Genome analysis in hops- a powerful method for improving an essential raw material for brewing

S. Seefelder, K. Kammhuber, A. Lutz, B. Engelhard and E. Seigner

Hop Research Center, Huell,
Institute for Crop Science and Plant Breeding,
Bavarian State Research Center for Agriculture,
Am Gereuth 2, D-85354 Freising-Weihenstephan,Germany
E-mail: Stefan.Seefelder@LfL.bayern.de

Descriptors:
Hops, breeding, raw material, polymerase chain reaction, marker assisted selection

SUMMARY
Genome analysis was established at the Hop Research Center in Hüll to accelerate the breeding work. The main aim is to develop molecular markers to increase the efficiency in selecting seedlings revealing powdery mildew-resistance and to estimate the genetic variability of the breeding material. AFLP markers were used for genome mapping, analyses of genetic diversity and trueness of type studies of varieties. Using DNA fingerprints a collection of 148 international accessions could be divided into subgroups reflecting their origin and quality. New markers for powdery mildew-resistance were mapped for the first time. Molecular markers for high cohumulone content were identified. The estimation of genetic diversity is essential for the selection of parents in specific breeding programs. Gene diagnosis is one way to improve powdery mildew-resistance without modifying the genome. This paper gives an overview on an efficient molecular tool to support classical hop breeding.

INTRODUCTION
Hop breeding at the Hop Research Center in Hüll has been directed traditionally towards satisfying the needs of the hop and brewing industry. Any new variety must produce high quality hops whilst requiring only low inputs from the grower. A specific spectrum of analytical compounds, high yield and resistance towards pests and diseases are the most important selection criteria. The efficiency of hop breeding can be considerably increased by the use of highly reproducible, consistent and easy to use molecular markers which allows precise identification of desired genotypes. These markers can be routinely applied for marker assisted selection (MAS) which is particularly useful in hops due to their high level of heterozygosity. Moreover, MAS can save labour and space for screening huge populations. These are the presuppositions to improve an important raw material for the brewing industry. The aim is to develop varieties with excellent brewing quality, good agronomic performance and a high resistance towards several diseases and pests. In order to meet the high demands of the hop and brewing industry it is necessary to reduce the long process of the selection and phenotypic assessments of promising breeding lines. In general, it takes 12 to 15 years to develop a new hop variety. In this study some examples are shown where genome analysis already supports the classical breeding procedure.

Fungal infections still represent the major economic risk factor in hop growing. Regular protective treatments with pesticides are indispensable for commercial hop

growing, but cause environmental problems. Therefore developing new disease resistant high quality varieties is a top priority in modern hop breeding. Molecular markers linked with inheritable traits such as resistance, quality and sex allow early evaluation of promising breeding material without laborious screening procedures.
In recent years, several applications of PCR (PCR = polymerase chain reaction) based methods were described in hop research (5, 6, 8, 9, 11, 16).

MATERIAL AND METHODS

Plant material

A set of 93 hop varieties, nine wild hops and 45 breeding lines from the Hop Research Center Hüll were used to study the genetic diversity (table 1).

Table 1: Hop accessions with different genetic background

Agnus	Galena	Japan 845	Premiant	Wye Challenger	89/2/55	91/53/15
Ahil	Ging Dao Do Hua	Kirin 1	Pride of Kent	Wye Northdown	89/2/25	93/53/33
Apolon	Glacier	Kirin 2	Pride of Ringwood	Wye Target	90/61/9	93/59/5
Atlas	Golden Star	Kitamidori	Progress	Yoeman	91/45/21	93/81/13
Aurora	Granit	Liberty	Saazer	Zatecki	91/59/25	93/88/3
Blisk	Green Bullet	Marynka	Saphir	Zenith	93/10/4	94/15/41
Bobek	Hallertauer Gold	Mt. Hood	Serebrianca	Zeus	93/10/17	94/29/15
Bor	Magnum	Neoplanta	Sladek	H18/97/4	93/10/34	94/45/15
Braustern	Merkur	Northern Brewer	Smaragd	H18/97/6	93/10/36	96/1/17
Brewers Gold	Hallertauer mfr	Nugget	Southern Star	H18/97/7	93/10/63	96/1/24
Buket	Taurus	NZ Hallertauer	Spalter	H18/97/8	94/75/758	96/8/14
Bullion	Hallertauer Tradition	Olympic	Spalter Select	H18/97/12	95/94/721	96/16/34
Cascade	Herald	Omega	Sticklebract	H30/97/16	95/94/730	96/30/11
Columbus	Herkules	Opal	Strisselspalter	WH25	97/40/3	96/35/26
Comet	Hersbrucker pure	Orion	Southern Brewer	WH30	97/60/11	96/37/25
Crystal	Hersbrucker spät	OT 48	Super Alpha	WH49	97/60/25	97/7/11
Density	Horizon	Pacific Gem	Tettnanger	82/36/28	97/65/753	97/26/6
Diva	Hüller Bitterer	Perle	Toyomidori	83/30/25	98/97/738	
Early Choice	Hüller Fortschritt	Phoenix	Urozani	83/30/54	99/60/11	
Eastern Gold	Hüller Start	Pilgrim	USDA 21055	83/69/8	99/61/9	
First Gold	Japan C-730	Pilot	Vojvodina	86/62/39	99/93/718	
Fuggle	Japan C- 827	Pioneer	Willamette	87/24/3	91/13/25	

For the development of molecular markers linked to powdery mildew resistance 120 plants from the progenies of three mapping populations (Table 2) were investigated. Resistance in all mapping populations is based on the *R2* gene deriving from the English cultivar Wye Target. The *R2* resistance is still effective against powdery mildew infections in the German hop growing regions.
Two of these populations were also used to identify markers linked to the cohumulone content.

Table 2: Mapping populations segregating for the *R2* gene and the cohumulone content

Wye Target (*R2* gene) x 96/09/01 (susceptible)
Wye Target (*R2* gene) x 93/36/02 (susceptible)
84/08/24 (*R2* gene) x 98/44/49 (susceptible)

To test the efficiency of sex specific markers 1807 plants were screened deriving from different crosses and from the wild hop collection at Hüll.

DNA extraction and AFLP assays
Genomic DNA was extracted using 150 mg of freeze-dried, finely ground leaves, hop cones or pellets following the protocol according to Doyle and Doyle (2). The AFLP procedure was performed as described by Vos et al. (11). These studies included the primer combinations used by Hartl and Seefelder (3) and Seefelder et al. (6). As *Mse*I specific primers for the preselective amplification step *M00* was used without selective nucleotides.

HPLC
The determination of the cohumulone content was carried out by HPLC (high performance liquid chromatography). The EBC method 7.7 was used for the sample preparation and for the analytical procedure.

Data analysis and linkage mapping
The AFLP patterns were analysed with the computer software Cross Checker ,Buntjer (1). The data matrix generated by this program was used as input data file for the software JoinMap 3.0 (Plant Research International BV, Netherlands) with which linkage mapping was performed. Linkage groups were determined using a minimum LOD (logarithm of the odds) threshold of 6.0. The map was constructed using the Kosambi mapping function (Kosambi, 1944).
Statistic analysis was performed using the SAS 8.0 statistical package.
Cluster analyses of the AFLP-fingerprints were done using the computer software NTSYSpc 2.0 (Exeter Software, NY, USA)

RESULTS AND DISCUSSION

Powdery mildew resistance linked to the *R2* gene
One hundred and twenty individuals of three full-sib mapping populations segregating for the powdery mildew (PM) resistance gene *R2* were screened for disease resistance in the laboratory and in the greenhouse. Resistance data did not deviate significantly from a 1:1 ratio of segregation and thus confirmed that a single dominant gene *R2* is involved in the resistance reaction of the seedlings.

Data scoring
Using 91 AFLP primer combinations 620 polymorphic fragments could be produced. 549 fragments were classified as 1:1 segregating markers (ab x aa), 414 contributed by the female (84 /08/24) and 135 by the male parent (98 /44 /49). 97 markers were heterozygous in both parents (ab x ab) segregating 3:1.
Segregation of 17 microsatellites (1, 13, 4) was also investigated in our mapping population.

Bulk segregant analysis to identify PM resistance markers
Among all AFLP primer pairs tested 20 could differentiate between the DNA bulks of 10 fully resistant and 10 highly susceptible hop plants. Chi-square analysis revealed that most of these AFLP markers followed a Mendelian segregation. AFLP markers were re-examined on all individuals of the progeny including the parents. Seven DNA fragments inherited from the resistant parent (markers in coupling phase) were identified as putative AFLP markers linked to the PM resistance locus *R2*.

Linkage analysis and mapping

Using 620 AFLPs (which include the above mentioned putative *R2* resistance markers) and 17 microsatellites, the *R2* resistance locus and the male sex locus *M* linkage analysis was performed and the male and female maps were constructed using JoinMap 3.0. Based on a LOD of six, 303 AFLP markers and all 18 SSRs (single sequence repeats) were assigned to nine female linkage groups covering 320.6 cM. In the male map 150 AFLPs and 15 SSRs were placed on eight linkage groups spanning a distance of 237.4 cM.

Located on the largest female group Gr-F1 covering 101 cM, the *R2* locus conferring PM resistance was flanked by two AFLP markers at a distance of 1.6 cM. This is for the first time that two markers adjacent to the *R2* PM resistance locus could be detected. Additional markers for PM resistance could be identified at a distance of 1.7 to 2.6 cM. This is also the first time that PM resistant markers could be assigned to a genetic map in hops.

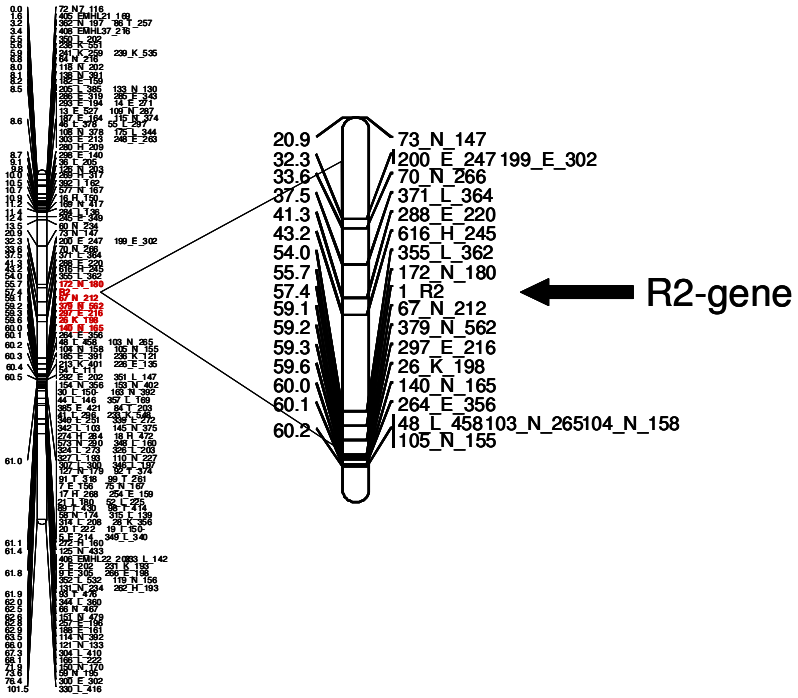


Fig. 1: Mapping of the *R2* resistance locus

AFLP markers for cohumulone

In screening the above mentioned mapping populations (table 2) with AFLPs, approximately 30 markers could be identified in linkage to a high cohumulone content. Regression analysis describing the relationship between selected markers and the cohumulone content of the hop plants revealed a correlation of 80% of the phenotypic variation. Analysis of the cohumulone content of the hop plants was continued over two additional seasons. Values were confirmed as being stable. Figure 2 shows a typical marker linked to a cohumulone content of more or equivalent to 29%.

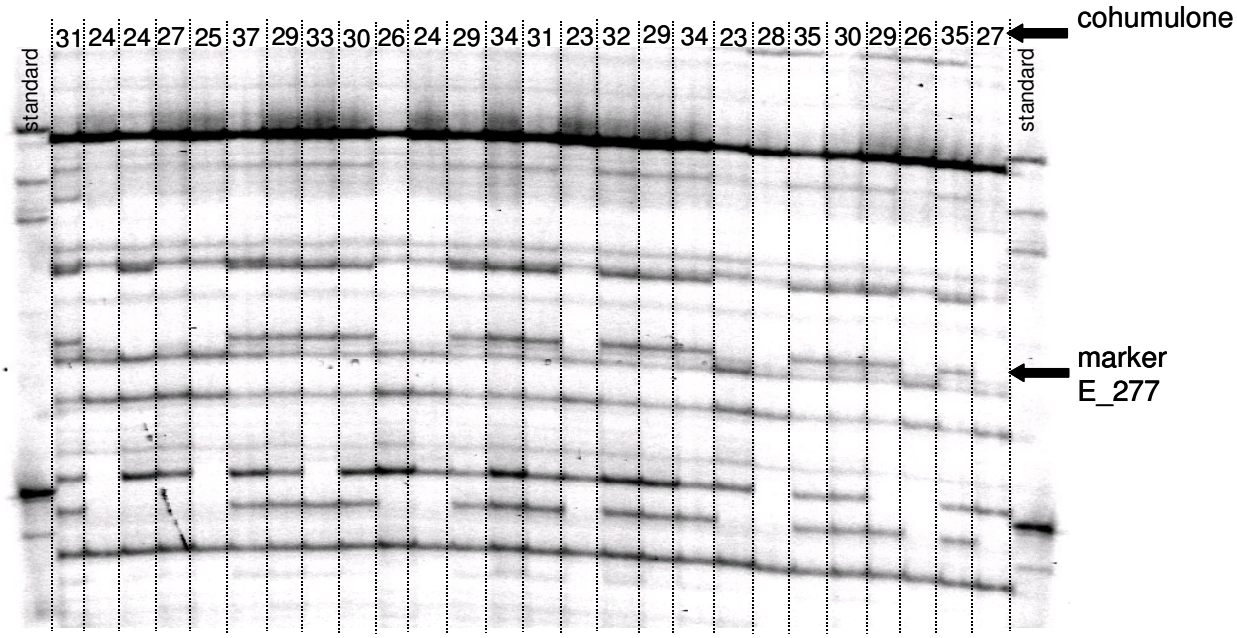


Figure 2: Marker for high cohumulone content

Sex specific markers

Leaves were taken from a total of 1807 hop seedlings of unknown sex. Care was taken that seedlings of various crossings were used, which allowed to test and evaluate the reliability of the sex markers in Hüll breeding material with different genetic background. DNA was extracted from all individuals and tested for the presence or absence of male specific fragments in their genetic fingerprint. During flowering these results were compared with the sex phenotype. Using the sex specific markers published in (9) and (12), only 7 out of 1807 plants tested showed differences between their phenotype and the presence or absence of the male marker fragment (Figure 3). An error rate of 0.7% during these investigations verifies the usefulness and reliability of these sex specific markers for quickly screening the sex of the Hüll breeding material. Further studies based on the international wild hop collection revealed that these DNA markers are not suitable for correct sex determination in the native North America germplasm. The error rate increased up to 50%. Therefore, before introducing North American hop germplasm into the Hüll breeding material it would be necessary to identify sex markers for the American gene pool.

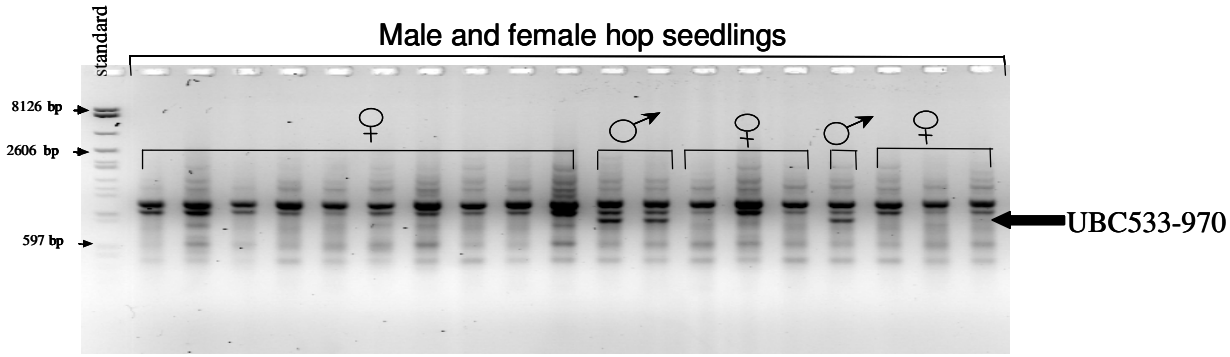


Figure 3: Sex specific marker

Genetic diversity

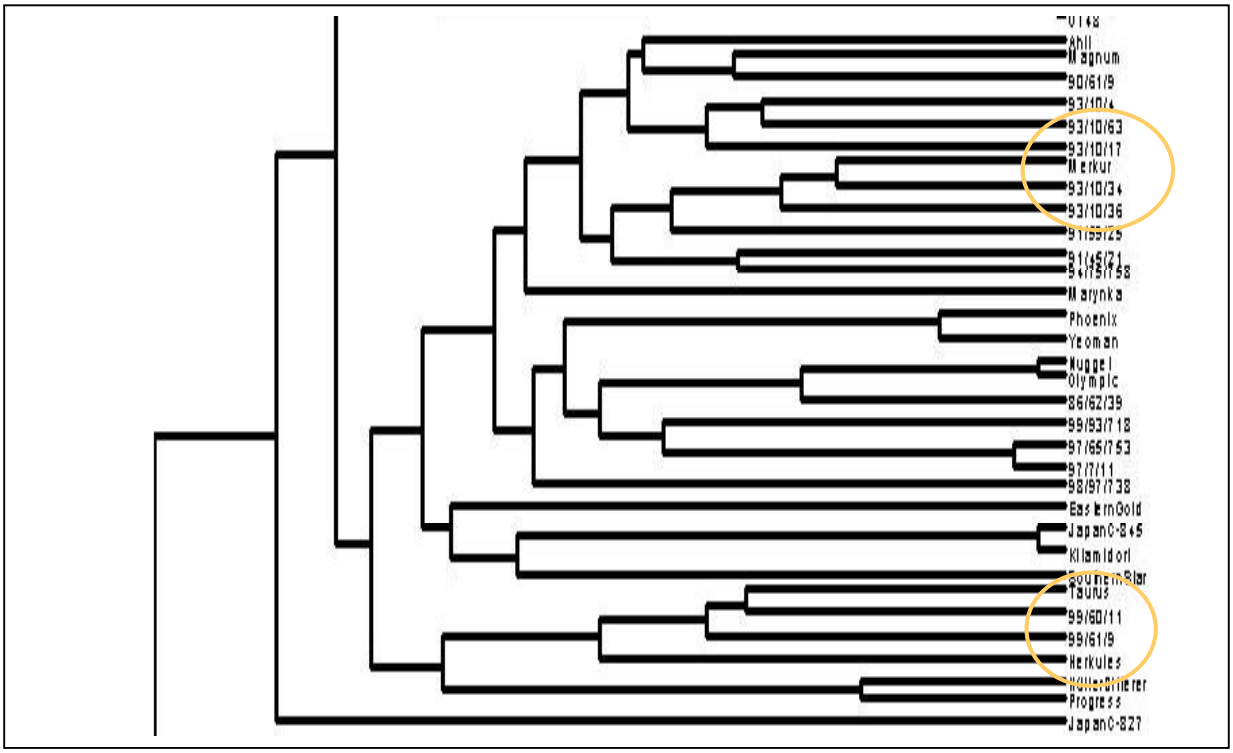
Fingerprint patterns of 148 international hop varieties and breeding lines were produced using 24 AFLP primer combinations. An average number of 20 polymorphic bands per combination was detected. Based on a total of 236 highly reproducible fragments, cluster analysis was conducted using NTSys 2.0.

The grouping of all varieties and breeding lines was correlated with their breeding history which confirmed and supported the efficiency of molecular characterisation.

In addition to former diversity studies with international hop cultivars and wild hops, this study included numerous current breeding lines. Figure 4 shows a cluster with varieties and breeding lines characterized by high alpha acids content. The breeding lines 93/10/17, 93/10/34, 93/10/36 and 93/10/63 are closely related to their sister the new variety Merkur. In addition, Herkules, the currently released Hüll high alpha variety, is grouped in the direct vicinity to its mother Taurus.

The extensive AFLP database built up during this work is also useful for a reliable and rapid identification of varieties

Figure 4: Main cluster with hops containing high alpha acids content



CONCLUSIONS

The above given overview shows that genome analysis is an efficient and sensitive method to support the classical hop breeding process. Using the AFLP technique the genetic relationship between the Hüll breeding lines and the world hop germplasm could be determined. Knowledge obtained from these studies allows a specific, more precise selection of crossing partners for special breeding strategies. In addition, the genetic fingerprint of hop varieties can help to ensure high quality which is crucial the hop and brewing industry. In some cases molecular characterization even excels chemical analysis. Often closely related varieties (e.g. Perle and Northern Brewer) can be discerned using AFLPs while gas chromatograph profiling fails. Moreover, all male specific markers are already in practical use to distinguish male and female hop plants without flowers available. The closely linked AFLP markers to the powdery mildew resistance gene *R2* will be transformed into STS (= sequence tagged site) markers for screening promising breeding lines.

ACKNOWLEDGEMENTS

The authors wish to thank Veronika Mayer, Jutta Kneidl and Luise Logothetis for their excellent technical assistance.

This study was funded by the Bavarian Ministry of Agriculture and Forestry and the Scientific Funding of the German brewing industry.

REFERENCES

1. Brady, J.L., Scott, N.S. and Thomas, M.R. 1996: DNA typing of hops (*Humulus lupulus*) through application of RAPD and microsatellite marker sequences converted to sequence tagged sites (STS). Euphytica, 91, 277-284.
2. Buntjer, J., B. (2000): Cross Checker: Computer assisted scoring of genetic AFLP data. Plant and animal Genome XII, San Diego, 2000.
3. Doyle,J.J. and J.L. Doyle (1990): Isolation of plant DNA from fresh tissue. Focus 12, 13-15
4. Hadonou, A. M., Walden, R. & Darby, P. 2004:Isolation and characterization of polymorphic microsatellites for assessment of genetic variation of hops (*Humulus lupulus* L.). *Molecular Ecology Notes* 4 (2), 280-282.
5. Hartl, L. and Seefelder, S. (1998): Diversity of selected hop cultivars detected by fluorescent AFLPs. *Theor. Appl. Genet*. 96: 112-116.
6. Henning, J., A. Haunold, G. Nickerson and U. Gamperst (1997) Estimates of genetic correlation for five traits in female hop accessions. J.Am. Soc. Brew. Chem. 55 (4), 161-165
7. Kosambi, D.D., 1944. The estimation of map distances from recombination values. Ann. Eugen. 12: 17-175.
8. Patzak J. (2001): Comparison of RAPD, STS, ISSR and AFLP molecular methods used for assessment of genetic diversity in hop (*Humulus lupulus* L.). Euphytica, 121: 9-18
9. Polley, A., E. Seigner, and M.W. Ganal (1997) Identification of sex in hop (Humulus lupulus) using molecular markers. Genome 40, 357-361
10. Saal, B. and Wricke, G. 2002: Clustering of amplified fragment length polymorphism markers in a linkage mape of rye. Plant Breeding 121, 117-123.

11. Seefelder, S., H. Ehrmaier, G. Schweizer and E. Seigner (2000): Genetic diversity and phylogenetic relationships among accessions of hop, Humulus lupulus, as determined by fragment length polymorphism fingerprinting compared with pedigree data. Plant breeding 119 (3), 257-264
12. Seefelder, S., H. Ehrmaier, G. Schweizer and E. Seigner (2000): Male and female genetic linkage map of hop, *Humulus* lupulus L. Plant breeding 119 (3), 249-255
13. Seefelder, S., Lutz, A., Seigner, E. and B. Engelhard (2002) Tools for hop breeding, DNA-marker, the hidden qualities of the hop plant. International Edition of the German Hop-Grower Magazine, Wolnzach, Hallertau 39-43
14. Seefelder, S., Ganal, M. and Seigner, E. 2002: Development of microsatellite markers. Annual report 2001– Society of Hop Research and Hop Research Institute Hüll, 46-47.
15. Seigner, E., Seefelder, S. and Felsenstein, F. (2002): Untersuchungen zum Virulenzspektrum des Echten Mehltaus bei Hopfen (*Sphaerotheca humuli*) und zur Wirksamkeit rassen-spezifischer Resistenzgene. Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 54: 147-151
Strommer, J., Peter, J., Zethof, J., de Keukeleire, P., and Gerats, T. 2002: AFLP maps of Petunia hybrida: building maps when markers cluster. Theor Appl Genet 105: 1000-1009
16. Sustar-Vozlic, J., and B. Javornik (1999) Genetic relationship in cultivars of hop, Humulus lupulus L. determined by RAPD analysis. Plant breeding 118, 175-181 Van Ooijen J.W. and Voorrips, R.E. 2001. JoinMap ® 3.0, software for the calculation of genetic linkage maps. Plant Research International, The Netherlands.
17. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acid Res 23:4407-4414