Genetic diversity within lamb's lettuce (*Valerianella locusta* L.) and across related species determined by AFLP markers

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Abstract: Amplified fragment length polymorphism (AFLP) analysis was employed to study the genetic diversity of elite germplasm of *Valerianella locusta* L and across related species. Twenty seven *Eco*RI/*Mse*I-based primer combinations with five selective bases (*Eco*RI-ANN, *Mse*I-CN) were screened individually in the elite, and fifteen of those in the exotic germplasm. Polymorphic AFLP marker bands were scored for calculation of Jaccard's coefficient of genetic similarities. Phenetic trees for both data-sets (elite and exotic) were constructed using UPGMA-cluster analysis, revealing genetic relationships among the old varieties, the recently bred varieties, and the genebank accessions. This study proved that the AFLP technique is both an efficient and powerful tool for the determination of genetic relationships in lamb's lettuce germplasm. Information emerging from this study will improve the breeding process by indicating the variability present in the existing breeding pools and devising the potential for its broadening. In addition, the management of the lamb's lettuce germplasm accessions in a genebank can be facilitated.

Keywords: Lamb's lettuce, *Valerianella locusta* L., AFLP, Genetic similarity, Germplasm, Cluster analysis

Introduction

Lamb's lettuce (*Valerianella locusta* L.), also known as corn salad, is member of the family Valerianaceae. This annual is a common weed in waste ground and cultivated land, but has also been cultivated for long time in gardens. It is not grown much outside continental Europe, where it is a favorite salad plant in France and Germany. The production area in greenhouses in Germany amounted to 207 ha, thus being the third most important greenhouse-grown vegetable crop, after tomato and cucumber (Bundessortenamt 1997). With an area of open field production of 1700 ha in Germany, lamb's lettuce is achieving more importance and, consequently, attracting an increasing interest of plant breeders. The basic breeding objectives are higher yield of leaves, growing type, plant height, leaf color and form, cold stress tolerance, as well as resistance to the major pathogens *Phoma* sp., *Peronospora* sp., and *Acidovorax valerianelle*. Lamb's lettuce breeding aims at producing fast growing varieties with round, dark green leaves. The genetics of the species is, however, not well known. It is a diploid, autogamous crop. No exact information on the chromosome number or the genome size are available. Closely allied to it are several members of the genus *Valerianella*, as well as some other species assigned to the genus *Fedia*. The possibilities of crossing cultivated lamb's lettuce to any other of its wild relatives, except *V. carinata*, are not known.

Information on the genetic diversity in breeding materials is essential for the optimal design of plant breeding programs. This applies, for example, to the choice of parents for establishing new breeding populations, recognition of heterotic groups, and the introgression of exotic germplasm. Another aspect of interest to breeders is cultivar registration and protection. Accurate estimation of genetic distances between varieties is of high importance in this respect. The utilization of morphological data is not recommended for this purpose in

lamb's lettuce, since the descriptors are highly subjective. In addition, little is known about the co-ancestry among lamb's lettuce varieties, because reliable and detailed pedigree records are ambiguous or not available.

Amplified Fragment Length Polymorphism (AFLP) marker technology has emerged as a powerful tool for germplasm characterization by DNA fingerprinting. In comparison to other molecular marker techniques, AFLPs allow the detection of large numbers of bands in one reaction (Vos et al. 1995) using small amounts of DNA without requiring prior sequence information. Compared to other fingerprinting techniques such as RAPDs, AFLPs are more reproducible and transferable between laboratories (Jones et al. 1997). AFLPs clearly assigned genotypes to known heterotic or other (e.g. winter–spring cereals) groups in maize (Lübberstedt et al. 2000), wheat (Bohn et al. 1999, Soleimani et al. 2002), barley (Schut et al. 1997), pea (Simioniuc et al. 2002), potato (Kim et al. 1998), rice (Zhu et al. 1998) and other crops. In Addition, AFLPs proved to be highly efficient for cultivar identification, in terms of their discrimination power (Lombard et al. 2000, Heckenberger et al. 2002).

Therefore, we chose AFLPs to assess the genetic diversity in lamb's lettuce. The objectives of our study were: (1) to establish and optimize the AFLP protocol for lamb's lettuce; to use AFLP fingerprinting to estimate the genetic diversity (2) among genotypes representing elite germplasm pool, (3) among *V. locusta* genotypes and related species representing exotic germplasm pool, as well as (4) among all investigated genotypes and accessions.

Material and methods

Plant material, morphological evaluation, DNA extraction, AFLP protocol

Two sets of materials were investigated. A set, further referred to as elite germplasm, contained 34 modern line varieties. The other set, further referred to as exotic germplasm, consisted of 12 varieties from former breeding periods, six genebank accessions of *V. locusta* wild types (Laterrade, Deutscher and *V. locusta* accession from the IPK Gatersleben, Germany; two wild forms from BAZ Braunschweig, Germany; one *V. locusta* accession originating from Sweden, obtained from Nunhems-Hild) and 12 accessions of lamb's lettuce wild relatives: *V. carinata* (IPK and Botanical Garden of Bordeaux, France), *V. coronata* (IPK), *V. dentata* (IPK), *V. echinata* (Botanical Garden, University of Göttingen, Germany), *V. eriocarpa* (IPK), *V. pumila* (IPK), *V. rimosa* (IPK), and three accessions of *Fedia cornucopia* (obtained from Nunhems-Hild). To test the reliability of the AFLP method, checks were included: (1) blind checks - genotypes already existing in each set (six in the elite and three in the exotic germplasm), sown twice under different numbers, (2) four duplicates - genotypes from the elite set added to the exotic set in order to allow comparison between the both sets, and (3) laboratory duplicates - genotypes duplicated after DNA extraction, and re-duplicated in consecutive steps of AFLP analysis (four per set), to test reliability and reproducibility of the AFLP protocol.

Fresh leaf tissue was ground to a fine powder in liquid nitrogen. The extraction of genomic DNA was done following the modified CTAB procedure (Hoisington et al. 1994). The concentration of extracted genomic DNA was adjusted to 125ng/µl.

AFLP fingerprints were produced according to Vos et al. (1995). An amount of 250ng DNA was digested with the restriction enzymes *Eco*RI and *Mse*I. Ligation of the restriction fragments to adapters was performed in one step. A 1:20 dilution (in water) of the restricted and adapter-ligated DNA was used as a template in the pre-amplification reactions with a total of two selective bases (*Eco*RI+A, *Mse*I+C). For the final selective amplification, a 1:20 dilution of pre-amplified DNA was amplified using γ-[^{33}P]-ATP labeled *Eco*RI-primer carrying three selective nucleotides in combination with a *Mse*I primer containing two

selective nucleotides. In total, 27 *Eco*/*Mse* primer combinations were employed for selective amplification in the elite germplasm, and 15 of those in the exotic germplasm. Following the final amplification, an equal volume of formamide loading dye was added to the PCR products. The amplified DNA fragments were denatured and separated by electrophoresis on a 6% denaturing polyacrylamide gel. After drying, gels were exposed to X-ray films for five to seven days before developing.

Statistical analysis

The bands ranging in length from 50 to 350 base pairs were scored manually as present (1) or absent (0), and transferred to a binary matrix. Only distinct and polymorphic major bands were analyzed using NTSYSpc (version 2.0, Rohlf 1998). Genetic similarity (GS) between the two genotypes *i* and *j* was calculated according to the formula of Jaccard (1908), using the SIMQUAL module of the NTSYSpc:

$GS_{ij} = N_{ij} / (N_{ij} + N_i + N_j)$

where N_i is the number of detected bands in the genotype *i* and not in genotype *j*, N_j is the number of detected bands in the genotype *j* and not in genotype *i*, and N_{ij} is the number of bands common to genotypes *i* and *j*. The standard errors (SE) of the calculated GS were estimated by the jackknife procedure (Miller 1974), using the Plabsim software (Frisch et al. 2000).

The generated similarity matrices were further analyzed using the UPGMA (unweighted pair group method using arithmetic averages, Sneath and Sokal 1973) clustering method in the SAHN module of the NTSYSpc. Dendrograms were created using the TREE module.

Results and discussion

Polymorphism detected by AFLPs

The 27 primer combinations tested in the elite germplasm resulted in a total number of 1315 bands, of which 211 (16%) were polymorphic. However, only 108 polymorphic bands (8% of the total number of bands) were selected as clearly distinct and reliable for data processing. The degree of polymorphism detected in the exotic germplasm set was much higher, mainly due to the presence of lamb's lettuce wild relatives. The 15 primer combinations applied for screening the exotic germplasm set did not yield a single monomorphic band in 1526 detected. As the most unambiguous, 287 bands (19%) were selected for data processing and GS estimation. The number of polymorphic bands per AFLP primer combination, selected for data processing, ranged from one to 12 in the elite germplasm, and from three to 48 in the exotic set.

Genetic similarities among genotypes

The high values of GS coefficients (>0.95) between replicated samples in both germplasm sets confirmed the reliability of AFLPs (Table 1). Comparable GS estimates between the replicated samples were found for RFLP, RAPD and AFLP marker data (Messmer et al. 1993, Hahn et al. 1995, Jones et al. 1997, Lübberstedt et al. 2000). In our study, the lowest estimate of GS in blind checks was 0.95 (Blind75 x CS-50), which was lower than GS estimates in laboratory duplicates with GS values > 0.98 (Table 1). Deviations from the expected level of 1.00 similarity detected between the blind checks and their respective original genotypes, could be explained by residual heterozygosity, especially in older varieties.

The laboratory duplicates showed very high levels of genetic similarity. The only two deviations from an absolute identity with the original sample were detected in L44 and L78, which might be due to incomplete digestion with restriction enzymes. Laboratory duplicates L46 and L47 were duplicates made out of L44 on the level of pre-amplification and selective

amplification, respectively, and indicated the reliability of the PCR protocol used. The same applied to the duplicates L79 and L80, which were the duplications of L77 in consecutive PCR reactions.

Table 1: Jaccard's coefficient of genetic similarities between replicates in AFLP reaction in the elite germplasm of lamb's lettuce (*Valerianella locusta* L.) and among related species

	Blind check		Laboratory duplicate	
	Genotype pair	GS (SE)	Genotype pair	GS (SE)
Elite set	Blind24 x Cirilla	1.00 (0.00)	Blind25 x L44	0.98 (0.05)
	Blind25 x Gala	0.98 (0.02)	L44 x L46	1.00 (0.05)
	Blind26 x CS-12	0.98 (0.03)	L44 x L47	1.00 (0.06)
	Blind41 x CS-27	0.99 (0.02)	L46 x L47	1.00 (0.02)
	Blind42 x CS-29	1.00 (0.02)	Blind41 x L45	1.00 (0.02)
	Blind43 x CS-31	0.96 (0.03)	-	-
Exotic set	Blind74 x CS-27	0.99 (0.05)	Blind76 x L77	1.00 (0.03)
	Blind75 x CS-50	0.95 (0.04)	L77 x L79	1.00 (0.04)
	Blind76 x CS-30	1.00 (0.05)	L77 x L80	1.00 (0.04)
	-	-	L79 x L80	1.00 (0.04)
	-	-	Blind75 x L78	0.99 (0.05)

Genetic similarities between elite germplasm genotypes ranged from 0.24 (CS-37 x Louvier) to 0.99 (CS-22 x CS-27), with a mean of 0.63. Standard errors for individual GS estimates varied from 0 to 0.11. In the exotic germplasm, the lowest GS values of 0.10 were detected between *V. coronata* and several other species: *V. carinata* (Gatersleben), *V. carinata* (Bordeaux), Louvier (Clause), Louvier (Le Paysan), Verte de Louvier and CS-46. The highest GS estimate in the exotic set of 1.00 was obtained between two *V. carinata* genebank accessions. The average GS value in the exotic germplasm set was 0.58. Standard errors of the individual GS estimates in this set varied from 0.02 to 0.07.

Cluster analysis of AFLP data

The dendrogram obtained with the UPGMA cluster analysis of GSs in the elite germplasm revealed genetically distinct groups within lamb's lettuce elite germplasm (Fig. 1). The clustering pattern partially agreed with the available morphological information. Some of the genotypes having stronger leaf structure ("veined types") tended to cluster together. Genotypes CS-37, CS-19, Trophy, and CLX3433 formed one cluster; CS-32, Armel, Verella and CS-31 formed the other; while CS-2, CS-17, and CS-18 formed the third cluster. Variety Louvier was divergent to all others, which was in accordance with the expectations from breeders' experience. Contrary to the expectations based on morphological traits, however, genotypes CS-27, CS-30, and Cirilla did not form a common cluster.

The prediction of the breeding behavior of offspring from parent combinations by the simultaneous use of AFLP-based genetic similarities and coefficients of co-ancestry has been suggested by numerous authors (Schut et al. 1997, Simioniuc et al. 2002). In a minor crop such as lamb's lettuce, breeding practices rely mostly on phenotypical selection, but whether morphological distances have any predictive value on breeding behavior remains questionable.

Genotypes belonging to the exotic germplasm created two major clusters in the UPGMA analysis, thus clearly separating formerly used breeding material and the *V. locusta* genebank accessions in one, and lamb's lettuce wild relatives in the other cluster (Fig. 2). The cluster-branch consisting of formerly grown breeding materials and several *V. locusta* genebank

accessions was further divided into two sub-clusters. Two *V. carinata* accessions clustered together, thus supporting the information that the accession from IPK Gatersleben originated from France. *V. locusta* genebank accession originating from Sweden clustered with *V.coronata* types, while the other *V. locusta* wild types, Laterrade and Deutscher clustered with the genotypes formerly used as breeding materials. In the other major cluster, consisting of wild relatives, three accessions of *Fedia cornucopia* formed a separate sub-cluster, which was expected because of their distinct morphology and cross pollinating pattern. The joint clustering of identical species can still not be supported with their genetics due to the lack of basic information on chromosome numbers and amount of DNA.

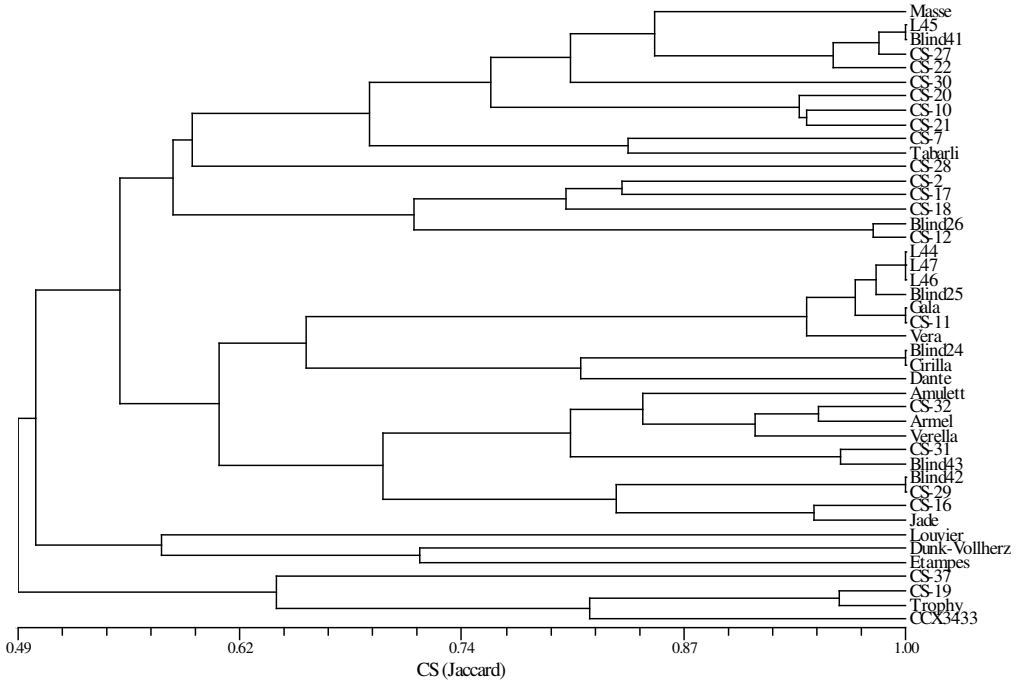

Figure 1. Association among 44 genotypes of lamb's lettuce elite germplasm revealed by average linkage (UPGMA) cluster analysis of Jaccard's genetic similarity (GS) coefficients calculated from AFLP data of 27 primer combinations

The results of our study indicated that wild forms of *V. locusta* clustered together with more developed breeding lines. Regarding the results of wild relatives obtained by AFLPs, *V. carinata* accessions were the closest to the cluster consisting of former elite material. If those genotypes carry desirable traits (e.g. resistance to pathogens), crosses with elite germplasm could be suggested for a limited broadening of lamb's lettuce breeding germplasm.

In conclusion, measurements of genetic diversity can be used in breeding programs to increase the genetic variation in base populations by crossing cultivars with a high level of genetic distance as well as for the introgression of exotic germplasm. Molecular genetic diversity estimates are extremely useful for intellectual property protection, particularly in the determination of essential derivation. The genetic diversity estimates based on molecular marker data may be compared to a minimum genetic distance which indicates that two varieties are not essentially derived (Lefebvre et al. 2001). Finally, our results can be applied in genebank management of *Valerianella* accessions, assisting in detection of duplicates in collections as well as in a more accurate classification of accessions, thus improving the utilization of germplasm conserved in genebanks.

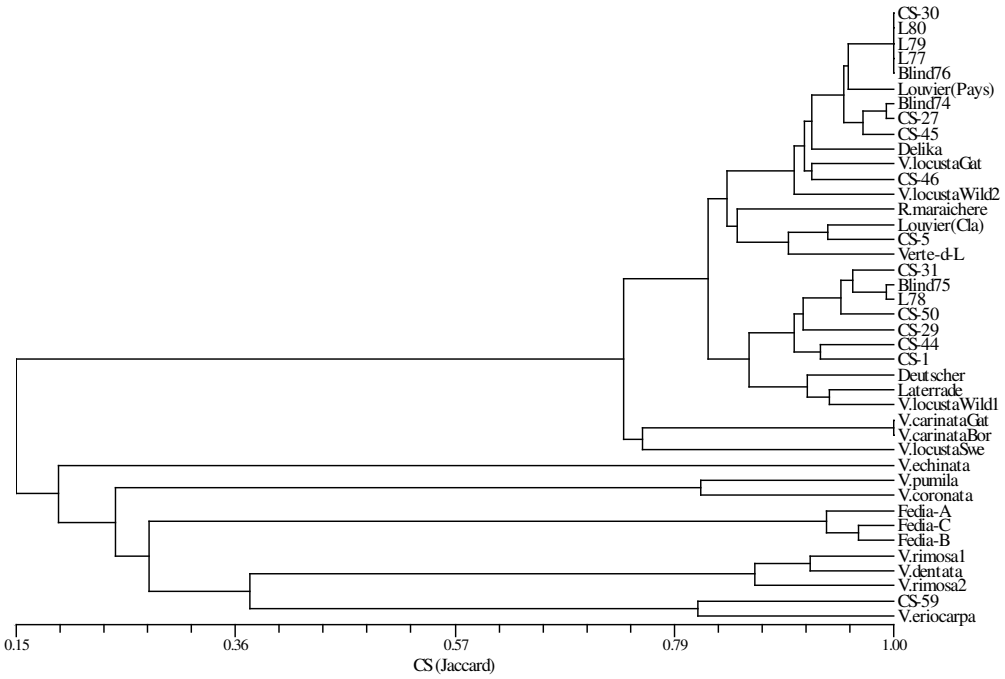

Figure 2. Association among 41 genotypes of lamb's lettuce exotic germplasm revealed by average linkage (UPGMA) cluster analysis of Jaccard's genetic similarity (GS) coefficients calculated from AFLP data of 15 primer combinations

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