Genetic Structure and Diversity among Radish Varieties as Inferred from AFLP and ISSR Analyses

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ABSTRACT. Twelve amplified fragment length polymorphism (AFLP) primer combinations and 10 inter-simple sequence repeat (ISSR) primers were applied to estimate genetic diversity among 68 varieties of cultivated radish (*Raphanus sativus* L.). The material consisted of open-pollinated varieties, inbred lines, diploid and a few tetraploid hybrid varieties of garden radish [*R. sativus* var. *sativus* DC. convar. *radicula* (DC.) Alef.] and black radish [*R. sativus* var. *niger* (Mill.) Pers.]. 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 fragments were scored for calculation of Jaccard's coefficient of genetic similarity (GS). Substantial level of genetic variability (average AFLP-based GS = 0.70; average ISSR-based GS = 0.61) was detected in the available germplasm of cultivated radish. Cluster analyses separated two weedy species from the cultivated germplasm. Within cultivated material, black radish and french breakfast radish types formed separate clusters. Based on AFLP data, a principal coordinate analysis (PCoA) and model-based approach revealed the genetic structure within cultivated radish germplasm and indicated 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 may be relevant for hybrid radish breeding.

Radish 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 (George and Evans, 1981; Wein, 1964). 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 garden radish and black 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 (*Raphanus sativus* var. *sativus* convar. *sinensis* Saz.) and *R. raphanistrum* L. Radish is open-pollinated, self-incompatible, diploid species with a chromosome number $2n = 18$, small genome size, and DNA amount in the unreplicated gametic nucleus (referred to as C-value) ranging from 0.55 to 1.45 pg (Dolezel et al., 1992; Olszewska and Osiecka, 1983).

Radish breeding was practiced for centuries, by means of mass or pedigree selection. In the past 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, which indicates 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* Tul. and *Albugo candida* (Pers.) Kuntze], and improved marketing conditions (Vogel, 1996). 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 the 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 varieties (Demeke et al., 1992; Ellstrand and Marshall, 1985; Huh and Ohnishi, 2003; Rabbani et al., 1998; Thormann et al., 1994). None of the currently available studies has focused on a wider set of European radish varieties. Molecular markers, such as AFLP (Vos et al., 1995) and ISSR (Zietkiewicz et al., 1994), were already successfully applied in genetic diversity analyses in various crops (Bohn et al., 1999; Simioniuc et al., 2002; Zhu et al., 1998), and confirmed the classification of germplasm into known heterotic groups (Lübberstedt et al., 2000; Pejic et al., 1998).

Our objectives were to establish the AFLP and ISSR protocols for radish, and apply them to 1) investigate the genetic diversity of a set of radish varieties that have currently been commercially produced in Europe, and 2) identify possible heterotic pools within

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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 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 the International Union for the Protection of New Varieties of Plants (UPOV).

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

Table 1. Varieties of garden radish and black radish^z, as well as the accessions of wild relatives of radish analyzed with amplified fragment length polymorphism and inter-simple sequence repeat molecular markers.

Variety name (or code)	Genetic constitution ^y	Production type ^x	Breeding company ^w	Variety name (or code)	Genetic constitution ^y	Production type ^x	Breeding company ^w
JW 6	Inbred line	OF	JE	Duro ^z	4n		Chr
JW 9	Inbred line	OF	JE	Fanal	4n		N
JW 14	Inbred line	OF	JE	Cherry Belle	OP		NZ
April Cross ^z	F ₁ hybrid		JE	Eiszapfen ^z	OP		JE
Cheriette	F ₁ hybrid		SS	Eterna	OP		JE
Content	F ₁ hybrid	UG	EZ	Falco	OP	OF	JE
Donar	F ₁ hybrid	UG	Syn	Flair	OP	UG/OF	RZ
Favorella	F ₁ hybrid	UG	NZ	Flamboyant	OP		JE
Florent	F ₁ hybrid	UG	EZ	Hilds Blauer Herbst ^z	OP		N
Fluo	F ₁ hybrid		Vil	Hilmar	OP	UG	N
Hyronda	F ₁ hybrid		JE	JW 30	OP		JE
Isar	F ₁ hybrid	OF	Syn	JW 31	OP		JE
JW 16	F ₁ hybrid	OF	JE	Karissima	OP	UG	N
JW 17	F ₁ hybrid	OF	JE	Marabelle	OP	OF/UG	NZ
JW 18	F ₁ hybrid	OF	JE	Neckarperle	OP	OF	N
JW 19	F ₁ hybrid	OF	JE	Neckarruhm rot ^z	OP		N
JW 20	F ₁ hybrid	OF	JE	Neckarruhm weiss ^z	OP		N
Masterred	F ₁ hybrid	OF	Sem	Nelson	OP		GG
Novella	F ₁ hybrid	UG	NZ	Parat	OP		JE
Picard	F ₁ hybrid	OF	RZ	Patricia	OP		N
Printo	F ₁ hybrid	OF	NZ	Raxe	OP		N
Radius	F ₁ hybrid	UG	EZ	Red Silk	OP		HM
Rondar	F ₁ hybrid	OF/UG	Syn	Ribella	OP	OF	NZ
R3	F ₁ hybrid	OF	JE	Riesenbutter	OP		JE
R6	F ₁ hybrid	OF	JE	Rondeel	OP		RZ
R16	F ₁ hybrid	OF	JE	Rota	OP		RZ
R49	F ₁ hybrid	UG	N	Rudi	OP	OF	JE
R50	F ₁ hybrid	UG	N	Runder Schwarzer Winter ^z	OP		N
R51	F ₁ hybrid	UG	N	Saxa – Rafine	OP	OF	RZ
Sunto	F ₁ hybrid	OF	NZ	Silva	OP	OF	JE
Tarzan	F ₁ hybrid	UG	EZ	Sirri	OP	OF	RZ
Trespa	F ₁ hybrid	UG	EZ	Sora	OP		N
Vitella	F ₁ hybrid	UG	NZ	Topsi	OP	UG/OF	N
Wernar	F ₁ hybrid	OF	Syn	Chinese small radish	(wild species)	weed	---
Boy	4n		N	<i>R. raphanistrum</i>	(wild species)	weed	---

^zBlack radish varieties.

^y4n and OP designate tetraploid variety and open-pollinated variety, respectively.

^xOF designates production in the open field; UG designates the production under glass.

^wJE = Juliwa-Enza, Heidelberg, Germany; SS = Sakata Seed Corp., Yokohama, Japan; EZ = Enza Zaden, Enkhuizen, The Netherlands; Syn = Syngenta, Wilmington, Del.; NZ = Nickerson-Zwaan, Made, The Netherlands; Vil = Vilmorin, La Ménétré, France; Sem = Seminis, Oxnard, Calif.; RZ = Rijk Zwaan, De Lier, The Netherlands; N = Nunhems Zaden BV, Haelen, The Netherlands; Chr = N.L. Chrestensen, Erfurt, Germany; GG = Gautier Graines SA, Eyragues, France; HM = Harris Moran, Modesto, Calif.

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 *EcoRI* and *MseI* primers carried three selective nucleotides. In total, 12 *EcoRI/MseI* primer combinations were employed. Amplified DNA fragments were denatured at 95 °C for 3 min and separated by electrophoresis in a 6% denaturing polyacrylamide 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 to 7 d before developing.

For ISSR amplification reactions a protocol of Ratnaparkhe et al. (1998) was modified: 100 ng genomic DNA, 1 μM ISSR primer (*Sigma-Ark*, Darmstadt, Germany), 0.5 mM dNTPs, 1 mM MgCl₂, 1 × PCR buffer, and 1 U Taq DNA polymerase (*Amersham Biosciences Europe GmbH*, Freiburg, Germany). 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. They differed in design (unanchored, 3'-end anchored primers, and 5'-end anchored primers) and the number of nucleotides in core sequence motif (di- and tri-nucleotide repeat primers). Amplified ISSR products were separated by electrophoresis on 2% agarose gels (*Biozym agarose DNA*, Oldendorf, Germany), stained in ethidium-bromide, and visualized under ultra-violet (UV) light.

AFLP fragments ranging from 50 to 350 base pair (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, 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 the 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 unweighted pair group method using arithmetic averages (UPGMA; Sneath and Sokal, 1973) clustering method were conducted with the NTSYSpc version 2.0 software (Rohlf, 1998). Reliability of dendrograms was tested by bootstrap analyses with 1000 replications to assess branch support, using the 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, F₁ hybrids and the two accessions of weedy species were excluded from the initial set of materials, and only AFLP data were used. A principal coordinate analysis (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 sub-populations (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 the Structure version 2.1 software (Pritchard et al., 2000) 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).

Table 2. Polymorphic information content (PIC) and marker index (MI) per amplified fragment length polymorphism primer combination in 70 accessions of cultivated radish, chinese small radish, and *R. raphanistrum*.

Primer combination	Total no. of bands	No. of selected polymorphic bands	PIC	MI
<i>EcoAAG / MseCTA</i>	51	42	0.21	9.01
<i>EcoAAG / MseCTC</i>	29	20	0.28	5.63
<i>EcoAAG / MseCTT</i>	35	28	0.24	6.72
<i>EcoACA / MseCTA</i>	31	31	0.23	7.00
<i>EcoACA / MseCTC</i>	43	35	0.22	7.84
<i>EcoACT / MseCAA</i>	30	19	0.18	3.46
<i>EcoACT / MseCAC</i>	23	17	0.29	4.90
<i>EcoAGA / MseCAC</i>	19	11	0.24	2.68
<i>EcoAGA / MseCAT</i>	29	19	0.25	4.72
<i>EcoAGC / MseCAA</i>	24	17	0.28	4.76
<i>EcoAGG / MseCTA</i>	18	16	0.27	4.39
<i>EcoAGG / MseCTG</i>	17	12	0.23	2.71
Total	349	267	---	---
Average	29.08	22.25	0.24	5.14

Table 3. Polymorphic information content (PIC) and marker index (MI) per inter-simple sequence repeat primer in 70 accessions of cultivated radish, 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

^zY = pyrimidine; B = C, G, or T; D = A, G, or T; H = A, C, or T; V = A, C, or G

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* × ‘April Cross’) to 1.00 (‘Wernar’ × ‘Isar’), with the mean of GS = 0.70. Standard errors for GS estimates varied from 0.00 to 0.07. GS estimates based on ISSRs ranged from 0.20 (‘JW 18’ × ‘April Cross’) to 1.00 (‘Wernar’ × ‘Isar’ and ‘JW 18’ × ‘JW 19’), with a mean of GS = 0.61. Standard errors for those GS estimates varied between 0.00 and 0.20. 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 did not create a clearly independent cluster, their separation from garden radish 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; Figs. 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’ and ‘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. In both molecular marker systems that we applied, the range of variation in GS estimates of blind checks was wider than of laboratory duplicates. In genetic diversity studies that included DNA marker controls, comparable GS estimates between replicated samples were found using restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), and AFLP marker data (Hahn et al., 1995; Lübberstedt et al., 2000; Messmer et al. 1993; Muminovic et al., 2004). Although the number of bands generated using ISSRs was lower than that detected with AFLPs, high GS estimates between samples duplicated on DNA level (laboratory duplicates) confirmed the reliability and reproducibility of both AFLP and ISSR protocols for radish. Slightly lower GS estimates between samples duplicated on plant level (blind checks), using either of the marker systems, may be due to errors during seed sowing or sampling leaf material for DNA extraction. Another explanation may be the presence of heterozygous individuals 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).

The 5'-end anchored primers yielded the highest average number of bands in our study with ISSRs, whereas the unanchored ISSR primers yielded the highest average number of polymorphic bands (Table 3). ISSR primers anchored at the 5'-end generate a higher number of fragments because they display broader specificity than the others (Bornet and Branchard, 2001). Di-nucleotide repeat ISSR primers produced the highest average number of bands and were generally more frequent in radish genome than tri-nucleotide repeats. However, they could not be used alone to efficiently differentiate between radish varieties. Similar results were found in accessions of wheat (*Triticum* L.) (Nagaoka and Ogihara, 1997), rice (*Oryza sativa* L.) (Akagi et al., 1997; Blair et al., 1998), and genus *Diplotaxis* DC. (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). ISSR 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 (*Pisum sativum* L.) (Lu et al., 1996), maize (*Zea mays* L.) (Gupta et al., 1994), and genus *Diplotaxis* (Martín and Sánchez-Yélamo, 2000). In general, the higher the density of repeats in a genome is, the more specific ISSR primers and more stringent PCR conditions should be used to limit the number of amplified products and to optimize their resolution on a gel (Fang et al., 1997).

An average PIC value of 0.24 across all scored AFLP bands

Fig. 1. Association among 68 varieties of cultivated radish and two related species was revealed by average linkage cluster analysis of Jaccard's genetic similarity coefficients calculated from amplified fragment length polymorphism 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.

(Table 2) agreed well with the results obtained in AFLP-based genetic diversity studies of allogamous species as maize (Lübberstedt et al., 2000) and ryegrass (*Lolium* L.) (Roldán-Ruiz et al., 2000). They also corroborated the PIC values calculated from AFLP data in autogamous crops, such as wheat (Bohn et al., 1999) and soybean [*Glycine max* (L.) Merr.] (Powell et al., 1996). 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 varieties 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 the genus *Amaranthus* L.

Fig. 2. Association among 68 varieties of cultivated radish and two related species was revealed by average linkage cluster analysis of Jaccard's genetic similarity coefficients calculated from inter-simple sequence repeat data of 10 primers. 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.

(Xu and Sun, 2001) and pumpkin (*Cucurbita pepo* L.) (Paris et al., 2003). AFLPs and ISSRs generated comparable ranges of variation in GS estimates within the studied radish germplasm, which were 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 related to one another than either was to *R. raphanistrum* (Figs. 1 and 2). The findings that GS estimates between *R. sativus* varieties and the accessions of *R. raphanistrum* and chinese small radish were low (Figs. 1 and 2) can be valuable for radish breeders who face serious problems in F₁ 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

bp 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 primer UBC 890 hybrid varieties did not produce a band at 800 bp as chinese small radish did, nor at 580 bp where a band was detected in *R. raphanistrum*. If further analysis and sampling of chinese small radish and *R. raphanistrum* accessions confirm our results, those simple ISSR assays could assist in estimating the level of seed purity before sowing hybrid radish varieties in the field, thus reducing 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

Fig. 3. Association among three inbred lines and 34 open-pollinated varieties of cultivated radish was revealed by principal coordinate analysis performed on genetic similarity estimates calculated from amplified fragment length polymorphism 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, using amplified fragment length polymorphism 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

^zInbred lines.

^yGiant radish variety.

^xBlack radish variety.

^wFrench breakfast radish variety.

relationships. Owing to a high degree of heterogeneity within any radish variety, the detected between-variety diversity is low, but there still is a substantial 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 F₁ 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, F₁ 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 allogamous species with a high degree of self-incompatibility (Banga, 1976; George and Evans, 1981), and populations are composed of heterozygous 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* Desf. landraces formed a

Fig. 4. Image shows (a) a sample of hybrid varieties of radish and (b) accessions of weedy species (A = chinese small radish; B = *Raphanus raphanistrum* L.) tested with inter-simple sequence repeat primer (CAA)₆. Only hybrid varieties produced a band of 1600 base pair (bp) in size.

cluster with *T. dicoccum* Schrank ex Schuebl. varieties and were clearly separated from *T. durum* varieties (Pujar et al., 1999). The authors supposed that a limited number of domestication events during the evolution of *T. dicoccum* varieties was the reason for the observed clustering. An analogous example was given in a study of red clover (*Trifolium pratense* L.), where Mattenkleee landraces were more closely related to field clover (*T. campestre* Schreb.) than to Mattenkleee varieties (Köllicker et al., 2003). The strong selection of Mattenkleee varieties targeted at a single trait (persistence) was postulated as the main justification of the unexpected clustering. Based on allozyme variation within and among varieties 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 red clover (Köllicker 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 as a clear and independent indication of germplasm grouping. The inferred sub-groups within garden radish germplasm may be employed as a basis 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 varieties of radish, which we detected with AFLPs and ISSRs. It could assist them in the choice of parents for crossing, defining priorities, and reducing the costs in radish variety improvement. However, for any further elucidation of garden radish complex and for a confirmation of division into heterotic groups within

its germplasm, additional studies involving a larger number of varieties, and preferably a co-dominant molecular marker system would be essential.

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