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Abundance and polymorphism of di-, tri- and tetra-nucleotide tandem repeats in chickpea (*Cicer arietinum* L.)

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Abstract The abundance and polymorphism of 38 different simple-sequence repeat motifs was studied in four accessions of cultivated chickpea (*Cicer arietinum* L.) by in-gel hybridization of synthetic oligonucleotides to genomic DNA digested with 14 different restriction enzymes. Among 38 probes tested, 35 yielded detectable hybridization signals. The abundance and level of polymorphism of the target sequences varied considerably. The probes fell into three broad categories: (1) probes yielding distinct, polymorphic banding patterns; (2) probes yielding distinct, monomorphic banding patterns, and (3) probes yielding blurred patterns, or diffused bands superimposed on a high in lane background. No obvious correlation existed between abundance, fingerprint quality, and the sequence characteristics of a particular motif. Digestion with methyl-sensitive enzymes revealed that simple-sequence motifs are enriched in highly methylated genomic regions. The high level of intraspecific polymorphism detected by oligonucleotide fingerprinting suggests the suitability of simple-sequence repeat probes as molecular markers for genome mapping.

Keywords Chickpea · Oligonucleotide fingerprinting · Simple-sequence repeats · Genetic diversity · DNA polymorphism

Introduction

Chickpea (*Cicer arietinum* L.) is an important self-pollinated grain legume crop in the Indian subcontinent, as well as in West Asia and North Africa. Unlike cereals, the productivity of this crop has not yet been markedly improved through conventional breeding. The main bottleneck for increasing yield is the susceptibility of the plant to many diseases and pests, most importantly to a fungal disease caused by the ascomycete *Ascochyta rabiei* (reviewed by Saxena and Singh 1987; Singh and Saxena 1992). Programs for breeding resistance genes into high-yielding cultivars are underway (Singh et al. 1992), but these are time consuming, and it is hard to achieve pyramidal resistance by conventional means.

In recent years, the use of molecular markers has speeded up plant breeeding for a variety of objectives, including resistance breeding (reviewed by Melchinger 1990). The establishment of molecular marker technology and the generation of a genetic map would also be desirable for marker-assisted selection and the positional cloning of resistance genes in chickpea. Cultivated chickpea shows only low levels of genetic polymorphism (see, e.g., Kazan and Muehlbauer 1991). Therefore, existing chickpea maps, comprising isozyme and morphological markers, are mainly based on interspecific crosses between *C. arietinum* and its wild relatives *C. reticulatum* or *C. echinospermum* (Gaur and Slinkard 1990 a, b; Kazan et al. 1993).

The availability of a sufficient number of polymorphic markers is a prerequisite for successful linkage studies. Commonly used markers, such as isozymes (Kazan and Muehlbauer 1991), restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs; our own unpublished results) have not to-date provided sufficient levels of polymorphism in chickpea. However, we recently demonstrated that considerable variation at the intraspecific level is detectable by oligonucleotide fingerprinting (Weising et al. 1989, 1991, 1992). In these experiments, restriction-digested, electrophoretically separated DNA samples from different chickpea accessions were in-gel hybridized to synthetic oligonucleotide probes complementary to simple tandem repeats. These probes recognized multiple, polymorphic DNA loci simulta-

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neously, thereby creating DNA fingerprints of probe-dependent informativeness (Weising et al. 1992).

Multilocus DNA fingerprints have already served to produce linkage maps in humans (Wells et al. 1989), animals (Georges et al. 1990; Julier et al. 1990) and fungi (Romao and Hamer 1992). In the present study, we investigated the potential of oligonucleotide fingerprinting for the generation of an intraspecific map in a plant species. To this end, we used oligonucleotide probes complementary to 38 different di-, tri- and tetra-nucleotide repeat motifs in combination with a set of restriction endonucleases to screen for polymorphisms in four accessions of chickpea. To the best of our knowledge, this is the first comprehensive investigation in any organism on the presence, abundance and polymorphism of DNA fingerprint target sequences, as visualized by di-, tri- and tetra-nucleotide repeat probes.

Materials and methods

Plant material

Seeds of chickpea accessions (*C. arietinum* ILC 482, ILC 1272, ILC 1929 and ILC 3279) were procured from ICARDA, Aleppo, Syria. The accessions were selected on the basis of high (ILC 3279), medium (ILC 482), or low (ILC 1272 and ILC 1929) resistance to *Ascochyta* blight. Plants were grown under greenhouse conditions at Frankfurt University.

Selection and labelling of oligonucleotide probes

Synthetic oligonucleotide probes complementary to di-, tri- and tetra-nucleotide repeats were used in the present experiments. Only about one-tenth of the 336 theoretically possible sequence combinations are practically useful, since (1) many probes are redundant and (2) some motifs are self-annealing. For the present study, 38 di-, tri- and tetra-nucleotide repeat probes were selected which are neither self-complementary nor able to detect overlapping target sequences (see Table 1). Oligonucleotides were end-labelled with polynucleotide kinase using gamma-^{32}P-ATP as phosphate donor, and purified on Whatman DE 52 columns (Ali et al. 1986).

DNA isolation

DNA was isolated from fresh leaves using a modified procedure for the isolation of intact nuclei from tissue-cultured cells (Willmitzer and Wagner 1981). Leaf material was either bulked from 20 to 50 individual plants of one accession, or isolated from individual plants.

Oligonucleotide fingerprinting

DNA was digested with 1 of 14 restriction enzymes (seven 4-base cutters: *Alu*I, *Dde*I, *Hin*fI, *Msp*I, *Rsa*I, *Sau*3A, *Taq*I; seven 6-base cutters: *Apa*I, B*am*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I) according to the supplier's instructions. Digested samples were electrophoresed in 0.9%–1.2% agarose gels in TBE buffer (0.1 M Tris, 0.1 M boric acid, 20 mM EDTA, pH8). After staining with ethidium bromide and photographing, gels were denatured, neutralized, dried on a vacuum gel dryer, and in-gel hybridized to the labelled oligonucleotide probes as described (Ali et al. 1986). T_m values were calculated according to Thein and Wallace (1986), and hybridization and stringent washes carried out at $T_m - 5\,°C$. In some experiments, T_m values were alternatively calculated by the OLIGO™ computer program (version 3.4 Rychlik and Rhoads 1989). After washing, gels were blotted dry, and exposed to X-ray films at −80 °C using intensifying screens. Before hybridization to a new probe, gels were regenerated by washing in 5 mM EDTA at 60 °C (2 × 30 min).

Results

In a first set of experiments, bulked DNA samples of 20–50 individuals of each accession were digested with 14 selected restriction endonucleases (see Materials and methods), and electrophoresed on agarose gels. In order to identify suitable enzyme/probe combinations and to analyze the distribution of different simple tandem repeats in methylated versus nonmethylated regions of the genome, the gels were dried, and hybridized to a variety of di-, tri- and tetra-nucleotide repeat probes. Figure 1 shows an example using a $(CAA)_5$ probe. In general, methyl-sensitive enzymes, such as *Apa*I, *Msp*I and *Bam*HI, consistently produced only few bands in the high-molecular-weight range with all probes, while digestion with methylation-insensitive enzymes (e.g., *Dra*I, *Hin*fI and *Taq*I) yielded many bands in the range between less than 1 and more than 8 kb, suggesting that simple sequences are predominantly located in extensively methylated regions of the chickpea genome. Interestingly, the fingerprint patterns derived from digestion with *Dra*I (5′-TTTAAA-3′) and *Eco*RI (5′-GAATTC-3′), both recognizing an At-rich 6-base target sequence, were distributed in the molecular weight range of a typical 4-base cutter.

Fingerprints derived from bulked DNA samples revealed two types of signals: (1) strong and distinct bands, and (2) somewhat blurred regions (Fig. 1). While the former type of bands is probably accession-specific, i.e, present in most if not all bulked individuals, the blurred regions are likely to be composed of several fragments showing intra-accessional length polymorphisms. On the basis of the strong and distinct bands revealed by $(CAA)_5$, all four chickpea accessions could be easily distinguished from each other with any enzyme except *Apa*I.

In a second set of experiments, DNA from three plants of each accession was analyzed with four enzymes and 8 probes. *Hin*fI, *Taq*I, *Dra*I and *Eco*RI were chosen for this study, but only *Taq*I was used with all the probes. Fragment numbers obtained with the different probes and enzymes are outlined in Table 1. Characteristic types of hybridization patterns are shown in Fig. 2. Signals were obtained with all probes except for $(CCG)_5$, $(GGAC)_4$ and $(GGCA)_4$. On the basis of hybridization patterns we classified the probes into the following categories (see Table 1 and Fig. 2):

Category-I probes [$(GATA)_4$, $(GCGT)_4$, $(GAAT)_4$ and $(GTGA)_4$ in Fig. 2]: distinct, polymorphic banding patterns. Different probes of this category show significant variation in the number of bands and the level of polymorphism.

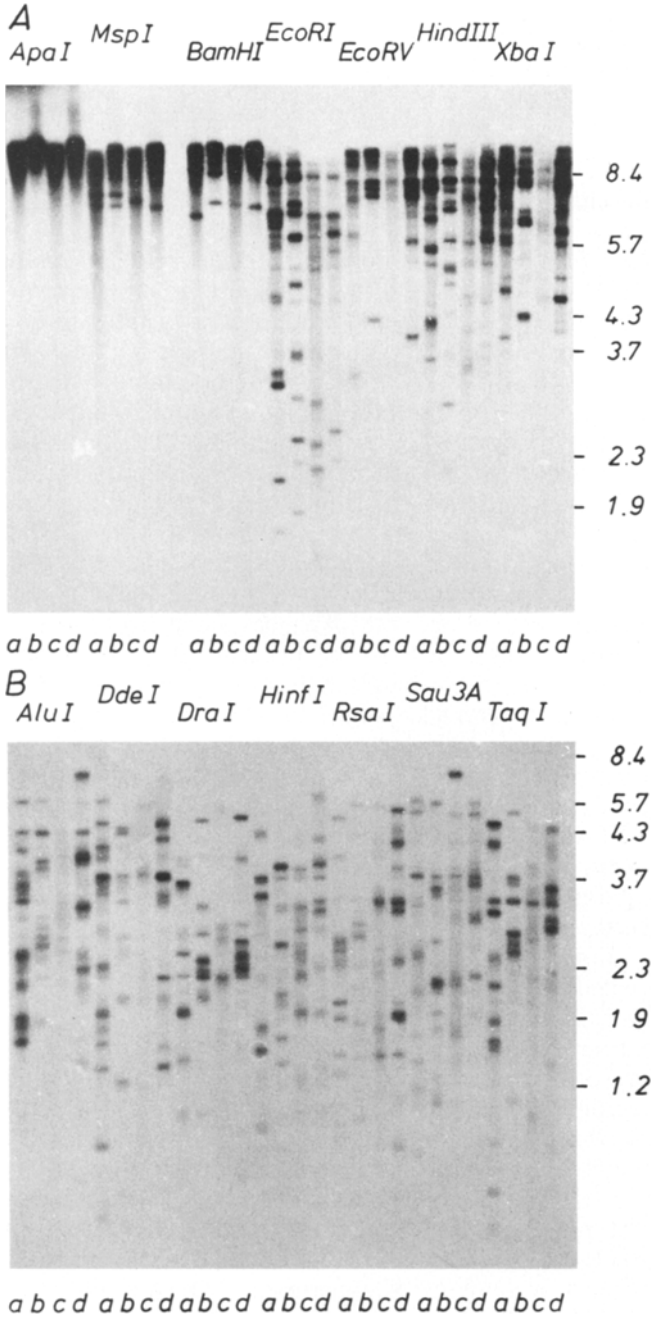

Fig. 1A, B $(CAA)_5$ fingerprints from bulked individuals of each of four chickpea accessions (*a*, ILC 482; *b*, ILC 1272; *c*,ILC 1929; *d*, ILC 3279). Total DNA was purified from leaves and digested with one of the indicated restriction enzymes. After electrophoretic separation of the resulting fragments in 0.9% (**A**) or 1.2% (**B**; 5 µg DNA per lane) agarose gels, the DNA was denatured, neutralized, the gel dried, and hybridized to radiolabelled $(CAA)_5$. Molecular weight markers are given in kb

Category-II probes [$(GATT)_4$ in Fig. 2]:distinct, monomorphic banding patterns.
Category-III probes [$(GTAA)_4$ in Fig. 2]: diffused bands superimposed on a high in-lane background, or very weak signals only.

The numbers of resolvable bands outlined in Table 1 are somewhat approximate, because of difficulties in counting faint bands. Nevertheless, the fragment numbers depended mainly on the probe, less so on the enzyme.

The "category-I" probes could be further subdivided according to the level of variability they detected (see Fig. 2). Thus, some probes [e.g, $(GATA)_4$ and $(CAA)_5$] revealed a considerable degree of between- as well as intra-accessional polymorphism. Others, such as $(GCGT)_4$, differentiated between, but not within, accessions. The number of polymorphisms detected by category-I probes was examined in more detail using the accessions ILC 482, ILC 1272 and ILC 3279, and the enzymes *Dra*I and *Taq*I. The results obtained for the different probes, the enzyme *Taq*I, and the accessions ILC 1272 and ILC 3279 are shown in Table 1. All data are summarized in Table 2. Only unequivocal polymorphisms were considered. For the determination of the numbers of between-accession polymorphisms, only bands that behaved monomorphic within accessions were counted. Table 2 illustrates that variation is generally higher between than within accessions.

Finally, we tested the influence of hybridization temperatures on the banding pattern specific for a particular probe. According to Thein and Wallace (1986), maximum stringency is obtained for oligonucleotide probes 15–20 bases in length, if melting temperatures (T_m) are calculated with 2 °C for each AT-pair, and 4 °C for each GC-pair (condition: 1 M NaCl), and hybridization is performed at $T_m - 5$ °C. For some of our oligonucleotide probes, however, considerably higher T_m values were computed by OLIGO^{TM} program (Rychlik and Rhoads 1989). To solve this apparent contradiction, we consecutively hybridized identical gels at both temperatures with results that were identical for all investigated probes (data not shown). Therefore, the temperature "window" that yields identical fingerprints is quite large, and hybridization temperatures calculated according to Thein and Wallace (1986) are stringent enough to ensure reprodu-cible fingerprint patterns.

Discussion

This paper reports on the presence and polymorphism of 38 different di-, tri- and tetra-nucleotide repeat motifs in the chickpea genome. Our results allow several conclusions:

(1) Informative fingerprints can be obtained with any enzyme, provided it is not sensitive to cytosine methylation. Taking the even distribution of distinct bands over a wide molecular weight range as criterion, best results were obtained with 4-base cutters (e.g., *Taq*I) and with 6-base cutters having AT-rich recognition sequence (e.g., *Dra*I).
(2) Digestion with methyl-sensitive enzymes suggests that simple tandem repeats are predominantly located

Table 1 Occurrence and polymorphism of di-, tri- and tetra-nucleotide repeats in the chickpea genome, as detected by oligonucleotide fingerprinting. See text for classification of probes into categories. No signals were obtained with $(CCG)_5$, $(GGAC)_4$ and $(GGCA)_4$. The minimum and maximum numbers of bands are indicated for each enzyme. The numbers of within- and between-accession polymorphisms are given for the combination of *Taq*I and the accessions ILC 1272 and 3279. ND, not determined

A: category-I probes

Probe	Number of bands				Number of polymorphisms		
	*Hin*fI	*Taq*I	*Dra*I	*Eco*RI	Between ILC 1272 and ILC 3279	Within	
						ILC 1272	ILC 3279
$(CA)_8$	ND	18–20	ND	ND	4	ND	ND
$(CAA)_5$	15–19	11–16	16–20	14–17	11	2	3
$(CAT)_5$	15–18	15–18	13–15	14–18	5	1	8
$(GAA)_5$	ND	13–14	ND	ND	9	ND	ND
$(CGA)_5$	ND	12–14	ND	ND	2	ND	ND
$(CTA)_5$	8–10	8–10	12–14	8–10	3	0	0
$(TCC)_5$	17–17	14–14	14–18	11–13	0	0	0
$(CTGT)_4$	10–13	8–10	8–11	6–9	5	0	2
$(GCGT)_4$	10–14	10–14	12–14	10–12	1	0	0
$(GAAT)_4$	13–15	13–15	13–15	12–14	12	2	4
$(GATA)_4$	12–16	12–16	12–16	8–10	6	5	9
$(GTTA)_4$	ND	12–15	ND	ND	7	ND	ND
$(CATA)_4$	ND	13–13	ND	ND	4	ND	ND
$(GAAA)_4$	9–11	10–11	9–10	9–11	0	ND	ND
$(GCGA)_4$	5–8	6–8	5–8	6–8	0	0	1
$(GGAT)_4$	8–10	11–12	10–12	8–10	0	0	0
$(GGGA)_4$	3–3	1–2	2–3	2–3	2	0	0
$(GGTA)_4$	2–4	4–5	3–5	3–4	2	1	1
$(GTTA)_4$	5–8	8–10	6–8	5–7	2	0	0
$(GTTC)_4$	4–4	4–5	4–6	4–6	3	1	1

B: category-II probes

Probe	Number of bands			
	*Hin*fI	*Taq*I	*Dra*I	*Eco*RI
$(GTC)_5$	23–23	24–24	18–18	20–20
$(CAG)_5$	16–18	18–20	18–20	16–18
$(GTTT)_4$	13–13	20–20	13–13	16–16
$(GATT)_4$	4–4	4–4	4–4	4–4
$(GGAA)_4$	2–2	4–4	ND	ND

C: category-III probes

$(GA)_8$, $(TAA)_5$, $(TAAA)_4$, $(GCAA)_4$, $(GGTC)_4$, $(GGCT)_4$, $(GTAA)_4$, $(GGGT)_4$, $(GGTT)_4$, $(GGGC)_4$

in highly-methylated regions of the chickpea genome, which are presumed to be transcriptionally silent.

(3) With few exceptions the number of bands detected by a particular probe is approximately identical, using any one of the enzymes *Eco*RI, *Taq*I, *Hin*fI and *Dra*I (Table 1). This suggests that most of the bands detected by hybridization are derived from separate loci in the genome, rather than from internal cuts within a cluster of repeats. However, clustering of simple repeats in large chromosomal regions [as observed for $(GATA)_n$ repeats on sex chromosomes of different organisms (Epplen 1988; Nanda etal. 1990)], or intermingling of different repeat motifs, cannot be precluded (Broun and Tanksley 1993; Lagercrantz et al. 1993). Since a high degree of clustering would considerably reduce the number of true polymorphc loci detected by probes this problem will have to be addressed by other techniques, e.g., linkage analysis or sequencing of distinct fingerprint bands.

(4) Our results further confirm that simple sequences are ubiquitous components of plant genomes, thus providing a broad collection of putatively informative probes. The three probes which gave no signal [$(CCG)_5$, $(GGAC)_4$ and $(GGCA)_4$] all have at least 75% GC. The others revealed different levels of polymorphism, which allowed us to classify them into different categories (Table 1). However, there was no obvious relationship between the properties of a particular probe (i.e., sequence characteristics, length of the repeat unit, base composition) and its informativeness. Therefore the level of polymorphism detected by a particular probe is unpredictable, and informative probes have to be determined empirically.

Informativeness is obviously not a property of a probe *per se*. Instead, fingerprints of individual chickpea plants often consisted of stable as well as variable bands.

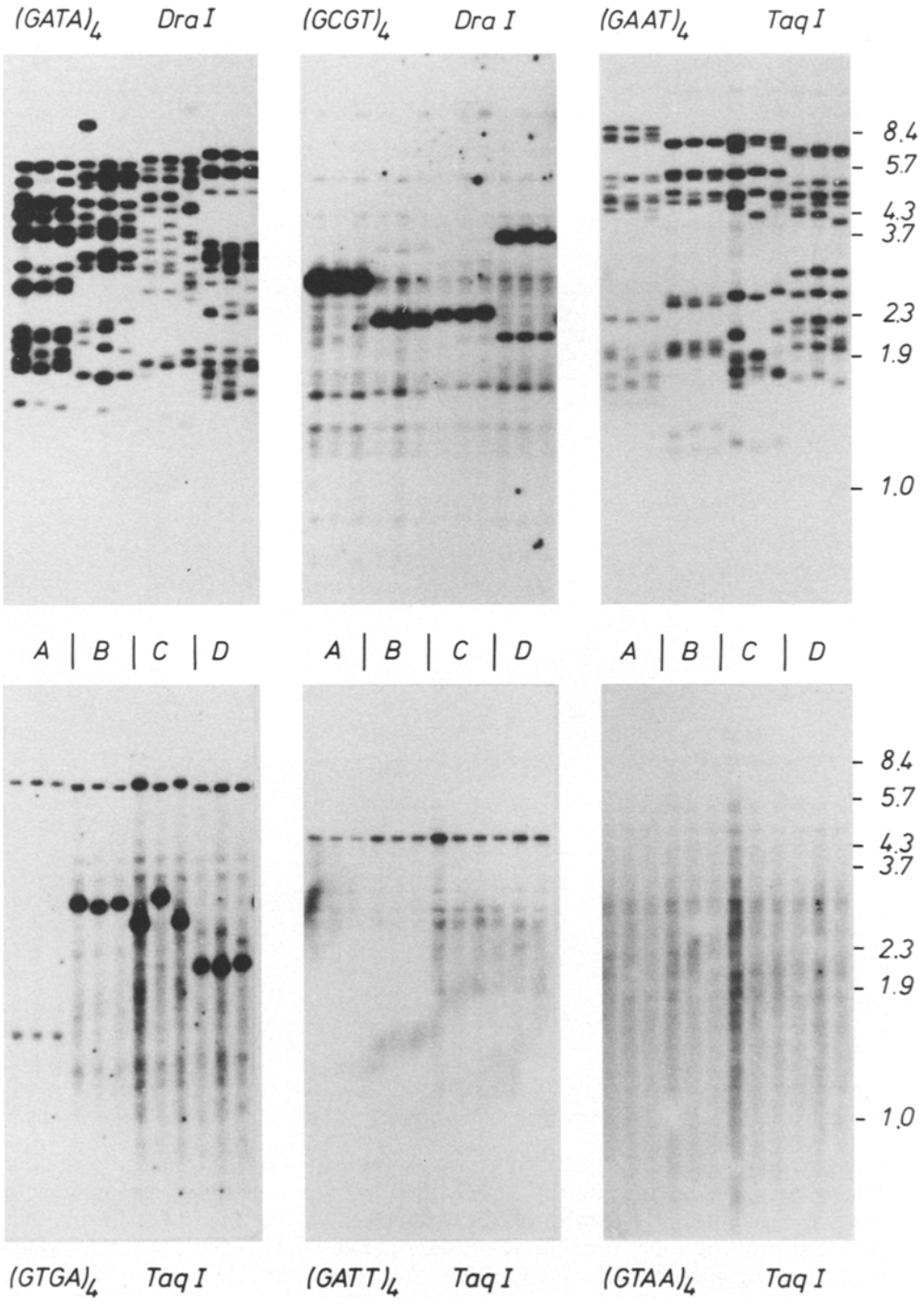

Fig. 2 Oligonucleotide fingerprints of three randomly selected individual plants of each of four chickpea accessions (*A*, ILC 482; *B*, ILC 1272; *C*, ILC 1929; *D*, ILC 3279). Total DNA was purified from leaves and digested with *Taq*I or *Dra*I. After electrophoretic separation of the resulting fragments in 1.2% agarose gels (5 μg DNA per lane), the DNA was denatured, neutralized, the gels dried, and consecutively hybridized to the indicated oligonucleotide probes. Molecular-weight markers are given in kb

In some cases, strong, polymorphic bands were found superimposed on a background of weaker, monomorphic bands [e.g., $(GCGT)_4$; Fig. 2], suggesting that longer repeats (resulting in stronger bands) are more variable than shorter ones. However, we also found exceptions to this rule. Therefore, additional factors (e.g., genomic localization, neighbouring sequences) may also govern the level of of variability. In any case, the sequence of the motif itself seems to play only a minor role.

The number of polymorphisms found within chickpea accessions was lower than between them (Table 2). Some probes [such as $(GGGA)_4$ and $(GCGT)_4$; Fig. 2] were perfectly stable within an accession, but polymorphic between accessions. A set of such probes may thus provide an invaluable tool for the identification of accessions and cultivars. The probes which we classified as category-I revealed 3.5–4.8 between-accession polymorphisms per probe, depending on the enzyme and the accessions investigated (Table 2). How many of these polymorphisms are actually useful for linkage analysis will depend on the extent of allelism and clustering, which

Table 2 Numbers of within- and between-accession polymorphisms detected by oligonucleotide fingerprinting of *Taq*I- and *Dra*I-digested DNA of three chickpea accessions with category-I probes

Enzyme	Accession(s)	Number of probes tested	Number of polymorphic bands per probe			
			Total	Min	Max	Average
*Taq*I	ILC 482	13	24	0	7	1.85
	ILC 1272	13	12	0	5	0.92
	ILC 3279	13	29	0	9	2.23
	ILC 482/ILC 1272	15	53	0	12	3.53
	ILC 482/ILC 3279	15	53	0	11	3.53
	ILC 1272/ ILC 3279	20	78	0	12	3.90
*Dra*I	ILC 482	13	21	0	5	1.61
	ILC 1272	13	14	0	6	1.08
	ILC 3279	13	20	0	7	1.54
	ILC 482/ILC 1272	14	54	0	10	4.57
	ILC 482/ILC 3279	14	59	1	12	4.21
	ILC 1272/ILC 3279	14	68	0	14	4.86

remain to be determined. However, the number of markers may be considerably increased, if parents are selected for maximum diversity of their fingerprint patterns, and if bands that are polymorphic within these accessions are also included.

Taken together, our results show that an intraspecific map of chickpea based on oligonucleotide fingerprinting is feasible. The availability of 50–100 markers generated by the 20 category-I probes would probably be sufficient for marker-assisted selection of *Ascochyta* resistance in breeding programs. Fingerprint-derived markers can, of course, also be used for the analysis of interspecific crosses involving *C. arietinum, C. reticulatum and C. echinospermum*, and then be integrated into the already-existing chickpea map (Kazan et al. 1993). For interspecific crosses, a high number of polymorphisms may be expected from both category-I and the category-II probes.

Linkage maps based on DNA fingerprinting exhibit advantages as well as limitations as compared to maps derived from other types of markers. Advantages include the high level of polymorphism detected, the rapid screening of the genome with few probes, the availability of universally applicable probes without cloning, and the higher reproducibility as compared to RAPDs. However, this approach may be limited by extensive clustering of simple repeats or by high mutation rates leading to unexpected fragments in the progeny (see, e.g., Jeffreys et al. (1988). Inclusion of parental and F_1 DNAs in the segregation analysis, and the preferred use of accession rather than (presumably less stable) individual-specific markers will help to minimize this problem. A disadvantage shared with RAPDs is that the allelic state of a fingerprint band is usually unknown, and its occurrence has to be treated as a dominant rather than a codominant marker. Despite these limitations, and in view of the advantages, we hold that oligonucleotide fingerprints will prove valuable for the establishment of maps in species, such as chickpea, with limited levels of genetic diversity.

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