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Genetic diversity in germplasm of cornsalad (*Valerianella locusta* L.), radish (*Raphanus sativus* L.), and celeriac (*Apium graveolens* L. var. *rapaceum*), investigated with PCR-based molecular markers

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Table of Contents

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Abbreviations

AFLP	amplified fragment length polymorphism
AMOVA	analysis of molecular variance
bp	base pair
CTAB	hexadecyltrimethylammonium bromide
DNA	deoxyribonucleic acid
dNTP	nucleotides
EDTA	ethylenediaminetetraacetic acid
f	coefficient of co-ancestry
GD	genetic distance
GS	genetic similarity
h	hour
ha	hectare
ISSR	inter simple sequence repeat
M	mole
MD	morphological distance
MI	marker index
min	minute
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
PIC	polymorphic information content
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SSR	simple sequence repeat
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
UPGMA	unweighted pair group method using arithmetic averages
UPOV	International Union for the Protection of New Varieties of Plants
UV	ultra-violet

1 General Introduction

Cornsalad, radish, and celeriac are vegetable crops whose cultivation in Central Europe has considerably increased over the last couple of decades. Cornsalad [*Valerianella locusta* (L.) Betcke, formerly *V. olitoria* (L.) Poll.; fam. Valerianaceae], also known as lamb's lettuce, is found in southern Europe, but probably originated in the Mediterranean area. It is a diploid crop with a chromosome number 2n = 14, but further genetic information is scanty. Of 181 species classified into the genus *Valerianella* (USDA 2003; The Royal Botanic Gardens Kew 2003), only *V. locusta* is cultivated. Owing to its mild and delicate taste as well as richness in iron, cornsalad is a favourite leafy salad vegetable in France, Germany, and The Netherlands. Based on the production area in greenhouses (200 ha), cornsalad is the third most important vegetable crop in Germany, after tomato and cucumber, whereas 1700 ha are planted in open fields (Bundessortenamt 1997).

Radish [*Raphanus sativus* L. var. *sativus* convar. *radicula* (DC.) Pers.; fam. Brassicaceae] is an ancient domesticate, native to the eastern Mediterranean and the Middle East, although some taxonomists consider China the centre of origin (Werth 1937; Rubatzky and Yamaguchi 1997). The first records about radish consumption in human nutrition date back to about 2000 BC in the ancient Egypt, whereas its cultivation started in China and Korea about 400 BC (Becker 1962; George and Evans 1981; Kaneko and Matsuzawa 1993). The first European variety of cultivated garden radish, however, was not recorded before the 16th century (Wein 1964; George and Evans 1981). Cultivated radish is a diploid species with a chromosome number 2n = 18 and small genome size (C-value = 0.55 pg; The Royal Botanic Gardens Kew 2003). Its economic importance is high and increasing. It is an important vegetable in Japan, Korea, China, India, and other East Asian countries (Kaneko and Matsuzawa 1993). Based on the latest available information, the production of radish in Europe amounts to 120000 t, with France, Greece, The Netherlands, Italy, and Spain being the main producers (Vogel 1996). From 1985 to 2000 in Germany, the area of radish cultivation under glass decreased from 74 to 52 to ha, whereas the open field cultivation increased almost six times, from 400 to 2700 ha (Bundessortenamt 1986, 2001; Statistisches Bundesamt Deutschland 2004).

The cultivation of celeriac (*Apium graveolens* L. var. *rapaceum*; fam. Apiaceae), the only cultivated species of the *Apium* genus (Quiros 1993), most likely started as early as

400 BC, when ancient Egyptians and Romans used it as a medicinal crop. First records on its domestication as a true vegetable date back to 16th century in the Mediterranean region (Smith 1979; Quiros 1993). Celeriac is a biennial crop with a chromosome number 2n = 22 and genome size of 3x10^9 bp DNA. Its flowers are small, with different developmental stages within the same umbel, which makes the emasculation of individual anthers difficult. A single genetic male sterile is reported as a spontaneous mutant in some weedy Iranian species (Quiros 1993). Therefore, nearly all celeriac varieties currently produced are open-pollinated. The area cultivated with celeriac is steadily increasing, and currently amounts to 1720 ha in Germany, which is approximately 4% of the total vegetable growing area in the country (Bundessortenamt 2001; Statistisches Bundesamt Deutschland 2004).

Cornsalad, radish, and celeriac are three economically important vegetable species in Europe, representing three different pollination types: autogamy (cornsalad; Ryder 1979), strict allogamy (radish; Kaneko and Matsuzawa 1993), and partial allogamy (celeriac; Quiros 1993). Establishment of the method to estimate genetic diversity in these three crops, using PCR-based molecular markers, can serve as a model in a wide range of vegetable crops.

Assessment of genetic relationships among cultivated plants is a fundamental component of crop improvement programs, as it serves to provide information about genetic diversity, and creates a platform for stratified sampling of breeding populations. Numerous studies have so far been conducted to analyse genetic relationships in a number of cultivated crops of major economic importance (for review, see Mohammadi and Prasanna 2003), but little has been done in so-called minor crops, such as cornsalad, radish, and celeriac.

Accurate assessment of levels and patterns of genetic diversity can be invaluable in crop breeding for various purposes, including (i) analysis of genetic variability of cultivars (Smith 1984; Cox et al. 1986), (ii) identification of diverse parental combinations to develop segregating progenies with maximum genetic variability for further selection (Barret and Kidwell 1998), and (iii) introgression of desirable genes or chromosome segments from diverse sources into elite germplasm (Thompson et al. 1998). Understanding genetic relationships among varieties can be particularly useful in planning crosses, in defining heterotic pools and assigning lines to specific heterotic groups (Hallauer and Miranda 1988), as well as for distinctiveness, uniformity and stability testing

and the protection of breeders' rights in the frame of essentially derived varieties (Lombard et al. 2000; Lefebvre et al. 2001; Heckenberger et al. 2002, 2003).

Furthermore, the knowledge of genetic diversity in the available germplasm is of an indispensable importance for plant genetic resources management in gene banks. Analysis of genetic diversity in germplasm collections facilitates reliable classification of accessions, detection of duplicates, and identification of useful accessions for specific breeding purposes (Engels et al. 2002; Mohammadi and Prasanna 2003).

Breeding objectives and genetic diversity in cornsalad, radish, and celeriac

Cornsalad breeding aims at the production of line varieties with round, dark green leaves, tolerant to extreme temperature stress, and resistant to *Phoma* sp., *Peronospora valerianelle*, and *Acidovorax valerianelle*, where new sources of resistance are required (Schieder and Hermens, personal communication). Radish breeding was practiced for centuries, by means of mass or pedigree selection. Since two decades, the production of F_1 hybrids using cytoplasmatic male sterility has widely replaced fairly simple breeding methods based on morphological traits (Schieder, personal communication) to generate genetically uniform varieties. The uniformity of a variety is becoming a high priority goal in radish breeding. Of 54 currently grown radish varieties in Germany, 19 (35.2%) are F_1 hybrids (Bundessortenamt 2001), thus indicating a significant increase during the last 15 years when only four of 41 (9.8%) registered radish varieties were hybrids (Bundessortenamt 1986). Most breeding work is aimed at further adaptation to different growing conditions, improved resistance to *Peronospora parasitica* and *Albugo candida* (Vogel 1996), specific market preferences, and improved marketing conditions. The main objectives in celeriac breeding are uniformity (in colour, size, and yield), quality, and disease resistance (Bundessortenamt 2001). The preferred celeriac varieties are characterised with round-shaped tubers, whitish colour of skin, and low insertion of roots (Schilling and Dijkstra, personal communication). Tubers of higher inner quality possess a firm consistency and weak internal rust spots of the flesh. Furthermore, varieties whose flesh remains white after cooking are favoured. Major diseases in celeriac are foliar late blight (*Septoria apiicola*) and celery root rot (*Phoma apiicola* Kleb.). Genes for resistance

to *Septoria* are found in some wild species, such as *Apium nodiflorum*. Nevertheless, the attempts to hybridize *A. gaveolens* with other species failed (Quiros 1993).

Based on breeders' knowledge and experiences, genetic diversity available in current assortments of cornsalad, radish, and celeriac can be described as narrow. Breeding programs in different companies rely on germplasm of geographically limited origin, and are based on phenotypic selection of varieties. Breeders preferably make crosses between already selected genotypes proven for their outstanding performance *per se* and/or as progenitors of superior cultivars. In consequence, the number of genotypes being recycled is small, thus reducing the genetic basis of the elite germplasm and ultimately increasing potential vulnerability to pests and abiotic stresses, which is also known from other major crops of agricultural importance (Graner et al. 1994). Improvements in breeding approaches applied for the selection of cornsalad, radish, and celeriac could be expected if hybrid breeding in open-pollinated species (radish and celeriac) or introgression of desirable traits from gene bank accessions (landraces and wild relatives) is made possible. Exploitation of heterotic patterns between different sources of germplasm (particularly in open-pollinated crops) is a major goal in hybrid breeding (Melchinger and Gumber 1998). A systematic and exact approach is necessary for the identification of heterotic patterns and concentrated introgression of new germplasm into breeding populations of cornsalad, radish, and celeriac.

The abundance of cornsalad, radish, and celeriac germplasm accessions in gene bank collections is relatively poor (Quiros 1993) and their relationship to commercially grown varieties is generally unknown. Wild species of *Valerianella*, *Raphanus*, and *Apium* might contribute to the introgression of new genes (such as resistance genes) into cultivated material. Species most closely related to the respective elite germplasm would be the most promising candidates for successful inter-specific crosses. Hence, wild species conserved in gene banks require an accurate description and classification.

Approaches to study relationships among varieties

Information on pedigree or geographic origin (passport data), as well as various kinds of phenotypic and genotypic descriptors (e.g., morphological, cytological, biochemical, and DNA markers), have so far been employed to study phylogenetic relationships among and

the genetic variation within varieties (Messmer et al. 1991; Messmer et al. 1993; Ben-Har et al. 1995; Schut et al. 1997; Lübberstedt et al. 2000; Roldán-Ruiz et al. 2001). Calculation of the co-ancestry coefficient *f* (Malécot 1948) requires reliable and detailed pedigree records (Bernardo 1993; Messmer et al. 1993; van Hintum and Haalman 1994). For remote ancestors, these prerequisites are often not fulfilled because breeding records are either incomplete or names of cultivars are ambiguous. For elite cultivars, pedigrees are also becoming increasingly protected as trade secrets by private breeding companies (Graner et al. 1994). Moreover, pedigree-based diversity measurements can result in an overestimation of the actual level of genetic diversity present in the genepool as a consequence of the assumptions that are made regarding genetic drift, selection pressure, and relatedness of ancestors without known pedigree (Cox et al. 1985; Messmer et al. 1993; Graner et al. 1994; Kim and Ward 1997; Barrett et al. 1998; Soleimani et al. 2002). Comprehensive genetic diversity studies have been conducted in major crops, using passport, morphological (Smith and Smith 1992; Ben-Har et al. 1995), and biochemical data obtained by analyses of isozymes (Cox et al. 1985; Hamrick and Godt 1997) or storage proteins (Smith et al. 1987). Nevertheless, their usefulness for obtaining reliable estimates of genetic similarity is limited because of the small number of marker loci available and the low degree of polymorphism generally found in elite breeding materials (Messmer et al. 1991).

The major strength of molecular markers is their ability to detect genetic diversity at levels of resolution that exceed by far those achievable with other, previously applied methods (Karp 2002). Owing to the great number of polymorphic marker loci, DNA markers possess the ability to bypass the assumptions inherent to pedigree analysis. Regarding their nature, DNA-assays are more robust and independent of environmental conditions. Nevertheless, the extent of their utility may depend on the nature of the marker system, their number, genome coverage, and the population under investigation (Karp 2002).

Development of the polymerase chain reaction (PCR; Mullis and Faloona 1987) was a technological breakthrough in genome analysis because it enabled the amplification of specific fragments from the total genomic DNA. Compared to other techniques, PCR-based DNA markers are less labour- and time-consuming, and provide an estimate of genetic similarity by direct sampling from the entire genome with unprecedented precision. Most widely applied DNA marker techniques differ not only in principle, but also in the

type and amount of polymorphism detected (Russell et al. 1997). Techniques such as non-PCR based restriction fragment length polymorphisms (RFLPs; Botstein et al. 1980) and PCR-based microsatellites or simple sequence repeat polymorphisms (SSRs; Tautz 1989) possess the ability to distinguish multiple bands (alleles) per locus, thus giving more information on a single locus. By contrast, individual bands detected with PCR-based fingerprinting techniques, such as randomly amplified polymorphic DNA (RAPDs; Williams et al. 1990) and amplified fragment length polymorphisms (AFLPs; Vos et al. 1995), are scored on a biallelic basis, as marker band present or absent. The major advantage of fingerprinting techniques is that multiple marker bands – fingerprints - are generated in a single assay.

Amplified fragment length polymorphism is a DNA fingerprinting technique based on selective amplification of restriction fragments. This PCR-based method is able to generate complex fingerprints of up to at least 100 DNA fragments in each reaction. AFLPs are highly reproducible (Jones et al. 1997), and possess a high multiplex ratio, which means that a large number of markers can be generated in a single reaction. By using different combination of selective primers, an almost unlimited number of markers can be obtained. The high multiplex ratio and the fact that no sequence information is needed represent big advantages of AFLPs over a number of other molecular marker methods. Therefore, they have successfully been employed for genetic diversity analysis of various plant species, including maize (Lübberstedt et al. 2000), wheat (Soleimani et al. 2002), barley (Turpeinen et al. 2003), soybean (Powell et al. 1996), and rice (Aggarwal et al. 2002).

Inter simple sequence repeats (ISSRs), as another PCR-based molecular marker method, involve anchoring of designed primers to a subset of microsatellite sequences, and amplify the region between two closely spaced, oppositely oriented microsatellites (Zietkiewitcz et al. 1994). Regarding the multiplex banding profile, high throughput, and relatively low cost, ISSRs have also been widely applied to estimate genetic diversity in various crops (Wu and Tanksley 1993; Gupta et al. 1994; Kantety et al. 1995; Charters et al. 1996; Sánchez de la Hoz et al. 1996; Yang et al. 1996; Nagaoka and Ogihara 1997; Parsons et al. 1997; Prevost and Wilkinson 1999; Métais et al. 2000).

Objectives

The objective of this PhD study was to investigate genetic diversity in germplasm of cornsalad, radish, and celeriac, using PCR-based molecular markers. More specifically, the objectives were to:

1. establish and optimise protocols for two DNA-marker systems (AFLPs and ISSRs) for cornsalad, radish, and celeriac,
2. evaluate the applicability and reliability of AFLPs and ISSRs in genetic diversity studies of cornsalad, radish, and celeriac,
3. analyse relationships and genetic diversity in breeding materials of cornsalad, radish, and celeriac (further referred to as elite material) using AFLPs,
4. define the genetic structure among radish varieties to establish heterotic pools for hybrid breeding,
5. analyse relationships and genetic diversity in formerly grown varieties and gene bank accessions of cornsalad, radish, and celeriac (further referred to as exotic material) using AFLPs, and
6. evaluate the usefulness of introducing exotic materials for broadening the genetic basis of the elite germplasm in cornsalad, radish, and celeriac.

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Genetic diversity in cornsalad (*Valerianella locusta*) and related species as determined by AFLP markers

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Abstract

Fifteen amplified fragment length polymorphism (AFLP) *Eco*RI/*Mse*I-based primer combinations with five selective bases (*Eco* RI-ANN, *Mse*I-CN) were used to estimate genetic diversity among 45 line varieties of cultivated cornsalad and 19 genebank accessions classified into nine different species related to cornsalad. Polymorphic fragments were scored for calculation of Jaccard's coefficient of genetic similarity (GS). The average GS estimate in elite germplasm (GS = 0.90) was substantially higher than in exotic germplasm (GS = 0.47). UPGMA-cluster analysis revealed genetic relationships among recently bred varieties, old varieties and genebank accessions. Analysis of molecular variance indicated almost threefold variability within sets compared with between sets due to a high level of polymorphism among wild species. Sources for increasing genetic diversity in elite germplasm of cornsalad were suggested and a duplicate among the genebank accessions was detected. AFLPs could be considered a powerful tool for genetic diversity estimation in cornsalad germplasm and are recommended for systematic fingerprinting of remaining cornsalad species.

Key words: *Valerianella locusta* — AFLP — AMOVA — cluster analysis — genetic similarity

Cornsalad, also known as lamb's lettuce, is a member of the family *Valerianaceae*. It is a diploid, autogamous crop, with the chromosome number 2n = 14 but otherwise little genetic information. Although 181 species are classified into the genus *Valerianella* (The Royal Botanic Gardens 2003), only *V. locusta* is cultivated and inter-species crosses have been established with *V. carinata* (Nunhems-Hild, personal communication).

Cornsalad is a favourite salad plant in Europe because of its mild and delicate taste and richness in iron. Based on its production area in greenhouses (200 ha), cornsalad is the third most important vegetable crop in Germany, after tomato and cucumber, while 1700 ha are planted in open fields (Bundessortenamt 1997, Juliwa-Enza personal communication). Breeding of cornsalad aims at the production of line varieties with round, dark green leaves, tolerant to extreme temperature stress, and resistant to *Phoma* sp., *Peronospora valerianellae*, and *Acidovorax valerianellae*, where new sources of resistance are required.

Estimation of genetic diversity in cultivated crops has important implications in breeding programmes and in the conservation of genetic resources (Soleimani et al. 2002). The currently available germplasm of cultivated cornsalad is assumed to be narrow as breeding programmes in different companies rely on germplasm of geographically limited origin (Nunhems-Hild and Juliwa-Enza, personal communication). According to the standard criteria of International Union for the Protection of New Varieties of Plants (UPOV), cornsalad varieties are described and differentiated using morphological traits such as seed size and shape, leaf length, shape, profile, glossiness, colour, thickness, and prominence of veins. Nevertheless, genetic diversity within cornsalad elite germplasm cannot be accurately described when based on these data. Information about co-ancestry, as another possibility of estimating genetic diversity, is ambiguous or not available in cornsalad varieties because pedigree records of modern cultivars are becoming increasingly protected as trade secrets of private breeding companies (Graner et al. 1994). Alternatively, molecular markers such as amplified fragment length polymorphisms (AFLPs) (Vos et al. 1995) can be employed to investigate more precisely genetic diversity within species, and to devise suggestions for broadening the germplasm used in breeding.

The AFLP marker technique generates complex banding patterns (DNA fingerprints) in one reaction, using small amounts of DNA without requiring prior sequence information. Compared with other fingerprinting techniques, AFLPs proved to be highly reproducible and transferable between laboratories (Jones et al. 1997). In intensively studied crops, such as maize (Marsan et al. 1998, Lübberstedt et al. 2000), wheat (Bohn et al. 1999, Soleimani et al. 2002), or barley (Becker et al. 1995, Ellis et al. 1997, Russell et al. 1997, Schut et al. 1997), AFLPs clearly assigned genotypes to known heterotic or other groups (e.g. winter and spring cereals). They were also applied in genetic diversity studies of other crops (Kim et al. 1998, Zhu et al. 1998, Abdalla et al. 2000, Lombard et al. 2000, Simioniuc et al. 2002), as well as in characterization of genebank accessions (Capo-chichi et al. 2001, McGregor et al. 2002). Finally, AFLPs proved highly efficient in cultivar identification due to their high discrimination power (Lombard et al. 2000, Lefebvre et al. 2001, Heckenberger et al. 2003).

The objectives of this new study were to establish and apply AFLP fingerprinting in cornsalad to estimate the genetic diversity among (1) currently used cornsalad varieties and (2) old cornsalad varieties and genebank accessions from the genus *Valerianella*, as well as (3) to facilitate a further systematic choice of sources for introgressing specific, valuable traits from exotic into elite germplasm.

Materials and Methods

Plant materials: Sixty-four accessions of cornsalad [*Valerianella locusta* (L.)] and related species were divided into two sets. The first set, further referred to as elite germplasm, contained 34 modern line varieties of *V. locusta*. Nineteen line varieties, originating from either of two collaborating breeding companies Nunhems-Hild and Juliwa-Enza, were designated with CS-codes ('CS-2', 'CS-7', 'CS-10', 'CS-11', 'CS-12', 'CS-16' to 'CS-22', 'CS-27' to 'CS-32', and 'CS-37'), whereas variety names were given for the remaining 15 line varieties. The second set, further referred to as exotic germplasm, consisted of 12 *V. locusta* line varieties from former breeding periods (designated with codes: 'CS-1', 'CS-5', 'CS-8', 'CS-44' to 'CS-46', 'CS-50', and 'CS-59', or variety names – depending on the origin), and 18 genebank or botanical garden accessions of *V. locusta* wild types and related species (Table 1). In addition, the reliability and reproducibility of the AFLP protocol was tested by (1) blind checks: genotypes sown twice under coded numbers (six in the elite and three in the exotic germplasm), and (2) laboratory duplicates: randomly chosen genotypes duplicated after DNA extraction and re-duplicated in consecutive steps of AFLP analysis (four per set). Four standard genotypes were used in both germplasm sets to ensure scoring of identical AFLP fragments in both sets of material.

DNA extraction, AFLP protocol: From a bulk of 20–30 plants per genotype, 2–3 g of fresh leaf material was ground to a fine powder in liquid nitrogen. The extraction of genomic DNA followed the modified CTAB procedure (Hoisington et al. 1994). The extracted genomic DNA was diluted in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and the final concentration adjusted to 125 ng/μl.

AFLP fingerprints were produced according to Vos et al. (1995), with modifications. Approximately 250 ng DNA was double-digested with 4.25 U *Eco*RI (Amersham Biosciences Europe GmbH, Freiburg, Germany) and 3 U *Mse*I (New England BioLabs GmbH, Frankfurt am Main, Germany) restriction enzymes at 37°C for 3 h, in 1 × One-Phor All Buffer Plus. To ligate the adapters, a ligation mixture consisting of 0.5 μM each *Eco*RI and *Mse*I adapter (both Metabion GmbH, Martinsried, Germany), 1 mM ATP, 2 × One-Phor All Buffer Plus, and 1 U of T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany) was added to the restriction digest, and incubated at 20°C (room temperature) for 2 h. The adapter sequences were:

*Eco*RI adapter: 5′-CTC GTA GAC TGC GTA CC
CAT CTG ACG CAT GGT TAA-5′

*Mse*I adapter: 5′-GAC GAT GAG TCC TGA G
A CTC AGG ACT CAT-5′

The ligation product was diluted 1 : 20 in sterile water and used as a template in the pre-amplification reaction with 1 × PCR buffer, 0.2 mM of each dNTP, 0.75 U *Taq* DNA polymerase (Amersham Biosciences Europe GmbH), and 0.4 μM of each of the two AFLP primers having a single selective base (5′GAC TGC GTA CCA ATT CA, 5′GAT GAG TCC TGA GTA AC). The PCR programme consisted of 20 cycles of 94°C for 30 s, 50°C for 60 s and 72°C for 60 s. Prior to a selective amplification, pre-amplification PCR products were diluted 1 : 20 in sterile water. *Eco*RI selective primers with three selective nucleotides were [^{33}P]-γ-ATP 5′-end labelled with T4 polynucleotide kinase (Invitrogen Life Technologies GmbH, Karlsruhe, Germany) and combined with *Mse*I primers carrying two selective nucleotides. In total, 15 *Eco*RI/*Mse*I primer combinations were employed for selective amplification reactions. The selective PCR was performed in 1 × PCR buffer, 0.25 mM each dNTP, 0.4 U Taq DNA polymerase, 0.45 μM *Mse*I primer, and 0.125 μM labelled *Eco*RI primer (Invitrogen Life Technologies GmbH). The following amplification profile was used: an initial cycle of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s, followed by 11 touchdown cycles in which the annealing temperature was reduced by 0.7°C per cycle. The annealing temperature was then kept constant at 56°C for the remaining 25 cycles, followed by an additional extension at 72°C for 2 min. After selective amplification, an equal volume of formamide-loading dye (98% formamide, 10 mM EDTA pH 8.0, and bromo-phenol blue and xylene cyanol, as tracking dyes) was added to PCR products. Amplified DNA fragments were denatured at 95°C for 3 min and separated by electrophoresis in a 6% denaturing polyacrylamide gel. One set of germplasm, genotyped with one AFLP primer combination, fitted to one gel. Each gel was run in 0.5 × TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA pH 8.0), at 23 W for 5 min, followed by 60 W for 2 h. After drying on Whatman paper, gels were exposed to X-ray films for 5–7 days before developing.

Table 1: Genebank and botanical garden accessions of cornsalad (*Valerianella locusta* L.) and related species analysed with amplified fragment length polymorphism markers[1]

Scientific name[2]	Cultivar name	Accession number	Country of origin	Original donor of accession	Maintainer of accession
Valerianella carinata Loisel.	–	–	France	Unknown	Nunhems-Hild
Valerianella carinata Loisel.	–	VALE 12	France	Bot. Garden, Bordeaux, France	IPK Gatersleben
Valerianella coronata DC.	–	–	Unknown	Bot. Garden, University of Göttingen, Germany	Nunhems-Hild
Valerianella dentata (L.) Pollich	–	VALE 4	Unknown	Bot. Garden, University of Cluj-Napoka, Romania	IPK Gatersleben
Valerianella echinata (L.) DC.	–	–	Unknown	Bot. Garden, University of Göttingen, Germany	Nunhems-Hild
Valerianella eriocarpa Desv.	–	VALE 13	France	Bot. Garden, Bordeaux, France	IPK Gatersleben
Valerianella locusta (L.) Laterrade	–	–	Unknown	Unknown	Nunhems-Hild
Valerianella locusta (L.) Laterrade	Valeriana Dolcetta	VALE 14	Italy	Unknown	IPK Gatersleben
Valerianella locusta (L.) Laterrade	Deutscher	VALE 3	Germany	DSG Quedlinburg, Germany	IPK Gatersleben
Valerianella locusta	–	–	Sweden	Unknown	Nunhems-Hild
Valerianella locusta	(Wild type 1)	BAZ 54288	Unknown	BAZ Braunschweig, Germany	BAZ Braunschweig
Valerianella locusta	(Wild type 2)	BAZ 58375	Unknown	BAZ Braunschweig, Germany	BAZ Braunschweig
Valerianella pumila DC.	–	VALE 8	Unknown	Bot. Garden, University of Bonn, Germany	IPK Gatersleben
Valerianella rimosa Bast.	–	VALE 10	Unknown	Bot. Garden, University of Mainz, Germany	IPK Gatersleben
Valerianella rimosa Bast.	–	VALE 11	Unknown	Bot. Garden, Antwerpen, Belgium	IPK Gatersleben
Fedia cornucopiae (L.) Gaertn.[3]	–	–	Unknown	Gehlsen, Halle, Germany	Nunhems-Hild

[1] All accessions supplied from the working collection of Nunhems-Hild.
[2] Taxonomical classification taken from http://www.rbgkew.org.uk.
[3] Three samples supplied for the study; syn. *Valeriana cornucopiae* Loisel.

Statistical analyses: Polymorphic bands in both genotype sets were first scored individually to estimate genetic diversity within both sets. Subsequently, data sets were combined using the DNA ladder and four standard genotypes, to identify common bands in both sets. For combined data analysis, monomorphic bands within elite germplasm were included if they were polymorphic in the exotic set. Only distinct major bands, ranging in length from 50 to 350 base pairs, were manually scored as present (1) or absent (0). As recommended for dominant marker systems in self-pollinated species or inbred lines (Link et al. 1995), genetic similarity (GS) between any of two genotypes i and j was estimated applying the formula of Jaccard (1908):

$$GS_{ij} = \frac{N_{ij}}{N_i + N_j - N_{ij}}$$

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. Estimation of GS values from the AFLP binary matrices, as well as the calculation of standard errors (SE) of GS estimates by the jackknife procedure with re-sampling over primers (Miller 1974), were done using the Plabsim software (Frisch et al. 2000). The GS matrix of combined data (elite and exotic germplasm) was further analysed using the unweighted pair group method using arithmetic averages (UPGMA) (Sneath and Sokal 1973) clustering method in the NTSYSpc version 2.0 (Rohlf 1998). Reliability of a dendrogram was tested by bootstrap analysis with 1000 replications to assess branching support, using the software package Winboot (Yap and Nelson 1996).

The average polymorphic information content (PIC) and the marker index (MI) were calculated for AFLP markers across assay units, assuming an AFLP primer combination an assay unit. Each polymorphic DNA fragment within an AFLP assay unit was considered a single dominant marker locus. The PIC value provides an estimate of the discriminatory power of a marker by taking into account not only the number of alleles at a locus but also the relative frequencies of these alleles. It was calculated by applying the formula of Roldán-Ruiz et al. (2000):

$$PIC_i = 2f_i(1 - f_i),$$

where PIC_i is the PIC of marker i, f_i is the frequency of the amplified allele (band present), and $(1-f_i)$ is the frequency of the null allele (band absent). Marker indices were calculated as the product of PIC and the number of polymorphic bands per assay unit, as suggested by Powell et al. (1996).

The co-ancestry coefficient f (Malécot 1948) was used to estimate the identity by descent of line pairs with known pedigree relationships, according to Falconer and Mackay (1996) and the assumptions proposed by Melchinger et al. (1991). Correlation between data matrices of f values and GS estimates from AFLP data, as well as the normalized Mantel test (Mantel 1967) were carried out in NTSYSpc.

To divide the molecular genetic variance into components attributable to the variance between and within sets, an analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed. Groups of material (elite and exotic germplasm) were considered sets and genetic data (AFLPs) were presented as haplotypes in the Arlequin ver. 2.000 software (Schneider et al. 2000).

Results

AFLP data

The 15 AFLP primer combinations tested in 64 accessions of cornsalad and related species revealed a total number of 1536 bands, with 285 selected as clearly distinct and reliable for data processing (Table 2). The number of polymorphic bands in the selected set of bands amounted to 54 in the elite germplasm. The degree of polymorphism detected in the exotic germplasm was substantially higher, with no monomorphic band detected. The number of polymorphic bands selected for data processing ranged from four to 46 per AFLP primer combination, with an average of 19. Average PIC values ranged from 0.19 to 0.34, whereas marker indices varied between 1.35 and 8.85 (Table 2).

Table 2: Polymorphic information content (PIC) and marker indices (MI) per amplified fragment length polymorphism primer combination in cornsalad (*Valerianella locusta* L.) and related species

Primer combination	Total no. of bands	No. of selected polymorphic bands	PIC	MI
EcoAAG/MseCT	104	4	0.34	1.35
EcoAAC/MseCC	112	23	0.22	5.08
EcoAAC/MseCG	132	30	0.22	6.45
EcoAAG/MseCA	97	10	0.27	2.65
EcoACA/MseCA	127	32	0.23	7.25
EcoACA/MseCC	76	25	0.23	5.72
EcoAGC/MseCG	77	11	0.25	2.72
EcoAGC/MseCT	97	18	0.25	4.48
EcoACG/MseCA	76	15	0.20	3.02
EcoACG/MseCC	66	46	0.19	8.85
EcoACT/MseCT	125	13	0.24	3.13
EcoACT/MseCC	132	12	0.32	3.85
EcoAGG/MseCA	124	16	0.29	4.69
EcoAGG/MseCT	99	12	0.27	3.27
EcoAGG/MseCC	92	18	0.25	4.50
Total	1536	285	–	–
Average	102	19	0.25	4.47

Reliability of AFLP data was confirmed by a significant correlation of 0.99 ($P < 0.05$) among four standard genotypes employed both in the elite and exotic germplasm. The lowest genetic similarity between genotypes duplicated at the plant level (blind checks) was 0.95, whereas the only deviation from an absolute identity ($GS = 0.99$) among laboratory duplicates was detected at the level of restriction digestion of DNA in a single duplicate (data not shown).

Genetic similarities among varieties and accessions

Genetic similarities within elite germplasm ranged from 0.79 ('CS-17' × 'Louvier') to 1.00 ('Gala' × 'CS-11'), with a mean of 0.90. In the exotic germplasm, the lowest GS value of 0.10 was detected between *V. coronata* and *V. carinata*, the accessions of the IPK genebank in Gatersleben. The highest GS estimate (1.00) in the exotic set was obtained between two *V. carinata* genebank accessions, one originating from IPK Gatersleben and the other from the Botanical Garden of Bordeaux. The average GS value in the exotic germplasm was 0.47. Genetic similarity in the whole set of 64 genotypes of elite and exotic germplasm ranged from 0.09 (*V. coronata* × 'CS-37') to 1.00 ('Gala' × 'CS-11'), with a mean of 0.63. Standard errors of the individual GS estimates were calculated only in the joint set and they varied from 0 to 0.08.

Pedigree information was available for only six modern line varieties in the study. The matrix of f estimates consisted of 21 data points in total. The association between f-matrix and a respective GS-matrix was low 0.19 ($P < 0.05$).

UPGMA cluster analysis and AMOVA

UPGMA cluster analysis using 15 AFLP primer combinations clearly separated the cultivated material of *V. locusta* from other *Valerianella* species and wild populations (Fig. 1). The first major cluster (Fig. 1a) consisted mainly of cultivated

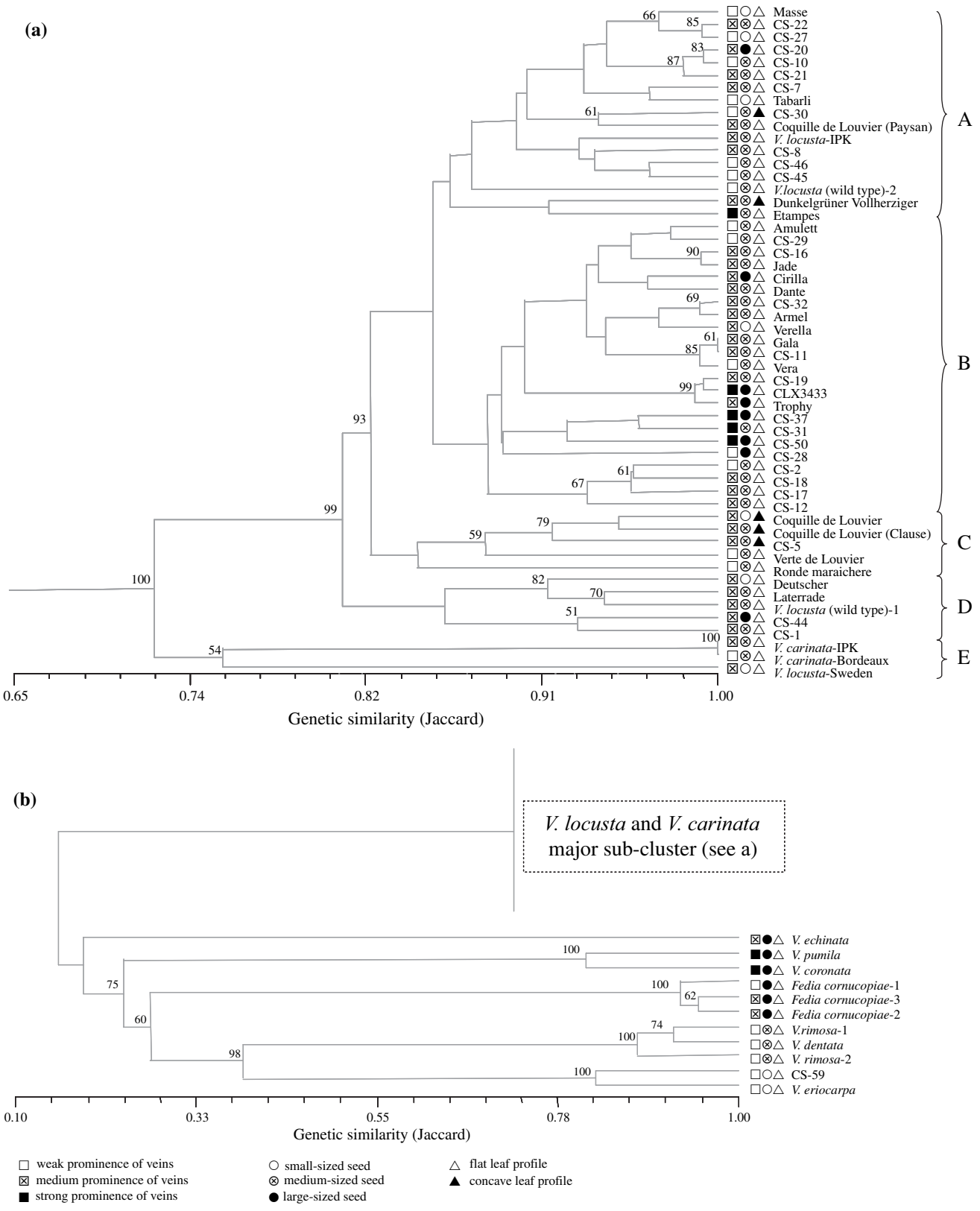

Fig. 1: Association among 64 genotypes of cornsalad (*Valerianella locusta* L.) and related species revealed by average linkage (UPGMA) cluster analysis of Jaccard's genetic similarity (GS) coefficients calculated from AFLP data of 15 primer combinations. Major sub-cluster a grouped *V. locusta* and *V. carinata* accessions, whereas the remaining species grouped to sub-cluster b. Letters A–E designate detected sub-clusters in the major sub-cluster a. Numbers at the nodes indicate the bootstrap values of the consensus tree obtained (branches lacking a value received < 50% bootstrap support). Squares, circles and triangles designate a rough evaluation of morphological traits

cornsalad and was further split into five divergent sub-clusters. The first and second sub-cluster (A and B) consisted exclusively of modern line varieties of cornsalad and former breeding materials, with the exception of one *V. locusta* wild form in the sub-cluster A. The third sub-cluster (C) gathered mostly the accessions of 'Louvier'-germplasm, while the fourth sub-cluster (D) grouped three *V. locusta* wild forms ('Deutscher', 'Laterrade' and *V. locusta* – wild form) and two older line varieties. Two genebank accessions of *V. carinata* and one *V. locusta* genebank accession formed the fifth sub-cluster (E).

The second major cluster (Fig. 1b) gathered *Valerianella* species different from *V. locusta* and *V. carinata*. Accessions of *V. echinata* and *Fedia cornucopiae* clearly clustered apart from other *Valerianella* species, forming two independent sub-clusters. Three additional sub-clusters were formed by (i) *V. pumila* and *V. coronata*, (ii) *V. dentata* and two accessions of *V. rimosa*, and (iii) 'CS-59' and *V. eriocarpa*.

The major clusters and sub-clusters were supported at 54–100% confidence interval limits in the bootstrap analysis (Fig. 1). AMOVA indicated that a higher proportion of variation was present within (76.4%) compared with between sets (23.6%).

Discussion

Reliability of the AFLP protocol established for cornsalad

DNA marker data are generally produced without replications or duplications because of high production costs and because they are considered (almost) error-free. Thus, experimental errors for marker data cannot be estimated for most published DNA marker studies. To establish the AFLP protocol for cornsalad, two control types were included and five and six selective nucleotides were tested in main amplification reactions using silver-staining and [^{33}P]-labelling approaches for visualization of AFLP fragments (data not shown). Five selective bases performed better than six selective bases in main amplification reactions, indicating the small genome size of the species, whereas the AFLP protocol using [^{33}P]-labelled primers resulted in a high reproducibility of laboratory duplicates (GS = 0.99–1.00). GS estimates lower than 0.99 for blind checks may be attributable to residual heterogeneity in older varieties (Nunhems-Hild and Juliwa-Enza, personal communication).

An average PIC value of 0.25 across all AFLP bands scored and an average marker index of 4.47 across all AFLP primer combinations (Table 2) corroborated results obtained in AFLP-based genetic diversity studies in other autogamous crops such as wheat (PIC = 0.32, MI = 3.41; Bohn et al. 1999) and soybean (PIC = 0.32, MI = 6.14; Powell et al. 1996). The studies on wheat and soybean additionally compared AFLPs to other molecular-marker systems. Owing to the high MI values of AFLPs, this marker system has been recommended for fingerprinting cultivars in plant variety protection, quality control, and the identification of essentially derived varieties (Bohn et al. 1999), which is an issue of a growing relevance in cornsalad breeding.

Genetic diversity in cornsalad elite germplasm

The range of AFLP-based genetic similarities in cornsalad elite germplasm (0.79 to 1.00) was comparable and slightly wider than in some other autogamous crops studied with AFLPs. Within autogamous crops, the available elite germplasms of cornsalad, barley, pea, and velvet-bean (Russell et al. 1997, Schut et al. 1997, Capo-chichi et al. 2001, Simioniuc et al. 2002) seem to be narrower than in wheat and soybean (Powell et al. 1996, Bohn et al. 1999, Soleimani et al. 2002), which may also be attributable to the differences in assortments of material in different studies (winter and spring cultivars of wheat, two- and six-row barley varieties, European and US soybean germplasm), or to the AFLP primer combinations used. The low variation in cornsalad might be the consequence of a narrow germplasm in the breeding programmes of different companies (Nunhems-Hild and Juliwa-Enza, personal communication).

The assumption that the currently used cornsalad germplasm relies on a rather narrow genetic base was supported with the apparent grouping of all modern line varieties studied (except the 'CS-5') in two sub-clusters of the major UPGMA cluster. Nevertheless, four old varieties interspersed the 'elite' cluster ('CS-8', 'CS-45', and 'CS-46' in the sub-cluster A, 'CS-50' in sub-cluster B), whereas some of the genebank accessions studied formed neighbouring sub-clusters D and E (Fig. 1). Morphologically similar varieties originating from the old variety 'Louvier' grouped in sub-cluster C. A high genetic similarity was confirmed between two old, phenotypically different but related lines 'CS-44' and 'CS-1' (GS = 0.93) in the sub-cluster D. Lines in sub-clusters C and D were known to be distinct from the rest of cornsalad germplasm spectrum (Nunhems-Hild and Juliwa-Enza, personal communication).

The matrix of co-ancestry estimates among six cornsalad genotypes was poorly correlated with the respective matrix of GS values ($r = 0.19$, $P < 0.05$), comparable with barley (Graner et al. 1994) and soybean (Cox et al. 1985). Low-to-moderate correlations between marker-based GS values and pedigree estimates may be due to a high 'background similarity' among unrelated accessions at the marker level (Graner et al. 1994). Marker-based GS estimates provide more information than pedigree data because they detect sequence variation and bypass assumptions inherent to pedigree analysis.

Morphological traits such as prominence of veins on the leaf surface, concave profile of the leaf ('spoon-forming' ability), and seed size have been selected as reliable descriptors of cornsalad. Line varieties characterized with a concave leaf profile, large seed sizes, and a strong prominence of veins on the leaf surface, were present in almost each AFLP-based sub-cluster, the latter two being moderately concentrated in sub-cluster B. Nevertheless, the strongly-veined leaf line 'Etampes' clustered with weak-veined lines 'CS-27' and 'CS-30' (sub-cluster A). In conclusion, AFLP-based groups in elite cornsalad germplasm revealed no apparent morphological pattern, which was in contrast to cereals (Russell et al. 1997, Bohn et al. 1999) or maize (Pejic et al. 1998, Lübberstedt et al. 2000).

Fingerprinting of *Valerianella* and *Fedia* species

Compared with elite germplasm of cornsalad, the average GS estimate in exotic germplasm was considerably lower (GS = 0.47). As a consequence, the second major cluster in UPGMA analysis demonstrated a greater genetic diversity within exotic germplasm accessions and contained mostly the species related to *V. locusta* (Fig. 1). Different species were clearly divided into separate sub-clusters, *V. echinata* being the most distant to all others. Three accessions of *F. cornucopiae* clustered

together and separately from *Valerianella* accessions, which is in accordance with allogamy and distinct morphology in *Fedia*. Formerly classified as *Valeriana cornucopiae*, *Fedia* was recently re-classified into a separate genus (USDA 2003), and is currently tested for broadening the genetic base of *V. locusta* (Nunhems-Hild, personal communication). The only cultivated variety clustering with other species was the formerly grown accession 'CS-59', which was consistent with its origin from *V. eriocarpa* (Juliwa-Enza, personal communication). The UPGMA clustering of *Valerianella* and *Fedia* species, established by AFLPs, generally confirmed their taxonomic classification. Closer clustering of one *V. rimosa* accession to *V. dentata* (GS = 0.92) than to the other accession of *V. rimosa* (GS = 0.89) might denote that either the classification of these accessions was not correct or that both species are genetically very close.

Molecular variance measured with AMOVA was three times higher within than between cornsalad germplasm sets. This is in disagreement with results in wheat (Soleimani et al. 2002) and with the genetic structure usually observed within self-pollinated plants (Hamrick and Godt 1997), but can be explained by the presence of different species in the study.

Application of molecular-marker technology is becoming a common practice for studying various aspects of plant genetic resources management (Bretting and Widrlechner 1995, Brown and Kresovich 1996, McGregor et al. 2002). In the present study, two independent *V. carinata* accessions (sub-cluster E) were identical (GS = 1). One of the accessions was obtained from the genebank at IPK Gatersleben, the other from the germplasm collection of Nunhems-Hild. However, both institutions obtained seed of the same accession from the Botanical Garden in Bordeaux, and named and propagated it independently. Thus, AFLP fingerprinting proved efficient in detecting duplicates in germplasm collections.

Application of AFLP fingerprinting in cornsalad breeding

Cornsalad breeding currently relies on phenotypic selection. AFLPs proved to be a robust and informative marker technique for detecting genetic diversity in cornsalad and can further be applied for (i) selection of divergent parent genotypes in order to develop segregating populations with increased genetic variation for subsequent selection, (ii) marker-assisted selection for the introgression of desirable chromosome segments from a wild species into an elite variety, (iii) distinctiveness, uniformity and stability (DUS) testing, as well as for the protection of breeders' rights regarding the approaches of essentially derived variety (EDV) concept.

Former breeding materials and *V. locusta* genebank accessions that carry favourable or phenotypically attractive traits, which clustered close to the cultivated cornsalad germplasm in the study, might serve as an immediate genetic resource for broadening the elite germplasm base, as those crosses are easy to perform. Alternatively, recently developed interspecific crosses might contribute to the introgression of new genes (such as resistance genes) from related species into cultivated material. Systematic fingerprinting of all 181 taxonomically classified *Valerianella* species should provide valuable information about those most closely related to *V. locusta* as the most promising candidates for successful interspecific crosses.

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3 Genetic structure and diversity among radish varieties as inferred from AFLP and ISSR analyses

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Abstract

Twelve AFLP (*Eco*RI/*Mse*I) primer combinations and ten ISSR primers were applied to estimate genetic diversity among 68 cultivated radish varieties. The material consisted of open-pollinated varieties, inbred lines, diploid and a few tetraploid hybrid varieties of garden radish (*Raphanus sativus* var. *sativus*) and Black radish (*R. sativus* var. *niger*). Two accessions of uncultivated relatives of radish that as weeds cause serious contamination during the process of hybrid radish production were added to the analyses. Polymorphic AFLP and ISSR fragments were scored for calculation of Jaccard's coefficient of genetic similarity (GS). Substantial levels of genetic variability (average AFLP-based GS = 0.70; average ISSR-based GS = 0.61) were detected in the available germplasm of cultivated radish. UPGMA cluster analyses separated two weedy species from the cultivated germplasm and indicated diversification within cultivated material. Black radish and French Breakfast radish types formed separate clusters, apart from the remaining germplasm. Based on AFLP data, a principal coordinate analysis (PCoA) and model-based approach revealed the genetic structure within cultivated radish germplasm and confirmed the existence of divergent pools. Although the model-based approach did not separate Black radish from French Breakfast radish varieties, it offered a clear sub-division within garden radish germplasm. The results of this study are relevant for the creation of heterotic pools for hybrid radish breeding.

Keywords: AFLP, Cluster analysis, Genetic diversity, Genetic structure, ISSR, Principal coordinate analysis, Radish

Introduction

Radish (*Raphanus sativus* L.) is an important commercial vegetable, consumed worldwide. It is an ancient domesticate, initially cultivated in China and Korea (Kaneko and Matsuzawa, 1993). The first European variety of cultivated radish was recorded in the 16th century (Wein, 1964; George and Evans, 1981). Based on the latest available information, the production of radish in Europe amounts to 120 000 t (Vogel, 1996).

Taxonomy classifies radish within the family Brassicaceae into the section *Raphanis* DC (Kaneko and Matsuzawa, 1993). Of six species in the section, only *Raphanus sativus* L. var. *sativus* convar. *radicula* (DC.) Pers. (garden or European radish) and *R. sativus* L. var. *niger* (Mill.) (Black or Spanish radish) are cultivated and commonly grown for their thickened fleshy hypocotyle and the upper part of the root. They cross freely and easily with related species, such as Chinese small radish (*R. sativus* L. var. *sativus* convar. *sinensis*) and *R. raphanistrum* L. Radish is a self-incompatible, open-pollinated, diploid species with a chromosome number 2n = 18 and small genome size (C-value = 0.55 pg, http://www.rbgkew.org.uk/cval/homepage.html).

Radish breeding was practiced for centuries, by means of mass or pedigree selection. Since two decades, the production of F_1 hybrids using cytoplasmatic male sterility has widely replaced simple breeding methods based on morphological traits (Banga, 1976). Uniformity of varieties is becoming a high priority goal in radish breeding. Over a third (35%) of currently grown radish varieties in Germany are F_1 hybrids, thus indicating a significant increase during the period of last 15 years (Bundessortenamt, 1986, 2001). Most breeding work is aimed at further adaptation to different growing conditions, improved resistance to pests (*Peronospora parasitica*, *Albugo candida*) (Vogel, 1996), and improved marketing conditions. Specific market preferences strongly influence the selection of morphological traits of root considered in the breeding process of radish (A. Schieder, personal communication). Thus, garden radish is bred for round, light-red colored roots, French Breakfast radish type has a unique oblong red root shading to white at tip, whereas Giant radish type possesses a stronger, red-fleshed root, wider in diameter and not prone to sponginess and glassiness.

Advanced practices in breeding major crops have demonstrated the superiority of inter-group over intra-group hybrids. For the optimum exploitation of heterosis, the parental lines should be derived from genetically unrelated germplasm pools, commonly referred to as

heterotic groups (Melchinger and Gumber, 1998). Only few studies have so far been conducted to estimate phenotypic (George and Evans, 1981) or genetic diversity of radish cultivars (Ellstrand and Marshall, 1985; Demeke et al., 1992; Thormann et al., 1994; Rabbani et al., 1998; Huh and Ohnishi, 2003). None of the currently available studies has focused on a wider set of European radish cultivars. Molecular markers, such as amplified fragment length polymorphisms (AFLPs, Vos et al., 1995) and inter simple sequence repeats (ISSRs, Zietkiewitcz et al., 1994), were already successfully applied in genetic diversity analyses in various crops (Zhu et al., 1998; Bohn et al., 1999; Simioniuc et al., 2002), and confirmed the classification of germplasm into known heterotic groups (Pejic et al., 1999; Lübberstedt et al., 2000).

Our objectives were to establish the AFLP and ISSR protocols for radish, and apply them to (i) investigate the genetic diversity of a set of radish varieties that have currently been commercially produced in Europe, and (ii) identify possible heterotic pools within available cultivated radish germplasm. Furthermore, a method for an early detection of weed (Chinese small radish and *R. raphanistrum*) in cultivated radish can be derived from our study.

Materials and Methods

Plant materials and DNA extraction

Sixty-eight accessions of cultivated radish grown in Europe were chosen for the study. The materials consisted of inbred lines, diploid and tetraploid hybrid varieties, and open-pollinated varieties of garden radish and Black radish. One accession of Chinese small radish (*R. sativus* var. *sativus* convar. *sinensis*) and one accession of *R. raphanistrum* were added to the study (Table 1). Two control types were included to test the reliability and reproducibility of the AFLP and ISSR protocols: (1) blind check – an accession sown twice under coded numbers, and (2) laboratory duplicate – a randomly chosen accession duplicated after DNA extraction and re-duplicated in consecutive steps of the analyses.

Plants were grown either in the open field or greenhouse, depending on their preferred growing conditions. Distinctive morphological traits for Giant radish, Black radish, and French Breakfast radish were estimated according to the standard criteria of UPOV (International Union for the Protection of New Varieties of Plants).

From a bulk of 20-30 plants per accession, 2-3 g of fresh leaf material 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).

AFLP and ISSR protocols

AFLP fingerprints were produced according to the original protocol of Vos et al. (1995), modified by Muminović et al. (2004). For selective amplifications, each of the *Eco*RI and *Mse*I primers carried three selective nucleotides. In total, 12 *Eco*RI/*Mse*I primer combinations were employed.

For ISSR amplification reactions a protocol of Ratnaparkhe et al. (1998) was modified: 100 ng genomic DNA, 1 µM ISSR primer (*Sigma-Ark*, Darmstadt), 0.5 mM dNTPs, 1 mM $MgCl_2$, 1 x PCR buffer, and 1 U Taq DNA polymerase (*Amersham Biosciences Europe GmbH*, Freiburg). Amplifications were performed with an initial step at 95 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s, and elongation at 72 °C for 2 min. The final step of an additional extension was at 72 °C for 10 min. In total, 10 ISSR primers were tested. Amplified products were separated by electrophoresis on 2% agarose gels (*Biozym agarose DNA*, Oldendorf), stained in ethidium-bromide, and visualized under UV light.

AFLP fragments ranging from 50 to 350 bp in length, and ISSR fragments between 350 and 2100 bp, were scored manually as present (1) or absent (0), and transferred to a respective (AFLP or ISSR) binary matrix to be separately analyzed. Only distinct and polymorphic major bands were chosen for the study.

Statistical analyses

To evaluate the discriminatory power of molecular markers, polymorphic information content (PIC) and marker index (MI) were calculated across assay units. Each single AFLP primer combination and ISSR primer was assumed an assay unit. PIC value was calculated applying the formula of Roldán-Ruiz et al. (2000): $PIC_i = 2f_i(1 - f_i)$, where f_i is the frequency of the amplified allele (band present), and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i. MI was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell et al., 1996).

To estimate the level of genetic diversity, genetic similarity (GS) between the two varieties *i* and *j* was calculated by applying the formula of Jaccard (1908): $GS_{ij} = N_{ij} / (N_i + N_j - N_{ij})$, where N_i is the number of detected bands in a variety *i* and not in variety *j*, N_j is the number of detected bands in a variety *j* and not in variety *i*, and N_{ij} is the number of bands common to varieties *i* and *j*. Estimation of GS values and the calculation of their standard errors (SE) by jackknife procedure with re-sampling over primers (Miller, 1974) were done with Plabsim software (Frisch et al., 2000), which is implemented as an extension of the statistical software R (Ihaka and Gentleman, 1996). The calculation of correlation between matrices of GS estimates based on AFLPs and ISSRs, as well as the UPGMA (unweighted pair group method using arithmetic averages, Sneath and Sokal, 1973) clustering method were conducted with the NTSYSpc version 2.0 (Rohlf, 1998). Reliability of dendrograms was tested by bootstrap analyses with 1000 replications to assess brunch support, using Winboot software (Yap and Nelson, 1996).

To obtain a clear view of the classification pattern in the available radish germplasm and thus detect possible heterotic groups, F_1 hybrids and the two accessions of weedy crops were excluded from the initial set of materials, and only AFLP data were used. A principal coordinate analysis (PCoA, Gower, 1966), was applied to graphically represent the relationship structure in radish germplasm. Computations were performed on the basis of $\sqrt{1-GS}$ matrix which possesses Euclidean distance properties (Gower and Legendre, 1986), applying the Plabsim software. Additionally, a model-based approach described by Pritchard et al. (2000) and extended by Falush et al. (2003) was used to infer population structure of the data set. Given a value for the number of subpopulations (clusters), this method assigns individuals from the entire sample to clusters in a way that Hardy-Weinberg disequilibrium and linkage disequilibrium are maximally explained. Three independent runs of STRUCTURE 2.1 software were conducted by setting the number of populations (*K*) from 1 to 6, and applying the "No admixture" model. For each run, we set 100000 repetitions for the burn-in time and additional 150000 for replication number. The run with the maximum likelihood was used to assign varieties to inferred clusters. The proportions of individual radish varieties assigned to inferred clusters were used to identify so-called meaningful groups. For each number of populations (*K*) a cluster was defined meaningful if it contained at least one variety represented with a proportion of 50% or more.

Results

AFLP and ISSR data

The 12 AFLP primer combinations tested revealed a total number of 349 bands, with 267 (76.5%) selected as clearly distinct and reliable for data processing (Table 2). Average PIC values ranged from 0.18 to 0.29, whereas MI varied between 2.68 and 9.01 (Table 2). Applying 10 ISSR primers 116 bands were detected, with 45 (38.8%) being clearly distinct and reliable for data processing (Table 3). Average PIC values were between 0.14 and 0.43, and MI varied from 0.37 to 2.56 (Table 3).

Reliabilities of established AFLP and ISSR protocols were confirmed by high GS estimates between blind checks (GS ranged from 0.89 to 0.98 for AFLPs, and from 0.81 to 1.00 for ISSRs), as well as between laboratory duplicates (GS from 0.98 to 1.00 for AFLPs, and from 0.95 to 1.00 for ISSRs) (data not shown).

Genetic similarities among varieties and UPGMA cluster analyses

Genetic similarities based on AFLPs ranged from 0.16 (*R. raphanistrum* x April Cross) to 1.00 (Wernar x Isar), with the mean of GS = 0.70. Standard errors for GS estimates varied from 0 to 0.07. GS estimates based on ISSRs ranged from 0.20 (JW 18 x April Cross) to 1.00 (Wernar x Isar and JW 18 x JW 19), with a mean of GS = 0.61. Standard errors for those GS estimates varied between 0 and 0.2. The correlation between AFLP- and ISSR-based GS estimates was high ($r = 0.80$, $P < 0.01$).

UPGMA cluster analyses of GS estimates based on AFLPs (Fig. 1) or ISSRs (Fig. 2) generated similar clustering patterns, although the cluster order in the dendrograms was not absolutely identical. Both analyses clearly separated *R. raphanistrum* from *R. sativus* (Fig. 1; Fig. 2). Although Black radish varieties (*R. sativus* var. *niger*) did not create a clearly independent cluster, their separation from garden radish (*R. sativus* var. *sativus*) was apparent. Within garden radish varieties, separate clusters were evident for French Breakfast radish and Giant radish types, with three Giant radish varieties (Falco, Rota, and Riesenbutter) apart from their main cluster. Additionally, garden radish varieties clustered closer if they originated from the same breeding company (Table 1; Fig. 1 and 2).

Principal coordinate analysis and analysis of genetic structure

First two principal coordinates in the PCoA accounted for 12.8 and 7.4% of the total variation (Fig. 3). Separate groups of Black radish, French Breakfast radish, and Giant radish could be detected. Only two Giant radish varieties (Falco and Rota) grouped away from the main Giant radish unit, and were closer to garden radish. The varieties and lines of garden radish made almost a compact group.

Applying the model-based approach, all varieties were assigned to meaningful clusters at the number of populations $K = 3$. The estimated likelihoods for $K = 3$ were comparable or higher than for $K = 4$-6 among the independent runs of the program. The run with highest ln-likelihood at $K = 3$ was used to define the model-based groups (Table 4). The largest group (49% of varieties) consisted of three inbred lines and 15 varieties of garden radish or Giant radish varieties. The second group contained 11 varieties (30%) and included Black radish, French Breakfast radish types, and two garden radish varieties (Neckarperle, Red Silk). The last model-based group had eight garden radish varieties (21%).

Discussion

Reliability of AFLP and ISSR protocols for radish

Molecular marker approaches are considered accurate in fingerprinting plant genome, so the inclusion of duplicates is not a general practice. To optimize our AFLP protocol for radish, we analyzed primers with a total of five and six selective nucleotides in the main amplification reactions, and tested silver-staining and radioactive labeling approaches for the visualization of amplified fragments. Main amplification reactions with six selective nucleotides produced a clear banding pattern with a reduced background "noise" on polyacrylamide gels. Compared with silver-staining for visualization of AFLP fragments, the protocol using radioactive labeling of the *Eco*RI primers resulted in a higher reproducibility of control types (data not shown).

Di-nucleotide repeat ISSR primers produced the highest average number of bands (Table 3) and were generally more frequent in radish genome than tri-nucleotide repeats. Nevertheless, they could not be used alone to efficiently differentiate between radish varieties.

Similar results were found in wheat (Nagaoka and Ogihara, 1997), rice (Akagi et al.,1997; Blair et al., 1998), and *Diplotaxis* (Brassicaceae) (Martín and Sánchez-Yélamo, 2000). Tri-nucleotide repeat ISSR primers yielded the highest amount of polymorphic bands and indicated their specificity within radish genome (Table 3). Primers having tetra-nucleotide repeats of the core sequence produced no fragments in radish (data not shown), which is in contrast to studies on pea (Lu et al., 1996), maize (Gupta et al., 1994), and *Diplotaxis* (Brassicaceae) (Martín and Sánchez-Yélamo, 2000). The 5'-end anchored primers yielded the highest average number of bands in our study, whereas the unanchored primers yielded the highest average number of polymorphic bands. Primers anchored at the 5'-end generate a higher number of fragments because they display broader specificity than the others (Bornet and Branchard, 2001). In general, the higher the density of repeats in a genome is, the more specific primers and more stringent PCR conditions should be used to limit the number of amplified products and to optimise their resolution on a gel (Fang et al., 1997).

In both molecular marker systems, the range of variation in GS estimates of blind checks was wider than of laboratory duplicates. This may be due to heterozygosity and heterogeneity present in radish varieties (A. Schieder, personal communication). Even F_1 hybrids in radish cannot be considered homogeneous because a critical level of inbreeding depression of parental inbreds is reached after only a few selfing generations (Kaneko and Matsuzawa, 1993).

An average PIC value of 0.24 across all scored AFLP bands (Table 2) agreed with the results obtained in AFLP-based genetic diversity studies of soybean, wheat, maize, and *Lolium* (Powell et al., 1996; Bohn et al., 1999; Lübberstedt et al., 2000; Roldán-Ruiz et al., 2000). The average PIC value of 0.26 across all scored ISSR bands (Table 3) was in harmony with the results obtained in an ISSR-based analysis of rice varieties (Nagaraju et al., 2002). A comparison of the two marker systems applied in our study indicated that the average MI of 5.14 in AFLPs (Table 2) was much higher than the average MI of 1.18 in ISSRs (Table 3). Thus, AFLPs proved to be a more informative marker system. This may also be the result of a comparatively low proportion of bands per ISSR analysis using agarose gels of low resolution (Charters et al., 1996; Fang and Roose, 1997). Additionally to their high multiplex ratio, AFLPs are highly reproducible and very efficient in detecting polymorphism (Powell et al., 1996). Owing to their high MI values, AFLPs have been recommended for fingerprinting cultivars in plant variety protection, quality control, and the identification of essentially

derived varieties (Bohn et al., 1999), which is an issue of a growing relevance in radish breeding.

Genetic diversity in radish germplasm

AFLPs and ISSRs differ in the nature of evolutionary mechanisms underlying their variation and their distribution in plant genome (Powell et al., 1996). Nevertheless, the results obtained from these marker systems were highly correlated, which was in accordance with similar studies on amaranth (Xu and Sun, 2001) and *Cucurbita pepo* (Paris et al., 2003). AFLPs and ISSRs generated comparable ranges of variation in GS estimates within the studied radish germplasm, and similar to the diversity of other members of the family Brassicaceae studied with AFLPs (Srivastava et al., 2001).

Distribution pattern of radish varieties into different clusters indicated the formation of well characterized and coherent groups that were in accordance with their taxonomical order – *R. sativus* var. *sativus* (convar. *radicula* and convar. *sinensis*), *R. sativus* var. *niger*, and *R. raphanistrum*. In addition, we confirmed that *R. sativus* var. *niger* and *R. sativus* var. *sativus* were more closely correlated to one another than either was to *R. raphanistrum* (Fig. 1 and 2). The findings that GS estimates between *R. sativus* varieties and the accessions of *R. raphanistrum* and Chinese small radish were low (Fig. 1 and 2) can be valuable for radish breeders who face serious problems in F_1 hybrid radish production. Out-crossings between cultivated radish and either of the weedy species (that are morphologically hard to differentiate from cultivated radish) is easy and frequent (A. Schieder, personal communication). In all hybrid radish varieties tested in our study, a band of 1600 kb was detected with the ISSR primer $(CAA)_6$, whereas neither Chinese small radish nor *R. raphanistrum* accession produced a band at the same position (Fig. 4). With the ISSR marker UBC 890 hybrid varieties did not produce a band at 800 kb as Chinese small radish did, nor at 580 kb where a band was detected in *R. raphanistrum*. Those simple ISSR assays could assist in estimating the level of seed purity before sowing hybrid radish varieties in the field, and could thus further reduce the costs of hybrid radish production.

The assumption that the currently used European radish germplasm relies on a narrow genetic basis (A. Schieder, personal communication) was supported with the apparent grouping of all studied radish varieties in sub-clusters with GS estimates higher than 0.70, both in AFLPs and ISSRs. Black radish did not mix with garden radish varieties in either

AFLP or ISSR cluster, whereas morphologically similar types (French Breakfast radish and Giant radish) formed distinct groups. The observation of high GS estimates between garden radish varieties originating from the same breeding company agrees with assumed closer pedigree relationships. Owing to a high degree of heterogeneity and heterozygosity within any radish variety, the detected between-variety diversity is low, but there still is a sufficient overall diversity in available radish germplasm. Of 267 AFLP bands used for the analyses, on average 156 (58.6%) were polymorphic among open-pollinated varieties, 150 (56.4%) among F_1 hybrids, and 138 (51.6%) among inbred lines (data not shown). Variation in average values of GS estimates within each of the variety groups did not indicate significant differences (open-pollinated varieties GS = 0.70, F_1 hybrids GS = 0.70, inbred lines GS = 0.60). For an accurate diversity study among or within radish varieties, a co-dominant marker system is recommended to compensate for a disadvantageous property of AFLPs and ISSRs in masking the detection of heterozygous individuals.

Genetic structure of radish germplasm

Radish is an autogamous species with a high degree of self-incompatibility (Banga, 1976; George and Evans, 1981), and populations are composed of heterogeneous individuals. PCoA based on AFLPs clearly separated radish varieties into morphologically diverse groups, such as Black radish, French Breakfast radish, Giant radish, and garden radish varieties (Fig. 3). Among Black radish, Duro was slightly separate. It is a unique cross between Black radish and garden radish in our study, which explains its close positioning to other garden radish varieties. The main reasons why PCoA did not indicate any further division within garden radish varieties may rest on a high heterogeneity assumed within them (A. Schieder, personal communication) and on the inability of a dominant marker system (such as AFLP) in detecting heterozygosity.

The model-based approach of Pritchard et al. (2000) clearly classified radish varieties into three groups (Table 4), comparable to PCoA analysis. The most compact group consisted of all Black radish varieties and French Breakfast radish types. Although those varieties are characterized with a similar root morphology (long strong roots), it may still be surprising that the model-based approach clustered them together when they belong to taxonomically different species. Similar results were obtained in wheat diversity study, where *Triticum durum* landraces formed a cluster with *T. dicoccum* cultivars and were clearly separated from

T. durum cultivars (Pujar et al., 1999). The authors supposed that a limited number of domestication events during the evolution of *T. dicoccum* cultivars was the reason for the observed clustering. An analogous example was given in a study of *Trifolium pratense* (Kölliker et al., 2003), where Mattenklee landraces were more closely related to field clover than to Mattenklee cultivars. The strong selection of Mattenklee cultivars targeted at a single trait (persistence) was postulated as the main justification of the unexpected clustering. Based on allozyme variation within and among cultivars of *Raphanus sativus*, domesticated radish retained a population structure similar to that of wild populations (Ellstrand and Marshall, 1985). Regarding the targeted selection of radish, the regional breeding of French Breakfast radish was aimed at elongated roots, very different to the small-size round roots of garden radish. Finally, another reason why taxonomically distant species grouped to the same inferred population may be that French Breakfast radish was represented by too few varieties to form a distinct cluster, whereas they are too divergent from garden radish varieties to fit into the common cluster.

Nevertheless, the model-based clustering method of Pritchard et al. (2000) based on three inferred populations revealed an unambiguous division within garden radish varieties. The available pedigree information (data not shown) was not sufficient to confirm the reliability of the applied model-based approach, as it did in studies on maize (Liu et al., 2003) and *Trifolium pratense* (Kölliker et al., 2003). Considering the fact that the model-based approach does not rely on a prior population information but only on genotypic data consisting of unlinked markers, it may be regarded a clear and independent indication of germplasm grouping. The inferred sub-groups within garden radish germplasm can be employed to establish heterotic pools in radish. This could serve breeders as a valuable information in the creation of further breeding approaches for utilization of the existing substantial level of genetic variation within European modern cultivars of radish, which we detected with AFLPs and ISSRs. It can assist them in the choice of parents for crossing, defining priorities, and reducing the costs in radish variety improvement. For any further elucidation of the garden radish complex and for a confirmation of division within its germplasm, additional studies involving a larger number of varieties, and preferably a co-dominant molecular marker system would be essential.

Acknowledgements

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Tables and Figures

Table 1: Varieties of garden radish and Black radish [z], as well as the accessions of wild relatives of radish analyzed with AFLP and ISSR molecular markers

Variety name (or code)	Genetic constitution	Production type [y]	Breeding company
JW 6	inbred line	OF	Juliwa - Enza
JW 9	inbred line	OF	Juliwa - Enza
JW 14	inbred line	OF	Juliwa - Enza
April Cross [z]	F_1 hybrid		Juliwa - Enza
Cheriette	F_1 hybrid		Sakata Seed
Content	F_1 hybrid	UG	Enza Zaden
Donar	F_1 hybrid	UG	Syngenta
Favorella	F_1 hybrid	UG	Nickerson-Zwaan
Florent	F_1 hybrid	UG	Enza Zaden
Fluo	F_1 hybrid		Vilmorin
Hyronda	F_1 hybrid		Juliwa – Enza
Isar	F_1 hybrid	OF	Syngenta
JW 16	F_1 hybrid	OF	Juliwa – Enza
JW 17	F_1 hybrid	OF	Juliwa – Enza
JW 18	F_1 hybrid	OF	Juliwa – Enza
JW 19	F_1 hybrid	OF	Juliwa – Enza
JW 20	F_1 hybrid	OF	Juliwa – Enza
Masterred	F_1 hybrid	OF	Royal Sluis
Novella	F_1 hybrid	UG	Nickerson-Zwaan
Picard	F_1 hybrid	OF	Rijk Zwaan
Printo	F_1 hybrid	OF	Nickerson-Zwaan
Radius	F_1 hybrid	UG	Enza Zaden
Rondar	F_1 hybrid	OF/UG	Syngenta
R3	F_1 hybrid	OF	Juliwa - Enza
R6	F_1 hybrid	OF	Juliwa - Enza
R16	F_1 hybrid	OF	Juliwa - Enza
R49	F_1 hybrid	UG	Nunhems - Hild
R50	F_1 hybrid	UG	Nunhems - Hild
R51	F_1 hybrid	UG	Nunhems - Hild
Sunto	F_1 hybrid	OF	Nickerson-Zwaan
Tarzan	F_1 hybrid	UG	Enza Zaden
Trespa	F_1 hybrid	UG	Enza Zaden
Vitella	F_1 hybrid	UG	Nickerson-Zwaan
Wernar	F_1 hybrid	OF	Syngenta
Boy	4n		Nunhems - Hild
Duro [z]	4n		Chrestensen
Fanal	4n		Nunhems - Hild
Cherry Belle	op		Nickerson-Zwaan
Eiszapfen [z]	op		Juliwa - Enza
Eterna	op		Juliwa - Enza
Falco	op	OF	Juliwa - Enza

Flair	op	UG/OF	Rijk Zwaan
Flamboyant	op		Juliwa - Enza
Hilds Blauer Herbst [z]	op		Nunhems - Hild
Hilmar	op	UG	Nunhems - Hild
JW 30	op		Juliwa - Enza
JW 31	op		Juliwa - Enza
Karissima	op	UG	Nunhems - Hild
Marabelle	op	OF/UG	Nickerson-Zwaan
Neckarperle	op	OF	Nunhems - Hild
Neckarruhm rot [z]	op		Nunhems - Hild
Neckarruhm weiss [z]	op		Nunhems - Hild
Nelson	op		Gautier Graines
Parat	op		Juliwa - Enza
Patricia	op		Nunhems - Hild
Raxe	op		Nunhems - Hild
Red Silk	op		Harris Moran
Ribella	op	OF	Nickerson-Zwaan
Riesenbutter	op		Juliwa - Enza
Rondeel	op		Rijk Zwaan
Rota	op		Rijk Zwaan
Rudi	op	OF	Juliwa - Enza
Runder Schwarzer Winter [z]	op		Nunhems - Hild
Saxa – Rafine	op	OF	Rijk Zwaan
Silva	op	OF	Juliwa - Enza
Sirri	op	OF	Rijk Zwaan
Sora	op		Nunhems - Hild
Topsi	op	UG/OF	Nunhems - Hild
Chinese small radish	(wild species)	weed	-
R. raphanistrum	(wild species)	weed	-

[z] Black radish varieties

[y] OF designates production in the open field; UG designates the production under glass

Table 2: Polymorphic information content (PIC) and marker indices (MI) per AFLP primer combination in 70 accessions of radish (*Raphanus sativus* L.), Chinese small radish, and *R. raphanistrum*

Primer combination [z]	Total No. of bands	No. of selected polymorphic bands	PIC	MI
E33/M59	51	42	0.21	9.01
E33/M60	29	20	0.28	5.63
E33/M62	35	28	0.24	6.72
E35/M59	31	31	0.23	7.00
E35/M60	43	35	0.22	7.84
E38/M47	30	19	0.18	3.46
E38/M48	23	17	0.29	4.90
E39/M48	19	11	0.24	2.68
E39/M50	29	19	0.25	4.72
E40/M47	24	17	0.28	4.76
E41/M59	18	16	0.27	4.39
E41/M61	17	12	0.23	2.71
Total	349	267	-	-
Average	29.08	22.25	0.24	5.14

[z] *Eco*RI and *Mse*I primer codes correspond to the official nomenclature available at http://www.keygene.com/html/nomenclature.htm

Table 3: Polymorphic information content (PIC) and marker indices (MI) per ISSR primer in 70 accessions of radish (*Raphanus sativus* L.), Chinese small radish, and *R. raphanistrum*

Primer	Sequence [z]	Total No. of bands	No. of selected polymorphic bands	PIC	MI
CAA	(**CAA**)$_6$	9	7	0.22	1.51
GCT-Y	(**GCT**)$_4$Y	11	6	0.14	0.81
UBC 811	(**GA**)$_8$C	16	6	0.43	2.56
UBC 825	(**AC**)$_8$T	8	2	0.24	0.47
UBC 855	(**AC**)$_8$YT	9	4	0.29	1.17
UBC 857	(**AC**)$_8$GG	18	2	0.18	0.37
UBC 864	(**ATG**)$_6$	8	6	0.27	1.64
UBC 866	(**CTC**)$_6$	11	5	0.33	1.67
UBC 889	DBD(**AC**)$_7$	15	3	0.29	0.86
UBC 890	VHV(**GT**)$_7$	11	4	0.19	0.75
Total	-	116	45	-	-
Average	-	11.6	4.5	0.26	1.18

[z] Primer motif is bolded; Y = pyrimidine; B = C, G, or T; D = A, G, or T; H = A, C, or T; V = A, C, or G

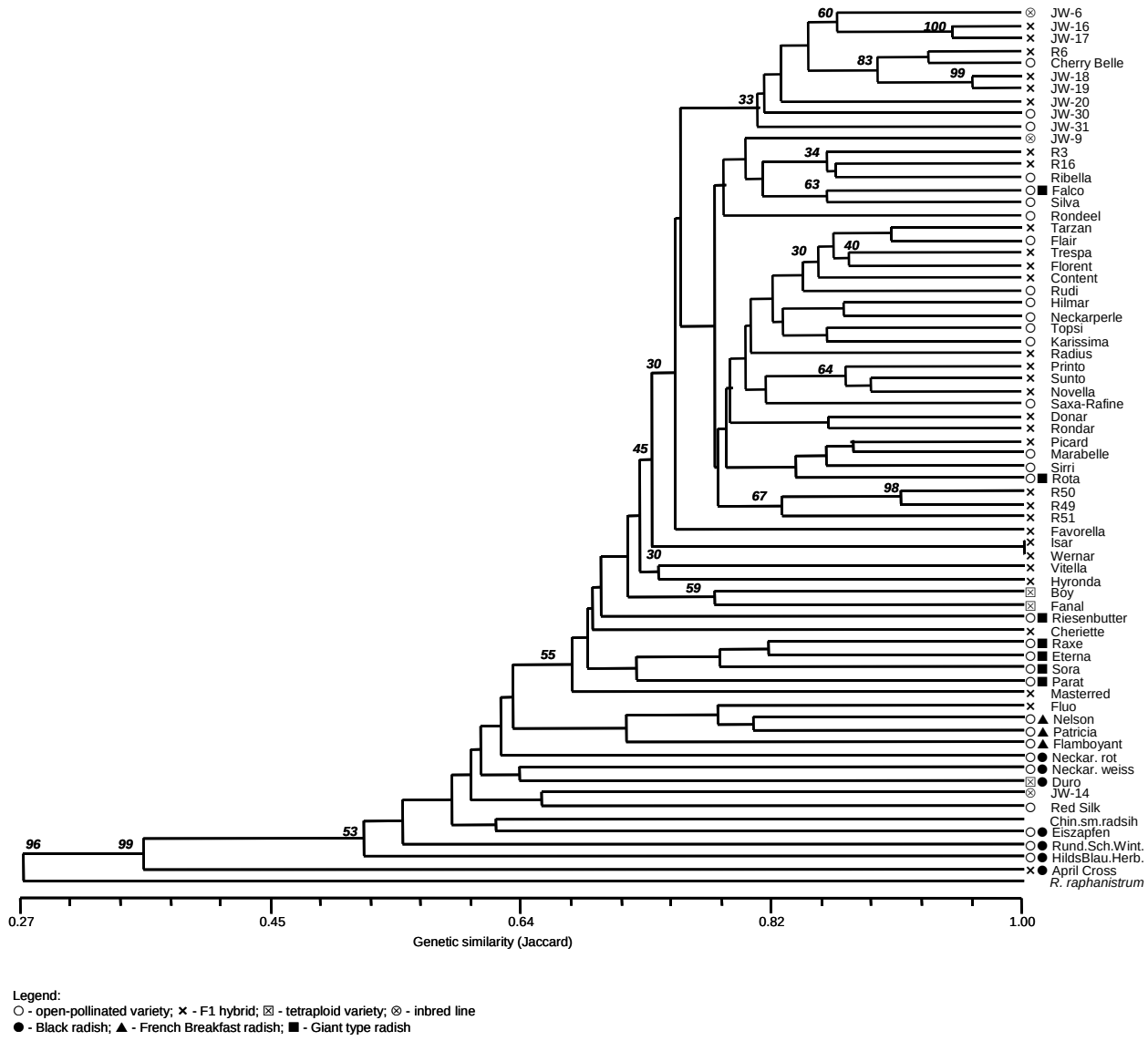

Fig. 1: Association among 68 varieties of radish (*Raphanus sativus* L.) and two related species revealed by average linkage (UPGMA) cluster analysis of Jaccard's genetic similarity (GS) coefficients calculated from AFLP data of 12 primer combinations. Numbers at the nodes indicate the bootstrap values of the consensus tree obtained (branches lacking the value received < 30% bootstrap support). Symbols designate genetic constitution of a variety and an evaluation of specific morphological traits.

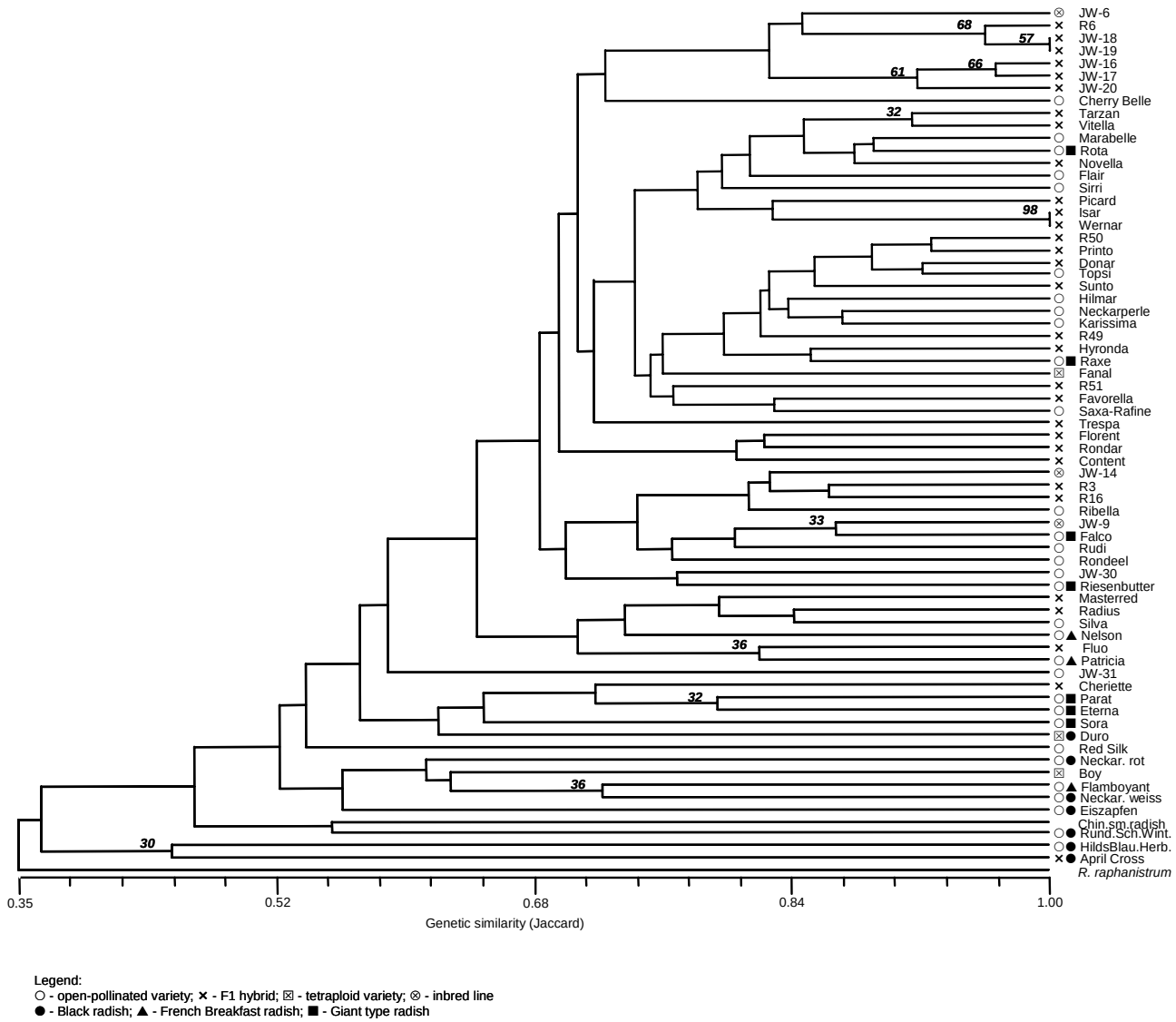

Fig. 2: Association among 68 varieties of radish (*Raphanus sativus* L.) and two related species revealed by average linkage (UPGMA) cluster analysis of Jaccard's genetic similarity (GS) coefficients calculated from ISSR data of 10 primer combinations. Numbers at the nodes indicate the bootstrap values of the consensus tree obtained (branches lacking the value received < 30% bootstrap support). Symbols designate genetic constitution of a variety and an evaluation of specific morphological traits.

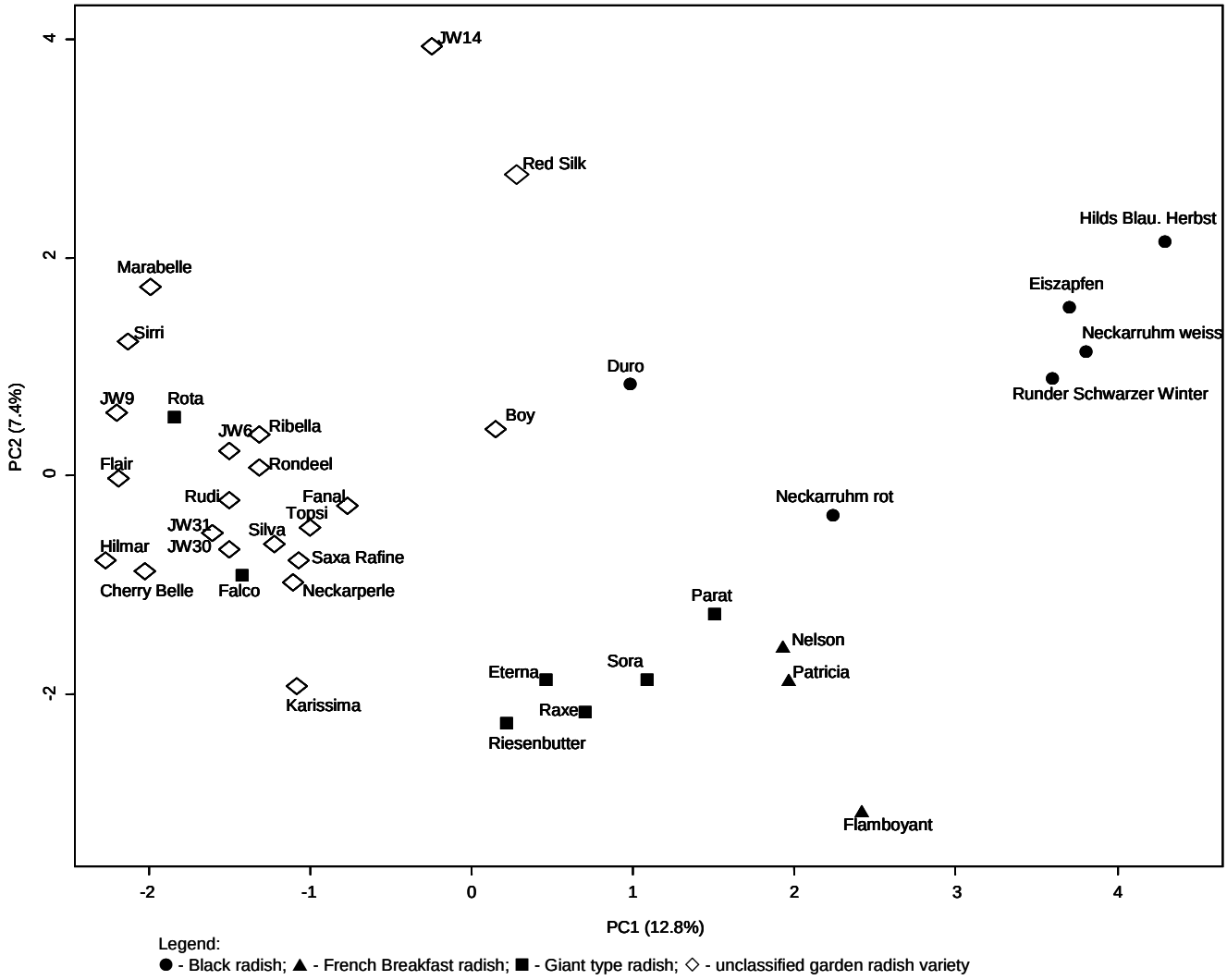

Fig. 3: Association among three inbred lines and 34 open-pollinated varieties of cultivated radish (*Raphanus sativus* L.) revealed by principal coordinate analysis (PCoA) performed on genetic similarity estimates calculated from AFLP data of 12 primer combinations. Symbols designate an evaluation of specific morphological traits.

Table 4: Model-based grouping of three inbred lines and 34 open-pollinated varieties of cultivated radish (*Raphanus sativus* L.), using AFLP data

Group	Variety
I	JW6 [z], JW9 [z], JW14 [z], Boy, Fanal, Falco [y], Flair, JW30, JW31, Marabelle, Parat [y], Raxe [y], Rota [y], Rudi, Silva, Sirri, Sora [y], Topsi
II	Duro [x], Eiszapfen [x], Flamboyant [w], Patricia [w], Hilds Blauer Herbst [x], Neckarperle, Neckarruhm rot [x], Neckarruhm weiss [x], Nelson [w], Red Silk, Runder Schwarzer Winter [x]
III	Cherry Belle, Eterna [y], Hilmar, Karissima, Ribella, Riesenbutter [y], Rondeel, Saxa-Rafine

[z] Inbred lines
[y] Giant radish variety
[x] Black radish varieties
[w] French Breakfast radish varieties

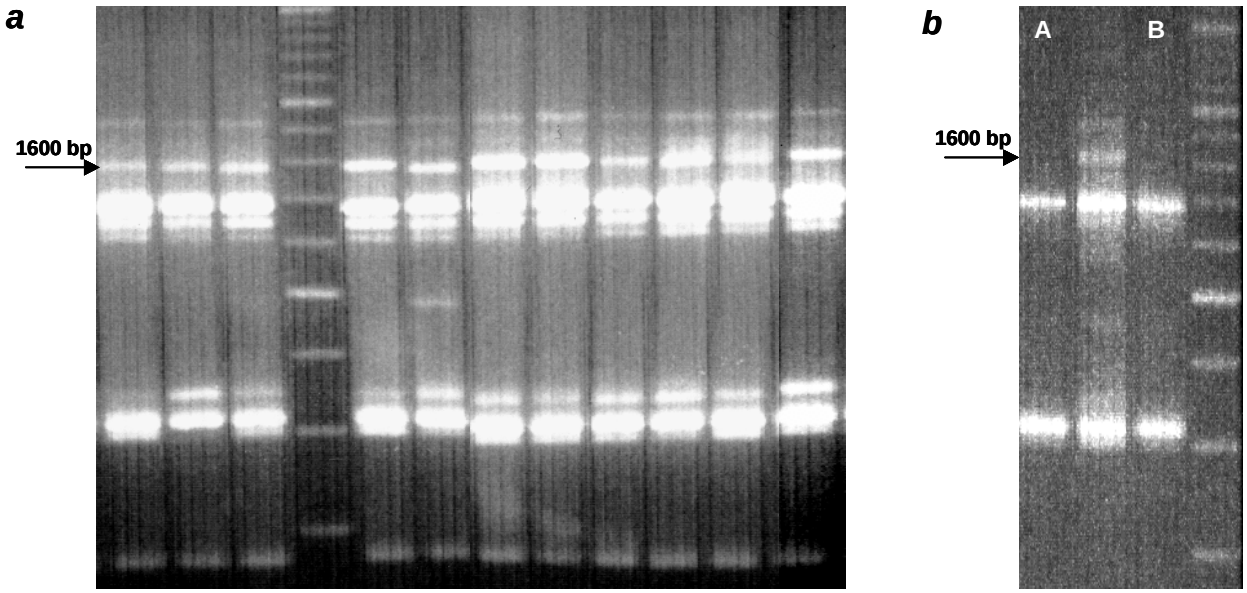

Fig. 4: Image of (*a*) a sample of hybrid varieties of radish and (*b*) accessions of weedy species (A – Chinese small radish; B – *Raphanus raphanistrum*) tested with ISSR marker $(CAA)_6$. Only hybrid varieties produced a band of 1600 kb in size.

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Prospects for celeriac (*Apium graveolens* var. *rapaceum*) improvement by using genetic resources of *Apium*, as determined by AFLP markers and morphological characterization

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Abstract

Genetic relationships among elite celeriac varieties and celeriac accessions conserved in genebanks are generally unknown. The objective of this study was to use amplified fragment length polymorphism (AFLP) markers and morphological characterization to identify material that could be of use in celeriac breeding. Genetic relationships were estimated in 34 elite celeriac varieties bred in Europe and 28 celeriac accessions conserved at the German genebank. Two varieties of celery, two varieties of leaf celery and three genebank accessions of wild *Apium* species were additionally analysed. Fifteen *Eco* RI/*Mse* I-based AFLP primer combinations were used. Polymorphic AFLP fragments were scored for calculation of Jaccard's coefficient of genetic similarity (GS). Morphological distances (MD) were determined based on 11 morphological traits. Average GS estimate in elite germplasm (GS = 0.90) was higher than in exotic germplasm (GS = 0.80). An AMOVA (analysis of molecular variance) revealed that a high proportion of variation was due to variation within elite celeriac varieties and genebank accessions. Although GS and MD matrices were poorly correlated ($r = 0.22$), UPGMA (unweighted pair group method using arithmetic averages) cluster analyses revealed clear genetic groupings of celeriac germplasm, which was supported by morphological traits. Elite, moderately bred and exotic varieties formed distinct clusters, indicating that only a part of the available genetic diversity in celeriac germplasm has been exploited in breeding. Distinct *Apium* species might be useful for the introgression of new genes into cultivated celeriac material. Broadening of celeriac collections in genebanks and detection of new genetic resources are vital for improvements in celeriac breeding.

Keywords: AFLP; AMOVA; *Apium graveolens*; cluster analysis; genetic resources; genetic similarity; morphological distance

Introduction

Apium graveolens L. (family *Apiaceae*) is the only cultivated species of the *Apium* genus (Quiros, 1993). Although the Mediterranean basin is considered the centre of origin, Argentina and Chile are the countries richest in *Apium* species. They are most commonly found in coastal areas, which implies that water currents may have played a significant role in their distribution. Cultivation of *A. graveolens* most likely started as early as 400 BC, when ancient Egyptians and Romans used it as a medicinal crop. First records on its domestication as a true vegetable date back to the 16th century in the Mediterranean region (Smith, 1979; Quiros, 1993).

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Very early, selection started in two directions: for solid and succulent petioles in one, and for enlarged hypocotyls in the other. *A. graveolens* L. comprises three distinct taxonomic varieties, grown for different economic purposes. Most widely grown is *A. graveolens* var. *dulce* (common name celery), which develops succulent, solid petioles. *A. graveolens* var. *rapaceum* (common name celeriac or root celery) is characterized by swollen hypocotyl and root tissue that results in a strongly flavoured, globe-like structure. The plants of *A. graveolens* var. *secalinum* (common name leaf celery or smallage) have slender, leafy petioles and are used mostly for their leaves as a condiment garnish or for medicinal purposes.

A. graveolens is a biennial crop ($2n = 22$; 3×10^9 bp DNA) with a very high degree of outcrossing (approximately 70%) due to wind and insect pollination (Quiros, 1993). Its flowers are small, with different developmental stages within the same umbel, thus making the emasculation of individual anthers difficult. A genetic male sterile genotype has been observed in weedy Iranian species (Quiros, 1993). Therefore nearly all celeriac varieties produced at the moment are open-pollinated. Regarding the latest available information (Bundessortenamt, 2001), the area cultivated with celeriac is steadily increasing, and currently reaches 1720 ha in Germany (approximately 4% of the total vegetable-growing area in the country).

The main objectives in celeriac breeding are uniformity (in colour, size and yield), quality and disease resistance (Bundessortenamt, 2001). The preferred celeriac varieties are characterized by round-shaped tubers, white skin colour and low insertion of roots. Tubers of high inner quality possess a firm consistency and weak internal rust spots of the flesh. Furthermore, varieties with white flesh after cooking are favoured. Major diseases in celeriac are foliar late blight (*Septoria apiicola*) and celery root rot (*Phoma apiicola* Kleb.). Genes for resistance to *Septoria* are found in some wild species, such as *A. nodiflorum*. Nevertheless, the attempts to hybridize *A. graveolens* with wild species have failed so far (Quiros, 1993).

The variability of germplasm used in celeriac breeding is assumed to be narrow and demands broadening (J. Dijkstra, personal communication). Nevertheless, celeriac accessions are poorly represented in genebanks (Quiros, 1993) and their relationships to commercially grown celeriac varieties have not been studied. In addition to the large working collection of *Apium* germplasm maintained at the University of California, USA (Quiros, 1993), the German genebank (Gatersleben, Germany) conserves a collection of 166 accessions of diverse *Apium* species, of which 36 are the accessions of celeriac.

Morphological, isozyme, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) studies (Quiros *et al.*, 1987; Huestis *et al.*, 1993; Yang and Quiros, 1993) have been conducted for the characterization of celery. Genetic variability of celeriac varieties that are either commercially grown or available in genebanks has not yet been studied. Development of reliable molecular marker systems such as amplified fragment length polymorphisms (AFLPs; Vos *et al.*, 1995) allows estimation of the diversity present in crop plants, thus facilitating future breeding and genebank management activities. AFLPs have been successfully applied to analyse genetic diversity in various crops (Kim *et al.*, 1998; Zhu *et al.*, 1998; Abdalla *et al.*, 2000; Lombard *et al.*, 2000; Simioniuc *et al.*, 2002), as well as to characterize genebank accessions (Capo-chichi *et al.*, 2001; McGregor *et al.*, 2002).

The objective of our study was to use morphological characterization and AFLP fingerprinting in celeriac to (i) estimate the genetic diversity among (a) European celeriac varieties and (b) a set of old celeriac varieties and genebank accessions from the genus *Apium* conserved at the genebank of the Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany), as well as to (ii) identify genetically diverse material that might have potential in celeriac breeding.

Materials and methods

Plant materials and DNA extraction

Sixty-nine accessions of celeriac and related species were included in the study. They were divided into two sets. The first set, further referred to as elite germplasm, contained 31 open-pollinated and three F_1 hybrid celeriac varieties that have undergone more or less intensive breeding in Europe. The second set, further referred to as exotic germplasm, consisted of 28 open-pollinated formerly grown celeriac varieties, two varieties of celery, two varieties of leaf celery and three genebank accessions of wild *Apium* species (Table 1).

To extract genomic DNA, 2–3 g of fresh leaf material (originating from a pool of 20–30 plants per accession) were frozen in liquid nitrogen and ground to a fine powder. The extraction of genomic DNA was done following the modified CTAB procedure (Hoisington *et al.*, 1994).

AFLP analysis

The AFLP fingerprints were produced according to the original protocol of Vos *et al.* (1995), modified by Muminović *et al.* (2004). *Eco*RI selective primers with three selective nucleotides were [^{33}P]γ-ATP 5′-end labelled and combined with *Mse*I primers carrying

Table 1. Elite and exotic germplasm of celeriac (*Apium graveolens* var. *rapaceum*), celery,[a] leaf celery[b] and related *Apium* species analysed with AFLP molecular markers

Elite germplasm		Exotic germplasm	
Variety	Breeding company	Variety	Acc. no.[c]
Alba	Nunhems-Hild	Ajax	Nunhems-Hild
Anita	Saatz. Quedlin.	Alabaster	API 19
Arvi	Nickerson Zwaan	Alabastrom	API 76
Bergers Weiße Kugel	Nunhems-Hild	Apfel	API 102
Brilliant[d]	Bejo	Apia	API 103
Cascade	Rijk Zwaan	Balder	Nunhems-Hild
Cesar[d]	Huizer	Ceva	API 66
Cisko[d]	Rijk Zwaan	Dresdner Markt	API 18
Diamant[d]	Bejo	Erfurter	Nunhems-Hild
Dolvi	Sperling	Eureka	API 65
Goliath[d]	Juliwa-Enza	Galina	API 73
Hans	GHG	Iram	API 121
Ibis	Juliwa-Enza	Kompakt	API 127
Ilona[d]	Bejo	Magdeburger Markt	API 22
Invictus	Sonnensaat	Marble Ball	API 70
Kojak	Juliwa-Enza	Montblanc	Nunhems-Hild
Luna (F_1)[d]	Bejo	Neckarland	Nunhems-Hild
Makar	Planti	Oderdörfer	API 21
Mars[d]	Nunhems-Hild	Phoenix	Nunhems-Hild
Mentor[d]	Royal Sluis	Pionier	API 72
Monarch[d]	Nunhems-Hild	Prager Riesen	API 101
Neve	Clause	Prazsky Obrovsky	API 24
Odrzanski	Planti	Robust	Nunhems-Hild
Ofir	Rijk Zwaan	Roka	API 26
Ortho	Demeter	Rokary	Nunhems-Hild
President[d]	Rijk Zwaan	Saxa	API 69
Prinz[d]	Nunhems-Hild	Tellus	API 67
Radiant[d]	Bejo	Wiener Markt	API 17
Regent	Nunhems-Hild	Giant Red[a]	Juliwa-Enza
Rex[d]	Nunhems-Hild	White Pascal[a]	Juliwa-Enza
Snehvide	Daehnfeldt	Aromatischer Schnitt[b]	Juliwa-Enza
Tania	Daehnfeldt	Gewöhnlicher Schnitt[b]	Juliwa-Enza
Terra (F_1)	Bejo	*A. graveolens* var. *lusitanicum*	API 32
Volltreffer	Terra	*A. inundatum*	API 145
		A. nodiflorum	API 146

[a] Celery variety (*Apium graveolens* var. *dulce*).
[b] Leaf celery variety (*Apium graveolens* var. *secalinum*).
[c] Refers to the accession numbers of the genebank at IPK (Gatersleben, Germany) or the company that supplied the accession from the breeders' collection.
[d] Commercial variety.

three selective nucleotides. In total, 15 *Eco*RI/*Mse*I primer combinations were employed for selective amplification reactions (Table 2). Amplified DNA fragments were denatured at 95°C for 3 min and separated by electrophoresis in a 6% denaturing polyacrylamide gel.

To test the reliability and reproducibility of the AFLP protocol, duplications were included in the form of (i) blind checks—accessions sown twice under coded numbers, and (ii) laboratory duplicates—randomly chosen accessions duplicated after DNA extraction and re-duplicated in consecutive steps of the AFLP analysis. To ensure scoring of identical AFLP fragments in both sets of materials, 20 bp DNA-ladder and four standard varieties were additionally used in both germplasm sets. One set of germplasm (including blind checks and laboratory duplicates), genotyped with one AFLP primer combination, fitted to one gel. Each gel was run in 0.5 × TBE buffer (100 mM Tris, 100 mM boric acid, 2 mM EDTA pH 8.0), at 23 W for 5 min, followed by 60 W for 2 h. After drying on Whatman paper, gels were exposed to X-ray films for 5–7 days before developing.

Evaluation of morphological traits

Morphological characterization of celeriac varieties was conducted at Nunhems-Hild in October 2001. Old variety Prazsky Obrovsky was not provided for the morphological evaluation due to a low seed germination rate.

Table 2. Polymorphic information content (PIC) and marker indices (MI) per AFLP primer combination in 69 accessions of celeriac (*Apium graveolens* var. *rapaceum*), celery (*A. graveolens* var. *dulce*), leaf celery (*A. graveolens* var. *secalinum*) and related *Apium* species

Primer combination[a]	Total no. of bands	No. of selected polymorphic bands	PIC	MI
E39/M53	25	20	0.26	5.14
E39/M57	33	8	0.24	1.89
E31/M48	39	6	0.30	1.79
E34/M48	28	15	0.13	1.93
E34/M61	55	25	0.11	2.74
E32/M59	61	52	0.12	6.45
E33/M54	27	17	0.04	0.76
E40/M47	68	22	0.20	4.37
E40/M54	59	34	0.14	4.73
E37/M60	48	31	0.11	3.56
E37/M62	52	35	0.21	7.44
E38/M49	50	4	0.24	0.96
E38/M56	39	8	0.20	1.58
E38/M57	37	10	0.24	2.37
E38/M48	39	5	0.23	1.16
Total	660	292	–	–
Average	44	19.47	0.18	3.12

[a] *Eco*RI and *Mse*I primer codes correspond to the official nomenclature available at http://www.keygene.com/html/nomenclature.htm

Evaluation of the traits was conducted on 10 randomly chosen plants per variety. Regarding the criteria of the International Union for the Protection of New Varieties of Plants (UPOV, 2002), the following morphological traits were evaluated: presence of anthocyanin coloration of the petiole, main colour of tuber skin (whitish or brown), tuber width (cm), tuber shape (irregular to round, on a scale of 3 to 9, where 9 designates round), presence of side shoots, presence of cracks, insertion of roots (low, medium or high), tuber uniformity, tuber inner cavity (low, medium or large), flesh discoloration after cutting, and yield (average weight of 10 tubers, expressed in kg).

Data analyses

Polymorphic bands in both germplasm sets were first scored separately to estimate genetic diversity within each of the sets. Subsequently, datasets were combined using the DNA-ladder and four standard varieties, to identify common bands in both sets. For combined data analysis, monomorphic bands within the first set were included if they were polymorphic in the second, exotic set. Only distinct major bands, ranging in length from 50 to 350 bp, were manually scored as present (1) or absent (0). The proportion of polymorphic bands per set, as well as within different types of material (celeriac, celery, leaf celery) was calculated.

To evaluate the discriminatory power of AFLPs, the polymorphic information content (PIC) and marker index (MI) were calculated across assay units, assuming that a single AFLP primer combination was an assay unit, and each polymorphic DNA fragment within an assay unit was a single dominant marker locus. The PIC value was calculated applying the formula of Roldán-Ruiz *et al.* (2000): $PIC_i = 2f_i(1 - f_i)$, where f_i is the frequency of the amplified allele (band present), and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i. MI was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell *et al.*, 1996).

To estimate the level of genetic variability, genetic similarity (GS) between the two varieties i and j was calculated by applying the formula of Jaccard (1908): $GS_{ij} = N_{ij}/(N_i + N_j - N_{ij})$, where N_i is the number of detected bands in a variety i and not in variety j, N_j is the number of detected bands in a variety j and not in variety i, and N_{ij} is the number of bands common to varieties i and j. Estimation of GS values and the calculation of their standard errors (SE) by a jackknife procedure with re-sampling over primers (Miller, 1974) were done with the Plabsim software (Frisch *et al.*, 2000), which is implemented as an extension of the statistical software R (Ihaka and Gentleman, 1996).

To divide the molecular genetic variance of celeriac varieties into components attributable to the variance between and within sets, an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed without celery and leaf celery varieties, as well as without wild *Apium* species. Groups of material (elite and exotic celeriac germplasm) were considered sets and genetic data (AFLPs) were presented as haplotypes in the Arlequin version 2.000 software (Schneider *et al.*, 2000).

For calculation of morphological distances (MD), observations for each trait were standardized by dividing with the phenotypic standard deviation of the particular trait. Euclidean distance (E_{ij}) was calculated based on standardized observations for each pairwise comparison of varieties, applying the formula $E_{ij}^2 = \Sigma_k(x_{ki} - x_{kj})^2$, where i and j designate varieties, and k is observed trait (Sneath and Sokal, 1973). The calculations were done using the NTSYSpc version 2.0 (Rohlf, 1998).

To estimate the correlation between matrices of AFLP-based GS estimates and MD values based on morphological traits, GS estimates were transferred to genetic distance (GD) estimates using the function $GD = 1 - GS$ (Gower and Legendre, 1986). Calculation of correlation between matrices of GD and MD estimates, normalized Mantel test (Mantel, 1967), as well as the UPGMA (unweighted pair group method using arithmetic averages; Sneath and Sokal, 1973) clustering method were conducted with the NTSYSpc version 2.0. Reliability

of the GS-based dendrogram was tested by bootstrap analyses with 1000 replications to assess the branching support, using the Winboot software (Yap and Nelson, 1996).

Results

AFLP data

The 15 *Eco*RI/*Mse*I primer combinations generated a total of 660 bands, with 292 (44%) selected as clearly distinct and reliable for data processing (Table 2). The number of polymorphic bands selected for data processing ranged from four to 52 per AFLP primer combination, with an average of 19. Average PIC values ranged from 0.04 to 0.30, whereas MI varied between 0.76 and 7.44 (Table 2).

Reliability of AFLP data was confirmed by a significant correlation of 0.99 ($P < 0.05$) among GS estimates of four standard varieties employed both in the elite and exotic set. Genetic similarities between genotypes duplicated on the plant level (blind checks) ranged from 0.93 to 0.97, and between laboratory duplicates from 0.98 to 1.00 (data not shown).

Genetic similarities among varieties

Genetic similarities within the elite set ranged from 0.68 (Goliath–Ofir; Mentor–Snehvide) to 0.95 (Monarch–President; Monarch–Diamant), with a mean of 0.90. In the exotic set, the lowest GS value of 0.05 was detected between Magdeburger Markt and the accession of *Apium inundatum*, and the highest (GS = 0.95) in two pairs of varieties (Ceva–Eureka; Marble Ball–Balder). The average GS value in the exotic set was 0.80. Genetic similarity in the whole set of 69 varieties ranged from 0.05 (Magdeburger Markt–*Apium inundatum*) to 0.96 (Ajax–President), with a mean of 0.75. Standard errors of the individual GS estimates varied from 0.01 to 0.06 in the elite, and from 0.02 to 0.07 in the exotic set.

AMOVA and UPGMA cluster analysis of AFLP data

According to the AMOVA, a higher proportion of variation was present within (88.6%) compared to between sets (11.4%). The UPGMA cluster analysis using the 15 AFLP primer combinations clearly separated the cultivated material of *A. graveolens* from the accessions of *A. nodiflorum* and *A. inundatum* (Fig. 1). The major *A. graveolens* cluster further split into six divergent subclusters (Fig. 1). Sub-clusters A, D and F consisted almost exclusively of celeriac varieties that have undergone substantial selection and breeding. The only varieties from the exotic set were Ajax, Iram and Montblanc in sub-cluster A, and Apia, Wiener Markt and Galina in sub-cluster D. Similarly, sub-clusters B and C consisted of only formerly grown celeriac varieties (exotic set), with minor exceptions of Neve in sub-cluster B and Odrzanski in sub-cluster C. Finally, sub-cluster E was composed of the two celery and two leaf celery varieties, with the accession of *A. graveolens* var. *lusitanicum* being more closely related to leaf celery varieties.

UPGMA cluster analysis of morphological data and correlation to AFLP data

Estimates of MD varied from 0.93 (Magdeburger Markt–Dresdner Markt) to 8.41 (Invictus–Ibis), with a mean of 4.7. Cluster analysis of MD values separated celeriac varieties into clusters that, similarly to the AFLP-based GS cluster, reflected the previous classification of varieties into elite or exotic set (Fig. 2). Exotic varieties Apfel and Iram (sub-cluster F) were most widely separated from the other materials. Sub-clusters A, C and D consisted mainly of elite materials, with the exception of five exotic varieties (Ajax, Robust, Apia, Dresdner Markt and Montblanc) in sub-cluster A. Consequently, subcluster B was composed of exotic varieties, with just one representative of the first set, variety Odrzanski. The single sub-cluster where varieties of elite and exotic sets were equally present was sub-cluster E (Fig. 2).

Regarding two morphological traits, anthocyanin coloration of the petiole and the main colour of tuber skin, varieties with white-coloured tubers clearly separated into sub-clusters A and E (Fig. 2). Consequently, all of the varieties in sub-clusters B, C and D had anthocyanin in the petioles and brown-coloured tubers (Fig. 2). Other morphological traits offered no support to the MD-based dendrogram.

Correlation between data matrices of GD estimates based on AFLP data and MD values based on morphological traits was low, with $r = 0.22$ ($P < 0.01$).

Discussion

Reliability of AFLP protocol established for celeriac

Inclusion of replications or duplications in molecular marker analyses is not a common practice because DNA marker data are considered highly accurate in fingerprinting plant genomes. In our study, the range of variation in AFLP-based GS estimates of blind checks was slightly wider than that of laboratory duplicates, which may be

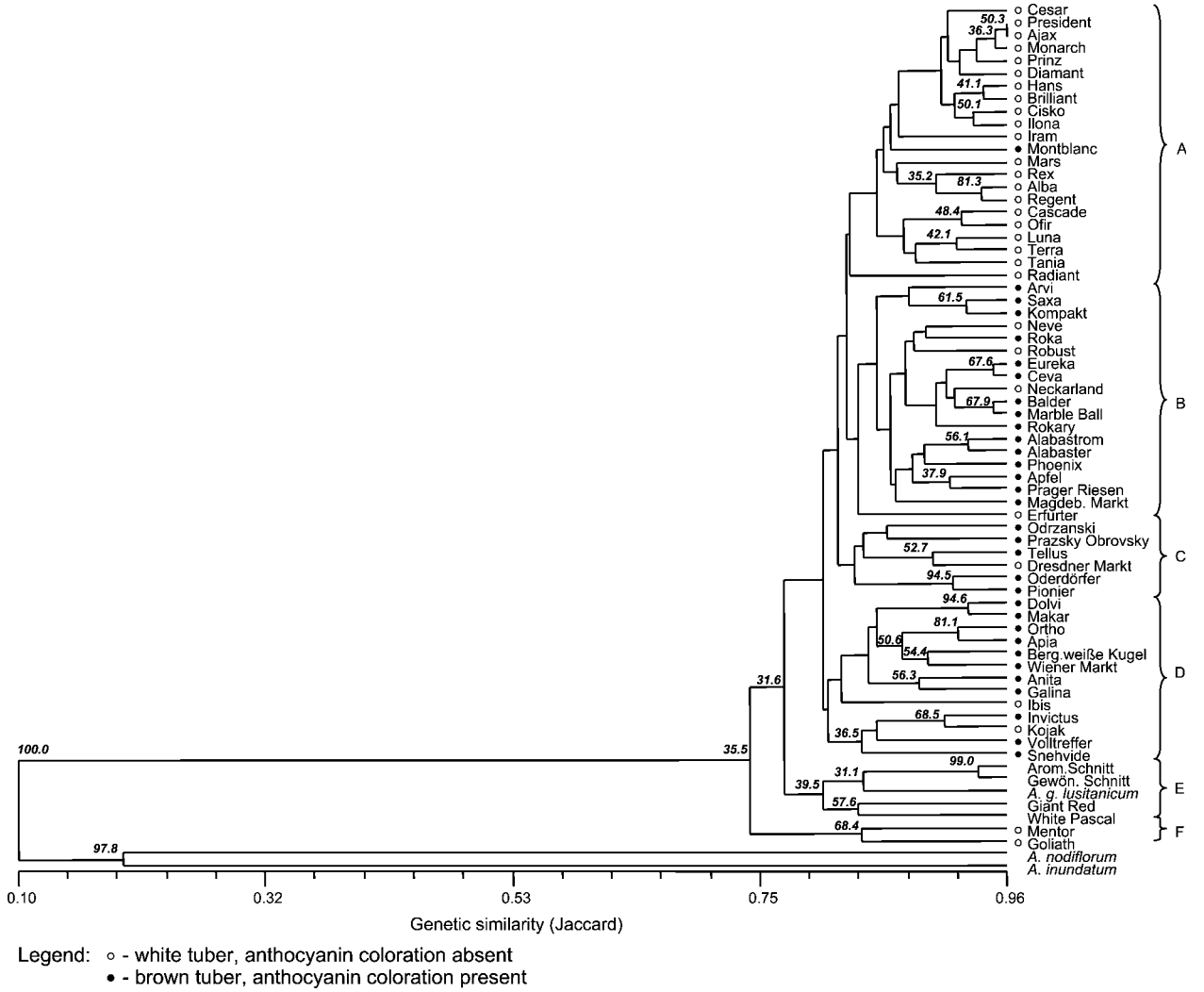

Fig. 1. Association among varieties of celeriac (*Apium graveolens* var. *rapaceum*), celery (*A. graveolens* var. *dulce*), leaf celery (*A. graveolens* var. *secalinum*) and related species, as revealed by average linkage (UPGMA) cluster analysis of Jaccard's genetic similarity (GS) coefficients calculated from AFLP data of 15 primer combinations. Numbers at the nodes indicate the bootstrap values of the consensus tree obtained (branches lacking the value received <30% bootstrap support). Letters A to F designate detected sub-clusters in the major *A. graveolens* cluster. Symbols designate colour of the tuber and presence of anthocyanin.

due to (i) errors during seed sowing and sampling leaf material for DNA extraction, or (ii) heterogeneity present in celeriac cultivars. Genetic similarity between laboratory duplicates was theoretically expected to be near to unity. Nevertheless, DNA quality differences may have affected the AFLP banding pattern (Vos *et al.*, 1995). The results of our study were comparable to those obtained in genetic diversity studies with RFLP, RAPD and AFLP markers (Messmer *et al.*, 1993; Hahn *et al.*, 1995; Lübberstedt *et al.*, 2000; Zeid *et al.*, 2003; Muminović *et al.*, 2004), where replicated samples were also used.

An average PIC value of 0.18 across all scored AFLP bands, as well as an average MI of 3.12 across all primer combinations (Table 2) were in harmony with other AFLP-based genetic diversity studies in various crops (Powell *et al.*, 1996; Bohn *et al.*, 1999; Lübberstedt *et al.*, 2000; Roldán-Ruiz *et al.*, 2000; Muminović *et al.*, 2004). Owing to their high multiplex ratio, AFLPs proved to be a highly informative molecular marker system, which recommends them for fingerprinting cultivars in cultivar identification, protection and quality control, as well as for the identification of essentially derived varieties (Bohn *et al.*, 1999; Heckenberger *et al.*, 2003), the latter being of increasing relevance in celeriac breeding.

Morphological diversity in celeriac germplasm

Regarding the presence of anthocyanin in the petioles and the colour of the tuber, dendrograms based both

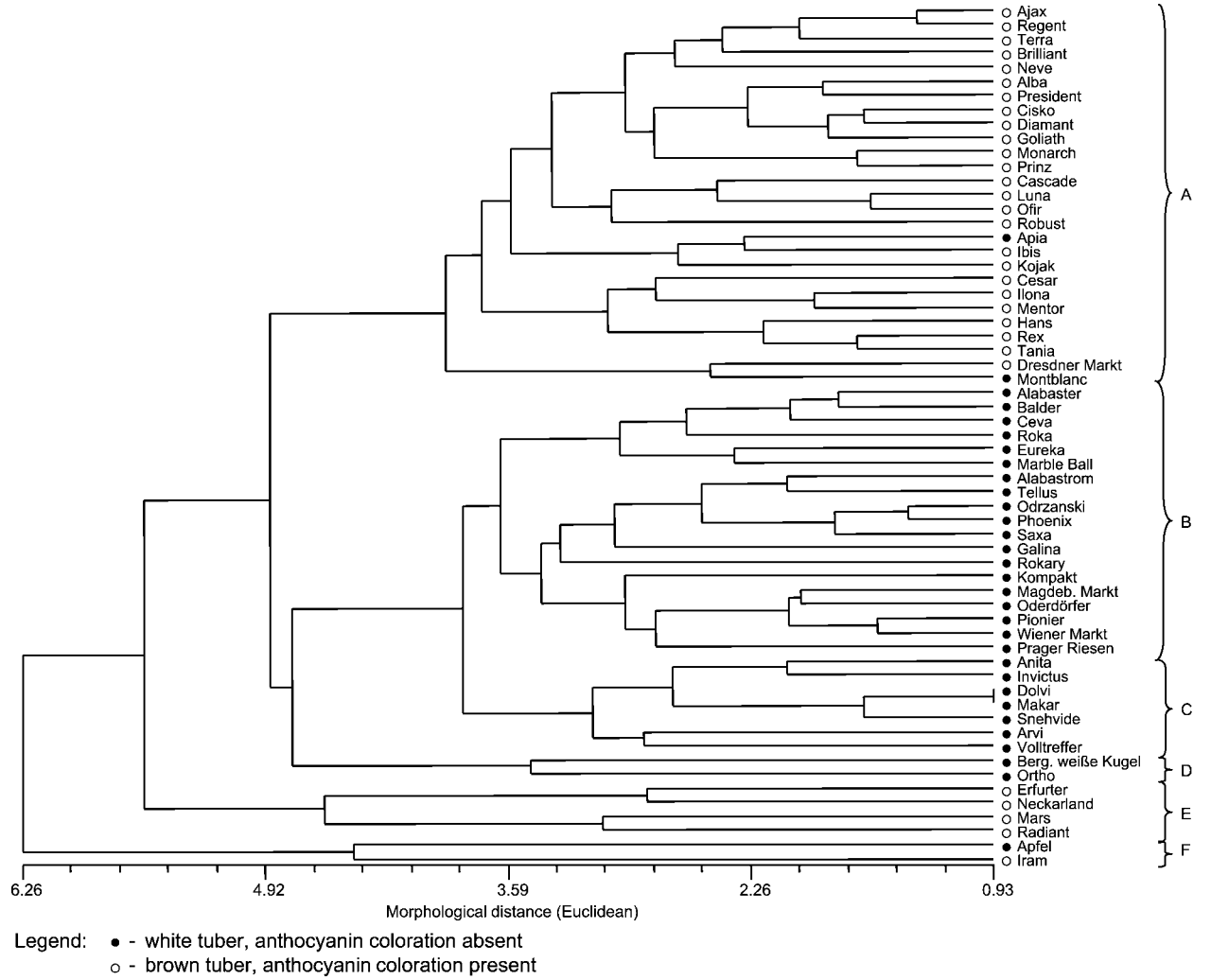

Fig. 2. Association among celeriac (*Apium graveolens* var. *rapaceum*) varieties, as revealed by average linkage (UPGMA) cluster analysis of Euclidean genetic distance (GD) coefficients calculated from morphological data. Letters A to F designate detected sub-clusters. Symbols designate colour of the tuber and presence of anthocyanin.

on GS and MD estimates indicated a clear morphological structure in celeriac germplasm (Figs 1 and 2). As expected, this was more clear in the MD-based dendrogram, where varieties with white-coloured tubers created distinct sub-clusters A and E (Fig. 2). In the GS-based dendrogram, varieties with white-coloured tubers were present in the sub-clusters A, E and F, but to a minor extent also in the clusters created mostly of varieties possessing brown-coloured tubers (sub-clusters B, C and D). Nevertheless, the distance matrices based on AFLPs and morphological data showed only a weak correlation ($r = 0.22$). This may be explained with the assumption that the observed morphological traits inadequately represented the underlying genetic relationships. Similarly to the results obtained in an AFLP study of barley (Schut *et al.*, 1997), the reasons for this could be (i) a limited number of traits evaluated, (ii) a limited variation of these traits, (iii) a limited number and irregular genome distribution of genes underlying these traits, and (iv) epistatic interactions among these genes. Morphological differentiation is the result of natural and/or artificial selection (Thierry d'Ennequin *et al.*, 2000). Although they are cheaper and easier to measure than diversity estimates using molecular markers, morphological traits cannot serve as unambiguous markers and should therefore be used with caution in systematic studies, especially in investigations of relationships between accessions.

Genetic diversity in celeriac germplasm

The range of AFLP-based genetic similarity estimates in elite celeriac germplasm (0.68–0.95) was comparable to AFLP-based diversity estimates in soybean, barley, pea and wheat (Powell *et al.*, 1996; Schut *et al.*, 1997; Simioniuc *et al.*, 2002; Soleimani *et al.*, 2002). Nonetheless,

the variation of GS estimates revealed with AFLP analysis in the exotic set of celeriac was significantly higher (GS from 0.05 to 0.95), indicating its high level of genetic diversity. Lower genetic variation measured among elite celeriac varieties is most likely the result of selection pressure and genetic drift in breeding programmes. Such practices, which are aimed at genetic homogenization and purity of cultivars, may result in an improvement in yield and other agronomically important traits at the expense of reducing the genetic base of crops (Soleimani *et al.*, 2002).

Dendrograms based on GS- and MD-estimates illustrate the intensity of selection and breeding which elite celeriac varieties have undergone (J. Dijkstra, personal communication). Varieties that are currently most important in the market and, therefore, considered commercially elite (Table 1) grouped closely together and were present only in sub-clusters A and F in the GS-based dendrogram, and in sub-clusters A and E in the MD-based dendrogram (Figs 1 and 2). Consequently, the celeriac varieties originally classified as the elite germplasm (Table 1), but which have not been intensively bred and are not widely represented on current markets (because they express unfavourable morphological traits, such as brown-coloured tubers) were dispersed in the sub-clusters consisting mainly of exotic materials (Figs 1 and 2). Although not morphologically distinct (Fig. 2), varieties Mentor and Goliath were distinct from other elite materials in GS-based dendrograms (Fig. 1, sub-clusters A and F). Varieties of these two sub-clusters might be considered to establish divergent germplasm pools (heterotic pools) in view of hybrid cultivar production.

Although the values of genetic similarity estimates did not seem significantly different and wide, and molecular variance measured with AMOVA was much higher within than between celeriac germplasm sets, elite and exotic celeriac varieties were clearly separated in distinct clusters using either AFLP or morphological data. Even if the order of varieties in GS- and MD-based clusters was not identical (Figs 1 and 2), those clustering patterns might indicate that current breeding of celeriac utilizes only a small fraction of genetic diversity available in celeriac. The proportion of polymorphic AFLP bands detected in elite celeriac varieties (32.2%) was lower than the proportion of polymorphic bands revealed in exotic celeriac germplasm (38%, data not shown), with 38 AFLP bands (13% of the total number of detected bands) being monomorphic in elite but polymorphic in the exotic germplasm. Thus, the inclusion of exotic varieties into celeriac breeding might substantially support the improvement of elite varieties.

This study demonstrated the ability of AFLPs to estimate accurately the distribution of diversity within and among *Apium graveolens* subspecies (celery, celeriac and leaf celery). The three cultivated forms of *A. graveolens* shared a large proportion (55.1%) of common AFLP bands, which implied a high degree of genetic similarity among these cultivated types and was in agreement with the findings of Yang and Quiros (1993). Nevertheless, clear separation was observed among celery, leaf celery and celeriac varieties, as in an RAPD-based study of celery diversity (Yang and Quiros, 1993). The values of the average GS estimates between elite celeriac and celery, and between elite celeriac and leaf celery varieties were 0.73 and 0.78, respectively, which is lower than the average GS estimate within elite celeriac varieties (GS = 0.90). Even though the number of varieties representing each of the three *A. graveolens* subspecies was not equal in our study, it can be concluded that a substantial genetic diversity is present among them. So far, celeriac was successfully used in celery breeding in the development of breeding lines resistant to a vascular disease incited by *Fusarium oxysporum* f. sp. *apii* (Quiros, 1993). According to our results, celery and leaf celery varieties could contribute substantially in broadening of the celeriac germplasm.

A high level of unexploited genetic diversity was detected in wild relatives of *A. graveolens*. The accessions of *A. inundatum* and *A. nodiflorum* were distinct from the cultivated *Apium* forms, whereas *A. graveolens* var. *lusitanicum* indicated a closer relationship to celery and leaf celery than to celeriac (Fig. 1). Regarding the high proportion of AFLP bands that were polymorphic in wild species but monomorphic in elite celeriac germplasm (62%; i.e. 181 'new' bands), a large potential for broadening the genetic base of celeriac is found in wild *Apium* species. Further breeding efforts should be undertaken to utilize them as genetic resources for celeriac breeding. In addition, these accessions of wild species could contribute to the introgression of new genes (such as resistance genes) into cultivated celeriac material, applying recently developed methods of interspecific crosses.

The average GS estimate in the analysed celeriac material was 0.75, whereas sub-clusters based on AFLPs started branching at the level higher than GS = 0.80 (Fig. 1). These results of our study indicate that the celeriac germplasm collection, conserved at the German genebank of IPK, covers a smaller part of variation compared to that usually expected in crop plants. A rising concern about possible genetic erosion of landraces and wild relatives due to current cultivation practices, as well as an increasing need for a steady flow of new germplasm (Scarascia-Mugnozza and Perrino, 2002), encourage the creation of comprehensive collections in which also minor crops, such as celeriac, must be better represented.

Understanding the significance or assessing the real (applicable) value of the diversity detected in plant genetic resources still remains a challenge (Karp, 2002), in terms of identifying alleles that may confer crop improvement. Celeriac germplasm collection at the German genebank demands broadening and use of molecular markers for detecting further valuable genetic resources, to the benefit of celeriac breeding.

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5 General Discussion

Reliability and reproducibility of the AFLP protocol established for cornsalad, radish and celeriac

The usual routine of molecular markers work does not include replications or duplications, because DNA marker data demand high production costs and are considered accurate in fingerprinting of plant genomes. Nevertheless, this approach does not allow an estimation of experimental error for marker data. To establish and optimize the AFLP laboratory protocol for cornsalad, radish, and celeriac the following steps were taken: (i) main amplification reactions with five (*Eco*RI-NNN x *Mse*I-NN) and six selective nucleotides (*Eco*RI-NNN x *Mse*I-NNN) were tested, (ii) silver-staining and [^{33}P]-labelling approaches for visualisation of AFLP fragments were applied, and (iii) two types of duplicates (on the plant level – blind checks, and on the DNA level – laboratory duplicates) were included. Furthermore, an additional, independent scoring was carried out by a third skilled person, to minimize errors due to scoring.

The AFLP primer combinations carrying a total of five selective nucleotides performed better than those with six selective nucleotides in the main amplification reactions with cornsalad DNA, suggesting a relatively small genome size of this crop. In both radish and celeriac, AFLP fragments produced clearer banding patterns with a significantly reduced background "noise" on polyacrylamide gels when primer combinations with six selective nucleotides were applied in the main amplification reactions.

In all three crops, the silver-staining protocol for visualization of AFLP fragments resulted in a lower reproducibility of the controls than the protocol using radioactive labelling of the *Eco*RI primers. Additionally, the documentation system applied with silver-staining AFLP protocol (direct scanning of dried gel from glass plates) was not optimal in terms of its handling and possible re-scoring feasibilities. Although significantly more expensive, radioactive labelling of the main amplification reaction primers resulted in clear images of AFLP fingerprints, whereas autoradiograms allowed a reliable long-term storage.

Applying the silver-staining AFLP protocol, genetic similarity (GS) estimates between blind checks and their respective original samples (GS = 0.74 to 0.77) did not reach sufficiently high levels, whereas laboratory duplicates indicated considerable error at the level of scoring. In contrast, the AFLP protocol using radioactive labelling of the main amplification primer resulted in a high reproducibility and reliability of the obtained data in all three studied crops, which was reflected in high GS values for blind checks (GS from 0.89 to 1.00) and laboratory duplicates (GS from 0.98 to 1.00). The obviously wider range of variation in GS estimates for varieties duplicated on a plant level (blind checks), particularly in radish, may be due to the heterozygosity and heterogeneity present in varieties. F_1 hybrids in radish, as in a number of other vegetable crops, can not be considered homogeneous and uniform because a critical level of inbreeding depression of parental inbreds is reached after only a few generations of selfing (Kaneko and Matsuzawa 1993; Schieder, personal communication). Finally, the reliability of the established AFLP protocol was re-confirmed by a high correlation ($r = 0.91$; $P < 0.01$) between the original and independently conducted scorings.

In a limited number of studies, where similar but not as numerous and reaction-specific DNA-controls were included (Messmer et al. 1993; Hahn et al. 1995; Lübberstedt et al. 2000), comparable GS estimates between the replicated samples were found for RFLP, RAPD and AFLP marker data in maize. The AFLP protocol established and optimized in this study proved to be reliable and reproducible in three vegetable crops of diverse pollination patterns, such as cornsalad, radish, and celeriac. It can, therefore, be suggested for further application of molecular marker technologies in a wide range of vegetable crops. If known, the genome size of a particular crop should to be taken into consideration when the choice of number of selective nucleotides in the main amplification reactions is made.

Genetic diversity in germplasm of cornsalad

Breeding practices in cornsalad rely mostly on phenotypic selection and on a narrow-based germplasm available in breeding companies. To investigate genetic diversity in cornsalad germplasm and to suggest possible ways of its broadening, 34 modern line varieties of cornsalad (referred to as elite germplasm) were analyzed, as well as 12 old cornsalad

varieties and 18 germplasm accessions of cornsalad and related species, conserved in gene banks and botanical gardens (referred to as exotic germplasm). Variability present in the elite cornsalad germplasm was comparable with the AFLP-based genetic diversity estimates in other autogamous crops, such as barley (Russell et al. 1997; Schut et al. 1997), pea (Simioniuc et al. 2002), or velvet-bean (Capo-chichi et al. 2001), though narrower than in wheat (Bohn et al. 1999; Soleimani et al. 2002) or soybean (Powell et al. 1996). The low variation among elite cornsalad varieties might be the consequence of a narrow-based germplasm in the breeding programs of different companies (Schieder and Hermens, personal communication), which is supported by the apparent grouping of the majority of studied modern line varieties in only two sub-clusters of the major UPGMA cluster. Additionally, the sub-clusters based on AFLP data revealed no apparent morphological pattern, even when reliable morphological descriptors of cornsalad (prominence of veins on the leaf surface, concave profile of the leaf, and seed size) were considered. This was in contrast to wheat (Bohn et al. 1999) or maize (Pejic et al. 1998; Lübberstedt et al. 2000), but in agreement with the results on barley (Schut et al. 1997), and may be a result of inadequate representation of genetic relationships by the observed morphological traits. To confirm the reliability of AFLP-based genetic diversity estimates, pedigree information of six elite cornsalad varieties was provided to calculate the coefficient of co-ancestry *f* (Malécot 1948). However, the matrix of co-ancestry estimates was poorly correlated with the respective matrix of GS values ($r = 0.19$, $P < 0.05$). This was comparable to barley (Graner et al. 1994) and soybean (Cox et al. 1985), and might be due to a high "background similarity" among unrelated accessions at the marker level (Graner et al. 1994). Furthermore, pedigree data should correspond to molecular marker-based GS estimates in cases of large populations produced by random-mating. Breeding processes, on the other hand, focus at populations of limited sizes, where the influences of drift and selection play a major role. Marker-based GS estimates provide more information than pedigree data because they detect sequence variation and bypass the assumptions common to pedigree analysis.

The average value of genetic similarity (GS) estimate in the exotic germplasm (GS = 0.47) was significantly wider than in the elite (GS = 0.90), thus indicating a substantial level of genetic diversity conserved in the material not yet utilized in cornsalad breeding. An extensively larger genetic diversity within exotic germplasm accessions was also demonstrated with the UPGMA analysis. Four old cornsalad varieties ('CS-8', 'CS-45',

'CS-46', and 'CS-50') were grouped within the "elite" cluster, whereas some of the studied gene bank accessions formed sub-clusters close to the elite germplasm. Only the species related to *V. locusta* were clearly divided from the elite germplasm.

These results indicated that old cornsalad varieties and *V. locusta* genebank accessions that clustered close to the elite germplasm might serve as a direct genetic resource for broadening the elite cornsalad germplasm base, as those crosses are easy to perform. Along with them, *V. locusta*-related species might contribute to the introgression of new genes (such as resistance genes) into cultivated cornsalad material by applying recently developed inter-specific crosses.

Genetic diversity in and genetic structure of radish germplasm

Since the discovery of cytoplasmatic male sterility in radish two decades ago, there has been a significant increase in the production of F_1 hybrid varieties. To optimally exploit heterosis in F_1 hybrid production, parental lines should be derived from genetically unrelated germplasm pools, commonly referred to as heterotic groups (Melchinger and Gumber 1998). However, none of the currently published studies on genetic diversity of radish (Ellstrand and Marshall 1985; Demeke et al. 1992; Thormann et al. 1994; Rabbani et al. 1998; Huh and Ohnishi 2003) has focused on the estimation of genetic diversity and determination of genetic structure of a wider set of cultivars representing European radish germplasm.

Once the AFLP protocol was optimized, 68 cultivated radish varieties were selected for the study. The material consisted of open-pollinated varieties, inbred lines, diploid and a few tetraploid hybrid varieties of garden radish (*Raphanus sativus* var. *sativus*) and Black radish (*R. sativus* var. *niger*). Additionally, two accessions of wild relatives of radish (*R. raphanistrum*, and Chinese small radish - *R. sativus* var. *sativus* convar. *sinensis*) that cause serious contamination during the process of hybrid radish production were included in the analysis. Variation in GS estimates within the studied radish germplasm was comparable to the diversity of other members of the family Brassicaceae studied with AFLPs (Srivastava et al. 2001). An apparent grouping of all studied cultivated radish varieties in sub-clusters with GS estimates higher than 0.70 supported the assumption that the currently used radish germplasm in Europe relies on a narrow genetic basis (Schieder,

personal communication). High GS estimates observed between garden radish varieties originating from the same breeding company were in agreement with the assumed closer pedigree relationships. Owing to a high degree of heterogeneity and heterozygosity within radish varieties, the detected between-variety diversity is low, but there still is a substantial overall diversity in the available radish germplasm. On average 58.6% of the detected AFLP bands were polymorphic among open-pollinated varieties, 56.4% among F_1 hybrids, and 51.6% among inbred lines.

In both UPGMA and PCoA analyses, morphologically similar garden radish varieties types (such as "French Breakfast radish" and "Giant radish") formed separate groups, and Black radish varieties were distinct from garden radish. Nevertheless, garden radish germplasm could not be further divided into possible heterotic groups by applying the PCoA approach. An unambiguous and independent separation within garden radish varieties was made with the model-based clustering method of Pritchard et al. (2000), which relies on genotypic data consisting of unlinked markers, and not on a prior population information. Although the available pedigree data were not sufficient to confirm the reliability of the applied model-based approach in radish, as they did in studies on maize (Liu et al. 2003) and *Trifolium pratense* (Kölliker et al. 2003), the inferred sub-groups can be employed for the establishment of heterotic pools within European modern cultivars of garden radish. An efficient utilization of the existing substantial level of genetic variation (detected with AFLPs) can facilitate the choice of parents for crossing, definition of priorities, and reduction of costs in hybrid radish variety improvement.

Genetic similarity estimates between cultivated radish varieties and the accessions of *R. raphanistrum* and Chinese small radish were low, which can be valuable for radish breeders facing serious problems in F_1 hybrid radish production. Out-crossings between cultivated radish and either of the weedy species (that are morphologically hard to differentiate from cultivated radish) is easy and frequent (Schieder, personal communication). Regarding the results of this study, two simple ISSR assays (with the ISSR primers $(CAA)_6$ and UBC 890) can assist in estimating the level of seed purity of hybrid radish varieties before their sowing in the field. This significantly reduces the costs of hybrid radish production.

Genetic diversity in germplasm of celeriac

Breeding of *Apium graveolens* germplasm started as early as 400 BC, when ancient civilizations used it as a medicinal crop. Selection was conducted in two directions: for solid and succulent petioles in one, and for enlarged hypocotyls in the other. Nowadays, *A. graveolens* comprises three distinct taxonomic varieties: celery (*A. graveolens* var. *dulce*), celeriac (*A. graveolens* var. *rapaceum*), and leaf celery (*A. graveolens* var. *secalinum*), grown for different economic purposes. Nearly all celeriac varieties produced at the moment are open-pollinated, but with a recently detected genetic male sterility in weedy Iranian species (Quiros 1993) progresses in hybrid celeriac production are expected (Dijkstra, personal communication). Along with the narrow-based germplasm used in celeriac breeding, the accessions of celeriac germplasm conserved in genebanks are not numerous (Quiros 1993) and their relationship to commercially grown varieties is unknown. Thirty-one open-pollinated and three F_1 hybrid celeriac varieties (elite germplasm), as well as 28 open-pollinated formerly grown celeriac varieties, two varieties of celery, two varieties of leaf celery, and three gene bank accessions of wild *Apium* species (exotic germplasm) were analysed morphologically (UPOV 2002) and with AFLPs.

Regarding only two of eleven morphological traits (presence of anthocyanin in the petioles and colour of the tuber), clear structure in celeriac germplasm was observed in a dendrogram based on morphological distance estimates. By contrast, varieties with white-coloured tubers and absence of anthocyanin coloration of the petiole were present in more than one sub-cluster based on GS estimates. Moreover, the matrices based on AFLPs and morphological data were poorly correlated (r = 0.22, $P < 0.01$). A disparity between genetic distances based on morphological traits and on molecular markers was reported by many authors (Ben-Har et al. 1995; Burstin and Charcosset 1997; Smith et al. 1997; Noli et al. 1997; Senior et al. 1998; Roldán-Ruiz et al. 2001). Such observations should not be regarded as indicating weakness or limitation of either of these systems. Varieties that display high phenotypic similarity need not be genetically similar as different genepools can be manipulated to create similar phenotypes (Roldán-Ruiz et al. 2001). Furthermore, when using morphological traits, the developmental and genotype-by-environment noise that is super-imposed on the genetic basis need to be accounted for, plus the various measurement limitations and inaccuracies. Therefore, consistency should only be expected

if varieties had shared genetic resources and parallel breeding objectives, or, conversely, were very different in both genepool source and selection targets (Roldán-Ruiz et al. 2001).

The ranges of AFLP-based genetic similarity estimates in elite celeriac germplasm (GS = 0.68-0.95) were significantly narrower than those in the exotic set (GS = 0.05-0.95). This is most likely due to selection pressure and genetic drift present in breeding programmes, where targeting genetic homogenisation and purity of cultivars cause a reduction in the genetic basis of crops (Soleimani et al. 2002). Nevertheless, commercial elite celeriac varieties clustered in two distinct clusters, thus pointing out the possibility that an undetected heterotic pool exists in the germplasm currently exploited in celeriac breeding. On the other hand, a higher proportion of polymorphic AFLP bands revealed in exotic celeriac germplasm, as well as the high level of genetic diversity "hidden" in exotic celeriac varieties and their clear separation from the elite varieties with UPGMA analysis indicate that current breeding of celeriac utilizes only a small fraction of the available germplasm diversity. Celeriac varieties might be considerably improved with the inclusion of exotic germplasm into breeding. Although the three *A. graveolens* subspecies (celery, celeriac, and leaf celery) were not equally represented in the study, a substantial genetic diversity was revealed among them. Values of the average GS estimates between elite celeriac and celery (GS = 0.73) and between elite celeriac and leaf celery varieties (GS = 0.78) were lower than the average GS estimate within elite celeriac varieties (GS = 0.90). On the other hand, a high degree of homology among these cultivated types (Yang and Quiros 1993) was confirmed with a large proportion (55.1%) of common AFLP bands. Consequently, celery and leaf celery varieties could be suggested as valuable contributors to the broadening of the celeriac germplasm. The highest level of unused genetic diversity was detected in wild relatives of *A. graveolens* in the study, which contributed 62% polymorphic AFLP bands that were absent (monomorphic) in the elite celeriac germplasm. Their utilization in celeriac breeding, either for germplasm broadening or introgression of new genes (such as resistance genes) into cultivated material, requires further efforts and the application of recently developed methods of inter-specific crosses.

A growing danger of a possible genetic erosion of landraces and wild relatives of crops due to ever-improving cultivation practices, along with a continual demand for a new germplasm, encourages the creations of broad and well-studied germplasm collections (Brown und Kresovich 1996; McGregor et al. 2002; Scarascia-Mugnozza and Perrino

2002). Sub-clusters containing celeriac gene bank accessions started branching at the level higher than GS = 0.80, thus confirming the reports of Quiros (1993) that celeriac germplasm collections worldwide are not abundant and cover a limited part of variation compared to that usually expected in crop plants. As the 20 gene bank accessions analysed in this study were good representatives of the celeriac collection (36 accessions, some of which are duplicates) conserved in the gene bank of the Institute of Plant Genetics and Crop Plant Research (IPK, Gatersleben, Germany), it can be concluded that celeriac germplasm collection demands immediate broadening. Furthermore, the application of molecular markers is recommended for detecting valuable genetic resources that could present an improvement for current and future celeriac breeding.

Conclusion and Outlook

Knowledge of genetic variation and genetic relationships is an important consideration for the optimal design of further breeding programs in cornsalad, radish, and celeriac, because it assists in the process of decision-making in breeding and helps addressing key issues of germplasm management. Genetic similarity estimates, based on the AFLP protocol optimized in this study, allowed a first insight into the genetic diversity present in the germplasm of the three vegetable crops and offered suggestions for their broadening. Furthermore, genetic structure of radish germplasm was revealed, which may influence the choice of genotypes to cross, thus being of an outstanding importance for successful hybrid radish breeding. Finally, an efficient rationalization and utilization of available germplasm resources was proposed.

An increasing number of studies has demonstrated the capacity of molecular markers to be highly discriminating between varieties in a wide range of species. Large progress made in molecular genetics, illustrated by the current rate of growth in complete genome sequence information, as well as important technical advances such as DNA chip technology allowing multi-parallel marker assays, have been astonishing and have revolutionized the possibilities of characterizing genetic diversity. Developments of genomics could further influence diversity studies by allowing the genetic profiling of the accessions, as to determine which have the highest potential for use in breeding programs (van Tienderen et al. 2002). This could be expected by developing marker systems for

functional genes (Andersen and Lübberstedt 2003), and by using the existing sequence information to develop markers that tag variation within the gene or in a flanking region (van Tienderen et al. 2002). Broadening the knowledge of the candidate genes for the traits of interest, understanding the linkage between molecular markers and traits, and the development of new statistical tools to handle the data can be considered successful strategies for the large-scale profiling of germplasm.

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6 Summary

During the last couple of decades, production and economic importance of cornsalad (*Valerianella locusta* L.; fam. Valerianaceae), radish (*Raphanus sativus* L. var. *sativus* convar. *radicula*; fam. Brassicaceae), and celeriac (*Apium graveolens* L. var. *rapaceum*; fam. Apiaceae) have been considerably increasing in Europe. Nevertheless, genetic diversity currently utilized for breeding cornsalad, radish, and celeriac is narrow, whereas their germplasm collections in gene banks are relatively poor.

Assessment of genetic diversity among breeding materials and genetic resources is an important consideration for the optimal design of further breeding programs. The major objective of this study was to investigate genetic diversity in germplasm of cornsalad, radish, and celeriac, applying amplified fragment length polymorphisms (AFLPs) and inter simple sequence repeats (ISSRs) molecular markers. In particular, the objectives were to (i) analyze relationships among breeding materials of the three vegetable crops (referred to as elite germplasm), as well as among their formerly grown varieties, gene bank and botanical garden accessions (referred to as exotic germplasm), (ii) reveal genetic structure of radish germplasm to establish heterotic pools for hybrid breeding, and (iii) evaluate the usefulness of introducing exotic materials for broadening of the elite germplasm in cornsalad, radish, and celeriac.

Average genetic similarity in 34 elite varieties of cornsalad was very high (GS = 0.90), which is comparable with other autogamous crops. The majority of elite varieties clustered closely applying the UPGMA analysis because of a narrow-based germplasm in cornsalad breeding. A substantial level of genetic diversity (GS = 0.47) was detected in 30 cornsalad varieties representing exotic germplasm. Exotic varieties that interspersed the sub-clusters of the elite may serve as a direct genetic resource for broadening the elite cornsalad germplasm base, whereas *Valerianella locusta*-related species that were distinct from cultivated germplasm can contribute to the introgression of new (resistance) genes.

Sixty-eight varieties of cultivated radish (garden and Black radish) created sub-clusters with GS estimates higher than 0.70, thus supporting the assumption that the currently used radish germplasm in Europe relies on a narrow genetic base. Owing to a high degree of heterogeneity and heterozygosity within radish varieties, the detected between-variety diversity was low, but there still was a substantial overall diversity in

available radish germplasm. Applying both UPGMA and principal coordinate analyses, Black radish varieties were distinct from garden radish. A further unambiguous division within garden radish germplasm was revealed with the model-based clustering approach. These sub-groups can be employed for establishment of heterotic pools within European modern cultivars of garden radish. In addition, ISSRs can substantially reduce hybrid radish production costs by an early detection of two closely related weed species (*R. raphanistrum* and *R. sativus* L. var. *sativus* convar. *sinensis*).

AFLPs and the evaluation of morphological traits were used to investigate genetic diversity in 34 varieties of elite celeriac germplasm and 35 accessions of exotic germplasm. Only two morphological traits supported the clustering pattern obtained with UPGMA analysis of morphological distance estimates. AFLP-based GS estimates offered a clearer view of diversity present in elite (GS = 0.68-0.95) and exotic germplasm (GS = 0.05-0.95), and clustered the two sets in distinct UPGMA-based sub-clusters. This indicated that only a small fraction of available genetic diversity is exploited for current breeding of celeriac. Exotic celeriac germplasm as well as varieties of celery and leaf celery might substantially improve commercial celeriac breeding. Wild relatives of *Apium graveolens* are valuable resources for the introgression of resistance genes. Regarding the generally high level of GS in celeriac germplasm conserved in the German gene bank, a broadening of the germplasm collection was suggested.

This study demonstrated the capacity of molecular markers to be highly discriminating among varieties of cornsalad, radish, and celeriac. AFLP-based genetic similarity estimates in the three vegetable crops (i) allowed the first insight into the genetic diversity and structure present in the germplasm, (ii) offered suggestions for germplasm broadening, and (iii) proposed a way of rationalization and utilization of available germplasm resources.

7 Zusammenfassung

Während der letzten Jahrzehnte ist in Europa die Produktion und wirtschaftliche Bedeutung von Feldsalat (*Valerianella locusta* L.; Fam. Valerianaceae), Radies (*Raphanus sativus* L. var. *sativus* convar. *radicula*; Fam. Brassicaceae) und Knollensellerie (*Apium graveolens* L. var. *rapaceum*; Fam. Apiaceae) beachtlich gestiegen. Allerdings ist die genetische Diversität dieser drei Kulturarten stark eingeschränkt.

Für eine Optimierung von Züchtungsprogrammen sind Informationen über die vorhandene genetischen Diversität innerhalb des Zuchtmaterials und die Verwandtschaft des Zuchtmaterials zu genetischen Ressourcen entscheidend. Ziel dieser experimentellen Arbeit war, die genetische Diversität von Feldsalat, Radies und Knollensellerie mit zwei unterschiedlichen DNA-Markersystemen, „amplified fragment length polymorphism (AFLPs)“ und „inter simple sequence repeats (ISSRs)“, zu untersuchen. Die einzelnen Ziele waren (i) die Untersuchung der Verwandtschaftsbeziehungen von Zuchtmaterial der drei Kulturarten (nachstehend als Elitematerial bezeichnet) sowie von historischen Sorten und Genbankmuster (nachstehend als exotisches Material bezeichnet), (ii) die Analyse von Möglichkeiten zur Etablierung von heterotischen Gruppen in Radies, und (iii) die Erörterung des Nutzens von exotischem Material zur Erweiterung des Elitezuchtmaterials in alle drei Kulturarten.

Die beobachtete durchschnittliche genetische Ähnlichkeit zwischen 34 Feldsalatelitelinien war sehr hoch (GS = 0.90). Dies ist vergleichbar mit Ergebnissen aus Studien anderer selbstbefurchtender Kulturarten. Dagegen verdeutlicht die niedrige durchschnittliche genetische Ähnlichkeit zwischen 30 exotischen Feldsalatlinien (GS = 0.47) eine hohe genetische Diversität des exotischen Materials. Exotische Feldsalatlinien, welche nahe mit dem Elitematerial verwandt sind, können direkt zur Erweiterung der genetischen Basis des Elitezuchtmaterials eingesetzt werden. Die mit Feldsalat weniger eng verwandten Arten könnten dagegen als Donor neuer (Resistenz-) Gene dienen.

Achtundsechzig Radiessorten (Radieschen und Rettich) bildeten Untergruppen mit einer hohen genetischen Ähnlichkeit (GS = 0.70). Diese Tatsache unterstützt die These, dass die genetische Basis von europäischem Radies eng ist. Infolge der hohen Heterozygotie und Heterogenität innerhalb von Radies war die Diversität zwischen Sorten gering. Die gesamte genetische Diversität des untersuchten Radiesmaterials war allerdings

ausreichend. Sorten von Rettich waren sowohl in einer Hauptkoordinaten- als auch in einer Clusteranalyse deutlich von Sorten von Radieschen getrennt. Eine weitere klare Untergruppierung innerhalb der Radieschensorten wurde mittels einer Modell-basierenden Clusteranalyse beobachtet. Diese Untergruppen können für die Etablierung heterotischer Gruppen von europäischem Radies benutzt werden. Weiterhin sind die Ergebnisse der ISSR-Studie hilfreich, die Kosten bei der Hybridproduktion von Radies zu reduzieren, da sie eine frühzeitige Erkennung zweier Unkrautarten (*R. raphanistrum* und *R. sativus* L. var. *sativus* convar. *sinensis*) in Radies ermöglichen.

AFLPs und morphologische Daten wurden dazu benutzt, die genetische Diversität in 34 Elite- und 35 exotischen Knollenselleriesorten zu untersuchen. Nur zwei morphologische Merkmale bestätigten die Gruppierung, die in einer Clusteranalyse basierend auf den morphologischen Distanzen beobachtet wurden. Andererseits kann mit AFLP-basierenden genetischen Ähnlichkeiten klar die Diversität des Elite- (GS = 0.68-0.95) und des exotischem Materials (GS = 0.05-0.95) beschrieben werden und eine UPGMA-Clusteranalyse erlaubt eine deutliche Trennung in Untergruppen. Diese eindeutige Unterteilung des Materials und der hohe Anteil polymorpher AFLP-Banden in exotischen Sorten deutet darauf hin, dass das aktuelle Knollenselleriezuchtmaterial nur einen kleinen Bruchteil der verfügbaren genetischen Diversität nutzt. Die Introgression von exotischen Knollenselleriesorten sowie von Stiel- und Schnittsellerie in das Knollenselleriezuchtmaterial könnte kommerziell genutzte Sorten verbessern. Die Wildarten von *Apium* können durch interspezifische Kreuzungen als Donoren für Resistenzgene genutzt werden. Die hohe genetische Ähnlichkeit zwischen exotischen Akzessionen von Knollensellerie der Genbank IPK deutet auf die Notwendigkeit hin, diese Sammlung zu erweitern.

Diese Studie belegt die Einsatzmöglichkeiten molekularer Marker im Feldsalat, Radies und Knollensellerie zur Unterscheidung von Sorten. AFLP-basierende genetische Ähnlichkeiten ermöglichten (i) erste Einblicke über die vorhandene genetische Diversität und Struktur der drei Kulturarten, (ii) Vorschläge zur Erweiterung der genetischen Basis, und (iii) eröffneten die Möglichkeit, vorhandene genetische Ressourcen effektiv zu nutzen.

8 Appendix

8.1 AFLP protocol

Restriction digestion of genomic DNA

A per reaction:

		final conc.
template DNA	2.00 µl	(250 ng)
10x OPA+ buffer (*Pharmacia*)	4.00 µl	1x
EcoRI (*Pharmacia*)	4.25 U	
MseI (*New England BioLabs*)	3 U	
ddH_2O	(up to volume)	
total:	40.00 µl	

B mix gently, centrifuge briefly to collect reaction

C incubate at 37°C, for 2-3 hours

Ligation of adapters

A per reaction:

		final conc. (in 40+10 µl)
10x OPA+ buffer (*Pharmacia*)	2.00 µl	0.4x
EcoRI – adapter (5 pmol/µl)	1.00 µl	0.1 µM
MseI - adapter (5 pmol/µl)	1.00 µl	0.1 µM
ATP (10mM)	1.00 µl	0.2 mM
T4 DNA ligase	1 U	
ddH_2O	(up to volume)	
total:	10.00 µl	
	(+ 40.00 µl digestion)	

B add mixture to each 40.0 µl of digestion reaction

C mix gently, centrifuge briefly to collect reaction

D incubate at 20°C (room temperature), for 2 hours

E dilute 1:20 in ddH_2O:

ligation products	10 µl
ddH_2O	190 µl
	mix well

F store unused portion of ligation products at –20°C

Pre-amplification reaction

A per reaction:

		final conc.
ligation products (dilution 1:20)	5.00 µl	
ddH_2O	8.35 µl	
10x PCR buffer (*Pharmacia*)	2.50 µl	1x
dNTP´s (1mM)	5.00 µl	0.2 mM
EcoRI + 1 (5 pmol/µl)	2.00 µl	0.4 µM
MseI + 1 (5 pmol/µl)	2.00 µl	0.4 µM
Taq DNA polymerase	0.15 µl	
total:	25.00 µl	

B mix gently, centrifuge briefly to collect reaction

C run 20 cycles in the PCR
1. 30 sec at 94°C
2. 60 sec at 50°C
3. 60 sec at 72°C

D dilute 1:20 in ddH_2O:

pre-amplification products	10 µl
ddH_2O	190 µl
	mix well

E store unused portion of pre-amplification products at –20°C

Primer labelling

A per reaction:

ddH_2O	0.10 µl
EcoRI + 3 (5 pmol/µl)	0.18 µl
5x kinase buffer	0.10 µl
T4 polynucleotide kinase	0.02 µl
[^{33}P]-γ-ATP (200 Ci/mmol)	0.10 µl
total:	0.50 µl

B mix gently, centrifuge briefly to collect reaction

C incubate at 37°C, for 1 hour

D heat inactivate the enzyme at 70°C for 10 min.

E centrifuge briefly to collect reaction contents

F store at –20°C until use

Main-amplification reaction

A per reaction:

pre-amplification products (dilution 1:20)	5.00	µl
Mse-mix:		
ddH_2O	0.20	µl
dNTP's (1mM)	5.00	µl
MseI + 2 (5 pmol/µl)	1.80	µl
sub-total 1:	7.00	µl
Eco-mix:		
ddH_2O	5.42	µl
10x PCR buffer (*Pharmacia*)	2.00	µl
Taq DNA polymerase	0.08	µl
EcoRI + 3 (5 pmol/µl)	0.50	µl
sub-total 2:	8.00	µl
total:	20.00	µl

B pipette 7.00 µl of the Mse-mix and 5.00 µl of diluted pre-amplification products, mix gently, centrifuge briefly to collect reaction

C add 8.00 µl of Eco-mix into the tube, and carefully pipette up/down several times (8-10) to mix reagents

D run the PCR

1. 2 min at 94°C
2. 30 sec at 94°C
3. 30 sec at 65°C (-0.7°C per cycle)
4. 1 min at 72°C
5. repeat steps 2 to 4, 11 times
6. 30 sec at 94°C
7. 30 sec at 56°C
8. 1 min at 72°C
9. repeat steps 6 to 8, 25 times
10. 2 min at 72°C
11. hold at 4°C

E load 20 µl of AFLP loading dye into the tube, pipette up/down several times (8-10) to mix

F denature for 3 min at 90°C and place immediately on ice

G load 2.5 µl into the gel (keep samples on ice during loading)

8.2 ISSR protocol

A per reaction:

		final conc.
template DNA (100ng/µl)	1.00 µl	
10x PCR buffer (*Pharmacia*)	2.50 µl	1 x
ISSR primer (5pmol/µl)	5.00 µl	1 µM
$MgCl_2$ (100mM)	0.25 µl	1 mM
dNTP's (10mM)	1.25 µl	0.5 mM
ddH_2O	14.80 µl	
Taq DNA polymerase (*Pharmacia*)	0.20 µl (1U)	1 U
	total amount: 25.00 µl	

B mix gently, centrifuge briefly to collect reaction

C run the PCR:

1. 95°C for 1min
2. 94°C for 30sec
3. 50°C for 30sec [1]
4. 72°C for 2min
5. repeat steps 2 to 4 35 times
6. 72°C for 10min

keep the PCR product at –20°C

[1] optimized annealing temperatures:

cornsalad	45.2°C
radish	48.0°C
celeriac	45.2°C

8.3 Basic solutions for DNA extraction, AFLP and ISSR analyses

(in alphabetical order)

AFLP-loading buffer

100 % formamide	20 ml
0.5M EDTA	400 µl
0.1% brom-phenol blue	10 – 20 mg
0.1% p-xylol blue	10 – 20 mg

10% APS (10 ml)

ammonium-persulfate	1 g
ddH_2O	10 ml

DO NOT autoclave

chloroform / isoamylalcohol (24:1)
(*work under the fume hood!!*)

chloroform	960 ml	480 ml	384 ml
isoamylalcohol	40 ml	20 ml	16 ml

CTAB

				final conc.
1M Tris-HCl (pH 7.5)	20 ml	50 ml	60 ml	100 mM
5M NaCl	28 ml	70 ml	84 ml	700 mM
0.5M EDTA (pH 8.0)	20 ml	50 ml	60 ml	50 mM
bi-destilled H_2O	up to 200 ml	up to 500 ml	up to 600 ml	
CTAB*	2.00 g	4.5 g	6.0 g	1%
autoclave				

* before adding CTAB, preheat the solution to 60-65°C and stir vigorously! Do not put the whole amount of ddH_2O at once, but add it at the end, when CTAB dissolves.

Na-disulfite is added to the extraction buffer just before use, directly into the extraction tube.

Developing solution

sodium-carbonate (Na_2CO_3)	48 g		
millipore H_2O	1.6 l		
(37%, 1 vial) formaldehyde	2.4 ml	-	ADD BEFORE USE
sodium-thiosulfate (10 mg/ml)	320 µl	-	ADD BEFORE USE

dNTP´s	(1mM)	(10mM)
dATP (100mM)	10 µl	100 µl
d CTP (100mM)	10 µl	100 µl
d TTP (100mM)	10 µl	100 µl
d GTP (100mM)	10 µl	100 µl
ddH_2O	960 µl	600 µl
total:	1000 µl	

0.5M EDTA (pH 8.0)

	for 1 l	for 2 l
EDTA	186.10 g	372.20 g

EDTA dissolves in pH 8.0. Add EDTA into H_2O (less than final volume), add NaOH pellets (approx. 20 g for 1 l of solution) and stir vigorously. Add H_2O up to the final volume, and adjust pH to 8.0.

autoclave

Fix/stop solution

glacial acetic acid	160 ml
millipore H_2O	1.44 l

Glacial acetic acid dilution (for Bind Silane)

100% Et-OH	99.5 ml
glacial acetic acid	0.5 ml

* 1 ml of dilution
* 5 µl of Bind – Silane

vortex, apply to short glass-plate
leave 4 – 5 min and wipe 2 times with ethanol

$MgCl_2$	(1M)	(100mM)
$MgCl_2$ x $6H_2O$	20.33 g	2.03 g
millipore H_2O	up to volume	up to volume
	100 ml	100 ml

autoclave

5M NaCl

NaCl	292.2 g
add millipore H_2O	up to 1000 ml

autoclave

2.5M Na-O-Ac

Na-acetate (anhydrous)	20.50 ml	51.25 ml	102.50 ml
bi-destilled H_2O	up to 100 ml	up to 250 ml	up to 500 ml
	autoclave		

Na-thiosulfate (10mg/ml)

Na-thiosulfate	1 g
ddH_2O	100 ml

1M NH_4-O-Ac

ammonium acetate (MW=77.08)	7.71 ml	19.27 ml	38.55 ml
bi-destilled H_2O	up to 100 ml	up to 250 ml	up to 500 ml
sterilize by filtration			

RNAse A (10mg/ml)

RNAse	100 mg
bi-destilled H_2O	10 ml
dissolve	

place in water bath and put to boil
let it boiling for 10-15 min
allow to cool down slowly to RT
dispense in 500 µl aliquots
store at –20°C

Staining solution (not to be used if older than 1 week)

silver-nitrate ($AgNO_3$)	2 g
millipore H_2O	1.6 l

50x TAE

	for 1 l	for 2 l
Tris	242.00 g	484.00 g
glacial acetic acid	57.10 ml	114.20 ml
0.5M EDTA (pH 8.0)	100 ml	200 ml
millipore H_2O	up to volume	up to volume

10x TBE

	for 1 l	for 2 l
0.5M Tris	121.13 g	242.26 g
0.45M boric acid	55.65 g	111.30 g
0.5M EDTA (pH 8.0)	20 ml	40 ml
millipore H_2O	up to volume	up to volume
set pH to 8.3		

TE (pH 8.0)

1M Tris-HCl (pH 8.0)	2 ml	2.5 ml	5.0 ml
0.5M EDTA (pH 8.0)	400 µl	500 µl	1.0 ml
bi-destilled H_2O	up to 200 ml	up to 250 ml	up to 500 ml
	_____	_____	_____
set pH to 8.0			
autoclave			

1M Tris – HCl (pH 7.5 or 8.0)

Tris-Base	121 g	
millipore H_2O	approx. 750 ml	
	_____	
dissolve		
add millipore H_2O	up to 1000 ml	
add HCl until desired pH is reached	75 ml	= pH 7.5
	42 ml	= pH 8.0
autoclave		

WASH – I (76% Et-OH, 0.2M Na-O-Ac)

Et-OH (absolute)	76 ml	152 ml	228 ml	304 ml	380 ml
2.5M Na-O-Ac	8 ml	16 ml	24 ml	32 ml	40 ml
bi-destilled H_2O	16 ml	32 ml	48 ml	64 ml	80 ml
	_____	_____	_____	_____	_____
	100 ml	200 ml	300 ml	400 ml	500 ml

WASH – II (76% Et-OH, 10mM NH_4-O-Ac)

Et-OH (absolute)	76 ml	152 ml	228 ml	304 ml	380 ml
1M NH_4-O-Ac	1 ml	2 ml	3 ml	4 ml	5 ml
bi-destilled H_2O	23 ml	46 ml	69 ml	92 ml	115 ml
	_____	_____	_____	_____	_____
	100 ml	200 ml	300 ml	400 ml	500 ml

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Curriculum Vitae

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Erklärung

Hiermit versichere ich an Eides statt, dass die vorliegende Arbeit von mir selbst verfasst und nur unter Zuhilfenahme von angegebenen Quellen und Hilfsmitteln angefertigt wurde.

Diese Arbeit wurde noch keiner anderen Prüfungsbehörde vorgelegt.

Stuttgart-Hohenheim,
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Jasmina Muminović