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Microsatellite fingerprinting in lettuce (*Lactuca sativa* L.) and wild relatives

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Abstract Southern hybridisation with a single microsatellite probe, $(TCT)_{10}$, sufficed to discriminate between a representative set of cultivars/accessions of lettuce, *Lactuca sativa* L., and its wild relatives *L. serriola*, *L. saligna* and *L. virosa*. Variability within cultivars was tested in a relatively modern cultivar (Hector), where no variation was found, and in an older and morphologically more variable cultivar (Madrilene), where heterogeneity was observed in the TCT fingerprint. $(TCT)_{10}$ fingerprinting should be useful for variety identification and homogeneity testing in lettuce.

Key words DNA fingerprinting · Lettuce · Variety identification · Microsatellites

Introduction

The cultivated lettuce, *Lactuca sativa* L., belongs to a genus of the Asteraceae family with about 100 species (Thompson et al. 1941). Together with *L. saligna*, *L. virosa* and its presumedly closest wild relative, *L. serriola*, it forms the subsection *Lactuca* of the section *Lactuca*. *L. sativa* and *L. serriola* are freely intercrossable; furthermore, *L. sativa* can be crossed with some difficulty with *L. saligna* and with even more difficulty with *L. virosa* (De Vries 1990).

Diversity and identity within the subsection *Lactuca* have been studied by several means: morphological characteristics by De Vries and Van Raamsdonk (1994) and Frietema de Vries et al. (1994), chromosome banding patterns by Koopman et al. (1993), isozymes by Kesseli and Michelmore (1986), RFLPs by Kesseli et al. (1991), and RAPDs by Waycott and Fort (1994). By selecting 22 polymorphic isozyme loci, it was possible to distinguish almost all members of a set of 18 accessions covering a broad range of crop types within cultivated lettuce, plus accessions from several other species in the genus *Lactuca* (Kesseli and Michelmore 1986). RFLPs could distinguish between most accessions of cultivated lettuce studied, except for sister lines from the same breeding population, using a combination of 55 probes and 3 restriction enzymes (Kesseli et al. 1991). RAPDs appeared to be able to distinguish between nearly identical germplasm accessions of cultivated lettuce, using a set of eight to ten primers scoring 40–55 bands, although in some cases morphological corroboration still proved to be necessary (Waycott and Fort 1994). Morphological markers (De Vries and Van Raamsdonk 1994; Frietema de Vries et al. 1994) and RFLPs (Kesseli et al. 1991) were also shown to be useful to study relationships between lettuce crop types as well as between *Lactuca* species. Chromosomal characteristics could not distinguish between *L. sativa* and *L. serriola*, or between accessions within these two species, but did show differences between this species pair and both *L. saligna* and *L. virosa* (Koopman et al. 1993).

In a comparative study by Kesseli et al. (1994), both RFLPs and RAPDs showed similar levels of polymorphism and error rate. However, RAPDs have been shown to be poorly reproducible between different laboratories (cf. Karp et al. 1997), and are therefore less useful for routine identification purposes. RFLPs do not suffer from this lack of reproducibility, but with closely related material a significant number of probe/enzyme combinations are needed. The probes also have to be isolated from genomic or cDNA banks, unless probes from other species can be used.

An alternative Southern-blotting-based technique may be more appropriate for identification purposes, namely microsatellite fingerprinting (Weising et al. 1995). Microsatellites are tandem repeats with a basic repeat unit of two to eight base pairs. They have been shown to be highly variable, mainly with respect to the number of repeat units.

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Table 1 *Lactuca* cultivars and accessions used in this study

Cultivar/accession	RKO or CGN number[a]	Crop type/species	Origin
Karif	RKO 92296	Crisp	
Van Sal	RKO 87454	Crisp	
Great Lakes 659	RKO 92160	Crisp	
Chou de Naples	RKO 86198	Crisp	
Webb's Wonderful	RKO 92180	Crisp	
Frisée de Beauregard	RKO 89448	Crisp	
Unikum	RKO 91107	Crisp	
Milly	RKO 91274	Butterhead	
Gotte Jaune d'Or	RKO 89353	Butterhead	
Balisto	RKO 93130	Butterhead	
Pierrot	RKO 92313	Butterhead	
Rougette du Midi	RKO 87447	Butterhead	
Madrilene	RKO 91323[b]	Latin	
Sudia	RKO 91239	Latin	
Sucrine	RKO 89431	Latin	
Gallega Original	RKO 89435	Latin	
Deer Tongue	RKO 88380	Latin	
Hohlblättriger Butter	RKO 87323	Cutting	
Prizehead	RKO 86195	Cutting	
Australische Gele	RKO 85432	Cutting	
Black Seeded Simpson	RKO 89235	Cutting	
Waldemann's Dark Green	RKO 90275	Cutting	
Oakleaf	RKO 91124	Cutting	
À Couper à Feuille de Chêne Blonde à Graine Noire	RKO 89428	Cutting	
Red Salad Bowl	RKO 92331	Cutting	
Lovina	RKO 93204	Cutting	
Monet	RKO 90207	Cutting	
Ruby	RKO 88102	Cutting	
Forellenschluß	RKO 91165	Cos	
Grise Maraîchère	RKO 89212	Cos	
Hector	RKO 91162[c]	Cos	
Little Leprechaun	RKO 88113	Cos	
Kasseler Strünkchen	RKO 87324	Cos	
Celtuce	RKO 89286	Stalk	
	CGN 13386	"Crisp" landrace (selection from a heterogeneous anthocyanin-rich sample)	The Netherlands
	CGN 05999	"Latin" landrace (possible hybrid with *L. serriola*)	Rumania
	CGN 05815	"Cutting" landrace	Via Botanical Garden Lisbon, Portugal
	CGN 04786	"Cos" landrace	Afghanistan
	CGN 11408	"Cos" (possible hybrid with *L. serriola*, white-seeded selection from mixed seed sample)	Afghanistan
Balady	CGN 05348	"Cos" landrace	Egypt
Gobekli Marul	CGN 04589	"Cos" landrace	Turkey
Kahu	CGN 04733	"Cos" landrace	Iran
Tianjin Big Stem	CGN 11387	Stalk	China
Cabagge Lettuce	CGN 10932	"Stalk" landrace	former USSR (through FIG Olomouc, Czechia)
	CGN 09356	"Oilseed"	Egypt
	CGN 05342	"Oilseed"	Egypt
Balady	CGN 05115	"Oilseed" (possible hybrid with *L. serriola*)	Egypt
	CGN 04897	"Oilseed" (possible hybrid with *L. serriola*)	
	CGN 04769	"Oilseed" (*L. serriola*)	Egypt
	CGN 11323	possible hybrid with *L. serriola* or *L. dregeana*	
	CGN 15684 (910401)	*L. serriola*	Hatay, Turkey
	CGN 15868 (910403)	*L. serriola*	Antalya, Turkey
	CGN 15695 (910412)	*L. serriola*	Eskesehir, Turkey
	CGN 17387 (900057)	*L. serriola*	Daghestan
	CGN 15683 (900052)	*L. serriola*	Daghestan
	CGN 15674 (900037)	*L. serriola*	Armenia
	CGN 15671 (900030)	*L. serriola*	Armenia
	CGN 10938	*L. serriola*	Bulgaria

Table 1 (Continued)

Cultivar/accession	RKO or CGN number[a]	Crop type/species	Origin
Balady	CGN 04667	*L. serriola*	Through Botanical Garden Rotterdam, The Netherlands
	CGN 05900	*L. serriola*[d]	Jerusalem, Israel
	CGN 10881	*L. serriola*[d]	Oudewater, The Netherlands
	CGN 15697 (910414)	*L. saligna*[e]	Balikesir, Turkey
	CGN 05310	*L. saligna*[d]	Raananna, Israel
	CGN 05327	*L. saligna*[d]	Gerona, Spain
	CGN 09316	*L. virosa*	United Kingdom
	CGN 14289	*L. virosa*	Asturia, Spain
	CGN 13339	*L. virosa*	Spain
	CGN 05793	*L. virosa*	Through Botanical Garden Szeged, Hungary
	CGN 05941	*L. virosa*	Israel
	CGN 15677 (900045)	*L. virosa*	Daghestan
	CGN 15680 (900049)	*L. virosa*	Daghestan
	CGN 04681	*L. virosa*[d]	Through Botanical Garden Amsterdam, The Netherlands
	CGN 04682	*L. virosa*[d]	Through Botanical Garden Warsaw, Poland
	CGN 13350	*L. virosa*[d]	Asturia, Spain

[a] Numbers in parentheses are the former CGN sampling numbers used in Frietema-de Vries et al (1994)
[b] For homogeneity tests we also used samples RKO 94319, 94334 and 95258
[c] For homogeneity tests we also used samples RKO 87178, 91162 and 95148
[d] CGN accessions used in this study only and not in Frietema-de Vries et al (1994)
[e] CGN accession misidentified as *L. serriola* in Frietema-de Vries et al (1994)

This variation can be probed by hybridising synthetic oligonucleotides complementary to microsatellite sequences to Southern blots. For example in tomato, Vosman et al. (1992) were able to distinguish 15 cultivars, including closely related ones, using a single $(GATA)_4$ probe. Tomato is a species in which the cultivars show very little genetic variation. The main condition that must be met before using this technique routinely is the selection of the optimal type of microsatellite for the species (group) under investigation.

In this study, microsatellite fingerprinting is shown to be useful for distinguishing cultivars in cultivated lettuce and accessions of the other wild species of the subsection *Lactuca*.

Materials and methods

Plant material

Plant material was obtained from the collections of the Centre of Genetic Resources, The Netherlands (CGN) and, for the commercially available lettuce cultivars, from the section for Registration and Plant Breeders Rights (RKO), both part of CPRO-DLO. From the 74 accessions studied, 67 were the same as those used by Frietema de Vries et al. (1994). The other 7 accessions of the species *L. serriola*, *L. saligna* and *L. virosa* were obtained from the CGN collection. All accessions are listed in Table 1.

DNA isolation

For comparison between accessions, DNA was extracted from pooled young plants. For comparison within cultivars, DNA was extracted from individual young plants as well as from pools of ten, to enable comparisons with the original results on all accessions. Leaves were collected and immediately frozen in liquid nitrogen and stored at –70 °C until use. DNA was isolated following a nuclear isolation protocol according to Vosman et al (1992).

Southern hybridisation

For Southern blotting, 2 µg of DNA was digested with 20 U of *Taq*I restriction endonuclease (LifeTechnologies), followed by Proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. A second round of *Taq*I restriction was performed to ensure complete digestion. DNA digests were separated on a 0.8% agarose gel and alkaline-blotted overnight onto Hybond N^+ membranes (Amersham). For hybridisation, oligonucleotides (10 pmol) were end-labelled using T4 polynucleotide kinase (LifeTechnologies) and 10 pmol [γ-^{32}P]ATP (5,000 Ci/mmol, Amersham). Hybridisation was performed at T_m –10 °C in a buffer consisting of 5×SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 0.5% blocking reagent (Boehringer Mannheim).

Results and discussion

Microsatellite fingerprinting

Oligonucleotides complementary to mini- and microsatellite sequences were assessed as a probe in Southern hybridisation to detect polymorphisms between cultivars of lettuce as well as genebank accessions of *L. serriola*, *L. virosa*, and *L. saligna*. A large part of the material has already been characterised morphologically by Frietema de Vries et al. (1994) who made their selection to represent the widest variation both in morphology and geographic origin of *L. sativa*, *L. serriola* and *L. virosa*. The following microsatellite motifs were tested: GA, GT, CAC, GGC, TAT, TCT, TGT, TCC, CTG, GACA, GATA, GGAT,

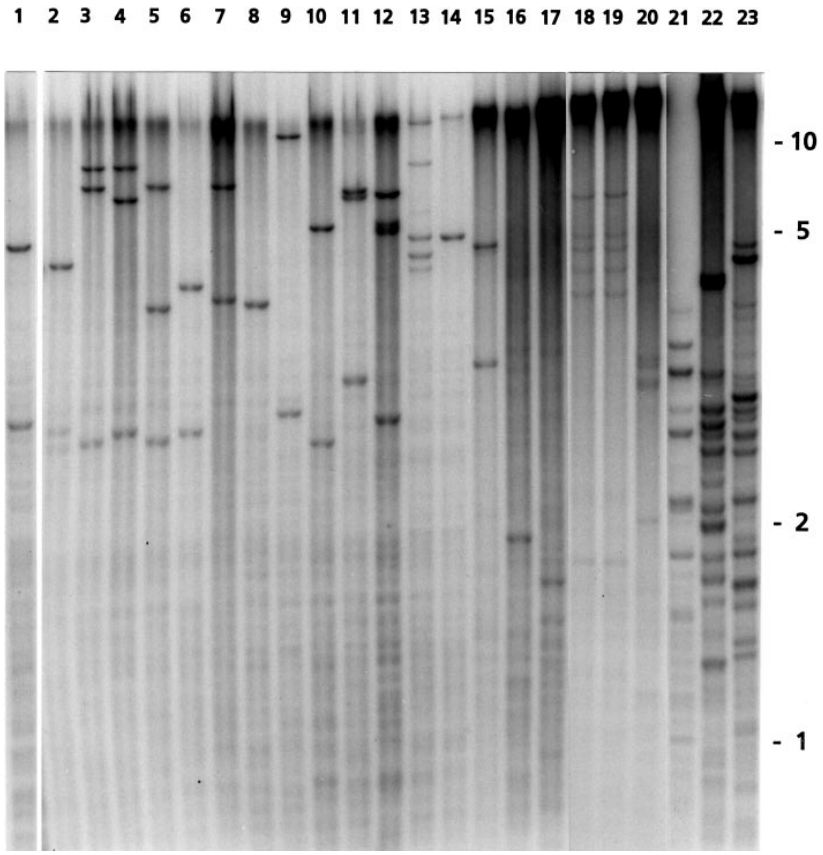

Fig. 1 Southern hybridisation of 23 cultivars and accessions of *Lactuca* using $(TCT)_{10}$ as a probe. DNA was digested with *Taq*I. Fragment sizes are indicated in kb in the right margin. Lanes contain *L. sativa* cultivars Hector (1), Madrilene (2), Karif (3), Van Sal (4), Frisée de Beauregard (5), Balisto (6), Pierrot (7), À Couper à Feuille de Chêne Blonde à Graine Noire (8), Monet (9), Tianjin Big Stem (10), Grise Maraîchère (11), CGN accessions 04786 (12), 05342 (13), 05115 (14), *L. serriola* CGN accessions 15686 (15), 05900 (16), 10881 (17), *L. virosa* CGN accessions 04681 (18), 04682 (19), 13350 (20), *L. saligna* CGN accessions 15697 (21), 05310 (22), 05327 (23)

TGTT and GGAAT. The minisatellite motifs tested were the human sequences 33.6 and 33.15 (Jeffreys et al. 1985), and the M13 repeat (Vassart et al. 1987). Most of the microsatellite motifs tested gave smears or non-interpretable banding patterns. The same was the case with the M13 minisatellite; the other minisatellites, 33.6 and 33.15, gave a very weak signal. The TCT microsatellite array proved to be the best scorable array for fingerprinting in lettuce.

Southern blots of a series of 74 *Taq*I-digested cultivars and accessions of *L. sativa*, *L. serriola*, *L. saligna* and *L. virosa* were hybridized to $(TCT)_{10}$. The accessions from lettuce included all the major crop types: crisp, butterhead, latin, cutting (looseleaf), cos (romaine), stalk (asparagus) and oilseed lettuce. In *L. sativa* and *L. serriola*, a striking pattern was obtained: mostly only two to three strong bands were visible in the high-molecular-weight range, yet this pattern was different between all the accessions tested. An example of bulk samples from a selection of accessions is shown in Fig. 1.

In *L. virosa* and *L. saligna* also, a distinct pattern, characteristic for each species, was visible, but with more bands than in *L. sativa* and *L. serriola* (Fig. 1). The species specificity of the TCT fingerprints was strikingly demonstrated by the finding among our sample of a *L. saligna* accession misidentified originally as *L. serriola* (accession CGN 15697, numbered 910414 in Frietema de Vries et al 1994, cf. Fig. 1). The reidentification was corroborated during the normal multiplication procedures of CGN and by studying the herbarium material of Frietema de Vries et al. (1994) and by sequencing the internal transcribed spacer 1 (ITS1) of the ribosomal DNA (Koopman et al., in press). In *L. saligna* and *L. virosa* also, all accessions tested could be properly distinguished, except *L. virosa* accessions CGN 4681 and 4682 which had an identical pattern (Fig. 1). These accessions were both derived from botanical gardens and of unknown origin (Table 1). They might have been exchanged between gardens.

The hypervariability combined with the low number of scorable bands makes it virtually impossible to use the TCT fingerprint for analyses of relatedness between cultivars/accessions. Since every accession appears to have the bands at a unique postion, there is no basis for comparison. Also, no obvious relationship could be established between the respective overall band patterns and known cultivar groups. In contrast, UPGMA clustering of RFLP data by Kesseli et al. (1991) largely conformed to the grouping into crop types. It has been suggested that microsatellites mutate by polymerase slippage during duplication (Schlötterer and Tautz 1992), making them less useful for phylogenetic purposes because of their hypervariability and the related problem of a high risk of homoplasy (cf Karp et al. 1997). Comparable microsatellite-containing sequences, for instance GATA/GACA/GATT in tomato (Arens et al. 1995), are known to be clustered in certain parts of the genome. They might also be associated with other types of repetitive sequences (e.g. Vosman and Arens 1997) that evolve at a different speed and in different ways than other parts of the genome (Lu et al. 1996).

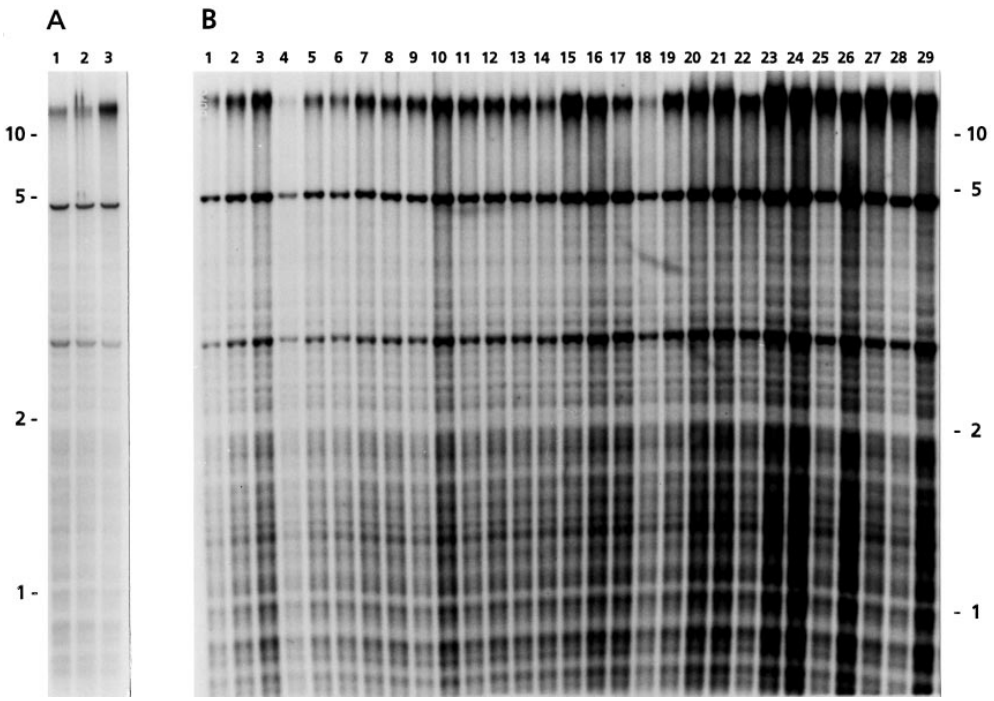

Fig. 2A, B Southern hybridisation of seed samples from three different years of lettuce cultivar Hector using $(TCT)_{10}$ as a probe. DNA was digested with *Taq*I. **A** Bulks of ten individuals each from seed samples RKO 87178 (1), 91162 (2), 95148 (3). Fragment sizes are indicated in kb in the left margin. **B** Individuals from seed samples RKO 87178 (1–10), 91162 (11–19), 95148 (20–29). Fragment sizes are indicated in kb in the right margin

At the species level, the close similarity in the behaviour of molecular markers, such as RFLPs (Kesseli et al. 1991) and the microsatellite fingerprinting in this study, of *L. sativa* and *L. serriola* as opposed to their closest relatives, *L. saligna* and *L. virosa*, once again raises the question whether these two can really be regarded as separate species. They are often regarded as a crop/weed complex (cf. De Vries 1990). Frietema de Vries et al. (1994), however, grouped them together into one species on the grounds that most distinguishing morphological characters show complete overlap between the two, except for characters immediately related to domestication. Although stating that the differences were large enough for warranting a separate species status for *L. sativa* and *L. serriola*, the principal-component analysis by De Vries and Van Raamsdonk (1994) also showed continuous variation between both species with differences mainly attributable to domestication. Thus, there do not appear to be compelling reasons to give them separate species status.

Stability of TCT fingerprints

The extreme variability in the lengths of only a few polymorphic high-molecular-weight bands in the TCT fingerprint prompted us to test whether the banding patterns were identical between individual plants of a cultivar and between different seed lots of a cultivar, i.e. whether the banding patterns were stable over different rounds of multiplication. For this purpose, a relatively modern, homogeneous cos cultivar, Hector, and an older relatively heterogeneous latin cultivar, Madrilene, were chosen from among the above test set of 74. For Hector, seed lots from 3 different years from the same source were used; for Madrilene, seed lots from three different sources. From each seed lot, a bulk sample and ten individuals were tested. The cultivar Hector is homogeneous for the TCT fingerprint (Fig. 2A, B). This is in line with the aforementioned homogeneity of this cultivar and indicates that the TCT fingerprint, despite its high variability, can be stably inherited within a group of plants. The cultivar Madrilene, however, shows a clear variability between the individuals of all seed lots tested (Fig. 3B). The bulk samples in Fig. 3A indicate that there is also some variation between seed lots, but the number of individuals used (ten) is too small to be certain about this. In addition, it becomes clear that the pattern of one relatively large and two relatively small bands in the bulk samples of Madrilene were misleading: in practically each individual only one relatively small band was visible that varied in molecular weight. Apparently, this variation tended to be limited to mainly two positions in the gel, i.e. the positions corresponding to the two bands seen in the samples containing mixtures of individuals (compare pattern of bulk samples in Fig. 1 and Fig. 3A to that of individuals in Fig. 3B).

The high level of discrimination that can be reached by TCT fingerprinting will allow it to be used for establishing a certain level of cultivar homogeneity. In addition, the $(TCT)_{10}$ fingerprint might also be useful for checking whether, after repeated cycles of propagation, cultivars still conform to the original plant material. However, it has to be borne in mind that apparently only a few hypervariable loci are tested that need not be independent.

Currently, we are isolating microsatellite sequences from lettuce to design primers for use in the sequence-tagged microsatellite site approach. This should not only be helpful in reproducibly identifying cultivars and accessions in *Lactuca* but also in further defining relationships between them.

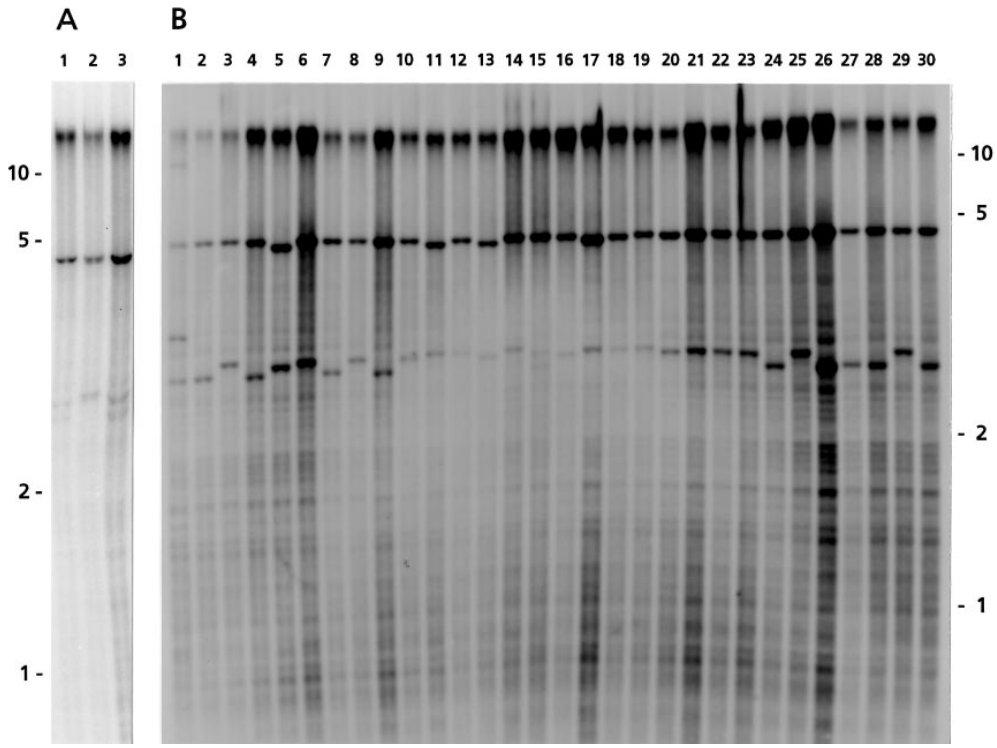

Fig. 3A, B Southern hybridisation of seed samples from three different origins of lettuce cultivar Madrilene using $(TCT)_{10}$ as a probe. DNA was digested with *Taq*I. **A** Bulks of ten individuals each from seed samples RKO 94319 (1), 94334 (2), 95258 (3). Fragment sizes are indicated in kb in the left margin. **B** Individuals from seed samples RKO 94319 (1–10), 94334 (11–20), 95258 (21–30). Fragment sizes are indicated in kb in the right margin

Conclusions

$(TCT)_{10}$ fingerprinting appears to be a powerful tool for identification of accessions and cultivars within at least the lettuce species of the subsection *Lactuca*. The fingerprints are well suited for analysing homogeneity of seed lots and verifying stability over different rounds of multiplication. The band patterns show too much variation to establish relationships between the accessions/cultivars.

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