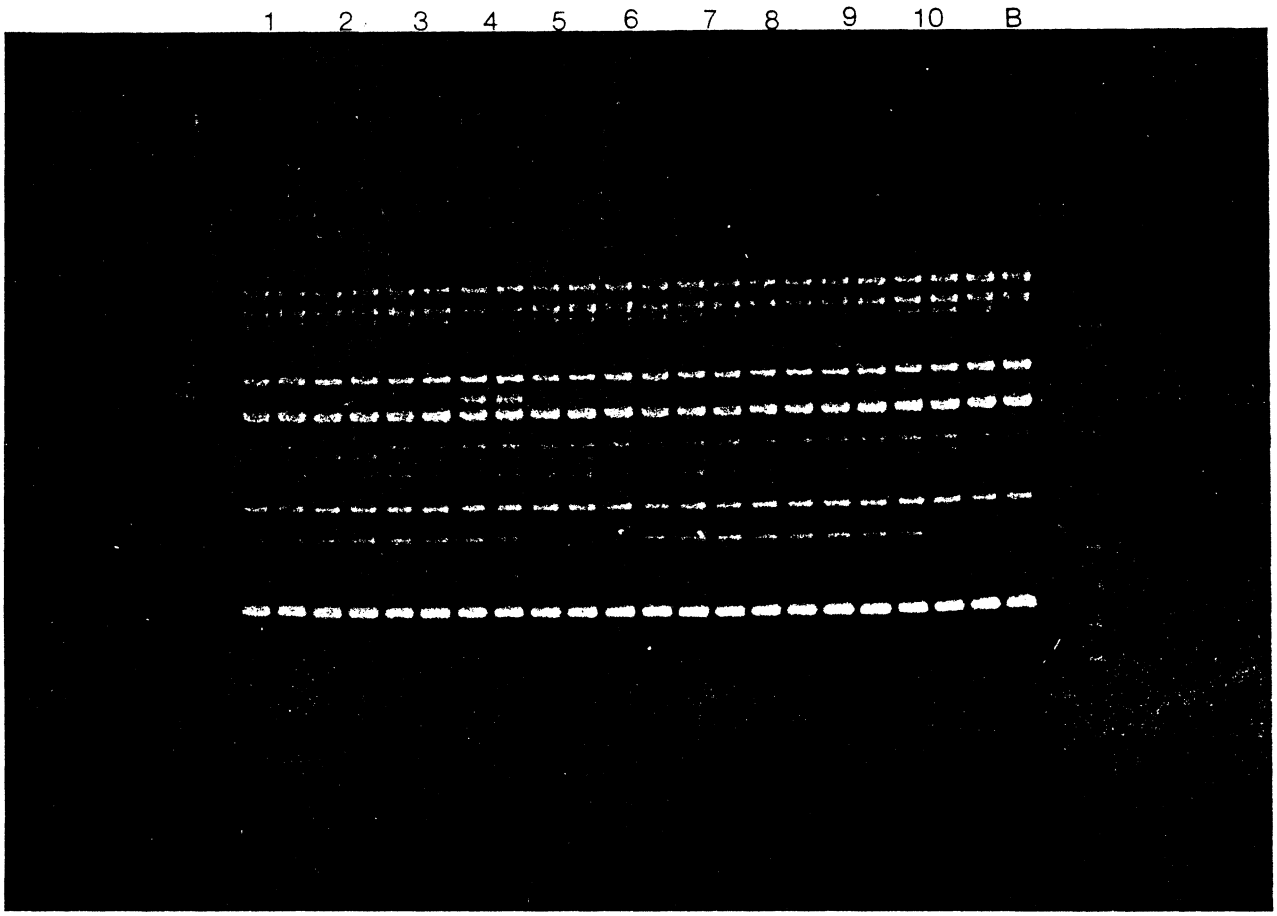


Figure 7. RAPDs profiles of 10 individual plants (in pairs), along with a bulk DNA preparation (B) from those 10 plants, of oilseed rape cultivar A (as in Table 2), using primer 33. Note the heterogeneity of RAPD profile within this variety - there are four profiles within this variety using this primer (pairs 1, 2, 4 and 6).

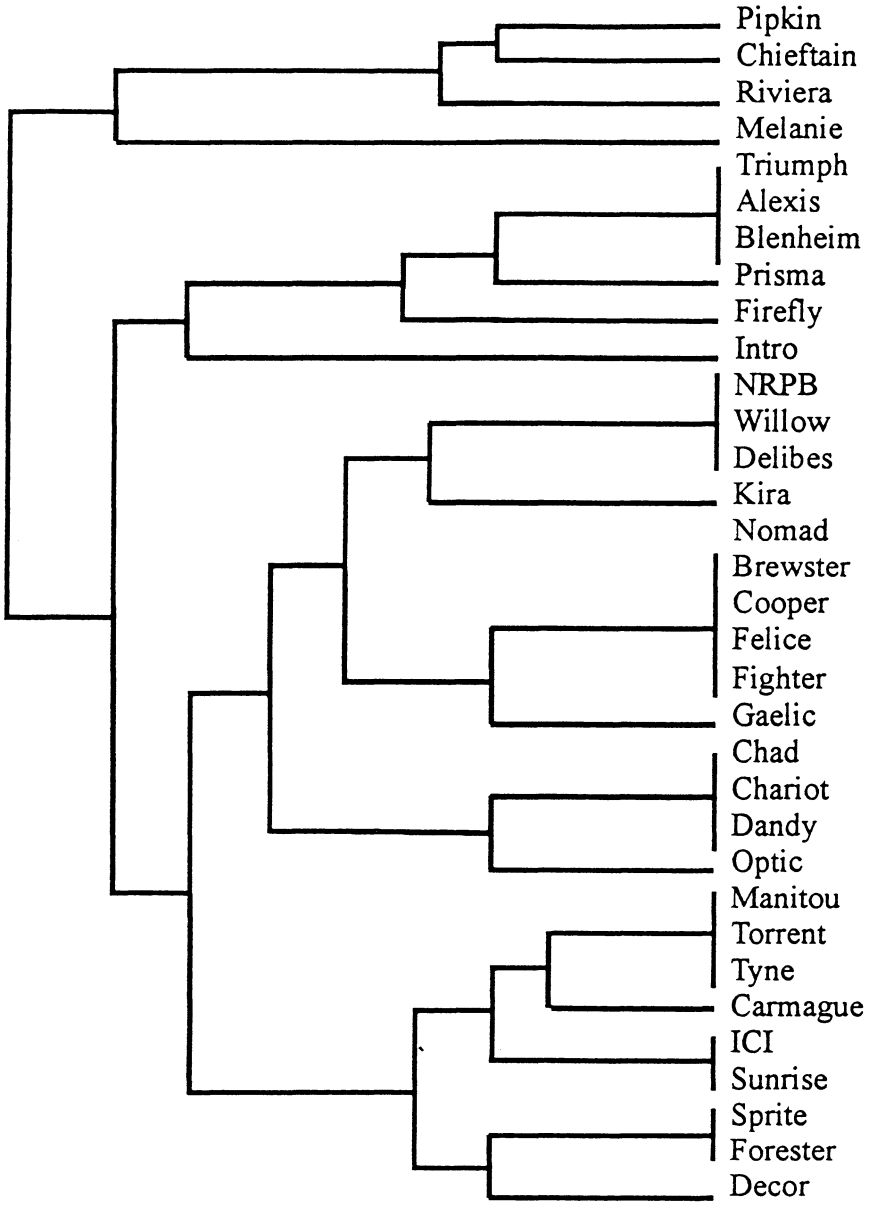


Figure 8. Cluster analysis (UPGMA) of 35 barley cultivars, using the combined data from three RAPD primers. The separation rate is 95%.

[End of document]